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—Original Article—

Time-Lapse Videomicrographic Observations of Blastocyst Hatching in Cattle

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Abstract. Morphological changes of cultured bovine blastocysts during hatching were observed using time-lapse videomicrography in order to investigate the patterns of the hatching process that occurred in the blastocysts and to determine whether the hatching patterns differed between blastocysts developed from fresh and cryopreserved embryos. Compacted morulae (CMs) were collected from superovulation-treated Japanese Black and Holstein dairy cattle and cultured in a medium in a CO_2 culture chamber equipped with an inverted microscope at 38.5 C. Images of resultant blastocysts during the period from blastocoel formation to completion of hatching were taken at 4-sec intervals by a CCD color camera connected to an inverted microscope and recorded by a time-lapse video cassette recorder. In blastocysts developed from fresh CMs, hatching was found to begin with protrusion of trophectoderm cells from zonae pellucidae at the expanded stage. Protrusion of the cells occurred in any site of the trophectoderm. After protrusion, a large or small slit was formed in the zona pellucida in all blastocysts as a result of blastocyst expansion or enlargement of the protrusion. Then, blastocysts completely escaped from the zona pellucida through the slit in the state of expansion. From these findings, the hatching patterns of cattle blastocysts could be classified into 5 types. In blastocysts developed from frozen-thawed CMs, the hatching pattern and length of time needed for hatching are similar to those in blastocysts developed from fresh CMs. In addition, the pregnancy rate of recipients following transfer of frozen-thawed CMs (52.4%) did not differ from that of recipients following transfer with fresh CMs (58.3%). These results suggested that the quality of frozen-thawed cattle embryos is comparable to that of fresh embryos and that there could be a relationship between the hatching pattern of blastocysts and the viability of embryos after transfer.

Key words: Cattle blastocyst, Cryopreserved embryo, Fresh embryo, Hatching pattern, Time-lapse videomicrography

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Lews and Gregory [1] first observed hatching in cultured rabbit blastocysts using time-lapse microcinematography. Since then, the process of blastocyst hatching has been morphologically observed not only in the rabbit [2, 3], but also in the golden hamster [4–6], guinea pig [7–9], rhesus monkey [10], cat [11], pig [12] and human [13–15]. The process of blastocyst hatching has been extensively studied in mice [16–23] and rats [23–26], and it was reported that the process of blastocyst hatching could be classified into 6 types in the mouse and 5 types in the rat, according to the site of protrusion of trophectoderm 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 [23].

In cattle blastocysts, the morphological changes during hatching have also been investigated by scanning electron microscopy [27] and time-lapse microcinematography [28]. Fléchon and Renard [27] reported that 31 out of 47 cultured blastocysts started hatching by protrusion of trophectoderm cells from zonae pellucidae after 24 h of culture and then formed a slit in the zona pellucida by blastocyst expansion. Massip *et al*. [28] reported that 22 out of 27 cattle blastocysts started hatching by protrusion of trophectoderm cells from zonae pellucidae at any site of the trophectoderm. Of these 22 cattle blastocysts that started hatching, 19 formed slits in the zona pellucida and completed hatching from the slit in the zona pellucida. The slits formed in the zonae pellucidae of cattle blastocysts are particularly large, and blastocysts forming small slits are reported to be unable to complete hatching [28]. In 3 blastocysts with incomplete hatching, 2 formed a small hole in the zona pellucida and stopped hatching when approximately half of the embryo had escaped from the zona pellucida [28]. However, there have been no reports with regard to the types of hatching patterns and time required for each process of hatching in cattle blastocysts based on morphological and dynamic observations.

On the other hand, Massip and Mulnard [29] have observed the morphological changes of frozen-thawed cattle blastocysts during hatching. In their observations, hatching was completed in only 1 of 11 cryopreserved early blastocysts, and its hatching was characterized by an initial protrusion of trophectoderm cells from a small hole in the zona pellucida, followed by expansion of the hole to form a larger slit in the zona pellucida up to the completion of hatching. Therefore, Massip and Mulnard [29] considered that the morphological changes during hatching of cryopreserved cattle embryos are identical to those of fresh embryos and that the ability to hatch in blastocysts after thawing is unaffected by the slow

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freezing procedure. Since the morphological changes during hatching in frozen-thawed blastocysts have only been examined in a single blastocyst by Massip and Mulnard [29], it is not clear what patterns of the hatching process occur in the frozen-thawed cattle embryos. Furthermore, there have been no reports with regard to the relationship between hatching patterns of blastocysts and their viability after transfer.

