A Morphological Study of Blastocyst Hatching in the Mouse and Rat

Sueo NIIMURA and Masayo FUJII¹⁾

Faculty of Agriculture, and ¹⁾Graduate School of Science and Technology, Niigata University, Niigata 950-21, Japan

Abstract. In cultured mouse and rat blastocysts, hatching was found to begin with protrusion of trophoctoderm cells from zonae pellucidae at the expanded stage. Protrusion of the cells occurred at the mural trophoctoderm and at the polar trophoctoderm. After protrusion, a slit was formed in the zona pellucida in all mouse and rat blastocysts. It was also confirmed that hatching was completed in the form of either expansion or contraction in both mouse and rat blastocysts. From these results, the process of blastocyst hatching could be classified into 6 types in the mouse. Since 5 of 6 types of hatching process, classified in mouse blastocysts, were observed in rat blastocysts, it is inferred that the process of hatching differs among blastocysts, but not remarkably between the mouse and rat. The mean duration of hatching from its start to completion was 22.4 h in mouse blastocysts and 40.9 h in rat ones. When the activity of trypsin-like proteinase was histochemically examined in mouse and rat embryos, this enzyme activity was detected in those from the compacted 16-cell stage to the expanded blastocyst stage. This enzyme activity was present in both mural and polar trophoctoderm cells of blastocysts, suggesting the reason for no polarity of protrusion of trophoctoderm cells in mouse and rat blastocysts.

Key words: Mouse, Rat, Blastocyst hatching, Time-lapse videomicrography, Trypsin-like proteinase. (J. Reprod. Dev. 43: 295–302, 1997)

Lewis and Gregory [1] first observed blastocyst hatching in rabbits using time-lapse cinematography. After that, the process of blastocyst hatching has been observed not only in the rabbit [2], but also in the golden hamster [3, 4], cattle [5, 6], guinea pig [7], rhesus monkey [8], cat [9] and pig [10] using time-lapse cinematography or time-lapse videomicrography.

The process of blastocyst hatching has been extensively studied in mice [11–17] and rats [18–20], and it is said that blastocyst hatching in these animals begins with protrusion of trophoctoderm cells out of zonae pellucidae [11, 12, 16, 18]. It is also

said that a slit is formed in the zona pellucida from the site of protrusion of trophoctoderm cells by enlargement of such protrusion [13, 15, 18, 20]. After that, the blastocyst repeats active contractions, leading to the enlargement of the slit, and then escapes from the zona pellucida in the state of expansion in mice [13, 15] and in the state of contraction in rats [19, 20]. Some investigators [11, 12] reported that protrusion of cells from zonae pellucidae occurred only in polar trophoctoderms, while others [16, 18] reported that such protrusion could occur in either mural or polar trophoctoderm. It was also reported that some mouse blastocysts completed hatching without slit formation in the zona pellucida [14, 17]. However, the results of previous studies may have been affected by a pro-

cedure of flushing or fixation because flushed blastocysts were observed without fixation [13, 16, 18, 19] or observed after fixation [17], or sections of fixed blastocysts with the uterus were observed [14, 15]. Particularly, cell protrusion and the state of contraction at the completion of hatching seem to be susceptible to the effects of these procedures. On the other hand, dynamic analyses on the process of hatching were performed only one [11] or 2 [12] blastocysts for the mouse and one blastocyst [20] for the rat using time-lapse cinematography. Accordingly, the results of previous studies on the hatching process differ among investigators.

It is generally accepted that blastocyst hatching requires regional dissolution of the zona pellucida by a hatching enzyme. However, demonstration of such enzyme has been made histochemically only in rabbit blastocysts [21] and in mouse embryos from the 8-cell to blastocyst stage [22, 23]. Namely, trypsin-like proteinase, called blastole-mase, has been detected from trophoctoderm cells of rabbit blastocysts from 6.5 to 7 days post copulation [21]. In the mouse, it has been confirmed that the activity of trypsin-like proteinase, called strypsin, appears in compacted 8- and 16-cell embryos, and is localized in the mural trophoctoderm of blastocysts [22]. However, it has been also reported that the activity of this enzyme is present in cells of both mural and polar trophoctoderms of mouse blastocysts [23], disclosing a discrepancy in localization of this enzyme among investigators. Such a hatching enzyme has not been demonstrated in rat embryos.

