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Basic Study of Assisted Hatching Using Mouse Embryo
—Comparison of the Effects with Partial Zona Dissection, Zona Drilling and Zona Thinning—

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Abstract: *[Purpose] Different six types of assisted hatching (AHA) were applied to mouse embryo and their effect on hatching was investigated. [Methods] The zona pellucida was treated as follows. (a) Piercingness (PIC): Pierce the zona with a very fine needle. (b) Partial zona dissection-small (PZD-small): Proceed the same as with PZD in microinsemination and make an incision about 1/8 the circumference in the zona. (c) Partial zona dissection-large (PZD-large): Enlarge the incision about 1/4 the circumference. (d) Zona drilling (ZD): Spray acid solution to the zona, which dissolves the zona to create a hole about 30 μm in diameter. (e) Zona thinning-partial (ZT-partial): Thin down a part of the outer area of the zona to about half the thickness by the same procedure as in ZD. (f) Zona thinning-total (ZT-total): Thin down the total outer area of the zona. [Results] When these AHA were each administered to 4-cell-stage embryos, the effects of PZD-large, ZD, ZT-partial and ZT-total on the hatching were observed, but the effects of PIC and PZD-small were not recognized. Furthermore the effects of AHA on 8-cell-stage embryo, morula and frozen-thawed embryo were recognized. With PZD-partial in which the opening of incision is small, the embryo in the halfway through hatching was trapped in the thick zona pellucida. With PZD-large or ZD in which an incision or a hole is large, hatching started at the earlier stage than normal (premature hatching). [Conclusion] With the role of the zona and the time to start hatching taken into consideration, zona thinning (ZT-partial or ZT-total) appears to become the most reasonable AHA.*

Key words: *Assisted hatching, Zona pellucida, Zona hardening, Frozen-thawed embryo, Mouse embryo.*

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Hatching of embryos is an important phenomenon in the process of reproduction. With the culturing conditions improved, embryos of good quality can be obtained even from eggs fertilized *in vitro*, but the implantation rate of *in vitro* fetilization and embryo transfer (IVF-ET) is far from satisfactory. Zona hardening occurs from various causes in embryos cultured *in vitro*. It is believed to be partly responsible for impairment of hatching and subsequent obstruction of implantation. Expectation is placed on assisted hatching (AHA) as a new technique to enhance the implantation rate. In this study we applied six kinds of AHA to mouse embryos and studied their effect on hatching along with their influence on the development of embryos.

Materials and Methods

Collection of mouse fertilized eggs

Five weeks old female B6C3F1 mice were induced to superovulate by consecutive intraperitoneal injections 48 hr apart of 8 IU pregnant mare serum gonadotropin (PMS; Teikokuzoki Co., Tokyo, Japan) and 8 IU human chorionic gonadotropin (hCG; Mochida Pharmaceutical Co., Tokyo, Japan). After hCG injection, the females were mated with male mice of the same strain (about 10 weeks after birth). Four-cell-stage embryos were collected from oviducts 52 hr after hCG administration.

Procedures for assisted hatching

The zona pellucida of mouse embryo was treated by six procedures as follows. A mouse embryo was put in 5 μl of Hepes-buffered human tubal fluid (Hepes-HTF; Irvine Scientific, Irvine, CA) placed under mineral oil, and micromanipulation was done for the zona pellucida.

(a) Piercingness (PIC): Pierce the zona pellucida with a very fine needle (8 μm in diameter).
(b) Partial zona dissection-small (PZD-small): Proceed the same as with partial zona dissection (PZD) in microinsemination and make an incision about 1/8 the circumference in the zona pellucida.
(c) Partial zona dissection-large (PZD-large): Enlarge the incision in the above-mentioned partial zona dissection to make an incision about 1/4 the circumference.
(d) Zona drilling (ZD): Spray acid Tyrode's solution (pH 2.5) to the zona pellucida, which dissolves the zona pellucida to create a hole. See that the diameter of the hole be about 30 μm.
(e) Zona thinning-partial (ZT-patial): Thin down a part of the outer area of the zona pellucida (circle about 30 μm in diameter) to about half the thickness by the same procedure as in zona drilling.
(f) Zona thinning-total (ZT-total): Thin down the total outer area of the zona pellucida to about 1/2 by immersing the embryo in an acid Tyrode's solution (pH 2.5) for about 3 sec. It is only this procedure that requires no micromanipulation.