In the present study, the morphological changes of a large number of cultured bovine blastocysts developed from compacted morulae (CMs) were observed using time-lapse videomicrography in order to investigate what patterns of the hatching process occurred in the blastocysts and to determine whether the hatching patterns differed between blastocysts developed from fresh and frozen-thawed CMs. The pregnancy rate of recipients following transfer of fresh or frozen-thawed CMs was also examined.

Materials and Methods

Animals and collection of embryos

For collection of embryos to culture and transfer, 30 Japanese Black cattle and 25 Holstein dairy cattle aged 3 to 8 years at the Live Stock Research Center of the Niigata Prefectural Agricultural Research Institute were used in the present study. They were primiparous or biparous cattle. The animals were handled according to the guide for care and use of laboratory animals of the center. At 9 to 13 days after the onset of estrous, the cattle were intramuscularly injected with 20 to 40 AU of FSH (Antrin®, Kawasaki, Kawasaki, Japan; 12- h intervals over 3 days) in decreasing doses and subsequently with 750 to 1,000 μg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$: Cloprostenol C®, Fujita Pharmaceutical, Tokyo, Japan) 3 days later to induce superovulation. Artificial insemination was carried out using frozen semen of Japanese Black or Holstein bulls 58 to 60 h after the $PGF_{2\alpha}$ injection.

In observing the process of blastocyst hatching, as in the present study, embryos should be cultured before development to the blastocyst stage because images of individual blastocysts over time in the period soon after blastocoel formation are necessary for analysis of blastocyst hatching. Therefore, CMs were collected from superovulated cows 7 days after insemination. The CMs were non-surgically collected by uterine flushing using a catheter and 500 ml Ringer's solution containing sodium lactate (Nippon Zenyaku Kogyo, Fukushima, Japan) with 1% bovine serum (BS; Invitrogen, Carlsbad, CA, USA).

Cryopreservation and thawing of embryos

Thirty-two CMs with morphologically excellent or good quality [30] were collected and loaded into 0.25-ml plastic straws (AAA201, Cassou straw; IVM Technologies, L'Aigle, France) with Dulbecco's phosphate buffered saline (PBS; Invitrogen) containing 1.8 M ethylene glycol (Wako Pure Chemical, Osaka, Japan), 0.1 M sucrose (Sigma-Aldrich, St. Louis, MO, USA) and 0.4% BSA (Sigma-Aldrich). Straws were placed into a programmable freezer (ET-1, Fujihira Industry, Tokyo, Japan) kept at 0 C and cooled at 1 C/min to –7 C. They were seeded at –7 C by pinching them with forceps cooled with liquid nitrogen and held for 10 min at –7 C. The seeded straws were cooled at 0.3 C/min to –30 C and then plunged and stored in liquid nitrogen.

After storage in liquid nitrogen, cryopreserved straws were held in air for 6 sec and immersed in a water bath of 30 C for 20 sec. After thawing, embryos were recovered from straws and immersed in PBS containing 20% FCS (Gibco BRL, Grand Island, NY, USA). Ethylene glycol was removed from the embryos by incubation in 500 μl PBS containing 20% FCS for 10 min at 38.5 C. Embryos were then washed 2 times in PBS containing 20% FCS and 3 times in a culture medium, TCM-199 (Gibco BRL) containing 20% FCS (Gibco BRL) and 0.0007% β-mercaptoethanol (Sigma-Aldrich), and then cultured in each well of a 4-well multidish (Nunc, Roskilde, Denmark) containing 600 μl/well of the culture medium, which had previously been covered with mineral oil (Sigma-Aldrich) and equilibrated in a CO_2 incubator (4020, Asahi Life Science, Tokyo, Japan).