In the present study, morphological changes in a large number of cultured mouse and rat blastocysts during the period from blastocyst formation to completion of hatching were observed using time-lapse videomicrography, in order to investigate what patterns of hatching process occurred in blastocysts of these animals, and also to determine whether hatching patterns differed between embryos with cell protrusion from polar trophoctoderms and those from mural trophoctoderms. The activity of trypsin-like proteinase was also histochemically demonstrated in mouse and rat embryos in the process of blastocyst formation, in order to determine the localization and the time of appearance of this enzyme.

Materials and Methods

Animals

Seventy female mature mice of the ICR strain and 40 female mature rats of the Wistar strain were used in the present study. These animals were kept and fed in a room at 24 C. This room was lit 14 h a day, from 0400 h to 1800 h. Mouse embryos were obtained by superovulations; the animals were subjected to peritoneal injections with 5 i.u. of PMSG (Serotropin, Teikoku Hormone Manufacturing Co., Japan) and with 5 i.u. of hCG (Gonotropin, Teikoku Hormone Manufacturing Co.) 48 h later, and then they were mated with ICR males. As for rats, embryos were obtained by spontaneous ovulation. Namely, rats were mated with males at the proestrous, and the next morning, the presence of sperm was ascertained in the smear. As it is known that ovulation occurs 13 h after hCG injection in mice [24] and at 0300 h on the day of sperm was ascertained in the smear in rats [25], the recovery hours of embryos were shown as the h estimated from ovulation time.

Observation of blastocyst hatching

In order to observe the process of blastocyst hatching, mouse and rat morulae were recovered from their uteri at 79 h or 90 h after ovulation, and were cultured in the M16 medium [26] or modified hamster embryo culture medium-1 [27] in a CO₂ culture chamber (CO₂ 5% in air : SK-1, Sankei, Japan) equipped with an inverted microscope (DIAPHOT, Nikon Corporation, Japan). Morphological observations were performed on the images which were taken by a CCD colour camera (Hitachi Electronic Co., Japan) connected with an inverted microscope and recorded by a time-lapse video cassette recorder (Victor Co., Japan). The analyses were done during the period from blastocyst formation to completion of blastocyst hatching.

Demonstration of trypsin-like proteinase

Embryos examined were at the stages of uncompact 8-cell, compacted 16-cell, morula, early blastocyst, expanded blastocyst and hatched blastocyst, and were recovered from oviducts or uteri at 55, 65, 79, 82, 87 and 107 h after ovulation in the mouse or recovered at 60, 77, 90, 101, 109 and 114 h after ovulation in the rat. Embryos at the stages of compacted 16-cell, morula and early blastocyst

were distinguished according to the following morphological features: both compacted 16-cell embryos and morulae shrunk, and the former had about 16 blastomeres, whereas the number of blastomeres could not be determined in the latter. Early blastocysts were slightly larger than morulae, and had a small cavity within the embryos. Collected embryos were washed twice with phosphate buffered saline (PBS, pH 7.4) [28] containing 0.4% polyvinyl pyrrolidone-40 (PBS-PVP) and fixed in PBS containing 10% formalin for 30 min at 4 C. In order to detect trypsin-like proteinase a slight modification of method used by Perona and Wasarman [22] was employed: the fixed embryos were incubated in a solution containing 4 mg N- α -benzoyl-DL-arginine β -naphthylamide (Sigma Chemical Co., MO, USA) which had been dissolved in 0.5 ml dimethyl sulfoxide, 6 mg Fast Garnet GBC salt (Sigma Chemical Co.), and 10.0 ml 50 mM phosphate buffer solution at pH 7.0, for 10 min at 37 C. Embryos incubated in a solution containing the substrate solvent, dimethyl sulfoxide, but devoid of the substrate were observed as controls. Treated embryos were washed 3 times in PBS-PVP and placed on glass slides to be photographed under a microscope (OPTIPHOT, Nikon Corporation). The same procedures of demonstration were repeated 3 times, using 20 embryos in all for every stage.

