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Intracytoplasmic Sperm Injection in Mammalian Oocytes

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포유동물난자의 난세포질내 정자직접주입법

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요 약

1960년대에 본격적인 연구가 시도된 난세포질내 정자직접주입법(Intracytoplasmic Sperm Injection ; ICSI)은 1976년에는 Uehara 등에 의한 Hamster의 연구에서 최초로 전핵의 형성에까지 성공하였다. 이후 계속된 연구를 통하여 여러 동물종에서 이 방법에 의한 난자의 수정 및 배발달에 성공하여, 1988년에 토끼, 1991년에 소, 1995년에는 생쥐에서 산자의 생산에 성공하였다. 한편, 사람에 있어서는 1988년 Lanzendorf 등에 의해 최초로 사람난자가 난세포질내 정자직접주입법에 의해 수정에 성공한 것이 보고되었으며, 1992년에는 Palermo 등에 의해 이 방법에 의해 수정된 수정란 이식을 통한 임신 및 성공적인 분만이 보고되었다. 난세포질내 정자직접주입법에 있어서는 정자의 운동성 및 첨체반응 등의 유무가 수정에 관여하는 중요한 요인이 아닌 것으로 알려지고 있으며, 성숙한 정자가 아닌 정소상체(미부-두부)정자 혹은 정소내의 미성숙정자를 사용하여 난세포질내 정자직접주입법을 시행하여도 수정 및 임신이 가능한 것으로 보고되었다. 또한 정자세포 (spematid)나 원형정자세포 (round spermatid)를 사용하여 난세포질내 정자직접주입법을 시행한 결과 Hamster (1993)와 사람 (1995)에서 난자의 수정과 배발달이 관찰되었으며 사람에 있어서는 분만까지 성공하였다. 현재까지 이 난세포질내 정자직접주입법은 학술적으로는 난자-정자가 결합하는 기전을 밝히는 연구에 이용되어 왔으며, 임상적으로는 시험관아기시술에 있어서 정자의 기능, 수 등이 문제가 되어 수정이 어려운 남성불임환자에게 적용하여 좋은 성과를 거두어 왔다. 향후 이 기술은 유전자진단이나 남성불임환자의 처치에 폭넓게 사용될 것으로 기대된다.

I. INTRODUCTION

Mammalian fertilization, whether it takes place within the female reproductive tract or within a laboratory dish (IVF), is comprized of many processes which must follow a specific sequence. The spermatozoon must bind to and path through the zona pellucida, fuse with the oolemma and become incorporated into the cytoplasm of the oocyte.

Gamete micromanipulative techniques have been investigated as a means for abnormal sperm to bypass the oocyte's investments and membranes and for understanding mechanism of fertilization processes. Micromanipulative techniques involve a set of techniques to be specific cases in which the fertilization process *in vitro* is likely to be inhibited. It enables the reproductive scientist to circumvent specific stages in the

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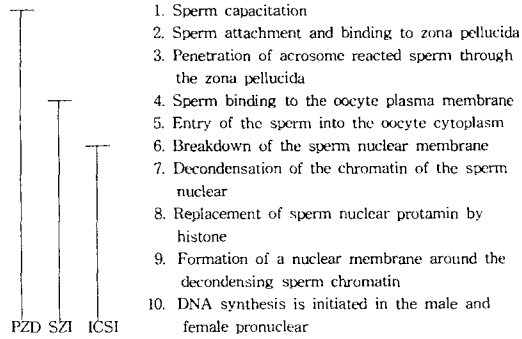

Fig. 1. Fertilization processes and micromanipulative technologies.

fertilization process, without correcting the actual basis for the disorder.

Four classes of microsurgical fertilization techniques, such as partial zona dissection (PZD), zona drilling, sub-zonal sperm insemination (SZI), and intracytoplasmic sperm injection (ICSI), have been explored in several mammalian species. The first two involve the creation of an artificial opening in the zona pellucida in order to allow spermatozoa to interact with the oocyte independant from the zona following insemination, procedures which are originally termed Zona drilling and PZD. A second category of assisted fertilization techniques directed at facilitating sperm-egg interaction is the subzonal placement or insertion of sperm (SZI). SZI completely bypasses the zona, and involves direct placement of the live sperm cell(s) into the perivitelline space. Finally, the third mode of microsurgical fertilization is the direct microinjection of a single sperm into the cytoplasm of the oocyte.

