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# Microtubule and Chromatin Organization in Bovine Oocytes following Intracytoplasmic Injection of Spermatozoon, Sperm Head and Tail

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# 소 난자에 있어서 세포질내 정자, 정자두부 · 미부 주입 후 미세소관과 염색질의 구조변화

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도정태 · 전수현 · 최종태 · 강영선* · 이보연** · 김승보**
김남형 · 이훈택 · 정길생

## = 국문초록 =

본 연구에서는 체외수정, 난자내 정자 직접주입, 난자내 정자 두부 · 미부 주입 후의 핵과 미세소관의 변화를 관찰하였다. 핵과 미세소관의 움직임은 형광염색을 실시한 후 공초점주사현미경을 이용하여 관찰하였다. 체외수정에서 관찰된 바와 동일하게 정자를 난자에 직접주입 한 직후 정자 중편부에서 성상체가 형성되었고, 이 성상체에 의해 자성 · 웅성 전핵이 융합되는 것으로 관찰되었다. 그러나 난자내 정자를 직접주입하였을 경우 웅성전핵으로 발달하는 비율이 낮았다. 이는 주입된 정자가 원형질막과 perinuclear theca에 싸인 체 난자내로 들어가 난자내의 sperm nucleus decondensing factor와 정자 핵과의 반응이 억제되기 때문으로 생각된다. 정자 두부 만을 주입하였을 경우 성상체가 형성되지 않았지만 자성 · 웅성 전핵 사이 또는 그 주위에서 두터운 미세소관층이 관찰이 되었다. 따라서 소에 있어서는 정자의 중편부에 위치하여 microtubule organizing center (MTOC)의 역할을 하는 중심립 또는 중심체 없이도 모계에서 유래된 미세소관이 형성되어 이것이 전핵의 융합과 세포분열에 관여하는 것으로 생각된다. 정자의 미부 만을 주입하였을 경우 성상체가 형성이 되지 않았으며, 자성핵 사이에 형성된 미세소관과 떨어져서 관찰되었다. 따라서 주입된 정자의 꼬리는 미세소관형성과 관련이 없는 것으로 생각된다. 이러한 결과는 소에 있어서, 수정 시 정자로부터 유래되는 중심립 또는 중심체가 없이도 미세소관을 형성하여 미세소관에 의해 이후의 배발달이 정상적으로 일어남을 보여주고 있다.

## INTRODUCTION

In a relatively short period, intracytoplasmic sperm injection (ICSI) technique has widely been used to overcome male factor infertility. Because direct injection of spermatozoon bypasses several steps during normal fertilization, such as acrosome reaction, sperm incorporation and egg activation procedures, the ICSI has also been used to investigate the early events of fertilization and to determine the roles of various sperm components during fertilization.

Recently, Pinto-Correia *et al.* (1994) de-

monstrated that the sperm midpiece contain the centrosomal material required for sperm aster formation. Because the isolated sperm head lack centriole, the additional insertion of centrosome may be required to successful fertilization with isolated sperm head. However, sperm head injected oocytes were normally developed to the blastocyst in cattle (Goto *et al.*, 1993) and to fetus in mice (Kuretake *et al.*, 1996). Furthermore, Heald *et al.* (1996) reported that the chromatin was sufficient to assemble microtubules and organize bipolar spindle in *in vitro* system. This prompt us to examine the possibility that isolated sperm head alone in the bovine oocytes can nucleate functional microtubules for pronuclear apposition. In the present study we determined microtubule assembly and chromatin configuration in bovine oocytes following intracytoplasmic injection of spermatozoon and isolated sperm head and tail.

## MATERIALS AND METHODS

### 1. Oocyte Collection

Bovine ovaries were obtained from an abattoir. The ovaries were arrived at an average temperature of 37℃, and were washed in warm 0.9% (W/V) saline solution (approximately 37-39℃) supplemented with penicillin G (75 μg/ml) and streptomycin (50 μg/ml). Follicles (2-8 mm in diameter) were aspirated using 10 ml syringe fitted with an 18 gauge needle. The follicular fluid and oocyte mixture were pooled onto the petridishes and the cumulus-oocyte complexes (COCs) with compacted cumulus cells and evenly pigmented cytoplasm were collected.

