Use of the Artificial Zona Pellucida Made of Calcium Alginate in the Development of Preimplantation Mouse Embryo

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Abstract: *Using calcium alginate as material for the artificial zona pellucida, the early embryonic development and the implantation of zona-free mouse eggs encapsulated with an artificial zona pellucida were investigated. In the group of two-cell embryos encapsulated with the artificial zona pellucida, 95.0% developed to the blastocyst. On the other hand, 93.1% in the zona intact group and 83.0% in the zona free group developed to the blastocyst. The rate of the embryo encapsulated with an artificial zona pellucida developing to the blastocyst was significantly higher than that in the non-capsulated zona-free embryo ($P<0.05$) and also exceeded that in the zona intact embryo. Twelve normal fetuses were obtained after the transfer of 75 blastocysts encapsulated with the artificial zona pellucida to recipients. These results indicates that calcium alginate used as an artificial zona pellucida is not detrimental to the development of the embryos but plays a protective role in the preimplantation embryo development, and the artificial zona pellucida dissolves timely in the uterus and dose not hinder implantation. If hardness of calcium alginate suitable for humans can be found, clinical application of IVF-ET with zona-free eggs making use of an artificial zona pellucida will become possible.*

Key words: *Artificial zona pellucida, Blastocyst formation, Calcium alginate, Implantation, Zona pellucida-free egg.*

Microinsemination is a therapeutic technique indispensable for severe oligozoospermia or severe asthenozoospermia, but it has a drawback in that special technical skill and equipments are required. Fertilization of eggs with the zona pellucida removed can be effected with a very small number of spermatozoa. Theoretically, therefore, fertilization similar to microinsemination can be expected if the zona pellucida is removed. However, the zona pellucida cannot be removed offhandedly since it has an important role of protecting the egg from physical and biological stimuli. Thinking that an artificial zona pellucida, if developed, would make it possible to effect *in vitro* fertilization and embryo transfer (IVF-ET) in the zona free eggs, we undertook this study. Using calcium alginate as material for the artificial zona pellucida, we studied its utility *in vitro* and *in vivo*.

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Materials and Methods

Making of an artificial zona pellucida and its in vivo solubility

An artificial zona pellucida was made of calcium alginate. Gelatinous masses of calcium alginate can be made by having a solution of sodium alginate acted on a $CaCl_2$ solution. Hardness of calcium alginate appropriate for the artificial zona pellucida was studied from the aspect of its solubility in the mouse uterus. When a solution of 2% or 3% sodium alginate (Alto; Kyosei Pharmaceutical Co., Otaru, Japan) is added dropwise to a solution of 1.5% or 3% $CaCl_2$, gelatinous masses of calcium alginate are formed. So the amount added was adjusted until the diameter became 1.5 mm and the masses were taken out of the $CaCl_2$ solution 30 sec later. The gelatiniform spheres were held at 37°C in 0.9% NaCl, then transferred to B6C3F1 mice. The female mice were anesthetized with ether during the gelatinous mass transfer. Using a scalpel, a small puncture was made in the end of uterine horn. Four gelatinous masses were injected through the puncture site into the uterine horn using a polyethylene catheter. The puncture site was closed with a single suture of silk thread. The mice were killed 24 h, 72 h, or 120 h after

the gelatinous masses transfer, and the number of intact gelatinous masses were determined.

Observations on the development of early embryos of zona-free fertilized eggs encapsulated with an artificial zona pellucida

1) Collection of mouse fertilized eggs: Five weeks old female B6C3F1 mice were induced to superovulate by consecutive intraperitoneal injections 48 h apart of 10 IU pregnant mare serum gonadotopin (PMS; Teikokuzoki Co., Tokyo, Japan) and 10 IU human chorionic gonadotropin (hCG; Mochida Pharmaceutical Co., Tokyo, Japan). After hCG injection, the females were mated with male mice of the same strain (about 10 weeks after birth). Two-cell embryos were collected from oviducts 45 h after hCG administration. The two-cell embryos were treated with acidic Tyrode's solution adjusted to pH 2.5 for 2 min to dissolve the zona pellucida. After confirming that the zona pellucida was dissolved completely, the embryos were washed three times with human tubal fluid (HTF; Irvine, USA) containing 3.5% bovine serum albumin (BSA; Fraction V; Sigma Chemical Co., USA).

2) Encapsulation with an artificial zona pellucida: Both sodium alginate and $CaCl_2$ were dissolved in distilled water to make a 3% sodium alginate solution and 1.5% $CaCl_2$ solution. The zona-free two-cell embryos were transferred to the 3% sodium alginate solution. One embryo with a small amount of the sodium alginate solution was aspirated into a pipette and injected into the $CaCl_2$ solution, whereupon it looked as if encapsulated in a gelatinous mass of calcium alginate about 1 mm in diameter at the tip of the pipette.

