ANIMAL EXPERIMENTATION

In Vivo Hatching Phenomenon of Mouse Blastocysts During Implantation

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Purpose: To determine whether the blastocyst zona shedding process within the murine uterine cornus in vivo is due to a global lytic process caused by uterine proteolytic enzyme, or is triggered by the blastocyst hatching process as observed in vitro.

Methods: Fifty-one female ICR mice aged 5–8 weeks were used for this study. From 8:00 p.m. of the 4th day postcoitus to 7:00 p.m. of the 5th day postcoitus, the uterine cornua of 51 mice were isolated at 30-min intervals. Blastocysts within the uterine cornua were flushed out with a balanced solution under the dissecting microscope. The stages of blastocyst development and the proportion of hatching or hatched blastocysts and the discarded zona pellucida (ZP) were inspected and counted.

Results: A total of 672 blastocysts were recovered from the uterine horns of the 51 mice. They were divided into six groups according to the blastocyst developmental stages (before or after ZP escape; before or after the initiation of implantation). Group I represents the earliest embryonic stage and Group VI represents the most advanced blastocyst developmental stage during the peri-implantation period. The empty ZP recovery rates (number of discarded ZP/all hatched blastocysts) were 52.3%, 21.5%, 17.2%, 6.6%, 1.6%, and 0% in Groups I–VI, respectively. Five hatching

blastocysts out of 199 embryos in Group I were found and a 100% of ZP recovery rate was obtained from 6 of 19 mice in Groups I and II.

Conclusions: The present study shows that active blastocyst hatching occurs in vivo because both hatching blastocysts and empty ZP can be found within the uterine cornua of ICR mice before implantation. The empty ZP recovery rate declined significantly, along with a progression of embryo development and implantation, implying that intrauterine zona lytic activity occurs during the peri-implantation stage.

KEY WORDS: Blastocyst; hatching; intrauterine.

INTRODUCTION

The zona pellucida (ZP) is a thick extracellular coat that surrounds mammalian eggs and preimplanted embryos. It supports communication between oocytes and follicle cells during oogenesis, protects oocytes and embryos during development, and regulates interactions between ovulated eggs and free-swimming sperm during as well as following fertilization (1). After fertilization, the preimplanted embryo undergoes a series of dynamic developmental changes leading to blastocyst formation, escape from the ZP, and attachment to the endometrium. These events, orchestrated by the embryo, may be mediated by uterine tissues and must take place if the embryo is to survive and develop normally (2). Zona pellucida penetration is a prerequisite for blastocyst attachment and subsequent implantation (3). Some embryos from human in vitro fertilization (IVF) may have difficulties escaping from their ZP, perhaps accounting for a proportion of the failure to establish pregnancy.

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Most of our knowledge about the blastocyst hatching mechanism comes from *in vitro* studies. Under culture conditions, the typical "hatching" or "shedding" behavior of late blastocysts involves expansion of the blastocoele cavity with concomitant stretching and thinning of the ZP, leading to focal rupture. This is immediately followed by extrusion of the embryo through the ruptured zona. Eventually, the empty zona is discarded by the embryo, remaining largely intact except for a distinct split delineating the original site of rupture. The process of *in vitro* hatching has been described in many species, including mouse (4), pig (5), cow (6), monkey (7), and human (8).

In addition to mechanical force, Perona and Wassarman (9) strongly suggest that a trypsin-like proteinase (74 kDa), called "strypsin," is directly involved in the *in vitro* hatching of isolated mouse blastocysts. Histochemical and autoradiographic evidence showed that, prior to the hatching of blastocysts, strypsin is found in association with cells of the mural trophoctoderm instead of the polar trophoctoderm or inner cell mass. Following hatching, strypsin is also associated with the empty ZP, specifically at the opening through which the embryo emerged. It is therefore suggested that *in vitro* hatching of mouse blastocysts is initiated by proteolysis of the region of ZP overlying the mural trophoctoderm (9).

The exact mechanism by which the embryo escapes from the ZP *in vivo* is poorly understood. Gonzales and Bavister (2) compared *in vitro* and *in vivo* timing and morphological differences of the escape of hamster blastocysts from the zona. Compared to their *in vivo* counterparts, *in vitro* zona hatching was delayed for more than 29 h in embryos cultured both from the 1-cell stage and blastocysts from uteri of pregnant female hamsters at 3.9 days postcoitus. Moreover, the morphology of blastocyst development and zona escape also differed between these two groups. The zona gradually thinned globally *in vivo* and eventually disappeared (global zona lysis). Retarded embryos also showed *in vivo* global zona lysis (2). The focally lysed ZP, trophoblastic "knobs" or "buds," and the discarded empty ZP which are usually observed in cultured hatching hamster blastocysts never occurred *in vivo* (10). The *in vitro* temporal and morphological derangement of events provides strong evidence for the participation of a uterine contribution during *in vivo* zona escape. Gonzales and Bavister (2) hypothesized that blastocyst hatching outside the uterine horns was an artifact caused by a blastocyst-derived proteolytic component, the

normal function of which related to early stages of implantation under physiological conditions.