### 2. *In vitro* Maturation

The collected oocytes were washed three times in Tyrode-Hepes medium (TL Hepes; Parrish *et al.*, 1985) and washed three times in equilibrated 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). The oocytes were cultured in 50 μl drops of TCM-199 under paraffin oil for 24 hrs at 39℃, 5% $CO_2$ in humidified atmosphere.

### 3. Sperm Preparation and *in vitro* Fertilization

*In vitro* fertilization was carried out by a method described by Sirard *et al.* (1988). Matured oocytes were washed twice with Sp-TALP and then with Fert-TALP (Rosenkrans *et al.*, 1993). After washing, five mature oocytes were pulled into 44 μl Fert-TALP droplets under paraffin oil. Bull spermatozoa recovered from frozen-thawed semen were separated on a discontinuous percoll gradient. Separated highly motile spermatozoa were added to a final concentration of $1.0 \times 10^6$ sperm/ml. Then 2 μl of heparin stock solution to induce sperm capacitation and 2 μl of PHE stock solution (2 mM phenicillamine 20 μM hypotaurine and 1 μM epinephrine) to stimulate sperm motility were added into Fert-TALP droplets. The fertilization media were incubated at 39℃ in 5% $CO_2$ in humidified atmosphere.

### 4. ICSI and subsequent culture

Spermatozoa for ICSI were prepared from frozen-thawed semen and treated by swim-up method. The sperm pellet was resuspended to 1 ml with heparin-containing (10 μg/ml) TL-HEPES in a effendorf tube and kept at 39℃ for 30-60 mins to induce capacitation. At 24 hrs after maturation, 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.

The *in vitro* capacitated spermatozoa were di-

luted (approximately 1 : 2) with 10% polyvinylpyrrolidone (PVP, Sigma) in TL-HEPES. The diluted spermatozoa and injection medium were placed nearby on a slide, and the slide was placed in Leitz fixed-stage microscope equipped with Leitz micromanipulators. Holding and injection pipettes were connected to a syringe system filled with fluorinert (Sigma, USA). Five to eight denuded oocytes were placed in the injection medium. Individual spermatozoon was picked up from the sperm drop with the injection pipette and the pipette was moved to the injection drop. Then, the sperm was injected into the ooplasm of an ovum held by a suction-controlled holding pipette.

After injection, the oocytes were transferred to a drop of 50 μM calcium ionophore A23187 (Sigma, USA) in CR1aa for 10 min 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, 1993). 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. Sperm head and tail injection

The sperm head and tail, spermatozoa were prepared from frozen-thawed semen as described in ICSI. In order to isolate sperm head and tail, the spermatozoa were transferred to polystyrene tube (2058, Falcon, USA) containing TL-HEPES and sonicated one min. The isolated sperm head and tail were injected and cultured with the same procedures as described in ICSI.

### 6. Immunolocalization of microtubule and chromatin

At specific time points following *in vitro* fertilization and injection of spermatozoon, sperm head and tail, the oocytes were permeablized in modified Buffer M (Schatten *et al.*, 1985; 25% glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM ethylenediamine- tetraacetic acid, 1 mM β-mercaptoethanol, 50 mM imidazol, pH 6.7, 3% Triton X-100, and 25 μM phenylmethylsulfonyl fluoride) for 15 mins, fixed in methanol at -20℃ for 10 mins, and stored in phosphate buffered saline (PBS) containing 0.02% sodium azide and 0.1% BSA for 2~7 days at 4℃.

Fixed oocytes were transferred to 4-well dish containing monoclonal anti-α-tubulin antibody (Sigma) diluted 1:200 in PBS, and incubated for 90 mins at 39℃. Oocytes were incubated in blocking solution (Albertini *et al.*, 1984; 0.1 M glycine, 1% goat serum, 0.01% Triton X-100, 1% powdered milk, 0.5% BSA, and 0.02% sodium azide) for 30 mins at 39℃. Then oocytes were incubated in fluorescein isothiocyanate (FITC)-labelled anti-mouse-IgG (Sigma) and followed by blocking. Propidium iodide (5 μg/ml) was used for the detection of DNA for by culturing oocytes for 1 hr. Stained oocytes were washed with PBS containing 0.5% Triton-X 100 and 0.5% BSA, and then mounted under a coverslip with antifade mounting medium (Universal mount; Fisher Scientific Co., Pittsbrugh. PA) to retard photobleaching. The slides were kept at 4℃ until examination. The immunolocalized oocytes were examined using laser- scanning confocal microscope (MRC-1024; Bio-Rad Corporation, Boston, MA). The images were recorded digitally and archived on an erasable magnetic optical disk.

