Plasminogen activator production and enhanced development in medium containing plasminogen or plasmin by rabbit embryos *in vitro*

M. A. Grobner and A. R. Menino, Jr*

Department of Animal Sciences, Oregon State University, Corvallis, OR 97331-6702, USA

A series of experiments was conducted to evaluate plasminogen activator production and effects of supplementing culture medium with plasminogen or plasmin on development of rabbit embryos *in vitro*. In Expt 1, 495 one- to two-cell embryos were cultured in Ham's F-12 with 15 mg BSA ml⁻¹ containing 0, 30, 60 or 120 µg porcine plasminogen ml⁻¹ or 0, 75, 150 or 300 µg rabbit plasminogen ml⁻¹. Percentages of embryos developing to the expanded blastocyst, hatching blastocyst and hatched blastocyst stages were greater ($P < 0.05$) in medium with 120 µg porcine plasminogen ml⁻¹ than in the absence of plasminogen. More ($P < 0.05$) embryos developed to the blastocyst, expanded blastocyst and hatching blastocyst stages in medium with 300 µg rabbit plasminogen ml⁻¹ than in the absence of plasminogen. In Expt 2, 216 one- to two-cell embryos were cultured in medium with 0, 30, 60 or 120 µg porcine plasminogen ml⁻¹ for 96 h, fixed and stained with haematoxylin and eosin, and the number of cells determined. No differences ($P > 0.05$) were observed in number of cells of morulae but blastocysts developing in medium with 120 µg porcine plasminogen ml⁻¹ had more ($P < 0.05$) cells (109.9 ± 10.4) than did blastocysts in medium with either 0 (69.4 ± 14.6) or 30 µg porcine plasminogen ml⁻¹ (73.3 ± 12.2). In Expt 3, 144 one- to two-cell embryos were cultured in medium with 0, 13 or 45 µg porcine plasmin ml⁻¹ or 120 µg porcine plasminogen ml⁻¹. Development to the hatched blastocyst stage was greater ($P < 0.05$) in medium with 45 µg porcine plasmin ml⁻¹ or 120 µg porcine plasminogen ml⁻¹ than in medium with 0 or 13 µg porcine plasmin ml⁻¹. In Expt 4, 101 embryos at days 3, 4 and 5 after mating were cultured to equivalent gestational age day 7, and medium was recovered at intervals of 24 h and assayed for plasminogen activator. Production of plasminogen activator was greater ($P < 0.05$) on days 6 and 7 than on days 4 and 5 for embryos collected on day 3, and was greater ($P < 0.05$) on day 7 than on day 6 for embryos collected on day 5. Although production of plasminogen activator progressively increased, no differences ($P > 0.05$) were observed by day in culture for embryos collected on day 4. These results suggest that rabbit embryos produce plasminogen activator during the peri-implantation period, and respond to plasmin and plasminogen in the culture medium with improved development.

Introduction

Several investigators have reported on the divergent morphology and biochemical characteristics of rabbit embryos developing *in vitro* compared with embryos developing *in vivo* (Maurer and Beier, 1976; Fischer, 1987; Hegele-Hartung *et al.*, 1988; Jung and Fischer, 1988; Fischer *et al.*, 1992). The retarded development *in vitro* can be ameliorated to a limited extent by including uterine flushings in the culture medium or it can be restored to normal development *in vivo* by transfer to pseudopregnant recipients (Fischer, 1987; Hegele-Hartung *et al.*, 1988; Jung and Fischer, 1988; Fischer *et al.*, 1990, 1992). One series of processes affected by culture *in vitro* are the changes in the extracellular embryonic coverings that occur

before implantation. Rabbit embryos possess extracellular embryonic coverings that undergo a dynamic remodelling process (Boving, 1963; Denker and Gerdes, 1979). Zygotes are initially surrounded by a zona pellucida to which a mucoprotein layer (mucocolemma) is added during oviductal transport (Boving, 1963). After uterine entry and development to the blastocyst stage, the zona pellucida becomes indiscernible, and a new layer, the neozona, is formed. The neozona immediately surrounds the embryo and is composed of material of both trophoblastic and maternal origin (Denker and Gerdes, 1979). By day 6 after coitus, a mucous layer of uterine origin, the gloiolemma, is added to the neozona and mucocolemma (Boving, 1963). Fischer *et al.* (1991) demonstrated that uterine components are required for dissolution of the zona pellucida and that formation of the neozona does not occur during development *in vitro*.

*Reprint requests.

Revised manuscript received 11 January 1994.

Remodelling of these extracellular embryonic coverings is considered to be important in the initiation of implantation (Denker and Hafez, 1975; Denker, 1977) and to be mediated by proteases from the uterus or blastocyst (Kirchner, 1972; Denker, 1974; Denker and Hafez, 1975; Denker, 1977; Denker and Gerdes, 1979). Complete identities of the proteases involved in the restructuring of the extracellular embryonic coverings in the rabbit are not known. Denker (1977) identified a trypsin-like serine protease, referred to as blastolemmase, that may participate in dissolution of the coverings at the blastocyst stage. Denker (1977) observed little proteolytic activity in blastocysts up to day 6 after coitus; however, blastolemmase activity increased dramatically in the trophoblast and extracellular embryonic coverings of blastocysts from day 6.5 to day 7 after coitus. Blastolemmase may be either directly synthesized by the trophoblast or synthesized by the endometrium as a proenzyme and subsequently activated by the trophoblast (Denker, 1977).

