Tohoku Journal of Agricultural Research
Vol. 28, No. 1, August 1977
Printed in Japan

The Development of the Rabbit Blastocyst in the Preimplantation after Removal of the Blastocyst Coat in Vitro

Kiyoshi TOTSUKAWA, Shichiro SUGAWARA and Saburo TAKEUCHI

Department of Animal Sciences, Tohoku University, Sendai 980

(Received May 10, 1977)

Summary

Experiments were made on the role of the blastocyst coat. The coverings of six-day pre-implanted rabbit blastocysts were removed by either the mechanical (A) or the enzymatical (B) method. In the mechanical method (A) the coat was cut off with a scissor-tip after the blastocyst was put into 1% saline solution and contracted. (B) In the enzymatical method the coat was dissolved with 0.25% pronase (for 2 minutes). Also, the blastocyst coat was holed with a fine needle. Immediately after the removal or holed treatment of coverings, the treated blastocysts were observed under a binocular microscope and the state of contracted blastocysts were examined by the culture during 5-6 hrs. Then, they were transferred to foster mothers. The holed blastocysts were implanted with the proportion of 15/19 (79%), and five fetuses were found at 28-days. In the treatment of the mechanical and pronase method, the proportions (No. of implanted recipient/No. of recipient) were 4/4 and 2/4, respectively. The rates of implantation were 42 and 33%, respectively. But, no fetuses were found in those two groups.

The mammalian ovum is surrounded by the zona pellucida, which consists of neutral or weakly acidic mucoprotein. In mammals, such as mouse, rat and hamster, this membrane must be removed or penetrated by the trophoblastic cells before implantation. In the rabbit, however, it is known that this outer membrane exists until implantation. Also, it is not clear whether this membrane is zona pellucida or not. Where marked expansion of the blastocyst occurs before implantation, as in the rabbit, the blastocyst coat become extremely attemuated and almost invisible. At this time, the blastocysts accumulates a lot of fluid containing verious substances. This phenomenon is worthy of note in relation to the action of blastocoelic fluid.

Edwards (1) reported that the cleavage in one-and two-cell rabbit eggs occurred even after removal of the zona pellucida, although the blastomeres exhibited a tendency to fall away from each other. Moore, Adams & Rowson (2) transplanted single naked blastomeres from 2- and 4-cell rabbit eggs to the

oviduct and found that no further development occurred. The study of zona pellucida has proceeded mainly in the mouse and rat, but the study of rabbit has not appeared up to this time. The function and significance of the zona pellucida has not been explained.

The purpose of the present study was to follow the development of the rabbit blastocyst in vitro after removal of the blastocyst coat and in vivo after transplanted to the uterus.

Materials and Methods

Female Japanese White rabbits were used as donor and recipient. The eggs were obtained from the uterus on 6 day p.c. Two methods were examined for the removal of the blastocyst coat.

(1) *Mechanical method*: blastocysts were put into 1% saline solution for a few minutes and contracted. The blastocyst coats were separated from the trophoblastic cells. The surface of the contracted portion was cut with scissortips and then the coats of the blastocysts were removed.

(2) *Chemical method* (*=resolution by pronase*): the coat was removed by placing the egg in 0.5 or 0.25% pronase solution at room temperature. In order to culture in vitro, the naked blastocysts were put into F_{12}+10% calf serum and were watched for 5 to 6 hr for recovery of their form (expansion). The incubator was maintained at about 37°C and gassed with 5% CO_2 in air. A hole was made on the opposite part of the inner cell mass of the blastocyst coat with a fine needle according to the previous report (3).

The naked and the holed blastocysts were cultured for 5–6 hr, and then transferred to the recipient for testing developmental ability. The recipients were mated with a vasectomized male to induce ovulation and pseudopregnancy. The sexual cycle between donor and recipient was synchronized. The transfer of naked and holed blastocysts was carried out under the anesthesia with nembutal, and eggs corresponded to the number of corpora lutea in the host were transferred to the uterine horn.

Recipients were killed 5 to 6 days or 22 to 23 days after the transfer and were examined for implantation sites and number of living fetus.

Results

1. *The effects of hypertonic saline or pronase treatment on the blastocyst coat.*

In order to separate the trophoblastic cells from the blastocyst coat, blastocysts were put into a hypertonic saline (1% saline solution) and the state of contraction was watched with the lapse of time (Fig. 1).

