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The Effects of Plasminogen on In Vitro Ovine Embryo Development[1]

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ABSTRACT

Plasminogen activator production by ovine embryos and the effects of plasminogen on ovine embryo development and zona pellucida integrity were evaluated. Eight-cell to sixteen-cell embryos were cultured in Whitten's medium containing 0, 60, or 120 μg/ml plasminogen. Plasmin and plasminogen activator concentrations in the medium were determined by a caseinolytic assay. More blastocysts hatched in medium containing 60 and 120 μg/ml plasminogen (33 and 21%, respectively) than 0 μg/ml plasminogen (0%; $p<0.05$). Zona pellucida dissolution time in acidified phosphate-buffered saline was less after incubation in medium with 60 and 120 μg/ml plasminogen (7.2 and 5.9 min, respectively) than 0 μg/ml plasminogen (9.4 min; $p<0.05$). Plasminogen activator production was low until the morula stage, increased during morula-blastocyst transition, and remained elevated through blastocoelic expansion and hatching. Zona pellucida solubility, plasminogen activator production, and plasminogen conversion to plasmin increased as embryonic stage advanced; however, plasminogen activator production and plasmin conversion to plasmin were poorly correlated with zona pellucida solubility. The results indicate that ovine embryos produce plasminogen activator, and plasmin can increase zona pellucida solubility; however, other factors may also be involved in altering zona pellucida integrity prior to hatching.

INTRODUCTION

Plasminogen activators (PAs) are serine proteases that convert the plasma zymogen plasminogen to the active serine enzyme plasmin (Christman et al., 1977). Embryonic production of PA has been identified in mice (Sherman et al., 1976; Strickland et al., 1976), rats (Leidholm and Astedt, 1975), swine (Mullins et al., 1980; Fazleabas et al., 1983), and cattle (Menino and Williams, 1987). Mouse and swine embryos exhibit temporally biphasic patterns of PA secretion (Sherman et al., 1976; Strickland et al., 1976; Fazleabas et al., 1983). Trophoblastic production of PA constitutes the first phase in mouse embryos and is believed to be involved in implantation, whereas the secondary phase is attributed to the parietal endoderm and is possibly involved in cellular migration (Sherman et al., 1976; Strickland et al., 1976). In swine embryos, the first phase corresponds to a period of tissue remodeling during the transition from a spherical to an elongated shape, and the secondary phase is associated with tissue proliferation occurring during the filamentous stages of blastocyst development (Fazleabas et al., 1983).

When mouse embryos are cultured in medium containing either plasminogen or plasmin, more embryos hatch or escape from the zona pellucida, attach to the plastic culture dish substratum, and generate trophoblastic outgrowth than embryos developing in medium lacking either protein (Menino and O'Claray, 1986). Cattle embryos liberate PA during blastocoelic expansion and hatching and, when cultured in medium with plasminogen, have a shorter interval to hatching than embryos developing in medium without plasminogen (Menino and Williams, 1987). Plasminogen is a luminal constituent of swine and mouse uteri and presumably enters the uterus as a serum transudate (Fazleabas et al., 1983; Finlay et al., 1983). Embryonic conversion of uterine plasminogen to plasmin by the secretion of PA may be a mechanism utilized by the blastocyst to cause a sublysis of the zona pellucida and facilitate hatching. To evaluate this model in sheep, the objectives of this study were to quantify embryonic plasminogen activator production and evaluate the effects of plasminogen on in vitro development and zona pellucida integrity.

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MATERIALS AND METHODS

Embryo Collection and Culture

Forty-three multiparous Polypay and crossbred ewes were estrus-synchronized with cloprostenol sodium (Estrumate®, Haver, Shawnee, KS) and superovulated with either pregnant mare's serum gonadotropin (PMSG; Calbiochem – Behring Corp., San Diego, CA) or porcine follicle-stimulating hormone (pFSH; Sigma Chemical Co., St. Louis, MO). Ewes received two 100-μg i.m. injections of cloprostenol sodium 10 days apart (Day 0 = first cloprostenol sodium injection) and either 1000 I.U. of PMSG i.m. on Day 8 or twice daily injections of pFSh i.m. at dosages of 5 mg, 4 mg, and 3 mg on Days 9, 10, and 11, respectively, for a total of 24 mg per ewe.