Embryos from several species including mice (Strickland *et al.*, 1976), pigs (Mullins *et al.*, 1980), cattle (Menino and Williams, 1987) and sheep (Menino *et al.*, 1989) produce the serine protease plasminogen activator. Plasminogen activator production has been implicated in trophoblastic penetration of the endometrium (Strickland *et al.*, 1976; Sappino *et al.*, 1989), metabolism of Reichert's membrane by parietal endoderm (Strickland *et al.*, 1976; Strickland, 1980), facilitation of hatching (Menino and Williams, 1987; Menino *et al.*, 1989) and tissue remodelling during changes in blastocyst shape (Fazleabas *et al.*, 1983). When plasminogen or plasmin is included in the culture medium, hatching or shedding of the zona pellucida is accelerated or increased in cattle and sheep embryos, respectively (Menino and Williams, 1987; Menino *et al.*, 1989), and hatching, attachment and trophoblastic outgrowth are enhanced in mouse embryos (Menino and O'Claray, 1986). Plasminogen is a uterine constituent (Fazleabas *et al.*, 1983; Finlay *et al.*, 1983) and would be available to the embryo as a source of protease for tissue remodelling or restructuring of extracellular embryonic coverings. Neither production of plasminogen activator nor the effects of the plasminogen-plasmin system on development *in vitro*, with the exception of a report by Kane (1986) with plasmin, have been investigated in early rabbit embryos. The objectives of this research were therefore to evaluate the effects of plasminogen and plasmin on development *in vitro* and to identify production of plasminogen activator by rabbit embryos.

Materials and Methods

Embryo collection and culture

New Zealand White rabbit does were superovulated by one of two methods. The first method was s.c. injections of 0.5 mg pig FSH (Sigma Chemical Company, St Louis, MO) twice daily for 3 days. The second method was a single s.c. injection of 150 iu of pregnant mares' serum gonadotrophin (PMSG; Calbiochem-Behring Corporation, San Diego, CA). Ninety-six hours after either the initial porcine FSH injection or single PMSG injection, does were naturally mated and injected i.p. with 100 iu hCG (Sigma Chemical Company). Embryos were

surgically collected from does 1, 3, 4 and 5 days after mating. For embryo collection, does were anaesthetized by injecting i.m. 60 mg ketamine hydrochloride (Vetalar: Park-Davis, Morris Plains, NJ) and 10 mg xylazine (Rompum: Haver, Shawnee, KS) kg^{-1} body mass. A ventral midline laparotomy was performed and the reproductive tract was exteriorized. Collection of embryos on days 1 and 3 (day 0: day of mating) was accomplished by retrograde flushing of the oviduct through a catheter inserted in the infundibulum. Collection of embryos on days 4 and 5 was accomplished by inserting a catheter proximal to the cervix of each uterine horn and flushing the uterus from the utero-tubal junction. Each individual oviduct or uterine horn was flushed with 5 and 10 ml, respectively, of Ham's F-12 (Sigma Chemical Company) buffered with 25 mmol Hepes l^{-1} (Sigma Chemical Company). Flushings were examined using a dissecting microscope, and embryos exhibiting normal morphology for their respective age were collected from the flushings by aspiration.

Embryos were washed once in Hepes-buffered Ham's F-12 supplemented with 15 mg BSA ml^{-1} (Sigma Chemical Company), placed into screw-cap test tubes maintained at 37°C and transported to the laboratory for culture. Embryos were washed three times and cultured in 50 μl Ham's F-12 with 15 mg BSA ml^{-1} under paraffin oil (Fisher Scientific Company, Tustin, CA) at 37°C in a humidified atmosphere of 5% CO_2 in air. Four to ten embryos, depending on the developmental age at collection, were cultured in each microdrop; culture duration varied from 48 to 192 h. Each microdrop within a treatment was considered as a replicate. The morphology of embryos was evaluated at intervals of 24 h at $\times 100$ –200 using an inverted-stage phase-contrast microscope, and stage of development was recorded. Where appropriate after evaluation embryos were transferred to fresh medium, and medium was recovered and stored at -20°C until assayed for plasminogen activator activity. Medium containing no embryos and incubated under identical conditions was collected to correct for spontaneous activation of plasminogen and to detect plasmin contamination in plasminogen-containing medium.

Experiment 1: development of rabbit embryos in medium containing plasminogen

The objectives of this experiment were to evaluate the effects of plasminogen on the development of one- to two-cell embryos to the morula and blastocyst stages, and to determine whether embryos produced plasminogen activator and activated plasminogen to plasmin. Three hundred one- to two-cell embryos (Fig. 1) were cultured in microdrops of Ham's F-12 with 15 mg BSA ml^{-1} containing 0 ($n = 82$), 30 ($n = 70$), 60 ($n = 69$) or 120 ($n = 79$) μg porcine plasminogen (Sigma Chemical Company) ml^{-1} for 192 h; each treatment comprised 14 replicates. These concentrations were selected because they approximate to physiological values (Fazleabas *et al.*, 1983; Finlay *et al.*, 1983) and developmental effects with porcine plasminogen have been demonstrated in cultured mouse (Menino and O'Claray, 1986), cattle (Menino and Williams, 1987) and sheep (Menino *et al.*, 1989) embryos. At intervals of 24 h, morphology of embryos was assessed and they were transferred to fresh microdrops. Medium was collected and

Fig. 1 One- to two-cell rabbit embryos collected 24 h after mating. (Scale bar represents 50 μm .)

stored at -20°C until assayed for plasminogen activator and plasmin activities. To determine whether the effects observed with heterologous plasminogen were physiological, a second group of embryos was cultured in medium containing homologous plasminogen. One hundred and ninety-five one- to two-cell embryos were cultured in microdrops of Ham's F-12 with 15 mg BSA ml^{-1} containing 0 ($n=40$), 75 ($n=40$), 150 ($n=39$) or 300 ($n=40$) μg rabbit plasminogen (Sigma Chemical Company) ml^{-1} or 60 μg porcine plasminogen ml^{-1} ($n=36$) for 168 h; each treatment comprised four replicates. Concentrations of rabbit plasminogen were selected on the basis of plasmin activities assayed for porcine plasminogen. At 24 h intervals, the morphology of the embryos was assessed and they were transferred to fresh microdrops. Medium was recovered and stored at -20°C until assayed for plasminogen activator and plasmin activities.