Observation of blastocyst hatching

In order to observe the process of blastocyst hatching, fresh CMs with morphologically good quality and frozen-thawed CMs were cultured in the medium in a CO_2 culture chamber (SK-1, Sankei, Tokyo, Japan; 5% CO_2 in air) equipped with an inverted microscope (DIAPHOT, Nikon, Tokyo, Japan) at 38.5 C. Observations were performed on images taken at 4-sec intervals by a CCD color camera (Hitachi Electronic, Tokyo, Japan) connected to an inverted microscope and recorded by a time-lapse video cassette recorder (BR-9050, Victor, Yokohama, Japan). The hatching process and length of time needed to hatch were observed in the images of both blastocysts developed from fresh and frozen-thawed CMs during the period from blastocoel formation to completion of hatching.

Assessment of viability in fresh and frozen-thawed embryos

In order to observe the development of CMs, fresh and frozen-thawed CMs were non-surgically transferred to the uteri of 24 and 21 cows (one embryo per recipient) on Day 7 of the cycle (Day 0, onset of estrus), respectively. Pregnancy was determined by ultrasound and rectal palpation 60 days after transfer.

Statistical analysis

The rate of hatching in blastocysts developed from fresh and frozen-thawed CMs and those of blastocysts showing each hatching type were statistically analyzed by the Chi-square test. The pregnancy rates of the recipients following embryo transfer were also statistically analyzed by the Chi-square test. The lengths of time needed by blastocysts to hatch were statistically analyzed by one-way analysis of variance. A value of $P<0.05$ was considered to be statistically significant.

Results

The rates of development of fresh and frozen-thawed CMs into blastocysts and the rates of hatching in the resulting blastocysts

When 34 fresh and 32 frozen-thawed CMs were cultured, a small cavity appeared within all the embryos, and the embryos developed into blastocysts. Of these 34 and 32 blastocysts, 34 and 31 of them, respectively, protruded trophectoderm cells from the zona pellucida and started hatching at the expanded stage (Fig. 1a–

