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Hormone Secretion by Preimplantation Embryos in a Dynamic In Vitro Culture System

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ABSTRACT

Bovine embryos recovered from superovulated donors on Days 8–18 postestrus were cultured in vitro in a tissue perifusion system to quantify hormone secretion. Embryos were cultured for 24 h at 37°C in Ham's F-10 medium supplemented 5% v/v with heat-treated, charcoal-stripped calf serum; 100 IU/ml penicillin; and 100 μg/ml streptomycin. The medium was saturated with 5% CO_2 in air and perifused at 50 μl/min (3 ml/h). Estrone (E_1), estradiol (E_2), progesterone (P_4), prostaglandin E_2 (PGE_2), and prostacyclin (PGI_2) were quantified by RIA in 6-h pools of perifusate fractions. Estrone was measurable (pg/h/embryo; mean ± SE) on Days 13 (10.80 ± 4.56) and 15 (34.80 ± 9.80); E_2 on Days 11 (36.80), 12 (81.28 ± 29.80), 13 (11.75 ± 4.09), 15 (157.20 ± 112.60), and 16 (30.26 ± 8.76); and P_4 (ng/h/embryo) on Days 13 (0.5–1.0) and 17 (~1.5). PGE_2 was secreted by Day 10 bovine embryos during the last 6 h of culture (19–24 h) and throughout culture for Day 11–18 embryos. The rate of PGE_2 secretion increased ($p < 0.05$) over the previous days(s) at Days 13 and 17. The mean (±SE) secretion rates (pg/h/embryo) for the 24-h culture by embryonic ages were as follows: Day 11 (63.39 ± 14.61), 12 (172.10 ± 30.90), 13 (3094.08 ± 283.35), 14 (1633.89 ± 49.98), 15 (3739.23 ± 1082.79), 16 (4955.37 ± 1381.83), 17 (11893.23 ± 1188.48), and 18 (13827.99 ± 3587.88). PGI_2 secretion, as measured by 6-keto-$PGF_{1\alpha}$, was also evident by Day 10, with increases ($p < 0.05$) occurring on Days 12, 16, and 17. The per-embryo mean (±SE) secretion rates (pg/h) for the culture period were as follows: Day 10 (18.30 ± 0.39), 11 (13.80 ± 0.75), 12 (45.18 ± 1.46), 13 (65.79 ± 1.56), 14 (73.80 ± 1.56), 15 (79.75 ± 2.13), 16 (210.09 ± 5.88), 17 (1160.81 ± 41.70), and 18 (1301.03 ± 60.06). Porcine embryos (Days 10 and 11), used to validate the ability of the assay to detect estrogens in perifusate fractions, secreted all hormones, but rates declined over time. These data indicate that bovine embryos secrete several hormones that may, because of quantity and timing, be involved in maternal recognition, establishment, and maintenance of pregnancy.

INTRODUCTION

Maternal recognition of pregnancy is the phrase used to describe the conceptus-uterus interaction that results in cessation of estrous cycle activity and maintenance of the fetal allograft. In early studies [1, 2], researchers described corpus luteum function and estrous activity in response to embryo removal at specific times postcoitus in sheep. In those studies blastocyst removal after Day 12 postestrus resulted in extended estrous cycles, whereas blastocyst removal before Day 12 had no effect. The secretory products of the conceptus act to establish the maternal-fetal relationship and other events necessary for maintenance of pregnancy.

Embryonic metabolism and secretion of specific substances are essential for conceptus survival and maternal recognition of pregnancy. Shemesh et al. [3] have shown that the bovine blastocyst can synthesize and secrete both steroids and prostaglandins, which appear to be involved in maternal recognition of pregnancy and other developmental processes. The trophoblast of the bovine and ovine blastocyst also synthesize and secrete proteins that have been implicated in the establishment of pregnancy [4–12]. In the pig, a significant event begins at approximately Day 11. The blastocyst changes from a spherical to a filamentous form. During this remodeling, the embryo begins to secrete estrogens, which have been shown to be necessary for pregnancy recognition [13]. The period of maternal recognition of pregnancy has also been described in cattle [14–16], horses [17, 18], and sheep [1, 2].

