Contractions of Mouse Blastocysts Whose Hatching Abilities were Suppressed by Soybean Trypsin Inhibitor

Sueo Niimura[1]* and Ryoko Wakasa[2]

[1]Faculty of Agriculture, and [2]Graduate School of Science and Technology, Niigata University, Niigata 950-2181, Japan

Abstract: *The role of contraction in blastocyst hatching was determined by time-lapse videomicrography in mouse blastocysts whose hatching ability had been suppressed by soybean trypsin inhibitor (STI). The hatching rate of blastocysts developed from morulae in a medium containing STI at a concentration of 1.0 mg/ml (STI-treated blastocysts) was 17.2%, which was significantly lower than the 63.9% for blastocysts developed from morulae in a medium without STI (non-treated blastocysts). Over the span of 32 hrs after blastocoel formation, the number of strong contractions was similar in STI-treated and non-treated blastocysts, but the total number of contractions and the number of weak contractions (less than 20% volume reduction) were significantly smaller in STI-treated blastocysts (4.22 and 2.94 times) than in non-treated blastocysts (5.80 and 4.50 times). These STI-treated blastocysts took a significantly longer time for weak contraction and for re-expansion after weak contraction (10.2 and 87.6 min), compared with non-treated blastocysts (7.8 and 58.2 min). It was also confirmed that the activity of trypsin-like proteinase was similar in STI-treated and non-treated blastocysts, and that a small hole was formed in the zona pellucida at the start of hatching in STI-treated blastocysts, as seen in non-treated blastocysts. Nevertheless, the rate of blastocysts with a slit resulting from enlargement of the small hole in the zona pellucida was 37.5% in STI-treated blastocysts, which was significantly lower than the 86.7% in non-treated blastocysts. These results suggest that weak contraction and re-expansion after weak contraction of blastocysts play an important role in hatching by slitting the region adjacent to the small hole in the zona pellucida.*

Key words: *Mouse, Blastocyst hatching, Contraction, Soybean trypsin inhibitor, Time-lapse videomicrography*

Received: September 5, 2000
Accepted: January 17, 2001
*To whom correspondence should be addressed.

The presence of contraction in mammalian blastocysts was first reported by Lewis and Gregory [1] in 1929. They observed the repeated contraction and expansion in cultured rabbit blastocysts by time-lapse microcinematography. The contraction has since been observed by time-lapse microcinematography and time-lapse videomicrography in cultured blastocysts of mice [2–8], rats [9], guinea pigs [10], hamsters [11–13] and cattle [14, 15]. Although the physiological role of contraction in blastocysts is unknown, the contraction has been considered to participate in the process of blastocyst hatching [5, 6, 9]. We recently reported that a few blastocysts showing no sign of contraction were present during the pre-hatching period, whereas all of the blastocysts repeatedly contracted and re-expanded during hatching and for 10 hrs post-hatching [16]. We also reported that the number of strong contractions (20% or more volume reduction) was less in blastocysts completing hatching than in those incompletely hatched until 32 hrs after blastocoel formation [16–18]. In view of these results, we suggested that contraction, particularly weak contraction, plays a role in hatching, whereas strong contraction is not related to hatching, but occurred in blastocysts unable to complete hatching [16–18].

On the other hand, Perona and Wassarman [19] reported that hatching was significantly inhibited in mouse blastocysts treated with the proteinase inhibitor, antipain, leupeptin or soybean trypsin inhibitor (STI), and that the hatching rate of STI-treated blastocysts was less than 15% of that observed in non-treated blastocysts. It was also reported that the inhibitory effect of STI on hatching was dose dependent, and the concentration of STI at which hatching was inhibited by 50% was about 0.4 mg/ml [19]. Therefore, it is inferred that the strength and number of contractions, which are thought to be associated with blastocyst hatching [5, 6, 9, 16–18],

might alter in the blastocysts in which hatching is inhibited by STI.

