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In Vitro Fertilization of Bovine Oocytes Matured *In Vitro* by Microinjection of Spermatozoa

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정자 미세주입에 의한 소 난포란의 체외수정

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밀양전문대학

적 요

미성숙 소 난포란을 20% FCS가 첨가된 TCM-199으로써 24시간 동안 5% CO_2, 8% O_2의 공기 조건하에서 체외성숙시킨 다음 난자의 위란강내로 정자를 미세주입하여 체외수정을 실시하였다. 미세주입하기전 정액을 0.75% BSA가 첨가된 Ham's F-10 배양액으로 2시간 동안 5% CO_2, 8% O_2의 공기 조건하에서 배양함으로써 수정능 획득을 하도록하였으며 이어 30분 동안 12mM의 dbcGMP와 10mM의 imidazol이 함유된 Ham's F-10 배양액으로 배양함으로써 첨체반응을 유도하였다.

본 실험에서 정자의 미세주입 후 제 2극체와 전핵형성이 일어난 난자의 비율은 각각 9.5, 5.4%였으며 2세포기 이후로 발달한 난자의 비율은 4.1% 이었다.

(Key Words : bovine oocytes, microinjection of spermatozoa, *in vitro* fertilization)

I. INTRODUCTION

Mammalian eggs are normally fertilized and developed in the Fallopian tube. In course of fertilization the spermatozoa penetrates into ooplasm through the zona pellucida of oocyte. The zona pellucida is thought to be a barrier against the penetration of the spermatozoa into the oocyte, although it protects the embryo during early developmental stage.

Microinjection of spermatozoa is a technique of mechanically inserting the spermatozoa into ooplasm or perivitelline space of an oocyte. This technique has been conducted in a number of species, including mice(Mann, 1988; Yamada et al., 1988), hamster(Uehara and Yanagimachi, 1976), rats(Thadani, 1979), rabbits(Keefer, 1989), cattle(Heuwieser, 1991) and human (Metka et al., 1987; Sathananthan et al., 1989).

After microinjection of spermatozoa, oocyte activation, sperm decondensation and pronuclei formation were observed. But a problem commonly encountered in this technique is the low fertilization rate of the microinjected oocytes. In addition, another problem is the inconsistent level of fertilization. One of the reason for low fertilization rate is thought to be the mechanical damage of oocyte during micromanipulation. Particulary, direct microinjection of spermatozoa into the ooplasm injures the gamates (Sathananthan et al., 1989). On the contrary

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spermatozoa injection into the perivitelline space by a fine micropette prevents injury to both the zona pellucida and the oolemma to ensure normal sperm-egg interaction(Ng et al., 1990). In addition, the sperm motility and the number of spermatozoa injected into perivitelline space may be factors influencing the fertilization of oocytes. But there is no definite evidence for such influences.

Yamada et al.(1988) reported that the fertilization was observed when acrosome-reacted motile spermatozoa were injected into the perivitelline space of mouse oocytes. In this study the microinjection technique developed from the mouse model was applied to bovine system to evaluate fertilization and cleavage of the bovine oocytes after microinjection of spermatozoa from frozen or fresh semen.

Ⅱ. MATERIAIS AND METHODS

1. Preparation of oocytes

The ovaries of heifers and cows were obtained at a local slaughterhouse and transported to the laboratory at room temperature in 0.9% physiological saline supplemented with 600 IU/ml of penicillin. The ovaries were washed three times in fresh medium. Oocytes were aspirated from the vesicular follicles with a 24 gauge needle.

Oocyte-cumulus complexes were isolated from sediments obtained by lefting follicle fluids settle for 15 min. These complexes were washed four times with TCM-199 and transferred to petri dishes containing 2 ml of TCM-199 suppplemented with 20% FCS for maturation *in vitro* for 24 hr at 39℃ under an atmosphere of 5% CO_2, 8% O_2 and maximum humidity.