The bovine embryo enters the uterus between Days 4 and 5 postestrus, hatches from the zona pellucida between Days 8 and 9, and begins to elongate at approximately Day 13 (estrus = Day 0). In cattle, the conceptus must be in the uterus by Days 16–17 for maternal recognition to occur, or another estrous cycle ensues. The objective of this study was to culture preimplantation bovine embryos in a dynamic in vitro culture system to quantify hormone secretion beginning on Day 8 and continuing through the period of maternal recognition of pregnancy.

MATERIALS AND METHODS

Cows were superovulated by means of a decreasing dose (6.5, 5, 4, 3 mg twice daily for 4 days) of FSH (FSH-P, Schering, Kenilworth, NJ) [19]. Embryos were recovered nonsurgically on Days 8–15 and at slaughter on Days 16–18 postestrus. After recovery, embryos (n = 152) were placed into PBS supplemented 1% v/v with antibiotics (penicillin, 100 IU/ml; streptomycin 100 μg/ml) and 0.4% v/v with BSA and evaluated at 70× magnification. Morphologically normal embryos were then cultured in either 0.5-ml or 1-

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ml chambers of a tissue culture perifusion system (Endotronics Multiple Chamber Module, Endotronics, Inc., Coon Rapids, MN) for 24 h at 37°C. One-to-four Day 8–14 embryos were cultured per chamber; Day 15–18 embryos were cultured individually. Embryo age and number of cultures per day of age were as follows; Day 8 (7), 9 (2), 10 (7), 11 (5), 12 (6), 13 (4), 14 (7), 15 (7), 16 (11), 17 (5), and 18 (8). Ham's F-10 medium supplemented 5% v/v with heat-treated, charcoal-stripped calf serum; 100 IU/ml of penicillin; and 100 μg/ml of streptomycin was used as the culture medium. The medium was saturated with a mixture of 5% CO_2 in air and perifused at a rate of 50 μl/min (3 ml/h). Perifusate fractions (4.5 ml) were collected every 90 min and stored at −20°C until assay. One milliliter from each fraction was pooled into four 6-h pools prior to assay. After the 24-h dynamic culture, embryos were placed into fresh medium in a static-culture, 37°C incubator with a 5% CO_2 in air atmosphere to be assessed for continued development.

Perifusate samples were thawed in an ice-water bath prior to RIA for estradiol (E_2), estrone (E_1), progesterone (P_4), prostaglandin E_2 (PGE_2), and 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$). E_2 and E_1 concentrations were determined by RIA in the laboratory of Dr. D.W. Forrest, as described by Forrest et al. [20]. The antiserum for E_2 was GDN #244 and the antiserum for E_1 was GDN #5325. Sensitivities of the E_2 and E_1 assays were 2.5 and 5.8 pg/ml, respectively. All samples for E_2 and E_1 were run in single assays with intraassay coefficients of variation of 10.7% and 2.7%, respectively. P_4 concentrations were determined with a solid-phase RIA kit (Diagnostic Products Corp., Los Angeles, CA). All samples were analyzed in a single assay. Sensitivity of the assay was 0.1 ng/ml with an intraassay coefficient of variation of 1.5%. Concentrations of PGE_2 in perifusate samples were determined in a solid-phase RIA kit (New England Nuclear, Billerica, MA). A single assay was utilized to determine PGE_2 concentration. Sensitivity of the assay was 0.5 pg/ml with an intraassay coefficient of variation of 5.2%. Stated cross-reactivity with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 6-keto-$PGF_{1\alpha}$ was less than 0.01 percent. Concentrations of 6-keto-$PGF_{1\alpha}$ were determined in a single assay in the laboratory of Dr. R.R. Magness according to the method of Magness et al. [21]. Sensitivity of the assay was 25.5 pg/ml with an intraassay coefficient of variation of 7.7%. Cross-reactivities of the 6-keto-$PGF_{1\alpha}$ antibody with $PGF_{2\alpha}$ and PGE_2 were 0.62% and 0.59%, respectively.