Experiment 2: numbers of cells of rabbit embryos cultured in medium containing plasminogen

The objective of Expt 2 was to evaluate a potential mitogenic effect of plasminogen on cultured rabbit embryos. Two hundred and sixteen one- to two-cell embryos were cultured in Ham's F-12 with 15 mg BSA ml^{-1} containing 0 ($n=53$), 30 ($n=54$), 60 ($n=54$) or 120 ($n=55$) μg porcine plasminogen ml^{-1} for 96 h; each treatment contained eight replicates. The morphology of the embryos was assessed at intervals of 24 h. At the end of culture, embryos were recovered from the microdrops, washed twice in 2.0% sodium citrate, transferred to 0.7% sodium citrate for 3 min, fixed onto microscope slides with 25% acetic acid in ethanol, air-dried (McGaughey and Chang, 1969) and stained with haematoxylin and eosin to determine the number of cells.

Experiment 3: development of rabbit embryos in medium containing plasmin or plasminogen

The principal objective of this experiment was to determine whether the improved development observed in medium with porcine plasminogen was due to the plasmin contaminant

Fig. 2. Rabbit morula collected 72 h after mating. (Scale bar represents 50 μm .)

accompanying it and whether such development could be supported by a similar amount of plasmin. A secondary objective was to evaluate the effects of plasminogen and plasmin on the rates of development to a particular cell stage. One hundred and forty-four one- to two-cell embryos were cultured in Ham's F-12 with 15 mg BSA ml^{-1} containing 0 ($n=39$) or 120 ($n=31$) μg porcine plasminogen ml^{-1} and 13 ($n=37$) or 45 ($n=37$) μg porcine plasmin ml^{-1} for 168 h; each treatment was composed of seven replicates. Plasmin concentrations were chosen to represent the amounts of plasmin contaminant assayed and total plasmin that could be activated in 120 μg porcine plasminogen ml^{-1} (13 and 45 μg plasmin ml^{-1} , respectively). The morphology of the embryos was assessed at intervals of 24 h.

Experiment 4: production of plasminogen activator by rabbit embryos on days 3, 4 and 5

The objective of Expt 4 was to identify plasminogen activator production by rabbit embryos collected as morulae and blastocysts and cultured to the equivalent of gestational age day 7. One hundred and one rabbit embryos were collected on days 3 ($n=37$), 4 ($n=24$) and 5 ($n=40$) after mating and cultured in Ham's F-12 with 15 mg BSA ml^{-1} (Fig. 2); each day was represented by 4–8 replicates. Embryos collected on days 3, 4 and 5 were cultured for 96, 72 and 48 h, respectively. At intervals of 24 h, the morphology of the embryos was assessed and they were transferred to fresh microdrops. Medium was recovered and stored at -20°C until assayed for plasminogen activator activity.

Plasminogen activator and plasmin assays

Plasminogen activator and plasmin activities were measured in culture medium using a casein-agar gel assay, as described by Menino and Williams (1987) and Kaekehuhi and Menino (1990) for cattle embryos. Briefly, activities of plasminogen

Table 1. Development of one- to two-cell rabbit embryos in Ham's F-12 containing 0, 30, 60 or 120 µg porcine plasminogen ml⁻¹

Plasminogen (µg ml ⁻¹)	Number of embryos	Number (%) of embryos at developmental stage				
		Morula	Blastocyst	Expanded blastocyst	Hatching blastocyst	Hatched blastocyst
0	82	65 (79) ^a	19 (23) ^a	3 (4) ^a	1 (1) ^a	0 (0) ^a
30	70	53 (76) ^a	25 (36) ^a	9 (13) ^b	2 (3) ^a	0 (0) ^a
60	69	54 (78) ^a	25 (36) ^a	18 (26) ^c	9 (13) ^b	3 (4) ^{ab}
120	79	63 (80) ^a	22 (28) ^a	19 (24) ^{bc}	9 (11) ^b	6 (8) ^b

^{abc}Numbers in the same column with different superscripts are significantly different ($P < 0.05$).

Calculated chi-squared values for development to the morula, blastocyst, expanded blastocyst, hatching blastocyst and hatched blastocyst stages with three degrees of freedom are 0.44, 4.32, 18.54, 12.27 and 10.86, respectively.

activator were determined with urokinase (E.C.3.4.21.31; Sigma Chemical Company) as the standard in concentrations of 0, 0.001, 0.005, 0.01, 0.05 and 0.10 mU ml⁻¹. Medium or urokinase standards (15 µl) were incubated with 15 µl of 120 µg human plasminogen ml⁻¹ (Sigma Chemical Company) at 37°C for 15 min. Aliquots of the incubated mixture (25 µl) were placed into wells 4 mm in diameter cut in an agar gel plate containing 1% nonfat dry milk (Carnation Company, Los Angeles, CA) and 1% agarose (Sigma Chemical Company) and incubated for 24 h at room temperature. At the end of incubation, plates were fixed for 10 min with 3% acetic acid, rinsed with tap water and the diameters of the caseinolytic zones were measured using a vernier calliper. Plasminogen activator concentrations in the medium were determined from the equation of the line calculated for the caseinolytic diameters by log urokinase concentrations. The quantity of plasminogen activator produced by the embryo (PA_E) was determined by deducting the amount in the medium without any embryos from the amount in the medium with an embryo for each 24 h interval. Plasminogen activator production (mU × 10⁻⁴ ml⁻¹) per day per viable embryo was calculated by dividing PA_E by the number of viable embryos observed at each 24 h interval.