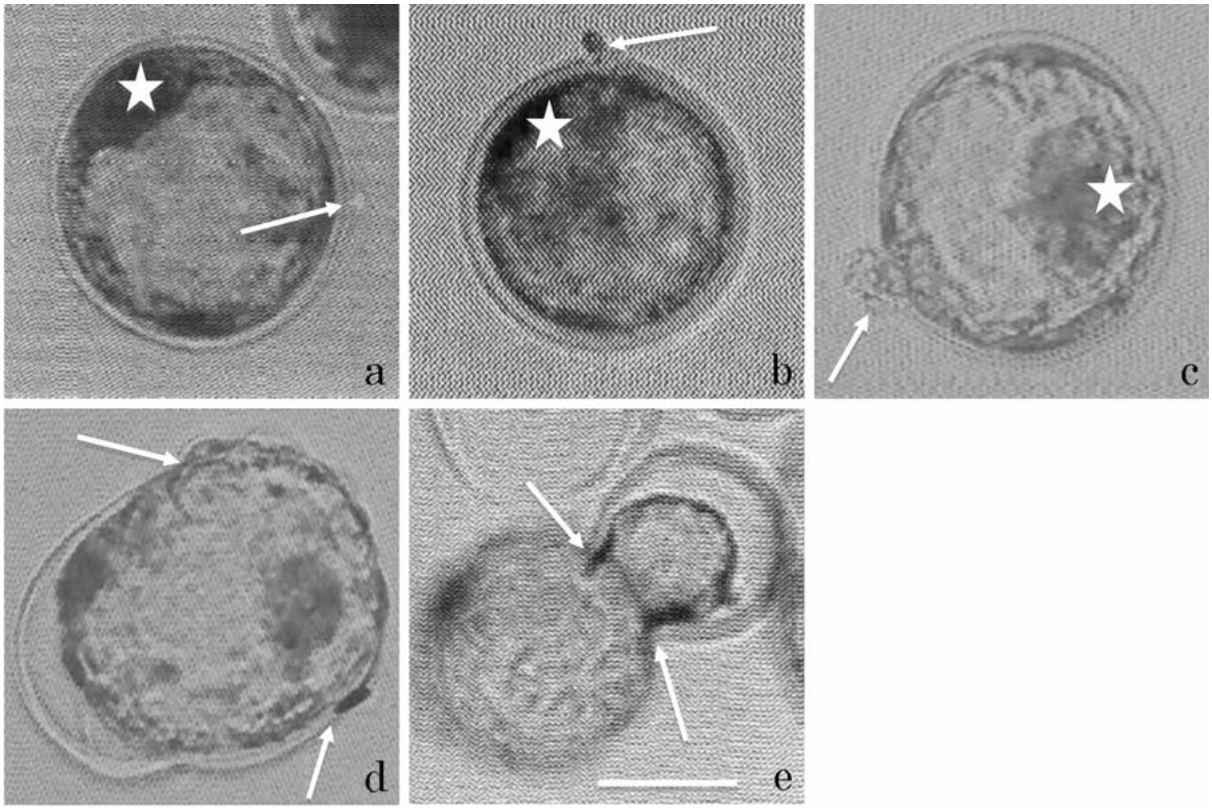

Fig.1. Time-lapse videomicrographs of cattle blastocysts developed from fresh CMs. Asterisks show inner cell masses. Scale bar indicates 80 μm. a: Protrusion of cells (arrow) from a small hole in the zona pellucida occurs at the mural trophectoderm. b: Protrusion of cells (arrow) from a small hole in the zona pellucida occurs at the embryonic polar trophectoderm. c: Protrusion of cells (arrow) from a small hole in the zona pellucida occurs at the abembryonic trophectoderm. d: A large slit (arrows) formed by blastocyst expansion is seen in the zona pellucida. e: A small slit (arrows) formed by enlargement of the protrusion is seen in the zona pellucida.

c). A slit was then formed in the zona pellucida in 31 blastocysts from each group (Fig. 1d and e). After that, all the blastocysts completely escaped through the slit in the zona pellucida and accomplished hatching.

The rate of development into blastocysts in frozen-thawed CMs did not differ from that in fresh CMs. There was also no significant difference in the hatching rates of blastocysts developed from fresh (91.2%) and frozen-thawed CMs (96.9%).

The process and patterns of hatching in blastocysts developed from fresh and frozen-thawed CMs

When the hatching patterns of the cattle blastocysts developed from fresh and frozen-thawed CMs were classified according to the site of protrusion of trophectoderm cells from the zona pellucida and the mode of slitting in the zona pellucida, the patterns could be classified into 5 types, as shown in Table 1.

In the 31 hatched blastocysts that developed from fresh CMs, the cells protruded out of zonae pellucidae from the mural trophectoderm in 11 embryos (35.5%, Types I and II, Fig. 1a), embryonic polar trophectoderm in 15 embryos (48.4%, Types III and IV, Fig. 1b) and abembryonic polar trophectoderm in 5 embryos (16.1%, Type V, Fig. 1c), respectively. These blastocysts with protrusion of trophectoderm cells from the zona pellucida increasingly protruded trophectoderm cells through the hole, and 25 (80.6%) formed a large slit in the zona pellucida by blastocyst expansion (Types I, III and V, Fig. 1d) and 6 (19.4%) formed a small slit by enlargement of the protrusion (Types II and IV, Fig. 1e). Then, these 31 blastocysts completely escaped from the zona pellucida through the slit in the state of expansion. The incidence of blastocysts showing Type I, III, IV or V was not significantly different.

In the 3 unhatched blastocysts that developed from fresh CMs, small holes were formed in the zona pellucida by cell protrusions from the mural trophectoderm in 1 embryo and from the embryonic polar trophectoderm in 2 embryos, but these small holes did not expand in all blastocysts. So, no slits were formed in the zona pellucida, and as a result, hatching could not be completed.

On the other hand, the 31 hatched blastocysts that developed from frozen-thawed CMs also protruded trophectoderm cells from a small hole in the zona pellucida at the expanded stage. Of the 31 blastocysts, 13 (41.9%, Types I and II), 11 (35.5%, Types III and IV) and 7 (22.6%, Type V) caused cell protrusions from the zona pellucida at the mural, embryonic polar and abembryonic polar trophectoderm, respectively. These blastocysts with a small hole in the zona pellucida increasingly protruded trophectoderm cells

Table 1. Hatching patterns of cultured cattle blastocysts developed from fresh and frozen-thawed CMs