Thirty sec after the encapsulated zona-free two-cell embryos were washed with fresh HTF and introduced into 10 μl of HTF (containing 3.5% BSA) placed under mineral oil in a plastic Petri dish. After incubation at 37°C under 5% CO_2 in air for 72 h, the embryos encapsulated with the artificial zona pellucida were observed using a stereoscopic microscope and evaluated the blastocyst formation. Non-capsulated zona-intact or zona-free two-cell embryos were used as the control.

Embryo transfer study

The blastocyst that grew while encapsulated with an artificial zona pellucida was transferred to a pseudopregnant mouse.

1) Preparation of pseudopregnant mouse: Twenty-four hours behind the time to administer gonadotropin to mice from which two-cell embryos are collected, PMS 5 IU and 48 h later hCG 5 IU were administered to female mice of B6C3F1 (8–10 weeks of age), and they were mated with vasectomized male mice on administration of hCG to prepare pseudopregnant mice.

2) Embryo transfer: The blastocyst that grew while encapsulated with an artificial zona pellucida was transferred into the uterus of a pseudopregnant mouse 72 h after encapsulation with the artificial zona pellucida. Eleven days after the blastocyst transfer the recipients were killed by cervical dislocation and examined for live fetuses.

Statistical analysis

The results of the experiment were studied by Chi-squared test and the difference was regarded as significant when the P value showed 0.05 or less.

Results

Solubility of calcium alginate masses in vivo

In Table 1 is presented the status of dissolution of calcium alginate masses in the mouse uterus. The calcium alginate mass made of a solution of 2% sodium alginate and a solution of 1.5% $CaCl_2$ began dissolving from 24 h after transfer and two-thirds of it dissolved 72 h after transfer. With the mass made of a solution of 2% sodium alginate and a solution of 3% $CaCl_2$, two-thirds dissolved at 72 h. The calcium alginate mass

Table 1. Disolution of the gelatinous masses of calcium alginate in uterine of mice (1.5 mm spherical masses of calcium alginate)

Concentration of sodium alginate	Concentratin of calcium chloride	Disolved gelatious masses / Injected gelatinous masses		
		24 h after transfer	72 h after transfer	120 h after transfer
2% (w/v)	1.5% (w/v)	4/12	8/12	12/12
	3% (w/v)	0/12	8/12	8/12
3% (w/v)	1.5% (w/v)	0/12	0/12	12/12
	3% (w/v)	0/12	0/12	8/12

made of a solution of 3% sodium alginate and a solution of 1.5% $CaCl_2$ did not dissolve 72 h after transfer but completely dissolved at 120 h.

Of the masses made of a solution of 3% sodium alginate and a solution of 3% $CaCl_2$, some did not dissolve even at 120 h. Usually, the zona pellucida starts hatching at the stage where the embryo developed to the expanded blastocyst and disappears rapidly thereafter. Therefore, thinking it appropriate for the zona pellucida to remain completely until 72 h after fertilization and to dissolve 96 to 120 h after, we decided to adopt the calcium alginate masses made of a solution of 3% sodium alginate and a solution of 1.5% $CaCl_2$ as an artificial zona pellucida.

Early embryonic developmet in mouse embryo encapsulated with the artificial zona pellucida

In Figs. 1 and 2 are shown photos of the embryos that developed in the artificial zona pellucida. In the group of embryos encapsulated with the artificial zona pellucida, 95.0% (229/242) developed to the blastocyst. On the other hand, 93.1% (108/116) in the zona intact group and 83.0% (100/121) in the zona free group developed to the blastocyst (Fig. 3). No difference was recognized between the artificial zona pellucida group and the zona intact group, but the rate of development was higher in the former group. The rate of development was poor in the zona-free group, there being a significant difference between the zona-free group on the one hand and the artificial zona pellucida group and the zona intact group on the other ($P<0.05$, $P<0.005$).

Results of blastocyst transfer

Seventy-five blastocysts encapsulated with the artificial zona pellucida were transferred to 15 recipients. Six recipients got pregnant and 12 normal fetuses were obtained (Fig. 4).