## RESULTS

### 1. Microtubule and chromatin configurations following *in vitro* fertilization and intracytoplasmic sperm injection

Oocytes were observed at 8~30 hrs post-IVF and 12~24 hrs post-ICSI to examine the microtubule and chromatin configurations during IVF and ICSI, and the sperm chromatin

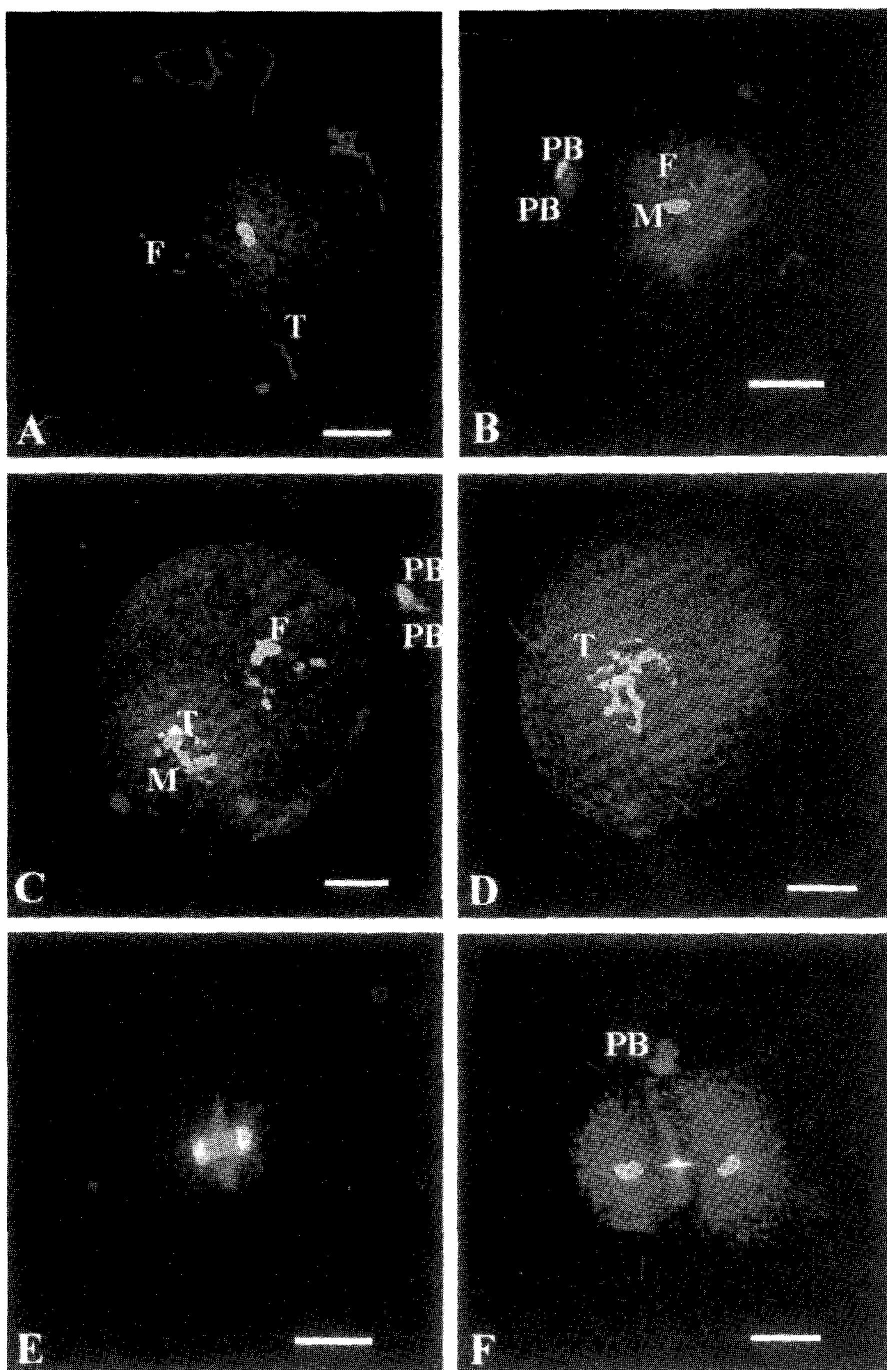


**Fig. 1.** Laser scanning confocal microscopic images of microtubules and chromatins in bovine oocytes following *in vitro* fertilization. F, female chromatin; M, male chromatin, or sperm head; T, sperm tail; PB, polar body. Bar = 25 μm. **A.** Sperm aster formed from the midpiece of inseminated sperm. **B.** Sperm aster dispersed from sperm neck. **C.** Dense microtubule network concentrated to male pronucleus. **D.** Astral microtubules dispersed to fill the cytoplasm at apposition stage. **E.** Anaphase stage. **F.