Data presented in figures and tables are per embryo; when multiple embryos were cultured, the mean secretion rate per embryo is presented.

Data were converted to logarithmic values and analyzed by one-way ANOVA to determine if differences in hormone secretion existed between embryonic ages. When differences were detected, Duncan's Multiple Range test was used to determine differences between means of hormone secretion [22].

TABLE 1. Secretion (pg/h) of E_2 and E_1 by Day 10 and Day 11 porcine embryos.[a]

Time in perifusion (h)[b]	Day 10		Day 11	
	E_2	E_1	E_2	E_1
6	233.6 ± 56.0	85.5 ± 18.4	375.4 ± 42.5	105.6 ± 9.3
12	16.6 ± 3.6	16.0 ± 8.4	19.2 ± 4.5	15.8 ± 7.4
18	6.0 ± 0.7	1.5 ± 2.2	9.5 ± 6.1	6.3 ± 4.7
24	2.2 ± 0.2	ND[c]	4.9 ± 1.8	15.1 ± 8.6

[a]Values represent mean (±SE) secretion rate per embryo (n = 4 cultures each).
[b]Times represent pools of perifusates collected and pooled every 6 h during the 24-h collection period.
[c]Nondetectable.

RESULTS

Approximately 60% of zona pellucida-intact embryos completed the hatching process during static culture. Hatched spherical embryos continued to expand, and elongated embryos (Days 13–15) tended to become more spherical with the formation of some vesicles. The more advanced (Days 16–18) embryos became segmented by the formation of multiple vesicles.

Small quantities of E_1 and E_2 were detectable in the perifusate from bovine embryos. However, the pattern of secretion was erratic: some embryos of the same gestational age secreted estrogens while other embryos did not. Still others secreted E_1 and/or E_2 at different time periods. Estrone was measurable in perifusate fractions from Day 13 and 15 embryos for most time periods. The secretion rates of E_1 during the first 6 h of culture were 10.80 ± 4.56 (mean ± SE) and 34.80 ± 9.80 (mean ± SE) pg/h/embryo for Day 13 and 15 embryos, respectively. E_2 was detected in media from Day 11 (36.80—single culture), 12 (81.28 ± 29.80), 13 (11.75 ± 4.09), 15 (157.20 ± 112.60), and 16 (30.26 ± 8.76) (mean ± SE; pg/h/embryo) embryos during the first 6 h of culture. Secretion either decreased or was not measurable during the remaining 18 h of culture with the exception of Day 15 embryos. These embryos secreted E_2 throughout the culture period, but at a declining rate.

Porcine embryos were cultured and samples were assayed using the same conditions as were used for bovine embryos to validate the results of either the presence or absence of estrogen secretion by bovine embryos under the conditions of the perifusion system. E_1 and E_2 were both detectable; however, the rate of secretion decreased throughout the culture period (Table 1).

Progesterone secretion by bovine embryos was also erratic and/or not measurable from all embryos during all time periods. However, coincidental with increases in PGE_2 on Days 13 and 17, P_4 was measurable in the perifusate. Day 13 embryos secreted between 0.5–1 ng/h/embryo, and Day 17 embryos secreted ~1.5 ng/h/embryo. P_4 was not measurable from other embryos. This erratic pattern of secretion for P_4 was also observed for porcine embryos (data not shown).

