Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium?

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Abstract

The steroid hormone progesterone (P_4) plays a key role in the reproductive events associated with pregnancy establishment and maintenance. High concentrations of circulating P_4 in the immediate post-conception period have been associated with an advancement of conceptus elongation, an associated increase in interferon-τ production and higher pregnancy rates in cattle. Using *in vitro* and *in vivo* models and ~8500 bovine oocytes across six experiments, the aim of this study was to establish the route through which P_4 affects bovine embryo development *in vitro* and *in vivo*. mRNA for P_4 receptors was present at all stages of embryo development raising the possibility of a direct effect of P_4 on the embryo. Exposure to P_4 *in vitro* in the absence or presence of oviduct epithelial cells did not affect the proportion of embryos developing to the blastocyst stage, blastocyst cell number or the relative abundance of selected transcripts in the blastocyst. Furthermore, exposure to P_4 *in vitro* did not affect post-hatching elongation of the embryo following transfer to synchronized recipients and recovery on Day 14. By contrast, transfer of *in vitro* derived blastocysts to a uterine environment previously primed by elevated P_4 resulted in a fourfold increase in conceptus length on Day 14. These data provide clear evidence to support the hypothesis that P_4-induced changes in the uterine environment are responsible for the advancement in conceptus elongation reported previously in cattle and that, interestingly, the embryo does not need to be present during the period of high P_4 in order to exhibit advanced elongation.
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Introduction

The steroid hormone progesterone (P_4) plays a key role in the reproductive events associated with pregnancy establishment and maintenance. High concentrations of circulating P_4 in the immediate post-conception period have been associated with an advancement of conceptus elongation, an associated increase in interferon-τ production and higher pregnancy rates in cattle (Lamming & Royal 1999, Mann & Lamming 2001, Inskeep 2004, Stronge *et al.* 2005, McNeill *et al.* 2006) and sheep (Ashworth *et al.* 1989, Satterfield *et al.* 2006).

The effects of elevated P_4 shortly after conception on the advancement of conceptus elongation have been convincingly demonstrated in cattle and sheep. Garrett *et al.* (1988) administered 100 mg P_4 on Days 1, 2, 3, and 4 of pregnancy which resulted in an increased peripheral plasma P_4 concentration on Days 2–5 and significantly larger conceptuses on Day 14. Using a P_4 implant on Day 3 of pregnancy, Carter *et al.* (2008) significantly elevated P_4 concentrations until Day 8 and this was associated with a larger conceptus recovered at slaughter on Day 16. Similarly, when ewes received daily injections of 25 mg P_4 from 36 h postmating, blastocyst

diameter increased by 220% on Day 9 and the time of elongation of blastocysts to a filamentous conceptus on Day 12 was advanced (Satterfield *et al.* 2006); these effects of P_4 treatment on blastocyst development were blocked by administration of RU486, a P_4 receptor (PGR) antagonist.

From the above, it is clear that the concentration of circulating P_4 has an effect on the developing embryo. This effect is likely as a result of downstream effects of P_4-induced changes in gene expression in the tissues of the uterus (Bauersachs *et al.* 2006, Satterfield *et al.* 2006, Forde *et al.* 2009) resulting in changes in the composition of histroph to which the developing embryo is exposed. Whether any of the effects of P_4 is directly on the embryo or whether the observed advancement of development is mediated solely through changes induced in the endometrium is, as yet, unknown. In an attempt to answer this question, some authors have added P_4 to embryo culture medium *in vitro* and examined development to the blastocyst stage. Results, however, have been varied and contradictory with some authors reporting positive effects of P_4 (Ferguson *et al.* 2005, Merlo *et al.* 2007) while others have reported no effect (Reggio *et al.* 1997, Goff & Smith 1998).

These contradictory observations may be partially explained by the culture system used, as steroids are known to be lipophilic and culture under oil, the system used in many laboratories, may result in depletion from the medium (Shimada *et al.* 2002).

Many of the actions of P_4 are mediated through binding to its nuclear receptor (PGR). This classical PGR was originally characterized in the rodent uterus (Milgrom *et al.* 1970), and then shortly after in humans (Rao *et al.* 1974). It was subsequently shown to exist in two isoforms, PGRA and PGRB, both of which similarly bind P_4 with high affinity (Lessey *et al.* 1983). Selective knockout experiments in mice have indicated that PGRA is both necessary and sufficient to elicit the P_4-dependent reproductive responses necessary for female fertility while PGRB is required to elicit normal proliferative responses of the mammary gland to P_4 (Conneely *et al.* 2002). Two related proteins with progestin binding activity were subsequently described, namely PGR membrane component (PGRMC) 1 and 2 (Falkenstein *et al.* 1996, Meyer *et al.* 1996, Gerdes *et al.* 1998). While only minimal information is available for PGRMC2, PGRMC1 has both high- and low-affinity P_4 binding sites (Meyer *et al.* 1996), has a single transmembrane spanning domain, and attenuates apoptosis in cultured rat (Peluso *et al.* 2006) and human (Engmann *et al.* 2006) granulosa cells in response to P_4 treatment. Whether these receptors function in bovine embryos is unknown.

Therefore, using ∼8500 bovine oocytes across six experiments, this study was designed to answer the following questions: 1) is there mRNA for the PGR on bovine embryos during early preimplantation development, a time when elevated P_4 *in vivo* is associated with an advancement in post-hatching development? 2) does addition of P_4 to the culture medium of bovine embryos under controlled conditions affect blastocyst development *in vitro* and subsequent embryo survival and elongation *in vivo* following transfer to synchronized recipient heifers? and 3) does elevated P_4 in the early days of the estrous cycle prepare the endometrium and uterine environment to advance the elongation of the conceptus following transfer of blastocysts produced *in vitro* in the absence of P_4?

Results

PGR mRNA relative abundance in bovine embryos

PGR mRNA was detected at all stages of early embryo development except the morula stage (Fig. 1A). The relative abundance of *PGR* mRNA was highest in immature oocytes and lowest at the 2- to 4-cell and 16 cell stages. Both *PGRMC1* and *2* were detected at all stages of development, decreasing from oocyte to embryonic genome activation and increasing at the blastocyst stage (Fig. 1B and C). A small number of mRNA copies of *PGR* were detected per blastocyst
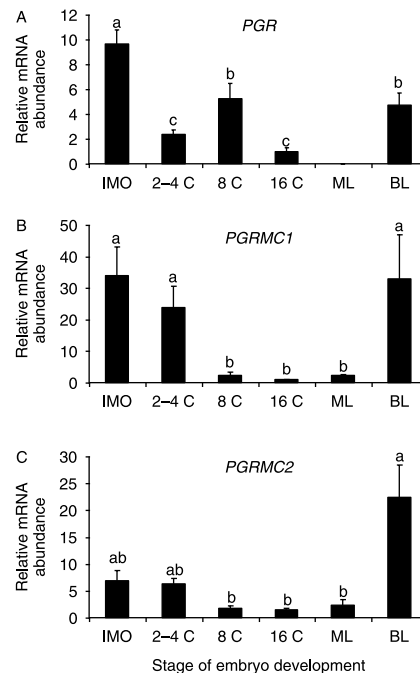
Figure 1 Presence of progesterone receptor mRNA on immature bovine oocytes and preimplantation embryos. RNA was extracted from pools of 10 immature oocytes (IMO), 2- to 4-cell, 8-cell, 16-cell, morula (ML) and blastocyst (BL) stage embryos. (A) Nuclear progesterone receptor (*PGR*); (B) progesterone receptor membrane component 1 (*PGRMC1*); and (C) progesterone receptor membrane component 2 (*PGRMC2*).