Experiments

Experiment 1: The effect of protein sources in culture medium on hatching of embryos: Mouse 4-cell-stage embryos were cultured in human tubal fluid (HTF; Irvine Scientific) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco Lab., Gland Island, NY) or HTF supplemented with 3.5% bovine serum albumin (BSA, Fraction V; Sigma Chemical Co., St Louis, MO). After incubation at 37°C under 5% CO_2, 5% O_2 and 90% N_2, the status leading to the hatching was observed over time using a stereoscopic microscope.

Experiment 2: Effect of AHA on mouse 4-cell-stage embryo: Assisted hatching in (a) to (f) was performed for mouse 4-cell-stage embryos immediately after collection. The embryos were cultured in a 3.5% BSA-added HTF and the status of hatching was observed at 48, 72 and 96 hr after collection of eggs.

Experiment 3: Effect of timing of AHA on result of AHA: Mouse 4-cell-stage embryos were cultured in a 3.5% BSA-added HTF. Assisted hatching of PZD-large (c) was performed for the 4-cell-stage embros, 8-cell-stage embyos after 10 to 15 hr culturing and morula after 18 to 22 hr culturing, and comparison was made of the status of hatching thereafter.

Experiment 4: Effect of AHA on frozen-thawed embryo: Mouse 4-cell-stage embryos were stored frozen immediately after collection. A mixture of 1.5 M propandiol (PROH; Sigma Chemical Co.) and 0.075 M treharose (Sigma Chemical Co.) was used as a cryoprotectant. The embryos were cooled rapidly down to −30°C with a programming freezer (ET-U3; Fujiya-Yano Kagaku Co., Tokyo, Japan) (rapid freezing) and were stored in liquid nitrogen for 2 weeks. The frozen embryos were rapidly thawed in tepid water of 37°C. Thereafter AHA of PZD-large (c) was performed, and using a 10% FCS-added HTF as a culture medium the status of hatching was observed over time.

Statistical analysis

The results of the experiment were studied by Chi-squared test and the difference was regarded as significant when the P value showed 0.05 or less.

Results

Effect of protein sources in culture medium on hatching of embryo: When the mouse 4-cell-stage embryos were cultured in HTF+ 10% FCS, the rates of partial hatching (halfway through hatching) 48, 72 and 96 hr after collection were 44%, 53% and 37% respectively. The rates of complete hatching at each prescribed hour were 0%, 30% and 48% respectively. The rates of partial hatching when the embryos were cultured in HTF+ 3.5% BSA were 9%, 42% and 28% and the rates of complete hatching were 0%, 14% and 38% (Fig. 1). When 3.5% BSA was added to the culture medium, the start of hatching was obviously delayed and suppression of hatching was recognized, as compared with the

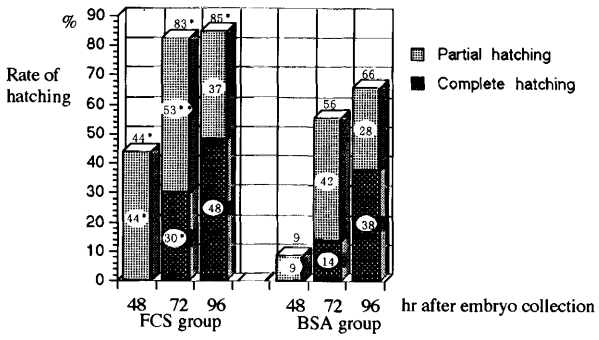

Fig. 1. Effect of protein sources in culture medium on hatching of embryo. FCS group: Embryos cultured in HTF+10% FCS (n=150). BSA group: Embryos cultured in HTF+3.5% BSA (n=150). * P<0.01: compared with BSA group. ** P<0.05: compared with BSA group.

addition of FCS. So embryos cultured in HTF+ 3.5% BSA were used as "the model of lower hatching rate" in this experiments to follow.

Effect of AHA on mouse 4-cell-stage embryo: When different types of AHA in (a) to (f) were each administered to mouse 4-cell-stage embryos, the rates of partial hatching and the rates of complete hatching 48, 72 and 96 hr after collection were summarized in Table 1. In Fig. 2 were graphically illustrated the rates of partial hathing 48 hr after and the rates of complete hatching 72 hr after.