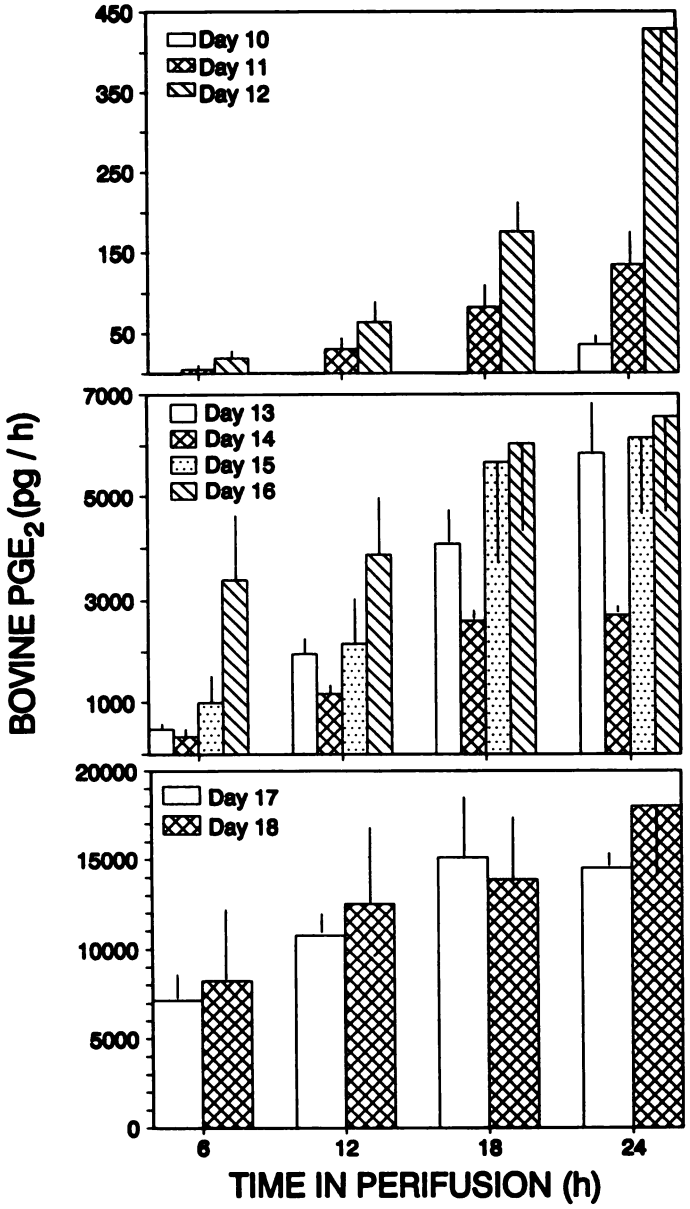

FIG. 1. Secretion of bovine PGE_2 (mean ± SE) by Day 10–12 (upper panel), 13–16 (middle panel), and 17–18 (lower panel) bovine embryos. Secretion rates were lower ($p < 0.05$) for Day 10–12 than for Day 13–16 embryos, while secretion was greater ($p < 0.05$) for Day 17–18 than Day 13–16 embros. Samples represent 6-h pools of perifusates collected over a 24-h period.

PGE_2 was detectable in the perifusate in consistently measurable quantities during the last 6 h of culture (19–24 h) from Day 10 bovine embryos. PGE_2 was measurable in the perifusate during the entire culture period for all embryos, Days 11–18. The mean (±SE) secretion (pg/h/embryo) rates for the 24-h culture by embryonic age were as follows: Day 11 (63.39 ± 14.61), 12 (172.10 ± 30.90), 13 (3094.08 ± 283.35), 14 (1633.89 ± 49.98), 15 (3739.23 ± 1082.79), 16 (4955.37 ± 1381.83), 17 (11893.23 ± 1188.48), and 18 (13827.99 ± 3587.88). Secretion rate between embryonic ages was analyzed separately for each time period (0–6, 7–12, 13–18, and 19–24 h). During the initial 6 h of culture when the embryos were adjusting to the culture condition, no distinct secretion patterns were detectable. However, beginning with the second time period (7–12 h) and continuing throughout the remainder of the culture period, differences in secretion rate by embryonic age were observed. The secretion rate for Day 11–12 embryos (Fig. 1, upper panel) was less ($p < 0.05$) than for other embryos. Day 13–16 embryos (Fig. 1, middle panel) secreted more ($p < 0.05$) PGE_2 than Day 11–12 embryos but less ($p < 0.05$) than Day 17–18 embryos. However, there was no difference ($p > 0.05$) in secretion rates between embryos recovered on Days 13–16. Secretion rates of Day 17–18 embryos (Fig. 1, lower panel) were not different ($p > .05$) from each other but were greater ($p < 0.05$) than those of the younger embryos. Porcine embryos also secreted PGE_2, but secretion decreased over time in a pattern similar to that observed for E_1 and E_2 (Table 2).

