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Significance of the Number of Embryonic Cells and the State of the Zona Pellucida for Hatching of Mouse Blastocysts In Vitro Versus In Vivo

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ABSTRACT

We investigated the course of mouse blastocyst hatching in vitro after experimental modulation of the hatching process by growth hormone or by laser treatment and compared it to embryos grown in vivo. When embryos were grown in vitro, successful hatching was dependent on blastocyst expansion and was based on a minimum number of embryonic cells. Embryos grown in the presence of growth hormone were more advanced in their development and hatched earlier. When an artificial opening was laser-drilled into the zona pellucida, hatching occurred at lower numbers of embryonic cells. In vivo, escape from the zona pellucida occurred earlier and independent of blastocyst expansion. However, when we isolated in vivo-grown blastocysts with intact zonae that had developed in vivo and then cultured them in vitro, blastocysts started to expand and hatched the following day when a sufficiently high number of embryonic cells was present. Our data show that successful hatching in vitro is dependent on a sufficiently high number of embryonic cells, which enables blastocyst expansion and zona shedding. In vivo, the lower number of embryonic cells detected in zona-free blastocysts indicates that the underlying mechanism of zona escape is different, does not depend on blastocyst expansion, and presumably involves lytic factors from the uterus.

INTRODUCTION

During early mouse embryonic development and up to the blastocyst stage in mice, the developing embryo is surrounded by the zona pellucida. This is composed of glycoproteins, carbohydrates, and other zona-specific proteins [1–3] and serves as an embryonic coat to protect the embryo. Prior to implantation, the embryo must escape from the zona pellucida in order to establish direct contact with the endometrium [4]. In vitro studies of the process of zona escape have been performed not only in the mouse but also in other species [4–6]. Typically, this process involves blastocyst expansion that causes a thinning of the zona pellucida prior to zona rupture at a distinct site [7]. Through the rather large opening caused by zona rupture the expanded blastocyst can hatch and leaves the zona behind almost intact. Several studies have been undertaken to identify enzymes that may be involved in the hatching process. The presence of a trypsin-like protease derived from the trophectoderm of mouse embryos was described and suggested to be a mediator of the zona thinning that is observed during hatching in vitro [8, 9]. However, at least for the hamster, Gonzales and Bavister [10] showed that escape from the zona pellucida in vivo is different from the hatching process observed in vitro, especially regarding the mode and timing. Blastocyst expansion, as well as focal lysis or perforation of the zona was not observed; instead, the zona showed global thinning and was completely lost within a few hours. This implicated an obvious uterine contribution to zona escape in vivo, most readily explained by the presence of uterine zona lysins to assist zona dissolution [11, 12].

We have studied the timing and course of blastocyst escape in vitro after experimental modulation of the hatching process. In an initial experiment, we determined the importance of the number of embryonic cells for the hatching process by growing mouse embryos in the presence or absence of growth hormone, which is known to promote embryonic cell growth [13–15]. In a second experiment we studied the role of the zona pellucida during the hatching process. This was achieved by introducing a laser-drilled opening through the zona to facilitate escape of the embryo without the need to overcome the resistance of the zona. Finally, we tried to determine whether our data have any implications for zona escape in vivo. Therefore and in view of the data presented by Gonzales and Bavister [10], we investigated the timing and mode of zona escape in the mouse in vivo.

MATERIALS AND METHODS

Experimental Design

We studied four experimental groups. In Group 1, mouse zygotes were isolated and cultured from the two-cell stage onward up to Day 5 in the presence of human recombinant growth hormone (hrGH) to determine the effect of enhanced embryonic growth on the course of hatching. In Group 2, a single opening was drilled into the zona pellucida of mouse two-cell embryos to determine the effect of the loss of zona resistance on the course of hatching. Groups 1 and 2 were specified as in vitro-grown embryos, and for both groups, the results were compared to untreated control groups. In Group 3, we isolated in vivo-grown embryos at different time points on Day 4 from the uterus. In Group 4, in vivo-grown embryos with intact zonae were isolated on Day 4 and cultured in vitro until Day 5.