When the blastocysts were transferred to hypertonic saline solution (1%), the contraction of blastocysts began 5 minutes later, and within 25 minutes 75%

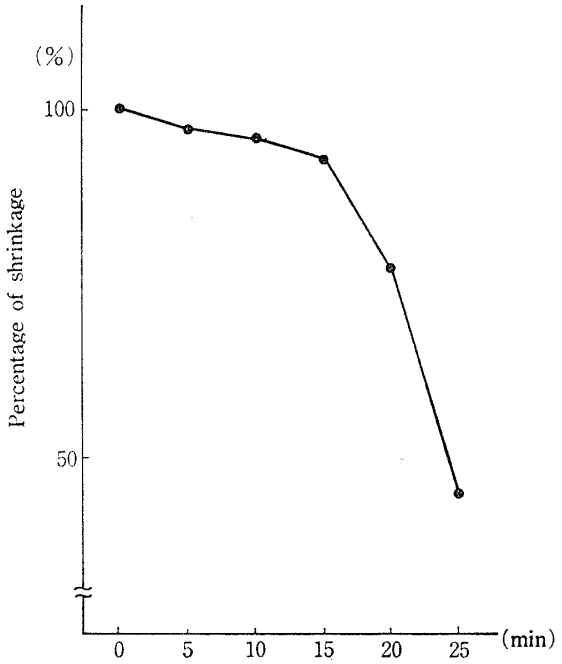


FIG. 1 Shrinkage of blastocysts by 1% saline solution

of the embryos were contracted to 75% of initial volume. Some of them were contracted to 20 or about 30% of their volume. The blastocysts which contracted over 30% were cultured in the medium (F_{12}+10% Calf serum), but none of them re-expanded naturally except to make a small sphere to trophoblastic cells.

Observation was made on the form of blastocyst. The separation of trophoblastic cell from the coat began from both sides of inner cell mass (ICM) portion, and then trophoblasts separated completely from the coat.

By this method, the volume of the naked blastocyst decreased to 30–70% of their initial size just after the removal of the blastocyst coat.

The results of the effects of 0.5 or 0.25% pronase on the removal of blastocyst coats are shown in Table 1.

By the 0.5% pronase, the resolution of the coat was observed after 2 minutes. All of them were solved after 5 minutes. On the other hand, the solution began after 3 minutes, and all of them were solved 6 minutes later. From those results, we decided to use 0.25% pronase (for 2 minutes) thereafter.

TABLE 1. *Dissolution of blastocyst coat by pronase*

	Time of dissolution (min).					
	1	2	3	4	5	6
0.5% pronase	0/12*	1/12	6/12	11/12	12/12	—
0.25% pronase	0/15	0/15	1/15	5/15	10/15	15/15

