

AN ABSTRACT OF THE THESIS OF

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The relationship between plasminogen activator (PA) production and cell stage, cell number and changes in overall diameter and zona pellucida thickness for bovine embryos developing in vitro was determined. Late morulae to blastocysts (n=80) were collected nonsurgically from naturally mated, estrous-synchronized and superovulated crossbred beef cows. Embryos were cultured, one embryo per 25- $\mu$ l microdrop, for 6 d. At 24-h intervals, embryos were evaluated for cell stage and transferred to fresh microdrops, media were recovered for PA analysis, and embryo diameters and zona pellucida thicknesses were measured with an ocular micrometer. Plasminogen activator production was determined using a caseinolytic assay with urokinase as the standard. Changes in diameter, zona pellucida thickness and PA production per 24-h interval for each embryo were plotted and the graphs cut out and weighed. Sixty-one (76%) embryos completed the hatching process. Total PA production was positively correlated to embryonic size ( $r=.40$ ;  $P<.001$ ), developmental stage ( $r=.35$ ;  $P=.001$ ) and cell number ( $r=.35$ ;  $P=.005$ ) and negatively, but weakly, correlated to zona

pellucida thickness ( $r=-.13$ ;  $P=.267$ ). Hatched embryos produced more total PA than embryos that did not hatch ( $.140 \pm .011$  vs  $.070 \pm .019$  g;  $P<.01$ ). The results suggest, as embryonic size and cell number increases and cell stage progresses, bovine embryos liberate more PA.

THE RELATIONSHIP BETWEEN PLASMINOGEN  
ACTIVATOR PRODUCTION AND VARIOUS ASPECTS OF  
BOVINE EMBRYO DEVELOPMENT IN VITRO

by

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## **DEDICATION**

To my dearest husband, Dale, in deepest gratitude for the faith, confidence, understanding, patience, support, but most of all, for all the love, strength and courage given me to carry me through each day. Also, his unselfish nature, always being by my side and believing in me has made this accomplishment an extra special one. There is no greater joy than knowing how blessed and lucky I am for having such a wonderful and special husband and one I love very much.

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# THE RELATIONSHIP BETWEEN PLASMINOGEN ACTIVATOR PRODUCTION AND VARIOUS ASPECTS OF BOVINE EMBRYO DEVELOPMENT IN VITRO

## Introduction

Accurate determination of bovine embryo viability prior to embryo transfer would improve the efficiency of the technique by affording only the transfer of embryos that have a high probability of survival. Although several investigators have attempted to develop assays that can measure embryo viability, rapid and dependable noninvasive procedures are limited (as reviewed by Butler and Biggers, 1989). Because of embryonic size limitations, few individuals have evaluated viability from an enzymatic perspective. Plasminogen activator (PA), the serine protease that activates the zymogen, plasminogen, to the enzyme, plasmin (Dano et al., 1985), is released by bovine embryos during blastocoelic expansion and hatching (Menino and Williams, 1987). Several potential roles for PA in development have been ascribed, including implantation (Sherman et al., 1976; Strickland et al., 1976), tissue remodelling and cell migration (Bode and Dziadek, 1979; Fazleabas et al., 1983) and facilitation of hatching (Menino and Williams, 1987); however, no one has yet correlated PA to specific aspects of embryo viability. Therefore, the objective of this research was to determine the relationship between PA production and cell stage, cell number and changes in overall diameter and zona pellucida thickness in bovine embryos developing in vitro.

## Review of the Literature

Success of embryo transfer in cattle depends heavily upon the quality of the embryos collected and the viability of the embryos transferred. An accurate and reproducible method for measuring embryo viability would afford the transfer of embryos that have a high probability of surviving to term. Application of such a method has the potential to increase conception rates in recipients and improve the overall efficiency of embryo transfer. Butler and Biggers (1989) reviewed methods developed by several investigators who attempted to measure embryo viability and classified the methods as: (1) morphological criteria, (2) development in culture, (3) fluorescent substrate metabolism, (4) fluorescence of dead cells and (5) metabolic/enzymatic measurements.

### Methods for Measuring Embryo Viability

Morphological criteria. Assessing embryo viability by morphological observation requires the use of light microscopy at 100 to 200X magnification to examine deviations in morphology from the ideal embryo. Shea (1981) used a three-point grading system to morphologically evaluate 6 to 8 d bovine embryos. Embryos above average in appearance (perfectly symmetrical, even granulation, no deformities in the zona pellucida, no extruded blastomeres and few if any degenerate cells) were assigned grade 4, average appearing embryos (small imperfections; some extraneous degenerate cells or abnormal shape) were assigned grade 3 and embryos below average in appearance (uneven blastomere size, extruded blastomeres, membrane rupture and more extraneous degenerate cells or abnormal shape,

but possessing a mass of viable cells) were assigned grade 2. Higher pregnancy rates were seen by bovine embryos ranked as above average (71%) and average (56%) than below average (44%) (Shea, 1981). Wright (1981) used a similar three-point grading scale and reported higher pregnancy rates by embryos ranked as above average (64%) than average (45%) and below average (33%). Similar results were observed by Lindner and Wright (1983) and Donaldson (1985) using four-point grading systems where 5 to 9 d bovine embryos were ranked as excellent, good, fair or poor and grades 1, 2, 3 or 4, respectively. Embryos ranked as excellent or grade 1 appeared ideal, and were spherical and symmetrical with cells of uniform size, color and texture. The appearance of embryos ranked as good or grade 2 included a few extruded blastomeres, an irregular shape and a few vesicles. Embryos ranked as fair or grade 3 had some imperfections, such as the presence of extruded blastomeres, vesiculation or few degenerated cells. Embryos ranked as poor or grade 4 had severe imperfections, such as numerous extruded blastomeres, degenerated cells, cells of varying sizes and large numerous vesicles, but a viable-appearing cell mass. Embryos graded as excellent (45%) and good (44%) produced significantly higher pregnancy rates than fair (27%) and poor (20%) embryos (Lindner and Wright, 1983). Donaldson (1985) reported higher pregnancy rates by embryos graded as 1 (56%) than embryos graded as 2 (49%), 3 (40%) and 4 (33%).

Development in culture. Short-term culture of embryos can be used to eliminate those embryos that fail to develop prior to transfer. Renard et al. (1980) attempted to use development in culture as an index for measuring embryo viability; however, they observed that bovine embryos

cultured for as short as 24 h produced fewer pregnancies than embryos that were transferred immediately after collection. Takahashi and Kanagawa (1986) cultured 7 and 8 d bovine embryos for 48 and 24 h, respectively, in Brinster's modified ovum culture medium (BMOC-3) using a test-tube system. At termination of culture, embryo viability was further examined by nonsurgical transfer to recipients. From the eleven heifers which received 7 d embryos cultured for 48 h, three recipients were diagnosed as pregnant and five of eleven heifers which received 8 d embryos cultured for 24 h were pregnant. Takahashi and Kanagawa (1986) concluded that 7 and 8 d bovine embryos could establish pregnancies following culture in BMOC-3.

Fluorescent substrate metabolism. Assessment of embryo viability can be accomplished through the metabolism of fluorescent substrates. Fluorescein diacetate (FDA) is converted to the fluorescing compound fluorescein in living cells by cytosolic esterases (Rotman and Papermaster, 1966). The use of fluorescent substrates in assessing embryo viability in the bovine was first examined by Schilling et al. (1979). Schilling et al. (1979) used a fluorescence microscopy technique with FDA as the substrate to evaluate viability of 5 to 8 d bovine embryos. Embryos were incubated for 3 to 5 min in phosphate buffered saline (PBS) containing FDA concentrations of 1:400,000 or 1:800,000 and were classified as having brilliant, partly, weak or negative fluorescence. After incubation in FDA, embryos which had a brilliant fluorescence had more mitotically active cells (33/39; 85%) than embryos which were partly (1/6; 10%), weakly (1/5; 20%) or negatively (0/36; 0%) fluorescent. Schilling et al. (1979) also examined 2 to 4 d rabbit embryos incubated

in Eagle's medium with FDA for 3 to 5 min. Embryos were transferred to recipients after incubation and at 17 to 20 d of pregnancy the reproductive tracts were evaluated for the number of fetuses. Schilling et al. (1979) did not observe a toxic effect due to FDA treatment; 63% of the FDA-treated embryos (22/35) compared to 46% of the control embryos (16/35) developed into fetuses. No differences were observed in mortality rates before implantation for either group; however, a lower mortality percentage after implantation was associated with FDA-treated embryos. Mohr and Trounson (1980) exposed 1-cell, 2-cell, morula and blastocyst stage mouse embryos to FDA for 1 min and assigned six grades relative to fluorescent intensity. Fluorescence was graded as follows: grade 5, very bright and uniform fluorescence within cells; grade 4, bright and uniform fluorescence; grade 3, uniform fluorescence; grade 2, pale and uniform, or patchy fluorescence within cells; grade 1, pale, often patchy fluorescence within cells, and just detectable visually; and grade 0, no visible fluorescence. Mohr and Trounson (1980) demonstrated that morphologically normal appearing embryos accumulated more intracellular fluorescein when exposed to FDA for 1 min than did developmentally retarded embryos. Niemann et al. (1981) also used FDA as a substrate to evaluate the viability of 6 to 8 d bovine embryos. These workers compared intensity of FDA staining to morphological evaluation and mitotic activity of the embryos. Embryos were morphologically classified as:

grade 1 - normal morphology; compact morula or blastocyst  
with a blastocoel, inner cell mass and trophectoderm,

grade 2 - some morphologic abnormalities; misshapen or uneven  
blastomeres,

grade 3 - embryos up to 16-cells with and without morphologic abnormalities (retarded),

grade 4 - unfertilized ova.