In the present study, the strength and the number of contractions were examined in mouse blastocysts whose hatching ability had been suppressed by STI (STI-treated blastocysts), and were compared with those in blastocysts cultured in a medium without STI (non-treated blastocysts), in order to determine the role of contraction in blastocyst hatching. The activity of trypsin-like proteinase was also histochemically demonstrated in STI-treated blastocysts to investigate the effect of STI on the synthesis of trypsin-like proteinase in such blastocysts.

Materials and Methods

Animal

Ninety-seven female mice of ICR strain prepared by mating at our laboratory were used in the present study. These mice were housed in autoclaved metal cages and were given a standard diet (MF, Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum* in an air-conditioned room (24°C), under controlled-lighting conditions (14L/10D; L: 04:00 hrs to 18:00 hrs). These females were superovulated with 5 i.u. PMSG (Serotropin®, Teikoku Hormone Manufacturing Co. Ltd., Tokyo, Japan), and with 5 i.u. hCG (Gonatropin®, Teikoku Hormone Manufacturing Co. Ltd.) injected 48 hrs later. Immediately after the hCG injection, these females were mated with mature males of the same strain.

Determination of STI concentration necessary to inhibit blastocyst hatching

In observing contraction of blastocysts, as in the present study, embryos before development to the blastocyst stage should be cultured because images of individual blastocysts over time soon after blastocoel formation are necessary to analyze the contractions. Therefore a concentration of STI at which STI had no effect on the development of morulae to blastocysts, but significantly inhibited the hatching of the resultant blastocysts, was first determined. Morulae were collected from oviducts and uteri of superovulated and mated females 72 hrs after the hCG injection, and were cultured in M16 medium [20] containing STI (Sigma Chemical Co., MO, USA) at 0.0, 0.5, 1.0, 5.0 or 10.0 mg/ml in a CO_2 incubator (5% CO_2 in air) at 37°C. The STI was used by directly dissolving it in M16 medium.

Development of morulae to blastocysts and completion of hatching in resultant blastocysts were observed after 24 and 100 hrs of culture, respectively.

Observation of contractions and morphological changes in blastocysts

In order to observe contractions and morphology in blastocysts, morulae were collected 72 hrs after the hCG injection and cultured in M16 medium without STI or in M16 medium containing STI at a concentration of 1.0 mg/ml. These morulae were cultured in a CO_2 culture chamber (SK-1, Sankei, Tokyo, Japan; 5% CO_2 in air) equipped with an inverted microscope (DIAPHOT, Nikon Corporation, Tokyo, Japan) at 37°C. To analyze the contraction of STI-treated and non-treated blastocysts, observations were performed on the images which were taken at 4-sec intervals by a CCD color camera (Hitachi Electronic Co., Tokyo, Japan) connected to an inverted microscope and recorded by a time-lapse video cassette recorder (Victor Co., Yokohama, Japan). The analyses of contractions were done until 32 hrs after blastocoel formation, according to the method described in our previous reports [16–18]. The degree of contraction was evaluated as follows: the volume of each blastocyst was calculated from its diameter on a display, and measured with a micrometer according to the method of Hurst and MacFarlane [21]. The percentage of volume reduction at the time of contraction from the volume before contraction was classified as weak when the percentage was less than 20% and as strong when 20% or more. Since the blastocysts were not completely spherical in shape, the mean value for the short and long axes of each embryo was used as the diameter of that embryo.

The lengths of time needed for contraction and re-expansion were analyzed in thirty images each for weak and strong contractions of STI-treated and non-treated blastocysts. The images were randomly selected from 32 hr recordings after blastocoel formation.

Morphological changes during hatching were also observed using the images of both non-treated and STI-treated blastocysts until 72 hrs after blastocoel formation.