** Cytokinesis is under way.

decondensation during ICSI. After sperm penetration, sperm aster was seen in the incorporated sperm midpiece (Fig. 1A). During sperm decondensation, the aster became larger and filled

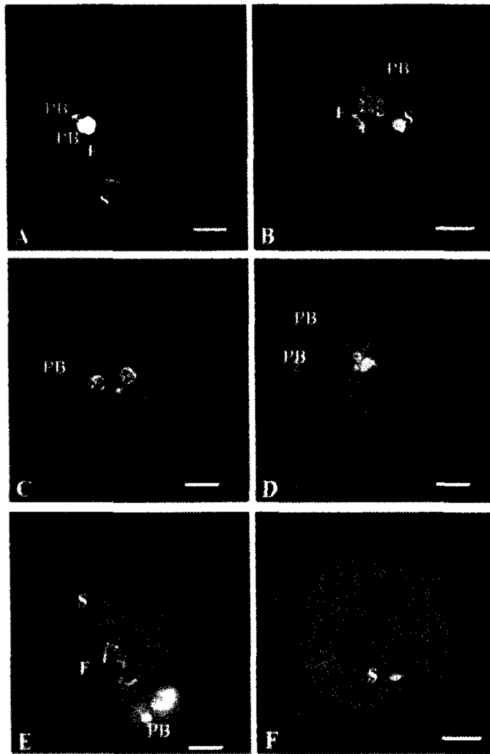


**Fig. 2.** Laser scanning confocal microscopic images of microtubules and chromatins in bovine oocytes following intracytoplasmic sperm injection. Green, microtubules; red, chromatin; yellow overalpping image of microtubule and chromatin; F, female chromatin; S, spermatozoon; PB, polar body. Bar = 25 μm. **A.** Sperm aster was formed around the injected sperm. **B.** Decondensed sperm head and enlarged microtubules. **C.** Male and female PN apposition stage. **D.** Bipolar spindle initiation. **E.** Sperm chromatin was not decondensed and sperm aster was not observed. **F.** Ruptured Ooplasm during Injection procedure.