As shown in Fig. 1, It can be possible to divide the fertilization process into 10 steps. In the conventional IVF and PZD, these 10 steps are needed for fertilization. However, SZI procedure skip several steps in fertilization process, and ICSI skip more steps than SZI. Therefore ICSI technique to be applied to specific cases in which the fertilization process *in vitro* is likely to be inhibited. It also enables the reproductive scientist to circumvent specific stages in the fertilization process, without correcting the actual basis for the disorder.

Ⅱ. EARLY STUDIES OF ICSI

An alternative method for IVF oocyte is to microsugically inject a sperm directly into the oocyte. In just theory, one could fertilize a great number of oocytes with a similar number of sperm, and possible increase fertilization rates by bypassing the cumulus mass and zona pellucida without increasing polyspermy. Significant results of ICSI have been documented in various species as summarized in Table 1.

The main purpose of early investigations into sperm injection conducted from 1960's is to investigate the early events of fertilization, such as membrane fusion between homologous and heterologous gametes, activation of the oocyte cytoplasm, and formation of male and female pronuclei.

Lille (1914) injected spermatozoa into starfish oocytes but with inconclusive results. Then, Hiramoto (1962) demonstrated that oocytes inseminated after sperm injection did undergo activation and sperm decondensation with 40 to 50% demonstrating polyspermic cleavage in sea urchin. Because only 10% of control oocytes exhibited polyspermy, Hiramoto reasoned that injected sperm could take part in the mitotic process.

Ⅲ. TECHNICAL ASPECTS OF THE ICSI PROCEDURE

1. Oocyte preparation for ICSI

Table 1. Significant results of ICSI in various species

Researcher		Species	Results
1962	Hiramoto	Sea urchin	No oocyte activation, pronucleus
1966	Lin	Mouse	Unsuccessful
1976	Uehara and Yanagimachi	Hamster oocyte Human sperm	Formation of pronucleus using normal, freeze-thawed, freeze dried sperm
1977 b	Uehara and Yanagimachi	Hamster	pronucleus formation in testicular, Cauda sperm
1980	Thadani	Mouse sperm Rat oocyte	Division(early stage)
1982	Perreault and Zirkin	Hamster	Sperm proteinase was not needed in ICSI for fertilization
1983	Markert	Mouse	Developed to blastocyst stage
1988	Hosoi et al.	Rabbit	Production of offspring
1988	Lanzendorf	Human	Pronucleus formation (6/20)
1989	Keefer	Rabbit	The fertilization rate was influenced by sperm treatment methods
1991	Goto et al.	Bovine	Production of offspring
1991	Yanagida et al.	Rabbit	ICSI with testicular sperm, Pronucleus formation, No pregnancy
1992	Palermo et al.	Human	Production of baby
1993	Ogura and Yanagimachi	Hamster	Embryo development by using round spermatid
1995	Kimura and Yanagimachi	Mouse	Production of offspring by using secondary spermatocyte
1995	Tesarik et al.	Human	Production of baby using round spermatid

The cumulus-corona cells attached to the zona pellucida are initially removed by exposure to PBS medium containing 1mg/ml of hyaluronidase (Type I-S ; Sigma Chemical Co.). Subsequently, −150 μm size of micropipette is pulled by microburner just before use and then removed corona cells attached tightly to zona-pellucida by pipetting. After removal of cumulus-corona cells, the oocytes are returned to the medium containing 10% of FCS until use.

2. Sperm preparation methods for ICSI

1) Preparation of ejaculated and epididymal sperm

Semen are treated by swim-up method or Percoll gradient method according to semen profile for ICSI. Ejaculated semen is washed twice (300×g, 10 minutes) and the pellet is resuspended with 0.5 ml of fresh Ham's F-10. Severe oligo- or oligo-asthenospermia are treated by swim-up method and astheno- or teratospermia are treated by two layer (50, 80%) Percoll centrifugation (250×g, 20min.). In some cases of severe asthenospermia human semen, pentoxiphylline (3.6mM) can be used to accelerate sperm motility.

2) Sperm preparation from testis tissue

Firstly, testicular tissue is cut into small piec-

es with a pair of fine scissors, then the tissue is minced and dispersed by repeated cutting of tissue with surgical blade and the tissue is pipetted with pulled pasteur pipette subsequently. The cell suspension is filtered through a mesh (53 μm) and centrifuged at 200×g for 5 min. The sedimented cells are resuspended in PBS and transferred onto the top layer of discontinuous Percoll solution (2-layer ; 50, 80%). After centrifugation, the bottom layer is washed twice with culture medium.

