Fertilization and *in vitro* development of bovine oocytes following round spermtid injection

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원형정자 미세주입에 의한 소 난자의 수정과 체외 배 발달

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=국문초록=

본 연구에서는 소 난자 세포질내 원형정자 및 원형정자 핵을 미세주입 후 수정과정과 체외 배발달을 조사하였다. 원형정자 주입 3 시간전에 인위적 자극을 주고 원형정자를 주입했을 때 난자들의 수정율이 주입 직후에 자극을 준 것들과 자극을 주지 않은 것들보다 높았다. 원형정자 주입과 원형정자핵을 주입한 후 전핵 형성율과 전핵 이동율을 조사하였으나 유의차를 발견할 수 없었다. 전핵의 형성과 이동중에 미세소관의 움직임을 간접형광면역법 및 공촛점 현미경을 사용하여 조사해본 결과, 난활성 직후 난자의 표층에서 미세소관이 발생해서 이것에 의해 웅성 및 자성전핵이 난자 중심부로 이전됨을 볼 수 있었다. 수정후 원형정자의 세포질내 Mitochondria의 분포상태를 알아보기 위해 MitoTracker에 노출시킨 원형정자를 주입한 후 형광현미경에서 관찰해 보았다. 그 결과 원형정자의 Mitochondria가 주입후 4-cell 이전까지는 관찰되었으나 그 이후에는 발견할 수 없었다. 원형정자를 미세주입한 후 소 난자를 체외에서 배양했을때 2.5%의 난자가 배반포로 발달하였다. 이상의 결과는 체외 배양한 소 난자 내에 원형정자 혹은 원형정자 핵을 미세 주입하여 정상적인 수정과 배발달이 가능한 것을 보여주는 것이다.

INTRODUCTION

More basic experiments in animals are of great significance for application and improvement of assisted conception methods such as intracytoplasmic sperm (ICSI) or round spermatid injection (ROSI). Because spermatids are unable to activate oocytes, mainly due to lack of the oocyte activation factors, additional activation procedure is required to allow fertilization following spermatid injection in the mouse (Kimura et al., 1995), rabbit (Sofikitis et al., 1996) and pig (Lee et al., 1998). Kimura and Yanagimachi (1995) reported that injection of 'undenuded' mouse spermatid resulted in 14% fertilization with a single electrical stimulation. In contrast, injection of spermatid nucleus into oocytes that have been parthenogenetically activated 1 h before injection resulted in much higher rate of fertilization. This suggests that experimental conditions employed affect fertilization rates following round spermatid or round spermatid nucleus injection.

It has been assumed strict maternal inheritance of mammalian mitochondrial DNA (mtDNA). However, paternal midpiece mitochondria enter the egg in the majority of mammals but their ultimate fate is unknown (Ankel-Simons & Cummins, 1996). The mechanisms by which the paternal mitochondria are eliminated are obscure and extremely diverse in eukaryotic organisms (Birky, 1995; Ankel-Simons & Cummins, 1996). If recognition and elimination of the paternal mitochondria are based on recognition by the embryo of elements of the sperm midpiece, then the microinjection of round spermatids, where these elements may not have yet fully formed, may allow paternal mtDNA to evade detection and elimination. This is potentially a concern for human infertility treatment, as the dominance of maternal transmission of mtDNA means that male fitness is irrelevant to the evolutionary 'interests' some of the mitochondrial genome, and some mitochondrial disorders (including perhaps some forms of infertility) are more severe and more prevalent in men than women (Johns, 1996; Cummins, 1997).

Despite importance of various animal models for the round spermatid injection in the human clinic, little information is available on this subject for any species other than the mouse. In the present study we determined fertilization processes and developmental ability following injection of round spermatid and round spermatid nuclei.