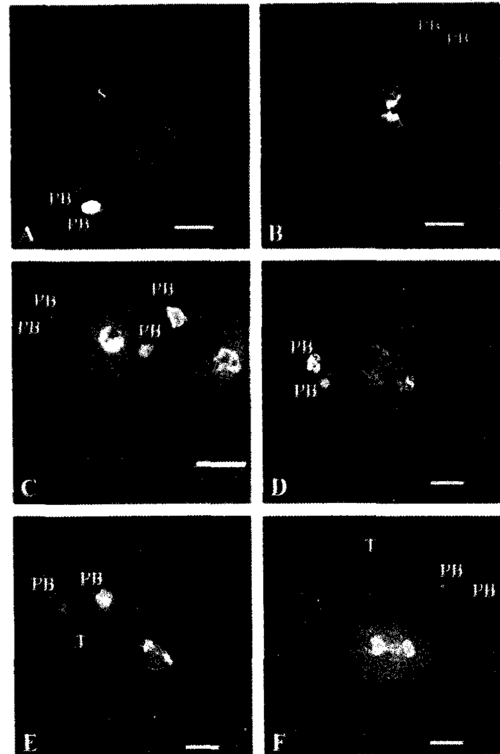


**Fig. 3.** Laser scanning confocal microscopic images of microtubules and chromatins in bovine oocytes following intracytoplasmic sperm head injection (**A-D**) and intracytoplasmic sperm tail injection (**E** and **F**). Green, microtubules; red, chromatin; yellow, overalpping image of microtubule and chromatin; F, female chromatin; S, spermatozoon; PB, polar body. Bar = 25 μm. **A.** Arrested oocyte at pronuclei apposition stage. Microtubules located beneath the oolemma. **B.** Microtubules organized between male and female pronuclei. **C.** Microtubules concentrated to two pronuclei (left and right). **D.** Parthenogenetic development. Injected sperm head was silent. **E, F.** Sperm tail was observed apart from the spindle.

the cytoplasm (Fig. 1B and C). Two pronuclei seemed to be apposed to the center of oocyte by the motor of microtubules. At the time of pronuclear apposition, astral microtubules filled the whole cytoplasm (Fig. 1D). At the time of syngamy, the microtubules were less detectable in the cytoplasm. Subsequently the embryo went through mitotic metaphase, anaphase (Fig. 1E) and telophase. At the end of the first cell cycle, the embryo underwent cytokinesis (Fig. 1F). These results indicate that microtubules are associated with the male derived centrosome and act as a functional spindle which is involved in the male and female pronuclei movement.

Following ICSI, microtubular aster was organized, which emanated from the sperm midpiece (Fig. 2A and B). And pronuclei were apposed at the center of the oocytes filled with microtubules throughout the cytoplams (Fig. 2C). Subsequently microtubules were concentrated to the apposed pronuclei and less detectable in the cytoplasm, where the bipolar

**Table 1.** Sperm Chromatin Configurations in bovine oocytes at 24 hr after intracytoplasmic sperm injection

| Examinations | No. of oocytes | (%) |
|---|---|---|
| Successfully injected | 35 | |
| With condensed sperm head[a] | 21 | (60.0) |
| With decondensed sperm head | 9 | (25.7) |
| Cleaved | 2 | ( 5.7) |
| Others | 3 | ( 8.6) |

[a] sperm head not decondensed.

spindle was organized (Fig. 2D). In many oocytes, microinjected sperm was not decondensed and did not organize sperm aster (Fig. 2E and Table 1). In this abnormal pattern, oocyte seemed to be activated by 50 μM calcium ionophore A23187 because second polar body was extruded and female pronucleus was formed. It might be a sperm nucleus decondensing factor (SNDF) that affect these abnormality. When injection was failed, ooplasm seep to the perivitellin space and sperm was not decondensed (Fig. 2F). Although in ICSI, male chromatin decondensation rate was very low, microtubule and chromatin configurations (Fig. 2, A-D) were similar to those in IVF.

## 2. Microtubule and chromatin configurations following injection of sperm head and tail

Oocytes were examined to figure out microtubule and chromatin configuration at 12 and 24 hrs after injection of sperm head and tail and the results were summarized in Table 2 and 3. Intracytoplasmic sperm head injection did not organize microtubular aster (Fig. 3, A-D, Table 2). Unexpectedly, following sperm tail injection sperm aster was not organized around the tail (Fig. 3E and F, Table 3). Abnormal microtubul configuration after sperm head injection was shown in Fig. 3A. The oocyte was arrested at pronuclear stage, forming female and male pronuclei (decondensed sperm chromatin). Since microtubules were distributed in the cortex region, this abnormal pattern may be due to centrosomal defect. Thus, it suggest that mitosis by the motor of microtubules could not occur. In normal cases, dense network of microtubules was organized between male and female pronuclei (Fig. 3B) or concentrated around both two pronuclei (Fig. 3C). Parthenogenetic development after sperm head injection was shown in Fig. 3D. The oocyte contained sperm chromatin not involved in syngamy after sperm head injection.