Prostacyclin, as measured by the stable metabolite 6-keto-$PGF_{1\alpha}$, was measured in perifusate samples during the same time intervals as PGE_2. The mean (±SE) secretion rates (pg/h/embryo) for the 24-h culture were as follows: Day 10 (18.30 ± 0.39), 11 (13.80 ± 0.75), 12 (45.18 ± 1.46), 13 (65.79 ± 1.56), 14 (73.80 ± 1.56), 15 (79.75 ± 2.13), 16 (210.09 ± 5.88), 17 (1160.81 ± 41.70), and 18 (1301.03 ± 60.06). Day 12 bovine embryos secreted more ($p < 0.05$) 6-keto $PGF_{1\alpha}$ than Day 10 or 11 embryos (Fig. 2, upper panel). However, secretion rates for Day 13–15 embryos were not different ($p > 0.05$) from that of Day 12 embryos (Fig. 2, middle panel). Secretion of 6-keto $PGF_{1\alpha}$ increased ($p < 0.05$) on Day 16, followed by an increase ($p < 0.05$) on Day 17. The Day 17 secretion rate was not different ($p > 0.05$) from that of Day 18 (Fig. 2, lower panel). Porcine embryos also secreted 6-keto $PGF_{1\alpha}$ in a manner similar to secretion of PGE_2 (Table 2).

DISCUSSION

The data reported here indicate that bovine and porcine embryos (gestational age Day 10 or greater) have the ability

TABLE 2. Secretion (pg/h) of PGE_2 and prostacyclin (6-keto $PGF_{1\alpha}$) by Day 10 and Day 11 porcine embryos.[a]

Time in perifusion (h)[c]	Day 10[b]		Day 11	
	PGE_2	$PGF_{1\alpha}$	PGE_2	$PGF_{1\alpha}$
6	3196.5	5.9	2760.75 ± 188.25	3.9 ± 0.06
12	69.9	3.2	423.25 ± 237.85	5.2 ± 0.15
18	ND[d]	3.5	ND[d]	5.2 ± 1.43
24	ND[d]	3.1	82.45 ± 35.15	10.7 ± 1.57

[a]Values represent mean secretion rate per embryo.
[b]Values represent mean secretion per embryo from single culture of 4 embryos.
[c]Times represent pools of perifusates collected and pooled every 6 h during the 24-h collection period.
[d]Nondetectable.