Estrus detection was initiated 24 h after the second injection of cloprostenol sodium and ewes were hand-mated by one of five rams starting at the onset of estrus and thereafter at 12-h intervals for as long as the ewe would accept a ram. Mated ewes were deprived of food and water for 24 h prior to surgical collection of ova. Three to four days after onset of estrus, anesthesia was induced in ewes by injection of 15 – 20 ml of 2.5% thiamylal sodium (Bio-tal®; Boehringer Ingelheim Animal Health, Inc., St. Joseph, MO) into the jugular vein and was maintained during surgery via inhalation with halothane (Fluothane®, Fort Dodge Laboratories, Inc., Fort Dodge, IA) and oxygen. The reproductive tract was exteriorized via ventral midline laparotomy and the oviducts and uteri were flushed in retrograde fashion with Whitten's medium (Whitten and Biggers, 1968) buffered with N-[2-hydroxyethyl] piperazine-N-′-[2-ethanesulfonic acid] (WM + H; Sigma) and lacking bovine serum albumin (BSA).

Flushings were examined with a dissecting microscope at 10 – 20× magnification and ova were recovered from flushings by aspiration. Ova were washed in 5 – 10 ml of WM + H with 15 mg/ml BSA (WM + H + BSA; Sigma) and transferred to screw-cap tissue culture tubes containing 5 ml of WM + H + BSA maintained at 37°C. Culture tubes were transported to the laboratory and ova were recovered from the tubes and washed in 50 μl microdrops of WM with 15 mg/ml BSA (WM + BSA) under paraffin oil (Fisher Scientific Co., Tustin, CA). Ova were evaluated for fertilization, cell stage, and overall morphology with an inverted stage phase-contrast microscope at 100 – 200× magnification. Morphologically normal eight-cell to sixteen-cell stage embryos were cultured in WM + BSA containing 0, 60, or 120 μg/ml porcine plasminogen (Sigma).

Embryos were cultured in 50-μl microdrops under paraffin oil in a humidified atmosphere of 5% CO_2 in air at 37°C and were observed daily for stage of development (Wright et al., 1976). At 24-h intervals, starting at 24 h of culture and continuing through 144 h of culture, embryos were transferred to fresh microdrops and the medium was recovered. Medium was frozen at – 20°C until assayed for PA and plasmin. To evaluate plasmin contamination in plasminogen-containing medium and to detect any nonspecific proteases in the culture medium, media without embryos were incubated and stored under identical conditions.

PA and Plasmin Assays

PA and plasmin levels in the culture media were determined by a caseinolytic assay as described by Menino and Williams (1987), with some modification. The modification consisted of preparing the casein-agar solution after the method described by Bjerrum et al. (1975), where warmed 2% nonfat dry milk (Carnation Co., Los Angeles, CA), dissolved in 0.038 M tris(hydroxymethyl)aminomethane – 0.10 M glycine buffer containing 0.195 g/l $CaCl_2 \cdot 2H_2O$ and 0.325 g/l sodium azide, was combined with an equal volume of 2% agarose (Sigma). Ten or fifteen milliliters of the warmed mixture was pipetted into 85 × 65 × 2 mm plastic diffusion plates for use in the plasmin and PA assays, respectively. For the PA assay, only medium from cultures with 0 μg/ml plasminogen were used. Urokinase (E. C. 3.4.21.31; Sigma) was used as the standard in the PA assay and standard concentrations were 0, 0.1, 0.5, 1.0, 5.0, and 10.0 mU/ml. For the plasmin assay, standard concentrations of plasmin (E. C. 3.4.21.7; Sigma) were 0, 5, 10, 50, 100, and 500 μg/ml. Plasmin activity was determined in all culture media used. Evaluation of plasmin activity in WM + BSA with 0 μg/ml plasminogen exposed to embryos was used to detect nonspecific embryonic proteases. Evaluation of plasmin levels in WM + BSA not exposed to embryos with 60 or 120 μg/ml plasminogen was used to determine the extent of plasmin contamination. Further details of the PA and plasmin assays and the methods used to calculate PA production/h/embryo and the quantity of plasmin produced by embryonic activation of plasminogen/h/embryo have been described (Menino and Williams, 1987).

