The supplementation of culture medium with protease improves the hatching rate of mouse embryos

Dong Ryul Lee1,3,4, Jeoung Eun Lee1, Hyun Soo Yoon1, Ho-Joon Lee2, Moon Kyoo Kim3 and Sung Il Roh1

1Infertility Research Center, Jeil Women’s Hospital, 2Infertility Research Laboratory, Samsung Cheil General Hospital and 3Department of Biology, Hanyang University, Seoul, Korea
4To whom correspondence should be addressed at: Infertility Research Center, Jeil Women’s Hospital, Daechi-dong 1021-4, Kangnam-ku, Seoul 135-280, Korea

Mammalian embryos are known to exhibit delayed development and have lower hatching rates in vitro than in vivo because of inadequate culture condition. These discrepancies may be due to a deficiency of the paracrine factors and proteolytic enzymes which exist in the oviduct and uterus. In order to evaluate the effects of proteases on embryonic development and hatching, 2-cell mouse embryos were cultured for 72 h with or without proteases. The addition of 1.0 μg/ml pronase (PE) and/or 0.1 μg/ml proteinase K (PK) did not affect embryonic development up to the blastocyst stage (94.1% versus 88.2%; 92.2% versus 90.2%, respectively) but significantly increased the hatching rate (60.4% versus 39.2%, 71.8% versus 35.3%, respectively). However, the addition of α-chymotrypsin (Chymo) was detrimental to embryonic development and hatching. Changes in the structure of the zona pellucida (ZP) structure of embryos which had been cultured in human tubal fluid (HTF) medium with PE and PK were assessed by fluorescein isothiocyanate-conjugated (FITC)-casein. Embryos cultured in HTF–PE and PK were not stained with FITC–casein. When these embryos were cultured within oviducts, their perivitelline space (PVS) became strongly stained with FITC–casein which was easily removed by phosphate-buffered saline washing. This suggests that PE and PK altered the structure of the ZP. We suggest that the addition of PE and PK to culture media may accelerate the hatching of embryo, by structurally altering the ZP and PVS. This may provide a valuable and effective assisted hatching technique for human in-vitro fertilization-embryo transfer.

Key words: assisted hatching/embryonic hatching/mouse embryo/protease/zona pellucida

Introduction

The zona pellucida (ZP) is the extracellular matrix that surrounds oocyte, and has a multi-role function. Prior to fertilization, it presents a species-specific sperm barrier. Immediately following fertilization, the ZP acts as a major block to polyspermey (Wassarman, 1992). The ZP may protect the oocyte against the infiltration of leukocytes (Modilinski, 1970) and infection by bacterial or fungal agents (Singh, 1987) in the reproductive tract. Also, it prevents the separation of blastomeres from cleaving embryos (Bronson and McLaren, 1970) and maintains the cell arrangement of early embryos to ensure successful development (Suzuki et al., 1995).

Hatching of the blastocyst from the ZP is prerequisite for implantation. If the blastocyst does not hatch within the time period that the uterus is receptive to implantation, pregnancy will fail. The mechanisms of hatching are not well characterized but are known to involve two factors. One is lysis of the ZP by substances secreted either by the embryo or the female reproductive tract. The second is pressure exerted on the ZP by expansion of the blastocyst. Perona and Wassarman (1986) reported that a trypsin-like protease was secreted by the mural trophoblast immediately before hatching. Further, a trypsin-like protease was identified in culture medium in which mouse embryos had been allowed to hatch (Sawada et al., 1990). Addition of protease inhibitors to culture medium inhibited hatching in vitro (Dabich, 1981; Yamazaki et al., 1985).

Recently, Gorden and Dapunt (1993) suggested that the main mediator of hatching was lysis of the ZP, rather than pressure applied against it, using a blastomere destruction and mineral oil droplet insertion model in the mouse.

