

## Plasminogen Activator Activity in the Bovine Oocyte-Cumulus Complex and Early Embryo

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**ABSTRACT.** In this study fibrinolytic assay systems were used to assess the plasminogen activator (PA) potential and plasmin generating ability of oocyte-cumulus complexes isolated from preovulatory bovine follicles (2–8 mm diameter) and of fertilized oocytes from the day of fertilization up to, and including, the hatched blastocyst stage (day 12). During embryo development, the culture medium was changed every 24 hr and samples examined for PA activity. Irrespective of the stage of maturity, no plasminogen or PA could be detected in unfertilized oocytes from which the cumulus layer had been removed. Both plasminogen and PA were found in the cumulus layer indicating that this, rather than the oocyte, was the source of these proteins in the oocyte-cumulus complex. Following oocyte fertilization, no PA activity was detected in either the developing embryo or in the culture medium before day 7. When the embryos had developed to the expanded blastocyst stage, days 7–8, PA production began with activity being detected in both the embryos and their culture medium. Between days 8 and 12, when embryos had reached the hatched blastocyst stage, the PA activity had increased significantly ( $p < 0.05$ ). Analysis of the culture media confirmed this increase in production of PA activity and, based on zymography, it was estimated that the molecular weight of the PA was 78 k daltons. — **KEY WORDS:** bovine, embryo, ovarian follicle, plasminogen activator.

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The plasminogen activator/plasmin system is now recognized to possess several physiological roles, including the regulation of extracellular matrix turnover and the activation of latent forms of certain growth hormones, in addition to its role in fibrinolysis [27]. Plasmin generating properties have been identified in or on the plasma membranes of endothelial cells, blood cells, neuronal cells and tumor cells [10]. Extracellular proteolysis by this system also appears to be involved with the tissue remodelling and cell migration associated with ovulation, implantation of early embryos and angiogenesis [20, 21, 25].

The proteolytic cascade of the plasmin system is initiated by plasminogen activators (PAs) which are serine proteases that act on the zymogen, plasminogen, converting it to the active form, plasmin. Two immunologically distinct activators have been identified: urokinase-type PA (u-PA) which is secreted as an inactive single chain molecule of 54 k daltons and tissue-type PA (t-PA) which is secreted in an active form with a molecular weight of around 70 k daltons [20, 27]. The u-PA tends to be localized on cell surfaces by the u-PA receptor while t-PA can interact with a number of surfaces such as fibrin, histidine-rich glycoproteins as well as cell membranes [10]. In both ovarian follicular fluid and cultured granulosa cells from women, rats and hens, t-PA is the predominant form of PA present [3, 7, 12, 15, 18, 22, 23, 28]. High levels of PA and plasminogen have also been reported in fluid from preovulatory follicles in the mare and cow [29, 30]. On the basis of these and other findings, it has been proposed that the PA system plays an important role in the process of ovulation [20].

It has shown that mouse gametes express plasminogen dependent proteolytic activities and that ovulated eggs contain and secrete t-PA [11]. Using the murine model, the expression of PAs by invasive embryonic cells *in vivo* has been documented in trophoblast cells which produce PA activity in a temporal pattern consistent with a role for this system in the endometrial disruption accompanying both implantation and early embryonic growth [27]. A role for the plasmin generating system appears to continue through pregnancy since it has been shown that in women, with complicated pregnancies, there is a correlation between both PA activity and specific PA inhibitory activity with both placental weight and infant birth weight [14].

Both ovine and bovine fertilized oocytes, cultured for 12–14 days have been shown to possess PA activity [2, 6, 13, 16, 17]. Based on the data from the murine studies, this would suggest that the PA/plasmin system also has a role to play in embryo development, an area of considerable importance in embryo transfer technology. Since there appears to be no information in the literature correlating PA activity in oocytes and embryos derived from the same animal, this study was designed to examine the development of PA activity in bovine embryos from the immature oocyte-cumulus complex stage through the hatched blastocyst stage. The PA activity was assessed by the extent of fibrin lysis induced by plasminogen conversion to plasmin using both fibrin slide and fibrin plate methods. The presence of PA activity was confirmed by electrophoresis of culture medium with both purified PA and molecular mass standards followed by the overlaying of fibrin-agarose gel plates on the polyacrylamide gels (zymography).