MATERIALS AND METHODS

1. In Vitro Maturation

The ovaries were collected from cows at a slaughterhouse and brought to the laboratory in saline at 37℃. The collected oocytes were washed three times in Tyrode-Hepes medium (TL HEPES; Parrish et al., 1985) and washed again three times in equibrated tissue culture medium (TCM-199; 400-1100, GIBCO BRL Co., USA) supplemented with 2.2 g/l sodium bicarbonate ($NaHCO_3$), 10% heat-treated fetal bovine

serum (FBS; 200-614, GIBCO), 0.22 μg/ml Na pyruvate, 25 μg/ml gentamycin sulfate, 1 μg/ml FSH-p (Schering Co., UK), and 1 μg/ml estradiol-17β (BCE-8875, Sigma, Missouri, USA). Then oocytes were cultured in 50 μℓ drops of TCM-199 under paraffin oil for 24 hrs at 39℃, 5% CO_2 in humidified atmosphere.

2. Isolation and preparation of Round Spermatids

The isolation of spermatids was performed using the method of Lee et al (1998). Briefly, testicular tissue was washed with Hank's Balanced Salt Solution (HBSS, Gibco, Grand Island, NY, USA) supplemented with 12.5 mM HEPES (Sigma). After washing, testicular tissue was minced into small pieces with a pair of scissors and the cells in the seminiferous tubules were released into the medium by repeated pipettings. The cell suspension was filtered through a mesh (50 μm, Nylon mesh) to remove cell aggregates and tissue debris, treated for 1 min with 0.1-0.2 mg/ml Pronase (Sigma) in HBSS and centrifuged at 400 x g for 5 min. This treatment eliminated most elongating (flagellum-generating) spermatids and mature spermatids from the cell population by agglutinating them in sticky masses.

3. Labeling of Living spermatid with MitoTracker

MitoTracker Green FM (Molecular Probes, Eugene, OR; catalogue no. M7514) was pepared as a stock 1M solution in anhydrous dimethyl sulphoxide (DMSO) and stored desiccated at -20℃. Spermatogenic cell fractions were incubated for 10min at a final concentration of 5 μM. The labeled spermatogenic cell fractions were washed by two cycles of resuspension and centrifugation in TL-HEPES.

4. Oocyte Activation

At 24 hrs after maturation, cumulus-oocytes complexes (COCs) were treated with 0.1% hyaluronidase in TL-HEPES and pipetted repeatedly to remove cumulus cells. The denuded oocytes were washed and transferred to drops of HEPES-buffered TCM 199 medium under paraffin oil. After or before injection, the oocytes were transferred to a drop of 50 μM calcium ionophore A23187 (Sigma, USA) in CR1aa for 10min at 39℃ to activate the oocytes. Then the same volume (50 μℓ) of PBS containing BSA (6 mg/ml) was added to stop the activation process (Keefer et al., 1990; Goto, 1996). Activated oocytes were transferred to drops of embryo culture medium, CR1aa (Rosenkrans and First, 1991) supplemented with 3 mg/ml fatty-acid-free BSA, 20 μℓ/ml MEM essential amino acid, 10 μℓ/ml MEM non-essential amino acid, 0.44 μg/ml Na pyruvate, 1.46 μg/ml glutamine, 25 μg/ml gentamycin.

5. Round Spermatid and Round Spermatid Nuclear Injection

Spermatogenic cell fractions were centrifuged (400 g, 5 min) and resuspended in TL HEPES : 10% polyvinylpyrrolidone solution (1:1). A microdrop (5 μℓ) of this suspension was placed in a slide, and the slide was placed in Leitz Differential Interference

Contrast inverted microscope equipped with Leitz micromanipulators. The oocytes were denuded cumulus cells by repeated pipetting. Oocytes with visible polar body and of excellent morphology were used for this experiment. Oocytes were centrifuged for 10 min in an Eppendorf centrifuge at 12,000 g in 50 ml TL-HEPES medium in 1.2 ml Eppendorf centrifuge tube. The injection of spermatid into the oocyte cytoplasm was performed using the method of Lee et al (1998). Briefly, the injection needle used was of 6-7 ㎛ inner and 8-9 ㎛ outer diameter. The polar body was at 6 or 12 o'clock and the point of injection at 3 o'clock. An oocyte was penetrated by the injecting micropipette, a small amount of cytoplasm was drawn into the micropipette, and then the cytoplasm together with the spermatid and a small amount of medium was expelled into the oocyte. In a series of experiments of round spermatid nuclear injection, a spermatid was drawn in and out of an 4-5 ㎛ inner diameter pipette until plasma membrane was ruptured. The spermtid nucleus without plasma membrane was then injected using same method mentioned above. Immediately after ooplasmic injection, the injecting micropipette was withdrawn quickly, and the oocytes were released from the holding pipette to reduce the intracytoplasmic pressure exerted to the oocyte.