**Table 2.** Chromatin Configurations in bovine oocytes following injection of sperm head

| Examinations | No. (%) of oocytes | |
|---|---|---|
| | 12 h post -injection | 24 h post -injection |
| successfully injected | 22 | 18 |
| 2PB + fPN + decondensed sperm head | 4 (18.2) | 8 (44.4) |
| 2PB + fPN + condensed sperm head | 11 (50.0) | 2 (11.1) |
| 1PB + fPN + decondensed sperm head | 2 ( 9.1) | 1 ( 5.6) |
| 1PB + fPN + condensed sperm head | 2 ( 9.1) | 1 ( 5.6) |
| Forming sperm aster | 0 ( 0.0) | 0 ( 0.0) |
| In mitotic metaphase to four cell | 0 ( 0.0) | 5 (27.7) |
| Others | 3 (13.6) | 1 ( 5.6) |

PB: polar body, fPN: female pronucleus.

**Table 3.** Chromatin Configurations in bovine oocytes following injection of sperm tail

| Examinations | No. (%) of oocytes | |
|---|---|---|
| | 12 h post -injection | 24 h post -injection |
| Tail observed | 8 | 10 |
| 2PB + female pronucleus | 6 (75.0) | 4 (40) |
| 2PB + the third meiotic metaphase[a] | 0 ( 0.0) | 1 (10) |
| 2PB + 2 pronuclei | 0 ( 0.0) | 3 (30) |
| 1PB + 2 pronuclei | 1 (12.5) | 1 (10) |
| Forming aster | 0 ( 0.0) | 0 ( 0) |
| Others | 1 (12.5) | 1 (10) |

PB: polar body
[a] Metaphase plate was formed again after meiotic metaphase II was compleated to form second polar body.

Female pronucleus was parthenogenetically divided and microtubules were spaned in the entire ooplasm. Table 2 shows the chromatin configuration in bovine oocytes following injection of sperm head and indicating that the incidence of sperm chromatin decondensation was higher than that of ICSI. When sperm tail was injected into oocytes, the oocyte was parthenogenetically developed (Fig. 3, E and F). Injected sperm tail was located apart from spindle and female chromatin.

## DISCUSSION

In the present study, we demonstrated the configurations of microtubule and chromatin during the first cell cycle following IVF, ICSI and injection of sperm head and tail. Microtubule and chromatin configurations of bovine oocyte fertilized by ICSI were similar to those obtained with *in vitro* fertilization of oocytes. Following ICSI and IVF, sperm aster was formed from male derived centrosome. The functional aster appeared to move the male and female pronuclei. When the isolated sperm head was injected into oocytes, microtubular aster was not formed. Instead, dense network of microtubules was organized around male and female pronuclei.

Microtubule and chromatin configurations of *in vitro* fertilized bovine oocytes have been reported by Navara *et al.* (1994). They demonstrated that sperm contributed the centrosome during fertilization. In this study, we also examined that male derived centrosome nucleated microtubule and form sperm aster from incorporated sperm. The microtubular aster moved both pronuclei into the center of oocyte and develops into bipolar spindles. As cytokinesis took place, the aster were filled in the cytoplasm and arrayed with each pole of the mitotic spindle serving as the centrosome for the daughter blastomeres.

During ICSI, microtubular aster was formed and enlarged from a spermatozoon as seen during normal fertilization. But the rate of male pronuclear formation in bovine oocytes fertilized by ICSI was lower than that observed in IVF (Table 1). In ICSI, because of absence of fusion procedure, the injected spermatozoon was different from the normally fertilized spermatozoa. The plasma membrane of injected spermatozoon was intact, in contrast *in vitro* fertilized spermatozoon was fused into ooplasm and the membrane cannot be incorporated into ooplasm (Yanagimachi *et al.*, 1994). Therefore, the sperm plasma membrane damaged by the process of immobilization may induce SNDF in ooplasm to reach and decondense the sperm nucleus (Dozortsev, 1996). In bovine ICSI, the oocytes are artificially activated to stimulate post-inseminational procedures; nevertheless, activation rates is very low (Goto *et al.* 1993, 1996). In our observation, many oocytes following ICSI extruded the second polar body but few of them formed male pronucleus (Fig. 2E). It seems that our activation method may not be sufficient to activate SNDF or sperm plasma membrane, even if it was broken by immobilization, retard the interaction between activated SNDF and sperm nucleus. Sperm nucleus decondensation rate following injection of isolated sperm head was higher than that following ICSI (Table 1, 2). Since the isolated sperm head could contact with ooplasmic factor more easily than immobilized sperm, sperm nucleus decondensation caused by SNDF could occur more easily in sperm head injection. Recently, Sutovsky *et al.* Suggested that perinuclear theca might prevent the access of cytoplasmic factors to the sperm DNA and the block the decondensation of the sperm nucleus.