With the procedure described in (a), piercing of the zona pellucida was all that was done. The effect of AHA was not obserbed, with no difference in the status of hatching between the manipulated embryos and the control. With the procedure in (b), an incision about 1/8 the circumference was made on the zona pellucida. While hatching was off to a good start early, most embryos were trapped in the zona pellucida because of the opening of incision being small, and hatching was not completed. With the procedure in (c), the incision was expanded to 1/4 the circumference. Hatching started early and the rate of complete hatching was high. With the procedure in (d), a hole was chemically made in the zona pellucida. The effect of AHA was the same as in the procedure (c). With the pocedures in (e) and (f), a part or total of the zona pellucida was thinned. The start of hatching was delayed compared with the procedures in (b), (c) and (d), but the rate of complete hatching was good.

Comparison was made of the thickness of the zona pellucida when hatching was taking place. In the control (intact zona pellucida) the zona pellucida was streched thin because hatching started after the blasto-

Table 1. Effect of AHA on hatching of mouse embryo

Type of AHA	No. of embryo	Status of hatching	No. of hatched embryo (%)		
			48 hr after embryo collection	72 hr after embryo collection	96 hr after embryo collection
(a) PIC	42	Partial	1 (2.4%)	21 (50%)	14 (33%)
		Complete	0 (0%)	6 (14%)	13 (31%)
		Total	1 (2.4%)	27 (64%)	27 (64%)
(b) PZD-small	63	Partial	41 (65%)*	57 (90%)*	51 (81%)*
		Complete	0 (0%)	1 (1.6%)*	7 (11%)*
		Total	41 (65%)*	58 (92%)*	58 (92%)*
(c) PZD-large	152	Partial	112 (74%)*	64 (42%)	40 (26%)
		Complete	0 (0%)	82 (54%)*	106 (70%)*
		Total	112 (74%)*	146 (96%)*	146 (96%)*
(d) ZD	154	Partial	125 (81%)*	60 (39%)	29 (19%)
		Complete	0 (0%)	86 (56%)*	117 (76%)*
		Total	125 (81%)	146 (95%)*	146 (95%)*
(e) ZT-partial	100	Partial	38 (38%)*	34 (34%)	10 (10%)*
		Complete	0 (0%)	60 (60%)*	85 (85%)*
		Total	38 (38%)	94 (94%)*	95 (95%)*
(f) ZT-total	211	Partial	26 (12%)**	42 (20%)*	21 (10%)*
		Complete	10 (5%)	120 (57%)*	160 (76%)*
		Total	36 (17%)*	162 (77%)*	181 (86%)*
Intact (Control)	417	Partial	31 (7%)	167 (40%)	104 (25%)
		Complete	0 (0%)	54 (13%)	167 (40%)
		Total	31 (7%)	221 (53%)	271 (65%)

PIC: Piercingness. PZD-small: Partial zona dissection-small. PZD-large: Partial zona dissection-large. ZD: Zona drilling. ZT-partial: Zona thinning-partial. ZT-total: Zona thinning-total. * $P<0.01$ compared with control. ** $P<0.05$ compared with control.

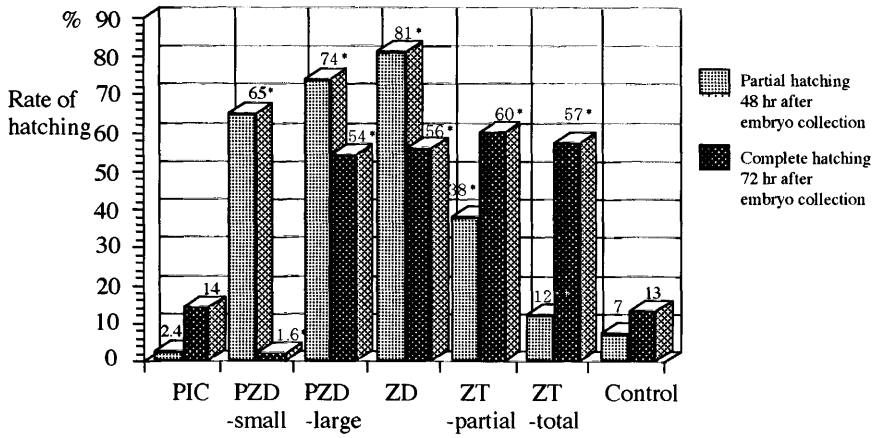

Fig. 2. Effects of different types of AHA on hatching of embryo. PIC: Piercingness (n=42). PZD-small: Partial zonadissection-small (n=63). PZD-large: Partial zonadissection-large (n=152). ZD: Zona drilling (n=154). ZT-partial: Zona thinning-partial (n=100). ZT-total: Zona thinning-total (n=211). Control: Intact zona pellucida (n=417). * P<0.01: compared with control. ** P<0.05: compared with control.