Sites of cell protrusion	Modes of slitting in the zona pellucida	Types of hatching	No. (%) of blastocysts showing each type	
			Fresh	Frozen-thawed
Mural trophectoderm	Blastocyst expansion	I	10 (32.3) [a]	8 (25.8) [a]
	Enlargement of protrusion	II	1 (3.2)	5 (16.1) [a]
Embryonic polar trophectoderm	Blastocyst expansion	III	10 (32.3) [a]	10 (32.3) [a]
	Enlargement of protrusion	IV	5 (16.1) [a]	1 (3.2)
Abembryonic polar trophectoderm	Blastocyst expansion	V	5 (16.1) [a]	7 (22.6) [a]

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

Table 2. Length of time needed by cattle blastocysts developed from fresh CMs to hatch

Types of hatching	From blastocoel formation to the onset of hatching (h)	From the onset of hatching to slit formation in the zona pellucida (h)	From slit formation in the zona pellucida to completion of hatching (h)	Duration of hatching from its start to completion (h)
I	23.4 ± 4.1*[a]	25.7 ± 6.7 [a]	7.8 ± 1.6 [a]	33.5 ± 6.6 [a]
II	75.5	22.1	14.6	36.6
III	34.0 ± 6.9 [a]	14.9 ± 4.2 [a]	7.5 ± 1.6 [a]	22.5 ± 4.2 [a]
IV	21.8 ± 3.2 [a]	23.0 ± 8.3 [a]	16.3 ± 7.5 [a]	39.3 ± 6.1 [a]
V	20.9 ± 1.8 [a]	13.2 ± 4.4 [a]	5.8 ± 1.4 [a]	19.1 ± 5.6 [a]

*Mean ± SE. Values with different superscripts in the same column are significantly different ($P<0.05$).

Table 3. Length of time needed by cattle blastocysts developed from frozen-thawed CMs to hatch

Types of hatching	From blastocoel formation to the onset of hatching (h)	From the onset of hatching to slit formation in the zona pellucida (h)	From slit formation in the zona pellucida to completion of hatching (h)	Duration of hatching from its start to completion (h)
I	32.9 ± 4.9* [a]	14.5 ± 2.2 [a]	10.3 ± 2.7 [b]	24.8 ± 2.7 [a]
II	20.4 ± 5.8 [a]	13.7 ± 3.1 [a]	26.9 ± 6.3 [a]	40.6 ± 7.6 [a]
III	29.4 ± 4.2 [a]	18.8 ± 3.8 [a]	10.9 ± 2.8 [b]	29.7 ± 4.8 [a]
IV	16.8	2.1	19.2	21.3
V	27.9 ± 4.3 [a]	15.7 ± 3.1 [a]	9.3 ± 3.3 [b]	24.9 ± 5.8 [a]

*Mean ± SE. Values with different superscripts in the same column are significantly different ($P<0.05$).

through the hole, and 25 embryos (80.6%, Types I, III and V) formed a large slit in the zona pellucida by blastocyst expansion and 6 embryos (19.4%, Types II and IV) formed a small slit by enlargement of the protrusion. Then, all the blastocysts completed escape from the zona pellucida through the slit in the state of expansion. The incidence of blastocysts showing Type I, II, III or V was not significantly different.

An unhatched blastocyst that developed from a frozen-thawed CM did not start hatching and degenerated at the expanded stage.

None of the hatched blastocysts that developed from fresh and frozen-thawed CMs, in which protrusion had occurred from the abembryonic polar trophectoderm, completed hatching through the small slit in the zona pellucida.

In Types I, III and V, there were no significant differences in the incidences between blastocysts that developed from fresh and frozen-thawed CMs.

Length of time needed by blastocysts to hatch

As shown in Table 2, the mean duration of hatching from its start to completion and mean length of time required for each hatching process in blastocysts that developed from fresh CMs did not differ among those in Types I, III, IV and V, although the mean duration of hatching from its start to completion tended to be longer in Type II and IV blastocysts with a small slit in the zona pellucida.

In blastocysts that developed from frozen-thawed CMs, although the mean duration from slit formation in the zona pellucida to completion of hatching was significantly longer in Type II blastocysts than in Type I, III and V blastocysts, the lengths of time required for another hatching process did not differ among the Type I, II, III and V blastocysts (Table 3). The mean duration of hatching from its start to completion tended to be longer in Type II blastocysts with a small slit in the zona pellucida.

The mean duration of hatching from its start to completion and mean length of time required for each hatching process in the same hatching types were similar for blastocysts that developed from

fresh and frozen-thawed CMs.

