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# Intactness of Zona Pellucida Does Not Affect the Secretion of a Trypsin-like Protease from Mouse Blastocyst

Assisted hatching (AH), which is known to improve the hatching potential of mammalian embryos, has been used to increase the pregnancy rate in in vitro fertilization cycles. However, the effect of AH on a trypsin-like protease, which is known to be associated with the hatching process, has not been studied. In this study, we evaluate whether the intactness of zona pellucida affects the secretion of a trypsin-like protease from mouse blastocyst. Four- to 8-cell stage mouse embryos were collected at 66- to 68 hr after hCG injection and divided into 3 groups according to the manipulation of zona pellucida. The groups are no treatment (control), drilling of zona pellucida (ZD) and thinning of zona pellucida (ZT). The activity of a trypsin-like protease, blastocyst development and hatching rate were compared among the three groups at 110 and 135 hr after hCG injection, respectively. The protease activity and blastocyst development were not significantly different among control, ZD and ZT groups at 110 and 135 hr after hCG injection, respectively. However, the hatching rate of ZD and ZT groups was significantly higher than that of control group at each time, respectively ($p$<0.001). Even in the zona pellucida removed embryos, the protease activity did not differ from the control group. In conclusion, the secretion of a trypsin-like protease from mouse blastocyst does not seem to be affected by the intactness of zona pellucida.

Key Words: *Protease Activity; Zona Pellucida; Mouse Embryo*

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## INTRODUCTION

Despite the efforts to improve the pregnancy rate, the implantation rate following in vitro fertilization-embryo transfer (IVF-ET) program remains low (1). One of the proposed limiting factors is impaired hatching caused by zona pellucida (ZP) hardening. Zona pellucida hardening is known to be the result of fertilization, cryopreservation, in vitro culture and aging process (2). Although ZP hardening can beneficial by blocking polyspermy, protecting the embryo from immunologic attack and aiding in oviductal transport, it can also be disadvantageous by impeding embryonic hatching and attributing to monozygotic twinning (3).

Mammalian embryos must escape from ZP to be able to implant in the uterus at the blastocyst stage (4, 5). One of the main mechanisms for embryonic hatching is lysis of the ZP by a trypsin-like protease secreted from either the embryo or female reproductive tract (6). A trypsin-like protease secreted from mouse blastocyst has been known to play an important role in embryonic hatching (7, 8). A few assisted hatching (AH) methods have been used to clinically improve the hatching potential and pregnancy rate (9, 10). However, the exact mechanism of AH has not been elucidated.

Under the hypothesis that AH treatment improves the hatching potential by increasing a trypsin-like protease secretion from mouse blastocyst, this study was undertaken to investigate whether intactness of ZP affects the secretion of a trypsin-like protease associated with embryonic hatching.

## MATERIALS AND METHODS

### Preparation of embryos

C57BL/6 (♀) × CBA (♂) F1 hybrid female mice at 7 weeks of age were superovulated with 5 IU of pregnant mare's serum gonadotropin (PMSG, Sigma). Forty-eight hours later, 5 IU of human chorionic gonadotropin (hCG, Sigma) was administrated intraperitoneally, followed by

mating overnight. At 66-68 hr after hCG injection, 4- to 8-cell stage embryos were collected by flushing of dissected oviducts of plugged female mice using Ham's F-10 supplemented with 3 mg/mL bovine serum albumin (culture medium). About 40 embryos in each group per experiment were cultured in a micro-droplet of the culture medium (embryo/0.5 μL) under paraffin oil at 37℃ in a humidified atmosphere of 5% $CO_2$ in air.

### Manipulation of zona pellucida

Two treatment methods (ZD: zona drilled; ZT: zona thinned) were used. Zona drilled method using acid Tyrode solution (pH 2.5) was performed as described by Gorden and Talansky (11). To make the ZT, the acid solution was applied to the ZP. The embryos that ruptured during ZT were excluded.

For the ZP-removed (ZR) embryos, four- to 8-cell stage embryos were collected and cultured by the same method. At 80 hr after hCG injection, half of the morula stage embryos were used as ZR embryos. The embryos were introduced to 0.1% (v/w) protease solution (P-8811, Sigma), washed 3 times with fresh medium, and then transferred to the culture drop. The last embryos were used as a control group.