Demonstration of trypsin-like proteinase activity in blastocysts

In order to demonstrate the activity of trypsin-like proteinase in STI-treated blastocysts, collected morulae were cultured for 48 hrs in M16 medium containing STI at a concentration of 1.0 mg/ml, in a CO_2 incubator (5% CO_2 in air) at 37°C until they developed to expanded blastocysts. As controls, expanded blastocysts cultured from morulae for 48 hrs in M16 medium without STI were used. Both STI-treated and non-treated blastocysts were washed twice with phosphate buffered saline (PBS, pH 7.4) [22] containing 0.4% polyvinyl pyrrolidone-

Table 1. Effects of soybean trypsin inhibitor (STI) on the development of mouse morulae and on the hatching of resulting blastocysts

Concentrations of STI (mg/ml)	No. of morulae cultured	No. and (%) of morulae developed into blastocysts	No. and (%) of blastocysts completed hatching
0.0	36	36 (100.0)[a]	23 (63.9)[a]
0.5	29	29 (100.0)[a]	10 (34.5)[b]
1.0	30	29 (96.7)[a]	5 (17.2)[b]
5.0	31	6 (19.4)[b]	1 (16.7)[b]
10.0	30	0 (0.0)[c]	0 (0.0)[c]

The development of morulae to blastocysts and the completion of blastocyst hatching were observed after 24 and 100 hrs of culture, respectively. Values with different superscripts in the same column are significantly different ($P<0.05$).

40 (PBS-PVP) and fixed in PBS containing 10% formalin for 30 min at 4°C. The activity of trypsin-like proteinase was detected by the method of Perona and Wassarman [19]. The fixed blastocysts were incubated in a solution containing 4 mg N-α-benzoyl-D, L-arginine β-naphthylamide (Sigma Chemical Co.) which had been dissolved in 0.5 ml dimethyl sulfoxide, 6 mg Fast Garnet GBC salt (Sigma Chemical Co.), and 10.0 ml of 50 mM phosphate buffer solution at pH 7.0, for 10 min at 37°C. Blastocysts incubated in a solution containing the substrate solvent, dimethyl sulfoxide, but devoid of the substrate were observed as controls. Treated blastocysts were washed 3 times in PBS-PVP and placed on glass slides to be photographed under a microscope (OPTIPHOT, Nikon Corporation). The same demonstration procedures were repeated 3 times, using 30 embryos in all.

Statistical analysis

The rate of development of morulae to blastocysts, the rate of blastocyst hatching, and the rate of blastocysts with different trypsin-like proteinase activity were statistically analyzed by Chi-square test. The number of contractions and the length of time needed for blastocysts to contract and re-expand were statistically analyzed by one-way analysis of variance.

Results

Blastocyst formation and hatching

When morulae were cultured in media containing STI at 0.5 and 1.0 mg/ml, they 100.0 and 96.7%, respectively developed to blastocysts, showing no difference from the developmental rate (100.0%) of control morulae cultured in a medium without STI (Table 1). The rate of development to blastocysts from morulae cultured with STI at 5.0 mg/ml was 19.4%, which was significantly lower than that in the control morulae. None of the morulae developed to blastocysts when they were cultured with STI at 10.0 mg/ml. Hatching rates for blastocysts developed from morulae in media containing STI at 0.5, 1.0 and 5.0 mg/ml were 34.5, 17.2 and 16.7%, respectively, which were all significantly lower than the 63.9% for non-treated blastocysts developed from morulae in a medium without STI. In view of these results, the medium containing 1.0 mg/ml STI was used in subsequent experiments because STI did not affect the development of morulae to blastocysts and had its maximum inhibitory effect on hatching of blastocysts at this concentration.

Number of contractions

When blastocysts were observed by time-lapse videomicrography, both STI-treated and non-treated blastocysts began to contract and expand repeatedly at the expanded stage from 6.31 and 6.34 hrs after blastocoel formation, respectively. The numbers of contractions until 32 hrs after blastocoel formation are shown in Table 2. Although the numbers of strong contractions (Fig. 1a-e) were similar for STI-treated and non-treated blastocysts, the total number of contractions and the number of weak contractions (Fig. 1f-j) were significantly smaller in STI-treated blastocysts (4.22 and 2.94 times) than in non-treated blastocysts (5.80 and 4.50 times).