3. Preparation of PVP for ICSI

To facilitate injection procedure in ICSI, polyvinylpyrrolidone (PVP) solution is used. After 1 g of PVP-360 is dissolved in Milli-Q water and subsequently dialyzed, lyophilized PVP is dissolved in T6 medium containing 0.3% BSA (Fraction V, Sigma) and filtered through an 0.8 m filter (Millipore). The commercial PVP solution is also available. The final concentration of 5~10% of PVP solution is used in injection procedure.

4. Pipette making and setting for ICSI

Different sizes of micropipette are used for micromanipulation according to the characteristics. For the PZD, the size of zona-cutting pipette is 1μm, and the I.D and O.D. of holding pipette are 20μm and 80μm, respectively. The size of sperm injection pipette in SZI is 8μm (I.D.) and 10μm (O.D.) The size of sperm injection pipette in ICSI is 5μm (I.D.) and 7μm (O.D.) To prepare the intracytoplasmic injection pipette the pulled capillary is opened on a microgrinder (Type EG-4, Narishige, Japan) to obtain an proper outer diameter and inner diameter. The microforge is used to make a sharp spike on the injection pipette and to bend the edge of the holding and injection pipettes to an angle of −70°. These pipettes are subsequently bend (−70°) once more apart −2 cm from first bending to facilitate the injection procedure.

The I. D and O. D. of holding pipette for ICSI are 10~20 μm and 80μm, respectively. The holding pipette is cut and fire-polished on a microforge (Alcatel Co., France) to obtain an outer diameter of 80μm and an inner diameter of 10~20μm.

5. ICSI procedures

A single sperm is immobilized by touching it with injection pipette and aspirated tail first into the injection pipette. The petri dish is then moved in order to visualize an oocyte in one of the T_6 droplets surrounding the sperm-PVP droplet. The oocyte is immobilized by negative pressure exerted on the holding pipette. The polar body is held 12 o'clock and injection pipette is injected deeply into the ooplasm at 3 o'clock. A single spermatozoon is released gently to the ooplasm. The injection pipette is withdrawn gently and the injected oocyte is released from the holding pipette. These all steps are repeated until all oocytes are injected. After sperm injection, the oocytes are transferred to the TCM 199 with 10% FCS culture medium and cultured.

Ⅳ. RESULTS OF ICSI IN ANIMAL MODELS

Uehara and Yanagimachi (1976) inject hamster sperm nuclei or whole human sperm into hamster oocytes. The hamster sperm nuclei are homogenized and filtered through tissue filter. Following injection, 68% of these hamster nuclei went on to form a male pronucleus. When human sperm are injected into hamster oocytes, 50% formed a male pronucleus, and when the human sperm had been lyophilized prior to injection, 72% of the injected sperm went on to form male pronuclei. In a later study, Uehara and

Yanagimachi (1977a) determined that even when hamster oocytes injected with sperm are not activated to form a female pronucleus or extrude a second polar body, the injected sperm did decondense but pronuclear formation did not occur. Furthermore, when hamster oocytes are injected with either testicular, caput epididymal or cauda epididymal sperm nuclei, testicular and cauda epididymal sperm nuclei went on to form pronuclei at a rate of 68 and 57%, respectively. Whereas very few caput (epididymal) sperm went on to form pronuclei (2%). Because testicular sperm nuclei are the least stable, most immotile sperm, the investigators could not explain why pronuclear formation is achieved in these oocytes, but not in oocytes injected with caput sperm. They suggested that hamster caput sperm nuclei may contain a factor that prevents pronuclear formation. This factor may be absent or inactive in testicular spermatozoa, added to or activated in caput nuclei and removed or inactivated in cauda nuclei.

Thadani (1979) demonstrated the importance of oocyte maturation in the process of fertilization. When rat oocytes at the germinal vesicle stage are injected with sperm heads, the sperm heads remained intact until the germinal vesicle broke down. The sperm head then decondensed yet did not form a male pronucleus. When ovulated rat ova are used (Thadani, 1980), uncapacitated mouse sperm formed male pronuclei in 67%, compared to a 73% rate of pronuclear formation when capacitated mouse sperm are used. In a similar study, Markert (1983) suggested that mouse sperm heads or whole sperm gave similar results when injected into mouse oocytes. Injected oocytes are reported to form pronuclei and develop normally to the blastocyst stage. Furthermore, it appears that morphologically defective sperm or apparently dead sperm will form a male pronucleus when injected an oocyte.