Embryos were also ranked for fluorescent intensity as positive, partly positive or negative. When bovine embryos were exposed to FDA for 3 to 5 min, 93% (131/141) of the grade 1 embryos were positively fluorescent compared to 23% (16/69) of the grade 2 and 9% (13/138) of the grade 3 embryos. Partly positive fluorescence was greater in grade 2 embryos (44/69; 64%) than grade 1 (10/141; 7%) and grade 3 (33/138; 24%) embryos; however, negative fluorescence was observed only for grade 2 (9/69; 13%) and grade 3 (92/138; 67%) embryos. Also, when bovine embryos were exposed to FDA for 3 to 5 min, those embryos which had a positive fluorescence (108/125; 86%) had more mitotically active cells than embryos which were partly positive (12/87; 14%) or negatively (0/80; 0%) fluorescent. Renard et al. (1982) exposed bovine blastocysts to FDA for 3 min and assigned grades of fluorescence, as bright, partially or non-fluorescent. These workers reported higher pregnancy rates (6/8; 75%) with brightly fluorescing bovine embryos than partially (2/6; 33%) or non-fluorescing (0/14; 0%) embryos. Hoppe and Bavister (1983) undertook a study to evaluate potential toxicity of the FDA viability assay in vitro and in vivo using eight-cell hamster embryos and bovine blastocysts and the relationship between fluorescence and viability using eight-cell hamster embryos. In the FDA toxicity trial using in vitro culture, eight-cell hamster embryos were assigned to three groups: (1) untreated controls (2) exposure to FDA and ultra-violet (UV) light and (3) UV light only. Hamster embryos were scored for development to the blastocyst stage after

24 h in culture. Bovine blastocysts were assigned to either an untreated control group or a FDA/UV group and development to the expanded blastocyst stage after 24 h in culture was recorded. Similar results were observed between the controls (60/174; 34%) and eight-cell hamster embryos developing to blastocysts following exposure to FDA and UV light (55/178; 31%) and UV light (62/175; 35%). No significant difference was observed between the controls (14/16; 88%) and the FDA and UV treated bovine blastocysts developing to expanded blastocysts (18/20; 90%) after 24 h in culture. In the FDA toxicity trial using embryo transfer, eight-cell hamster embryos and bovine blastocysts were assigned to two groups: (1) untreated controls and (2) exposure to FDA and UV light. In the hamster, 10 control embryos were placed into one horn and 10 FDA/UV treated embryos were placed into the other horn of each recipient whereas, in the bovine a single embryo was transferred to each recipient. In both the hamster and bovine, no significant difference was detected between FDA/UV treated embryos (49/79; 62% and 5/10; 50%, respectively), and the controls (52/77; 67% and 10/14; 71%, respectively). The final experiment consisted of an experimental group containing eight-cell hamster embryos that did not cleave during 24 h of culture and a control group of freshly-recovered eight-cell hamster embryos. The fluorescent intensity in the control group was consistently brighter while that of the experimental group varied from weak to bright. In the freshly-recovered group, 83% were brightly fluorescent whereas in the cultured, developmentally arrested group, only 56% were fluorescent. Hoppe and Bavister (1983) concluded that a poor relationship between fluorescent intensity and apparent viability exists because 56% of the developmentally arrested embryos



showed positive fluorescence. Although not a fluorescent stain, cells without intact membranes take up trypan blue. Hutz et al. (1985) exposed hamster zygotes to FDA and trypan blue and used freezing to provide a degenerate group of zygotes for comparison. Intensity of fluorescence and trypan blue exclusion were compared to rates of RNA synthesis with [<sup>3</sup>H]-uridine. Hutz et al. (1985) observed that exclusion of trypan blue and fluorescence with FDA were associated with embryos that actively incorporated [<sup>3</sup>H]-uridine and synthesized RNA.

Fluorescence of dead cells. Diamino-phenyl-indole (DAPI) is a fluorescent vital dye which binds specifically to cellular DNA. Niemann et al. (1981) exposed 6 to 8 d bovine embryos to DAPI for 10 to 15 min and compared intensity of DAPI staining to morphological classification. Niemann et al. (1981) observed that grade 3 or retarded embryos (43/61; 71%) with poor membrane integrity would take up DAPI sooner than grade 2 (6/37; 16%) or grade 1 (0/63; 0%) embryos with intact membranes.

Metabolic/enzymatic measurements. Metabolic or enzymatic measures of embryo viability have used glucose uptake or metabolism and enzyme activity in the culture medium as diagnostic criteria. Renard et al. (1982) surveyed enzymes released into the medium by cultured 7 to 8 d bovine blastocysts and observed that glucose-6-phosphate dehydrogenase, succinodehydrogenase and  $\beta$ -hexosaminidase were released by both degenerating and actively developing embryos; however, lactate dehydrogenase was released only by degenerating embryos. Rieger (1984) measured (1-<sup>14</sup>C)-glucose metabolism by 7 d bovine embryos as <sup>14</sup>CO<sub>2</sub> evolved. This procedure involved incubation of single embryos in small microwells with .5  $\mu$ Ci of labelled substrate in 30  $\mu$ l of phosphate-buffered medium.

Carbon dioxide evolved was trapped by an encompassing bath of 400  $\mu$ l of .1N NaOH and was mixed with scintillation fluid and counted following incubation. Rieger (1984) reported a 3-fold difference between normal and degenerating embryos. Gardner and Leese (1987) used an ultra-microfluorometric technique to measure glucose uptake by single mouse blastocysts before transfer to pseudopregnant recipients and found greater glucose uptake by male ( $4.4 \pm .2 \text{ pmol}\cdot\text{embryo}^{-1}\cdot\text{h}^{-1}$ ) and female ( $4.9 \pm .4 \text{ pmol}\cdot\text{embryo}^{-1}\cdot\text{h}^{-1}$ ) embryos that resulted in pregnancies, than by embryos that did not produce pregnancies ( $3.6 \pm .5 \text{ pmol}\cdot\text{embryo}^{-1}\cdot\text{h}^{-1}$ ). Butler et al. (1988) used a noninvasive fluorescence technique to measure glucose uptake in 165 morula and blastocyst stage mouse embryos in two different culture media. Eighty-four embryos were assayed in M2 medium and 81 embryos were assayed in M16 medium. Embryos were morphologically evaluated and assigned a quality grade. Morphologically normal embryos were assigned grade 1; embryos displaying minor abnormalities, such as extruded cells, were assigned grade 2; and embryos with major morphological defects were assigned grade 3. Glucose uptake by grade 3, or degenerate embryos, was significantly less than by grades 1 and 2. Moreover, glucose uptake by embryos incubated in M2 medium per 4-h interval was lower than that in M16 medium. Menino and Williams (1987) reported that plasminogen activator (PA) was released by bovine embryos during blastocoelic expansion and hatching. Plasminogen activator has been detected in other species, including the rat (Liedholm and Astedt, 1975), mouse (Sherman et al., 1976; Strickland et al., 1976) and pig (Mullins et al., 1980; Fazleabas et al., 1983); however, the physiologic significance of this enzyme is not known. Because PA is readily secreted

into the extracellular environment, it may be an easily accessible index of embryo viability.