For all experimental groups, the course of hatching was assessed by documentation of the type of embryonic stage (morula, blastocyst, expanded blastocyst, hatching/hatched blastocyst) reached by Day 4/5 (in vivo-grown embryos; Groups 3, 4) or by Day 5 (in vitro-grown embryos; Groups 1, 2). We assessed the thickness of the zona pellucida of all embryos at Day 4/5 in vivo and at Day 5 in vitro. We further investigated the zona characteristics by laser drilling of an opening into the zona of these embryos. The size of

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TABLE 1. The number of embryonic cells and the course of embryonic development in embryos grown in vitro with or without hrGH (Group 1).

Day 5	Morula	Blastocyst	Expanded blastocyst	Hatching
With hrGH				
No. of embryos	5/138	29/138	70/138[a]	34/138[b]
	3.6%	21.0%	50.8%	24.6%
MNEC (mean ± SD)	n.d.[c]	36.0 ± 3.8[d]	52.9 ± 9.0[d]	70.8 ± 11.8
Without hrGH				
No. of embryos	12/118	54/118	47/118[a]	5/118[b]
	10.2%	45.7%	39.8%	4.2%
MNEC (mean ± SD)	n.d.[c]	34.7 ± 8.8[d]	55.5 ± 7.5[d]	n.d.[c]

[a] Significantly different from hrGH treatment group versus controls ($P < 0.005$; χ^2-test).
[b] Significantly different from hrGH treatment group versus controls ($P < 0.001$; χ^2-test).
[c] The MNEC was not determined due to loss of embryos during staining procedure.
[d] Not significantly different from hrGH treatment group versus controls (n.s. ANOVA).

TABLE 2. The number of embryonic cells and embryonic development in embryos grown in vitro with or without laser drilling at the zygote stage (Group 2).

Day 5	Morula	Blastocysts	Expanded blastocyst	Hatching
With laser				
No. of embryos	0/35	2/35	0/35	33/35[a]
	0%	5.7%	0 %	94.3%
MNEC (mean ± SD)	n.d.	20.0 ± 0	n.d.	46.7 ± 15.0[b]
Without laser				
No. of embryos	0/34	11/34	18/34	5/34[a]
	0%	32.4%	52.9%	14.7%
MNEC (mean ± SD)	n.d.	33.3 ± 5.9	49.5 ± 12.6	68.8 ± 19.7[b]

[a] Significantly different from laser-treated group versus controls ($P < 0.01$; χ^2-test).
[b] Significantly different from laser-treated group versus controls ($P < 0.001$; ANOVA).

this opening was measured immediately after laser drilling. Following the drilling of this first opening, we measured the thickness of the zona again and introduced another laser-drilled opening next to the first one to investigate a possible change in zona structure.

Finally, we determined the number of embryonic cells of embryos on Day 4/5 (in vivo) and on Day 5 (in vitro) in each experimental group by using a differential staining technique.

Reagents and Culture Medium

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. The medium used for isolation and culture of embryos was M16 medium [16] supplemented with BSA (4 mg/ml).

Isolation and Culture of Embryos

According to federal law, permission was obtained to perform the animal experimentation described in this study (K32 147-2737/3203). Zygotes were collected from oviducts of unstimulated CB6F1 female mice (7–12 weeks old) that were mated over night with NMRI males (8–14 weeks old) and that presented with a vaginal plug on the next morning. This day was considered as Day 1. Females were sacrificed on that day, and zygote cumulus complexes were isolated out of the swollen ampullae. Zygotes were denuded by hyaluronidase treatment (60 IU in M16 culture medium), washed thoroughly, and incubated in 50-μl droplets of medium covered with mineral oil in a plastic culture dish (3004; Falcon, Becton Dickinson, Heidelberg, Germany). The medium was replaced every other day. In vivo-grown blastocysts were flushed with PBS from the uterus of pregnant females at 10 AM and at 2 PM on Day 4. Flushings were screened for the presence of blastocysts and/or empty zonae using a stereo microscope. Blastocysts were collected, washed briefly in culture medium, and incubated as described above.

Culture with Human Recombinant Growth Hormone

For experimental Group 1, zygotes were grown in M16 medium up to the 2-cell stage and then randomly allocated to a treatment group and a control group. In the treatment group, hrGH (N70931; Novus Molecular Inc., San Diego, CA) was added to the culture medium to give a final concentration of 100 ng/ml. Control embryos were grown in M16 culture medium without hrGH. Culture was continued for another 3 days until Day 5.