Assay for Changes in Zona Pellucida Solubility

After 144 h of culture, embryos were recovered from the microdrops and washed in Dulbecco's phosphate-

buffered saline (DPBS) containing 0.1% BSA (DPBS + BSA). Five microliters of DPBS + BSA containing 3 – 6 embryos were transferred to 50-μl microdrops of DPBS acidified to pH 2.5 with 1 N HCl. Zona pellucidae were continually observed for dissolution at room temperature with an inverted stage phase-contrast microscope. The time between placement of the embryos in acidified DPBS and when the zona pellucida of an embryo was no longer visible at 200× magnification was designated as the zona pellucida dissolution time (ZPDT) for that embryo.

Statistical Analysis

For the analysis of embryo development, each cell stage was assigned a developmental code: eight-cell (0), sixteen-cell (1), morula (2), early blastocyst (3), blastocyst (4), expanded blastocyst (5), hatching blastocyst (6), and hatched blastocyst (7). Differences in coded embryo development (CED) and ZPDT due to treatment, and PA production and plasminogen conversion to plasmin by time were determined by analysis of variance and Fisher's least-significant-difference procedures. Differences in the percentages of embryos developing to a particular cell stage were determined by Chi-square analysis. PA production and plasminogen conversion to plasmin were plotted by time for each microdrop, and the graphs were cut out and weighed with an analytical balance. Graph weights with the corresponding CED means and mean ZPDT for each microdrop within a level of plasminogen were evaluated for significance of relationship using correlation-regression analysis (Steel and Torrie, 1980).

RESULTS

One, 86, and 13 percent of the embryos collected 3 – 4 days after onset of estrus were at the four-cell, eight-cell (Fig. 1), and sixteen-cell stages, respectively. Ninety-four, 96, and 94 eight-cell and 12, 18, and 12 sixteen-cell embryos were cultured in WM + BSA containing 0, 60, or 120 μg/ml plasminogen, respectively. No significant differences in mean CED were observed among embryos cultured in WM + BSA with 0, 60, or 120 μg/ml plasminogen (2.2, 2.0 and 2.2, respectively; error mean square = 1.4). However, as shown in Table 1, more blastocysts hatched (Figs. 2 and 3) in medium containing 60 and 120 μg/ml plasminogen than 0 μg/ml plasminogen ($p<0.05$). In fact, WM + BSA with 0 μg/ml plasminogen failed to support in vitro hatching within 144 h of culture. Table 2 reports the time required for embryos cultured in WM + BSA containing 0, 60, or 120 μg/ml plasminogen to develop to the morula, blastocyst, expanded blastocyst, and hatched blastocyst stages. Although a trend was observed where embryos cultured in medium with plasminogen attained a particular developmental stage earlier than embryos developing in medium without plasminogen, no significant differences were observed.

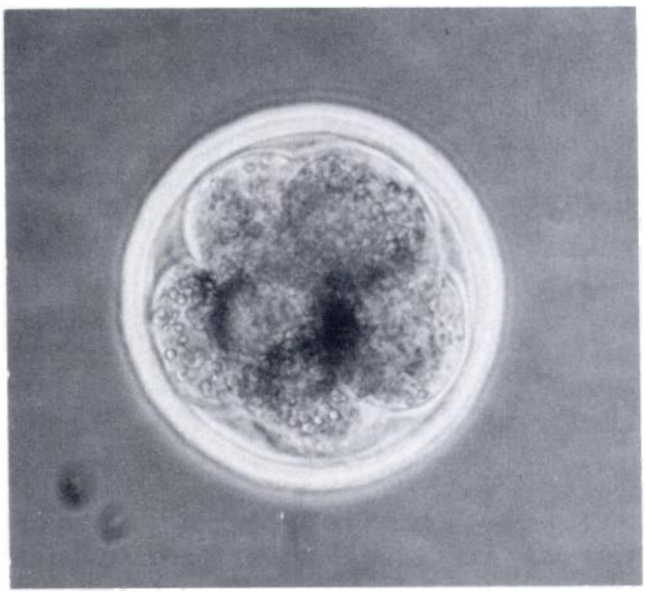

FIG 1. Eight-cell ovine embryo collected 3 days after onset of estrus (×300).