After maturation oocyte-cumulus complexes were treated with 800 IU/ml of bovine testicular hyaluronidase(Serva, Heidelberg) for 10 min and washed three times in TCM-199. The cumulus cells surrounding the oocytes were removed by gentle pipetting. The presence of polar bodies was checked carefully. The oocytes without polar bodies were cultured in TCM-199 containing 100 μM of dibutyryl cyclic adenosine 3′, 5′-monophosphate(dbcAMP) for 2~3 hr.

For the microinjection two oocytes were placed in the droplets of culture medium in a glass chamber just beside the droplet for spermatozoa, and covered with silicon oil (Mettler et al., 1988).

2. Preparation of spermatozoa

Frozen or fresh semen were used for microinjection. The fresh semen was collected from the epididymis of slaughtered bulls. Frozen semen was thawed at 39℃ of water for 30 sec. Semen was put into Ham's F-10 medium containing 0.75% of BSA. Spermatozoa were kept for 2 hr at 39℃ under an atmosphere of 5% CO_2 and 8% O_2 for capacitation, and then only upward swimming spermatozoa underwent induction of the acrosome reaction.

They were, continuously, left in Ham's F-10 medium containing 12 mM of dbcGMP(N^2-2′-0-dibutyryl guanosine 3′, 5′-cyclic monophosphate, Sigma, Munich) and 10 mM of imidazole(Sigma, Munich). After 30 min of culture, the spermatozoa were put into a droplet of medium containing 1.5% of BSA in a glass chamber just beside the droplets of oocytes for microinjection.

3. Microinjection of spermatozoa

All the micromanipulatory procedures were performed under an inverted microscope (Olympus, Tokyo) fitting two micropanipulator (Leitz, Hamburg). The air injection and suction systems were regulated by an electronic

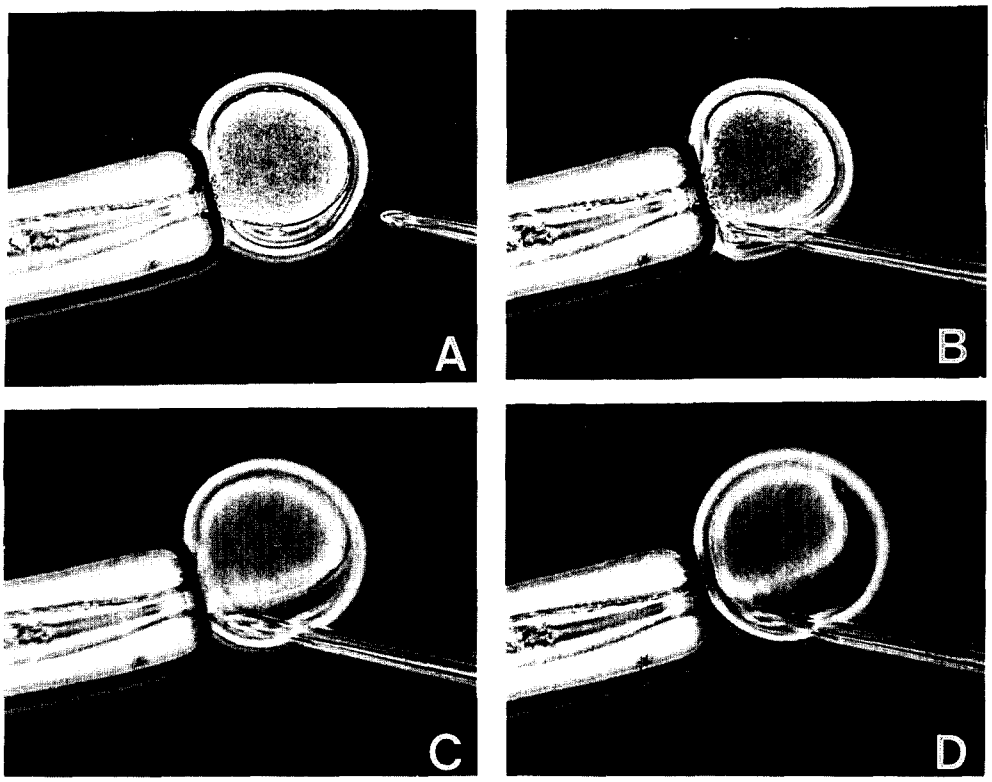

Fig. 1. Procedures of microinjection of spermatozoa to bovine oocytes(× 250).
A : The oocyte is held by the holding suction pipette, and 1 spermatozoon has been aspirated into the injection pipette, which is brought to the oocyte.
B : Injection pipette is introduced to the zona pellucida.
C : Injection pipette is introduced into the perivitelline space.
D : The spermatozoon is injected into the perivitelline space.