### Protease activity assay

The conditioned medium was collected at 110 and 135 hr after hCG injection, respectively, and frozen at -70℃ until protease assay. The protease activity was measured as described previously (8) with a slight modification. The reaction of the protease was immediately started after adding 10 μL of the thawed medium into 0.5 mL of 0.1 mM t-butoxycarbonyl (Boc)-Leu-Ser-Thr-Arg-4-methyl-coumaryl-7-amide (MCA) (Peptide Institute, Inc., Japan) in 50 mM Tris/HCl (pH 9.0) and the initial velocity was detected at 37℃. The protease activity was measured by spectrofluorophotometer (excitation: 380 nm, emission: 460 nm; RF-5301PC, Shimadzu, Japan). In the control study, the protease activity showed good correlation with the MCA, the reaction product (data not shown). And the protease activity was not increased by the substrate only.

### Statistical analysis

Using chi-square analysis, development and hatching rate were compared, and the enzyme activity was compared by ANOVA. $p$ value of less than 0.05 was considered as statistically significant ($p<0.05$).

## RESULTS

At 110 hr after hCG injection, the protease activity of ZP-treated groups was not significantly different compared to that of control group. However, the hatching rate of ZP-treated groups was significantly higher than that of control group ($p<0.001$, Fig. 1). Particularly, ZT group showed higher hatching rate than ZD group ($p<0.01$).

At 135 hr after hCG injection, the protease activity of ZP-treated groups was not significantly different compared to that of control group. However, the hatching rate of ZP-treated groups was significantly higher than that of control group ($p<0.001$, Fig. 2). Unlike at 110 hr, the hatching rate between ZP-treated groups was not

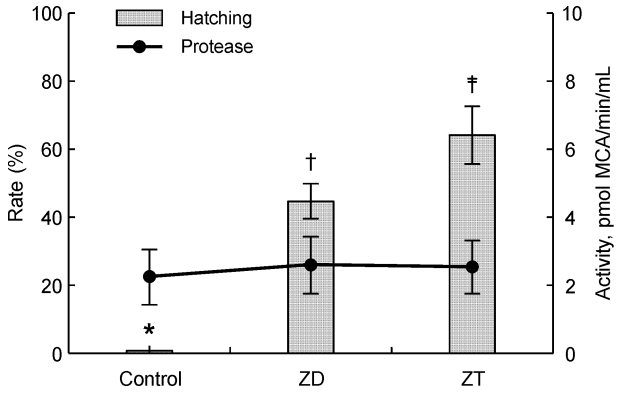


Fig. 1. Comparison between hatching rate and activity of a trypsin-like protease in the control, ZD and ZT groups at 110 hr after hCG injection. The value of each column was obtained from three replications of experiments and expressed as mean ±standard error. ZD, drilling of zona pellucida; ZT, thinning of zona pellucida; * vs †,‡: $p<0.001$; † vs ‡: $p<0.01$

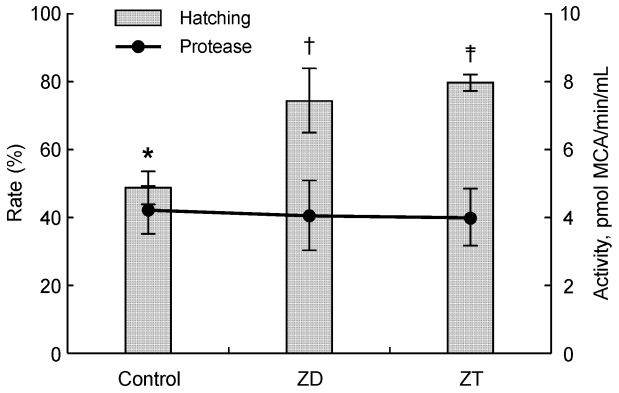


Fig. 2. Comparison between hatching rate and a protease activity in control, ZD and ZT groups at 135 hr after hCG injection. The value of each column was obtained from three replications of experiments and expressed as mean±standard error. ZD, drilling of zona pellucida; ZT, thinning of zona pellucida; * vs †,‡: $p<0.001$; † vs ‡: $p>0.05$