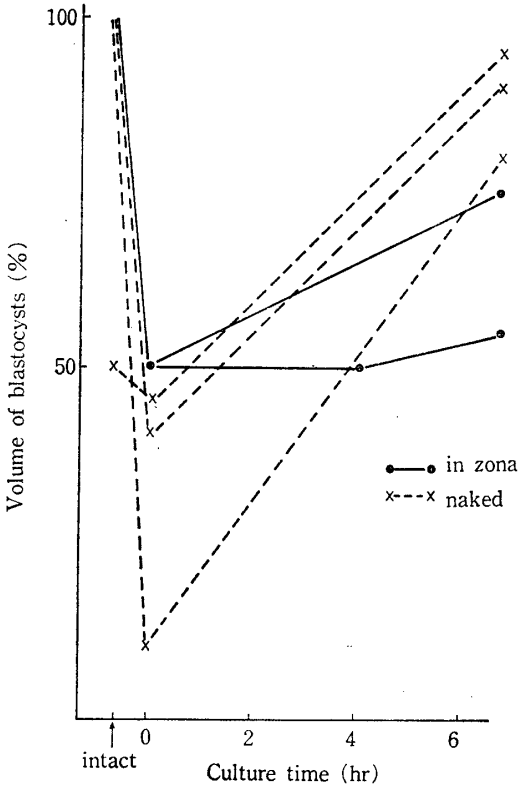


FIG. 2. Re-expansion of naked and in zona blastocyst during culture

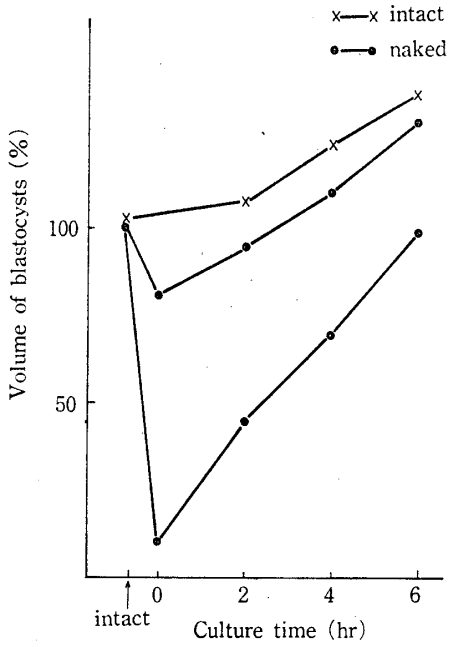


FIG. 3. Expansion of intact and naked blastocysts during culture

The process of the resolution of the coat was shown as follows; A part of the upper sides of the ICM begins to form a protuberance. At the same time, the blastocysts, which are in ball form, change to an elliptical form. The blastocyst coat of protuberance part begins to resolve, then the other part succeeds. The naked blastocyst returns to a spherical form, and then contracts gradually.

2. *Culture of naked blastocyst.*

The results are shown in Fig. 2 and 3. The blastocyst, which contracted to the volume of 80 to 90% of intact size, re-expanded to their initial size during 4 hours of incubation and continued to expand to the initial blastocyst size.

The blastocyst, which contracted considerably, started to expand by the culture, and re-expanded to initial size after 6 hours (Plate 1–4).

The result are shown in Fig. 3.

The re-expansion of the blastocysts, which decreased to the volume of 40 to 50%, was slower than that of the naked blastocyst.

The naked blastocysts re-expanded rapidly just after the culture. Also, 20% of naked blastocysts started to re-expand and reached 70% recovery during 6 hrs.

TABLE 2. *Survival of blastocysts after treatment of zona pellucida*

Group	Treatment of zona	No. of recipients	No. of showing implantation sites (R)	No. of eggs transferred	No. of implantation sites (%)	No. of living fetuses (%)
I	Making a hole in zona	4	4	19	15(79)	5(26)
II	Removed by pronase	4	4	19	8(42)	0
III	Removed mechanically	4	2	15	5(33)	0

3. *Transplantation test of the treated blastocyst.*

In order to examine the activity and the ability to grow the treated blastocysts, they were transferred to a recipient female. The results are summarized in Table 2.

It is noted that all of the recipients, which had been received the holed blastocyst, had implantation sites.

The recipients having implantation sites were 4/4 (implantation rate: 79 %). Five fetuses were found at 28-days. In comparing the treatments with pronase and the mechanical methods, the No. of implanted recipient / No. of recipients were 4/4 and 2/4, respectively.

The rates of implantation (No. of implanted egg/No. of transferred egg) were 42 and 33%, respectively.

However, no fetuses were found in those two groups.

Discussion

We have studied the physiological significance of the zona pellucida (or blastocyst coat) in rabbit which is not removed from the egg till implantation, unlike mouse and rat.

In fact, it seems possible that the blastocyst coat plays an important role in the pre-implantation in the rabbit.

Unlike other animals, the blastocyst in rabbit was expanded rapidly, accumulating a lot of blastocoelic fluid in the blastocoel during the pre-implantation. So, the blastocyst coat may protect the embryo from various harmful effects, till cell-contact with uterine epithelium occurs.

Recently, so-called "Blastokinin" was discovered in rabbit uterine fluid secreted from the pre-implanted endometrium and it was demonstrated that it promotes the growth of blastocyst and hatching.

The blastocysts coats became thin with the development of the blastocyst.

The blastokinin might be related to the disappearance of the coat. But,

little is known about the mechanism.

From our previous experiment (3), it was demonstrated that the contracted blastocyst re-expanded and a small ball of trophoblastic cells were exposed from the incision when the blastocyst was holed into the coat.