Statistical analysis

The number of blastocysts with different hatching patterns and of embryos with different enzyme activities was statistically analyzed by the Chi-square test. Concerning the duration of blastocyst hatching in the mouse, statistical analysis was performed using the One-way analysis of variance.

Results

Process of blastocyst hatching

When mouse morulae were cultured, a small cavity appeared within the embryos, and the embryos developed into blastocysts (Fig. 1). When these early blastocysts were further cultured, the trophoblast cells protruded out of zonae pellucidae at the expanded stage (Figs. 2 and 3), and the blastocysts started hatching. After 12 to 42 h, the blastocysts completely escaped from the zona pellucida, and accomplished hatching (Figs. 4 and 5).

Of the embryos cultured, 50% completed hatching. When hatching patterns of 30 mouse blastocysts were classified according to the site of protrusion of trophoblast cells from the zona pellucida, the mode of slitting in the zona pellucida and the state of blastocyst contraction at the time of escape from the zona pellucida, the process could be classified into 6 types, as shown in Fig. 6 and Table 1. Protrusion of trophoblast cells out of the zona pellucida occurred at 10.5 to 31 h after blastocyst formation from the mural tro-

Videomicrographs of cultured mouse blastocysts. 200 $\times$.

Fig. 1. Early blastocysts with small blastocoels.

Fig. 2. An expanded blastocyst with a protrusion (an arrow) from mural trophoblast.

Fig. 3. An expanded blastocyst with a protrusion (an arrow) from polar trophoblast.

Fig. 4. A hatched blastocyst with a vacant zona pellucida. The embryo completes hatching in the state of expansion.

Fig. 5. Videomicrographs of a cultured mouse blastocyst (arrows) showing completion of hatching in the state of contraction. 200 $\times$. a: before contraction; b: during contraction; c: at the completion of hatching.

Fig. 6. A diagram of the process of blastocyst hatching in the mouse. *Types I to VI: See text for classification of each type of blastocyst hatching.

phectoderm in 20 embryos (Types I to IV, Fig. 2) and from the polar trophectoderm in 10 embryos (Types V and VI, Fig. 3). There was no significant difference in the number of blastocysts with the

protrusion from mural trophectoderm cells and those from polar trophectoderm cells. After protrusion, a slit in the zona pellucida was observed in all embryos. This slit was formed by blastocyst

Table 1. Patterns of hatching in mouse blastocysts *in vitro*

Sites of protrusion	Modes of slitting in zona pellucida	States of blastocyst contraction at the time of completion of hatching	Types of hatching	No. and (%) of blastocysts showing each type	Duration of hatching (h)
Mural trophoctoderm	Blastocyst expansion	Expansion	I	9 (30)	18.7 ± 8.31*
	Blastocyst expansion	Contraction	II	2 (7)	20.0 ± 1.41
	Enlargement of protrusion	Expansion	III	5 (17)	22.4 ± 5.97
	Enlargement of protrusion	Contraction	IV	4 (13)	29.5 ± 13.87
Polar trophoctoderm	Blastocyst expansion	Expansion	V	3 (10)	18.6 ± 7.49
	Enlargement of protrusion	Expansion	VI	7 (23)	25.4 ± 16.37

* Mean ± S.D. There are no significant differences in the duration of hatching between each Type.

Table 2. Patterns of hatching in rat blastocysts *in vitro*

Sites of protrusion	Modes of slitting in zona pellucida	States of blastocyst contraction at the time of completion of hatching	No. and (%) of blastocysts showing each hatching pattern	Duration of hatching (h)
Mural trophoctoderm	Blastocyst expansion	Expansion	2 (25)	35.8
	Blastocyst expansion	Contraction	2 (25)	53.0
	Enlargement of protrusion	Contraction	1 (13)	55.0
Polar trophoctoderm	Blastocyst expansion	Expansion	2 (25)	44.5
	Enlargement of protrusion	Expansion	1 (13)	6.0

expansion in 14 embryos (Types I, II and V) and by enlargement of the protrusion in 16 embryos (Types III, IV and VI). A significantly more number of blastocysts completed hatching in the state of expansion (Types I, III, V and VI, Fig. 4), compared with those in the state of contraction (Types II and IV, Fig. 5) ($P < 0.01$). None of blastocysts, in which protrusion had occurred from the polar trophoctoderm, completed hatching in the state of contraction. Compared with the mean duration (22.4 h) of hatching from its start to completion in the 30 embryos, hatching was shorter in Types I, II and V, and longer in Types IV and VI.