## MATERIALS AND METHODS

**Collection and preparation of ovaries:** Reproductive tracts were obtained from mature Holstein cows at a local slaughter house. The ovaries were transported to the laboratory in 0.85% NaCl at 30°C within 2 hr of slaughter. Oocytes were aspirated from small antral follicles (2–8 mm in diameter) with a 21 × 5/8" gauge needle attached to a 10 ml plastic syringe. The aspirated material was transferred to a plastic test tube and the cumulus-oocyte complexes allowed to sediment under gravity for 10 min at 37°C. The sedimented complexes were transferred to a petri dish using a glass micropipette and intact cumulus-oocyte complexes selected on the basis of microscopic examination. Only intact cumulus-oocyte complexes exhibiting a compact, multilayered cumulus were utilized in this study. The fibrinolytic activity was determined in a group of these oocytes and the remainder were used for the maturation-fertilization experiment.

**In vitro oocyte maturation, fertilization and embryo culture:** Ten of the selected cumulus-oocyte complexes were cultured together in Medium 199 with Earle's salts (Gibco-BRL, NY, U.S.A.) supplemented with 10% heat-treated calf serum (CS, Gibco-BRL, NY, U.S.A.), 0.2 mM Na pyruvate, 0.12 IU/ml porcine follicle stimulating hormone (Antrin, Denka Seiyaku, Japan) and 50 µg/ml gentamicin sulfate (Sigma Chem. Co., MO, U.S.A.) at 38.5°C under 5% CO<sub>2</sub> humidified air for 22 hr. The matured oocytes were transferred to a medium consisting of Hepes-modified Tyrode's buffer (mTALP) supplemented with 10 mg/ml bovine serum albumin (BSA, A-6003, Sigma Chem. Co., MO, U.S.A.), 10 µg/ml heparin and 5 mM caffeine for fertilization. Several of the matured complexes were separated for determination of fibrinolytic activity and the remainder were used for fertilization and embryo development.

Groups of ten matured oocyte-cumulus complexes were fertilized by combination with bovine spermatozoa (5 × 10<sup>6</sup>/ml). The spermatozoa were prepared by thawing, washing twice with BSA-free mTALP with centrifugation at 500 × g for 5 min between each wash, and resuspension in BSA-free mTALP with 10 mM caffeine. Six hr after insemination, each group of 10 fertilized oocytes was introduced into a 100 µl drop of Medium 199 with 10% CS under mineral oil (Squibb, NJ, U.S.A.) and cultured at 38.5°C under 5% CO<sub>2</sub> in humidified air with daily changes of medium. At 24 hr intervals, up to 12 days post insemination, fertilized embryos were harvested for the determination of fibrinolytic activity and the medium collected and stored in small aliquots at –80°C. Six to 7 days after insemination the embryos were determined to be at the morulae and blastocyst stage. The cumulus-cell layer was removed from a group of these embryos which were then cultured for a further 24 hr. At the end of this period the stage of development of the embryos was recorded, the embryos separated from the culture media, and the media frozen in aliquots at –80°C.