6. Immunofluorescence Microscopy

Microtubules and DNA were detected by indirect immunocytochemical techniques described by Kim et al (1996[a]). Briefly, the oocytes were permeabilized in a modified Buffer M (Simerly and Schatten, 1993; 25% glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid, 1 mM β-mercaptoethanol, 50 mM imidazol, pH 6.7, 3% Triton X-100, and 25 mM phenylmethylsulfonyl fluoride) for 20 min, fixed in methanol at -20℃ for 10 min, and stored in phosphate buffered saline (PBS) containing 0.02% sodium azide and 0.1% bovine serum albumin for 2 to 7 days at 4℃. Microtubule localization was performed using anti-α-tubulin monoclonal antibody (Sigma). Fixed oocytes were incubated for 90 min at 39℃ with antibody diluted 1:300 in PBS. After several washes with PBS containing 0.5% Triton X-100 and 0.5% BSA, oocytes were incubated in a blocking solution (0.1 M glycine, 1% goat serum, 0.01% Triton X-100, 1% powdered milk, 0.5% BSA, and 0.02% sodium azide) at 39℃ for 1 h. The blocking was followed by incubation in FITC-labeled goat antimouse antibody (Sigma). DNA was fluorescently detected by exposure to 5 mg/ml propidium iodide (Sigma) for 1 h. Stained oocytes were then mounted under a coverslip with antifade mounting medium (Universal Mount, Fisher Scientic Co., Huntsville, AL) to retard photobleaching. Slides were examined using laser-scanning confocal microscopy. Laser-scanning confocal microscopy was performed using a Bio-Rad MRC 1024 equipped with a Krypton-argon ion laser for the simultaneous excitation of fluorescein for microtubules and of propidium iodide for DNA. The images were recorded digitally and archived on an erasable magnetic optical disk.

7. Statistical Analyses

The data were pooled from at least four replications. Differences in the percentages of oocytes developing to a particular stage were determined by Chi-square procedures.

RESULTS

At 18 to 20 h following round spermatid injection, the chromatin configuration was determined in the oocytes in either activated at 3 h before injection or activated immediately after injection. The oocytes with closely apposed two pronuclei, 2-cell and two pronuclei and two polar body were classified as normal fertilization. As shown in Table 1, the incidence of normal fertilization following activation at 3 h before spermatid injection was higher than that with no stimulation or with stimulation immediately after injection. Table 2 summarizes chromatin configuration in bovine oocytes at 18 to 20 h after round spermatid injection or denuded spermatid injection. The incidence of normal fertilization was similar that following spermatid and denuded spermatid injection.

The microtubules were organized from the cortex and then filled the whole cytoplasm in all cases in normally fertilized oocytes (Fig 1A, B, C). This microtubules seem to move both pronuclei into the oocyte center. Following pronulear apposition, microtubules were disappeared from oocytes. This organization is similar to what has been shown previously in the parthenogenetically activated cattle oocytes (Navara et al, 1994). Fig 1D showed diploid chromosome spread at 22 h following injection, suggesting complete fertilization of bovine oocyte by round spermatid.

Following injection of round spermatid, which had exposed to the 5μM MitoTracker for 1 h, paternal derived Mitochondria were seen in the oocytes (Fig 1E). This paternal mitochondria appeared to eliminate from during fertilization (Fig 1F).

As shown in Table 3, the staining intensity tended to decline with time, so that while in the 1-cell embryos at 10-12 h 20 of 26 had bright (4) fluorescence this declined to 10 of 21 (47.6%) in the 1-cell at 20-22 h, 0 of 6 (0%) in the 2-cell and 0 of 5 (0%) in the 4-cell embryos. The final fate of mitochondria appeared generally to be small points of fluorescence within single cells.

Table 4 showed developmental ability of bovine oocytes following injection of round spermatid. Two point five percent oocytes were developed to the blastocyst stage at 7 days following injection.