However, there are different opinions. In cattle, expanded blastocysts flushed from the uterus had zona no thinner than that of blastocysts grown and expanded *in vitro*, and sometimes an empty zona was found in flushings from superovulated cows (11). In addition, Bergstrom (12) fixed peri-implantation stage mouse uteri *in situ* by perfusion of a fixative solution through the abdominal aorta to study the shedding of ZP in normal pregnancy. The absence of any partially lysed zonae surrounding blastocysts *in situ* together with the presence of cracks in otherwise seemingly intact zonae surrounding such blastocysts in Epon-embedded material are regarded as indirect evidence in favor of hatching as the method whereby ZPs are usually shed from mouse blastocysts (12).

It remains to be clarified whether ZP escape by blastocysts *in vivo* is based on a global lysis process caused by a uterine proteolytic component as claimed by Gonzales and Bavister (2), or is triggered by the blastocysts as has been observed *in vitro* (4–8). The purpose of this study, therefore, was to demonstrate the precise blastocyst zona shedding process within mouse uterine cornua in normal pregnancy by flushing peri-implantation blastocysts from the uterine cornua and to look for discarded empty ZP or even the blastocysts themselves in the process of hatching.

MATERIALS AND METHODS

In this study, female ICR mice (aged 5–8 weeks) in estrus were used. The project was evaluated and approved by the Institutional Research and Animal Ethics Committees. If the mice were not naturally in estrus, superovulation was induced by intraperitoneal injection of 10 IU pregnant mare serum gonadotrophin (PMSG; Sigma, USA), followed by 10 IU human chorionic gonadotrophin (hCG; Serono, Italy) 46–48 h later. The animals were then placed with males of proven fertility overnight and checked for the presence of vaginal mucus plugs the next morning. Under a controlled lighting schedule (14 h light/10 h darkness; lights on at 5 a.m.), mating was expected at midnight, which was taken as time zero.

Fifty-one mice were sacrificed by cervical dislocation and the uterine horns were isolated every 30 min, from 3 days and 20 h postcoitus to 4 days and 19 h postcoitus (102–125 h after hCG injection). Blastocysts within the uterine cornua were flushed out with Leibovitz's L-15 medium under the dissecting microscope. The osmolality of L-15 medium was

adjusted to 290 mOsm/kg. The stages of blastocyst development and the proportion of hatching or hatched blastocysts and discarded ZP were inspected and counted.

The 51 mice were divided into six groups according to the stages of blastocysts that were flushed out of the uterine cornua: Groups I and II included mice with hatching and hatched blastocysts in the same uterine horns (Group I, 1–50% hatched blastocysts; Group II, 51–99% hatched blastocysts). Mice with all the blastocysts hatched out were divided into four groups (III–VI) according to the percentage of implanting blastocysts demonstrated by projections of trophoblast cytoplasm (Fig. 1(b)) (13): Group III, 0% implanting; Group IV, 1–50% implanting; Group V, 51–99% implanting; Group VI, 100% implanting.

RESULTS

A total of 672 blastocysts were obtained from 51 mice during the peri-implantation period (Table I). Five hundred thirty-five of the blastocysts had escaped from the ZP (Fig. 1(a)). Two hundred twelve of the blastocysts presented “cytoplasmic projections of trophoblast,” which indicated implanting blastocysts and were probably due to remodeling of the intracellular cytoskeleton (Fig. 1(b)) (13). As the first attachment is extremely superficial and easily broken, the implanting blastocyst can still be recovered by

Table I. Blastocysts Recovered from the Uterine Cavities of 51 Mice During the Peri-Implantation Period

Developmental stage of blastocysts	Number of blastocysts
Blastocysts in intact zona pellucida	115
Blastocysts in opened zona pellucida	17
Hatching blastocysts	5
Hatched blastocysts	323
Implanting blastocysts	212
Total	672

Note. Thirty-five retarded blastocysts within intact zona pellucida were also recovered.

^aDuration of peri-implantation period was from 3 days and 20 h postcoitus to 4 days and 19 h postcoitus.

flushing (14). One hundred thirty-seven of the blastocysts were still in the ZP, including 17 blastocysts within opened ZP and 5 hatching blastocysts (Fig. 1(c); Table I). Most of the hatching blastocysts and the blastocysts within broken (or focally lysed) ZP hatched out after brief culture in vitro. Thirty-five retarded embryos within intact ZP were also recovered from the uterine cavities.

Sixty-three of discarded empty ZP with a distinct opened site (Fig. 1(d)) were also flushed out of the uterine horns along with the 535 hatched blastocysts. Thus, the overall empty zona recovery rate (number of discarded empty ZP/number of hatched blastocysts) was 11.78%. The empty ZP recovery rate (number of discarded ZP/all hatched blastocysts) were 52.3%, 21.5%, 17.2%, 6.6%, 1.6%, and 0% in Groups I–VI, respectively (Table II). In other words, the empty ZP recovery rate declined significantly as evidence of embryo development and implantation increased (Table II). The ZP recovery rate from 6 of 19 mice in groups I and II was 100%. Some of the empty ZP recovered from the uterine cavity were thinned out and soft and with collapsed cavities (Fig. 1(e)), which is different from what we observed in vitro (Fig. 1(f)).