**Determination of fibrinolytic activity:** (a) Fibrin slide

method. The fibrinolytic activity of the immature and mature oocyte-cumulus complexes, the fertilized oocytes and the cumulus-free oocyte (oocyte/embryo) was determined by a modification of Todd's fibrin slide method [26]. Briefly, each oocyte/embryo was washed three times with 0.85% NaCl before being placed on a 57 × 26 mm glass slide which had been covered with 20 µl of bovine thrombin (50 units/ml, Mochida Pharmaceut. Co., Ltd., Japan). The glass slide was then overlaid with 100 µl of 0.5% bovine fibrinogen (Daiichi Pure Chem. Co., Japan) in 0.07 M phosphate buffer, pH 7.4 containing 1.0% NaCl. Two different types of fibrinogen solution were used: half of the oocyte/embryo preparations were covered with plasminogen rich fibrinogen (Type 1) and the other half covered with plasminogen free fibrinogen (Type 2). The glass slides were then placed horizontally in a moist chamber at 37°C for up to 4 hr to allow fibrin formation to occur on the slide and fibrinolysis to develop around the oocyte/embryos. To stop the fibrinolytic reactions, the glass slides were placed in a chamber filled with formalin gas for 12 hr. The slides were then stained with Harris's hematoxylin solution for 15 min, destained under running water for 15 min and then mounted with Bioleit (Koken, Tokyo, Japan).

(b) The fibrin plate method was used to determine the fibrinolytic activity in the medium collected from the 24 hr culture of the cumulus-free embryos and in the medium collected from the fertilized embryos at days 2–3 and day 7–8 of culture. Fibrin plates were prepared with plasminogen free bovine fibrinogen as previously described [29]. Before application of the media samples to the plate 5 µl of the sample was mixed with 10 µl of bovine plasminogen (0.25 units/ml, Sigma Chem. Co., MO, U.S.A.) and either urokinase (UK, 25 IU/ml, Green cross, Osaka, Japan) or 5 µl of tissue plasminogen activator (t-PA, Ak-124, Kowa Co., Tokyo, Japan). This mixture was spotted, in duplicate, on the fibrin plate and the plate incubated at 37°C in a humidified chamber for 18 hr. The area of lysis around each spot was measured in mm<sup>2</sup> and the average of the duplicate spots used to assess the fibrinolytic activity of the sample. A minimum of 20 assays on each sample of media were completed.

**Zymography:** Plasminogen activator (PA) activity in the culture media of the fertilized embryos was assessed by a combination of one-dimensional sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions and a modification of the zymographic method of Granelli-Piperno and Reich [9]. Briefly, a 60 µl sample of medium was applied to 7.5% polyacrylamide gel and electrophoresis conducted for 4 hr. The gels were then washed with a 2.5% solution of Triton X-100 (Sigma Chem. Co., MO, U.S.A.) for 90 min to remove the SDS, rinsed with H<sub>2</sub>O and blotted to remove excess solution. The gel was then applied to the surface of a fibrin-agar plate (14 × 16 × 0.15 cm) prepared with a mixture of 15 ml agarose (2.5%, type L, Behring, Germany), 15 ml plasminogen rich fibrinogen (0.4%, human, Chromogenix AB, Sweden) and 10 µl thrombin (50 IU/ml, bovine, Mochida, Tokyo, Japan). The polyacrylamide gel

with the agarose underlay was incubated in a moist chamber at 37°C for 5 hr. The gel was then dried at 37°C for 24 hr and stained with 0.5% Coomassie Brilliant Blue R-250. The gels were destained with ethanol/acetic acid (25%–8%) and dried. As controls, t-PA and molecular weight markers (LMW Kit E, Pharmacia, Sweden) were run in parallel with the media samples.