In domestic animals the hatching rate is lower in vitro than in vivo. This may be caused by zona hardening (Cohen, 1991; Zhang et al., 1991) or by reduction of zona lysin (Gordon and Dapunt, 1993; Schiewe et al., 1995). Failure of hatching leads to a failure of implantation. In human in-vitro fertilization (IVF)–embryo transfer programmes, women aged >38 years or with elevated basal follicle stimulating hormone (FSH) level were shown to have oocytes with abnormally thick ZP and low hatching ability, resulting in low pregnancy rates. To improve the hatching potential of blastocysts, assisted hatching using methods such as partial zona dissection (PZD, Malter and Cohen, 1989b) and zona drilling (Cohen et al., 1992) has been proposed. Recently, it has been reported that assisted hatching techniques can enhance success in many cases of human IVF-embryo transfer. However, use of a micromanipulator and/or acid Tyrode solution may be harmful to embryonic development by exposing it to external environments for a long period, or through toxicity.

The purpose of this study was to evaluate the effect of the addition of proteases to culture medium on embryonic development and hatching, and hence to develop a new method for assisted hatching.
Materials and methods

Recovery of mouse embryos
Female mice (ICR strain) were superovulated using a 5 IU i.p. injection of pregnant mare’s serum gonadotrophin (G-4877; Sigma Chemical Co., St Louis, MO, USA) followed by injection of human chorionic gonadotrophin (HCG, CG-5; Sigma) and then mated with 12 week old males. Mating was confirmed by the presence of a copulation plug on the morning following the HCG injection. The 2-cell stage embryos were recovered from the oviduct at 48–50 h after HCG injection and washed in human tubal fluid medium (HTF; Quin et al., 1985) supplemented with 0.5% bovine serum albumin (BSA; Gibco BRL, Grand Island, NY, USA).

Culture of mouse embryos in media containing proteases
In order to determine the concentration of proteases which would thin rather than completely remove the ZP during 72 h incubation, 2-cell mouse embryos were cultured in 0.5% BSA–HTF supplemented with various concentrations of proteases: pronase (PE; from Streptomyces griseus, type XIV, 4 units/mg, P-5147; Sigma), proteinase K (PK; from Tritirachium album, 10–20 units/mg, P-6556; Sigma) or α-chymotrypsin (Chymo; bovine pancreas, type IL, 40–60 units/mg, C-4129; Sigma). The control group was cultured in HTF supplemented with 0.5% BSA. Each protease was added to 0.5% BSA–HTF at a concentration of 1 mg/ml, and then diluted with 0.5% BSA–HTF to give concentrations of 10, 1, 0.1 or 0.01 μg/ml.

All media used in culture were preincubated for 6–8 h at 37°C under 5% CO₂ in air. The 2-cell mouse embryos were cultured in 0.5% BSA–HTF supplemented with various concentrations of PE, PK or Chymo, and embryonic development and state of ZP were examined at 24 h intervals under an inverted microscope (Diaphot 300; Nikon, Tokyo, Japan).

Evaluation of proteases effects on embryonic development and hatching
After determination of appropriate protease concentrations, the 2-cell mouse embryos were cultured in 30 μl drops of 0.5% BSA–HTF, or 0.5% BSA–HTF supplemented with PE, PK or Chymo at 37°C under 5% CO₂ in air. Blastocyst development and hatching was examined at 72 h after culture under an inverted microscope. Partially hatched blastocysts were judged to ‘hatching’.

To evaluate the effects of combined proteases on embryonic development and hatching, the 2-cell mouse embryos were cultured in 30 μl drops of 0.5% BSA–HTF, or 0.5% BSA–HTF supplemented with PE+PK or PE+PK+Chymo. Blastocyst development and hatching was examined 72 h later.

Evaluation of protease effects on embryonic development and hatching according to the incubation period
To determine the effect of protease treatment time on embryonic development and hatching, the 2-cell mouse embryos were cultured in 0.5% BSA–HTF supplemented with 1.0 μg/ml PE, 0.1 μg/ml PK, and 1.0 μg/ml PE and 0.1 μg/ml PK for 24 or 48 h and then transferred to 0.5% BSA–HTF. In control groups, the 2-cell embryos were cultured in 0.5% BSA–HTF or 0.5% BSA–HTF supplemented with 1 μg/ml PE for 72 h. Blastocyst development and hatching was examined 72 h later.