DISCUSSION

In this study, we have demonstrated that hatching blastocysts, blastocysts within opened ZP, and discarded empty ZP with a well-delineated open site can be flushed out of the uterine horn during the peri-implantation period. These data support our speculation that the active hatching process occurs in the uterine cavity of ICR mice in normal pregnancy. In addition, the 100% empty ZP recovery rate in some Group I and Group II mice suggests that the blastocyst hatching phenomenon can be observed in the uterine cavity if the timing is correct.

Fig. 1. Embryos and the discarded empty ZP recovered from the mouse uterus during the peri-implantation period. (a) Escaped blastocysts; (b) implanting blastocysts with trophoblast cytoplasmic projections; (c) hatching blastocysts; (d) discarded empty ZP with a distinct open site and a collapsed cavity; (e) a thinned out, discarded, empty ZP; (f) empty ZP discarded by in vitro hatched blastocysts.

Table II. Empty Zona Pellucida (ZP) Recovery Rates in Mice with Different Embryonic Developmental Stages

Embryonic stage	% Hatched blastocysts			% Implanting blastocysts		
	1–50 ^a	51–99 ^b	100 ^c	1–50 ^d	51–99 ^e	100 ^f
Total blastocysts	119	169	87	91	185	21
No. of hatched blastocysts	21	130	87	91	185	21
No. of empty zona pellucida	11	28	15	6	3	0
Empty ZP recovery rate (%) ^g	52.3	21.5	17.2	6.6	1.6	0

Note. Fifty-one mice were divided into two parts: Groups I–III, before the onset of implantation; Groups IV–VI, after the initiation of the implantation process. A 100% zona pellucida recovery rate was obtained from 6 of 19 mice in groups I and II.

^aGroup I contained eight mice; Five hatching blastocysts were found in this group.

^bGroup II contained 11 mice.

^cGroup III contained 10 mice.

^dGroup IV contained seven mice.

^eGroup V contained 11 mice.

^fGroup VI contained four mice.

^gEmpty ZP recovery rate represents “the number of empty ZP/hatched blastocysts.”

On the other hand, the degeneration of some of the empty ZP recovered from the uterine cavity and decline in the empty ZP recovery rate as the proportion of hatched and implanting blastocysts increased indicates that uterine zona lytic activity exists during the peri-implantation period. As hatching and implantation occur within a short period of time (2), it is difficult to determine whether the *in vivo* uterine zona lytic activity physiologically accounts for the attachment and implantation process or for assistance in the hatching process.

In this regard, we hypothesize that during the *in vivo* peri-implantation period, both blastocyst hatching and uterine zona lytic activity are involved in the ZP escape process. The uterine proteolytic activity may weaken the zona but a blastocyst-derived internal force splits the zona. After the attachment and implantation processes are initiated, proteolytic activity dissolves the discarded empty ZP.

Our data on mouse blastocyst zona shedding are not totally compatible with the results from hamster zona escape studies (2,10), which led to the conclusion that active blastocyst hatching is an *in vitro* artifact and that global zona lysis appears to be the precise mechanism of *in vivo* zona escape. First, focally lysed ZP, trophoblastic “knobs” or “buds,” and discarded empty ZP, which have never been observed in *in vivo* hamster studies (10), were indeed recovered from mouse uterine cornua during the peri-implantation period in our investigation.

Second, the hamster blastocyst ZP were expanded and lysed globally in the uterus, similar to the effects of being dipped into trypsin solution *in vitro* (2), but this type of global zona lysis was not observed in our study. Instead, both blastocysts within

focally lysed zona and hatching blastocysts were recovered.

Finally, retarded embryos in the hamster uterine cavity escaped from the ZP by lysis of zona, suggesting that the embryo itself did not involve in the zona-escape process (2). In our study, we recovered degenerated embryos within intact ZP even when all of the viable blastocysts had escaped from their ZP. Therefore, we suggest that in the mouse, the uterine factor on its own does not completely remove the zona and that an active blastocyst hatching process is still involved in the zona escape mechanism *in vivo*.

The differences mentioned previously between mice and hamsters indicate that there may be species differences in the zona-escape process. We speculate that both active blastocyst hatching and uterine proteolytic activity are involved in the intrauterine ZP escape process in most species. This hypothesis has been supported by observations that a thinned out, discarded, opened ZP can be recovered from the uterine cavity of the cow (12) and pig (Prof. Winston T. K. Cheng, personal communication). The variations in timing, the relative strength of active blastocyst hatching activity, and uterine proteolytic activity may explain a number of the differences in zona escape among different species.

CONCLUSIONS

The present study demonstrates that an active blastocyst hatching process occurs *in vivo* because hatching blastocysts and empty ZP can be found within the uterine cornua of ICR mice before implantation. On the other hand, the empty ZP recovery rate declined

significantly as embryo development and implantation progressed, implying that uterine zona lytic activity also occurs during the peri-implantation stage.

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