(10 ± 2.7). For *PGRMC1*, the number of mRNA copies per blastocyst was higher $(10\,111\pm2548)$ while for *PGRMC2* the number was intermediate (1366 ± 160).

P_4 concentration in the mid-luteal bovine oviduct

Mean P_4 concentration in the oviduct flushings was 4.0 ± 0.44 ng/ml $(n=23)$. P_4 was undetectable in the PBS used for flushing. In the stock solution used as a control for the assay, 17.29 ± 1.02 ng/ml P_4 was detected from the expected 20 ng/ml concentration.

Disappearance of P_4 during culture

P_4 concentration in synthetic oviduct fluid (SOF) on Day 1 (first day of *in vitro* embryo culture) was as expected (∼35 ng/ml). Culture in 25 µl droplets under mineral oil resulted in the rapid depletion of P_4 such that by Day 3 it was barely detectable (0.1–1.6 ng/ml). When culture took place in a larger volume of medium (500 µl) in the absence of oil, P_4 concentrations remained unchanged (39–40 ng/ml) throughout the 7 days of culture (Fig. 2A); however, in the presence of embryos the concentration decreased gradually over the subsequent 7 days such that by Day 7 approximately half of the original concentration remained (∼15 ng/ml; Fig. 2A). Similarly, the presence of a bovine oviduct epithelial cell (BOEC)
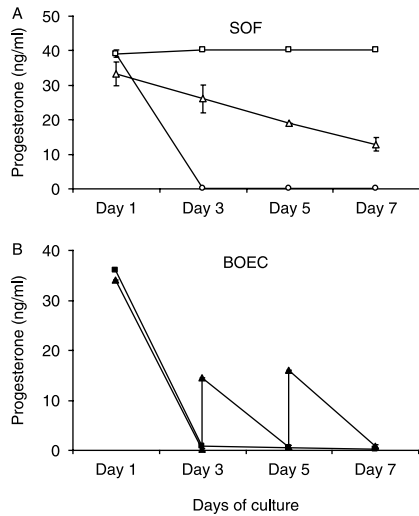
Figure 2 Effect of culture system on the rate of disappearance of progesterone from culture medium in the absence (A) or presence (B) of a bovine oviduct epithelial cell (BOEC) monolayer. Following addition of a known quantity (1×10^{-7} M) of progesterone to the culture medium on Day 1, the concentration remaining on Days 3, 5 and 7 of culture was assessed. Culture conditions tested included: i) 25 µl droplets of SOF under mineral oil, the system of culture routinely used on our laboratory, open circles; ii) 500 µl SOF in wells (without embryos), open squares; iii) 500 µl SOF in wells with embryos, open triangles; iv) 500 µl SOF in co-culture with BOEC (without embryos), closed squares; and v) 500 µl SOF in co-culture with BOEC with replacement of half of the media every 2 days (without embryos), closed triangles. (Experiment 3).

monolayer resulted in the rapid depletion of P_4 from the medium (Fig. 2B); when the medium was replenished every 2 days by removing 250 µl and replacing it with 250 µl medium containing the original concentration of P_4, concentrations temporarily increased but rapidly returned to low levels (Fig. 2B). Based on these results, all embryo culture in subsequent experiments took place in 500 µl medium without oil overlay.

Effect of P_4 supplementation on embryo development *in vitro*

Ethanol at the concentrations used to prepare the working solutions of P_4 had no effect on any of the parameters studied. Addition of exogenous P_4 at any of the concentrations tested did not affect cleavage rate or blastocyst development, irrespective of the absence (Table 1) or presence (Table 2) of a BOEC monolayer. Similarly, there was no evidence of any P_4-induced difference in the relative transcript abundance of any of the genes studied in either blastocysts or the cultured oviduct cells (Fig. 3). The cell number and allocation (inner cell mass (ICM), trophectoderm (TE)) within Day 7 blastocysts was not affected by culture with P_4 (Table 3).

Effect of exposure to P_4 in vitro *on blastocyst elongation* in vivo *following embryo transfer*

Data on the production of embryos *in vitro* in the absence or presence of progesterone for embryo transfer and on the recovery of embryos after transfer are shown in Table 4. As expected, there was no difference in the P_4 profile of the recipient animals which received control or P_4-treated embryos (Fig. 4A). The recovery rate of embryos on Day 14 was similar for both groups (control: $50.4 \pm 5.35\%$ versus P_4-treated: $65.0 \pm 6.75\%$; Table 4). There was large variation in conceptus size on Day 14, even within the same recipient (Fig. 5). Exogenous P_4 in culture up to Day 7 had no effect on embryo dimensions on Day 14 (length: control: 3.80 ± 0.46 mm, $n = 121$ versus P_4-treated: 4.46 ± 0.53 mm, $n = 130$; Fig. 4B).

Effect of elevated P_4 in vivo *prior to embryo transfer on blastocyst elongation*

Data on the production of embryos *in vitro* for transfer to progesterone-primed recipients and on the recovery of embryos after transfer are shown in Table 5. Insertion of a P_4 releasing intravaginal device (PRID) on Day 3 increased P_4 concentration four- to fivefold within 24 h (Fig. 6A). The concentration remained significantly higher than control animals until Day 7 after which time concentrations were no longer different between control and treated heifers. Elevation of P_4 prior to embryo transfer on Day 7 resulted in a significant increase in mean conceptus length and area on Day 14 compared to those recovered from control animals (length: control: 4.27 ± 0.42 mm, $n = 112$ versus P_4-treated: 15.4 ± 1.61 mm, $n = 79$, $P < 0.001$; Fig. 6B).

Table 1 Effect of supplementation with progesterone (P_4) during *in vitro* culture in synthetic oviduct fluid on the cleavage rate and blastocyst yield of bovine embryos *in vitro* (Experiment 4, 9 replicates).

			Blastocyst yield		
	N	**Cleaved, *n*** (mean±s.e.m.%)	Day 6, *n* (mean±s.e.m.%)	Day 7, *n* (mean±s.e.m.%)	Day 8, *n* (mean±s.e.m.%)
Control	410	315 (77.1±3.1)	58 (16.2±4.2)	121 (31.6±4.7)	138 (36.2±5.6)
0.1×10^{-7} M P_4	402	323 (80.9±4.3)	66 (17.5±2.4)	118 (31.0±3.2)	147 (38.2±3.4)
1×10^{-7} M P_4	408	329 (81.8±3.7)	61 (16.8±2.9)	127 (33.8±4.3)	152 (40.0±4.0)
10×10^{-7} M P_4	407	329 (82.1±2.7)	71 (18.8±1.8)	138 (35.3±3.1)	154 (39.36±3.5)
Control (ethanol)	194	161 (82.9±2.7)	19 (9.5±4.5)	57 (29.1±5.7)	68 (34.9±4.5)

$P > 0.05$.

Table 2 Effect of supplementation with progesterone (P_4) during *in vitro* culture in synthetic oviduct fluid in the presence of a bovine oviduct epithelial cell monolayer on the cleavage rate and blastocyst yield of bovine embryos *in vitro* (Experiment 4, 7 replicates).