cyst had expanded fully (Fig. 3-A), while with (b) PZD-small, (c) PZD-large and (d) ZD in which a hole was made in the zona pellucida the zona did not become thin and remained thick because hatching started at the morular or early blastocyst stage (Fig. 3-B). With (b) PZD-small in which the opening of incision is small, the embryo in the halfway through hatching was trapped in the thick zona pellucida (Fig. 3-C). With the zona thinning in (e) ZT-partial and (f) ZT-total, the zona pllucida became thin because hatching started when the blastocyst had expanded to some extent (Fig. 3-D).

Effect of timing of AHA on the result of AHA: When AHA of PZD-large (c) was performed at the 4-cell-stage, 8-cell-stage and morular stage, the proportion of embryos in the halfway through hatching 48, 72 and 96 hr after collection of eggs (4-cell-stage embryos) and the rates of complete hatching were as follows. Assisted hatching for 4-cell-stage embryos: The rates of partial hatching were 72%, 49% and 29%. The rates of complete hatching were 0%, 47% and 70%. Assisted hatching for 8-cell-stage embryos: The rates of partial hatching were 83%, 49% and 30%. The rates of complete hatching were 0%, 47% and 68%. Assisted hatching for morula: The rates of partial hatching were 84%, 53% and 31%. The rates of complete hatching were 5%, 45% and 67%. Control: The rates of partial hatching were 7%, 42% and 31%. The rates of complete hatching were 0%, 20% and 43%. Hatching was facilitated in the AHA-treated embryos but differences according to the time to perform AHA were not recognized (Table 2).

Effect of AHA on frozen-thawed embryo: Of the frozen-thawed 4-cell-stage embryos, the proportion of embryos halfway through the hatching 48, 72 and 96 hr after starting culture were 22%, 29% and 23%, and the rates of complete hatching were 0%, 27% and 41% respectively. Of the control (fresh 4-cell-stage embryos), the rates of partial hatching were 47%, 48% and 34%, and the rates of complete hatching were 0%, 31% and 47%. When AHA (PZD-large) was performed for frozen-thawed 4-cell-stage embryos, the rates of partial hatching were 51%, 34% and 15%, and the rates of complete hatching were 17%, 62% and 82% respectively (Fig. 4). Hatching of frozen-thawed embryos tend to be delayed compared with fresh embryos. Assisted hatching was indicated to facilitate the hatching of frozen-thawed embryos.

Discussion

With a remarkable advance in assisted reproductive technology in recent years, the fertilization rate and cleaving rate in IVF-ET have obviously increased, but the implantation rate remains low. Impairment of hatching due to the *in vitro* induced zona hardening is thought to be one of the reasons for the low implantation rate in IVF-ET. The details of the mechanism of hatching have yet to be elucidated, but two factors are believed to be