The blastocyst coat, however, did not increase in volume and only the exposed ball of the trophoblastic cells expanded. Those phenomena are most interesting in pre-implantation rabbit blastocysts in vitro.

So, in order to make clear the mechanism of re-expansion of the blastocyst and the role of the coat at this stage the blastocyst coat was removed artificially in the present study.

At the beginning, all the blastocysts were put into a hypertonic salt solution in order to examine the effects of a hypertonic solution on blastocysts.

In the hypertonic medium the blastocysts started to contract after a few minutes, and most of them (11/15) contracted to 70% in volume. The contraction of blastocyst after removal of the coat might show the physiological role of the coat at this stage, but still little is known. The rapid re-expansion of the naked blastocysts shown in Fig. 1 and 2, might indicate that the mechanism of the control of the uptake of the substances in the blastocyst coat and trophoblastic cells were deranged.

Speed of re-expansion in the naked blastocyst was faster than that of the holed blastocyst. Sugawara (4) reported that the growth of the pre-implanted blastocysts was rapid and that some substances in the blastocoelic fluid were accumulated during the stage. It is interesting what sort of substance was taken up into the blatocoelic fluid.

Between the two methods for removal of blastocyst coat the mechanical method was more harmful than the chemical one. As the coats dissolved gradually in the case or the chemical method, the speed of contraction of blastocyst as well as the breaking or disappear of the blastocyst coat might have disturbed the blance. It is suggested that the blastocyst coat plays an important role such as the protection from environmental factors and the transport of some substance, which are concerned with taking in and out of various ions and organic substances between uterine and blastocoelic fluids.

The naked blastocysts received the physical effects easily and adhered to the apparatus which was made from steel or glass. On the experiments of egg transfer, the naked blastocysts were more difficult for transplantation into the uterus than the intact blastocyst.

In the study using mouse eggs, Modlinski (5) observed that naked eggs transplanted to the oviducts adhered to the wall and that the immobilizing of the egg caused inhibition or disturbance of their cleavage. Moore, Adams & Rowson (2) also transplanted single naked blastomeres from 2- and 4-cell rabbit eggs to the oviduct and found that no further development occurred. In this

experiment it may be assumed that naked blastocysts adhered to the uterus wall under abnormal conditions, and so normal growth was inhibited.

The implantation rate of naked blastocysts was ranged from 33 to 42%. It is estimated that the transferred blastocyst could grow in some degree but no further development occurred. On the contrary, it was found that mouse blastocyst, which had no zona pellucida, possessed a specific adherence at the preimplantation stage and, it was estimated that the adherence of blastocyst gave some effects to the phase of contact at the time of implantation. In this connection, Jones and Kemp (6) observed sediments of mucoprotein and shell acid on the outer surface of trophoblastic cells of pre-implantation (6 days) in mouse blastocyst. Brodburg (7) found the phase of mucoprotein on the outer surface of human trophoblastic cells. The estrogen surge at the preimplantation stage related to the synthesis of the adherence factor of trophoblastic cell, and at this time the activity of tyrosine amine transferase (TAT) was found to increase (8). Otherwise, as mentioned above the role of the blastocyst coat in rabbit appeared to differ from other animals during the stage of implantation. But, further studies are necessary to clarify the role and function of the blastocyst coat.