Plasmin and nonspecific protease activities in the culture medium were determined with porcine plasmin (E.C.3.4.21.7; Sigma Chemical Company) as the standard in concentrations of 0, 5, 10, 50, 100 and 500 µg ml⁻¹. Medium or plasmin standard (15 µl) were added to wells cut in the casein-agar gel plates and incubated for 64 h at room temperature. At the end of incubation, plates were fixed with 3% acetic acid, and diameters of the caseinolytic zones were measured using a vernier calliper. Plasmin concentrations or nonspecific protease activities in the medium were determined from the equation of the line calculated for caseinolytic ring diameters by log plasmin concentrations.

Statistical analyses

The effects of the various culture conditions on numbers (percentages) of embryos that developed to the morula, blastocyst, expanded blastocyst, hatching blastocyst and hatched blastocyst stages were determined using chi-squared

procedures for tests of independence in $r \times 2$ contingency tables (Steel and Torrie, 1980). When a significant ($P < 0.05$) calculated chi-squared value resulted, 2×2 comparisons were conducted where appropriate. Differences in numbers of nuclei, times required for embryos to develop to a particular cell stage, and activities of plasminogen activator and plasmin were detected by analysis of variance and least significant differences procedures using the NCSS statistical software program (Number Cruncher Statistical System, Version 4.1, 1984, J. L. Hintze, Kaysville, UT).

Results

A total of 990 embryos was collected from 60 does, giving a mean embryo recovery of 16.5 embryos of which 956 were cultured.

Experiment 1: development of rabbit embryos in medium containing plasminogen

Numbers of rabbit embryos developing *in vitro* to the morula and blastocyst stages did not differ ($P > 0.10$) among the four concentrations of porcine plasminogen given (Table 1). More ($P < 0.05$) embryos developed to the expanded and hatching blastocyst stages in medium with 60 µg porcine plasminogen ml⁻¹ compared with 0 and 30 µg ml⁻¹. Development to the expanded, hatching and hatched blastocyst stages was greater ($P < 0.05$) in medium with 120 µg porcine plasminogen ml⁻¹ than with 0 µg ml⁻¹ (Fig. 3). Complete divesting of the zona pellucida was observed only for embryos cultured in either 60 or 120 µg porcine plasminogen ml⁻¹ (Fig. 4).

Development of rabbit embryos to the morula stage did not differ ($P > 0.10$) when the various concentrations of plasminogen were administered (Table 2). More ($P < 0.05$) embryos developed to the blastocyst stage in medium with 300 µg rabbit plasminogen ml⁻¹ compared with 0 µg ml⁻¹. More ($P < 0.05$) embryos attained the expanded blastocyst stage in medium with 150 and 300 µg rabbit plasminogen ml⁻¹ compared with 0 µg ml⁻¹. Fewer ($P < 0.05$) embryos commenced hatching in medium containing no rabbit plasminogen

Fig. 3. Hatching blastocyst that developed from a one- to two-cell rabbit embryo after 120 h in Ham's F-12 containing 15 mg BSA ml⁻¹ and 120 µg porcine plasminogen ml⁻¹. (Scale bar represents 50 µm.)

Fig. 4. Hatched blastocyst that developed from a one- to two-cell rabbit embryo after 144 h in Ham's F-12 containing 15 mg BSA ml⁻¹ and 120 µg porcine plasminogen ml⁻¹. (Scale bar represents 50 µm.)

than in medium containing either porcine or rabbit plasminogen, and development overall was similar ($P > 0.05$) in medium with either plasminogen. In this experiment, no embryos were observed to have completed hatching within the 168 h of culture.

Plasminogen activator activity and plasmin production from plasminogen were not detected in medium recovered from cultured one- to two-cell rabbit embryos (data not shown). Although some plasmin activity was detected in plasminogen-containing medium, this activity was due to an inherent plasmin contaminant and did not differ between medium recovered from embryos and control medium lacking embryos.

Experiment 2: numbers of cells of rabbit embryos cultured in medium containing plasminogen

Development to the morula, blastocyst and expanded blastocyst stages by 96 h of culture did not differ ($P > 0.10$) among the treatments tested and were similar to the results of Expt 1. However, the number of blastocysts hatching by 96 h of culture was greater ($P < 0.05$) for embryos cultured in medium containing porcine plasminogen (20 of 163), and no embryos (of 53) had initiated hatching in medium with no porcine plasminogen. At the end of the 96 h culture, embryos were graded for developmental stage before fixation. Numbers of cells of morulae did not differ ($P > 0.10$) with the various concentrations of porcine plasminogen administered (Table 3). However, blastocysts developing in medium with 120 µg porcine plasminogen ml⁻¹ had more cells than did blastocysts in medium with 0 or 30 µg ml⁻¹ ($P < 0.05$).