microinjector(Eppendorf, Hamburg) connected through polyethylene tubes.

The injecting pipettes were prepared by pulling glass capillaries with a horizontal pipette puller(Leitz, Hamburg) and were sharpened with a sharpener(Bachofer, Reutlingen). The inner diameter of the pipette was about 10 μm. The oocyte-holding pipettes were prepared by drawing out capillary glass tubes and smoothing the tips with a small flame.

Motile spermatozoa were picked up by the injection pipette from the spermatozoa tail end, and injected into the perivitelline space of an oocyte that was held by a holding pipette (Mettler, 1988). If the injected spermatozoa were no longer motile in the perivitelline space, another active one was injected. After that the oocyte was transfered individually into the TCM-199 supplemented with 20% FCS and cultured further at 39℃ under an atmosphere of 5% CO_2, 8% O_2.

4. Observation of oocytes

The oocytes were observed after 16~18hr under the inverted microscope, and were examined for the presence of 2nd polar bodies and pronuclei. The control of the 2~4 cell stage embryo followed after 48hr.

Ⅲ. RESULTS

Total 302 bovine oocytes were microinjected with spermatozoa. The oocytes of which ooplasm were damaged during the microinjection could be recognized immediately. About 80% of oocytes(242 of 302) were microinjected successfully. However, 7.4% of oocytes were