Evaluation of ZP alteration by fluorescence microscopy
To evaluate changes induced by protease treatment, ZP and perivitelline space (PVS) were assessed by fluorescein isothiocyanate-conjugated casein staining (FITC-casein, C-0528; Sigma; Kim and Schultz, 1993). Briefly, the 2-cell mouse embryos were divided into two groups: one was cultured in 0.5% BSA–HTF supplemented with 1 μg/ml PE and 0.1 μg/ml PK for 24 h, the other was cultured in 0.5% BSA–HTF for 24 h. Germinal vesicle-stage oocytes were matured in 0.5% BSA–HTF and used as a control group. Some embryos and oocytes were used for staining and others were used for organ culture. For staining, embryos and oocytes were incubated in 200 μg/ml FITC–casein in Ca²⁺-free PBS for 15 min at room temperature. After washing in PBS, they were placed on clean slides and examined under a fluorescence microscope (Optiphot-2; Nikon). For organ culture, embryos and oocytes were transferred into oviduct, cultured together for 20 h and then stained with FITC–casein in PBS for 15 min. After PBS washing, they were placed on the clean slides, and examined under a fluorescence microscope.

Statistical analysis
Differences in rates of development and hatching between protease-treatment groups and control group were analysed by χ²-test. P < 0.05 was defined as statistically significant.

Results
The effects of PE, PK or Chymo on mouse embryonic development and hatching are summarized in Tables I–III. The inclusion of 1 μg/ml PE in the medium induced neither ZP loss nor reduced embryonic development, but increased the hatching rate after 72 h of culture (60.4% versus 39.2%, P < 0.01, Table I). 0.1 μg/ml PE had no significant effect on blastocyst development or hatching. The inclusion of PK at a lower concentration of 0.1 μg/ml did not affect blastocyst development, but significantly increased the hatching rate (71.8% versus 35.3%, P < 0.01, Table II). 0.01 μg/ml PK had no significant effect on blastocyst development or hatching. However, inclusion of 1 μg/ml Chymo led to a significantly

### Table I. Development and hatching of 2-cell mouse embryos cultured in HTF containing 0.5% BSA with or without pronase (PE) for 72 h

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of embryos (%)</th>
<th>No. of embryos (%)</th>
<th>No. of blastocysts hatching (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>102</td>
<td>90 (88.2)</td>
<td>40 (39.2)</td>
</tr>
<tr>
<td>1.0 μg/ml PE</td>
<td>101</td>
<td>95 (94.1)</td>
<td>61 (60.4)*</td>
</tr>
<tr>
<td>0.1 μg/ml PE</td>
<td>102</td>
<td>84 (82.4)</td>
<td>21 (20.6)</td>
</tr>
</tbody>
</table>

*P < 0.01 compared to control.

HTF = human tubal fluid; BSA = bovine serum albumin

### Table II. Development and hatching of 2-cell mouse embryos cultured in HTF containing 0.5% BSA with or without proteinase K (PK) for 72 h

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of embryos (%)</th>
<th>No. of embryos (%)</th>
<th>No. of blastocysts hatching (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>102</td>
<td>92 (90.2)</td>
<td>36 (35.3)</td>
</tr>
<tr>
<td>0.1 μg/ml PK</td>
<td>103</td>
<td>95 (92.2)</td>
<td>74 (71.8)*</td>
</tr>
<tr>
<td>0.01 μg/ml PK</td>
<td>103</td>
<td>96 (93.2)</td>
<td>40 (38.8)</td>
</tr>
</tbody>
</table>

*P < 0.01 compared to control.