Experiment 3: development of rabbit embryos in medium containing plasmin or plasminogen

More ($P < 0.05$) embryos developed to the morula stage in medium with 45 µg plasmin ml⁻¹ or 120 µg porcine plasminogen ml⁻¹ compared with 13 µg plasmin ml⁻¹ (Table 4). However, development to the blastocyst stage was similar ($P > 0.05$) for embryos cultured in medium with 0, 13 or 45 µg plasmin ml⁻¹ or 120 µg porcine plasminogen ml⁻¹. More ($P < 0.05$) embryos developed to the expanded blastocyst stage in medium with 45 µg plasmin ml⁻¹ or 120 µg porcine plasminogen ml⁻¹ than with 0 or 13 µg plasmin ml⁻¹. Onset and completion of hatching were greater ($P < 0.05$) in medium with 45 µg plasmin ml⁻¹ or 120 µg porcine plasminogen ml⁻¹ compared with medium with 0 or 13 µg plasmin ml⁻¹. Only embryos cultured in medium with 120 µg porcine plasminogen ml⁻¹ or 45 µg plasmin ml⁻¹ completed hatching. The time taken for morula formation was shorter ($P < 0.05$) for embryos cultured in medium with either porcine plasminogen or plasmin compared with medium with 0 µg plasmin ml⁻¹ (Table 5). No differences ($P > 0.10$) in developmental rates to subsequent cell stages were observed owing to plasmin or porcine plasminogen concentration in the medium.

Experiment 4: plasminogen activator production by rabbit embryos on days 3, 4 and 5

Developmental stages of embryos collected on days 3, 4 and 5 after mating were 16 cell to morula, morula to blastocyst and blastocyst to expanded blastocyst, respectively. Embryos from days 3 and 4 that commenced hatching by the end of culture were observed to have left the zona pellucida but remained within the confines of the mucolemma (Fig. 5). Embryos collected on day 3 exhibited little plasminogen activator activity by 48 h of culture but showed a tenfold increase ($P < 0.05$) by 72 h and a 20-fold increase ($P < 0.05$) by 96 h of culture (Table 6). Plasminogen activator production for embryos collected on day 4 exhibited a similar increase over time, although no significant differences were observed. Embryos collected on day 5 demonstrated the greatest activity of plasminogen activator and exhibited a tenfold increase

Table 2. Development of one- to two-cell rabbit embryos in Ham's F-12 containing 60 µg porcine plasminogen ml⁻¹ and 0, 75, 150 or 300 µg rabbit plasminogen ml⁻¹

Plasminogen (µg ml ⁻¹)	Number of embryos	Number (%) of embryos at developmental stage			
		Morula	Blastocyst	Expanded blastocyst	Hatching blastocyst
Porcine					
60	36	33 (92) ^a	18 (50) ^{ab}	13 (36) ^{ab}	11 (30) ^b
Rabbit					
0	40	35 (88) ^a	11 (28) ^a	10 (25) ^a	4 (10) ^a
75	40	28 (70) ^a	19 (48) ^{ab}	19 (48) ^{ab}	15 (38) ^b
150	39	32 (82) ^a	22 (56) ^{ab}	22 (56) ^b	16 (41) ^b
300	40	35 (88) ^a	26 (65) ^b	26 (65) ^b	20 (50) ^b

^{ab}Numbers in the same column with different superscripts are significantly different ($P < 0.05$).

Calculated chi-squared values for development to the morula, blastocyst, expanded blastocyst and hatching blastocyst stages with four degrees of freedom are 8.06, 12.40, 16.05 and 16.13, respectively.

Table 3. Numbers of nuclei in rabbit morulae and blastocysts developing from one- to two-cell embryos after 96 h in Ham's F-12 containing 0, 30, 60 or 120 µg porcine plasminogen ml⁻¹

Plasminogen (µg ml ⁻¹)	Morulae		Blastocysts	
	<i>n</i>	Mean ± SEM	<i>n</i>	Mean ± SEM
3	10	29.6 ± 3.2 ^a	6	69.4 ± 14.6 ^a
30	15	29.5 ± 2.6 ^a	8	73.3 ± 12.2 ^a
60	13	29.3 ± 2.8 ^a	14	88.8 ± 9.2 ^{ab}
120	13	27.3 ± 2.8 ^a	11	109.9 ± 10.4 ^b

^{ab}Values in the same column with different superscripts are significantly different ($P < 0.05$).

($P < 0.05$) in production between 24 and 48 h of culture. Plasminogen activator production by day 5 was several times greater than that on comparable gestational days of embryos collected on days 3 or 4 (Fig. 6). Nonspecific protease activity was not observed in medium recovered from cultured rabbit embryos on days 3, 4 and 5 and all caseinolytic activity was plasminogen dependent.

Discussion

The results suggest that addition of plasminogen or plasmin to culture medium for rabbit embryos at early cleavage stages improves development *in vitro* and that rabbit blastocysts produce plasminogen activator. The improved development was observed as accelerated development to the morula stage, increases in percentages of embryos developing to the expanded and hatching blastocyst stages, and complete escape from the zona pellucida occurring only in medium with plasminogen or plasmin. These results compare favourably with increased incidences of hatching by mouse (Menino and O'Claray, 1986) and sheep embryos (Menino *et al.*, 1989), and accelerated development by cattle embryos (Menino and

Williams, 1987) in medium with similar concentrations of plasminogen. There are two possible explanations, which are not mutually exclusive, for the improved development in medium with plasminogen or plasmin. The effects of plasminogen or plasmin could be the result of a direct effect of the protease on the embryo, or an indirect action through weakening of the zona pellucida.