## RESULTS

A preliminary experiment was conducted with the fibrin slide method to determine the effect of the time of exposure of fertilized embryos, at different stages of development, on the extent of lysis. In the plasminogen-rich fibrin slides, lysis was observed in all samples of embryos at the blastocysts stage up to the hatched blastocyst stage after 4–6 hr of incubation (Table 1). In the plasminogen-free fibrin slides no lysis was observed after 6 hr of incubation and only trace lysis were visible in four of the six samples when the incubation was extended up to 10 hr. Since no lysis was detected in the less developed 16 cell embryos even after 32 hr of incubation irrespective of whether plasminogen was present or absent in the sample (Table 1), it was concluded that a 4 hr incubation time would be adequate to detect fibrinolytic potential in the developing embryos. The fibrinolytic potential of the unfertilized and fertilized embryos was compared in fibrin plates prepared with and without plasminogen (Table 2). Fibrinolysis was consistently observed in both the immature and matured oocytes when cumulus layer was also present. When the cumulus cells and oocytes were evaluated separately only the cumulus cells exhibited the ability to induce lysis on the fibrin plate (Table 2). As had been observed in the preliminary experiment with fertilized embryos, the extent of fibrinolysis was dependent on both the stage of embryonic development and on the presence of plasminogen in the fibrinogen solution (Table 2). In the absence of plasminogen less than 20% of the embryos examined exhibited fibrinolytic activity even when they had developed to the expanded-hatched blastocyst stage (day 8–12). By this stage of development greater than 95% of the embryos exhibited lysis when plasminogen was present (Table 2).

To further evaluate the PA activity secreted by the developing embryos, the culture media collected from each stage of development of the fertilized oocytes was examined by the fibrin plate method for the presence of PA activity. Fibrinolytic activity was detected in all samples of media collected from the fertilized oocytes but no activity was detected in the control samples containing fresh, unused media (Table 3). The activity in the media samples collected from the embryos at the 4 to 8 cell stage exhibited a four fold increase in the lysis area when t-PA was included in the incubation mixture compared to those samples supplemented with urokinase (Table 3). This difference was consistent in all media samples tested. In both the fibrin slide and fibrin plate assays the level of activity in media collected from the expanded blastocyst to hatching-hatched blastocyst stage (days 8–12) was similar and

significantly greater ( $p < 0.05$ ) than the activity at the 4–8 cell stage (days 2–3). These results suggest that the activity present in the culture media is, at best, a weak activator of urokinase induced plasmin formation but is effective in augmenting t-PA induced plasmin formation. To confirm this conclusions, the culture medium collected from the fertilized embryos at days 8–12 was subjected to zymography (Fig. 1). Media from the expanded blastocyst stage exhibited a single weak band of activity which migrated at the 78 k dalton region, while media collected from the hatching-hatched blastocyst stage showed a single strong band of activity in the same region. Activity was only found in culture media, not in control media, and only at a location close to that of the 74 k dalton observed for the purified t-PA (Fig. 1).

## DISCUSSION

It has been shown that follicular fluid aspirated from bovine preovulatory ovarian follicles contains both

Table 1. Effect of incubation time on the extent of lysis induced by fertilized oocytes on a fibrin slide

Stage of development	Incubation hr	Number of samples exhibiting lysis	
		Plasminogen free fibrin	Plasminogen rich fibrin
16 cell	32	0 (7)	0 (7)
Blast-Hatch	4–6	0 (5)	6 (6)
Blast-Hatch	8–10	4 (6)*	6 (6)

16 cell: embryos collected after 2–3 days of culture; Blast-Hatch: embryos collected at days 6–7.

Number of samples exhibiting lysis: compared to the total number, in brackets, of samples analysed.

\* Only trace lysis observed.

Table 2. Fibrinolytic activity in unfertilized and fertilized oocytes evaluated by the fibrin slide method

Sample	Number of samples exhibiting lysis*	
	Plasminogen free fibrin	Plasminogen rich fibrin
Intact immature oocyte	9 (10)	10 (10)
Cumulus cells alone	5 (5)	5 (5)
Oocyte alone	0 (5)	0 (5)
Intact mature oocyte	9 (10)	10 (10)
Cumulus cells alone	5 (5)	5 (5)
Oocyte alone	0 (5)	0 (5)
**Fertilized oocyte Day 1	0 (10)	0 (10)
2	0 (10)	1 (10)
3	0 (10)	1 (10)
6	1 (10)	0 (10)
7	1 (15)	2 (14)
8	0 (12)	12 (12)
9	2 (16)	19 (20)
12	2 (11)	12 (12)

\* Number of samples exhibiting lysis compared to the total number, in bracket, of samples analysed.