HTF = human tubal fluid; BSA = bovine serum albumin

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**Notes:**
- **No. of embryos examined.**
- **Control:** HTF = human tubal fluid; BSA = bovine serum albumin
- **Two groups:** one was cultured in 0.5% BSA–HTF supplemented with 1 μg/ml PE and 0.1 μg/ml PK for 24 h, the other was cultured in 0.5% BSA–HTF for 24 h. Germinal vesicle-stage oocytes were matured in 0.5% BSA–HTF and used as a control group. Some embryos and oocytes were used for staining and others were used for organ culture. For staining, embryos and oocytes were incubated in 200 μg/ml FITC–casein in Ca²⁺-free PBS for 15 min at room temperature. After washing in PBS, they were placed on clean slides and examined under a fluorescence microscope (Optiphot-2; Nikon). For organ culture, embryos and oocytes were transferred into oviduct, cultured together for 20 h and then stained with FITC–casein in PBS for 15 min. After PBS washing, they were placed on the clean slides, and examined under a fluorescence microscope.

**Statistical analysis**
Differences in rates of development and hatching between protease-treatment groups and control group were analysed by χ²-test. P < 0.05 was defined as statistically significant.
hatching rate after 24 h inclusion was not different from the control group. After 48 h, the hatching rate was slightly lower for all groups cultured in 0.1 μg/ml PK, and 1.0 μg/ml PE and 0.1 μg/ml PK significantly increased the hatching rate, mouse embryos must be cultured in medium containing 1.0 μg/ml PE for at least 48 h. A similar result was obtained following culture in 0.1 μg/ml PK, and 1.0 μg/ml PE and 0.1 μg/ml PK (data not shown).

Table III. Development and hatching of 2-cell mouse embryos cultured in HTF containing 0.5% BSA with or without α-chymotrypsin (Chymo) for 72 h

<table>
<thead>
<tr>
<th>Group</th>
<th>No. a</th>
<th>No. of embryos (%)</th>
<th>Morula</th>
<th>Blastocyst</th>
<th>No. of blastocysts hatching (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>103</td>
<td>14</td>
<td>89 (86.4)</td>
<td>25 (24.3)</td>
<td></td>
</tr>
<tr>
<td>1.0 μg/ml Chymo</td>
<td>103</td>
<td>32</td>
<td>71 (68.9)*</td>
<td>7 (6.8)*</td>
<td></td>
</tr>
<tr>
<td>0.1 μg/ml Chymo</td>
<td>101</td>
<td>7</td>
<td>94 (93.1)</td>
<td>29 (28.7)</td>
<td></td>
</tr>
</tbody>
</table>

*aNo. of embryos examined.
*bProtease supplementation: 1 μg/ml pronase plus 0.1 μg/ml proteinase K; Chymo = α-chymotrypsin.

Table IV. Developmental and hatching rate of 2-cell mouse embryos cultured in human tubal fluid medium (HTF) supplemented with combined proteases for 72 h

<table>
<thead>
<tr>
<th>Group</th>
<th>No. a</th>
<th>No. of embryos (%)</th>
<th>Morula</th>
<th>Blastocyst</th>
<th>No. of blastocysts hatching (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTF + 0.5% BSA</td>
<td>103</td>
<td>8</td>
<td>95 (92.2)</td>
<td>38 (36.9)</td>
<td></td>
</tr>
<tr>
<td>1.0 PE + 0.1 PK</td>
<td>103</td>
<td>19</td>
<td>84 (81.6)</td>
<td>62 (60.2)*</td>
<td></td>
</tr>
<tr>
<td>0.1 PE + 0.01 PK</td>
<td>103</td>
<td>9</td>
<td>94 (91.3)</td>
<td>64 (62.1)*</td>
<td></td>
</tr>
<tr>
<td>1.0 PE + 0.1 PK</td>
<td>102</td>
<td>37</td>
<td>65 (63.7)*</td>
<td>65 (63.7)*</td>
<td></td>
</tr>
<tr>
<td>+ 1.0 Chymo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Protease concentrations are given as μg/ml.

