韓國家畜繁殖學會誌 23(4) : 359~364(1999)
Korean J. Animal Reprod.

Recent Advances in Intracytoplasmic Sperm Injection of Mammalian Oocytes

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Ⅰ. INTRODUCTION

Recent advances in intracytoplasmic sperm (ICSI) and round spermatid injection (ROSI) would provide exciting opportunities not only for the male infertility but also for studying gamete physiology during fertilization and early development. Furthermore, intracytoplasmic sperm injection could be used to produce transgenic animals (Perry et al., 1999). However, it is not clear in the fertilization processes in mammalian oocytes following intracytoplasmic injection of spermatozoon, isolated sperm head or round spermatid. During fertilization, sperm introduced paternal genetic materials as well as various epigenetic factors such as, sperm born activation factors, centrosome, mitochondria and male specific imprinted genes. However, little information is available on this subject for any species other than the mouse. Recently, I have investigated fertilization processes and early *in vitro* development of poricne oocytes following injection of a spemratozoon, various sperm components, round spermatid, its nucleus and various foreign species spermatozoa. The result obtained could provide insight into strategies for the enhancement of assisted reproductive technology as well as of techniques for the production of transgenic animals.

Ⅱ. EPIGENETIC FACTORS

1. Activation Factors

During fertilization the sperm cell activates oocytes by releasing an oocyte activating factor(s) (OAF) into oocytes. Parrington (1996) reported that OAF is a 33 kDa protein residing in the equatorial segment region of the acrosome. Intracytoplasmic sperm injection (ICSI) has become a routine clinic procedure for the treatment of male factor infertility. Because injection of a single spermatozoon into an oocyte bypasses contact and fusion between the plasma membrane of both gametes, concerns have been raised how the oocytes can be activated following ICSI. It has been known that, in the human, direct injection of spermatozoa induces an oscillatory pattern of Ca^{2+} rise by introduction of sperm born oocyte activating factors (OAF, probably oscillin, Tesarik et al., 1994). In the mouse, the OAF appears to be a 33 kDa protein residing in the equatorial segment region of the acrosome (Parrington et al., 1996). It has also been known that in the mouse OAF appears (or becomes active) at spermiogenesis and is located in the perinuclear material (Kimura et al., 1998). Activation process is essential for the fertilization procedures such as second meiosis and cortical granule reaction (Kim et al., 1997c&d). Because injection of foreign species spermatozoa, such as hamster, rabbit, pig, human or sea

urchin into mouse oocytes activates oocytes, OAF is not strictly species specific for the mouse oocyte (Wakayama et al., 1997; Kimura et al., 1998). We determined activation of pig oocytes follwoing injection of a spermatozoon, various sperm components, round spermatid or foreign species spermatozoa.

2. Centrosome

In most animal, the penetrating sperm introduces the centrosome, which organize an aster of microtubules called sperm aster. The sperm aster appeared to be involved in the process of pronuclear movement and mitosis. Microtubule mediated events in pig oocytes have been studied during fertilization and parthenogenesis (Kim et al., 1996a&b; 1997). Following sperm penetration the microtubular aster was organized in the sperm neck area in combination with maternal centrosomal material, which moves male and female chromatin toward the center of oocytes. After electrical activation, in contrast, cytoplasmic centrosomal material is activated and organizes a network of microtubules which moves pronuclei to the center of eggs.

3. Paternal Mitochondria

Mitochondria are semi-autonomous organelles found in all eukarytic cells. Mitochondria have a prfound role to play in mammalian tissue bioenegetics in growth, in aging and in apoptosis, and yet they descend from an asexually reproducing independent life form. Recently, Cummins et al. (1997, 1998) demonstrated fate of microinjected spermatid and sperm mitochondria in the mouse oocyte and embryos. Mouse sperm or spermatid mitochondria following ICSI or ROSI, disappeared during the 4 to 8 cell transcription. The paternal mitochondria appear to be specifically targeted for destruction, as other thiol-rich sperm components such as the perforatorium and axonemal coarse fibre persist to the blastocyst stage and possibly later. The mechanism by which the mitochondria of one parent (usually paternal but occasionally maternal) are eliminated are illusive at present.