Table 1. Fertilization of bovine oocytes at 18-20 h following round spermatid injection

Parameter assessed	Activation time		
	None	3 h before injection	After injection
No. of oocytes[a]			
with successfully injected	33	51	48
with closely apposed two PN (%)	2(6.1)	8(15.7)	3(6.25)
with 2-cell (%)	1(3.0)	5(9.8)	3(6.25)
with two PN and two PB (%)	0(0)	7(13.7)	7(14.6)
with three PN + one PB (%)	0(0)	8(10.2)	12(25)
with metaphase II (%)	19(57.6)	10(19.6)	10(20.8)
with others[b] (%)	11(33.3)	12(23.5)	13(27.1)

[a]Abbreveation: PN, pronucleus; polar body, [b]others include with of without unidentified multiple nuclei.

Table 2. Fertilization of bovine oocytes at 18-20 h following round spermatid injection and round spermatid nucleus injection

Parameer assessed	ROSI	ROSNI
No. of oocytes[a]		
with successfully injected	54	51
with closely apposed two PN (%)	5(9.3)	3(5.9)
with 2-cell (%)	6(11.1)	4(7.8)
with two PN and two PB (%)	10(18.5)	12(23.5)
with three PN + one PB (%)	9(16.7)	9(17.6)
with metaphase II (%)	11(20.4)	13(25.5)
with others[b] (%)	12(22.2)	10(19.6)

[a]Abbreveation: PN, pronucleus; polar body, [b]others include with or without unidentified multiple nuclei.

Table 3. Persistence of MitoTracker-stained spermatid mitochondria in the bovine oocyts following injection

Stage	Time	No. of oocytes examined	Intensity of Green Fluorence			
			4	3	2	1
1-cell (%)	10-12 h	26	20(76.9)	4(15.4)	2(7.7)	0(3.8)
	20-22 h	21	10(47.6)	6(28.6)	4(19.0)	1(4.8)
2-cell (%)		6	0(0)	0(0)	4(66.7)	2(33.3)
4-cell (%)		5	0(0)	0(0)	2(40)	3(60)

Table 4. In vitro development of bovine oocytes following a spermatid injection

Injection	No (%). of oocytes			
	examination	cleavage	morula	Blastocyst
sham	52	5(9.6)	0(0)	0(0)
spermatid	79	25(31.6)	11(13.9)	2(2.5)

DISCUSSION

Present study demonstrated that additional activation procedure is required for the successful fertilization with round spermatid. This result extended to the cattle the requirement of activation procedure following round spermatid injection in mice (Kimura et al., 1995), humans (Fishel et al., 1997), rabbit (Sofikitis et al., 1996) and pig (Kim et al., 1998). Previous studies indicated that the oocyte activation factors, probably oscillin, is a 33 kDa protein residing in the equatorial segment region of the acrosome (Parrington et al., 1996). In the mouse, pig OAF appears (or becomes active) in spermiogenesis and is located in the perinuclear material of sperm head (Kimura et al., 1998).

Chemical stimulation at 3 h before injection enhanced the incidence of fertilization of oocytes following round spermatid injection. This suggests that the timing of spermatid injection and oocyte activation is important for the normal development of pronuclei and for the formation of syngamy. While the injected spermatid is in G1, the unfertilized oocytes at metaphase II is M phase. Unlike the sperm DNA, the spermatid DNA is not protected against an immediate action of oocyte cytoplasmic factors by the association with protamines. When spermatids are injected into oocytes, metaphase promoting factor (MPF, Masui et al., 1971), which maintains the oocyte chromosomes in the metaphase of the second meiotic division, may also drive the spermatid nuclei to condense. Fishel et al (1997) suggested that the problem of cell cycle imbalance between the spermatid and the metaphase II oocyte can be avoid by artificially activating the oocytes several hours before injection. Therefore, synchronization of cell cycle following spermatid injection by activating several hours before injection would enhance the incidence of fertilization and subsequent embryonic developments in bovine oocytes.