PA production and plasminogen conversion to plasmin by cultured ovine embryos are reported in Figure 4. Caseinolytic activity was not observed in WM + BSA with 0 μg/ml plasminogen, indicating the absence of contaminating proteases associated with BSA. No caseinolytic activity was detected in WM + BSA containing embryos with 0 μg/ml plasminogen unless plasminogen was added, suggesting the absence of nonspecific

TABLE 1. Incidence of hatching among ovine blastocysts developing in Whitten's medium containing 15 mg/ml BSA and 0, 60, or 120 μg/ml plasminogen.

Plasminogen (μg/ml)	Number of blastocysts	Number (%) of blastocysts hatching
0	19	0 (0)[a]
60	15	5 (33)[b]
120	14	3 (21)[b]

[ab]Values in the same column without common superscripts are different ($p<0.05$).

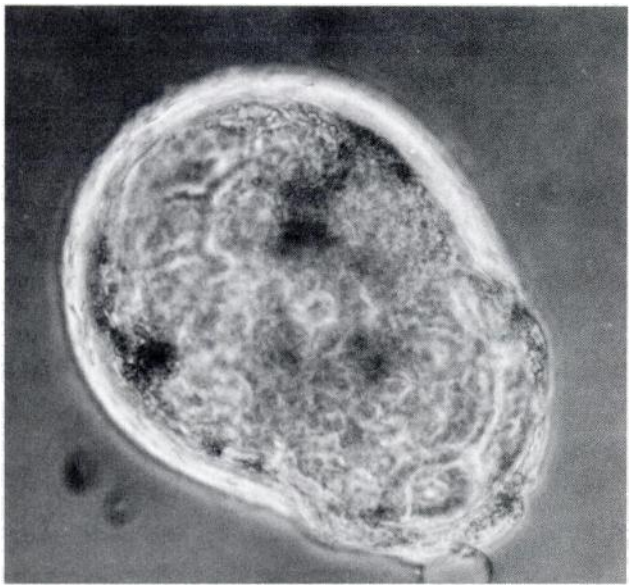

FIG 2. Ovine blastocyst hatching after 96 h in WM + BSA and 60 μg/ml plasminogen (×300).

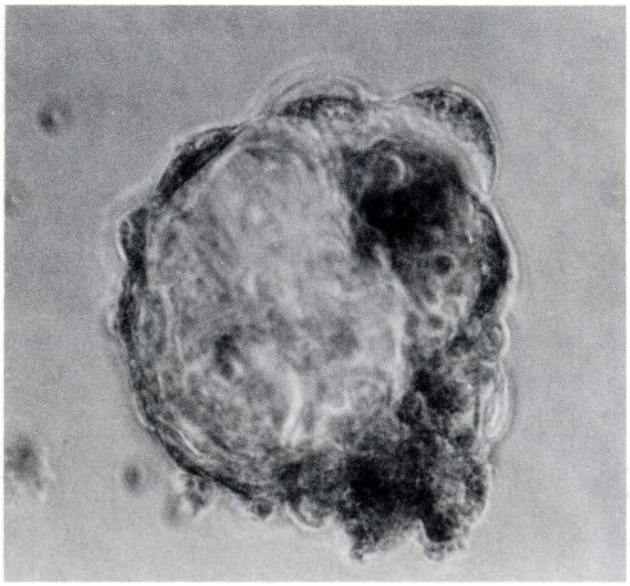

FIG 3. Hatched ovine blastocyst after 96 h in WM + BSA and 120 μg/ml plasminogen (×300).