Recently I traced the fate of male derived mitochondria in porcine oocytes following injection of pig spermatozoon or spermatid labelled with MitoTracker into pig oocytes (unpublished data). As shown in the mouse oocytes, either sperm or spermatid mitochondria disappreared from the early 2 to 4-cell stage embryos following ICSI and ROSI. These results suggested that either sperm or round spermatid injection for infertile men will not pose a significant risk to offspring by transmitting abnormal mitochondria genomes.

4. Male Genomic Imprinting

The paternal sperm specific mark (imprint) on the sperm derived alleles of certain genes is implied in transcription control and is required for normal development. Differential expression paternal and maternal alleles of implinted genes is related to differential DNA methylation patterns in the genes and their promotor. Ariel et al. (1994) examined the methylation of DNA during mouse spermatogenesis. All the gene examined are apparently unmethylated in the spermatogenic cells in the testes, but were remethylated in immature spems. Although genomic implinting of gametes occurs sometime duirng gametogenesis the exact time when it occurs remains unknown. The injection of mature oocyte with spermatogenic cells at various stages of differentiation would be a means of determining when implinting of male germ cells is completed. Sucessful fertilization and *in vitro* development of porcine oocytes follwoing injection of round spermatid suggested that genomic implinting were occured at least early than round spermatid

stage in the pig (Lee et al., 1997).

Ⅲ. RESULT AND DISCUSSION

1. A Spermatozoon and Various Sperm Components Injection

When a spermatozoon or Triton X-100 treated porcine sperm head was injected into porcine oocytes, the oocyte was activated, whereas the sperm tail did not induce activation. Injection of either a trypsin treated or NaOH treated sperm head failed to induce activation. A male pronucleus was formed in the activated oocytes following injection of Triton X-100 treated sperm head. Neither a trypsin nor NaOH treated sperm head was decondensed. Transmission electron microscopy was used to observe sagittal sections of isolated sperm heads after sonication in the presence of triton X-100 or trypsin. While Triton X 100 treatment left perinuclear material around the nucleus, trypsin removed perinuclear material extensively. Therefore, like in the mouse (Kimura et al., 1998), in the pig some substance in perinuclear material, which is firmly attached to the sperm plasma membrane, may activate porcine oocytes during fertilization or following ICSI.

Following ICSI, the sperm aster was organized from the neck of spermatozoon, and filled the whole cytoplasm. In contrast, the sperm aster was not organized following isolated sperm head injection. Instead, microtubules were organized from the oocyte cortex and then filled the whole cytoplasm in all cases in normally fertilized oocytes (n=35). This organization is similar to what has been shown previously in the parthenogenetically activated oocytes (Kim et al., 1996 b&c, 1997a,b) or in the oocytes following round spermatid injection (Lee et al., 1998).

At 20 to 24 h after spermatozoon or isolated sperm head injection, the incidence of pronuclear apposition, mitosis and two cell division was considered as normal fertilized. Following ICSI and head injection, 49% and 43% were normally fertilized, respectively. During pronuclear movement the sperm aster filled the whole cytoplasm following ICSI, suggesting their role for the pronuclear apposition. In contrast, following sperm head injection, microtubules organized from maternal sources filled the whole cytoplasm, which seems to move male and female chromatin. After pronuclear apposition the microtubules were less detectable in the cytoplasm in the oocytes following ICSI or isolated sperm head injection (data are not shown). During mitotic prometaphase, microtubules were detected mainly in the condensed chromatin mass following ICSI or following isolated sperm head injection. At mitotic metaphase, microtubules were concentrated around chromatin as shown previously during fertilization (Kim et al., 1996b). At anaphase, asters were assembled at each spindle pole, and then large asters were seen, illustrating the role of microtubules in mitosis. In some oocytes (6/21, 29%) injected isolated tails organized aster.