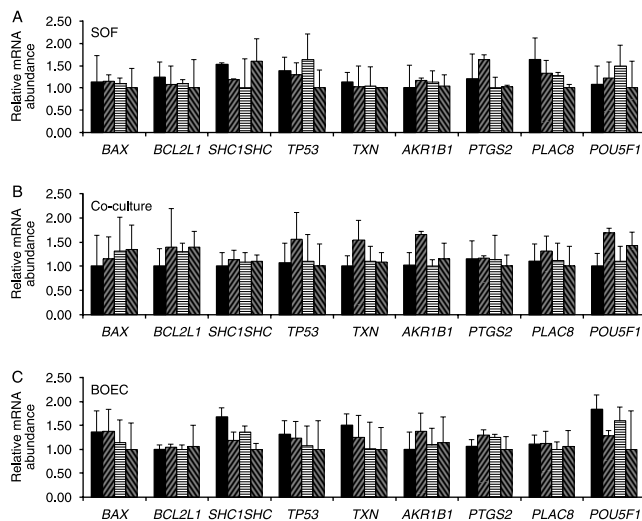|			Blastocyst yield		
	N	**Cleaved, n** (mean±S.E.M.%)	**Day 7, n** (mean±S.E.M.%)	**Day 8, n** (mean±S.E.M.%)	**Day 9, n** (mean±S.E.M.%)
Control	328	245 (74.3±2.6)	74 (25.6±4.9)	99 (30.2±5.2)	102 (31.2±4.9)
0.1×10^{-7} M P_4	328	248 (75.1±3.0)	68 (20.6±1.1)	92 (28.1±1.7)	97 (29.6±1.6)
1×10^{-7} M P_4	328	259 (78.5±1.9)	89 (27.3±2.0)	108 (33.1±2.0)	109 (33.4±1.8)
10×10^{-7} M P_4	328	253 (76.8±1.9)	75 (22.8±1.9)	98 (29.9±2.3)	101 (30.9±2.2)

$P>0.05$.

Discussion

The main findings of this study are i) mRNA for PGRs exists on the bovine embryo, raising the possibility of a direct effect of P_4 on the embryo ii) supplementation with P_4 during *in vitro* embryo culture does not affect the proportion of zygotes reaching the blastocyst stage *in vitro*, cell number and allocation within the blastocyst, mRNA abundance of selected transcripts or post-hatching elongation *in vivo*, iii) elevation of P_4 *in vivo* prior to embryo transfer leads to a dramatic increase in conceptus length on Day 14. Taken together, these results suggest that the well described association between early elevation of P_4 post conception and advanced conceptus length (Garrett *et al*. 1988, Satterfield *et al*. 2006, Carter *et al*. 2008) is not due to a direct effect of P_4 on the embryo but rather due to P_4-induced changes in the environment in which the embryo is developing. Furthermore, the early embryo does not need to be present in the uterus when P_4 concentrations are elevated in order to benefit from their consequences.

Figure 3 Relative transcript abundance of selected transcripts in bovine blastocysts (A and B) and cultured oviduct cells (C) in the absence (black bars) or presence of increasing concentrations of progesterone. (A) Blastocysts produced in synthetic oviduct fluid (SOF); (B) blastocysts produced in SOF in co-culture with a bovine oviduct epithelial cell (BOEC) monolayer; and (C) BOEC. No differences between progesterone treatment and control ($P>0.05$).

Several studies have reported lower P_4 concentrations in cows that failed to become pregnant although the degree and timing of the association between P_4 concentrations and embryo survival are still unclear. For example, some authors have reported a positive association between milk or plasma P_4 concentration and embryo survival on Days 4–6 of the estrous cycle (Green *et al*. 2005, McNeill *et al*. 2006) while others found no association until Days 14–17 (Chagas e Silva & Lopes da Costa 2005). Despite these inconsistent results there is an indication that low P_4 concentrations during the early post-ovulatory luteal phase, or a delay in the normal rise in P_4 at this time are associated with reduced embryo survival (Diskin & Morris 2008).

Expression of PGRs in embryos has been demonstrated in several species including mouse (Hou & Gorski 1993), pig (Ying *et al*. 2000) and horse (Rambags *et al*. 2008). To our knowledge, the present study shows for the first time *PGR* mRNA expression in bovine preimplantation embryos. However, the number of transcripts per embryo was low and the functionally is, as yet, unclear. Expression of PGRMC1 has previously been shown in bovine preimplantation embryos (Dode *et al*. 2006) but, to date, little is known about its function.

Culture of embryos *in vitro* in droplets of medium necessitates the use of oil to prevent evaporation. The problem of absorption of steroids by the oil overlaying the culture media drops is well known (Shimada *et al*. 2002, Zheng *et al*. 2003). One approach to overcome this problem is the pre-equilibration of oil with the appropriate steroid (Zheng *et al*. 2003). Alternatively, by using a high concentration of P_4 at the beginning of incubation (almost 3000 ng/ml), the same authors reported that the capacity of the oil overlay to absorb the steroid was close to being saturated before the oocytes were added, thus sustaining an adequate concentration of between 211 and 48 ng/ml for the duration of oocyte culture (Zheng *et al*. 2003). In the present study, having established that P_4 was depleted rapidly from the medium in the presence of oil, we chose to eliminate the oil completely to avoid any ambiguity in the results.

Irrespective of the concentration used, P_4 supplementation during *in vitro* culture had no effect on the proportion of zygotes developing to the blastocyst stage or on the quality of the blastocysts as assessed in

Table 3 Total, inner cell mass (ICM) and trophectoderm (TE) cell numbers (mean±s.e.m.) in Day 7 bovine blastocysts produced *in vitro* in the absence or presence of progesterone (1×10^{-7} M).

	Control	**Progesterone**
No. of blastocysts	42	32
Total nuclei	145.28±4.71	137.91±6.18
No ICM nuclei	34.33±1.15	36.19±1.83
% ICM	24.13±0.81	26.64±1.11
No TE nuclei	110.95±4.30	101.72±5.30
% TE	75.87±0.81	73.36±1.11
ICM/TE	0.33±0.01	0.37±0.02

$P > 0.05$.

terms of cell number and allocation within the blastocyst or relative transcript abundance for a selected number of genes. Similarly, Pereira *et al.* (2009) failed to demonstrate a direct association between high P_4 concentrations and embryo survival. This apparent lack of an effect on blastocyst development is consistent with previous observations from our group. For example, Carter *et al.* (2008), reported no effect on the recovery of blastocysts on Day 7 from single-ovulating animals with normal or elevated P_4 concentrations despite a clear advancement of post hatching elongation. In addition, Rings *et al.* (2008) reported no effect on blastocyst yield following the endoscopic transfer of *in vitro* produced zygotes to the ipsilateral oviduct of heifers with normal or elevated P_4 concentrations. Taken together, these results would strongly suggest that P_4 is not associated with the ability of the oocyte to form a blastocyst.