embryonic caseinolytic proteases. Correlation coefficients for standard curves of ring diameters of the lytic zones by log urokinase or log plasmin concentration used to quantify PA or plasmin, respectively, were 0.96 – 0.99. No differences in PA production (Fig. 4, Panel A) were detected between 24, 48, 72, 120, and 144 h or between 72, 96, 120, and 144 h of culture ($p>0.05$); however, production of PA was greater at 96 h than at 24 and 48 h of culture ($p<0.05$). PA production was low during the first 48 h of culture, or until the morula stage; production increased during morula-blastocyst transition and remained elevated throughout the blastocyst and expanded blastocyst stages. Evaluation of WM + BSA containing 60 or 120 μg/ml plasminogen in which embryos were not included demonstrated that 2 – 10% of the plasminogen was contaminated with plasmin. Plasminogen conversion to plasmin by ovine embryos cultured in WM + BSA with 60 μg/ml plasminogen (Fig. 4, Panel B) was not different between 24, 48, and 144 h or between 48, 72, 96, and 144 h of culture ($p>0.05$). No differences in plasmin production were observed between 72, 96, 120, and 144 h of culture ($p>0.05$); however, ovine embryos activated significantly less plasminogen at 24 h when compared to 72, 96, and 120 h, and at 48 h when compared to 120 h of culture. Plasminogen conversion to plasmin by embryos cultured in WM + BSA with 120 μg/ml plasminogen (Fig. 4, Panel C) was not different between 24, 48, and 72 h or between 72, 96, 120, and 144 h of culture ($p>0.05$); however, embryos produced more plasmin

TABLE 2. Time (h) to morula and blastocyst formation, blastocoele expansion, and hatching for ovine embryos cultured in Whitten's medium containing 15 mg/ml BSA and 0, 60, or 120 μg/ml plasminogen.[a]

Plasminogen (μg/ml)	Development to the following stages: Morula n	Morula mean	Blastocyst n	Blastocyst mean	Expanded blastocyst n	Expanded blastocyst mean	Hatched blastocyst n	Hatched blastocyst mean
0	81	48.6	19	92.2	16	106.5	0	–
60	84	41.4	15	75.2	9	85.3	5	129.6
120	83	44.8	14	77.1	9	82.7	3	112.0

[a]Error mean squares for analyses of variance at the morula, blastocyst, expanded blastocyst, and hatched blastocyst stages are 362.5, 1159.9, 1382.1, and 563.2, respectively.