Discussion

Since the first success in human IVF-ET [1] , IVF-ET is widely applied in the treatment of infertility. However, it is well known that some couples with impaired sperm characteristics, e.g. severe oligozoospermia or severe asthenozoospermia, can not receive the benefit by the conventional IVF-ET. Recently, in order to work out such problem, micromanipulation technology has been applied. In practice methods such as zona drilling (ZD), partial zona dissection (PZD), subzonal insemination (SUZI) and intracytoplasmic sperm injection (ICSI) have been planned and successful cases of pregnancy have been reported [2–12]. Although the microinsemination is a useful technology for severe oligozoospermia or severe asthenozoospermia, it requires expensive equipment and specially trained technicians. If the zona pellucida can be removed, the same fertilization as with PZD and SUZI can be effected. Actually, mouse oocytes, freed from zonae pellucidae, were fertilized with a extremely small number of spermatozoa *in vitro* [13]. Since, under ordinery conditions, the zona pellucida is essential for normal fertilization, however, removing it is ill-advised. The zona pellucida is made up of sulfated glycoprotein, having functions such as inducing acrosome reaction, blocking polyspermy, protecting the egg from physical and biological stimuli and suppressing dispersion of the blastomeres. Moreover, it causes hatching timely on implantation and thereafter disappears rapidly in the uterus. If an artificial zona pellucida

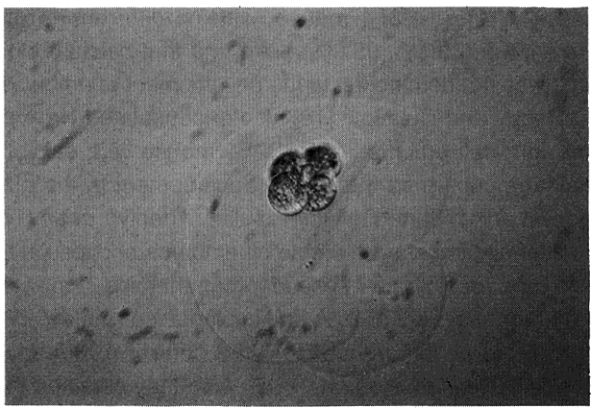

Fig. 1. The four-cell mouse embryo developed in the artificial zona pellucida.

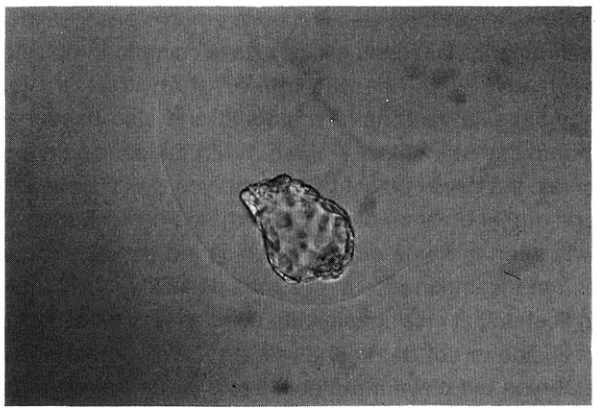

Fig. 2. The mouse blastocysts developed in the artificial zona pellucida.

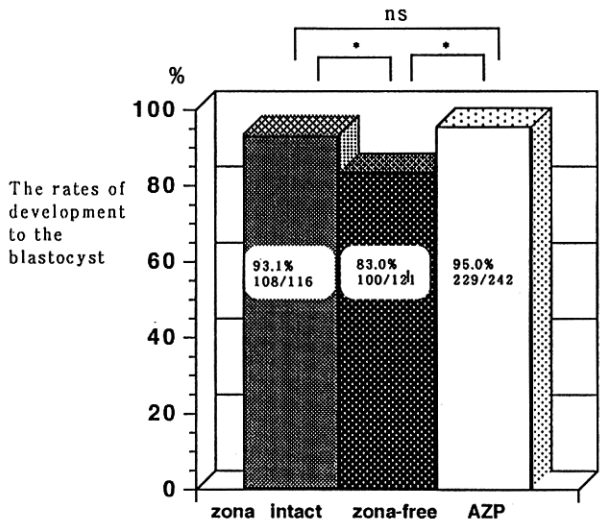

Fig. 3. The rates of development to the blastocyst. zona intact: zona intact embryos. zona-free: zona-free embryos. AZP: embryos encapsulated with the artificial zona pellucida. *: $P<0.05$ n.s: not significant. 95.0% in the group of embryos encapsulated with the artificial zona pellucida, 93.1% in the zona intact group, and 83.0% in the zona-free group developed to the blastocyst, respectively.

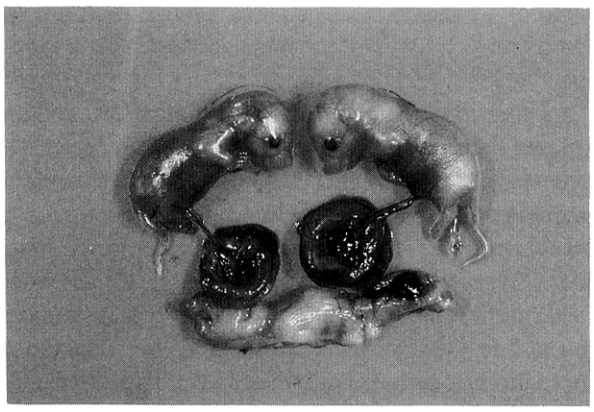

Fig. 4. Normal fetuses obtained 11 days after the blastocysts encapsulated with the artificial zona pellucida transfer.

having functions close to physiological ones can be encapsulated without impairing the egg, it might be possible to effect *in vitro* fertilization and embryo transfer even with zona-free eggs. Based on this idea, we decided to conduct a fundamental study. Calcium alginate having a network structure in which chain polymers are entwined together like strands was selected as the material, and calcium alginate prepared by a solution of 3% sodium alginate and a solution of 1.5% $CaCl_2$ was used as an artificial zona pellucida after its solubility in the mouse uterus was studied.