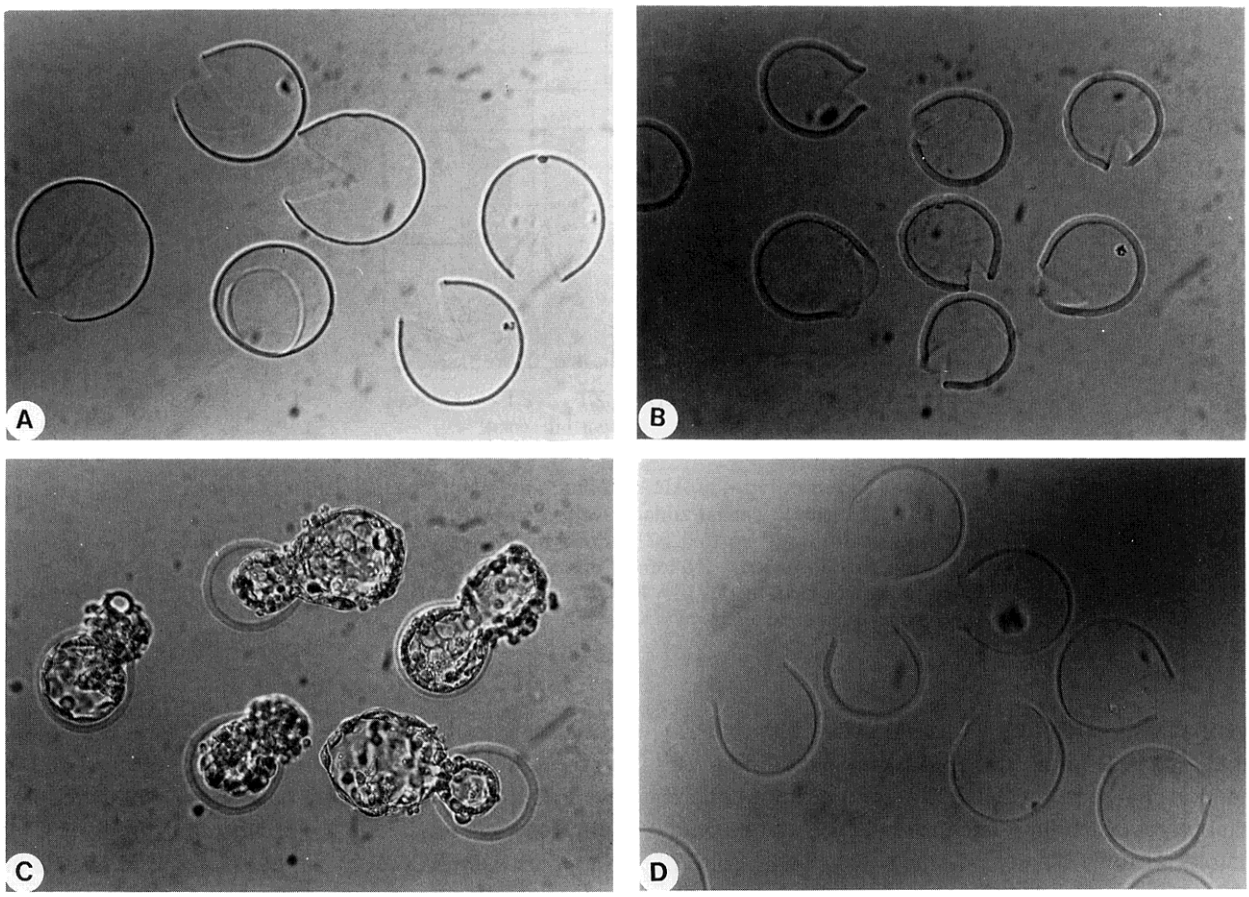

Fig. 3. Zonae pellucidae of mouse embryos (× 200). (A) Zonae of intact embryos (control): Zonae were streched thin because hatching started after the blastocysts had expanded fully. (B) Zonae of embryos administered PZD-large: Zonae did not become thin and remained thick because hatching started at the morular or early blastocyst stage. (C) Zonae of embryos administered PZD-small: Embryos in the halfway through hatching were trapped in the thick zona pellucida. (D) Zonae of embryos administered ZT-total: Zonae became thin because hatching started when the blastocysts had expanded to some extent.

concerned with that mechanism. One is zona lysins that are secreted from the embryo itself or from within the female genital ducts, and the other is the pressure from within arising from expansion of the blastocyst [1].

It is said that the blastocyst expands the zona pellucida from within while repeating expansion and contraction cyclically [2] and eventually dissolves the zona pellucida by the action of lysins to cause rupture. Gordon and Dapunt [1] have maintained that of the two factors, zona lysins play a leading role and have demonstrated the importance of lysins being available in sufficient quantity during the implantable period in mouse. Persona and Wasserman [3] found a trypsin-like protease that was secreted from mural trophectoderm immediately before hatching, named it "strypsin" and showed that it played an important role in the progress of hatching. Thereafter, this protease was identified also in the culture medium containing embryos that started hatching [4]. Additionally, it was also made clear that hatching is obstructed by a protease inhibitor [5], so the importance of the role of this substance as zona lysin was confirmed. Also it has been proved that the hatching rate of mouse embryos is correlated with the concentration of essential amino acid in the culture medium [6]. The necessity of essential amino acid and serum in hatching has also been pointed out. In the present study too, the hatching rate was significantly low in the embryos cultured in the medium with only albumin (BSA) added compared to the embryo in the medium containing serum, which enabled us to confirm the importance of serum.