\*\* Day refers to the number of days in culture.

plasminogen and PA activity [1] and that the content of these proteins is higher than that found in circulating plasma [30]. The proteins found in ovarian follicular fluid are believed to be derived from two sources: transudates of plasma and secretory products of the cumulus cells [8, 24]. The results of this study confirm that the cumulus cells, but not unfertilized oocytes, are capable of producing and secreting both PA activity and plasminogen. Rat granulosa cells in culture produce fibrinolytic activity predominantly

Table 3. Plasminogen activator activity observed in culture medium with the fibrin plate method

Stage of development	Area of lysis** ( $10^{-1}$ mm <sup>2</sup> /embryo/hr)	
	tPA	UK
Control media	0	0
4-8 cell stage	$1.72 \pm 0.67$	$0.41 \pm 0.64$
Expanded blastocyst	$3.82 \pm 1.64^{a)}$	$1.07 \pm 0.22^{b)}$
Hatching-Hatched blastocyst	$3.00 \pm 1.55^{a)}$	$0.94 \pm 0.39^{b)}$

Number of samples evaluated at each stage of development  $\geq 20$ .

\* Media collected at different days of culture: 4-8 cell stage  $\equiv$  day 2; expanded blastocyst to hatched blastocyst  $\equiv$  days 8-12.

\*\* Area of lysis observed in the presence of tissue plasminogen activator, tPA, or urokinase, UK.

a,b) represents  $p < 0.05$  compared to values at 4-8 cell stage respectively as determined by a non-paired student's t-test.

in the form of t-PA [1, 3, 8, 15]. Similar activity has also been detected in human and hen granulosa cells [7, 22]. It has been suggested that a role of PA in the developing follicle may be to convert the zymogen, plasminogen, to its proteolytic active form, plasmin, which, in turn, can activate collagenases in the follicular wall leading to rupture of the wall and the release of the oocyte at ovulation [20, 21]. Although this may not be the only role for plasmin in follicular development, the importance of potential plasmin production in follicular development is indicated by the findings in this study that the bovine follicular cumulus layer produces both plasminogen and PA. Indeed, it has been shown that the plasmin generating potential is higher in human follicular fluids and granulosa cells that can be fertilized *in vitro* compared to follicles containing oocytes that fail to fertilize [19].

In contrast to the absence of PA activity in the unfertilized oocyte, developing bovine embryos require the ability to produce and secrete PA activity. In this study no PA activity could be detected in either the embryo or the culture medium until they had reached the 4-8 cell stage which occurred around day 7 post fertilization. By the time the embryos had reached the expanded blastocyst stage, the level of PA activity, as estimated by the area of lysis of fibrin per embryo per hr, doubled and the activity remained elevated through the hatched blastocyst stage. A similar plateau of PA activity at this stage of developing bovine embryos has previously been reported [2, 6]. It has been proposed that