Laser Drilling of Openings into the Zona Pellucida

In experimental Group 2, two-cell embryos were randomly allocated to a treatment or a control group. The zona pellucida was drilled with a 1.48-μm diode laser system (Fertilase; MTM Medical Technologies Montreux SA, Clarens, Switzerland) coupled to an inverted microscope (DMIRB, Leica, Bensheim, Germany) that was further equipped with a 40× Hofman modulation contrast objective (Leica) and a heated microscope stage. Both the laser beam path and the mode of laser action have been described earlier [17–19]. Laser drilling was performed in the culture dish. For the laser treatment of two-cell stage embryos (Group 2), we used a single laser pulse of 6 ms that gave an opening of 12–15 μm in diameter that was drilled at a position next to the second polar body [20]. For the laser perforation of the zona pellucida at later developmental stages (blastocysts and expanded blastocysts), the laser drilling in all experimental groups (Groups 1–4) was performed under standardized conditions using a 6-ms laser pulse length. In later developmental stages, the openings were drilled on a randomly located position next to each other. In Group 2, these openings were drilled next to the opening initially drilled at the two-cell stage.

Assessment of the Mean Number of Embryonic Cells

The mean number of embryonic cells (MNEC) was determined with the differential nuclear staining method described by Lane and Gardner, [21], except that the zona pellucida was removed by consecutive laser shots instead of using pronase. For the evaluation of differential color staining we used an inverted microscope (DMIRB; Leica) equipped with appropriate filter sets for UV and green/blue fluorescence (Leica).

Statistical Data Analysis

Comparisons of the number of embryos in Groups 1 and 2 that were in the course of hatching were made by the chi-square test. Comparisons of the mean number of embryonic cells, zona thickness, and size of laser-drilled openings were made with ANOVA.