When the sperm-egg fusion occurs, cortical gran-

Table 2. Effect of timing of AHA on result of AHA

Timing of AHA	No. of embryo	Status of hatching	No. of hatched embryo (%)		
			48 hr after embryo collection	72 hr after embryo collection	96 hr after embryo collection
4-cell stage	70	Partial	50 (72%)*	34 (49%)	20 (29%)
		Complete	0 (0%)	33 (47%)*	49 (70%)*
		Total	50 (72%)*	67 (96%)*	69 (99%)*
8-cell stage	80	Partial	66 (83%)*	39 (49%)	24 (30%)
		Complete	0 (0%)	38 (47%)*	54 (68%)*
		Total	66 (83%)*	77 (96%)*	78 (98%)*
Morula stage	91	Partial	76 (84%)*	48 (53%)	28 (31%)
		Complete	5 (5%)	41 (45%)*	61 (67%)*
		Total	81 (89%)*	89 (98%)*	89 (98%)*
Intact (Control)	124	Partial	9 (7%)	52 (42%)	38 (31%)
		Complete	0 (0%)	25 (20%)*	53 (43%)*
		Total	9 (7%)	77 (62%)	91 (73%)

*: P<0.01 compared with control.

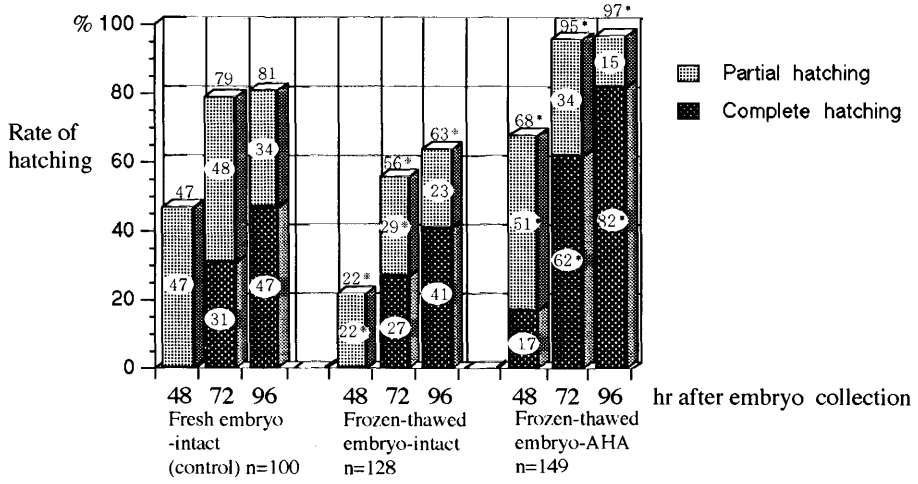

Fig. 4. Effect of AHA on frozen-thawed embryo. ※ P<0.01: compared with intact fresh embryo group. * P<0.01: compared with intact frozen-thawed embryo group.

ules are released and thereby the zona pellucida undergoes a physical and chemical change (zona reaction), which leads to zona hardening [7, 8]. This change prevents polyspermy, protects the embryo from physical, chemical and immunological impairment [9] and plays an important role in the development of early embryo after fertilization. However it has been known that *in vitro* induced zona hardening due to the culturing conditions different from *in vivo* takes place in addition to this physiological zona hardening in the *in vitro*-cultured embryo [10, 11]. Furthermore, the absence of lysins originating from female genital tracts in the *in vitro* culture system also makes for the impairment of hatching. Assisted hatching (AHA) is aimed to assist hatching by manipulating the zona pellucida of such embryos in various ways with a micromanipulator. This time we made a comparative study as to the effect of various types of AHA using the mouse 4-cell stage embryos. As a result, we found that creating a relatively large opening in the zona pellucida or thinning of it facilitated hatching