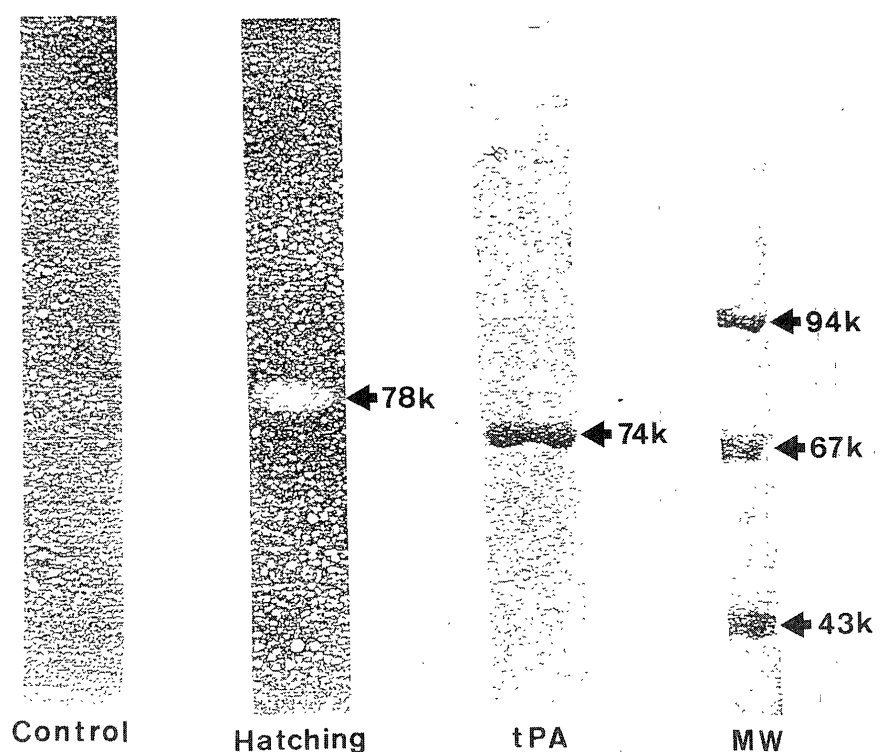


Fig. 1. Identification of plasminogen activator in fertilized oocyte culture medium by SDS-PAGE zymography. Control: 24 hr media sample, between days 1 and 2 of culture, when embryo at expanded blastocyst stage. Hatching: 24 hr media sample, between days 8 and 9 of culture, when embryos at hatching blastocyst stage. tPA: Purified human tissue plasminogen activator. MW: molecular weight standards.

one of the roles of PA in the developing embryo is to facilitate the hatching process [13] especially since hatched embryos produce more total PA than non-hatched [6, 13]. Under *in vitro* conditions, the number of blastocysts which hatch increases and the timing of hatching more closely resembles that under *in vivo* conditions when plasminogen is present in the medium [16, 17]. Since the results of this study indicate that, at least up to the hatched blastocyst stage, the developing embryo does not produce detectable levels of plasminogen, it is likely that other tissue sources provide the plasminogen substrate for the PA released from the developing embryo.

A combination of SDS-PAGE and zymography was used to characterize the PA activity secreted by the embryos at the expanded blastocyst and hatching blastocyst stage. This technique showed that the PA activity was located in a single band which migrated in the 78 k dalton region close to that of the lung derived t-PA used as a control marker. This finding, that the predominant form of PA is t-PA, is in contrast to previous reports in which u-PA has been identified as the primary form of PA in cultured bovine embryos [6]. A major difference between this and previous studies is the stage of development of the cultured embryos at which PA activity was evaluated. In the present study the embryos were examined only up to the hatched blastocyst stage while the earlier reports examined embryos at the trophoblast stage [6]. Further studies are in progress to investigate whether the developing bovine embryo switches from t-PA to u-PA production after the hatched blastocyst stage [2].

Bovine embryos cultured in the presence of the u-PA inhibitor, PA inhibitor-2 (PAI-2), exhibited a hatching rate similar to embryos cultured in the absence of the inhibitor [5]. However, PAI-2 is less effective as an inhibitor of t-PA than u-PA. Depending on the relative levels of t-PA and PAI-2, t-PA activity could still be functional in the embryo at a level sufficient to permit hatching. This conclusion is compatible with the results of this study and with murine studies which have demonstrated that ovulation can occur in the absence of either u-PA or t-PA but is reduced with a combined deficiency of u-PA and t-PA [4]. Further, transgenic mice with single or combined deficiencies of t-PA and/or u-PA can survive embryonic development and are viable at birth [4]. Hence, it is clear that the biological function of the PA system in mammalian development is complex and has not yet been fully elucidated.

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