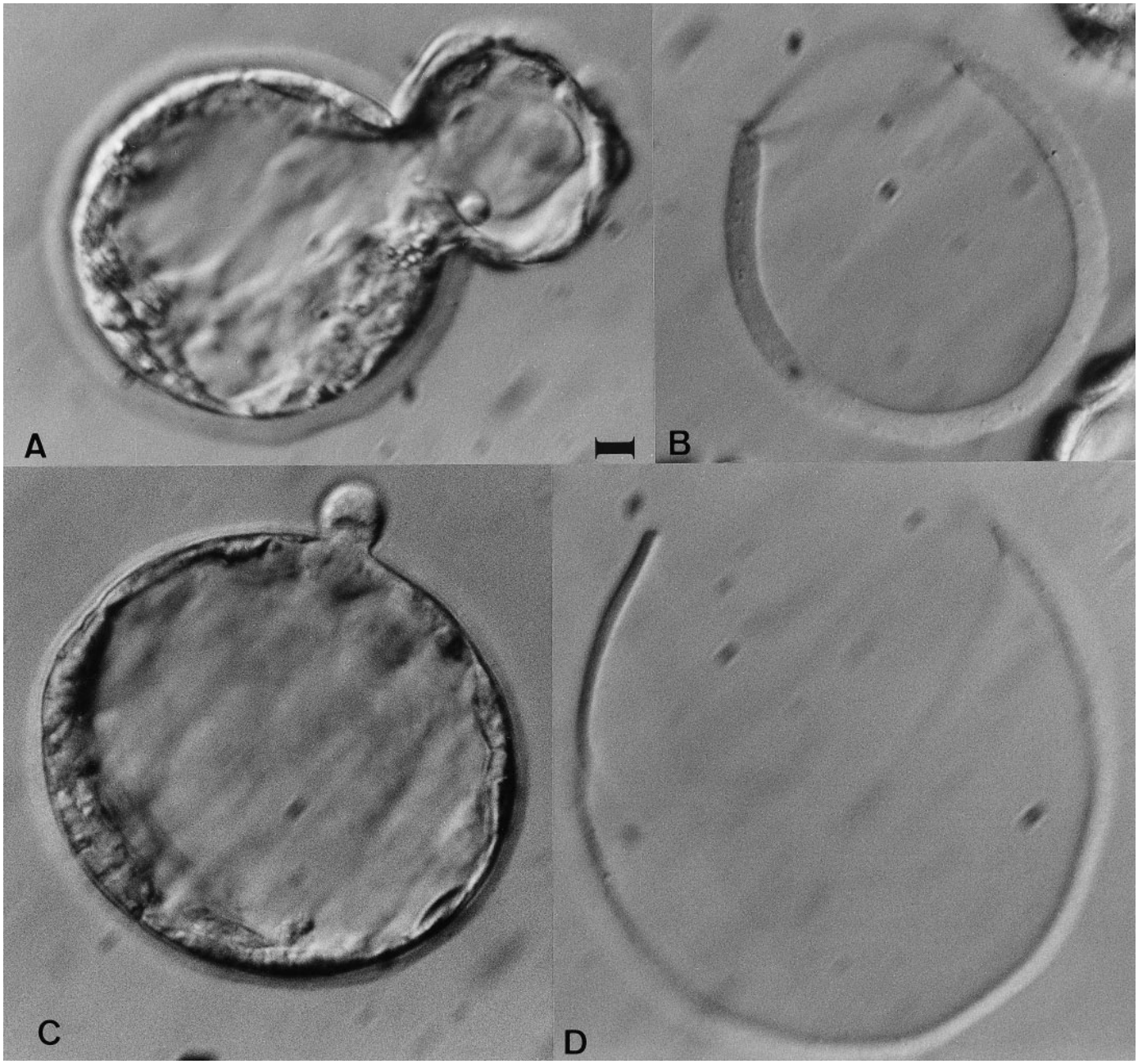

FIG. 1. Hatching blastocyst (Group 2) and an empty zona (**A, B**) derived from laser-treated zygotes. Hatching blastocyst and empty zona (**C, D**) from an untreated zygote (**C**). Note the difference in the thickness of the zona in blastocysts derived from laser-treated (**A**) versus untreated zygotes (**C**) and the size and shape of empty zonae (**B, D**). Bar in A is 15 μm for A–D.

RESULTS

The Number of Embryonic Cells and the Timing of Hatching In Vitro

We first investigated the developmental progression as well as the MNEC from isolated embryos grown in a standard medium in the presence or absence of hrGH from Day 2 (two-cell stage) up to Day 5 (experiment in Table 1). In the presence of hrGH, significantly more embryos had reached the stage of expanded blastocysts (70/138) and hatching blastocysts (34/138) by Day 5 than in the absence of hrGH (47/118, $P < 0.005$ and 5/118, $P < 0.001$, respectively). There were no significant differences in the number of embryonic cells at comparable stages between the hrGH group and the controls. However, in the hrGH group more embryos were of an advanced developmental stage and possessed the high number of embryonic cells characteristic for that stage. This shift in the developmental progression is due to the improvement of mouse embryo development in vitro by hrGH [13].

Influence of a Laser-Drilled Opening in the Zona on the Hatching Process

The artificial creation of a laser-drilled opening in the zona pellucida of mouse embryos at the two-cell stage did not interfere with further embryonic development (experiment in Table 2; also compare the results reported by Montag et al. [20] and Germond et al. [22]). The majority of laser-drilled embryos had started hatching (33/35; 94.3%)

TABLE 3. Zona thinning and zona hardness in embryos grown in vitro or in vivo.

	First laser-drilling		Second laser-drilling	
	ZP thickness 1	Opening 1	ZP thickness 2	Opening 2
Day 5 In vitro				
With laser (Group 2)	7.7 ± 0.7[a,c]	13.0 ± 1.1[b,d]	7.8 ± 0.7[a]	13.5 ± 0.6[b]
Without laser	3.6 ± 1.9[c,e]	7.9 ± 2.5[d,f]	5.0 ± 1.5[e]	12.9 ± 0.8[d,f]
Day 4 in vivo				
10 AM (Group 3)	6.9 ± 1.3[g,i]	13.1 ± 1.9[h]	7.0 ± 1.3[g]	14.2 ± 0.8[h]
Flushed on Day 4 and cultured until Day 5 (Group 4)	3.2 ± 2.0[i,j]	9.2 ± 2.6[k]	4.6 ± 1.6[j]	13.7 ± 0.8[k]

[a,b,g,h] Not significantly different between first and second laser drilling. All values are mean ± SD.
[c,d] Significantly different between laser-treated embryos and controls ($P < 0.001$).
[e] Significantly different between first and second laser drilling ($P < 0.01$).
[f,k] Significantly different between first and second laser drilling ($P < 0.001$).
[i] Significantly different between Group 3 and Group 4 ($P < 0.001$).
[j] Significantly different between first and second laser drilling ($P < 0.05$).

