Epigenetic Factors During Fertilization and Early Development Following Intracytoplasmic Sperm or Round Spermatid Injection in the Pig

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=국문초록=

포유동물의 수정은 정자가 난자내로 침입함으로써 시작되는데 이때 정자는 부계의 유전물질 이외에도 다양한 후성적 요소들 (epigenetic factors), 즉 난활성 인자, 중심체, 부계 유래의 mitochondria 및 부계 특이 삽입 유전자 등을 난자에 전달해 준다. 하지만 수정 및 초기 배발달동안 정자에 의해 전달된 후성적 요소들의 역할과 기능적 발현 및 억제 기전에 관해서는 명확히 알려져 있지 않다. 수정보조기법인 ICSI 및 ROSI의 개발은 남성불임치료에 혁신적인 기술로 자리잡고 있을 뿐만 아니라 포유동물의 수정과정을 이해 하는데 많은 도움을 주고 있다. 본 연구실에서는 최근 몇 년간 돼지난자에 정자, 다양한 정자 구성 요소들, 정낭세포, 및 이종의 정자 등을 미세주입하여 수정을 유도한 후 핵질 및 세포질의 변화과정과 배 발달과정을 살펴 봄으로써, 수정시 정자에 의해 전달되는 후성적 요소들의 기능과 발현 기작을 규명하고자 하였다. 이러한 연구의 결과들은 체외수정, ICSI, ROSI 등의 임상치료기술의 개선에 기초자료로 활용될 수 있으리라 생각한다.

INTRODUCTION

Assessment of normal fertilization and offspring production by intracytoplasmic sperm (ICSI), round spermatid (ROSI) or round spermatid nucleus injection (ROSNI) would provide exciting opportunities not only for the male infertility but also for studying gamete physiology during fertilization and early development. 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. Concerns have been raised on the lack of knowledge from basic scientific researches using animal models with the increase in the successful pregnacy by assisted reproduction techniques. However, little information is available on this subject for any species other than the mouse. Recently, we investigated fertilization processes and early in vitro development of porcine oocytes following injection of a spemratozoon, various sperm components, round spermatid, its nucleus and various foreign species spermatozoa. We have focused to the epigenetic events during fertilization. The result obtained could provide insight into strategies for the enhancement of assisted reproductive technology, such as conventional in vitro fertilization, ICSI, ROSI or ROSNI.

ACTIVATION FACTOR

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.

1. A spermatozoon and various sperm components injection

Table 1 summarizes the incidence of activation and male pronuclear formation between 10 to 12 h following injection of various sperm components. 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

Table 1. Activation and male pronuclear formation in porcine oocytes at 10 to 15 h following various sperm components

Sperm heads treated	No. (%) of oocytes		
	successfully injected (r)	activated	MPN
none	42 (5)	7 (17)	–
spermatozoa	25 (4)	19 (76)	15 (60)
sperm head			
1% triton	46 (5)	41 (89)	29 (63)
0.1% trypsin	44 (5)	10 (23)	6 (14)
100 mM NaOH	35 (4)	3 (9)	0 (0)
sperm tail	19 (3)	2 (11)	–

MPN: male pronucleus

Table 2. Activation and male pronuclear formation in porcine oocytes at 10 to 15 h following mouse, bovine, human and porcine sperm injection

Source of spermatozoa	electrical stimulation	No. (%) of oocytes		
		successfully injected (r)	activated	MPN
none	+	28 (4)	26 (93)	0 (0)
(sham injection)	−	26 (4)	6 (23)	0 (0)
pig	+	55 (6)	53 (96)	34 (62)
	−	50 (6)	42 (84)	29 (58)
cow	+	52 (6)	50 (96)	31 (60)
	−	52 (6)	43 (83)	23 (44)
human	+	54 (6)	48 (89)	25 (46)
	−	59 (6)	39 (66)	31 (53)
mouse	+	50 (6)	49 (98)	36 (72)
	−	51 (6)	37 (73)	29 (57)

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.

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 (Table 2). 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.

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 (Table 3). 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 (Table 4). Electrical stimulation at 2 h before spermatid injection significantly enhanced the incidence of normal fer-

Table 3. Chromatin configuration of porcine oocytes at 9 to 12 h following intracytoplasmic injection of round spermatid

Electrical stimulation	No. of oocytes				
	Injected (r)	Activated	2PN + 2PB	1PN + sPN*	Others
None	95 (10)	25 (26)[a]	5 (20)	15 (60)	5 (20)
2 h before injection	96 (10)	82 (85)[b]	36 (44)	16 (20)	30 (37)
after injection	97 (10)	85 (88)[b]	20 (24)	19 (22)	46 (54)

Table 4. Fertilization of porcine oocytes at 15 to 18 h following intracytoplasmic injection of round spermatid or round spermatid nuclei

Type of cell injected	No. (%) of oocytes				
	Successfully injected (r)	2PN + 2PB	apposed PN + 2PB	1PN + sPN*	Others**
Round spermatid	123 (7)	42 (34)	32 (26)	9 (7)	40 (33)
Round spermatid nuclei	102 (7)	38 (37)	30 (29)	12 (12)	22 (22)

tilization 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.

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.

1. A spermatozoon and sperm head injecton

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)

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, human, mouse spermatozoa into pig oocytes

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 injection

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 t 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; 1997), following spermatid (Lee *et al.*, 1998) or isolated sperm head injection (present study) 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 re-

plicate 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.

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 we 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.

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 differentail 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. Sucesful 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).

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