A single uncapacitated rabbit sperm is injected into the cytoplasm of a series of superovulated rabbit oocytes. Sixty-three percent of the oocytes survived and forty-six developed to the pronuclear stage. Half of the fertilized zygotes cleaved to 2~4 cell stage 24h after microinjection (Hosoi et al., 1988). However, Keefer (1989) reported that injection of capacitated sperm yielded a significantly higher number of pronuclear eggs than did injection of uncapacitated sperm.

In cattle, microinjection of *in vitro* capacitated bovine spermatozoa into cytoplasm only 2% of the injected oocytes cleaved, but when spermatozoa are injected into oocytes activated by 5 min incubation in calcium ionophore, decondensation of sperm nuclei occurred in 37% of the injected oocytes, and 28% cleaved to the 2~4 cell stage 48h after injection. These results indicate that bovine oocytes are not sufficiently stimulated by the injection procedure itself to complete meiosis, but calcium ionophore caused activation and normal cleavage following fertilization by sperm injection. Goto et al. (1990) matured bovine follicular oocytes in culture, activated them by in the calcium ionophore A 23187 and then a single spermatozoon is injected into their cytoplasm. Cleavage rate to the 6~12 cell stage is 15%, and a calf is born after transfer of a blastocyst derived from ICSI (1991).

V. RESULTS OF ICSI IN HUMAN IVF

Since the human oocytes were fertilized by ICSI technique by Lanzendorf and coworkers (1988), the use of ICSI technique is gradually increased in the human IVF program to treat male factor infertility and idiopathic infertility to induce fertilization. The first ICSI-baby was

Table 2. Clinical Results of ICSI in Several Centers

Center Name	ICSI Results (%)		Reference
	Fertilization rate	Pregnancy rate	
CHA Hospital	1878/2868 (65.5)	146/408 (35.8)	1994. AFS[23]
Brussel group	12091/20171(59.9)	688/1770(38.9)	1994. AFS[39]
Cornel Univ.	1920/2968 (64.7)	148/355 (41.7)	1995. Theriogenology[9]

born in 1992 by Brussel's group. To date, more than 1000 babies were born by this technique. In human IVF, the ICSI technique is applied in the following criteria : 1) very low (or no) fertilization rate in the previous IVF cycle(s) 2) present of very high antisperm antibody 3) in the cases of microepididymal sperm aspiration and testicular sperm extraction 4) <500,000 sperm in the total ejaculate. The main three groups in Asia, Europe and America were compared in Table 2. There was no significant difference in the fertilization rates and pregnancy rates among the three groups. The fertilization and pregnancy rates were comparable with those of convention IVF. Recently, Oum et al. (1995, CHA Hospital) reported high fertilization rate (~75 %) by improvment of techniques and ICSI system. It could be supposed that the fertilization rate could be more improved by the future research.

VI. DEVELOPMENT OF EMBRYOS FERTILIZED BY ICSI

The fertilization is assessed about 16~18hrs after ICSI, the oocytes are observed under the inverted microscope(×200) for the presence of pronuclei and polarbody. Fertilization is considered normal when two clearly distinct pronuclei containing nucleoli are present. The further development *in vitro* of the normally fertilized oocytes was assessed after the ICSI procedure and conventional IVF.

In conventional IVF, 832 human oocytes were inseminated, and 570 (68.5%) oocytes were fertilized. In ICSI, 526 human oocytes were sperm injected, and 319 (60.6%) were successfully fertilized. The pregnancy rate was 21.2% (22/104) in conventional IVF group and 23.8% (20/84) of transferred cases were pregnant in ICSI group. In the cleavage rates (Fig. 2), there was no significant difference between embryos of IVF and ICSI. However, there were statistical differences between two groups in embryo quality. Many good quality embryos were observed in conventional IVF group than ICSI group. Most of the embryos were G2~G4 grade in both group, and the ratio of G2, G3 grade in conventional group were higher than ICSI group ($P<0.05$). It seemed that the ICSI procedure may give some damage to oocytes, and resulted in production of many low grade oocytes. However, there was no difference in pregnancy rate between IVF and ICSI group (21.2% and 23.8%, respectively). Therefore, these results suggest that possible damage from ICSI procedure

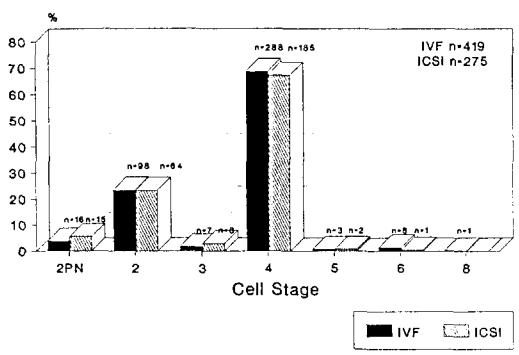

Fig. 2. Comparison of cell stage in ICSI and IVF.