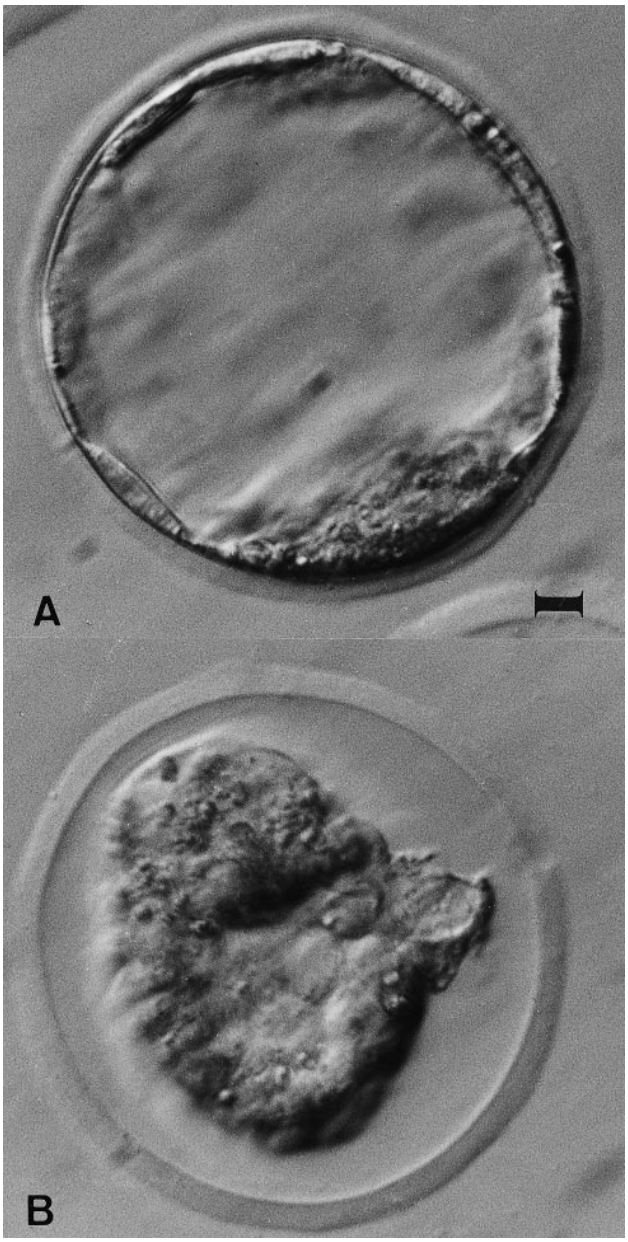

FIG. 2. An expanded blastocyst grown in vitro is shown before (**A**) and after (**B**) laser drilling of the zona. Both photographs were taken within 3 min. Bar in A is 15 μm for A and B.

by Day 5 compared with the controls (5/34; 14.7%). This difference was significant ($P < 0.01$). Hatching always occurred through the laser-drilled opening. We analyzed the MNEC in hatching embryos, and the values were 46.7 ± 15.0 in the laser group and 68.8 ± 19.7 in the controls, and this difference was significant ($P < 0.001$).

Blastocyst Expansion and Zona Thinning in Untreated Versus Laser-Drilled Embryos

The previous experiment showed that, in the presence of a laser-drilled opening in the zona, hatching was initiated and occurred through the artificial opening as soon as the blastocyst started to expand. During the course of embryonic development, initially laser-treated embryos were not enlarged (Fig. 1A and compare with other published results [17, 20, 22]), and empty zonae from hatched embryos were not enlarged (Fig. 1B). For comparison, in the untreated control, blastocyst expansion occurred prior to hatching, and this process was accompanied by global zona thinning (Fig. 1C). Empty zonae from hatched blastocysts were always enlarged and remained thin (Fig. 1D).