### Biochemistry of Plasminogen Activator

Plasminogen activators (PA) are serine proteases which activate the zymogen, plasminogen, to the active enzyme, plasmin (as reviewed by Dano et al., 1985). Plasminogen activators can be categorized into two types based primarily on their molecular weight (MW): urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). Both uPA and tPA are immunologically distinct, produced by different genes and have MW of 30 to 50 kD and 70 kD, respectively. Urokinase-plasminogen activator can either be in a single-polypeptide chain (scuPA) form as the inactive proenzyme or a two-polypeptide chain form (tcuPA) as the active enzyme. Both scuPA and tcuPA have molecular weights equal to 50 kD. The heavy chain (B-chain) of tcuPA is 30 kD and the light chain (A-chain) is 20 kD; both chains are held together by a disulfide bridge. Two-chain urokinase-plasminogen activator also exists in an enzymatically active form with a MW of 30 kD. The 30 kD form of uPA consists of a heavy chain and a light chain; however, the light chain is comprised of 21 amino acids from the C-terminal end of the light chain from the native 50 kD form. The amino acid sequence of the heavy chain in the 30 kD uPA is the same as the two-chain 50 kD form. The reduction in the A-chain in the 30 kD form is believed to be due to proteolysis of the native 50 kD form. The active site of uPA is located on the heavy chain, and is composed of serine, histidine and aspartic acid residues.

Tissue-plasminogen activator has a MW of 70 kD as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Tissue-plasminogen activator, like uPA, consists of a single-polypeptide chain or zymogen form and a two-polypeptide chain or active enzyme form; both molecular forms are stabilized by a disulfide bridge. The two chains are of equal length, unlike that of uPA, where the A-chain is shorter than the B-chain. The A-chain in human tPA is longer than the A-chain in human uPA; however, the A-chains of tPA and uPA are homologous. The B-chain of tPA has a similar amino acid sequence to that of uPA; however, differences between the B-chains of tPA and uPA reside in a six-residue insertion near the C-terminal of tPA and a six-residue extension at the C-terminal of uPA. Like uPA, plasminogen is the only known substrate for tPA; however, tPA has a strong affinity for fibrin, unlike uPA. Both uPA and tPA have growth factor-like domains that are homologous with epidermal growth factor (Pennica et al., 1983; Ny et al., 1984).

The principal physiologic role of PA is the induction of the fibrinolytic protease, plasmin, from the zymogen, plasminogen, which functions in clot lysis (Christman et al., 1977). Various cells and tissues produce PA and the subsequent formation of plasmin may not be aimed primarily at clot breakdown. Plasminogen activators are involved in several reproductive processes. During the preovulatory period, elevated levels of PA induce the conversion of plasminogen to plasmin which participates in the degradation of the follicle wall at ovulation (Reich et al., 1985). The granulosa cells in response to gonadotropin stimulation are the source of ovarian PA (Shimada et al., 1983; Canipari and Strickland, 1986). Plasminogen activator also functions in cumulus

cell detachment and granulosa cell proliferation (Shimada et al., 1983; Espey et al., 1985). Granulosa cells isolated from mouse ovaries produce uPA; however, rat granulosa cells produce primarily tPA (Canipari et al., 1987).

Plasminogen activators are produced by other reproductive organs, such as the uterus (Kwaan and Albrechtsen, 1966; Harpel et al., 1966, 1967; Mullins et al., 1980) and the testes (Lacroix et al., 1977, 1981). Although a specific function for PA in the uterus has not been defined, production of this enzyme may be involved in tissue remodelling at implantation (Mullins et al., 1980). In the testes, the Sertoli cells serve as the chief source of PA and, depending upon hormonal or cell-mediated conditions, can produce either uPA or tPA (Marzowski et al., 1985; Hettle et al., 1986). Hettle et al. (1986) reported that PA may be involved in restructuring of the seminiferous tubule during spermatogenesis and migration of cytoplasmic extensions of Sertoli cells between spermatocytes and the basal lamina.

### Biochemistry of Plasminogen and Plasmin

Plasminogen is an inactive proenzyme. In its native form, plasminogen is composed of a single polypeptide chain and is approximately 92 kD (as reviewed by Dano et al., 1985). Because glutamic acid (Glu) serves as the N-terminal amino acid, plasminogen is often referred to as Glu-plasminogen. Glu-plasminogen can be proteolytically transformed to Lys-plasminogen, another single chain form of the proenzyme. Lys-plasminogen is approximately 8 kD less than Glu-plasminogen and results from the proteolytic cleavage of the N-terminal activation peptide of Glu-

plasminogen by plasmin. Cleavage of the Arg 560-Val 561 peptide bond by plasminogen activator converts Glu-plasminogen and Lys-plasminogen to the active enzymes Glu-plasmin and Lys-plasmin, respectively. Conversion of Glu-plasmin into Lys-plasmin is catalyzed by plasmin and also results in the liberation of the activation peptide. Plasmin is a serine protease containing two-polypeptide chains held together by a single disulfide bond. Plasmin contains a light chain (B-chain) with a MW of 25 kD and a heavy chain (A-chain) with a MW of 63 kD. The active site is located on the light chain and the amino acid sequence is similar to other serine proteases such as trypsin, chymotrypsin and pancreatic elastase (Robbins and Summaria, 1976; Christman et al., 1977; Dano et al., 1985). Plasmin, like trypsin, hydrolyzes proteins and peptides at lysyl and arginyl bonds. Several protease inhibitors are present in plasma that inhibit plasmin. The two most important inhibitors of plasmin are  $\alpha_2$ -antiplasmin and  $\alpha_2$ -macroglobulin. Alpha<sub>2</sub>-antiplasmin is considered a fast-reacting plasmin inhibitor whereas  $\alpha_2$ -macroglobulin inhibits plasmin only when there is a local depletion of  $\alpha_2$ -antiplasmin.

#### Plasminogen Activator in the Embryo

Rat embryos. Embryonic production of plasminogen activator was first detected by Liedholm and Astedt (1975) using a histochemical fibrinolytic technique. Briefly, 1 to 5 d rat embryos (where detection of the vaginal plug is d 1) were washed in physiological saline, placed on fibrin slides for 30 min in a 23°C moist chamber, fixed in formaldehyde and stained with Giemsa solution and Harris' hematoxylin. Day 6 uteri were removed, washed in physiological saline and frozen immediately in liquid nitrogen.

Cryostat sections of the uteri were cut to locate implantation sites and the sections were placed on fibrin film and fixed and stained as described. Fibrinolytic activity displayed by 1, 2, 3 and 4 d oviductal embryos were  $49 \pm 10(X10^3)$ ,  $70 \pm 4(X10^3)$ ,  $61 \pm 5(X10^3)$  and  $84 \pm 9(X10^3)\mu^2$ , respectively. Fibrinolytic activity decreased when the embryos entered the uterus  $27 \pm 4(X10^3)\mu^2$  and disappeared at the time of implantation. Liedholm and Astedt (1975) also examined the fallopian tubes and endometrium from nonpregnant rats and decidua from pregnant rats and observed fibrinolysis in tissues from nonpregnant rats but no fibrinolytic activity in decidua. When tranexamic acid (AMCA), a competitive inhibitor of PA, was added to the fibrin film, no fibrinolysis was detected. Loss of fibrinolysis in the presence of AMCA validated that the fibrinolytic activity of the embryo was due to PA. Liedholm and Astedt (1975) proposed that fibrinolytic activity during ovum transport prevents its adhesion to fibrin deposits lining the mucosa. They also proposed that loss of fibrinolysis when the embryo enters the uterus serves as a prerequisite for adhesion to the endometrium and implantation. Huarte et al. (1985) investigated PA production in primary and secondary rat oocytes using SDS-PAGE and zymography. Plasminogen activator was not detectable in primary oocytes; however, tPA was detectable in the transition of the primary oocyte to the secondary oocyte and in the secondary oocyte. Antibodies to tPA inhibited PA activity in secondary oocytes. Huarte et al. (1985) concluded that tPA was present in rat secondary oocytes and its appearance was triggered by resumption of meiotic maturation. Liu and Hsueh (1987) identified PA in rat cumulus-oocyte complexes by SDS-PAGE and zymography. Tissue-plasminogen activator activity was localized to the oocyte and low

Levels of tPA were detected in cumulus-oocyte complexes from immature rats before and after PMSG injection; however, uPA was not observed. After hCG injection, tPA levels in the cumulus-oocyte complexes increased in a time-dependent manner and were correlated with the extent of cumulus cell expansion and dispersion. Cumulus-oocyte complexes incubated with FSH or hCG exhibited dramatic increases in tPA activity. Liu and Hsueh (1987) concluded that gonadotropin stimulation of ovulation induced increases in oocyte content of tPA which were related to cumulus cell expansion and dispersion.