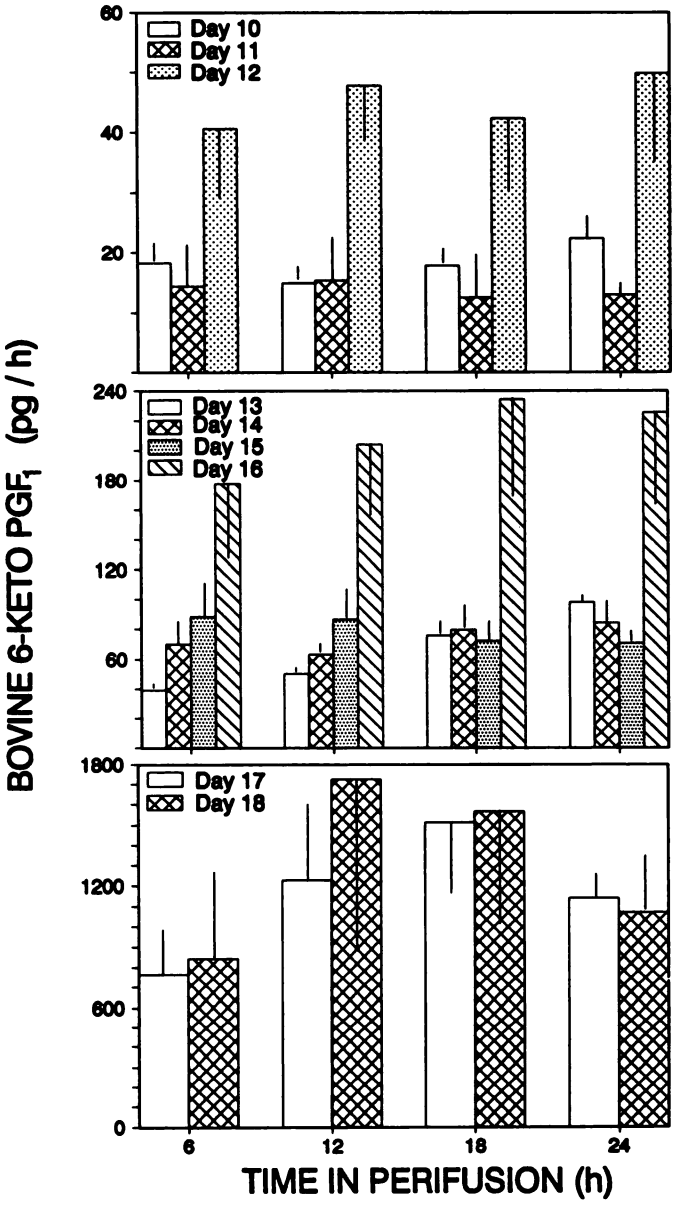

FIG. 2. Secretion of bovine prostacyclin (mean ± SE) as measured by the stable metabolite 6-keto-$PGF_{1\alpha}$, by Day 10–12 (upper panel), 13–16 (middle panel), and 17–18 (lower panel) bovine embryos. Day 12 embryos secreted more prostacyclin ($p < 0.05$) than did Day 10–11 embryos (upper panel). Secretion rate for Days 13–15 was not different ($p > 0.05$) from that for Day 12 (middle panel), but increased on Day 16 ($p < 0.05$). Day 17 and Day 18 embryos (lower panel) had greater ($p < 0.05$) secretion rates than did Day 10–12 (upper panel) or Day 13–16 (middle panel) embryos. Samples represent 6-h pools of perifusates collected over a 24-h period.

to synthesize and secrete both steroids and prostaglandins in vitro. Estrogen secretion (E_1 and E_2) by bovine embryos was low and variable between embryos of the same gestational age. These data are similar to those reported by Shemesh et al. [3], in which estradiol-17β was undetectable in uncultured blastocysts and detectable in only some cultured blastocysts. Aromatase activity has been shown in Day 16 bovine blastocysts [23] and Day 6 rabbit blastocysts [24]. To validate the release of E_1 and E_2 into the perifusate by the bovine embryo, porcine embryos were cultured identically to the bovine embryos. Porcine embryos have been shown to secrete estrogens in measurable quantities by Day 10 [13]. In this study, porcine embryos secreted E_1 and E_2 into the medium throughout culture, although the quantity secreted decreased over time. This suggests a lack of substrate, since embryos appeared morphologically normal and continued to develop in static culture. These data are consistent with previous work as reported in a review by Bazer et al. [25]. P_4 secretion by bovine embryos was undetectable for most embryos. However, some embryos of similar gestational ages secreted measurable quantities of P_4. Moreover, all Day 13 and Day 17 embryos secreted P_4. Shemesh et al. [3] have also demonstrated the presence of progesterone in bovine blastocysts, the content of which increased in culture. The significance of the embryonic secretion of P_4 is not clear. However, some researchers [26] have indicated that the uterine oxytocin receptor appears to be a limiting factor in the oxytocin-induced release of $PGF_{2\alpha}$. These researchers have also shown that concentrations of oxytocin receptors in the sheep uterus coincided with increases and decreases in circulating concentrations of estrogens and progesterone. Estrogen appears to increase and progesterone to decrease uterine oxytocin receptors.