In order to analyze changes in zona thickness and structure, we introduced two laser-drilled openings into the zona and measured the size of these openings as well as the thickness of the zona immediately before and after laser drilling (experiment in Table 3).

In embryos that were derived from laser-treated two-cell stages, the mean thickness of the zona at Day 5 was 7.7 ± 0.7 μm. The size of a newly laser-drilled opening at Day 5 was 13.0 ± 1.1 μm. This laser drilling at Day 5 did not lead to a change in zona thickness (7.8 ± 0.7), and the diameter of another drilled opening (13.5 ± 0.6 μm) also did not differ significantly.

In untreated controls (Group 2) the thickness of the zona pellucida of expanded blastocysts on Day 5 was 3.6 ± 1.9 μm (Table 3). When we introduced a laser-drilled opening into the zona of these blastocysts, the size of the first opening measured 7.9 ± 2.5 μm. These values were both significantly different ($P < 0.001$) compared with the treatment group, where the zona was opened at the two-cell stage. Laser drilling of expanded blastocysts (Fig. 2) caused an immediate collapse of the blastocyst (Fig. 2B). After this initial collapse, the thickness of the zona increased significantly ($P < 0.01$) to 5.0 ± 1.5 μm (Fig. 2A versus B), and the size of another laser-drilled open was significantly larger (12.9 ± 0.8 μm; $P < 0.001$).

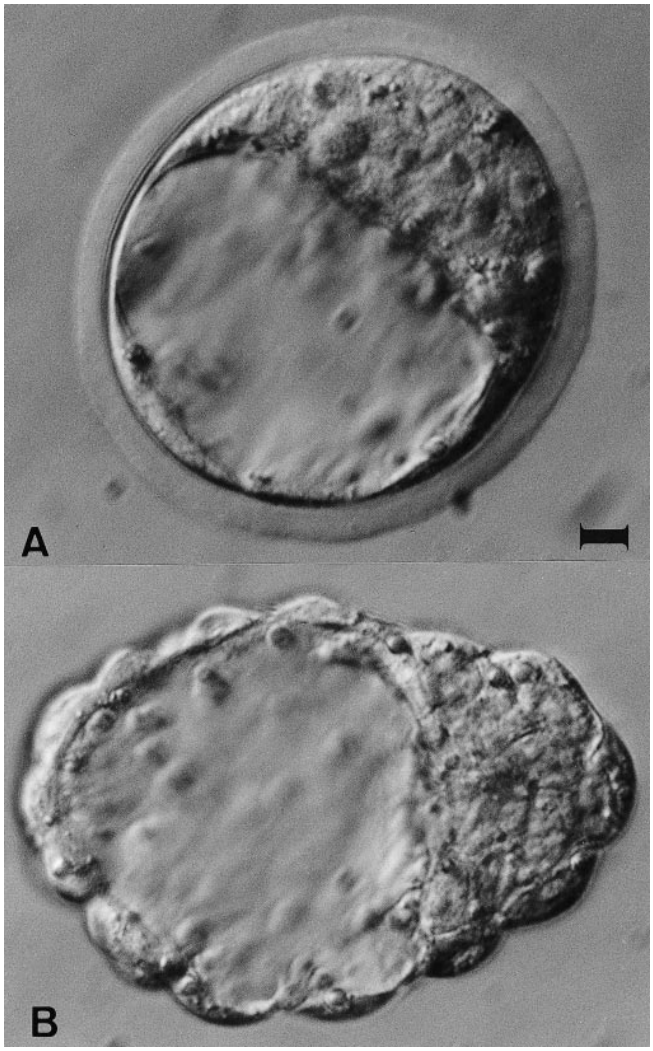

FIG. 3. In vivo-grown embryo flushed at Day 4 at 10 AM (**A**) or at 2 PM (**B**). Bar in A is 15 μm for A and B.

Mode and Timing of Hatching In Vivo

Embryos grown in vivo (Group 3) were flushed at day 4 of development in the morning (10 AM), and by that time, all embryos (n = 38) had reached the blastocyst stage. These blastocysts were not expanded and presented with an intact zona pellucida (Fig. 3A). When we flushed embryos later the same day (2 PM), we only found blastocysts that had escaped from their zonae (n = 38; Fig. 3B). The mean number of embryonic cells was not significantly different between the early or late flushed embryos (36.8 ± 5.7 versus 37.1 ± 7.8, $P = 0.89$; Table 4). We never found empty zonae in our flushings at 2 PM.