Extending the above data, we addressed the question of whether an effect of P_4 could be manifested post hatching by transferring blastocysts produced in the absence or presence of P_4 to synchronized recipients. We chose Day 14 to slaughter the animals as it represents the initiation of elongation in cattle, a critical checkpoint in development. In addition, previous personal experience has shown that in the case of multiple transfers of embryos as carried out here, separating rapidly elongating conceptuses after Day 14 can be extremely difficult. Lastly, but perhaps most importantly, it is known that very little embryonic loss occurs after Day 14 in cattle (Dunne *et al.* 2000) and thus survival to Day 14 represents a useful measure of developmental competence. Consistent with the data on blastocyst formation described above, culture in the presence of P_4 to the blastocyst stage and subsequent

transfer to recipients did not lead to an advancement in conceptus length by Day 14. Blood samples from all recipients from Day 0 to Day 14 indicated no difference between those receiving control or P_4-treated embryos, thus ruling out any potential effect of recipient P_4 concentration on embryo development as previously described (Lonergan *et al.* 2007). These observations are perhaps not that surprising when one considers that it is possible to transfer embryos produced *in vitro* in the absence of P_4, and achieve acceptable pregnancy rates.

Given that we have previously shown that elevating P_4 in heifers in a manner identical to that described in the present study leads to a significant alteration in the transcriptome of the endometrium (Forde *et al.* 2009), the final experiment addressed the question of whether elevating P_4 *in vivo* for a short window (Day 3–7) prior to embryo transfer would create an environment capable of advancing the development of blastocysts transferred to the uterus on Day 7. In other words, does the embryo need to be present during the period of elevated P_4 *in vivo* in order to benefit from it? In contrast to the results of Experiment 5, transfer of blastocysts to a high P_4 environment on Day 7 lead to a fourfold increase in conceptus length on Day 14. This provides clear evidence to support the hypothesis that P_4-induced changes in the uterine environment are responsible for the advancement in conceptus elongation reported previously in cattle (Garrett *et al.* 1988, Carter *et al.* 2008) and sheep (Satterfield *et al.* 2006) and, interestingly, the embryo does not need to be present during the period of high P_4 in order to exhibit advanced elongation.

In conclusion, taking these results together with those from previous studies, it would appear that P_4 either *in vivo* (Carter *et al.* 2008, Rings *et al.* 2008) or *in vitro* (present study) does not affect the ability of embryos to develop to the blastocyst stage in cattle. Furthermore, exposure to P_4 *in vitro* does not affect the post-hatching elongation of the embryo following transfer to a recipient. In contrast, however, transfer of a blastocyst to a uterine environment previously primed by elevated P_4 results in a significant advancement in conceptus elongation consistent with an increase in interferon-τ production and a stronger signal around the critical time of maternal recognition of pregnancy. Given our knowledge of P_4-induced changes in endometrial gene expression, future studies will focus on the composition of uterine histotroph in an effort to identify secreted factors associated with the stimulation of conceptus growth.

Table 4 Development of bovine blastocysts produced *in vitro* in the absence or presence of progesterone (P_4, 10×10^{-7} M) following transfer (in groups of 20 blastocysts) to synchronized recipients on Day 7 and recovery on Day 14 (Experiment 5).

	N	**Cleaved, *n*** (mean±s.e.m.%)	**Blastocyst yield, *n*** (mean±s.e.m.%)	**No. of recipients**	**No. of embryos recovered** (recovery rate %)
Control	1659	1208 (72.7±1.91)	412 (24.6±1.60)	12	121 (50.4±5.35)
+P_4	1876	1372 (72.9±2.08)	486 (25.7±1.46)	10	130 (65.0±6.75)
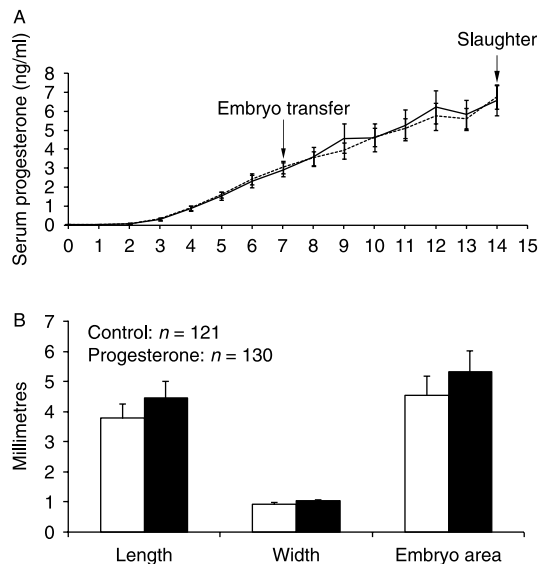
Figure 4 Mean ($\pm$s.e.m.) serum progesterone concentration in recipient heifers (A) and Day 14 embryo dimensions (B) following transfer of Day 7 blastocysts produced *in vitro* in the absence or presence of progesterone (1×10^{-7} M) to synchronized recipients and recovery at slaughter on Day 14. Each recipient received 20 blastocysts which had been produced *in vitro* in the presence (dashed line, black bars) or absence (solid line, white bars) of exogenous progesterone. Note that, as expected, the progesterone profiles for both heifer groups are similar, irrespective of embryo type transferred. Exposure to progesterone *in vitro* did not affect the embryo dimensions on Day 14 ($P > 0.05$).

Materials and Methods

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the Cruelty to Animals Act (Ireland 1897) and European Community Directive 86/609/EC.

In vitro *embryo production*

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Company. The techniques for producing embryos *in vitro* have been previously described in detail (Rizos *et al.* 2002). 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 h in TCM-199 supplemented with 10% (v/v) FCS and 10 ng/ml epidermal growth factor at 39 °C under an atmosphere of 5% CO_2 in air with maximum humidity. For IVF, matured COCs were inseminated with frozen–thawed percoll-separated bull sperm at a concentration of 1×10^6 spermatozoa/ml. Gametes were co-incubated at 39 °C under an atmosphere of 5% CO_2 in air with maximum humidity. Semen from the same bull was used for all experiments.

At $\sim$20 h post insemination (pi), presumptive zygotes were denuded, divided into groups of 40–50, and transferred to 500 µl culture wells. The basal medium for all embryo culture was SOF supplemented with 5% FCS. Depending on the experiment (see below), this was supplemented or not with P_4

and/or with BOECs. Cleavage rate was recorded at 48 hpi and blastocyst development recorded at Days 6, 7, and 8 pi. All Day 7 blastocysts were snap frozen in groups of ten in liquid nitrogen and stored at -80 °C for mRNA extraction and real-time quantitative RT-PCR.

Preparation of BOEC monolayer

Ipsilateral oviducts were collected from slaughtered heifers at the mid-luteal phase of the estrous cycle and transported to the laboratory on ice. Each oviduct was flushed with 10 ml of SOF+5% FCS. Oviduct cells were centrifuged at 750 ***g*** for 9 min and the resulting pellet was washed by centrifugation at 100 ***g*** for 5 min in 5 ml of the same medium. The final pellet was re-suspended in culture medium and the cells separated by pipetting. Oviduct cells were counted in a hemocytometer and diluted in the appropriate volume of culture medium to give a final concentration of 1×10^6 cells/ml. Culture wells (500 µl) were then prepared from this solution and used for embryo culture 4–5 days later. Half of the media was replenished every 48 h before and during embryo culture.