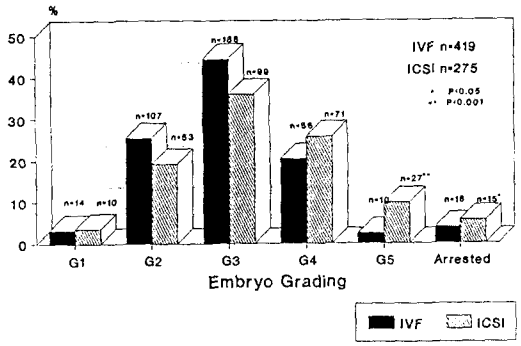

Fig. 3. Comparison of embryo quality in ICSI and IVF.

was not detrimental factor for embryo development and pregnancy (Oum, K. B., 1995).

Ⅶ. EFFECTS OF ANTISPERM ANTIBODIES IN ICSI

Research in animal models has demonstrated that sperm antibodies can interfere with the fertilization process (Russo and Matz, 1974; Tzartos, 1979; Yanagimachi, 1981; Saling et al., 1985 ; Srivastava et al., 1986). The incubation of serum from men that contain antisperm Ab with sperm free of antisperm Ab will decreased sperm survival and motility (Mathur et al., 1988). However, Bronson et al. (1990) reported that antisperm Ab promoted sperm penetration of zona-free hamster eggs also increased the numbers of spermatozoa adherent to the oolemma.

The effects of antisperm antibodies on fertilization was examined in conventional IVF and ICSI. Conventional IVF was performed in four cases with high levels of antisperm antibodies. The ranges of sperm IgA and IgG detected by immuno-bead test were 23~90% and 60~97%, respectively.

No oocytes were fertilized at all in conventional IVF. ICSI was performed in the next trial in the same cases. As results, 10 out of the 27 oocytes were successfully fertilized. This results showed that anti-sperm antibodies (IgA and IgG class) interfere fertilization in conventional IVF, but anti-sperm antibodies did not a detrimental role in ICSI (Oum et al., 1994)

The incidence of antisperm Ab in fertile and infertile men and women is not known but is approximately <2% in serum, sperm, and cervical mucus in fertile men and women with a range of 5 to 25% in serum, sperm and cervical mucus in infertile couples (Kutteh et al., 1992; Bronson and Tung, 1992).

Ⅷ. EFFECT OF SPERM MOTILITY ON THE FERTILIZATION, DAMAGE RATES IN ICSI

The sperm motility is one of most important factor for fertilization in conventional IVF. When the sperm is fused to oocyte membrane, the sperm already lost it's motility. To compare natural fertilization and ICSI, motile (highly progressive with hyperactivation and mild progressive or shaking movement) sperm or artificially immobilized sperm by touching it with micropipette was injected to the oocytes.

The highest fertilization was obtained when immobilized sperm was used (69.3%). In contrast, the rates of damage to oocytes during ICSI increased according to sperm motility. When the sperm with high progressive motility was injected, about half (47%) of the sperm injected oocytes were damaged and finally degenerated by sperm movement. This vigorous sperm movement may affect on micro-organelle of sperm injected oocyte, resulted in oocyte degeneration. Results suggested that sperm must be immobilized before ICSI as natural fertilization process to obtain good fertilization rate and to prevent oocytes damage (Oum et al., 1995).

Ⅸ. DEVELOPMENTAL POTENTIAL OF TESTICULAR SPERMATOZOA, SPERMATOCYTE AND SPERMATID

Testicular spermatozoa was tested the fertilizing capacity by sperm microinjection. Uehara and Yanagimachi (1977b) observed male pronucleus formation after injection of testicular hamster sperm and cauda and caput epididymal spermatozoa. Decondensed nuclei of testicular and cauda epididymal spermatozoa could transform into pronuclei in activated eggs, whereas those of the vast majority of caput epididymal spermatozoa failed to do so at least within 9 hrs. This failure of nuclei of caput epididymal spermatozoa might be due to the presence of a factor in the nuclei which prevents transformation of decondensed sperm nuclei into pronuclei. This factor might be absent or inactive in the nuclei of testicular spermatozoa and removed or inactivated again by another factor while the spermatozoa reside within the cauda epididymis.