Acknowledgement

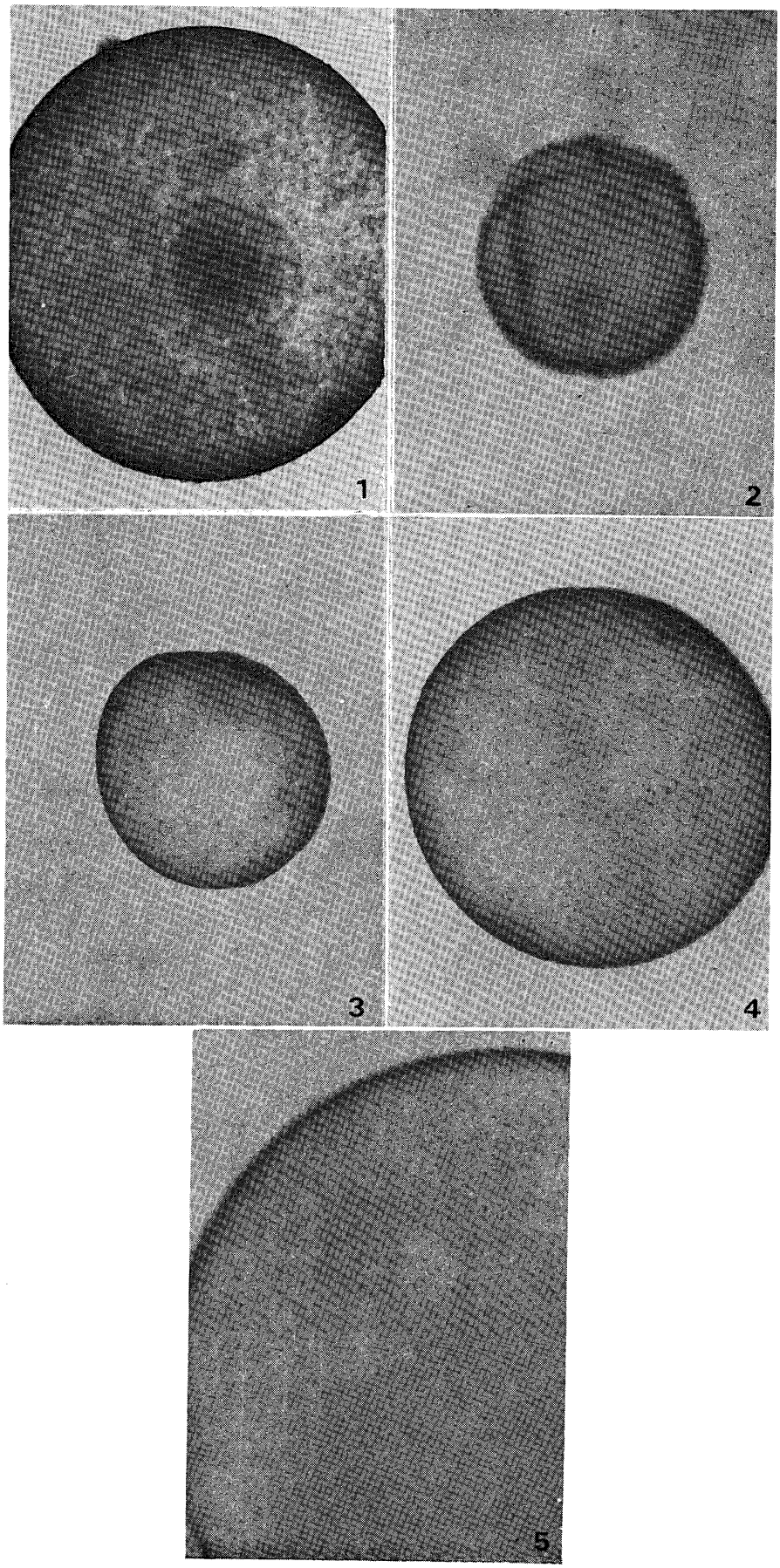
We wish to express my thanks to Professor J. Masaki for his advice on the manuscript.

References

1) Edwards, R.G., *J. Reprod. Fert.* **7**, 413 (1964)
2) Moore, N.W., C.E. Adams and L.A.E. Rowson, *J. Reprod. Fert.* **17**, 527 (1968)
3) Totsukawa, K., S. Sugawara and S. Takeuchi, *Jap. J. Zool. Sci.* **45**, 319 (1974)
4) Sugawara, S., *Jap. Soc. Anim. Reprod.* **15**, xii (1970)
5) Modlinski, J.A., *J. Embryol. exp. Morph.* **23**, 539 (1970)
6) Jones, B.M. and R.B. Kemp, *Nature* (London) **221**, 829 (1969)
7) Brodburg, S., W.D. Billington, D.R.S. Kirby and E.A. Williams, *Am. J. Obstet Gynec.* **104**, 416 (1969)
8) Holiems, P.V. and A.D. Dickson, *J. Embryol. exp. Morph.* **29**, 639 (1973)

Explantation of plate

1. intact blastocyst (initial size). ×70. 2. naked blastocyst immediately after treatment.
3. re-expansion blastocyst (4 hr after culture in vitro).
4. full expansion blastocyst (6 hr after culture in vitro).
5. more expansion blastocyst (8 hr after culture in vitro).


1
2
3
4
5