In cultured 8 rat blastocysts, hatching began during the expanded stage at 8.5 to 20.5 h after blastocyst formation. Of the blastocysts cultured, 28.6% completed hatching. In contrast to hatching patterns in mouse blastocysts, those of rat blastocysts could be classified into 5 types shown in Table 2. Although the difference was not statistically significant, protrusion of trophoctoderm cells out of the zona pellucida appeared more often from mural trophoctoderms (5 embryos), compared with that from polar trophoctoderms (3 embryos). A slit in the zona pellucida was formed in all embryos as a result of expansion in 6 blastocysts and

enlargement of the protrusion in 2 blastocysts. Hatching was completed in the state of expansion in 5 embryos and in the state of contraction in 3 embryos. None of blastocysts, in which protrusion had occurred from the polar trophoctoderm, completed hatching in the state of contraction. Compared with the mean duration (40.9 h) needed to complete hatching in the 8 embryos, the shortest time (6 h) was observed in one embryo showing the Type VI hatching process, as classified in the mouse blastocysts, and the longest time (55 h) was observed in another embryo showing the Type IV process.

Activity of trypsin-like proteinase

When mouse and rat embryos were treated by the method of Perona and Wassarman [22], brown granules of Fast Garnet GBC were deposited in the cytoplasm of embryos (Figs. 7 and 8). The granules were regarded as showing the presence of trypsin-like proteinase activity, because such granules did not appear in the control embryos incubated in the solution devoid of the substrate (Fig. 9). In the present study, the activity of trypsin-like proteinase was judged by the amount of the granules, which indicates the strength of activity.

Fig. 7. A mouse morula. Brown granules of Fast Garnet GBC showing the presence of trypsin-like proteinase activity are seen in the cytoplasm of blastomeres. 400 $\times$.

Fig. 8. An expanded rat blastocyst. Brown granules of Fast Garnet GBC showing the presence of trypsin-like proteinase activity are seen in the cytoplasm of both mural and polar trophoblast cells. 400 $\times$.

Fig. 9. An expanded control mouse blastocyst incubated in a substrate-free solution. No brown granules are seen in the cytoplasm of any trophoblast cell. 400 $\times$.

Table 3. Trypsin-like proteinase activity in mouse and rat embryos during the course of blastocyst formation

Animals	Activities	Developmental stages					
		uncompacted	Compacted	Morula	Blastocyst		
		8-cell	16-cell		Early	Expanded	Hatched
Mouse	Strong	0*(0)	6 (30)	8 (40)	7 (35)	6 (30)	0 (0)
	Weak	0 (0)	14 (70)	12 (60)	13 (65)	14 (70)	0 (0)
	Negative	20 (100)	0 (0)	0 (0)	0 (0)	0 (0)	20 (100)
Rat	Strong	0 (0)	10 (50)	15 (75)	13 (65)	13 (65)	0 (0)
	Weak	0 (0)	10 (50)	5 (25)	7 (35)	7 (35)	0 (0)
	Negative	20 (100)	0 (0)	0 (0)	0 (0)	0 (0)	20 (100)

*The number of embryos with percentages in parentheses. There are no significant differences in the number of mouse or rat embryos with a strong enzyme activity among the stages of compacted 16-cell, morula, early blastocyst and expanded blastocyst.

As shown in Table 3, the activity of trypsin-like proteinase at first appeared in some blastomeres of compacted 16-cell mouse and rat embryos. The activity was retained up to expanded blastocysts. In mouse and rat blastocysts, the activity was present in the cytoplasm of both mural and polar trophoblast cells, while not in the cytoplasm of inner-cell-mass cells.