In another experiment, we isolated in vivo-grown blastocysts with intact zonae (n = 45) on Day 4 at 10 AM and cultured them until Day 5. We observed that all blastocysts started to expand, as did the in vitro-grown embryos, and by Day 5, 68.9% (31/45) were expanded blastocysts and presented with 52.0 ± 10.5 cells (Table 4). The remaining blastocysts were in the course of hatching (14/45; 31.1%) and presented with an MNEC of 67.8 ± 9.8.

Zona Hardness of In Vivo-Grown Blastocysts

As with in vitro-grown embryos, we determined the thickness of the zona and the size of laser-drilled openings in flushed embryos after two subsequent laser shots in Group 3 (10 AM) and in Group 4 (Table 3). In Group 3 embryos, the thickness of the zona (6.9 ± 1.3 μm versus 7.0 ± 1.3 μm) as well as the size of consecutively drilled openings (13.1 ± 1.9 μm versus 14.2 ± 0.8 μm) did not differ significantly.

When embryos were flushed on Day 4 and cultured until Day 5, the onset of blastocyst expansion from Day 4 to Day 5 was accompanied by a significant zona thinning (3.2 ± 2.0 μm; $P < 0.001$). On Day 5, the size of a first laser-drilled opening was 9.2 ± 2.6 μm. Subsequently, zona thickness was determined to be 4.6 ± 1.6 μm and another drilled opening measured 13.7 ± 0.8 μm. We found statistically significant differences for the increase in zona thickness ($P < 0.05$) and the change in the size of the second laser-drilled opening ($P < 0.001$).

TABLE 4. The number of embryonic cells in embryos grown in vivo.

Day 4 (group 3)	Blastocysts	Hatched blastocysts
Early flushed (10 AM)		
No. of embryos	38/38	0/38
MNEC (mean ± SD)	36.8 ± 5.7[a]	n.d.
Late flushed (2 PM)		
No. of embryos	0/38	38/38
MNEC (mean ± SD)	n.d.	37.1 ± 7.8[a]
Flushed on Day 4 (10 AM) and cultured until Day 5 (Group 4)	Expanded blastocysts	Hatching blastocysts
No. of embryos	31/45	14/45
	68.9%	31.1%
MNEC (mean ± SD)	52.0 ± 10.5	67.8 ± 9.8

[a] Not significantly different between embryos flushed at 10 AM and at 2 PM ($P = 0.89$; ANOVA).

DISCUSSION

Hatching In Vitro Is Dependent on a Sufficiently High Number of Embryonic Cells

A primary aim of our study was to investigate the factors that affect hatching in vitro. Our data implicate that the number of embryonic cells and the zona pellucida itself have the most significant influence on hatching efficacy.

Blastocyst expansion in vitro has been observed in most species studied to date [7]. The initiation of this process depends on the number of embryonic cells. We found that embryos grown in the presence of hrGH were more advanced in their development compared to untreated controls. Although the number of embryonic cells at comparable developmental stages did not differ between treated embryos and controls, embryos grown in the presence of hrGH produced more embryonic cells within the 5-day interval. This improvement in embryonic development led to a high proportion of hatching blastocysts that presented with an MNEC of 70.8 ± 11.8. Similar values were found for in vitro-hatching blastocysts in all other experimental groups (Group 2 control: 68.8 ± 19.7; Group 4: 67.8 ± 9.8), except for laser-treated embryos in Group 2. These data imply that in vitro hatching is initiated as soon as a sufficient number of embryonic cells is available to overcome the resistance of the zona. If this resistance of the zona is not present, e.g., after laser drilling of an opening at a very early stage of development (e.g., laser-treated embryos in Group 2), hatching can already be initiated with fewer cells (46.7 ± 15.0) and at a time point that presumably reflects the onset of blastocyst expansion.

Undoubtedly, the hatching process in vitro requires the active participation of the trophectoderm [5]. Some authors described a trypsin-like proteinase, called strypsin, that is thought to act as a trophectodermally produced zona lysin [8, 9]. Morphological studies show that zona escape in vitro is usually initiated by a penetration of trophectodermal projections into the zona pellucida at a certain distinct site, this becoming known as the site of focal lysis [10]. Whether or not trophectodermal enzymes are involved in this focal lysis is at present unknown.