As shown in Table 3, the mean lengths of time required by STI-treated blastocysts for strong contraction and for re-expansion following strong contraction were similar to those for non-treated blastocysts. But the mean lengths of time required by STI-treated blastocysts for weak contraction and for re-expansion following weak contraction were 10.2 and 87.6 min, respectively, which were significantly longer than the 7.8 and 58.2 min for non-treated blastocysts.

Table 2. Numbers of contractions of cultured mouse blastocysts

Blastocysts	No. of blastocysts examined	No. and (%) of blastocysts completed hatching	Degrees of contraction		
			Weak	Strong	Total
Non-treated	30	19 (63.3)[a]	4.50 ± 0.26*[a]	1.30 ± 0.17[a]	5.80 ± 0.26[a]
STI-treated	32	5 (15.6)[b]	2.94 ± 0.28[b]	1.28 ± 0.22[a]	4.22 ± 0.34[b]

*Mean ± S.E. Blastocysts observed were developed from morulae in M16 medium without STI (non-treated) or in M16 medium containing STI at a concentration of 1.0 mg/ml (STI-treated). The degree of contraction was divided into two, based on the percentages of volume reduction of blastocysts; Weak: less than 20%, Strong: more than 20%. The number of contractions was counted for 32 hrs after blastocoel formation. Values with different superscripts in the same column are significantly different ($P<0.05$).