Mouse embryos. Strickland et al. (1976) surveyed PA production using both a fibrin-agar overlay assay and an  $^{125}\text{I}$ -fibrinolytic assay in cultured mouse embryos during the early stages of development and differentiation. When embryos were cultured at the early cleavage stages (2- to 8-cell) PA production was not detected until the late blastocyst stage (equivalent gestational age equal to 6 d, where detection of the vaginal plug is d 1). A biphasic pattern of PA production was observed where PA levels peaked between d 7 to 9, declined to d 11 and increased at d 13. Strickland et al. (1976) identified the tissue sites of embryonic PA production using microdissection and immunosurgery. To obtain cultures of trophoctodermal vesicles free of inner cell mass (ICM) four- to eight-cell embryos were microdissected into individual blastomeres or pairs of blastomeres, respectively, and cultured. Alternatively, d 5 blastocysts were treated with  $10^{-4}\text{M}$  5-bromo-2'-deoxyuridine (BUdR), which selectively destroys the ICM (Sherman and Atienza, 1975), to obtain trophoctodermal vesicles. Trophoctodermal vesicles prepared by either technique and cultured to an equivalent gestational age of 14 d exhibited a single peak in PA



production between d 8 to 9 which was similar to the first phase of PA secretion seen with intact embryos. Isolated ICM were prepared following the immunosurgical method described by Solter and Knowles (1975) where d 6 mouse blastocysts were exposed to rabbit anti-mouse serum then treated with guinea pig complement. Cultured isolated ICM initiated PA production at an equivalent gestational age of 9 d and continued to liberate PA until d 14. When isolated ICM were cultured to allow differentiation of the parietal endoderm and subjected to a second round of immunosurgery, no PA production was observed in cultures of ICM cores. These data suggest that the parietal endoderm was responsible for the secondary phase of PA production. To further validate the source of PA production as the parietal endoderm, 10 d embryos were recovered and dissected into four component tissues: parietal endoderm, yolk sac (containing visceral endoderm and mesoderm), amnion and embryo proper. Plasminogen activator was not associated with the amnion, yolk sac and embryo proper; however, the parietal endoderm exhibited considerable PA activity. From these data, Strickland et al. (1976) concluded that mouse embryos exhibited a biphasic pattern of PA production where trophoblast was responsible for the first phase and parietal endoderm the second phase. Because trophoblastic production of PA corresponded to the time of implantation, Strickland et al. (1976) suggested that PA was involved in endometrial penetration by the trophoblast. Parietal endoderm cells migrate and secrete an extracellular matrix beneath the trophectoderm to form Reichert's membrane. Reichert's membrane follows the growth of the yolk sac and requires remodelling as the yolk sac enlarges. Strickland et al. (1976) have suggested that the production of PA by parietal endoderm is

involved in cellular migration beneath the trophoblast and the metabolism of Reichert's membrane. Sherman et al. (1976) compared PA production in mouse embryos and teratocarcinoma cells using techniques similar to those described by Strickland et al. (1976). In cultures of mouse embryos, the biphasic pattern of PA production was evidenced where trophoblast cells contributed to the first phase and parietal endoderm to the second phase. The teratocarcinoma cell line used, known as F-9 embryonal carcinoma cells, resemble undifferentiated stem cells and do not produce PA. Sherman et al. (1976) induced the F-9 embryonal carcinoma cells to differentiate by aggregate formation in bacteriological culture dishes. The differentiated cells developed into cells that were morphologically and histologically similar to parietal endoderm cells and positive for PA production. Sherman et al. (1976) concluded that PA production by the embryo and the differentiated F-9 embryonal carcinoma cells is internally programmed and common to cells actively involved in migration and connective tissue degradation. Bode and Dziadek (1979) dissected d 7 to 10 mouse embryos (where detection of the vaginal plug is d 1) into their major embryonic tissues and assayed each for PA production using either a caseinolytic assay or a fibrin-agar overlay assay. Plasminogen activator production by the parietal endoderm was persistent from d 7 through d 10 of gestation. On d 7 and 8, the ectoplacental cone and extraembryonic ectoderm exhibited plasminogen-dependent proteolytic activity. The embryonic ectoderm began to secrete PA on d 8 during the latter stages of embryo development. On d 7, there was no indication of PA secretion by the visceral endoderm; however, there was a marked increase in PA production between early and late stage embryos on d 8 and

by d 9 PA production had decreased. Visceral extraembryonic endoderm was consistently negative for PA production; however, onset of PA secretion was induced on d 9 during a 28-h culture period. Some embryonic mesoderm fragments secreted PA in early and mid d 8 embryos and by the late stages all the embryonic mesoderm fragments were secreting PA. Plasminogen activator was not secreted by the extraembryonic mesoderm tissues until d 8 in late-stage embryos and persisted through 28 h of culture on d 9. On d 10, PA production by the visceral yolk sac (VYS) endoderm, VYS mesoderm and the amnion remained constant through 48 h of culture. Bode and Dziadek (1979) concluded that PA could not be used as a biochemical tissue-specific marker for the parietal endoderm; however, PA could still be involved in tissue remodelling during mouse embryogenesis. Apparent contradictions between the results of Strickland et al. (1976) and Bode and Dziadek (1979) are present because Strickland et al. (1976) was only able to detect PA production by the parietal endoderm from d 10 embryos. The differences may be a result of primary cultures used by Strickland et al. (1976) whereas whole tissue sections were used by Bode and Dziadek (1979). Rizzino and Sherman (1979) cultured mouse blastocysts in the serum-free medium, EM-2 [50% National Cancer Institute Culture Medium (NCTC-109) and 50% Preimplantation Culture Medium (PCM)], and assayed the embryos for PA production using the fibrin agar overlay method. Rizzino and Sherman (1979) observed endoderm-like cells growing out from the ICM after 8 d of culture in EM-2 which were positive for PA. Embryonic cells that were negative for PA were either secondary trophoblast or extra-embryonic ectoderm (Rizzino and Sherman, 1979). These authors concluded that EM-2 supported the development and differentiation of parietal

endoderm which was morphologically and biochemically similar to parietal endoderm developing either in serum-supplemented medium or in utero. Kubo and Spindle (1980), using the fibrin-agar overlay procedure, observed that trophoblast from 4 d mouse blastocysts was either negative or weakly positive for PA during the period of attachment to the culture dish substratum. Enhanced PA levels were associated with vigorous trophoblastic outgrowth and spreading (Kubo and Spindle, 1980). Kubo and Spindle (1980) also examined the effects of various protease inhibitors on PA activity in the trophoblast. Nitrophenyl-p-guanidino benzoate (NPGB), a potent inhibitor of PA, inhibited trophoblastic PA at a concentration of  $1 \times 10^{-4}$  M and also significantly reduced trophoblastic outgrowth. Kubo and Spindle (1980) concluded that cultured mouse embryos depended on fibrinolytic activity for trophoblastic outgrowth but not attachment. Sellens and Sherman (1980) cultured 4 d mouse blastocysts (detection of the vaginal plug is d 1) for 7 d in various media designed as either optimal or suboptimal to support blastocyst development and surveyed PA production using the fibrin-agar overlay method. The different culture media used were: PCM, PCM supplemented with .05% fetuin (PCM + FET), PCM with amino acids and vitamins (PCM + AA + VIT), PCM with AA, VIT and FET (PCM + AA + VIT + FET), EM-2, EM-2 with fetuin omitted (EM-2-FET), NCTC with serum (cNCTC) and cNCTC with an agarose substratum (cNCTC/agarose). Embryos cultured in the enriched cNCTC/agarose, cNCTC, EM-2-FET and EM-2 had substantially more cells, greater quantities of total protein and DNA and produced more PA throughout the 7 d culture period than embryos cultured in PCM, PCM + FET, PCM + AA + VIT and PCM + AA + VIT + FET. Plasminogen activator production per embryo was similar

for embryos cultured in PCM and PCM + FET and for embryos cultured in PCM + AA + VIT and PCM + AA + VIT + FET. Based on these results, Sellens and Sherman (1980) concluded that mouse blastocysts developing in suboptimal medium reflect their culture conditions and produce less PA than blastocysts developing in optimal medium. Sherman (1980) investigated fibrinolytic activity in 1, 2, 3, 4 and 5 d cultured mouse embryos and zonae pellucidae using the fibrin-agar overlay method. Sherman (1980) reported that fibrinolytic activity was observed in all stages of preimplantation embryo development. Sherman (1980) also reported that: 1) fibrinolytic activity was present in zona-enclosed embryos that failed to develop; 2) fibrinolytic activity was not evident in embryos treated with pronase to dissolve the zona pellucida; 3) empty zonae pellucidae or zonae pellucidae containing debris from degenerate embryos, possessed fibrinolytic activity; and 4) when zonae pellucidae were manually removed, fibrinolytic activity was evident in the empty zonae pellucidae and the embryos were negative or weakly positive. Sherman (1980) suggested that fibrinolytic activity associated with preimplantation mouse embryos was confined to the zona pellucida and was not of embryonic origin. During d 5 of gestation, small amounts of fibrinolytic activity were associated with unhatched blastocysts; however, activity ceased when the zonae pellucidae were removed with pronase. Hatched blastocysts showed no indication of fibrinolytic enzymes. Alternatively, on d 4 of gestation, a mixture of hatched and unhatched blastocysts was obtained and removal of the zonae pellucidae from unhatched blastocysts resulted in no fibrinolytic activity; however, during the culture period, half of the spontaneously hatched blastocysts possessed fibrinolytic activity.