The importance and role of cytoplasmic components of the spermatid during

fertilization are poorly understood. Since the spermatid has just completed meiosis and contains a complete haploid set of the chromosome in it's nucleus, the nucleus could mingle with female chromatin and develop further to offspring. In the mouse injection of spermatid nuclei resulted in 77 % fertilization while injection of undenuded nuclei resulted in the 14% fertilization (Kimura et al., 1995). This suggested that some cytoplasmic components, which persist some time after injection, inhibit male pronuclear formation.

Concerns have been raised towards the fertilization processes with spermatid or spermatid nucleus. Unlike mouse and hamster, most animals including pig and human showed paternal inheritance of centrosome/centrioles during fertilization (Kim et al., 1996[a,b]; Schatten et al., 1994). Recently we reported successful fertilization in porcine oocytes following injecting either isolated sperm head or spermatid (Kim et al., 1998; Lee et al., 1998). The functional microtubules for male and female pronuclear movements were organized from the oocyte cytoplasm during fertilization with spermatid or isolated sperm head (Kim et al., 1998; Lee et al., 1998). Fertilization with spermatid nucleus confirmed our previous findings that fertilization processes can be occurred in the absence of paternal derived centrosome in the pig.

Our results demonstrated that spermatid mitochondria are, if anything, less viable in the oocyte and embryo than those of mature spermatozoa. This would therefore seem to alleviate the concerns that the microinjection of immature germ cells in the treatment of human male infertility might lead to mitochondria disease in offspring (Cummins, 1997; Houshmand et al., 1997). While it is generally assumed that mitochondrial segregation is random at cell division and that replication occurs independently of the cell cycle, in fact there is a complex species-specific and even cell-specific system of communication between the mitochondrial and nuclear genomes (Poyton & McEwen, 1996). It has been known for some years that mitochondrial replication in hybrid cell lines is strongly influenced by the nuclear genome (De Francesco et al., 1980), and mitochondrial genomes in such heteroplasmic situations can behave in either a co-dominant or a dominant/recessive mode (Hayashi et al., 1987). Jenuth et al. (1997) found strong tissue-specific and age-related selection for defferent mitochondrial genotypes in heteroplasmic mice created by embryo-cytoplast fusion. This again highlights the complex nature of nucleo-cytoplasmic interactions for mitochondrial segregation and tissue energetics.

In summary, our study shows successful fertilization and in vitro development of bovine oocytes following injection of round spermatid. Incidences of fertilization in bovine oocytes were not different thoses following injection round spermatid or round spermatid nucleus. Fertilization processes was similar with those observed previously in porcine and mouse oocytes.

SUMMARY

In this study we determined fertilization processes and developmental ability of porcine oocytes following injection of round spermatid in the presence of artificial activation. Electrical stimulation at 3 h before spermatid injection significantly increased the incidence of normal fertilization as compared to those following injection without stimulation or with stimulation immediately after injection. The incidences of two pronuclear formation and apposition were not different in oocytes between following intracytoplasmic spermatid and spermatid nucleus injection. Indirect immunocytochemistry and laser scanning confocal microscopy study revealed that microtubules were organized from the oocyte cortex following round spermatid injection, and this seemed to move both male and female pronuclei into the oocyte center. Paternal mitochondria which are introduced with spermatid have been observed up to 4-cell. Our study indicated that either round spermatid or it's nucleus can be used to produce viable bovine embryos by injection into unfertilized oocytes.

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Figure 1. **A, B, C, D.** Chromatin, micrutubule and chromosome assembly in bovine oocytes following round spermatid injection. **A, B, C** : Laser scanning confocal microscopy ; green, microtubules ; red, DNA ; yellow, overlaped image of green and red. **D** : Hoechst dye of chromosome. **A**. two male and female nuclei were seen at 12 h following injection. Microtubules were organized from the cortex and seemed to more both pronuclei into the oocyte center. **B**. Two pronuclei were apposed in the oocyte center at 18 h following round spermatid injection. **C**. Two cell division was occurred at 20 h following round spermatid injection. **D**. Chromosome spread in bovine oocytes at 20 h following round spermatid

injection. **E, F.** Mitochondria and chromosome in bovine oocytes following round spermatid injection. green, mitochondria ; red, DNA ; yellow, overlaped image of green and red. **E**. Mitochondria adjacent to male pronuclei. **F**. Mitochondria in bovine syngamy.