Lacham and Trounson (1991) found sperm motility pattern (shaking, progressive) were affect the fertilization rates even the sperm were injected under zona pellucida. Better fertilization were achieved in corpus, cauda epididymal sperm group which had more progressive motility than testicular and caput epididymal spermatozoa. Yanagida et al. (1991) were also found no difference between the fertilization rates in ICSI using testicular intact sperm and sperm head only and early cleavage rates. However, the cleavage rates to 3-cell stage were very low (2~7%), and no pregnancies were achieved. Recently, Devroey and colleagues (1994) reported successful fertilization and cleavage of human oocytes after human testicular sperm using ICSI. The fertilization rates (45%) were comparable to those of ICSI using matured normal sperm.

Male and female gametes both contribute to the genome of the zygote, but their morphology and behavior prior to fertilization are quiet different. Mammalian oocytes are released from the ovary during methaphase of the second meiotic division, which is stimulated to completion by the fertilizing spermatozoon. Male germ cells on the other hand, complete their meiosis long before fertilization, and the youngest haploid stage, the round spermatid, then undergoes a series of profound structural and biochemical modifications in the testis and epididymis to become a functional spermatozoon.

However, since the spermatid nucleus contains a haploid set of chromosomes that are almost certainly imprinted, one might expect zygotes of full developmental potential when such nuclei are introduced directly into the cytoplasm of mature oocytes. Ogura and Yanagimachi (1993) demonstrated the potential capacity of round spermatid as a paternal genetic material instead of mature spermatozoon. One year later, Ogura et al.,(1994) reported the birth of normal young after electrofusion of mouse oocytes with round spermatid. In 1995, Kimura and Yanagimachi reported successful fertilization and production of offspring by using secondary spermatocyte in mouse. In the same year, Tesarik and coworkers (1995) reported successful delivery of babies after transfer of human embryos fertilized by using human round spermatid by ICSI technique. These results indicated that animal and human testicular sperm and round spermatid have developing potential, and also the genomic imprinting of male germ cells that is essential for normal development of embryos is completed by the beginning of spermiogenesis.

Ⅹ. DOES ICSI INCREASE MALFORMATION ?

There have been worries regarding possible increased embryo abnormalities following ICSI. With direct intracytoplasmic injection of sperm,

there is theoretically an even higher risk of chromosomal or genetic abnomalities, because the selection barriers present in natural fertilization process or other micromanipulation techniques are absent in ICSI.

In 1995, Van Steirteghem and collegues reported 1160 pregnancies, including 64 pregnancies after replacement of frozed-thawed embryos. Through prenatal diagnosis by chorionic villus sampling or amniocentesis, a total of 491 karyotypes was determined. Of these, 479 were normal 46XX or 46XY, 6 were benign structure aberrations inherited from one of the parents and 6 or 1.2% were abnormal and 669 children were born. Of these children, eighteen, or 2.7%, major malformations had been observed. This malformation rate is compatible with that of conventional IVF.

It must be noted that sperm may be able to transfer foreign transcripts through *in vitro* fertilization in the mouse (Camaioni et al, 1992). However, there does not seem to be an increase in congenital malformations or genetic diseases as a result of in vitro fertilization. But with ICSI, there is a theoretical risk of transferring foreign transcripts (viral or bacterial) into the egg and therefore, of their becoming incorporated into the embryonic genome (Ng et al., 1993). Based on the above results, the future large scale study is required to prove the safety of ICSI technique.

XI. CONCLUSION

ICSI has been utilized by investigators to study processes occuring during oocytes maturation, sperm decondensation, pronuclear formation and DNA synthesis.

Embryo development following ICSI has been attempted in the mouse, bovine, rabbit, monky, and human. While all models allowed for pronuclear formation and cleavage, until recently, the production of live born offspring was reported in rabbit (1988), cattle (1991), human (1992) and mouse (1995). Live born offspring was also reported by using round spermatid instead of mature sperm by this technique in mouse (1993) and human (1995).

The use of ICSI to study fertilization events has led to many important insights into sperm-egg interaction and early development in mammals. This technique may also prove to be valuable in future investigations into such areas genetic diagnosis, teratology and infertility.

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