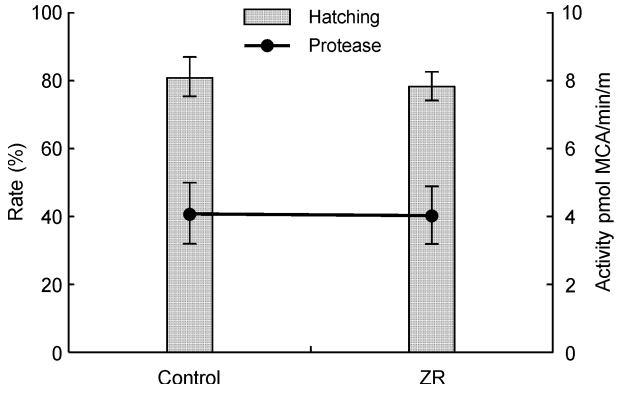


Fig. 3. Comparison between blastocyst development and a protease activity in the ZR embryos compared to control group. The value of each column was obtained from three replications of experiments and expressed as a mean±standard error. ZR, zona pellucida removed

different at 135 hr after hCG injection ($p>0.05$).

To evaluate whether protease secretion was related with the presence of ZP, the protease activity of the ZP-removed (ZR) embryos was assessed. The protease activity of the control and ZR embryos was not significantly different at 135 hr after hCG injection (Fig. 3).

The percentage of embryos developed to blastocyst was not different among the three groups at each time point (Table1).

## DISCUSSION

Mammalian embryos are known to exhibit delayed development, and they have lower hatching rates in vitro than in vivo because of inadequate culture condition. Because in vitro culture system may induce ZP hardening and reduce hatching potential, AH methods have been used to improve the hatching potential. Although effects of AH on hatching and implantation remain to be elucidated, AH methods have improved pregnancy rate in patients with old age ≥38, elevated basal FSH, increased ZP thickness, prior IVF failure in any program and excessive fragmentation (2). Also, AH methods have been reported to advance the hatching time (9, 12). Liu et al. (13) suggested that the efficiencies of AH could be postulated as follows: First, AH facilitated implantation by allowing earlier embryo-endometrial contact. Second, earlier contact might increase chances of endometrial exposure and enhance embryo developmental potential by permitting earlier exposure of the embryo to vital growth factors derived from the endometrium. Last, earlier contact might also optimize synchronization between embryo and endometrium, resulting in improved implantation efficiency. Therefore, earlier hatching time is important to higher implantation in in vitro culture system.

In the present study, we reconfirmed that the ZP-treated groups had higher hatching rate and earlier hatching time than the control group. Particularly, between the two treated methods, ZT method may be more effective than ZD method, because the ZT group showed significantly higher hatching rate than the ZD group at 110 hr after hCG injection.

Hatching is an indispensable procedure to implantation. One of the main hatching mechanisms is lysis of ZP by enzyme secreted from either embryo or female reproductive tract (6). A trypsin-like protease secreted from mouse blastocyst has been known to play an important role in hatching. Perona and Wassarman (7) showed that the protease (strypsin) was found in cells of mural trophectoderm, not in polar trophectoderm or inner cell mass, and that the hatching of mouse blastocyst in vitro was initiated by the ZP region overlying mural trophectoderm. However, there are some controversies about the location of the protease expression (14, 15). Sawada et al. (8) revealed that a trypsin-like protease, which most efficiently hydrolyzed t-butoxycarbonyl-Leu-Ser-Thr-Arg-4-methylcoumaryl-7-amide, detected in hatching medium played a key role in mouse blastocyst hatching, probably as a hatching protease. Although the protease did not completely dissolve the ZP, it partially digested or weakened the ZP at a stage before hatching. Therefore, the protease secreted from mouse blastocyst might be a prerequisite for embryo hatching. In in vitro culture system, however, the in vitro hatching rate was lower than in vivo due to the dilution effect of culture medium (6, 16).