Differential staining of blastocysts

Differential staining of ICM and TE cells was carried out using published procedures (Thouas *et al.* 2001) with minor modifications. For partial permeabilization and staining of TE, blastocysts were incubated in 300 µl of PBS with 0.2% Triton X-100 and 100 µg/ml propidium iodine in the dark for 50 s at room temperature. For fixation and total cell staining, blastocysts were then immediately transferred into 300 µl 100% ethanol containing 25 µg/ml bisbenzimide (Hoechst 33342) for 3 min at room temperature. Fixed and stained blastocysts were then transferred directly into glycerol, taking care to avoid carryover of excessive amounts of fixative
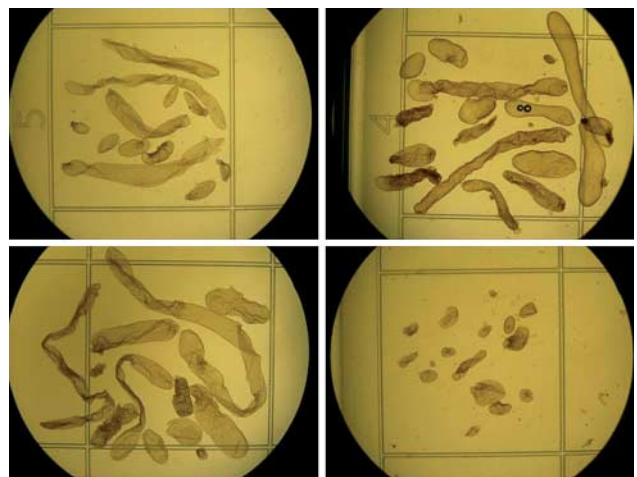
Figure 5 Representative images of Day 14 elongating bovine embryos recovered from four individual heifers following the transfer of 20 blastocysts on Day 7. The images show the type of variation and recovery one can expect following such multiple embryo transfer. For scale, the square grid in the dish is 13×13 mm.

Table 5 Development of bovine blastocysts produced *in vitro* in the absence of progesterone (P_4) following transfer on Day 7 (in groups of 20 blastocysts) to synchronized recipients with normal or elevated P_4 and recovery on Day 14 (Experiment 6).

N	Cleaved, *n* (mean±s.e.m.%)	Blastocyst yield, *n* (mean±s.e.m.%)		No. of recipients	No. of embryos recovered, *n* (recovery rate %)
1817	1527 (84.2±0.92)	527 (29.7±1.53)	Normal P_4	9	112 (61.7±5.89)
			High P_4	9	79 (43.9±7.49)

Elevated progesterone was achieved by insertion of a PRID on Day 3 of the estrous cycle. See Materials and Methods for a detailed description.

solution. Finally, blastocysts were mounted onto a glass microscope slide in a drop of glycerol, gently flattened with a cover slip and visualized for cell counting under a fluorescence microscope.

P_4 assays

Following collection, blood samples were refrigerated (4 °C) for 12–24 h before being centrifuged at 1500 ***g*** at 4 °C for 20 min. Serum was separated and stored at −20 °C until assayed to determine P_4 concentration by solid phase RIA using a Coat-A-Count Progesterone kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). The sensitivity of the assay was 0.03 ng/ml. The intra- and interassay coefficients of variation for assays of P_4 (low, medium, and high samples) were 8.5, 4.8, 4.9, 10, 4.9, and 5% respectively.

In order to determine P_4 concentrations in spent culture media and in oviductal flushings an ELISA kit, prepared for human serum or plasma, (DE1561 DIMEDITEC Diagnostics GmbH, Kiel, Germany) was used. Inter and intra-assay coefficients of variation were 6.63 and 6.42% respectively.

RNA extraction, RT, and quantification of mRNA transcript abundance

For the analysis of the expression of mRNA for PGRs across development, RNA was extracted from pools of ten immature oocytes, 2- to 4-cell, 8-cell, 16-cell, morula and blastocyst stage embryos. To assess the effect of culture with P_4 on mRNA abundance of candidate transcripts in blastocysts and in co-cultured oviduct cells, three pools of ten Day 7 blastocysts or oviduct cells from each experimental group were snap frozen.

Poly(A) RNA was prepared following the manufacturer's instructions using the Dynabeads mRNA Direct Extraction KIT (Dynal Biotech, Oslo, Norway). To take account of potential differences in RNA content between different developmental stages, after adding the lysis buffer, 0.1 pg rabbit globin mRNA (Sigma) was added to each tube as an internal standard. The RT reaction was carried out following the manufacturer's instructions (Promega) using poly(T) anchored primers and AMV reverse transcriptase enzyme in a volume of 20 µl to prime the RT reaction and to produce cDNA. Tubes were heated to 70 °C for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 5 units of Superscript RT enzyme. They were then incubated at room temperature for 10 min and then at 42 °C for 60 min to allow the RT of RNA, followed by 70 °C for 10 min to denature the RT enzyme.

The quantification of all mRNA transcripts was carried out by real-time quantitative PCR (qPCR) as previously described (Bermejo-Alvarez *et al*. 2008). Three replicate qPCR experiments were conducted for all genes of interest. To examine mRNA expression in blastocysts and BOEC, experiments were conducted to contrast relative levels of each transcript and histone (*H2AFZ*) in every sample. qPCR was performed by adding a 2.5 µl aliquot of each sample to the PCR mix containing the specific primers to amplify *H2AFZ*, BCL2-associated X protein (*BAX*), BCL2-like 1 (*BCL2L1*), Src homology 2 domain containing transforming protein C1 (*SHC1SHC* or *P66*), tumor protein p53 (*TP53*), genes related to apoptosis, thioredoxin (*TXN*), aldo-keto-reductase family 1-member 1 (*AKR1B1*), prostaglandin-endoperoxidase-2 (*COX-2* or *PTGS2*), genes related to metabolism, placenta-specific 8 (*PLAC8*), related to implantation, and POU class 5 homeobox 1 (*OCT3/4* or *POU5F1*)
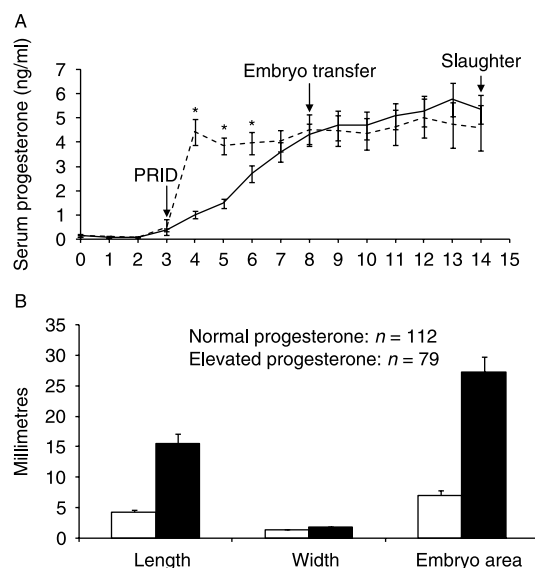
Figure 6 Mean (±s.e.m.) serum progesterone concentration in recipient heifers (A) and Day 14 embryo dimensions (B) following transfer of Day 7 *in vitro* produced blastocysts to synchronized recipients with normal (solid line, white bars) or elevated (dashed line, black bars) progesterone concentrations and recovery at slaughter on Day 14. Elevated progesterone concentrations were achieved using a previously validated model (Carter *et al*. 2008) involving insertion of a PRID on Day 3 of the estrous cycle. Each recipient received 20 blastocysts which had been produced *in vitro* in the absence of progesterone. Elevated progesterone was associated with a significant increase in mean embryo dimensions (*P* < 0.05).

related to pluripotency. To compare mRNA expression of PGRs (*PGR*, *PGRMC1*, and *PGRMC2*) in embryos at different developmental stages, the experiments were conducted to contrast relative levels of each transcript and rabbit globin (*HBA*) in each sample. Primer sequences and the sizes of the amplified fragments of all transcripts are shown in Table 6. The comparative C_T method was used to quantify expression levels (Rizos *et al*. 2007). Quantification was normalized to the endogenous control, *H2AFZ* (when expression between blastocysts and BOEC monolayer was carried out) or to an exogenous control (*HBA*; when expression between embryos at different developmental stages was carried out). Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative C_T method, the ΔC_T value was determined by subtracting the control gene (*H2AFZ* or *HBA*) C_T value for each sample from each gene C_T value of the sample. Calculation of $\Delta\Delta C_T$ involved using the highest sample ΔC_T value (i.e. the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔC_T sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta C_T}$.