Blastocyst expansion in vitro leads to zona thinning, and it has been suggested that this is due to the pressure of the expanding blastocyst and that trophectodermal lysins mediate a global zona lysis [23]. We observed zona thinning in expanding blastocysts in vitro. When we created artificial openings in the zona of expanded blastocysts, these openings caused a release of the pressure exerted by the embryo on the zona. In all experiments, the pressure release led to an increase in zona thickness, and subsequently, we observed that enlarged zonae were softer, as a second laser-drilled opening had a larger size. Therefore, we conclude that zona thinning during blastocyst expansion is not due to a loss of zona material but results from the stretching of the zona. This is further supported by the findings from Chan [24] who reported that zygotes that do not cleave show no change in zona thickness during culture in vitro. From this and from our data we conclude that blastocyst expansion is the only underlying reason for global zona thinning in vitro. During this process, the zona becomes not only harder but also more brittle. Further, if this in vitro process of global zona thinning would involve the active participation of an embryonic zona lysin, one should also observe zona softening. This was not observed in our experiments, and we may speculate that an embryonic lysin could only be involved in a focal lysis. We do not know whether global zona thinning in vitro is also accompanied by structural changes of the zona. This question deserves further investigation especially in view of the observation that after complete hatching of an embryo in vitro the empty zona remains thin and shows no further increase in zona thickness despite the pressure release.

Hatching In Vivo Is Accomplished by Uterine or Trophectodermal Factors Absent In Vitro

We found striking differences in the course of hatching in vivo compared to in vitro. Firstly, embryos reached the blastocyst stage in vivo 1 day earlier and possessed fewer cells than blastocysts derived from in vitro culture. These data are in accordance with those reported by Gonzales and Bavister [10] for the hamster. Secondly and even more important, in vivo-grown embryos lost the zona pellucida completely on Day 4 between 10 AM and 2 PM during a time period of 4 h. When we isolated in vivo-grown embryos in between, i.e., during this time window, we found non-expanded blastocysts with a slightly enlarged but globally thinner zonae (data not shown). The rapid loss of the zona cannot be explained by blastocyst expansion and zona shedding, because the low number of embryonic cells on Day 4 would not support such a process. This hypothesis is further supported by our observation that in vivo-flushed blastocysts with an intact zona and that are subsequently cultured in vitro are not able to escape from the zona within 4 h after isolation. Instead, these blastocysts continued their growth and, after having reached a certain threshold in the mean number of embryonic cells (67.7 $\pm$ 9.8), underwent blastocyst hatching. Therefore, the mode of hatching of these blastocysts exhibited similar characteristics to the hatching of embryos grown in vitro since the zygote stage. As in vitro, the expansion of these blastocysts caused zona thinning, and we could demonstrate by investigation of subsequently drilled openings that the thinning of the zona was accompanied by zona hardening, as in the case of in vitro-grown embryos. This points to the fact that in the mouse and as in the hamster [10] the complete loss of the zona pellucida in vivo can only be accomplished by the constant presence of uterine lysins.

That uterine lysins are involved in the hatching process in vivo has been shown by others in the mouse [11, 25] and in the hamster [10, 26]. It is interesting to note that, in vivo, this lytic activity is established just before implantation and occurs in a timely, orchestrated manner [10, 25]. The work presented by others [10, 25] showed that in some species zona lysis can occur at any embryonic stage and also on preimplantation embryos if these are present in the uterus at that time point. We do not know what can happen to an embryo that is not ready for implantation at the time when the lytic activity occurs. All available data suggest that the zona of this embryo will be lysed [25]. Whether such an embryo denuded from its surrounding zona will still grow to an intact and viable blastocyst and thereafter implant is not known. However, it should be noted that in other species, e.g., cattle, zona pellucida-intact embryos, as well as empty zonae can still be found in uterine flushing up to Day 16 after ovulation [27]. Therefore our findings in the mouse cannot be generally adapted to other species.

In conclusion our data show that in the mouse there are substantial differences between the course of hatching in vitro and in vivo. Further understanding of these processes is essential in order to optimize in vitro fertilization treatment.

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