2. Injection of Pig, Cattle, Mouse or Human Spermatozoa

Most oocytes were activated at 10 to 12 h following injection of sperm cell regardless of electrical stimulation. Our study also showed that intracytoplasmic sperm injection of foreign species such as bovine, mouse or human activated porcine oocytes. Some oocytes (23%) were activated in the oocytes following sham injection, probably due to parthenogenetic stimulation. The incidence of activation and pronuclear formation was not different in oocytes following injection of porcine, bovine, mouse or human spermatozoa (Table 2) Previous results showed that the mouse oocytes are readily

activated by injection of hamster, human, rabbit, pig or sea urchin spermatozoa (Rybouchkin et al., 1995; Wakayama et al., 1997, Kimura et al., 1998). Collectively, the substance causing oocyte activation seems not to be species specific for the mouse or porcine oocyte.

Pronuclear apposition was observed in all oocytes following injection of porcine, bovine, mouse or human sperm. Following porcine sperm injection, the microtubular aster was organized from the neck of spermatozoon, and filled the whole cytoplasm as shown earlier. In contrast, following bovine, mouse or human spermatozoa injection, the sperm aster was not seen in porcine oocytes, but decondensed male chromatin or male pronucleus was presented at 9 to 12 h following injection of bovine (n=19), mouse (n=18) and human (n=15) spermatozoa regardless of electrical stimulation. Instead, maternal derived microtubules were organized from the cortex to the center of all oocytes, which have male and female pronuclei.

3. Round Spermatid or Round Spermatid Nucleus Injection

Activation was determined as either second polar body extrusion or pronuclear formation. The spermatid injection alone did not induce activation of oocytes as compared with sham injection. The oocytes with two large pronuclei and two polar bodies (2PN + 2PB) were classified as normal fertilization at 9 to 12 h following round spermatid injection. Electrical stimulation at 2 h before spermatid injection significantly enhanced the incidence of normal fertilization as compared to those following injection with no stimulation or with stimulation immediately after injection. Table 2 showed pronuclear formation and apposition in porcine oocytes following injection of round spermatid and spermatid nucleus. The incidences of two pronuclear formation and apposition were not different between following intracytoplasmic spermatid and spermatid nucleus.

At 6 h following round spermatid injection, the microtubules were organized from the oocyte cortex and then filled whole oocyte cytoplasm in all case of normally fertilized oocytes (Lee et al., 1998). This organization is similar to what has been shown previously in the parthenogenetically activated oocytes. In non-activated oocytes, meiotic spindle was organized around male condensed chromatin. In some case (2/6), the small microtubular aster was seen around male chromatin. However, it did not enlarge nor filled the whole cytoplasm. Instead, a dense network of micortubules moved both pronuclei the center of the oocytes. During pronuclear movement, the materal derived microtubules filled whole cytoplasm, which seems to move both male and female pronuclei. After pronuclei movement, the microtubules are less detectable in the cytoplasm.

The mechanism whereby the maternal derived microtubules organize and move pronuclei to the center of oocytes during parthenogenesis (Navara et al., 1994; Kim et al., 1996 b&c; 1997c, 1999b), following spermatid (Lee et al., 1998) or isolated sperm head injection (1999a,b) is elusive at present. Heald et al. (1996) developed an *in vitro* system in which beads coated with artificial chromosomes in Xenopus eggs. In the absence of centrosome, chromatin beads induce the assembly of functional microtubules in interphase, competent to transport nuclear substrates and replicate DNA. More recently Rodionov and Borisy (1997) observed formation of the radial microtubule array in the fish melanophore cells in the absence of centrosome. Their result suggested that self organization mechanism for microtubule assembly would be presented in the cytoplasm, which possibly arrange chromatin in

proper position during mitosis. Taken together, the cell cytoplasm may have the ability to organize the appropriate microtubules for the chromatin dynamics during pronuclear apposition or mitosis, although it is poorly understood.

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