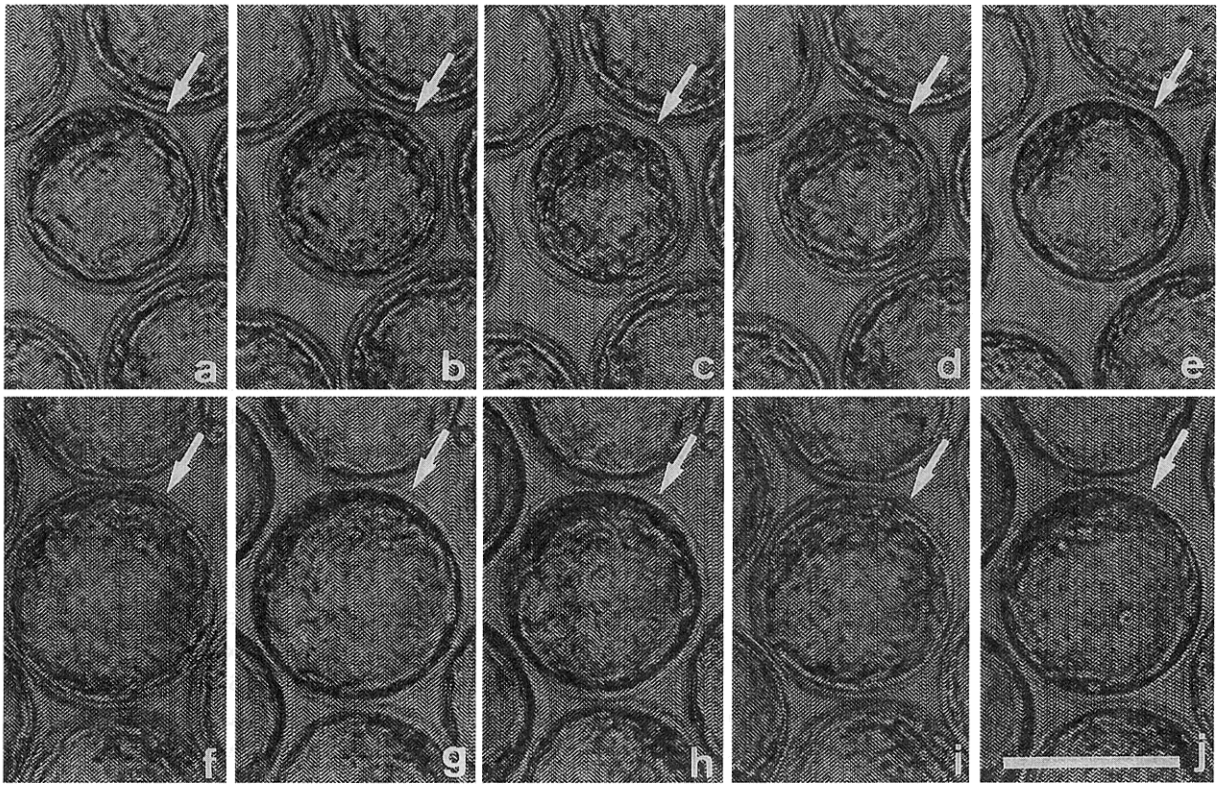

Fig. 1. Time-lapse videomicrographs of non-treated mouse blastocysts (arrows) showing a strong contraction (a-e) and a weak contraction (f-j). The blastocysts showing the strong and weak contractions required 10 min (a to c) and 6 min (f to h) to contract maximally, and 5.1 hrs (c to e) and 2.2 hrs (h to j) to expand to the size before contraction, respectively. Scale indicates 100 μm. (a, f) Before contraction. (b, g) During contraction. (c, h) At the time of contraction. (d, i) During re-expansion. (e, j) At the time of re-expansion.

Process of blastocyst hatching

When morphological changes were observed in 30 non-treated blastocysts, a small hole was formed in the zona pellucida of 28 blastocysts (93.3%) 25.3 hrs after blastocoel formation (Fig. 2a), and these blastocysts started hatching by protruding trophectoderm cells through the hole, whereas no hole was formed in the zonae pellucidae of the remaining 2 blastocysts. Blastocysts with a small hole in the zona pellucida increasingly protruded trophectoderm cells through the hole, and then 26 blastocysts (86.7%) formed a slit in the zona pellucida by enlarging the hole 12.4 hrs after the start of hatching (Fig. 2b). In the blastocysts that had formed a slit in the zona pellucida, trophectoderm cells continuously escaped through the slit, and hatching was completed in 19 blastocysts (63.3%) 23.6 hrs after the

Table 3. Length of time needed for cultured mouse blastocysts to contract and re-expand

Blastocysts	Weak contraction			Strong contraction		
	No. of contractions examined	Time required for contraction (min)	Time required for re-expansion (min)	No. of contractions examined	Time required for contraction (min)	Time required for re-expansion (min)
Non-treated	30	7.8 ± 1.2*[b]	58.2 ± 8.4[b]	30	16.8 ± 3.6[a]	171.0 ± 15.6[a]
STI-treated	30	10.2 ± 0.6[a]	87.6 ± 9.6[a]	30	21.6 ± 4.2[a]	232.2 ± 38.4[a]

*Mean ± S.E. Blastocysts observed were developed from morulae in M16 medium without STI (non-treated) or in M16 medium containing STI at a concentration of 1.0 mg/ml (STI-treated). Weak and strong contractions examined were randomly selected in 30 non-treated and 32 STI-treated blastocysts until 32 hrs after blastocoel formation, respectively. Values with different superscripts in the same column are significantly different ($P<0.01$).