Despite the time of removal from the reproductive tract, cultured blastocysts which gave rise to trophoblastic outgrowth almost invariably secreted PA. Sherman (1980) concluded that PA secretion in mouse embryos was not involved with hatching or acquisition of adhesiveness by trophoblast cells, but was involved with trophoblast invasiveness. Marotti et al. (1982) examined cultured embryonic tissues (parietal endoderm, visceral endoderm and extraembryonic mesoderm) from d 12 mouse embryos (where day of the vaginal plug was d 1) for PA using SDS-PAGE and zymography. When the parietal endoderm, visceral endoderm and extraembryonic mesoderm were cultured in serum-free media, all cell types secreted PA. When the cells were exposed to acid-treated serum before assay or were mixed with serum and then acid-treated, PA activity in the parietal endoderm was only partially inhibited, whereas PA activity in the visceral endoderm and extraembryonic mesoderm was completely inhibited. These results implied that mouse embryonic cells produced two types of PA. When all three embryonic cell types were cultured in medium containing actinomycin D or cycloheximide, PA in the conditioned medium was completely inhibited, indicating that the cells were actively transcribing mRNA for PA and synthesizing PA. Using SDS-PAGE and zymography, Marotti et al. (1982) demonstrated that parietal endoderm produced two types of PA; a major form at 79 kD and a minor form at 45 kD. Visceral endoderm PA migrated as a single form at 48 kD; however, extraembryonic mesoderm PA migrated as a doublet at 48 kD. Because other systems have identified antigenically different PA, the enzymes produced by the mouse embryonic cells were analyzed using two antibodies, anti-human melanoma PA (anti-tPA) and anti-Lewis lung carcinoma cell PA

(anti-uPA). In the parietal endoderm, anti-tPA completely inhibited the two forms of PA; however, no effect was observed on the PA from the visceral endoderm or extraembryonic mesoderm. Plasminogen activator in the visceral endoderm and extraembryonic mesoderm was inactivated by anti-uPA; however, anti-uPA had no effect on parietal endoderm PA. Marotti et al. (1982) also treated F-9 teratocarcinoma stem cells with retinoic acid and dibutyryl cAMP (RA-DBC) to characterize the PA produced by differentiated teratocarcinoma cells. Retinoic acid-DBC-treated cells produced two forms of PA, a minor form at 45 kD and a major form at 79 kD, which are both completely inhibited by anti-tPA but unaffected by anti-uPA. The results suggest that F-9 teratocarcinoma stem cells when induced to differentiate by RA-DBC treatment secrete PA similar to the parietal endoderm. Marotti et al. (1982) concluded that the parietal endoderm produced a tPA, whereas the visceral endoderm and extraembryonic mesoderm produced a uPA during mouse embryogenesis. Huarte et al. (1985) examined primary and secondary oocytes and 1, 2, 3 and 4 d mouse embryos (where mating was designated as d 0) for PA activity using SDS-PAGE with zymography. Plasminogen activator was not detected in primary oocytes, but was detected in secondary oocytes and transiently in zygotes; although PA activity was not present for very long in zygotes. Zymography indicated that only tPA was present in mouse oocytes and uPA could not be detected. When secondary oocytes were treated with cycloheximide, protein synthesis was inhibited and tPA was not detectable. When secondary oocytes were treated with actinomycin D and  $\alpha$ -amanitin, RNA synthesis was inhibited; however, tPA was still detectable, indicating that new mRNA synthesis was not required for the induction of tPA activity. Huarte et

al. (1985) concluded that tPA mRNA was present in primary oocytes but not translated until after meiotic maturation is resumed. Huarte et al. (1985) also concluded that most tPA is secreted at fertilization and tPA production stops at this time until synthesis resumes at d 6. Menino and O'Claray (1986) evaluated development of two-cell mouse embryos (where the presence of the vaginal plug was d 0) cultured for 10 d in various media. The media used were Whitten's medium with 10 mg/ml bovine serum albumin (WM) as the control, WM with 2.3, 4.6, 23.1 or 46.2  $\mu\text{g/ml}$  plasmin, WM with 14.6, 29.1 or 145.7  $\mu\text{g/ml}$  plasminogen, WM with .1, .2, 1.1 or 2.2  $\mu\text{g/ml}$  trypsin, WM with .2, .3, 1.6 or 3.3  $\mu\text{g/ml}$  pronase and WM with 10% heat-treated bovine serum (HTBS). Proteolytic activities in the culture medium were determined using a caseinolytic agar gel assay. Pronase and WM with 1.1 and 2.2  $\mu\text{g/ml}$  trypsin significantly decreased blastocyst formation when compared to WM. Whitten's medium with 2.3 or 23.1  $\mu\text{g/ml}$  plasmin and WM with 14.6  $\mu\text{g/ml}$  plasminogen resulted in more embryos developing to the blastocyst stage than in WM. Significantly less embryos hatched in WM than in all plasminogen- and plasmin-supplemented media with the exception of WM with 29.1  $\mu\text{g/ml}$  plasminogen. Media with plasmin, plasminogen and HTBS supported significantly greater incidences of attachment and subsequent trophoblastic outgrowth than other protease supplemented media or WM. Menino and O'Claray (1986) suggested that the enhanced embryo development observed in medium supplemented with plasmin or plasminogen was due to the provision of a protease in the culture medium and an additional trophic effect. Proteolytic activity was significantly increased in plasminogen supplemented medium containing embryos thereby validating the activation of plasminogen to plasmin by mouse embryos.



Huarte et al. (1987) evaluated molecular control of PA production in secondary mouse oocytes using Northern blot analysis. Huarte et al. (1987) proposed that meiotic maturation of mouse oocytes triggers the polyadenylation and translation of the dormant tPA mRNA and the 3' noncoding region is critical for translational recruitment. Strickland et al. (1988) demonstrated that during meiotic maturation of mouse oocytes, stored maternal mRNA for tPA is polyadenylated and translated but degraded by the time fertilization occurs. Injection of antisense RNA molecules that hybridized with complementary tPA mRNA prevented polyadenylation, translation and destabilization of the mRNA and blocked the appearance of tPA activity in secondary mouse oocytes. Strickland et al. (1988) proposed that oocyte tPA may be involved in the dispersion of the cumulus oophorus cells.

Pig embryos. Mullins et al. (1980) were the first to detect PA production by pig blastocysts. Mullins et al. (1980) cultured 12 d pig blastocysts for 48 h and measured PA production using the  $^{125}\text{I}$ -fibrin plate assay. Large quantities of PA were secreted into the medium by cultured 12 d blastocysts in a time-dependent manner over 48 h. Plasminogen activator levels in the uterine flushings were high at the beginning and end of the estrous cycle and low during the luteal phase. In uterine flushings from pregnant animals, PA levels peaked between 10 to 12 d and remained low thereafter. In cycling gilts, the presence of an uterine PA inhibitor was also demonstrated. By assaying flushings from ovariectomized gilts administered daily injections of progesterone, estrogen, progesterone and estrogen, or corn oil, Mullins et al. (1980) substantiated that the PA inhibitor was progesterone-induced and active

against both urokinase and PA produced by d 12 conceptuses. Because the placentation of the pig blastocyst is not invasive, Mullins et al. (1980) concluded that the progesterone-induced inhibitor may be involved in controlling PA induced proteolysis in the uterus. Fazleabas et al. (1983) investigated isolated pig blastocysts between 10 and 16 d of gestation (where onset of estrus was designated as d 0) and measured PA production using the  $^{125}\text{I}$ -fibrin plate assay. Changes in PA release by cultured 13 to 16 d filamentous blastocysts before and after washing were evaluated. On d 13, PA production was low in both washed and unwashed blastocysts. Washed blastocysts on d 14 and 15 produced relatively large amounts of PA, whereas the unwashed blastocysts produced very little PA. Large quantities of PA were released by both washed and unwashed blastocysts on d 16 of gestation. The variable results obtained due to washing suggested that variable quantities of a PA inhibitor were associated with the blastocysts. Plasminogen activator production by washed blastocysts at 6, 12, 24, 36 and 48 h of culture was also examined. Plasminogen activator was released into the medium in a time-dependent fashion and was linear in most cultures for at least 24 h. Plasminogen activator release by d 10 to 16 washed blastocysts, which ranged in morphology from spherical to filamentous, was also examined. Plasminogen activator production was similar among spherical, tubular and elongated blastocysts (d 10 to 11.5). The DNA content per blastocyst remained fairly constant and cell number was not greatly increased during this period. In the early filamentous stage (d 12 and 13) PA release into the medium was very low. Marked increases in PA release were associated with large increases in DNA content as observed in filamentous (d 14 to 16) blastocysts. The quantity