With respect to a direct effect of the protease, cell-surface receptors for both plasmin and plasminogen have been identified on human carcinoma cells (Burtin and Fondaneche, 1988) and rat glioma cells (Hall *et al.*, 1989), and proteases have been shown to induce growth-factor-like effects on various cell types *in vitro* (Cunningham, 1981). Blumberg and Robbins (1975) found that some proteases, including plasmin, stimulated deoxyglucose uptake and induced cell division in chick embryo fibroblasts. Pienkowski *et al.* (1974) and Konwinski *et al.* (1978) both observed mitogenic effects of proteases on cultured mouse embryos. The increase in the number of nuclei in blastocysts developing in medium with 120 µg porcine plasminogen ml⁻¹ (albeit small and not more than twice the number in medium without protease) may be one explanation for the improved hatching observed in medium with plasminogen or plasmin. Kane (1983a) observed that hatched blastocysts had twice as many cells as did unhatched blastocysts. The increase in numbers of cells coupled with the accelerated development observed suggest that the added plasminogen or plasmin may have exerted a mitogenic effect on one- to two-cell rabbit embryos developing *in vitro*.

Exposure to pronase or trypsin promotes hatching by rabbit blastocysts *in vitro* (Onuma *et al.*, 1968; Kane, 1983b, 1986). Kane (1986) suggested that proteases probably soften the zona pellucida by partial digestion, thereby allowing the blastocyst to grow by removing a block to expansion. Although plasminogen activator could not be detected in medium recovered from one- to two-cell embryos, it is possible that the quantity of activator produced was beyond the detection limit of the assay. Minute quantities of plasmin may have been generated that were sufficient to induce partial zonolytic effects. Although hatching does not occur *in vivo* in rabbits, the potential for

Table 4. Development of one- to two-cell rabbit embryos in Ham's F-12 containing 0, 13 or 45 µg porcine plasmin ml⁻¹ or 120 µg porcine plasminogen ml⁻¹

Plasmin/ plasminogen (µg ml ⁻¹)	Number of embryos	Number (%) of embryos at developmental stage				
		Morula	Blastocyst	Expanded blastocyst	Hatching blastocyst	Hatched blastocyst
Plasmin						
0	39	26 (66) ^{ab}	13 (33) ^a	3 (8) ^a	2 (5) ^{ab}	0 (0) ^a
13	37	21 (57) ^a	7 (19) ^a	3 (8) ^a	1 (3) ^a	0 (0) ^a
45	37	31 (84) ^b	17 (46) ^a	12 (32) ^b	12 (32) ^c	10 (27) ^b
Plasminogen						
120	31	26 (84) ^b	12 (39) ^a	9 (29) ^b	6 (19) ^{bc}	4 (13) ^b

^{ab}Numbers in the same column with different superscripts are significantly different ($P < 0.05$).

Calculated chi-squared values for development to the morula, blastocyst, expanded blastocyst, hatching blastocyst and hatched blastocyst stages with three degrees of freedom are 8.39, 6.42, 12.58, 17.01 and 21.22, respectively.

Table 5. Time (h) to the morula and blastocyst stages for one- to two-cell rabbit embryos cultured in Ham's F-12 containing 0, 13 or 45 µg porcine plasmin ml⁻¹ or 120 µg porcine plasminogen ml⁻¹

Plasmin/ plasminogen (µg ml ⁻¹)	Morula		Blastocyst	
	<i>n</i>	Mean ± SE	<i>n</i>	Mean ± SE
Plasmin				
0	26	68.3 ± 2.6 ^a	13	108.9 ± 5.4 ^a
13	21	59.4 ± 2.9 ^b	7	106.3 ± 7.4 ^a
45	31	58.1 ± 2.3 ^b	17	110.1 ± 4.8 ^a
Plasminogen				
120	26	55.4 ± 2.6 ^b	12	118.0 ± 5.7 ^a

^{ab}Values in the same column with different superscripts are significantly different ($P < 0.05$).

zonalytic effects observed with embryos developing *in vitro* in this study suggests that these proteins may be involved in remodelling of the extracellular embryonic coverings. Despite potential mitogenic and zonalytic roles for plasminogen and plasmin in this study, no effect of plasmin on rabbit embryo development was observed in a report by Kane (1986). However, the highest concentration of plasmin (25 µg ml⁻¹) used by Kane (1986), was below that (45 µg ml⁻¹) producing the greatest developmental enhancement in this study. In addition, medium with 13 µg plasmin ml⁻¹ failed to support development any more than medium containing neither plasminogen nor plasmin.

Development of rabbit embryos in rabbit plasminogen confirmed that the enhanced development attributed to porcine plasminogen was physiological. All concentrations of rabbit plasminogen examined resulted in development that was as good as or better than 60 µg porcine plasminogen ml⁻¹. Rabbit embryos may respond more to a homologous rather than to a heterologous plasminogen, assuming that it is indeed plasminogen that produces the developmental enhancement.

Fig. 5. Hatching blastocyst that developed from a rabbit blastocyst at day 4 after 48 h in Ham's F-12 containing 15 mg BSA ml⁻¹. (Scale bar represents 50 µm.)

When medium was analysed for plasminogen activator production by one- to two-cell embryos cultured for 7 or 8 days, no activity could be detected. Production of the activator was detected only by embryos collected on days 3, 4 or 5 after mating and cultured to an equivalent gestational age of day 7. Although similar patterns of plasminogen activator production were detected for embryos collected at different equivalent gestational ages, greatest activity was observed by embryos collected on day 5 and cultured for 48 h. Rabbit embryos developing *in vitro* lag behind embryos developing *in vivo*; this suggests that failure to detect plasminogen activator when zygotes were subjected to culture may be related to development. By ultrastructural comparison, Van Blerkom *et al.* (1973) found no differences between development *in vivo* and *in vitro* over the first 4 days. However, embryos cultured from the one- to two-cell stage *in vitro* lag behind comparably aged embryos *in vivo* in both number of cells and blastocyst diameter, beginning at day 3. Embryos developing *in vitro* do not usually have more than 500 cells (Kane, 1987). It seems evident that rabbit

Table 6. Plasminogen activator production (milliunits $\times 10^{-4}$ ml $^{-1}$ per embryo) by rabbit embryos on days 3, 4 and 5 after mating cultured to equivalent gestational age day 7

Equivalent gestational age (days)	Day of embryo collection		
	3	4	5
4	1.07 ^a	—	—
5	1.28 ^a	5.48 ^a	—
6	15.85 ^b	10.37 ^a	51.13 ^a
7	25.62 ^b	38.69 ^a	544.71 ^b
SEM	4.67	11.91	133.18

^{ab}Means in the same column with different superscripts are significantly different ($P < 0.05$).