PGR mRNA copy number quantification in Day 7 blastocysts

In addition to the above, an absolute quantification method was used to measure the presence and copy number of *PGR*, *PGRMC1*, and *PGRMC2* in Day 7 blastocysts. Three groups of ten blastocysts from different experimental replicates were used. Quantification of *PGR* mRNA copy number in each sample was done using a standard curve for each receptor type. To generate the copy number standard curve for cDNA standards, *PGR* cDNA was amplified by PCR from mRNA purified from BOECs. PCR products were purified from an agarose gel according to the manufacturer's protocol (Elu-quick DNA purification kit, Whatman, Stanford, ME, USA). Following the confirmation of the PCR product by ethidium bromide-stained 2% agarose gel electrophoresis, the expected fragments were eluted and the concentration of the PCR fragments was determined spectrophotometrically by measuring absorbance at 260 nm. The standards in triplicate and cDNA samples (equivalent to 0.66 embryos per sample) were then co-amplified in the same reaction prepared from a master mix. Copy numbers of fragments were empirically determined using the following equation: copy number of PCR standard $=$ mole $\times$ Avogadro Constant $=$ grams/fragment molecular weight $\times$ Avogadro Constant (Avogadro Constant $= 6.0221367 \times 10^{23}$). The quantified PCR fragments were later serially diluted in five 10-fold serial-dilutions

Table 6 Details of primers used for quantitative real-time PCR.

Gene symbol	MGI official name	Primer sequence (5′–3′)	Fragment size	GenBank accession no.
H2AFZ	H2A histone family, member Z	5′-AGGACGACTAGCCATGGACGTGTG 5′-CCACCACCAGCAATTGTAGCCTTG	212	NM_174809
BAX	BCL2-associated X protein	5′-CTACTTTGCCAGCAAACTGG 5′-TCCCAAAGTAGGAGAGGA	158	NM_173894.1
BCL2L1	BCL2-like 1	5′-GGAGCTGGTGGTTGACTTTC 5′-CTAGGTGGTCATTCAGGTAAG	517	BC147863.1
SHC1SHC	Src homology 2 domain containing transforming protein c1 (*P66*)	5′-GTGAGGTCTGGGGAGAAGC 5′-GGTTCGGACAAAGGATCACC	334	NM_001075305
TP53	Tumor protein p53	5′-CTCAGTCCTCTGCCATACTA 5′-GGATCCAGGATAAGGTGAGC	364	U74486
TXN	Thioredoxin	5′-ATGGTGAAACAGATTGAGAGCA 5′-CGTTGGAATACTTTTCAGAGAGAGAA	154	AF104105
AKR1B1	Aldo-keto-reductase family 1-member 1	5′-CGTGATCCCCAAGTCAGTGA 5′-AATCCCTGTGGGAGGCACA	152	M31463.1
PTGS2	Prostaglandin-endoperoxide synthase 2 (*COX2*)	5′-ATCTACCCGCCTCATGTTCCT 5′-GGATTAGCCTGCTTGTCTGGA	187	AF031698
PLAC8	Placenta-specific 8	5′-CGGTGTTCCAGAGGTTTTTCC 5′-AAGATGCCAGTCTGCCAGTCA	163	NM_016619
POU5F1	POU class 5 homeobox 1 (*OCT3/4*)	5′-CGAGTATCGAGAACCGAGTG 5′-CAGGGTTCTCTCCCTAGCTC	440	NM_174580.1
HBA	Alpha-hemoglobine	5′-GCAGCCACGGTGTCGAGTAT 5′-GTGGGACAGGAGCTTGAAAT	257	X04751
PGR	Progesterone receptor	5′-GAGAGCTCATCAAGGCAATTGG 5′-CACCATCCCTGCCAATATCTTG	227	XM_583951.4
PGRMC1	Progesterone receptor membrane component 1	5′-TGTGTGTCACAAATCCAGAAAG 5′-AATCATGCAGTTAGGTCAATCG	265	AF254804
PGRMC2	Progesterone receptor membrane component 2	5′-AGGAGAGGTGAGGGTGTCCATC 5′-TTTCAAATGATCTTGCTGTTGGG	206	NM_001099060

increments and were used as known copy-number template standards in qPCR. Fluorescence was acquired at each cycle in order to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence rises above background for each sample. The Rotor-Gene quantification software generates a best-fit line and determines unknown concentrations by interpolating the noise-band intercept of an unknown sample against the standard curve of known concentrations. Reverse-transcriptase negative controls (samples containing RNA to which reverse transcriptase was not added) were included to exclude PCR amplification of contaminating genomic DNA.

Statistical analysis

Data were analyzed using the SigmaStat (Jandel Scientific, San Rafael, CA, USA) software package. One-way repeated-measures ANOVA (followed by multiple pair-wise comparisons using Student–Newman–Kleus method) was used for the analysis of differences in embryo development (cleavage rate, blastocyst yield), ratio of ICM to TE cells, recovery rate of embryos on Day 14 and also for the analysis of differences in mRNA expression. Blastocyst cell numbers, serum P_4, and embryo dimensions on Day 14 were compared with Students *t*-test.

Experiment 1: PGR mRNA in bovine embryos

The aim of this experiment was to determine the presence of mRNA for three PGRs (*PGR*, *PGRMC1*, and *PGRMC2*) during early bovine embryo development. Three groups of ten immature oocytes and *in vitro* produced bovine embryos were removed from culture at the 2- to 4-cell, 8-cell, 16-cell, morula and blastocyst stage, snap-frozen in groups of 10 in liquid nitrogen and stored at $-80\ °C$ until analysis. In addition, three groups of ten blastocysts from different experimental replicates were used to determine the copy number of *PGR*, *PGRMC1*, and *PGRMC2* in Day 7 blastocysts.

Experiment 2: P_4 concentration in bovine oviduct flushings

In order to get an estimate of the concentration of P_4 to which the embryo might normally be exposed *in vivo* (as opposed to serum concentrations), bovine oviducts ($n=23$) were collected from animals slaughtered at the mid-luteal phase of the estrous cycle (assessed by the presence of a large functional corpus luteum) and transferred on ice to the laboratory. All oviducts were trimmed and flushed slowly with 2 ml PBS; to maximize P_4 recovery, the PBS was left in the oviduct for up to 5 min before recovery. P_4 concentration was determined as described above.