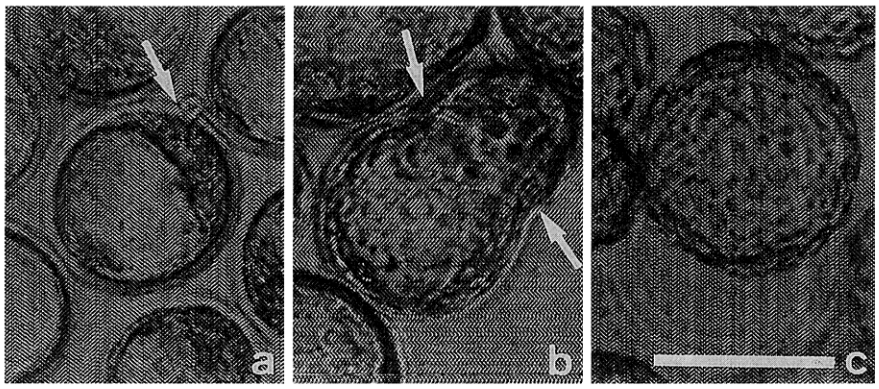

Fig. 2. Time-lapse videomicrographs of a non-treated mouse blastocyst showing completion of hatching. Scale indicates 100 μm. (a) At the onset of hatching. A trophectoderm cell protrudes from a small hole (arrow) in the zona pellucida. (b) During hatching. A slit (arrows) formed by enlarging a small slit is seen in the zona pellucida. (c) Completion of hatching. The blastocyst completely escapes from the slit in the zona pellucida.

start of hatching (Fig. 2c). On the other hand, a small hole was also formed in the zona pellucida in 24 (75.0%) of 32 STI-treated blastocysts 24.0 hrs after blastocoel formation, and hatching was started. The rate of STI-treated blastocysts with a small hole in the zona pellucida was similar to that of non-treated blastocysts. Of these 24 STI-treated blastocysts, 12 formed a slit in the zona pellucida by enlarging the hole 11.8 hrs after the start of hatching, and 5 of the 12 blastocysts completed escape through the slit 26.6 hrs after the start of hatching. The rate of STI-treated blastocysts with slit formation in the zona pellucida and that of STI-treated blastocysts completing hatching were 37.5 and 15.6%, respectively, which were significantly lower than those of non-treated blastocysts.

Activity of trypsin-like proteinase

When STI-treated blastocysts and non-treated blastocysts (Fig. 3a,b) were treated by the method of Perona and Wassarman [19], brown granules of Fast Garnet GBC were deposited in the cytoplasm of both lots of blastocysts. The granules were regarded as showing the presence of trypsin-like proteinase activity, because such granules did not appear in the control blastocysts incubated in the solution devoid of the substrate (Fig. 3c). In STI-treated and non-treated blastocysts, the activity was present in the cytoplasm of both mural and polar trophectoderm cells, but not in the cytoplasm of inner-cell-mass cells. In the present study, the activity of trypsin-like proteinase was judged by the amount of deposited granules of Fast Garnet GBC and estimated on a 2-point scale, as strong or weak, as shown in Figs. 3a and 3b. The percentages of STI-treated blastocysts with strong or weak activity were similar to those of non-treated blastocysts (Table 4).

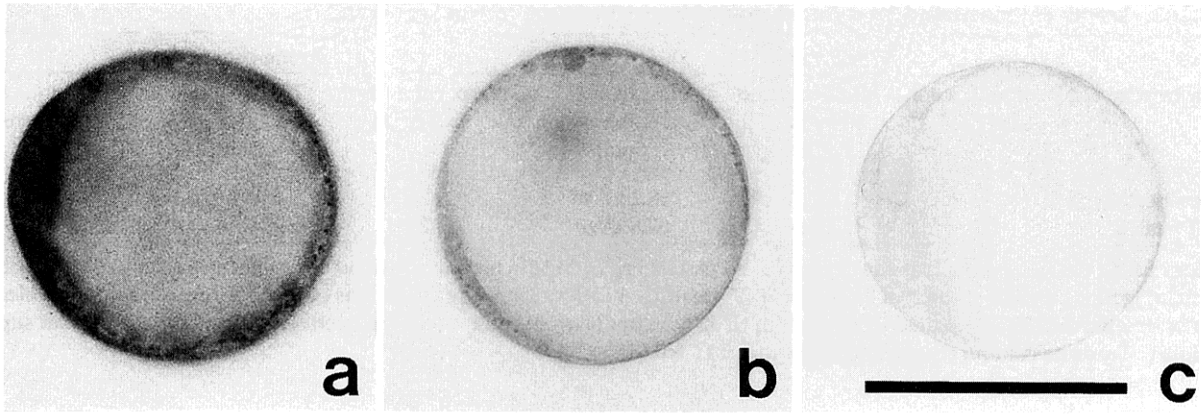

Fig. 3. Brown granules of Fast Garnet GBC showing the presence of the activity of trypsin-like proteinase are deposited in the cytoplasm of trophectoderm cells of non-treated mouse blastocysts (a,b), but not in the cytoplasm of any trophectoderm cells of a non-treated blastocyst (c) incubated in a substrate-free solution. Scale indicates 100 μm. (a) Strong activity. (b) Weak activity. (c) No activity.