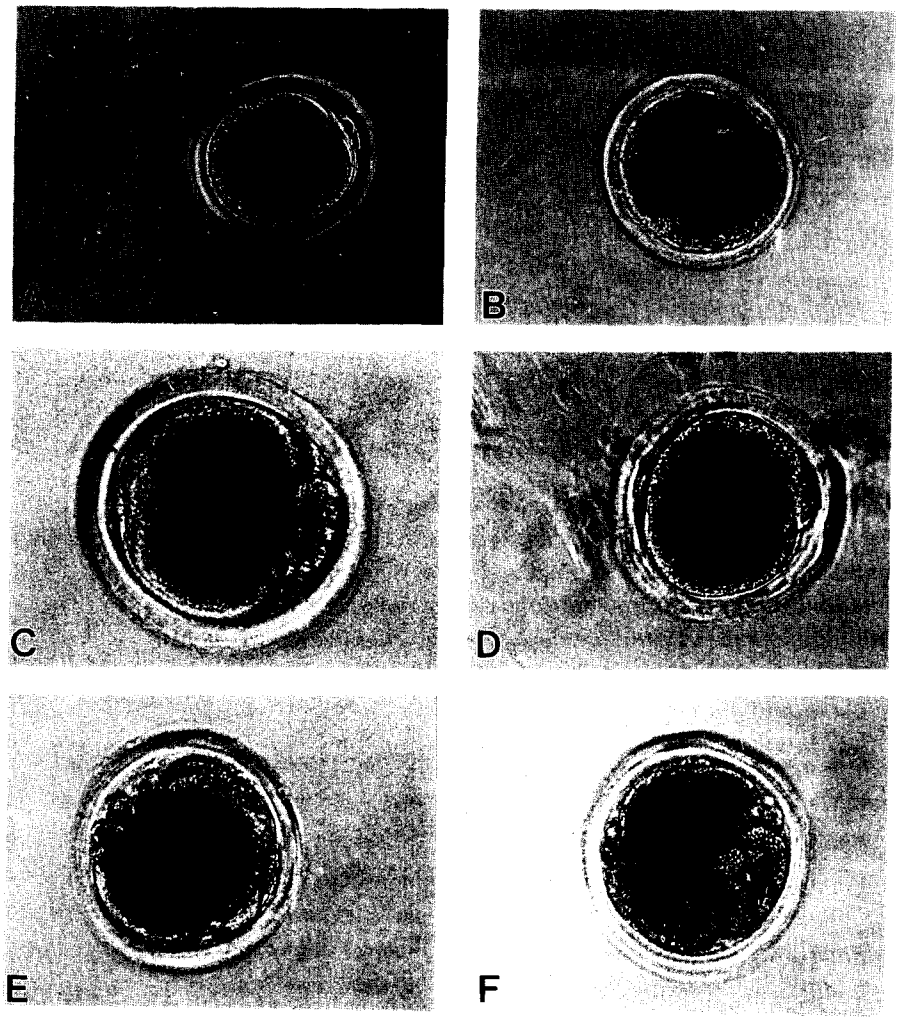

Fig. 2. Bovine oocytes fertilized *in vitro* by microinjection of spermatozoa and developed *in vitro* to the stage of 2nd polar body(A), pronuclei(B), dividing(C), partial division(D), 2-cell(E), and 4-cell(F)(× 250).

Table 1. *In Vitro* fertilization of bovine oocytes matured *in vitro* by microinjection of spermatozoa

Source of Spermatozoa	No. of Oocytes Microinjected	No. and(5) of Oocytes Developed to the Stage of			
		2nd Polar Body	Pronuclei	Partial Division	Cleavage
Frozen Semen	112	12(10.7)	3(2.7)	2(1.8)	4(3.6)
Fresh Semen	130	11(8.7)	10(7.7)	15(11.4)	6(4.6)
Total	242	23(9.5)	13(5.4)	17(7.0)	10(4.1)

degenerated during the culture after microinjection. The number of oocytes with 2nd polar body, visible pronuclei, partial division and cleavage over 2 cells are shown in table 1. The first group of oocytes was microinjected with frozen-thawed semen. The motility of those spermatozoa was decreased rapidly after treatment of acrosome reaction. By using the fresh semen, motile active spermatozoa were able to be supplied, and the rate of fertilization was slightly higher.

The occurence rates of 2nd polar body and visible pronuclei were 9.5% and 5.4%, respectively. The oocyte were observed without process of fixation or staining. So, it is considered that the occurence rates of pronuclei would be, in fact, higher than those of this result. On the other hand, 11.2% of oocytes(27 of 242) showed cell division however partial division was observed in 7% of oocytes. The rates of cleavage over 2 cells was 4.1%.

Ⅳ. DISCUSSION

Microinjection of spermatozoa is a suitable method for fertilization with small amount of spermatozoa(Lasalle et al., 1987), and may be used as a useful technique for treatment of oligo-/asthenozoospermia in human *in vitro* fertilization(Yamada et al., 1988; Sathanantnan et al., 1989). It also offers the possibility of investigating many aspects of spermatozoa-oocytes interaction. However, the results of microinjection of spermatozoa have not been satisfactory yet.

Keefer et al.(1990) reported that bovine oocytes were less sensitive to the injection process of spermatozoa than hamster or rabbit oocytes. Heuwieser(1991) indicated that bovine oocytes were not sufficiently activated by the injection process of a motile spermatozoa into the perivitelline spaces. The result of this study also showed low rates of fertilization and cleavage, but it suggests the possibility of fertilization of bovine oocytes by microinjection of spermatozoa.

Particulary, an attention was given to the partial division. It means that the oocytes were activated after microinjection even if they could not develope to compact 2 cell stage. It may be supposed to be due to the action of flagella of spermatozoa, mechanical stimuli during microinjection or chemical effects of maturation medium. By increasing the exposure time of oocytes to dbcAMP in maturation medium, it was observed that zona pelucida become hard and perivitelline spaces were widely enlarged, and some of oocytes were degenerated. It is known that dbcAMP modulated the maturation, activation, and degeneration of oocytes in culture, and frequent segmentation of bovine oocytes was observed when they were cultured in the medium containing high concentration of

dbcAMP(Sato et al., 1988).