The rate of the two-cell mouse embryo encapsulated with an artificial zona pellucida developing to the blastocyst was significantly higher than that in the non-capsulated zona-free embryo and also exceeded that in the zona intact embryo. With the zona-free embryos, the development of the embryos was suppressed and a phenomenon of the blastomeres being dispersed was seen in some embryos, which led to re-confirmation of the importance of the zona pellucida. This result indicates that calcium alginate used as an artificial zona pellucida is not detrimental to the development of the embryos but plays a protective role in the preimplantation embryo development. When the blastocyst encapsulated with an artificial zona pellucida is transferred to pseudopregnant recipients, implantation and normal development to fetuses can be recognized. This indicates that the artificial zona pellucida dissolves timely in the uterus and dose not hinder implantation.

The method of coating embryos with a gelatinous substance to protect the fertilized eggs has from old times been studied by many investigators. In 1979, Willadsen [14] put the blastomeres separated from the sheep two-cell embryos back into the zona pellucida, embedded them in agar for protection, made them develop to the blastocysts and then transferred them to the recipients, reporting that pregnancy was established in 11 sheep.

Adaniya *et al.* [15] coated the embryos with calcium alginate in order to protect *in vitro* fertilized embryos in the uterus and compared the development of embryos *in vitro* with the control. They stated that the rate of development to the blastocyst was 37.4% in SW mouse and 91.7% in CB6F1 mouse, with no difference from the control (33.3%, 95.0%) seen and that calcium alginate was not detrimental to the development of embryos.

Cosby and Dukelow [16] also encapsulated two-cell and four-cell embryos of B6D2F1 mouse with calcium alginate, confirmed the rate of development to the blastocyst and reported similar results. Adaniya *et al.* [17] additionally transferred eight-cell embryos encapsulated with calcium alginate to pseudopregnant mice, achieved the pregnancy rate of 20% and implantation rate of 9.6% and found no difference from the control (pregnancy rate 24%, implantation rate 9%). Also they obtained the second generation of 72 mice from 12 (8.6%) mouse neonates obtained by transferring 139 embryos encap-

sulated anew with calcium alginate to 20 mice. On the basis of these results, they have maintained that calcium alginate is not detrimental to the development of embryos, implantation and the growth of fetuses since it dissolves within 48 h after transfer. Setting an eye on the use of 0.1% Poly-L-Lysine or calcium alginate for the purpose of protecting frozen-thawed embryos where each blastomere has been separated, Krentz *et al.* [18] had the mouse morula encapsulated with it as a fundamental experiment and reported that there was no difference in the development of embryos from the control.

These studies were aimed at protecting the embryos in the uterus in IVF-ET. They differ in the objective from our present study that dealt with the use of calcium alginate as a substitute for the zona pellucida.

If calcium alginate can actually be used in human IVF, it will make complete removal of the zona pellucida possible and facilitate fertilization. If the solubility of calcium alginate in the uterus is studied further and if hardness of calcium alginate suitable for humans can be found, clinical application of IVF-ET with zona-free eggs making use of an artificial zona pellucida will become possible.

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マウス初期胚発生におけるアルギン酸カルシウム製人工透明帯の応用

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アルギン酸カルシウムを用いて人工透明帯を作成し，人工透明帯を装着したマウス２細胞期胚の初期発育と着床への効果を検討した．人工透明帯装着群の胚盤胞への発育率は95.0％を示し，透明帯除去群の83.0％に比べ有意（P<0.05）に高値であった．透明帯無処置群の胚盤胞への発育率は93.1％で，これは人工透明帯装着群と差をみなかった．人工透明帯を装着したまま発育した75個の胚盤胞を受卵雌に移植して12匹の正常な胎仔が得られた．これらの成績は，人工透明帯に用いられたアルギン酸カルシウムは初期胚の発育に有益であり，また子宮の中でタイムリーに溶解して着床を妨げないことを示している．透明帯除去卵によるヒト体外受精に，このアルギン酸カルシウムを用いた人工透明帯の応用が期待される．

キーワード：人工透明帯，胚盤胞，アルギン酸カルシウム，着床，透明帯除去卵．