well. Presumably PZD is the most widely used AHA, almost all of the embryos becoming trapped in the thick zona pellucida when the opening of incision is small. And in this case, complete hatching rate become lower than non-treated intact embryos (control) (Fig. 2). That is to say, PZD-small rather makes detrimental effect to the process of embryo hatching. Reports also have it that the separation and rupture of inner-cell masses occur when the embryo passes through a slit-like narrow opening of incision, resulting in an increase of enzygotic twins [12]. In contrast, a relatively large hole (30 μm) is formed with ZD [13, 14] so the hatching-facilitating effect is satisfactory. In this case, concern is felt about the influence of acid Tyrode's solution used for opening the zona pellucida on the egg cell membrane, but it appears to pose no problem about the development and implantation of the embryo in the case of fertilized eggs [15], while its detrimental effect on unfertilized eggs has been confirmed [16, 17]. When, however, a large hole is created in the zona pellucida, (either with PZD or ZD), the hatching starts at the earlier stage —morula or early blastocyst— than normal (premature hatching), and additionally the risk of the embryo being trapped in the zona pellucida becomes greater due to the thinning of the zona pellucida being insufficient. If the embryo transfer is performed at such a stage, it might bring about serious events such as protruding of a part of the blastomeres from the opening during manipulation or in the uterus, entering of immunocytes present in the uterus from the opening of incision and flowing out of zona lysins secreted from the embryo [18]. Zona thinning (ZT) is a method that was contrived to make up for such drawbacks of the zona pellucida-opening technique. It is a new AHA whereby a part of the outside or the entire circumference of the zona pellucida is thinned by using an acidic solution or enzymes such as pronase. The effect of ZT has already been reported [18, 19]. With this method, hatching starts in a nearly physiological condition when the blastocyst is expanded and the zona pellucida has become thin to some extent. In the present study using mice, we also were able to show its efficacy.

When the embryo implanted by IVF-ET was examined retrospectively, thinning of the zona pellucida was already in progress [20, 21] and the implantation rate for the embryos subjected to microinsemination by zona opening for fertilization was good [22]. These facts paved the way to clinical application of AHA. Cohen [22] performed AHA by PZD for human embryos two days after collection of eggs, reporting a significantly high pregnancy rate (46%) over the pregnancy rate (26%) in the non-AHA group. According to Cohen *et al.* [23], however, it is not that AHA is effective for any embryos, but that AHA is rather counterproductive and causes a delay in the development of embryo and incomplete implantation in embryos, of which development is good and in which thinning has advanced. Mentioning items as follows as indications for AHA on the basis of the retrospective study, they have proposed a "selective assisted hatching". (a) Embryos whose zonae had overall thickness of more than 15 μm on the embyo transfer, (b) embryos obtained from patients age 39 or older, or whose basal FSH level is high, (c) embryos that show a delay in development or excessive (over 20%) fragmentation, (d) where no implantation occurred despite embryos of good quality having been transferred.

Embryos from elderly or high FSH patients and embryos showing excessive fragmentation appear to cause hatching impairment easily [23, 24]. They performed AHA for 790 patients, 2,569 embryos according to the indications and obtained good results with the pregnancy rate 43% (for patients) and implantation rate 22% (for embryos) [25]. Opinions are varied on the stage of embryo at which to perform AHA. According to our present study, no difference was seen in the effect of AHA from the 4-cell stage to the morula stage. It is reasonable to perform AHA as late as possible when the function of the zona pellucida and the time to start hatching are taken into consideration.

It has been suggested that freezing-thawing of an egg changes the property of the zona pellucida [26]. With unfertilized eggs, freezing-thawing causes the entry of the sperm into the zona pellucida to decline markedly [27]. In our present study too, hatching tended to be delayed and suppressed compared with the control, and good hatching occurred with AHA. For the frozen-thawed embryos, it would be better to perform AHA positively [12, 26].

In our present study we were able to obtain a good rate of complete hatching by any procedures of AHA except small incision. And with the role of the zona pellucida and the time to start hatching taken into consideration, zona thinning appears to become the most reasonable AHA. However, the biophysical properties of the zona pellucida are different between mice and humans (almost uniform structure over the full thickness of the zona pellucida in mice vs a close double structure inside in humans) [28, 29], so further studies are required with respect to the clinical application. Recently, a combinational use of co-culture and AHA [30, 31], and AHA using lasers have been reported [32, 33], each showing good results. We intend to pursue the

most effective AHA in human IVF-ET on the basis of the results obtained in this study and also by taking the morphological and chemical structure of the human zona pellucida into consideration.

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