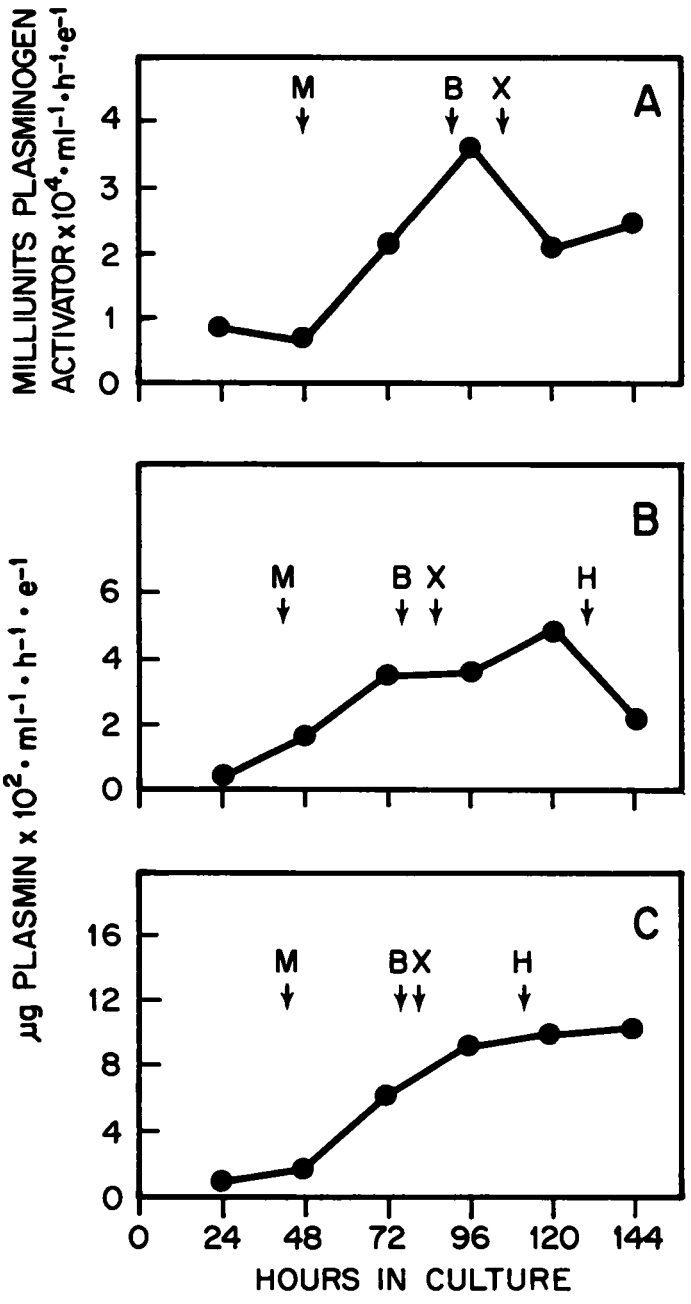

FIG 4. PA production (*Panel A*) and plasminogen conversion to plasmin in WM + BSA containing 60 (*Panel B*) or 120 (*Panel C*) µg/ml plasminogen. Each point in Panel A is the mean of 15 replicates containing 4 – 7 embryos per replicate (error mean square for Panel A is 12.81). Each point in Panels B and C is the mean of 18 replicates containing 4 – 7 embryos per replicate (error mean squares for Panels B and C are 13.22 and 64.64, respectively). The *letters* and *arrows* in each *panel* denote the times required for embryos to develop to the morula (*M*), blastocyst (*B*), expanded blastocyst (*X*), and hatched blastocyst (*H*) stages, as presented in *Table 2*.

from plasminogen at 96, 120, and 144 h than at 24 and 48 h of culture ($p<0.05$). Plasminogen conversion to plasmin followed the profile observed for PA, where embryos activated little plasminogen until the morula stage.

TABLE 3. Zonae pellucidae dissolution times (min) for ovine embryos cultured for 144 h in Whitten's medium containing 15 mg/ml BSA and 0, 60, or 120 µg/ml plasminogen.

Plasminogen (µg/ml)	Number of replicates[a]	Zona pellucida dissolution time (min)[b]
0	15	8.4[c]
60	18	7.2[d]
120	17	5.9[d]

[a]Each replicate contained 4 – 7 embryos.

[b]Values are expressed as means; error mean square = 7.7.

[cd]Means without common superscripts are different ($p<0.01$).

Times required for complete dissolution of the zona pellucida in acidified DPBS for embryos cultured in WM + BSA containing 0, 60, or 120 µg/ml plasminogen are reported in Table 3. Embryos developing in WM + BSA containing 60 or 120 µg/ml plasminogen had lower ZPDT than embryos cultured in WM + BSA without plasminogen ($p<0.05$).

Correlation coefficients for ZPDT, CED, and PA production or plasminogen conversion to plasmin for the culture periods are reported in Table 4. Correlation coefficients for ZPDT with CED were negative for all concentrations of plasminogen and were significant for embryos cultured in WM + BSA with 0 and 120 µg/ml plasminogen, indicating that the zona pellucida became less resistant to dissolution in acidified DPBS as cell stage advanced. Zona pellucida dissolution time was poorly correlated with PA production and plasminogen conversion to plasmin although a negative, but nonsignificant, correlation coefficient was observed for cultures in WM + BSA with 120 µg/ml plasminogen. Correlation coefficients for PA production and plasminogen conversion to plasmin with CED were positive and significant, suggesting that greater quantities of PA in the culture medium were associated with more developmentally advanced embryos.

TABLE 4. Correlation coefficients for ZPDT, CED, and PA production or plasminogen conversion to plasmin (P) for ovine embryos cultured in Whitten's medium containing 15 mg/ml BSA and 0, 60, or 120 µg/ml plasminogen.

		Plasminogen (µg/ml)		
X-value	Y-value	0	60	120
ZPDT	CED	–0.90 (0.01)[a]	–0.30 (0.28)	–0.62 (0.05)
	PA/P	0.05 (0.92)	0.04 (0.48)	–0.34 (0.33)
PA/P	CED	0.47 (0.06)	0.55 (0.01)	0.66 (<0.01)

[a]Values in parentheses indicate level of statistical significance.