Discussion

The results of the present study revealed that protrusion out of zonae pellucidae occurred from either mural trophoblast or polar trophoblast in mouse and rat blastocysts, showing the absence

of polarity in blastocyst hatching of these animals. Cole *et al.* [11, 12] reported that protrusion of trophoblast cells occurred only from the polar trophoblast in mouse blastocysts, while Bergström [16] and Dickmann and DeFeo [18] observed protrusion from either polar or mural trophoblast in mouse and rat blastocysts, as with those observed in the present study. The reason for this discrepancy can be explained by that Cole *et al.* [11, 12] observed only 2 embryos. The fact that none of blastocysts having protrusion from the polar trophoblast completed hatching in the state of contraction is thought to be characteristic of blastocyst hatching in the mouse and rat.

In all mouse and rat blastocysts observed in the present study, a slit was formed in the zona pellu-

cida, indicating that formation of such slit is essentially required for the blastocyst hatching of these animals. It was also proved that there are two modes of slit formation; namely, formation of a larger slit by blastocyst expansion and formation of a small slit by the enlargement of protrusion. Since blastocysts, in which a small slit was formed, included those whose slit in the zona pellucida was very difficult to detect because of its small size, embryos with such a small slit might have been missed in the reports [14, 17] describing the completion of hatching in mouse blastocysts without slit formation in the zona pellucida.

It has been reported that blastocyst hatching completes in the state of expansion in the mouse [13, 15] and in the state of contraction in the rat [19, 20], while the present study is the first to reveal that hatching can complete in the form of either expansion or contraction in both mouse and rat blastocysts. Both mouse and rat blastocysts are known to have contractions [11–13, 20, 29], and the number of contractions per h in a mouse blastocyst is greater during hatching than before or after hatching [29]. In the present study, the ratio of blastocysts completing hatching in the state of expansion was 80% in the mouse and 63% in the

rat. From these results, it is thought that blastocysts repeatedly contract and expand frequently during hatching, but many maintain the state of expansion before the completion of hatching.

It was confirmed that the process of blastocyst hatching can be classified into 6 types in the mouse and 5 types in the rat. When the process of blastocyst hatching in the mouse was compared with that in the rat, rat blastocysts were found to have the Type I, II, IV, V or VI process, as classified in mouse blastocysts. Therefore, it is inferred that the process of blastocyst hatching is comparable between the mouse and rat.

In the present study, the activity of trypsin-like proteinase, a hatching enzyme, was histochemically demonstrated in mouse and rat embryos from the 16-cell stage to the expanded blastocyst stage, suggesting the production of trypsin-like proteinase by embryos during these stages. Since the activity of this enzyme was observed in both polar and mural trophoctoderm cells of blastocysts, no localization of this enzyme in trophoctoderm cells seems to be the reason for no polarity of protrusion of trophoctoderm cells in either mouse or rat blastocyst.

References

1. **Lewis WH, Gregory PW.** Cinematographs of living developing rabbit eggs. *Science* 1929; 69: 226–229.
2. **Kane MT.** Inhibition of zona shedding of rabbit blastocysts in culture by presence of a mucin coat. *J Reprod Fertil* 1975; 44: 539–542.
3. **Bavister BD.** Studies on the developmental blocks in cultured hamster embryos. In: Bavister BD (ed.), *The Mammalian Preimplantation Embryo: Regulation of Growth and Differentiation In Vitro*. New York: Plenum Press; 1987: 219–249.
4. **Gonzales DS, Bavister BD.** Zona pellucida escape by hamster blastocysts in vitro is delayed and morphologically different compared with zona escape in vivo. *Biol Reprod* 1995; 52: 470–481.
5. **Massip A, Mulnard J.** Time-lapse cinematographic analysis of hatching of normal and frozen-thawed cow blastocysts. *J Reprod Fertil* 1980; 58: 475–478.
6. **Massip A, Mulnard J, Vanderzwalmen P, Hanzen C, Ectors F.** The behaviour of cow blastocyst *in vitro*: cinematographic and morphometric analysis. *J Anat* 1982; 134: 399–405.
7. **Blandau RJ.** Observation on implantation of the guinea pig. *Anat Rec* 1949; 103: 19–48.
8. **Enders AC, Schlafke S.** Differentiation of the blastocyst of the rhesus monkey. *Am J Anat* 1981; 162: 1–21.
9. **Roth TL, Swanson WF, Wildt DE.** Development competence of domestic cat embryos fertilized in vivo versus in vitro. *Biol Reprod* 1994; 51: 441–451.
10. **Gary ML, Wright RW.** Morphological and quantitation aspects of the development of swine embryos *in vitro*. *J Anim Sci* 1978; 46: 711–719.
11. **Cole RJ, Paul J.** Properties of cultured preimplantation mouse and rabbit embryos, and cell strains derived from them. In: Wolstenholme GEW, O'Connor M (eds.), *Preimplantation Stage of Pregnancy*. London: J & A Churchill Ltd; 1965: 82–112.
12. **Cole RJ.** Cinematographic observations of the mouse blastocyst. *J Embryol Exp Morph* 1967; 17: 481–490.
13. **Orsini WM, McLaren A.** Loss of zona pellucida in mice, and the effect of tubal ligation and ovariectomy. *J Reprod Fertil* 1967; 13: 485–499.
14. **Potts DM, Wilson IB.** The preimplantation conceptus of the mouse at 90 hours postcoitum. *J Anat*