The times of the measurable secretions of P_4 observed in this study (Days 13 and 17) correspond to the times of elongation and maternal recognition of pregnancy, respectively, in the cow. Betteridge et al. [14] and Northey and French [15] have shown that the bovine embryo must be present in the uterus prior to Day 17 for pregnancy to be maintained. The secretion of P_4 on Day 13 may be in response to a cellular remodeling similar to the cellular remodeling of the pig embryo at the same gestational age, as reported by Geisert et al. [13].

The PGE_2 secretion by the bovine blastocyst was consistently measurable from Day 10 embryos during the last 6 h of perifusion culture and from Day 11–18 embryos throughout culture. Secretion increased at Day 13 and Day 17, which correspond to elongation and maternal recognition of pregnancy, respectively. Several researchers have measured synthesis and/or secretion of PGE_2 from ovine [27–30], bovine [3, 31–34], and porcine [35, 36] embryos. Niimura and Ishida [37] have demonstrated the presence of PGE_2 synthesis in preimplantation mouse embryos. The synthesis and secretion of PGE_2 by bovine embryos may have endocrine, paracrine, and autocrine effects. Khurana and Wales [38] have shown that PGE_2 and $PGF_{2\alpha}$ stimulated glucose incorporation into nonglycogen macromolecules in preimplantation mouse embryos in vitro. Lewis [39] suggested that prostaglandins may be involved in Na^+ transport and accumulation of fluid in ovine blastocysts. PGE_2 increases vasodilation and vascular permeability and is involved in immunoregulation [40, 41]. Kennedy et al. [42] have suggested that in the pseudopregnant rat, the onset of en-

dometrial responsiveness to PGE_2 is temporally correlated with the appearance of PGE_2 binding sites between Days 4 and 5. Increases in vascular permeability prior to decidualization in rats and other species may be in response to PGE_2. Silvia et al. [43] have shown that PGE_2 will inhibit the effect of $PGF_{2\alpha}$ on luteal cells (ovine) in vitro. These researchers also demonstrated a dose-dependent increase in P_4 secretion by luteal cells in vitro up to 10 ng/ml of PGE_2, after which increases in PGE_2 decreased secretions of P_4. Schwall et al. [44] have suggested that the stimulation of P_4 secretion by PGE_2 is probably mediated via the large luteal cells. The antiluteolytic properties of PGE_2 in vivo have been demonstrated in the ewe [45–50] and the cow [51, 52]. Data from this study indicate that the bovine embryo secretes PGE_2 prior to and throughout the period of maternal recognition of pregnancy.

Prostacyclin secretion has been shown for bovine [33, 53] and ovine [30] embryos. Prostacyclin has been implicated in the reduced systemic pressor response to angiotensin II and an increased refractoriness of the uterine vascular bed during pregnancy [21]. In this study, 6-keto-$PGF_{1\alpha}$ was measurable by Day 10 with significant increases in secretion rates occurring at Days 12, 16, and 17. The secretion pattern for 6-keto-$PGF_{1\alpha}$ was parallel to that of PGE_2, with secretions increasing with embryonic age. Whether this was an effect of an increase in embryonic size or an increase in synthesis and secretion was not determined. However, data from this study indicate that prostanoid synthesis at these embryonic ages is preferentially directed toward PGE_2.

Embryos cultured independently of maternal tissue may not represent in vivo secretion rate or relative hormone balance. However, previous studies [31–35] have not demonstrated an inhibitory effect of endometrial tissue on embryos in coculture. Lewis and Waterman [32] and Lewis [34] have shown that coincubation of embryos and endometrium may alter prostaglandin secretion away from $PGF_{2\alpha}$ toward PGE_2. Although $PGF_{2\alpha}$ was not measured in this study, these data demonstrate that secretion of PGE_2 was 10-fold greater than for 6-keto-$PGF_{1\alpha}$. Other studies [32, 53] have indicated that endometrial tissue may modulate the synthesis and secretion of prostaglandins. However, the effect of maternal tissue on the secretion of steroids and prostanoids was not determined in this study.

These data define a temporal increase in prostanoid secretion from bovine embryos during early embryonic development (Days 8–18). This suggests a role for prostanoids in uterine preparation for pregnancy and luteostasis in cattle.

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