SEM: pooled standard error of the mean.

Fig. 6. Production of plasminogen activator (log mU $\times 10^4$ ml $^{-1}$) by rabbit embryos collected on days 3 (●), 4 (■) and 5 (○) after mating and cultured to equivalent gestational day 7.

embryos require factors of uterine origin for normal expansion and cell division.

Jung (1989) reported a tenfold increase in protein synthesis between blastocysts developing *in vivo* on days 4 and 5 after mating. In fact, protein content of rabbit embryos *in vivo* increases from 0.2 μ g for day 3 morulae to 50.4 μ g for peri-implantation blastocysts on day 7 (Morgan and Kane, 1993). Rabbit blastocysts cultured *in vitro* also exhibit reduced amino acid incorporation, dependent on the duration of culture (Jung *et al.*, 1987). Rabbit embryos that were cultured on days 3 and 4 after mating have been shown to have more dead cells and an increase in secondary lysosomes with a corresponding enhancement in protein degradation compared with embryos that were not cultured (Hegele-Hartung *et al.*, 1988; Fischer, 1989; Fischer *et al.*, 1992). Hegele-Hartung *et al.* (1988) concluded that development *in vitro* was slowed to a rate where a period of 24 h matches only a few hours of development *in vivo*.

In this study, embryos exposed to the day 5 uterine environment may be assumed to be more vigorous and to have the necessary mechanisms for protein production, and hence

are able to produce greater quantities of plasminogen activator. Although the functional significance of the activator to the rabbit blastocyst was not elucidated in this study, several investigators have suggested a role in implantation in the mouse (Strickland *et al.*, 1976; Sappino *et al.*, 1989). Sappino *et al.* (1989) demonstrated the presence of urokinase-type plasminogen activator mRNA in invasive and migrating trophoblast cells of mouse embryos on days 5.5 and 6.5 after mating that eventually becomes undetectable by day 10.5. Because rabbit blastocysts increase production of plasminogen activator on day 7, the time of implantation, it may have a similar role in facilitating endometrial penetration in rabbits.

These results suggest that addition of plasminogen or plasmin to culture medium enhances rabbit embryo development *in vitro*. Although the mechanism is not clear, the improved development may not necessarily depend upon proteolytic activity for induction of the mitogenic effect. The observation that rabbit blastocysts produce plasminogen activator would provide a role for plasminogen of uterine origin as a zymogen that could be activated by the embryo to provide a source of protease. This protease could help remodel the extraembryonic coverings and implantation. Similar to suggestions for mice, the finding that production of plasminogen activator was greatest by blastocysts on day 7 after mating suggests that this enzyme may be involved in implantation in rabbits.

The authors wish to thank D. Martin for typing the manuscript. Technical Paper No. 10 080, Oregon Agricultural Experiment Station.

References

- Blumberg PM and Robbins PW (1975) Effect of proteases on activation of resting chick embryo fibroblasts and on cell surface proteins *Cell* **6** 137–147
- Boving BG (1963) Implantation mechanisms. In *Conference on Physiological Mechanisms Concerned with Conception* pp 321–396 Ed. CG Hartman. Pergamon Press, New York.
- Burtin P and Fondaneche M (1988) Receptor for plasmin on human carcinoma cells *Journal of the National Cancer Institute* **80** 762–765
- Cunningham DD (1981) Proteases as growth factors. In *Tissue Growth Factors* pp 229–248 Ed. R. Baserga. Springer-Verlag, New York.
- Denker H-W (1974) Trophoblastic factors involved in lysis of the blastocyst coverings and in implantation in the rabbit: observations on inversely oriented blastocysts *Journal of Embryology and Experimental Morphology* **32** 739–748
- Denker H-W (1977) Implantation: the role of proteases and blockage of implantation by protease inhibitors *Advances in Anatomy, Embryology and Cell Biology* **53** 1–123
- Denker H-W and Gerdes H-J (1979) The dynamic structure of rabbit blastocyst coverings. I. Transformation during regular preimplantation development *Anatomy and Embryology* **157** 15–34
- Denker H-W and Hafez ESE (1975) Proteases and implantation in the rabbit: role of trophoblast vs uterine secretion *Cytobiologie* **11** 101–109
- Fazleabas AT, Geisert RD, Bazer FW and Roberts RM (1983) Relationship between release of plasminogen activator and estrogen by blastocysts and secretion of plasmin inhibitor by uterine endometrium in the pregnant pig *Biology of Reproduction* **29** 225–238
- Finlay TH, Katz J, Kirsch L, Levitz M, Nathoo SA and Seiler S (1983) Estrogen-stimulated uptake of plasminogen by the mouse uterus *Endocrinology* **112** 856–861
- Fischer B (1987) Development retardation in cultured preimplantation rabbit embryos *Journal of Reproduction and Fertility* **79** 115–123
- Fischer B (1989) Effects of asynchrony on rabbit blastocyst development *Journal of Reproduction and Fertility* **86** 479–491