Experiment 3: effect of culture system on P_4 concentration in culture medium

It is known that steroids are lipophilic in nature and use of oil during culture can result in their depletion from the medium (Shimada *et al*. 2002). Before progressing to experiments examining the addition of P_4 to culture on embryo

development, the aim of this experiment was to assess the effect of culture system on the rate of disappearance of P_4 from culture medium. Following addition of a known quantity (1×10^{-7} M) of P_4 to the culture medium on Day 1, the concentration remaining on Days 3, 5, and 7 of culture was assessed. Culture conditions tested included: i) 25 μl droplets of SOF under mineral oil, the system of culture routinely used on our laboratory ii) 500 μl SOF in wells (without embryos), iii) 500 μl SOF in wells with embryos, iv) 500 μl SOF in co-culture with BOEC (without embryos) and v) 500 μl SOF in co-culture with BOEC with replacement of half of the media every 2 days (without embryos).

Experiment 4: effect of P_4 supplementation of culture medium on embryo development and quality

The aim of this experiment was to determine the effect of exogenous P_4 added during *in vitro* culture on the proportion of embryos reaching the blastocyst stage and on blastocyst quality measured as the relative mRNA abundance of selected genes related to quality. Bovine zygotes produced by IVM/IVF were cultured in SOF alone ($n=1627$; 9 replicates) or in co-culture with BOEC ($n=1312$; 7 replicates) in the absence or presence of P_4 at one of three concentrations (0.1, 1 or 10×10^{-7} M, equivalent to 3.15, 31.5, and 315 ng/ml respectively). A vehicle control containing an equivalent amount of ethanol used to prepare the P_4 stock solution was included initially to eliminate the effect of ethanol ($n=194$). Day 7 blastocysts from each group were snap-frozen in liquid nitrogen in groups of 10 for mRNA extraction. Also, BOEC from each group were isolated on Day 7 using 100 μl lysis buffer and snap-frozen for mRNA extraction. Quantification of transcripts for *BAX*, *BCL2L1*, *SHC1SHC*, *TP53*, *TXN*, *AKR1B1*, *COX2*, *PLAC8*, and *POU5F1* was carried out by real-time qRT-PCR. These genes have been previously associated with likelihood of pregnancy after embryo transfer in cattle (El-Sayed *et al*. 2006).

Experiment 5: effect of exposure to P_4 in vitro *on survival and elongation of blastocysts* in vivo *following embryo transfer*

As mentioned above, there is clear evidence for an association between circulating P_4 concentration and advanced conceptus elongation (Garrett *et al*. 1988, Carter *et al*. 2008). In order to test the hypothesis that exposure to P_4 during early development *in vitro* (zygote to blastocyst stage) would affect post-hatching elongation, zygotes produced by IVM/IVF were cultured in the absence ($n=1659$) or presence ($n=1876$) of 10×10^{-7} M P_4 (4 replicates). Although higher than normal physiological concentrations, this dose was chosen to maximize the likelihood of observing an effect if one should exist. Based on the results of Experiment 3, medium was replenished every 2 days to maintain P_4 concentrations. Blastocyst development was recorded and Day 7 blastocysts ($n=440$) were transferred to the uteri of synchronized recipients (20 blastocysts per recipient). Twenty-two crossbred beef heifers were used as recipients (predominantly Charolais or Limousin cross; 18–24 months old). 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 a standard beef ration. Estrous cycles were synchronized by administration of two i.m. injections of the prostaglandin $F_{2\alpha}$ analogue, cloprostenol (PG; Estrumate; Chanelle, Loughrea, Galway, Ireland), 11 days apart. Standing estrus was defined as Day 0. A total of 20 *in vitro*-produced blastocysts produced in the presence or absence of P_4 were transferred to each recipient on Day 7 (control, $n=12$; P_4-treated, $n=10$) and all recipients were slaughtered on Day 14 to assess embryo survival. A daily blood sample was taken from all animals by jugular venipuncture from Day 0 to Day 14 to establish the recipient P_4 profile in order to ensure that all animals had similar endogenous P_4 concentrations. Following slaughter, the reproductive tract was removed, sealed in a plastic bag and placed in a sealed polystyrene box for transportation to the laboratory (within 60 min). After removal of the ovaries and the oviducts the uterine horns were trimmed free of excess tissue before being flushed with 40 ml PBS. Embryos were located under a stereomicroscope, measured and then snap frozen individually in liquid nitrogen and stored at $-80\,°C$. From each replicate a representative number of Day 7 blastocysts from both groups was processed as described above for assessment of cell number (control: $n=42$; P_4: $n=32$).

Experiment 6: effect of elevated P_4 in vivo *on survival and elongation of bovine blastocysts following embryo transfer*

In order to test the hypothesis that a modified uterine environment induced by artificially elevated circulating P_4 is capable of advancing the post-hatching elongation of Day 7 blastocysts, groups of 20 blastocysts produced *in vitro* in the absence of P_4 were transferred to synchronized recipient heifers with normal ($n=9$) or elevated ($n=9$) P_4 concentrations. To obtain divergent P_4 concentrations in heifers, a validated model was used where approximately half of the animals received a PRID (containing 1.55 g P_4; CEVA Animal Health Ltd, Chesham, UK) on Day 3 of the estrous cycle as previously described (Carter *et al.* 2008). A daily blood sample was taken from all animals by jugular venipuncture from Day 0 to Day 14 to establish the recipient P_4 profile. All heifers were slaughtered on Day 14 and elongating conceptuses were recovered and measured as described above.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

Ashworth CJ, Sales DI & Wilmut I 1989 Evidence of an association between the survival of embryos and the periovulatory plasma progesterone concentration in the ewe. *Journal of Reproduction and Fertility* **87** 23–32.

Bauersachs S, Ulbrich SE, Gross K, Schmidt SE, Meyer HH, Wenigerkind H, Vermehren M, Sinowatz F, Blum H & Wolf E 2006 Embryo-induced transcriptome changes in bovine endometrium reveal species-specific and common molecular markers of uterine receptivity. *Reproduction* **132** 319–331.

Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P & Gutierrez-Adan A 2008 Epigenetic differences between male and female bovine blastocysts produced *in vitro*. *Physiological Genomics* **32** 264–272.

Carter F, Forde N, Duffy P, Wade M, Fair T, Crowe MA, Evans AC, Kenny DA, Roche JF & Lonergan P 2008 Effect of increasing progesterone concentration from Day 3 of pregnancy on subsequent embryo survival and development in beef heifers. *Reproduction, Fertility, and Development* **20** 368–375.

Chagas e Silva J & Lopes da Costa L 2005 Luteotrophic influence of early bovine embryos and the relationship between plasma progesterone concentrations and embryo survival. *Theriogenology* **64** 49–60.

Conneely OM, Mulac-Jericevic B, DeMayo F, Lydon JP & O'Malley BW 2002 Reproductive functions of progesterone receptors. *Recent Progress in Hormone Research* **57** 339–355.

Diskin MG & Morris DG 2008 Embryonic and early foetal losses in cattle and other ruminants. *Reproduction in Domestic Animals* **43** 260–267.

Dode MA, Dufort I, Massicotte L & Sirard MA 2006 Quantitative expression of candidate genes for developmental competence in bovine two-cell embryos. *Molecular Reproduction and Development* **73** 288–297.

Dunne LD, Diskin MG & Sreenan JM 2000 Embryo and foetal loss in beef heifers between day 14 of gestation and full term. *Animal Reproduction Science* **58** 39–44.