of plasminogen in uterine flushings was elevated on d 12; however, on d 14 to 16 little or no plasminogen was detected. Quantities of plasminogen ( $\mu\text{g}/\text{uterine horn}$ ) were calculated to accurately determine the amount of plasminogen present on each day. On d 10 to 12 high levels of plasminogen were detected; however, low levels were detected between d 14 and 16. Inhibition of plasmin activity by uterine flushings on d 10.5, 11.5, 13 and 16 were studied. Between d 10.5, when the blastocysts were spherical, and d 12 when the blastocysts became filamentous, the quantity of plasmin inhibitor activity rose 7-fold; however, after d 13, plasmin inhibitor activity dropped drastically to less than one-fifth the previous levels. In the nonpregnant gilt, a similar release of plasmin inhibitor can be initiated by administering an i.m. injection of estradiol valerate on d 11 of the estrous cycle. Uterine flushings were recovered at 1, 4, 12, 24, 48 and 72 h after either estradiol valerate or corn oil injection. In the estrogen-treated animals, more protein and plasmin inhibitor were recovered over the 72 h period than in corn oil-treated gilts. A significant difference in plasmin inhibitor levels were observed between 4 and 24 h post-injection. Between 24 and 72 h there was a 6- to 10-fold increase in the total amount of plasmin inhibitor recovered from the flushings of estrogen-treated gilts when compared to flushings from corn oil-injected gilts. Based on these results, Fazleabas et al. (1983) suggested that estrogen release by the elongating (d 12) blastocyst triggers a complex secretory response in the encompassing endometrium which results in an adjustment of the uterine environment to reconcile with the growing and potentially invasive embryo. Estrogen production by the blastocyst, during a period of time when blastocysts are releasing PA

and uterine plasminogen is available, causes the endometrium to liberate a plasmin inhibitor to control the embryonic-induced proteolysis. Fazleabas et al. (1983) reported that pig embryos experienced a biphasic pattern of PA production, where the initial phase corresponds to blastocyst elongation (d 10 to 12) and the second phase corresponds to a time when DNA content of the embryo is increasing (d 14 to 16). Fazleabas et al. (1983) further indicated that the first phase of PA production was attributed to tissue remodelling, whereas the second phase corresponded to tissue proliferation.

Bovine embryos. Menino and Williams (1987) evaluated PA production and plasminogen conversion to plasmin in cultured 5 to 6 d bovine embryos (onset of estrus was designated as d 0) using a caseinolytic agar gel assay. Plasminogen activator production was low or undetectable until the blastocyst stage increased during blastocoelic expansion and initiation of hatching, and remained high throughout and after hatching. Plasminogen conversion to plasmin was detected in all media containing plasminogen. Embryonic activation of plasminogen, for all plasminogen levels (15, 30, 60 and 120  $\mu\text{g/ml}$ ), was similar to the PA profile and was lowest during blastocyst formation. Plasminogen conversion to plasmin increased markedly between the blastocyst and expanding blastocyst stages and plateaued during hatching and thereafter. Embryos cultured in medium with 120  $\mu\text{g/ml}$  plasminogen expanded and hatched sooner than embryos developing in 0  $\mu\text{g/ml}$  plasminogen; however, no differences were observed in the number of embryos hatching due to the level of plasminogen in the medium. Menino and Williams (1987) concluded that plasminogen activation to plasmin may serve as a mechanism for the bovine blastocyst to effect a sublysis of the zona pellucida and thereby facilitate hatching.

## Materials and Methods

Fifteen crossbred beef cows were estrous-synchronized with two 25-mg injections of prostaglandin  $F_{2\alpha}$ <sup>1</sup> ( $PGF_{2\alpha}$ ) administered i.m. 14 d apart (Day 0=first  $PGF_{2\alpha}$  injection) and superovulated with twice-daily i.m. injections of porcine follicle-stimulating hormone<sup>2</sup> at dosages of 5, 4, 2 and 1 mg on Days 12, 13, 14 and 15, respectively (Menino and Williams, 1987). Estrus detection was started 24 h after the second  $PGF_{2\alpha}$  injection and conducted at 12-h intervals thereafter. Cows exhibiting estrus were naturally mated, using one of two bulls, at the beginning of estrus and at 12-h intervals until the cow would no longer accept the bull. Cows were nonsurgically flushed 7 d after mating with Dulbecco's phosphate buffered saline (PBS; Dulbecco and Vogt, 1954) containing .5% bovine serum albumin<sup>3</sup> (BSA) and 10 ml/l of an antibiotic-antimycotic solution<sup>3</sup> (10,000 units penicillin, 10 mg streptomycin and 25  $\mu$ g amphotericin B per ml in .9% sodium chloride). Uterine flushings were examined using a dissecting microscope for the presence of embryos. Late morulae to blastocysts were collected from the flushings by aspiration and washed three times in several microdrops of Ham's F-12<sup>3</sup> with 15 mg/ml BSA under paraffin oil<sup>4</sup>. Embryos were morphologically evaluated with an inverted stage-phase contrast microscope using the grading system described by Lindner and Wright (1983). Embryos were individually cultured in 25- $\mu$ l microdrops of

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<sup>1</sup>Lutalyse<sup>®</sup>, The Upjohn Co., Kalamazoo, MI.

<sup>2</sup>Schering Corp., Kenilworth, NJ.

<sup>3</sup>Sigma Chemical Co., St. Louis, MO.

<sup>4</sup>Fisher Scientific Co., Tustin, CA.

Ham's F-12 containing 15 mg/ml BSA under paraffin oil in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C for 6 d. At the initiation of culture and at subsequent 24-h intervals, embryonic cell stage was recorded and overall embryo diameter (OD) and zona pellucida thickness (ZPT) were measured with an ocular micrometer affixed to the right eyepiece of the microscope (Lindner and Wright, 1978). Overall embryonic diameter included the embryo proper and the zona pellucida, until the embryo hatched. Overall embryonic diameter post-hatching was the diameter of the hatched blastocyst for the remaining culture period. After hatching was completed, a final measurement of the zona pellucida thickness was recorded. Measurements of overall embryonic diameter and zona pellucida thickness were based on one micrometer unit equalling 38.2  $\mu$  (magnification X200). At 24-h intervals, embryos were transferred to fresh microdrops and 15  $\mu$ l of the culture medium were recovered and frozen at -20°C until assayed for PA. To correct for spontaneous activation of plasminogen due to the culture conditions, media from drops not containing embryos were also recovered and frozen. At termination 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 hematoxylin and eosin.

Concentrations of PA in the culture medium were determined using a caseinolytic assay (Menino and Williams, 1987) with modifications. The modifications consisted of preparing the casein-agar solution after the method described by Bjerrum et al. (1975) where warmed 2% nonfat dry milk<sup>5</sup>,

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<sup>5</sup>Carnation Co., Los Angeles, CA.

dissolved in .038 M tris (hydroxymethyl)-aminomethane-.10M glycine buffer containing .195 g/l  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  and .325 g/l sodium azide, was combined with an equal volume of 2% agarose<sup>3</sup>. Fifteen milliliters of the warmed mixture were pipetted into 85 x 65 x 2 mm plastic diffusion plates for use in the PA assay. Also, 15  $\mu\text{l}$  of 120  $\mu\text{g}/\text{ml}$  human plasminogen<sup>3</sup> as the substrate was combined with 15  $\mu\text{l}$  of either recovered culture medium or 0, .0009, .0045, .0091 and .0455 milliunits/ml urokinase<sup>3</sup> (E.C. 3.4.21.7) as the standard and incubated for 15 min at 37°C. Following incubation, 25  $\mu\text{l}$  of the incubated mixture were pipetted into 4 mm diameter wells cut in a casein-agar gel plate and incubated at room temperature for 24 h. The casein-agar gel plates were fixed for 15 min with 3% acetic acid, rinsed in tap water and the diameters of the caseinolytic zones measured with an electronic digital caliper. Concentrations of PA in the culture medium were determined by equation of the line calculations for caseinolytic ring diameter by log urokinase concentration. The amount of PA produced by the embryo was determined by subtracting the amount of PA in the medium without an embryo from the amount in the medium with an embryo for a 24-h interval. Preliminary experiments using human plasminogen as the substrate demonstrated a 10-fold increase in sensitivity compared to porcine plasminogen (Menino and Williams, 1987).