In this study, although the hatching rate of the ZP-

Table 1. Development rate in the control, ZD and ZT groups according to culture time

| Group | No. of embryos examined | No. of embryos developed to blastocyst (%) | |
|---|---|---|---|
| | | At 110 hr* | At 135 hr* |
| Control | 117 | 90 (76.9) | 96 (82.1) |
| ZD | 121 | 95 (78.5) | 99 (81.8) |
| ZT | 114 | 99 (86.8) | 102 (89.5) |

The value of each column was obtained from three replications of experiments
ZD, drilling of zona pellucida; ZT, thinning of zona pellucida; *, time after hCG injection

treated groups was significantly higher than that of control group at both 110 and 135 hr after hCG injection, respectively, the protease activity among control, ZD and ZT groups was not different. Even in the ZR embryos, there was no difference in the protease activity compared to the control group. These results suggest that the hatching rate is not affected by the secretion of a trypsin-like protease in in vitro culture system and that the protease is secreted regardless of intactness of ZP.

In conclusion, AH treatment improves the hatching potential not by the mechanism of an increased trypsin-like protease secretion from mouse blastocyst.

## REFERENCES


1. Cohen J, Elsner C, Kort H, Malter H, Massey J, Mayer MP, Wiemer K. *Impairment of hatching process following IVF in the human and improvement of implantation by assisting hatching using micromanipulation. Hum Reprod 1990; 5: 7-13.*
2. Schlenker TW, Schoolcraft W. *Assisted hatching: techniques and results. Annual Meeting Postgraduate Course Evaluation Form. Course VII, 1997; 103-13.*
3. Alikani M, Noyes M, Cohen J, Rosenwaks Z. *Monozygotic twinning in the human is associated with the zona pellucida architecture. Hum Reprod 1994; 9: 1318-21.*
4. McLaren A. *The embryo. In: Austin CR, Short RV, eds. Reproduction in mammals. Cambridge University Press, 1982; 2: 1-25.*
5. Renfree MB. *Implantation and placentation. In: Austin CR, Short RV, eds. Reproduction in mammals. Cambridge University Press, 1982; 2: 26-69.*
6. Gorden JW, Dapunt U. *New mouse model for embryos with a hatching deficiency and its use to elucidate the mechanism of the blastocyst hatching. Fertil Steril 1993; 59: 1296-301.*
7. Perona RM, Wassarman PM. *Mouse blastocysts hatch in vitro by using a trypsin-like proteinase associated with cells of mural trophectoderm. Dev Biol 1986; 114: 42-52.*
8. Sawada H, Yamasaki K, Hoshi M. *Trypsin-like protease from mouse embryos: evidence for the presence in culture medium and its enzymatic properties. J Exp Zool 1990; 254: 83-7.*
9. Khalifa E-AM, Tucker MJ, Hunt P. *Cruciate thinning of the zona pellucida for more successful enhancement of blastocyst hatching in the mouse. Hum Reprod 1992; 7: 532-6.*
10. Lee DR, Lee JE, Yoon HS, Kim MK, Roh SI. *The supplementation of culture medium with protease improves the hatching rate of mouse embryos. Hum Reprod 1997; 12: 2493-8.*
11. Gorden JW, Talansky BE. *Assisted fertilization by zona drilling: a mouse model for correction of oligospermia. J Exp Zool 1986; 239: 347-54.*
12. Malter H, Cohen J. *Blastocyst formation and hatching in vitro following zona drilling of mouse and human embryos. Gamete Res 1989; 24: 67-80.*
13. Liu HC, Cohen J, Alikani M, Noyes N, Rosenwarks Z. *Assisted hatching facilitates earlier implantation. Fertil Steril 1993; 60: 871-5.*
14. 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.*
15. Yamazaki K, Kato Y. *Sites of zona pellucida shedding by mouse embryo other than mural trophectoderm. J Exp Zool 1989; 249: 347-9.*
16. Schiewe MC, Hazeleger NL, Sclimenti C. *Physiological characterization of blastocyst hatching mechanisms by use of a mouse antihatching model. Fertil Steril 1995; 63: 288-94.*