It can't be asserted that dbcAMP of medium exerted an influence upon the partial division of oocytes, however, this possibility, also, cannot be neglected.

The advantage of this technique over other techniques such as zona drilling, zona dissection and microinjection into ooplasm is that injection of spermatozoa can be done with less damage for the oocytes, and with much better survival. The perivitelline space can be widened to facilitate spermatozoa injection(Yang et al., 1988).

It is still questionable whether the injected spermatozoa under the zona pellucida need their motility to penetrate into ooplasm. Barg et al. (1986) indicated that acrosome reacted and motile spermatozoa are required for fertilization in mice. While the fertilization of human oocytes was observed by microinjection of immotile spermatozoa(Bongso et al., 1989). It may be due to a species difference. Markert(1983) observed that the phenotype of the spermatozoa did not reflect the genotype in terms of fertility after microinjection, and immotile and defective spermatozoa produced the same results that were produced by fertilization with healthy, robust spermatozoa. In this study compared with frozen semen, when fresh semen was used slightly higher rates of fertilization and cleavage of oocytes were obtained.

There is a tendency that increasing the number of injected spermatozoa results in higher penetration rate of oocytes. Ng et al.(1990) indicated that multiple spermatozoa injection had a theoretical advantage because acrosome reacted spermatozoa could be still more recepted. In this study only one motile spermatozoon was injected into each oocyte. Sometimes, one or two immotile spermatozoa were injected with motile spermatozoon. Heuwieser (1991) observed that only one spermatozoon per oocyte showed evidence of decondensation even if multi-spermatozoa were injected into perivitelline space of bovine oocytes. After all, the more important thing is how to exactly select and inject the acrosome-reacted spermatozoa.

With regard to development of oocyte after microinjection, Lacham(1989) observed the fertilized eggs to develope to the blastocyst stage in mice. Mann(1988) got live young of mice from the microinjected oocytes. Evsikov (1990) reported that by an electric pulse after microinjection, more than 60% of human oocytes cleavaged. Recently, Keefer et al. (1990) indicated that microinjected bovine oocytes divided to the two or eight-cell stage. Goto et al.(1991) reported that bovine oocytes matured *in vitro* underwent cleavage to the blastocyst after sperm microinjection directly into ooplasm, and normal calves were obtained by the embryo transfer, but it showed lower fertility.

In our study the number of normal cleavage oocytes after spermatozoa injection was 4.1%(10 of 242). Heuwieser et al.(1991) reported that 9~22% of fertilization rate following spermatozoa injection into perivitellin space were obtained in bovine system. These results are comparable to the result of our study.

The aim of this study is to determine the rates of fertilization and cleavage of bovine oocytes by microinjection of spermatozoa. The rates of fertilization and cleavage were low. However, this method might be used as a useful technique for studying the spermatozoa-oocyte interaction.

V. SUMMARY

Capacitated and acrosome-reacted spermatozoa were microinjected into the perivitelline space of bovine oocytes matured *in vitro*. Oocytes obtained from the ovaries of slaughtered heifers and cows were cultured *in vitro* in the TCM-199 supplemented with 20% FCS for 24 hr at 39℃ under an atmosphere of 5% CO_2, 8% O_2. Fresh or frozen spermatozoa were incubated for 2 hr at 39℃ under an atmosphere of 5% CO_2, 8% O_2 in Ham's F-10 medium containing 0.75% BSA for capacitation, and kept for 30 min in culture medium containing 12 mM of dbcGMP and 10 mM of immidazol for acrosome resction.

One motile spermatozoon was injected into the perivitelline space of each oocyte. The 2nd polar body and the pronuclei were observed in 9.5% and 5.4% of oocytes, respectively. The rate of cleavage of oocyte over 2-cell stage was 4.1%(10 of 242). These results indicate that the microinjection may be a useful technique to study sperm-oocyte interaction.

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