Pregnancy rates of recipient cows

When 21 frozen-thawed CMs were transferred into 21 recipients, 11 (52.4%) of them became pregnant, showing no difference from the pregnancy rate (58.3%, 14/24) of recipients that received fresh CMs.

Discussion

In the present study, the detailed hatching process in a large number of cultured cattle blastocysts and the lengths of time needed by blastocysts to hatch were observed during the period from blastocoel formation to completion of hatching using time-lapse videomicrography. As a result, it was confirmed that the hatching patterns of cattle blastocysts could be classified into 5 types according to the site of protrusion of trophectoderm cells from the zona pellucida and the mode of slitting in the zona pellucida. The incidences of Type I and III blastocysts, in which hatching was found to begin with cell protrusions from the zona pellucida at either the mural or polar trophectoderm and hatching was completed through a large slit in the zona pellucida formed by expansion of blastocysts, were higher (32.3 and 32.3%) than in the remaining type blastocysts, although the incidences of blastocysts among Types I, III, IV and V were not significantly different.

The present study revealed that protrusion of cells from the zona pellucida occurred at any site of the trophectoderm, showing the absence of polarity in the hatching of cattle blastocysts. Concerning the occurrence of cell protrusion from the trophectoderm, the results of the present study are similar to those of previous investigations in cattle [27, 28], mouse and rat [23] blastocysts. It is generally accepted that blastocyst hatching requires regional dissolution of the zona pellucida by a hatching enzyme, trypsin-like proteinase. Demonstration of such an enzyme has been performed histochemically in blastocysts of rabbits [31], mice [23, 32, 33], rats [23] and cattle [34]. Since the activity of a hatching enzyme was reported to be present in cells of the embryonic polar, abembryonic polar and mural trophectoderm in cattle blastocysts [34], no localization of this enzyme in trophectoderm cells seems to be the reason for no polarity of protrusion of trophectoderm cells in cattle blastocysts.

In most of the blastocysts that formed small holes in the zona pellucida in the present study, a slit was formed in the zona pellucida at the same site, and then hatching was completed through the slit. These results suggest that formation of such a slit is essentially required for the hatching of cattle blastocysts. It has been reported that cattle blastocysts forming small slits in the zona pellucida are unable to complete hatching [28], while the present study is the first to reveal that 19.4% (6/31) of blastocysts can complete hatching through a small slit in the zona pellucida. The reason for this discrepancy can be explained by the fact that Massip *et al*. [28] observed only 19 blastocysts. The fact that none of the blastocysts having protrusion from the abembryonic polar trophectoderm completed hatching through the small slit in the zona pellucida is thought to be characteristic of blastocyst hatching in cattle.

Concerning the time required for hatching, there was no significant difference among the Type I, III, IV and V blastocysts. However, the time required for hatching from its start to completion tended to take longer in the Type II and IV blastocysts, which formed small slits in the zona pellucida. This result suggests that the large slit in the zona pellucida contributes to hatching by facilitating the escape of blastocysts from the zona pellucida and that the size of the slit formed in the zona pellucida influences the time required for hatching in cattle blastocysts.

On the other hand, Massip and Mulnard [29] observed by time-lapse microcinematography that one frozen-thawed cattle blastocyst completed hatching through the same morphological changes as fresh blastocysts. From their results, they concluded that the slow freezing and thawing treatments have no influences on the hatching of cattle blastocysts.

In the present study, we revealed that cattle blastocysts developed from frozen-thawed CMs have the same hatching types as those in blastocysts developed from fresh CMs. The present study also revealed that the site of protrusion of the trophectoderm from the zona pellucida, the mode of slit formation in the zona pellucida and the length of time needed for hatching in blastocysts developed from frozen-thawed CMs are similar to those in blastocysts developed from fresh CMs. In addition, we found no significant difference in the pregnancy rate between recipients following transfer of frozen-thawed and fresh CMs. This result for the pregnancy rate in recipients with frozen-thawed CMs may support the view that there are no differences in the hatching patterns between blastocysts developed from fresh and frozen-thawed CMs, as mentioned above.

The results of the present study suggest that the developmental competence of frozen-thawed cattle embryos is comparable to that of fresh embryos and that there could be a relationship between the hatching patterns of blastocysts and the development of embryos after transfer.

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