Table 4. Activity of trypsin-like proteinase in cultured mouse blastocysts

Blastocysts	No. of blastocysts examined	No. and (%) of blastocysts showing	
		Strong activity	Weak activity
Non-treated	30	9 (30.0)[a]	21 (70.0)[a]
STI-treated	30	6 (20.0)[a]	24 (80.0)[a]

Blastocysts observed were cultured from morulae for 48 hrs in M16 medium without STI (non-treated) or in M16 medium containing STI at a concentration of 1.0 mg/ml (STI-treated). Values with different superscripts in the same column are significantly different ($P<0.05$).

Discussion

In the present study it was revealed that STI-treated mouse blastocysts had a low hatching rate and fewer weak contractions than non-treated blastocysts. These results indicate that weak contractions are particularly closely related to hatching of blastocysts, as suggested by previous reports [16–18]. A reason for the decrease in the number of weak contractions in STI-treated blastocysts is thought to be that both weak contraction and re-expansion after weak contraction took a longer time, than in non-treated blastocysts. It was also confirmed that the failure to complete hatching in STI-treated blastocysts resulted from no slit formation in the zona pellucida, although the activity of trypsin-like proteinase and the formation of a small hole in the zona pellucida in the STI-treated blastocysts were similar to those in the non-treated blastocysts. Although it is unclear whether STI inhibits the slit formation in the zona pellucida, the results suggest that STI has no effect on the synthesis and secretion of trypsin-like proteinase in blastocysts. In addition, the results of the observation on contractions and morphological changes in STI-treated blastocysts in the present study suggest that weak contraction and re-expansion after weak contraction play an important role in hatching by slitting the region adjacent to the small hole in the zona pellucida.

It has been reported that strong contractions increase in blastocysts that fail to complete hatching [16–18]. A reason for the failure to complete hatching in blastocysts with a large number of strong contractions is thought to be that embryos fail to complete hatching in time because a strongly contracted blastocyst requires a much longer time for re-expansion to the size before contraction [16,18]. It has also been observed that strong contractions increase in number in blastocysts in which hatching is inhibited by treatment with indomethacin, probably because the permeability of trophectoderm cells alters through inhibition of prostaglandin biosynthesis in embryos, resulting in an increase in the number of strong contractions [17]. In view of these results, it was postu-

lated that strong contractions also increased in number in STI-treated blastocysts in the present study because hatching was significantly inhibited in these blastocysts, but the number of strong contractions remained unchanged, whereas weak contractions decreased in number. Although the reason for this discrepancy in the number and strength of contraction is not clear, the realities of contractions in STI-treated and indomethacin-treated blastocysts were thought to differ because the hatching roles of trypsin-like proteinase and prostaglandin biosynthesis differ.