- Fischer B, Jung T, Hegele-Hartung C and Beier HM (1990) Development of preimplantation rabbit embryos in uterine flushing-supplemented culture media *Molecular Reproduction and Development* **27** 216–233
- Fischer B, Mootz U, Denker HW, Lambertz M and Beier HM (1991) The dynamic structure of rabbit blastocyst coverings. III. Transformation of coverings under non-physiological developmental conditions *Anatomy and Embryology* **183** 17–27
- Fischer B, Lambertz M and Hegele-Hartung C (1992) Ultrastructural and autoradiographic study of preimplantation rabbit embryos grown in conventional or uterine flushing-supplemented culture media *In Vitro Cellular and Developmental Biology* **28A** 337–347
- Hall SW, Vandenberg SR and Gonias SL (1989) Expression of plasminogen receptors on C6 glioma cells *Brain Research* **495** 373–376
- Hegele-Hartung C, Fischer B and Beier HM (1988) Development of preimplantation rabbit embryos after *in-vitro* culture and embryo transfer: An electron microscopic study *Anatomical Record* **221** 31–42
- Jung T (1989) Protein synthesis and degradation in non-cultured and *in vitro* cultured rabbit blastocysts *Journal of Reproduction and Fertility* **86** 507–512
- Jung T and Fischer B (1988) Correlation between blastocyst diameter and DNA or protein synthetic activity in rabbit blastocysts *Biology of Reproduction* **39** 1111–1116
- Jung T, Fischer B and Beier HM (1987) Quantitative aspects of protein synthesis in non-cultured and cultured rabbit blastocysts *Human Reproduction* **2** 23–27
- Kaekuahiwi MA and Menino AR Jr (1990) Relationship between plasminogen activator production and bovine embryo development *in vitro* *Journal of Animal Science* **68** 2009–2014
- Kane MT (1983a) Variability in different lots of commercial bovine serum albumin affects cell multiplication and hatching of rabbit blastocysts in culture *Journal of Reproduction and Fertility* **69** 555–558
- Kane MT (1983b) Evidence that protease action is not specifically involved in the hatching of rabbit blastocysts caused by commercial bovine serum albumin in culture *Journal of Reproduction and Fertility* **68** 471–475
- Kane MT (1986) A survey of the effects of proteases and glycosidases on culture of rabbit morulae to blastocysts *Journal of Reproduction and Fertility* **78** 225–230
- Kane MT (1987) *In vitro* growth of preimplantation rabbit embryos. In *The Mammalian Preimplantation Embryo: Regulation of Growth and Differentiation* *In Vitro* pp 193–217 Ed. BD Bavister. Plenum Press, New York.
- Kirchner C (1972) Uterine protease activities and lysis of the blastocyst covering in the rabbit *Journal of Embryology and Experimental Morphology* **28** 177–183
- Konwinski M, Solter D and Koprowski H (1978) Effect of removal of the zona pellucida on subsequent development of mouse blastocysts *in vitro* *Journal of Reproduction and Fertility* **54** 137–143
- McGaughey RW and Chang MC (1969) Meiosis of mouse eggs before and after sperm penetration *Journal of Experimental Zoology* **170** 397–410
- Maurer RR and Beier HM (1976) Uterine proteins and development *in vitro* of rabbit preimplantation embryos *Journal of Reproduction and Fertility* **48** 33–41
- Menino AR Jr and O'Claray JL (1986) Enhancement of hatching and trophoblastic outgrowth by mouse embryos cultured in Whitten's medium containing plasmin and plasminogen *Journal of Reproduction and Fertility* **77** 159–167
- Menino AR Jr and Williams JS (1987) Activation of plasminogen by the early bovine embryo *Biology of Reproduction* **36** 1289–1295
- Menino AR Jr, Dyk AR, Gardiner CS, Grobner MA, Kaekuahiwi MA and Williams JS (1989) The effects of plasminogen on *in vitro* ovine embryo development *Biology of Reproduction* **41** 899–905
- Morgan PM and Kane MT (1993) Protein content of rabbit embryos: one-cell to peri-implantation blastocyst *Journal of Reproduction and Fertility* **97** 101–106
- Mullins DE, Bazer FW and Roberts RM (1980) Secretion of a progesterone-induced inhibitor of plasminogen activator by the porcine uterus *Cell* **20** 865–872
- Onuma H, Maurer RR and Foote RH (1968) *In vitro* culture of rabbit ova from early cleavage stages to the blastocyst stage *Journal of Reproduction and Fertility* **16** 491–493
- Pienkowski M, Solter D and Koprowski H (1974) Early mouse embryos: growth and differentiation *in vitro* *Experimental Cell Research* **85** 285–290
- Sappino AP, Huarte J, Belin D and Vassalli JD (1989) Plasminogen activators in tissue remodeling and invasion: mRNA localization in mouse ovaries and implanting embryos *Journal of Cell Biology* **109** 2471–2479
- Steel RDG and Torrie JH (1980) *Principles and Procedures of Statistics: A Biometrical Approach* (2nd edn). McGraw-Hill Book Co, New York.
- Strickland S (1980) Plasminogen activator in early development. In *Development in Mammals* pp 81–100 Ed. MH Johnson. Elsevier/North Holland Biomedical Press, New York.
- Strickland S, Reich E and Sherman MI (1976) Plasminogen activator in early embryogenesis: enzyme production by trophoblast and parietal endoderm *Cell* **9** 231–240
- Van Blerkom J, Manes C and Daniel JC Jr (1973) Development of preimplantation rabbit embryos *in vivo* and *in vitro*. I. An ultrastructural comparison *Developmental Biology* **35** 262–282