DISCUSSION

The pattern of PA production typified by the low level at the morula stage, the increase during blastocoel formation and maintenance of the elevated level during blastocoelic expansion and hatching probably reflects transcriptional and translational events occurring during activation of the embryonic genome in sheep. Crosby et al. (1988) have indicated that the wave of transcriptional activity associated with activation of the ovine embryonic genome occurs during the fourth cell cycle or the period preceding the sixteen-cell stage. PA, therefore, may be one of the many gene products induced during the transition from maternal to embryonic control of early development (Flach et al., 1982). In mice, mRNA for tissue PA (tPA) accumulates during oogenesis and is stored as a dormant, stable form until resumption of meiosis, when the transcripts are polyadenylated, translated, and destabilized (Huarte et al., 1985, 1987; Strickland et al., 1988). Synthesis of tPA stops at fertilization (Huarte et al., 1985), and mouse embryos do not elaborate PA until Day 6 postcoitum (Strickland et al., 1976; Sellens and Sherman, 1980). Work in our laboratory has demonstrated that bovine embryos liberate significant quantities of PA during blastocoelic expansion and hatching (Menino and Williams, 1987).

The physiologic role of PA in early embryo development is not well defined in the domestic species. Embryonic PA may convert uterine plasminogen to plasmin, which can then proteolytically degrade the zona pellucida and facilitate hatching. Domon et al. (1973) demonstrated uterine effects on zona pellucida integrity when they reported a decreased resistance to solubilization in sodium isothiocyanate by mouse zona pellucidae recovered from the uterus versus those recovered from the oviducts. Menino and Wright (1982) also observed a decreased resistance to pronase by porcine zonae pellucidae as embryonic cell stage advanced or as embryos moved from the oviducts to the uterus. In the present study, zonae pellucidae of embryos cultured in medium with plasminogen had reduced resistance to solubilization in acidified DPBS. The increased incidences of hatching in medium with plasminogen may have been due to a sublytic effect of plasmin, which weakened the zona pellucida sufficiently to allow more embryos to hatch.

Bovine embryos cultured in medium with plasminogen expanded and initiated and completed hatching sooner than embryos developing in medium without plasminogen, presumably due to plasmin-mediated effects on the zonae pellucidae (Menino and Williams, 1987). A similar trend was observed in the present study; however, blastocyst formation occurred earlier in medium with plasminogen, suggesting that development was accelerated. The accelerated development may have been due to a growth factor-like effect exerted by some proteases, including plasmin (Cunningham, 1981).

The significant correlations between CED and PA production and plasminogen conversion to plasmin were somewhat expected, since only embryos as advanced as morulae elaborated PA. The negative correlations between CED and ZPDT for embryos cultured in WM + BSA with 0 μg/ml plasminogen suggests that the zona pellucida undergoes cell-stage-associated changes in integrity without any reproductive tract influence. Alternative substrates for PA have been detected in other tissues (Baron-Van Evercooren et al., 1987) and it is possible that the embryonic PA uses the proteins of the zona pellucida as its substrate. The poor correlation between ZPDT and either PA production or plasminogen conversion to plasmin for embryos developing in WM + BSA with 0 or 60 μg/ml, respectively, detracts from the hypothesis that these enzymes are directly involved in sublysis of the zona pellucida. The negative correlation between ZPDT and plasminogen conversion to plasmin for embryos cultured in WM + BSA with 120 μg/ml suggests that, at least at the higher concentration of plasminogen, the zona pellucida becomes more soluble as the embryo activates greater quantities of plasminogen.

On the basis of our observations, we have concluded that the ovine embryo produces PA and converts plasminogen to plasmin. Plasmin is capable of causing a change in solubility of the zona pellucida; however, a change in solubility occurs in the zona pellucida in the absence of plasminogen or plasmin. PA and plasmin may participate in hatching, but other factors must exist that are involved in changing the solubility of the zona pellucida. These other factors may include either physical forces resulting from blastocoelic expansion or even other enzymes, such as strypsin in mice (Perona and Wassarman, 1986), that as yet are unidentified in sheep.

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