References

1) Lewis, W.H. and Gregory, P.W. (1929): Cinematographs of living developing rabbit-eggs. Science, 69, 226–229.
2) Kuhl, W. and Friedrich-Freksa, H. (1936): Richtungskörperbildung und Furchung des Eies sowie das Verhalten des Trophoblasten der weißen Maus. Zool. Ans. Suppl., 9, 187–195.
3) Borghese, E. and Cassini, A. (1963): Cleavage of mouse egg. In: Cinematography in Cell Biology (Rose, G.G., ed.), pp. 263–277, Academic Press, New York and London.
4) Cole, R.J. and Paul, J. (1965): Properties of cultured preimplantation mouse and rabbit embryos, and cell strains derived from them. In: Preimplantation Stages of Pregnancy (Wolstenholme, G.E.W. and O'Connor, M., eds.), pp. 82–112, J. & A. Churchill LTD, London.
5) Cole, R.J. (1967): Cinematographic observations on the trophoblast and zona pellucida of the mouse blastocyst. J. Embryol. Exp. Morph., 17, 481–490.
6) Mulnard, J.G. (1967): Analyse microcinématographique du développement de l'œuf de souris du stade II au blastocyste. Arch. Biol. Liége, 78, 107–138.
7) Bin, L. and Mulnard, J. (1980): Analyse cinématographique et morphométrique du comportement *in vitro* du blastocyste de la souris. Arch. Biol. Bruxelles, 91, 37–48.
8) Checiu, M., Schlechta, B., Checiu, I. and Sandor, S. (1990): *In vitro* studies on normal and pathological preimplantation development. I. Events of normal mouse preimplantation development as revealed by microcinematography. Morphol- Embryol., 36, 101–111.
9) Bitton-Casimiri, V., Brun, J.L. and Psychoyos, A. (1970): Comportement *in vitro* des blastocystes du 5e jour de la gestation chez la Ratte; étude microcinématographique. C.R. Acad. Sc. Paris, 270, 2979–2982.
10) Blandau, R.J. (1971): Culture of guinea pig blastocyst. In: The Biology of the Blastocyst (Blandau, R.J., ed.), pp. 59–70, The Univ. of Chicago Press, Chicago and London.
11) Bavister, B.D. (1987): Studies on the developmental blocks in cultured hamster embryos. In: The Mammalian Preimplantation Embryo: Regulation of Growth and Differentiation *In Vitro* (Bavister, B.D., ed.), pp. 219–249, Plenum Press, New York and London.
12) Kane, M.T. and Bavister, B.D. (1988): Vitamin requirements for development of eight-cell hamster embryos to hatching blastocysts in vitro. Biol. Reprod., 39, 1137–1143.
13) Gonzales, D.S. and Bavister, B.D. (1995): Zona pellucida escape by hamster blastocysts in vitro is delayed and morphologically different compared with zona escape in vivo. Biol. Reprod., 52, 470–480.
14) Massip, A. and Mulnard, J. (1980): Time-lapse cinematographic analysis of hatching of normal and frozen-thawed cow blastocysts. J. Reprod. Fert., 58, 475–478.
15) Massip, A., Mulnard, J., Vanderzwalmen, P., Hanzen, C. and Ectors, F. (1982): The behaviour of cow blastocyst *in vitro*: cinematographic and morphometric analysis. J. Anat., 134, 399–405.
16) Niimura, S. and Takahashi, E. (1995): Time-lapse videomicrographic observations of the contraction in cultured mouse blastocysts. Anim. Sci. Technol., 66, 713–719.
17) Niimura, S. and Takahashi, E. (1996): The in vitro influence of indomethacin and prostaglandins on the contraction of mouse blastocysts. Jpn. J. Fertil. Steril., 41, 13–17.
18) Niimura, S., Takeuchi, T. and Matsuyama, H. (2000): Time-lapse videomicrographic observations of the contraction in cultured blastocysts collected from mice of different ages. J. Reprod. Dev., 46, 1–7.
19) Perona, R.M. and Wassarman, P.M. (1986): Mouse blastocysts hatch *in vitro* by using a trypsin-like proteinase associated with cells of mural trophectoderm. Dev. Biol., 114, 42–52.
20) Whittingham, D.G. (1971): Culture of mouse ova. J. Reprod. Fert. Suppl., 14, 7–21.
21) Hurst, P.R. and MacFarlane, D.W. (1981): Further effects of nonsteroidal anti-inflammatory compounds on blastocyst hatching in vitro and implantation rates in the mouse. Biol. Reprod., 25, 777–784.
22) Dulbecco, R. and Vogt, M. (1954): Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med., 99, 167–182.