Goto (1993) showed that bovine oocytes can develop normally to the blastocyst stage after being fertilized by the injection of sperm head. Kuretake *et al.* (1996) reported sperm-head-injection derived offspring in the mouse. Therefore, tail components may not be essential for normal embryo development at least in the mouse. Furthermore, recent work shows that

DNA is sufficient to nucleate microtubules (Heald *et al.*, 1996). Plasmid DNA-coated bead, contain no MTOCs and kinetochores, nucleate microtubules and form bipolar spindle in Xenopus egg extracts. And once spindle is formed, a cell absence of chromosomes can develop to anaphase and cytokinesis (Zhang *et al.*, 1996). Thus, they suggested that stage-specific changes occur at an appropriate time, despite the absence of chromosomes. In this study, we observed microtubule and chromatin configuration in bovine oocytes after injection of sperm head. Following sperm head injection, sperm aster was not formed from sperm head, but we observed microtubule dense network around male and female pronuclei. These results suggest that male derived centrosome is not essential to nucleate microtubular spindle in bovine oocytes.

During bovine fertilization, sperm DNA and sperm centrioles were transformed but sperm tail (principal piece, the fibrous sheath) was destructed in the ooplasm at the third mitotic cycle (Sutovsky *et al.*, 1996). Pinto-Correia *et al.* (1994) suggested that sperm midpiece injected into ooplasm form aster in rabbit oocytes. However our observation is not consistent with the result from Ponto-Correia *et al.* (1994). In the present study microtubular aster or microtubule network was not formed around isolated tail. We might miss the critical time point for forming aster, but at least the aster from tail could not be a functional aster because sperm tail was found apart from the spindle and chromatin.

In summary, the present study and our data shows that microtubule configuration after ICSI is not different from that during normal fertilization, but different from intracytoplasmic sperm head injection (ICSHI). Sperm aster was not formed, but dense microtubule network was seen between female PN and decondensed sperm head. In conclusion, one obvious fact from this study is that male derived centrosomes are not essential for microtubule nucleation in bovine oocytes. Further studies are required to determine the roles of sperm centrioles/centrosomes and sperm tail in mammal.

## SUMMARY

The objective of this study was to determine the microtubule assembly and chromatin configuration during the first cell cycle in bovine oocytes following injection of spermatozoon, sperm head and tail. The microtubule and chromatin configuration was imaged with fluorescent labeled monoclonal α-tubulin antibody and propidium iodide under laser scanning confocal microscope. Microtubule and chromatin dynamics in bovine oocytes following intracytoplasmic sperm injection (ICSI) were not different from those observed during *in vitro* fertilization (IVF). Following ICSI, the microtubular aster was observed around sperm midpiece. During pronuclear formation, the sperm aster was enlarged and seen around male and female pronuclei. At mitotic metaphase, the microtubular spindle assemble astral poles and chromosomes were aligned on the spindle equator. At mitosis, asters were concentrated to each spindle pole and they filled the cytoplasm. After injection of the isolated sperm head, the microtubular aster was not seen around sperm head in any cases (0/18). Instead, microtubules were organized from the cytoplasm, which filled the whole cytoplasm during pronuclear apposition. These microtubules seem to move male and female pronuclei. These results suggest that isolated sperm head can develop into normal pronucleus in mature bovine oocytes, and competent to participate syngamy with the ootid chromatin. The functional microtubules following isolated sperm head injection in bovine oocytes appeared to be organized solely from maternal stores.

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