Changes in PA production, overall embryonic diameter and zona pellucida thickness per 24-h interval for each embryo were plotted over the culture period and the resultant graphs cut out and weighed. Cell stages that embryos attained in culture were assigned the following developmental codes: morula to early blastocyst=1, blastocyst=2, expanded blastocyst=3, hatching blastocyst=4 and hatched blastocyst=5. Embryonic

cell numbers were determined from a mean of three counts of the stained nuclei using bright field microscopy (magnification X100-320). Analysis of variance and Fisher's least significant difference were used to detect differences by cell stage for overall diameter, zona pellucida thickness and PA production for bovine embryos developing in vitro. Differences in total PA production, changes in embryonic size and zona pellucida thickness, and cell number due to cow source and development to a particular cell stage were determined by analysis of variance and orthogonal contrasts. Correlation-regression analysis was conducted to determine the relationship between total PA production and changes in embryonic size and zona pellucida thickness, cell number and developmental cell stage.



## Results

A total of 174 ova were recovered from 13 cows with ova and embryo recovery rates of  $13.4 \pm 2.8$  and  $9.6 \pm 1.8$ , respectively. Morphologically normal embryos were recovered from 11 cows for a recovery rate of  $7.5 \pm 1.6$  embryos. Sixty-one of 80 (76%) embryos completed the hatching process in vitro. Differences in overall diameter were not observed at the morula, early blastocyst (Figure 1) and blastocyst (Figure 2) stages; however, overall diameter increased when blastocoealic expansion, hatching and completion of hatching occurred ( $P < .05$ ; Table 1). No difference in overall diameter was observed among hatched blastocysts (Figure 3) except between 24 and 48 h after hatching. Mean zona pellucida thickness was larger for morulae, early blastocysts and blastocysts than for other cell stages ( $P < .05$ ). Zona pellucida thickness was similar for hatching and hatched blastocysts; however, expanded blastocysts possessed the thinnest zonae pellucidae ( $P < .05$ ). The amount of PA produced was similar for the morula, early blastocyst, blastocyst, expanded blastocyst and hatched blastocyst stages at 72- and 96-h post-hatching ( $P > .05$ ). Plasminogen activator production did not differ between hatching blastocysts and blastocysts at 72-h post-hatching or between hatching blastocysts and blastocysts 48 h after hatching ( $P > .05$ ). The greatest amount of PA production occurred at hatching and 24-h post-hatching ( $P < .05$ ).

Total PA production and changes in overall diameter and zona pellucida thickness, expressed as graph weights, and cell numbers for embryos developing up to the morula to early blastocyst, blastocyst, expanded blastocyst, hatching blastocyst and hatched blastocyst stages

are reported in Table 2. Embryos that developed to hatched blastocysts produced greater amounts of PA than embryos that remained as morulae to early blastocysts, or developed to blastocysts and hatching blastocysts ( $P < .05$ ); however, differences in total PA production could not be detected between embryos that developed to expanded and hatched blastocysts. Cell numbers were greater for hatched blastocysts (Figure 4) than blastocysts, expanded blastocysts and hatching blastocysts ( $P < .05$ ). Analysis for the number of nuclei in the morula to early blastocyst stage could not be determined due to this group being composed of a single embryo. Changes in overall diameter were greater for hatched blastocysts than morulae to early blastocysts, blastocysts, expanded blastocysts and hatching blastocysts ( $P < .05$ ). Changes in zona pellucida thickness differed in expanded and hatched blastocysts compared to morulae and early blastocysts ( $P < .05$ ); however, differences in changes in zona pellucida thickness could not be detected among blastocysts, expanding blastocysts, hatching blastocysts and hatched blastocysts, or among morulae to early blastocysts, blastocysts and hatching blastocysts ( $P > .05$ ). Cow source exerted a significant effect on changes in overall diameter, zona pellucida thickness, developmental cell stages, cell numbers and the amount of plasminogen activator liberated by bovine embryos developing in vitro (Table 3).

Total plasminogen activator production was positively correlated to changes in overall diameter, developmental stage and cell number and negatively, but weakly, correlated to changes in zona pellucida thickness (Table 4). Positive correlations were observed for changes in overall diameter with cell stage and cell number. Changes in zona pellucida

thickness were negatively correlated with changes in overall diameter, cell stage and cell number. Cell number was positively correlated to cell stage validating that more nuclei were present with advanced stages.

TABLE 1. OVERALL DIAMETER (OD), ZONA PELLUCIDA THICKNESS (ZPT) AND PLASMINOGEN ACTIVATOR PRODUCTION (PA) FOR BOVINE EMBRYOS DEVELOPING IN VITRO

	Cell stage <sup>a</sup>										Error mean square
	M	EB1	B1	X	I	H(0)	H(1)	H(2)	H(3)	H(4)	
OD ( $\mu$ )	156.0 <sup>b</sup>	157.8 <sup>b</sup>	163.7 <sup>b</sup>	185.7 <sup>c</sup>	215.0 <sup>d</sup>	254.6 <sup>e,f</sup>	264.2 <sup>f</sup>	249.2 <sup>e</sup>	262.1 <sup>e,f</sup>	260.2 <sup>e,f</sup>	1396.9
ZPT ( $\mu$ )	11.7 <sup>b</sup>	11.6 <sup>b</sup>	11.7 <sup>b</sup>	6.3 <sup>c</sup>	8.1 <sup>d</sup>	7.4 <sup>d</sup>	-	-	-	-	5.8
PA ( $\text{mU}\cdot\text{ml}^{-1}\cdot\text{d}^{-1}$ )	1.0 <sup>b</sup>	1.7 <sup>b</sup>	1.9 <sup>b</sup>	12.4 <sup>b</sup>	24.2 <sup>c,d</sup>	39.7 <sup>e</sup>	45.9 <sup>e</sup>	24.4 <sup>d</sup>	12.4 <sup>b,c</sup>	6.0 <sup>b</sup>	683.9

<sup>a</sup>Cell stages are abbreviated as morula (M), early blastocyst (EB1), blastocyst (B1), expanding blastocyst (X), hatching blastocyst (I) and hatched blastocyst (H) at 0 (0), 24 (1), 48 (2), 72 (3) and 96h (4) after hatching.

<sup>b,c,d,e,f</sup>Values in the same row without common superscripts are different ( $P < .05$ ).

TABLE 2. PLASMINOGEN ACTIVATOR PRODUCTION (PA) AND CHANGES IN OVERALL DIAMETER (OD) AND ZONA PELLUCIDA THICKNESS (ZPT) EXPRESSED AS GRAPH WEIGHTS (g), AND CELL NUMBERS (CN) FOR BOVINE EMBRYOS DEVELOPING TO VARIOUS CELL STAGES IN VITRO

Parameter	Cell stage					Error mean square
	Morula-early blastocyst	Blastocyst	Expanded blastocyst	Hatching blastocyst	Hatched blastocyst	
PA (g)	.009 <sup>a</sup>	.029 <sup>a</sup>	.114 <sup>a,b</sup>	.044 <sup>a</sup>	.140 <sup>b</sup>	.007
CN (nuclei)	24.0	37.8 <sup>a</sup>	84.1 <sup>a</sup>	65.8 <sup>a</sup>	236.5 <sup>b</sup>	13141.4
OD (g)	.237 <sup>a</sup>	.229 <sup>a</sup>	.266 <sup>a</sup>	.259 <sup>a</sup>	.341 <sup>b</sup>	.002
ZPT (g)	.175 <sup>a</sup>	.138 <sup>a,b</sup>	.130 <sup>b</sup>	.135 <sup>a,b</sup>	.123 <sup>b</sup>	.001

<sup>a,b</sup>Values in the same row without common superscripts are different (P<.05).

TABLE 3. RESULTS OF ANALYSES OF VARIANCE EVALUATING THE EFFECTS BETWEEN INDIVIDUAL COWS ON CHANGES IN OVERALL DIAMETER (OD) AND ZONA PELLUCIDA THICKNESS (ZPT), CELL STAGES (CS), CELL NUMBERS (CN) AND PLASMINOGEN ACTIVATOR PRODUCTION (PA) FOR CULTURED BOVINE EMBRYOS

Parameter	F-ratio	Probability
OD	4.67	<.01
ZPT	10.29	<.01
CS	2.13	.04
CN	3.29	.01
PA	5.03	<.01

TABLE 4. CORRELATION COEFFICIENTS (r) FOR CHANGES IN OVERALL DIAMETER (OD) AND ZONA PELLUCIDA THICKNESS (ZPT), CELL STAGES (CS), CELL NUMBERS (CN) AND PLASMINOGEN ACTIVATOR PRODUCTION (PA) FOR BOVINE EMBRYOS DEVELOPING IN VITRO

X-value	Y-value	r	Probability
OD	ZPT	-.12	.278
	CS	.63	<.001
	CN	.79	<.001
	PA	.40	<.001
ZPT	CS	-.26	.021
	CN	-.23	.068
	PA	-.13	.267
CS	CN	.54	<.001
	PA	.35	.001
CN	PA	.35	.005



Figure 1. Early blastocyst collected 7 d after mating (320X).