Table VI. The binding patterns of fluorescein isothiocyanate–casein to perivitelline space of mouse oocytes and embryos

<table>
<thead>
<tr>
<th>Group</th>
<th>No. a</th>
<th>After culture for 24 h in control or protease-supplemented medium b</th>
<th>After further culture in isolated oviduct for 24 h</th>
<th>After subsequent washing with PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control MII oocytes</td>
<td>3</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Control embryos</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Treated embryos</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*aNo. of experiments performed, each with >10 oocytes or embryos.
*bProtease supplementation: 1 μg/ml pronase plus 0.1 μg/ml proteinase K. MII = in-vitro matured metaphase II; PBS = phosphate-buffered medium.

To evaluate whether PE and/or PK in culture media had any effect on ZP and PVS, the mouse embryos treated with 1 μg/ml PE and 0.1 μg/ml PK for 24 h were assessed by FITC–casein (Table VI). In vitro-matured MII oocytes were not stained with FITC–casein. However, after culture within an oviduct, a distinct fluorescence staining within the PVS could be seen which did not change after PBS washing. When 2-cell embryos were cultured in 0.5% BSA-HTF for 24 h and stained with FITC–casein, the PVS became stained and was not removed by PBS washing. However, the 8-cell embryos cultured in media containing PE and PK were not stained with FITC–casein (Figure 1). When these embryos were co-cultured in an oviduct, the PVS became strongly stained with FITC–casein, which was easily removed by PBS washing (Table VI).

Discussion

In mammals, embryonic development up to the blastocyst stage and hatching from the ZP are prerequisites for implantation and subsequent pregnancy. In vitro, inadequate culture conditions may give rise to a reduction of zona lysin by delayed embryonic development, which may induce zona hardening and result in failure of implantation.

It has been suggested that assisted hatching techniques may enhance implantation not only by facilitating the embryonic hatching process but also by allowing earlier embryo–endometrium contact (Liu et al., 1993), and may also promote the transport of growth factor(s) to enhance embryonic

Proteases improve hatching rate in mouse embryo
development. Artificial alteration of the ZP has been induced both by PZD using mechanical force and by ZD using acidic Tyrode's solution. In many studies, it has been reported that these assisted hatching techniques were able to increase the hatching rate and pregnancy rate in both human IVF-embryo transfer programmes and animal experiments. But, Malter and Cohen (1989a) suggested that PZD could inhibit the embryonic development: the natural expansion of the blastocyst with the thinning of the ZP did not occur. The possibility also arises of loss of blastomeres or of the whole embryo during contractions of the female reproductive tract (Nichols and Gardner, 1989). To avoid these drawbacks, Khalifa et al. (1992) attempted cruciate thinning of the mouse ZP, and obtained a significant enhancement of blastocyst hatching. Zona thinning using an Er:YAG laser on embryos at 48 h after oocyte retrieval also significantly increased the implantation and pregnancy rates (Antinori et al., 1996).

Cohen et al. (1992) reported that assisted hatching was effective in patients aged >38 years and in those with elevated basal FSH levels, but that patients whose embryos had thin ZP might be jeopardized by assisted hatching. However, Bider et al. (1997) did not observe increased implantation rates in women of advanced age, despite differences in the number of embryos transferred. These observations suggest that chemical zona drilling may be useful in a selective aetiology of infertility.

Gorden and Dapunt (1993) developed a mouse embryo model with a hatching deficiency, using blastomere destruction and mineral oil replacement. Because embryos with cells reduced by 1/2 or 1/4 were able to develop normally up to blastocyst but had reduced hatching ability, zona lysis appeared to be more important to the hatching process than pressure against the zona induced by blastocyst expansion.