- 1967; 102: 1–11.
15. **McLaren A.** The fate of zona pellucida in mice. *J Embryol Exp Morph* 1970; 23: 1–19.
16. **Bergström S.** Shedding of the zona pellucida in normal pregnancy and in various hormonal states in the mouse. *Z Anat Entwickl Gesch* 1972; 136: 143–167.
17. **Bergström S.** Shedding of the zona pellucida of the mouse blastocyst in normal pregnancy. *J Reprod Fertil* 1972; 31: 275–277.
18. **Dickmann Z, DeFeo VJ.** The rat blastocyst during normal pregnancy and during delayed implantation, including an observation on the shedding of zona pellucida. *J Reprod Fertil* 1967; 13: 3–9.
19. **Dickmann Z.** Shedding of the zona pellucida. *Adv Reprod Physiol* 1969; 4: 187–206.
20. **Bitton-Casimiri V, Brun JL, Psychos A.** Comportement *in vitro* des blastocystes du 5e jour de la gestation chez la Ratte; étude microcinématographique. *C R Acad Sci Paris* 1970; 270: 2979–2982.
21. **Denker HW.** Implantation: the role of proteinases and blockage of implantation by proteinase inhibitors. *Embryol Cell Biol* 1977; 53: 1–123.
22. **Perona RM, Wassarman PM.** Mouse blastocysts hatch *in vitro* by using a trypsin-like proteinase associate with cells of mural trophectoderm. *Dev Biol* 1986; 114: 42–52.
23. **Sawada H, Yamazaki K, Hojo E, Kato Y, Someno T, Hoshi M.** Period of action of mouse blastocyst hatching enzyme and its histochemical localization in embryos. *Zool Sci* 1987; 4: 1044 (abstract DB68).
24. **Gates AH.** Maximizing yield and developmental uniformity of eggs. In: Daniel JC Jr (ed.), *Methods in Mammalian Embryology*. San Francisco: WH Freeman and Company; 1971: 4–85.
25. **Pederson ES.** Histogenesis of lutein tissue of the albino rat. *Am J Anat* 1951; 88: 397–427.
26. **Whittingham DG.** Culture of mouse ova. *J Reprod Fertil Suppl* 1971; 14: 7–21.
27. **Miyoshi K, Funahashi H, Okuda K, Niwa K.** Development of rat one-cell embryos in a chemically defined medium: effects of glucose, phosphate and osmolarity. *J Reprod Fertil* 1994; 100: 21–26.
28. **Dulbecco R, Vogt M.** Plaque formation and isolation of pure lines with poliomyelitis viruses. *J Exp Med* 1954; 99: 167–182.
29. **Niimura S, Takahashi E.** Time-lapse videomicrographic observations of the contraction in cultured mouse blastocysts. *Anim Sci Technol (Jpn)* 1995; 66: 713–719 (In Japanese).