Figure 2. Blastocyst collected 7 d after mating (320X).

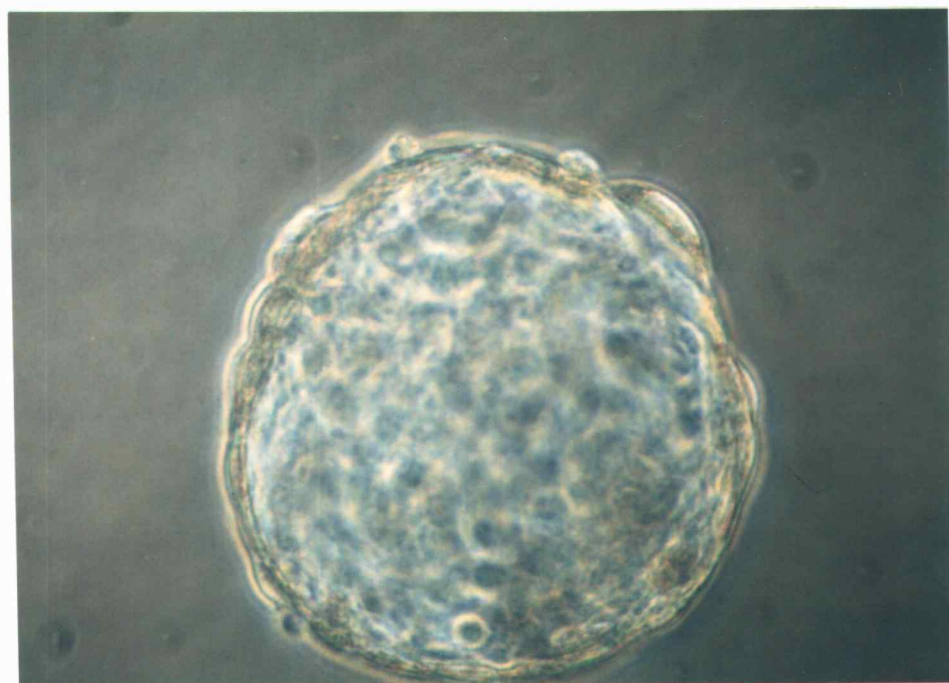


Figure 3. Hatched blastocyst that developed from an early blastocyst after 4 d of culture (320X).

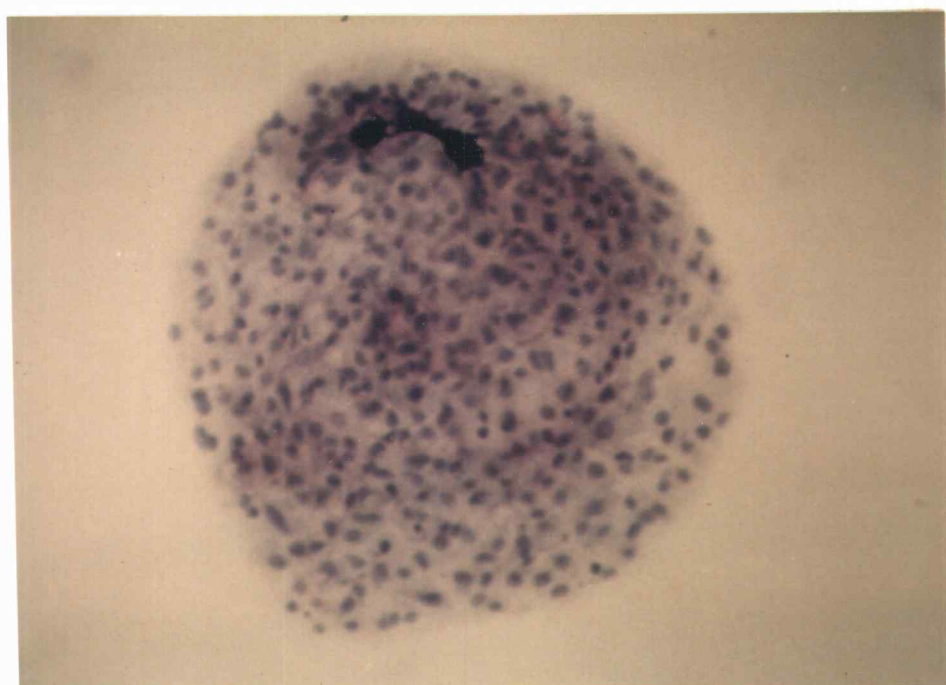


Figure 4. Hatched blastocyst stained with hematoxylin and eosin after 6 d in culture (160X).

## Discussion

Overall diameters and zona pellucida thicknesses of bovine blastocysts were similar to measurements reported by Linares and King (1980) and Renard and Heyman (1979). Sizes of d 10 and 11 embryos collected by Renard and Heyman (1979) were similar to embryos of equivalent gestational age cultured in the present study; however, d 11 embryos cultured for 24 h and d 12 embryos either freshly collected or cultured for 24 h were 2- to 3-fold greater in overall diameter than embryos of comparable gestational age. Similar size differences between cultured embryos and embryos developing in vivo have been reported in swine (Lindner and Wright, 1978). Lindner and Wright (1978) observed marked differences in overall diameter in expanded blastocysts and to a lesser extent in hatched blastocysts developing in vivo and in vitro. Measurements of zona pellucida thickness paralleled those reported by Lindner and Wright (1978) where the zona pellucida was thinnest at the expanded blastocyst stage, validating the elasticity of the zona pellucida. Size differences between embryos developing in vivo and in vitro suggest the uterine environment provides some nutritive or growth factors that are absent in vitro. Jung and Fischer (1988) observed high correlations in embryonic volume with DNA and protein synthesis in noncultured rabbit blastocysts and with rabbit blastocysts cultured in uterine flushings; however, changes in volume in rabbit blastocysts cultured in serum-supplemented medium were poorly correlated to DNA and protein synthesis. Jung and Fischer (1988) have suggested that the uterine components are involved in trophoblastic transport which stimulates blastocoealic expansion.

The pattern of PA production was similar to that reported by Menino and Williams (1987) with the exception of peak levels of PA associated with hatched blastocysts or blastocysts 24 h after hatching. Menino and Williams (1987) cultured d 5 to 6 embryos and observed that peak PA levels were associated with blastocysts initiating the hatching process; however, if the periods between blastocyst formation and peak PA levels are examined similar intervals of 120 to 144 h are observed for the two studies. Quantities of PA produced by the embryo were also 2- to 4-fold lower in this study compared to the report by Menino and Williams (1987). Part of this difference can be readily attributed to the volume of the microdrops used because the present study cultured one embryo in 25  $\mu$ l whereas Menino and Williams (1987) cultured 4 to 6 embryos in 50  $\mu$ l. The embryo to microdrop volume ratio may also have contributed to discrepancies between the two studies. The greater volume of medium may have caused a sparing effect on the embryo to inhibit the production or release of PA. Alternatively, the greater the number of embryos per microdrop may be synergistic and stimulate the release of PA. Some evidence exists which suggests that the embryo to microdrop volume ratio or the number of embryos in a microdrop can affect in vitro development. Wright et al. (1978) observed a subtle effect on hatching in mice with more embryos cultured per microdrop. Menino and Wright (1982) observed differences in cleavage indexes between swine embryos cultured in 50- $\mu$ l microdrops and tubes with 4.0 ml of medium. Significant differences in total PA produced could not be detected between expanded blastocysts and hatching blastocysts or between expanded blastocysts and hatched blastocysts, yet hatched blastocysts produced more total PA than hatching

blastocysts. Because only four embryos developed to the hatching blastocyst stage the number of observations may be limiting; however it is possible that these embryos could not sustain PA production. Similar PA production between expanded and hatched blastocysts suggests that although the expanded blastocysts did not hatch these embryos may have remained viable throughout culture and continued to produce PA. Plasminogen activator production was positively correlated to cell number, cell stage and overall diameter suggesting that PA secretion may be an indicator of embryo viability. Validation of the relationship between PA and embryo viability would be the production of live offspring by embryos that liberate substantial quantities of PA.

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