It has been reported that proteolytic activity is present in uterine fluid (Orsini and McLaren, 1967; McLaren, 1970). Some investigators have also proposed that an embryonic hatching enzyme termed 'strypsin' (Perona and Wassarman, 1986) and a uterine proteolytic component (Bavister, 1995; Gonzales and Bavister, 1995) may contribute to the hatching process. Therefore, in order to evaluate the effect of proteolytic enzymes on embryonic development and hatching, we cultured 2-cell mouse embryos in HTF supplemented with the proteases PE, PK and Chymo for 72 h and evaluated their developmental and hatching ability. At concentrations of 1.0 µg/ml PE, 0.1 µg/ml PK, 1.0 µg/ml Chymo, the ZP of embryos were not removed but were thinned. The inclusion of PE and PK in culture media did not affect development up to blastocyst
stage but significantly increased hatching rate (Table I–II). However, the inclusion of Chymo was detrimental to embryonic development and hatching. Our study also showed that a combination of PE and PK could increase hatching rate whereas Chymo disturbed embryonic development. Hatching rate after 24 and 48 h incubation was not significantly different. Therefore, to increase the hatching rate in the mouse, we suggest that the embryos be cultured in 0.5% BSA-HTF containing 1.0 μg/ml PE, 0.1 mg/ml PK, or 1.0 mg/ml PE and 0.1 mg/ml PK for at least 48 h.

Kim et al. (1996) reported that, in mouse, the biochemical properties in PVS of oocyte and embryo within oviduct underwent change. These changes were due to proteinaceous material(s) secreted by the oviduct and became strongly stained with FITC-casein. The staining of PVS by FITC-casein continued to the morula stage and then gradually disappeared at blastocyst stage, and was not removed by washing. In order to evaluate whether the presence of PE and/or PK in the culture media changed the ZP and PVS, we cultured mouse embryos in medium supplemented with 1 μg/ml PE and 0.1 μg/ml PK for 24 h, and then stained them with FITC-casein. Oocytes and embryos that had been co-cultured with oviduct showed a distinct fluorescence staining within their PVS, while embryos cultured in HTF supplemented with PE and PK were not stained with FITC–casein. However, when these embryos were cultured with oviduct, their PVS became strongly stained with FITC–casein but this was easily removed by PBS washing (Table VI). It is considered that proteinaceous material(s) secreted by the oviduct may gain access to PVS following alteration of ZP structure by treatment with PE and PK.

Recently, a new mechanism of hatching has been suggested. Gonzales and Bavister (1995) compared hatching time and behaviour of hamster embryos developing in vivo and in vitro. In vivo, zona lysis occurred progressively and uniformly around the ZP (global zona lysis), and small blastocoele cavities appear in escaping and zona-escaped embryos. Therefore, the hatching mechanism in vivo may be different from in vitro due to uterine factor(s) that are absent from in-vitro culture conditions. These uterine factor(s) may be proteolytic enzyme(s) or signalling molecule(s) which allow the blastocyst to secret zona lysis.

Recently, an ongoing pregnancy was achieved following transfer of zona-free human blastocysts obtained by treatment with 0.5% pronase (Fong et al., 1997). It was suggested that removal of the ZP removed one more obstacle to implantation and that pronase treatment may be safe. However, there may be problems in the application of this technique to human assisted reproduction programmes. We think that removal of ZP from embryo before transfer may not protect it against blood contamination and damage during transfer into the uterus, although loss of blastomeres or the whole embryo during contractions of the female reproductive tract is overcome by blastocyst transfer.

In the present study, we obtained good development and a higher hatching rate in mouse embryos, possibly through alteration of ZP structure. These results are similar to reports of other assisted hatching techniques. In our human IVF–embryo transfer programme, we reported good pregnancy rates and normal deliveries following addition of a low dosage of protease to the culture medium (Roh et al., 1996). Also, Fong et al. (1997) have reported that day 5 or 6 blastocysts whose ZP were removed enzymatically, or day 5 or 6 blastocysts that hatched naturally on their own in vitro, were able to attach tightly, spread out and grow on a variety of feeder layers irrespective of the source of supporting cells. Therefore protease treatment may be a very safe and convenient technique for improving hatching and implantation.

In conclusion, we propose that the addition of proteases, similar to those of the female reproductive tract, may improve embryonic development and hatching. The inclusion of PE and/or PK in culture media did not affect development up to blastocyst but significantly increased hatching rate in mouse. The morphology of the resulting blastocysts was similar to that of escaping blastocyst in vivo reported by Gonzales and Bavister (1995). Therefore PE and/or PK may overcome a deficiency of zona lysin, and be a valuable and effective assisted hatching technique in human assisted reproduction programmes.

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