El-Sayed A, Hoelker M, Rings F, Salilew D, Jennen D, Tholen E, Sirard MA, Schellander K & Tesfaye D 2006 Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. *Physiological Genomics* **28** 84–96.

Engmann L, Losel R, Wehling M & Peluso JJ 2006 Progesterone regulation of human granulosa/luteal cell viability by an RU486-independent mechanism. *Journal of Clinical Endocrinology and Metabolism* **91** 4962–4968.

Falkenstein E, Meyer C, Eisen C, Scriba PC & Wehling M 1996 Full-length cDNA sequence of a progesterone membrane-binding protein from porcine vascular smooth muscle cells. *Biochemical and Biophysical Research Communications* **229** 86–89.

Ferguson CE, Davidson TR, Mello MRB, Lima AS, Kesler DJ, Wheeler MB & Godke RA 2005 Evidence for a direct effect of P_4 on IVF-derived bovine 8-cell embryos. *Reproduction, Fertility, and Development* **17** 219.

Forde N, Carter F, Fair T, Crowe MA, Evans AC, Spencer TE, Bazer FW, McBride R, Boland MP, O'Gaora P et al. 2009 Progesterone-regulated changes in endometrial gene expression contribute to advanced conceptus development in cattle. *Biology of Reproduction* [in press]. DOI: 10.1095/biolreprod.108.074336.

Garrett JE, Geisert RD, Zavy MT & Morgan GL 1988 Evidence for maternal regulation of early conceptus growth and development in beef cattle. *Journal of Reproduction and Fertility* **84** 437–446.

Gerdes D, Wehling M, Leube B & Falkenstein E 1998 Cloning and tissue expression of two putative steroid membrane receptors. *Biological Chemistry* **379** 907–911.

Goff AK & Smith LC 1998 Effect of steroid treatment of endometrial cells on blastocyst development during co-culture. *Theriogenology* **49** 1021–1030.

Green MP, Hunter MG & Mann GE 2005 Relationships between maternal hormone secretion and embryo development on day 5 of pregnancy in dairy cows. *Animal Reproduction Science* **88** 179–189.

Hou Q & Gorski J 1993 Estrogen receptor and progesterone receptor genes are expressed differentially in mouse embryos during preimplantation development. *PNAS* **90** 9460–9464.

Inskeep EK 2004 Preovulatory, postovulatory, and postmaternal recognition effects of concentrations of progesterone on embryonic survival in the cow. *Journal of Animal Science* **82** E24–E39.

Lamming GE & Royal MD 1999 Ovarian hormone patterns and subfertility in dairy cows. *Proceedings of an International Symposium Organized by the British Society of Animal Science Entitled 'Fertility in the High-Yielding Dairy Cow', Galway, Ireland, September 1999. BSAS, Occasional Publication 2001.* **26** 105–118.

Lessey BA, Alexander PS & Horwitz KB 1983 The subunit structure of human breast cancer progesterone receptors: characterization by chromatography and photoaffinity labeling. *Endocrinology* **112** 1267–1274.

Lonergan P, Woods A, Fair T, Carter F, Rizos D, Ward F, Quinn K & Evans A 2007 Effect of embryo source and recipient progesterone environment on embryo development in cattle. *Reproduction, Fertility, and Development* **19** 861–868.

Mann GE & Lamming GE 2001 Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. *Reproduction* **121** 175–180.

McNeill RE, Diskin MG, Sreenan JM & Morris DG 2006 Associations between milk progesterone concentration on different days and with embryo survival during the early luteal phase in dairy cows. *Theriogenology* **65** 1435–1441.

Merlo B, Iacono E & Mari G 2007 Effect of progesterone and epidermal growth factor on *in vitro*-produced eight-cell bovine embryos in a serum-free culture medium. *Reproduction, Fertility, and Development* **19** 211.

Meyer C, Schmid R, Scriba PC & Wehling M 1996 Purification and partial sequencing of high-affinity progesterone-binding site(s) from porcine liver membranes. *European Journal of Biochemistry* **239** 726–731.

Milgrom E, Atger M & Baulieu EE 1970 Progesterone in uterus and plasma. IV. Progesterone receptor(s) in guinea pig uterus cytosol. *Steroids* **16** 741–754.

Peluso JJ, Pappalardo A, Losel R & Wehling M 2006 Progesterone membrane receptor component 1 expression in the immature rat ovary and its role in mediating progesterone's antiapoptotic action. *Endocrinology* **147** 3133–3140.

Pereira RM, Marques CC, Baptista MC, Vasques MI & Horta AE 2009 Embryos and culture cells: a model for studying the effect of progesterone. *Animal Reproduction Science* **111** 31–40.

Rambags BP, van Rossem AW, Blok EE, de Graaf-Roelfsema E, Kindahl H, van der Kolk JH & Stout TA 2008 Effects of exogenous insulin on luteolysis and reproductive cyclicity in the mare. *Reproduction in Domestic Animals* **43** 422–428.

Rao BR, Wiest WG & Allen WM 1974 Progesterone "receptor" in human endometrium. *Endocrinology* **95** 1275–1281.

Reggio BC, Lynn JW & Godke RA 1997 The effect of progesterone on the development of IVF-derived bovine embryos cultured in a semi-defined culture medium. *Theriogenology* **47** 284.

Rings F, Carter F, Holker M, Kuzmany A, Besenfelder U, Havlicek V, Tesfaye D, Schellander K & Lonergan P 2008 Effect of elevated circulating progesterone concentration on development of *in vitro* produced bovine zygotes *in vivo*. *Reproduction, Fertility, and Development* **20** 147–148.

Rizos D, Ward F, Duffy P, Boland MP & Lonergan P 2002 Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. *Molecular Reproduction and Development* **61** 234–248.

Rizos D, Pintado B, de la Fuente J, Lonergan P & Gutierrez-Adan A 2007 Development and pattern of mRNA relative abundance of bovine embryos cultured in the isolated mouse oviduct in organ culture. *Molecular Reproduction and Development* **74** 716–723.

Satterfield MC, Bazer FW & Spencer TE 2006 Progesterone regulation of preimplantation conceptus growth and galectin 15 (LGALS15) in the ovine uterus. *Biology of Reproduction* **75** 289–296.

Shimada M, Kawano N & Terada T 2002 Delay of nuclear maturation and reduction in developmental competence of pig oocytes after mineral oil overlay of *in vitro* maturation media. *Reproduction* **124** 557–564.

Stronge AJ, Sreenan JM, Diskin MG, Mee JF, Kenny DA & Morris DG 2005 Post-insemination milk progesterone concentration and embryo survival in dairy cows. *Theriogenology* **64** 1212–1224.

Thouas GA, Korfiatis NA, French AJ, Jones GM & Trounson AO 2001 Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. *Reproductive Biomedicine Online* **3** 25–29.

Ying C, Yang YC, Hong WF, Cheng WT & Hsu WL 2000 Progesterone receptor gene expression in preimplantation pig embryos. *European Journal of Endocrinology* **143** 697–703.

Zheng P, Si W, Bavister BD, Yang J, Ding C & Ji W 2003 17Beta-estradiol and progesterone improve *in-vitro* cytoplasmic maturation of oocytes from unstimulated prepubertal and adult rhesus monkeys. *Human Reproduction* **18** 2137–2144.