—Review—

Intracytoplasmic Sperm Injection (ICSI) in Cattle and Other Domestic Animals: Problems and Improvements in Practical Use

Toshitaka Horiuchi[1] and Takashi Numabe[2]*

[1]*Laboratory of Animal Reproductive Sciences, Department of Bioresources, Hiroshima Prefectural University, Shoubara, Hiroshima 727-0023 and*
[2]*Miyagi Prefectural Animal Industry Experiment Station, Miyagi 989-6455, Japan*

Intracytoplasmic sperm injection (ICSI) is the microfertilization technique of the direct injection of a single spermatozoon or sperm head (nucleus) into ooplasm. In mammals, Uehara and Yanagimachi [1, 2] first reported that sperm nuclei injected into hamster oocytes could transform into male pronuclei. Since then, many investigations have aimed at successful production of offspring following ICSI. Normal live offspring resulting from ICSI have been born in rabbits [3], cattle [4], humans [5–7] and mice [8–10].

In human ICSI, high fertilization and pregnancy rates were achieved when a motile spermatozoon was injected after immobilization [6]. Dramatic advances in the treatment of severe male-factor infertility have been achieved by using ICSI, and at present, the technique of ICSI is being widely used in clinical applications for human infertility.

In cattle, the first ICSI calves were born in 1990 in Japan [4]. These results showed that bovine embryos produced by ICSI with a dead (killed) bull spermatozoon could develop into live calves. The normality of the calves (growth, behavior and reproduction) was also reported after five years [11]. However, the cleavage rate of oocytes after ICSI was very low (12 percent, 61/507) [4], the production rate of transferable embryos was low for practical use [4, 12–24]. In other domestic animals (pig, horse and sheep), the cleavage rate of ICSI oocytes was also low [20, 24–32].

There are many excellent reviews on the current status and future of ICSI in humans [33], mice [34] and domestic animals including cattle [35–40], and this paper reviews the problems with and improvements to ICSI for practical use in domestic animals, especially in cattle.

Received: December 8, 1998
Accepted: January 21, 1999
*To whom correspondence should be addressed.

History of ICSI in Cattle and Other Domestic Animals

In cattle and other domestic animals, the aims of ICSI are (1) the effective utilization of spermatozoa for livestock improvement and the multiplication of excellent animals; and (2) to provide another option to get fertilized oocytes, if in vitro fertilization (IVF) rates are low. Furthermore, there is the possibility of utilizing of defective spermatozoa from infertile animals, if the animals are excellent for productivity or possess a special ability.

Histories of ICSI in cattle, pig, horse and sheep are shown Tables 1 and 2. In comparison with human [6, 7, 33] and mouse [8–10] ICSI, fertilization rates, cleavage rates and the production number of offspring in bovine, porcine, equine and ovine ICSI are extremely low.

The first live calves following ICSI were reported by Goto *et al.* (1990) [4]. Their success was mainly dependent on the progress of in vitro maturation and in vitro fertilization of bovine oocytes and the establishment of an in vitro culture system by co-culture with cumulus cells [41]. Following the previous reports of Westusin *et al.* (1984) [12] and Younis *et al.* (1989) [14], Goto *et al.* (1990) [4] used capacitated bull spermatozoa killed by repeated freezing and thawing without cryoprotectants. In early reports on bovine ICSI, many researchers selected dead spermatozoa for practical use or for basic information on the fertilizability of sperm nuclei (Table 1). Their results showed that sperm nuclei transformed into male pronuclei, even if spermatozoa

Table 1. ICSI in cattle

Sperm	Fertilization	*In vitro* development		Preg.or Offspring	Reference
		Cleavage	Blastocyst		
Dead	0~25%	4%	-	-	Westhusin *et al.* (1984) [12]
Dead (Freeze-dried)	13%	-	-	-	Kameyama *et al.* (1985) [13]
Dead					Younis et al. (1989) [14]
Dead	15~76%	4~38%	-	-	Keefer *et al.* (1990) [15]
Dead	-	12%	2%	Offspring	Goto *et al.* (1990) [4]
Dead	22~39%	-	-	-	Heuwieser *et al.* (1992) [16]
Dead	46%	15~71%	7~11%	-	Li *et al.* (1993) [17]
Live?	67%	67%	9%	-	Pavasuthipaisit *et al.* (1994) [18]
Dead	-	36%	4%	-	Iwasaki *et al.* (1994) [19]
Live?	25%	-	-	-	Catt *et al.* (1995) [20]
Dead	5%	-	-	-	Qian *et al.* (1996) [21]
Live?	-	38~55%	0~33%	-	Chen *et al.* (1997) [22]
Live/Dead	-	38~40%	8%	-	Gordon *et al.* (1997) [23]
Live	81%	-	-	-	Katayose *et al.* (1997) [46]
Dead	50%	11%	-	-	Hosoi *et al.* (1998) [24]
Live	78%	74%	29%	-	Horiuchi *et al.* (1998) [42]
Live (DTT-treated)	60%	61%	24%	Preg.38% (6/16:~d54)	Rho *et al.* (1998) [59]
Live	-	72%	28%	-	Horiuchi *et al.* (1999) [43]

Table 2. ICSI in pig, horse and sheep

Species	Sperm	Fertilization	*In vitro* development		Preg.or Offspring	Reference
			Cleavage	Blastocyst		
Pig	Dead	60%	-	-	-	Catt *et al.* (1995) [20]
Pig	Dead	66%	10%	-	-	Hosoi *et al.* (1998) [24]
Horse	Live?	-	39%	21% (Morula)	No Preg. (0/2)	Meintjes *et al.* (1996) [25]
Horse	Live	-	25% (1/4)	-	Preg. (1/1)	Squires *et al.* (1996) [26]
Horse	Live	30%	13%	5% (16 cell)	-	Dell'Aquilia *et al.* (1997) [27]
Horse	Live	38%	48%	-	-	Dell'Aquilia *et al.* (1997) [28]
Horse	Live	50%	8-20%	8% (6-8 cell)	-	Gondahl *et al.* (1997) [29]
Horse	Live	-	5-21%	-	No Preg. (0/7)	Kato *et al.* (1997) [30]
Sheep	Live	63%	48%	7%	-	Catt *et al.* (1995) [20]
Sheep	Live	30%	-	-	-	Gomez *et al.* (1997) [31]
Sheep	Live	34%	-	-	-	Gomez *et al.* (1998) [32]

did not survive. However, the fertilization rates were various and the cleavage rates were very low.

Van Steirteghem *et al.* (1993) [6] reported a high fertilization rate in ICSI with human spermatozoa by the aspiration of ooplasm, the use of motile spermatozoa and immobilization by tail scoring prior to injection. Particularly, the injection of a motile spermatozoon after immobilization by tail scoring was the key to success. These excellent results were confirmed by many medical doctors and were accepted. Recently, also, in cattle, horse and sheep, motile spermatozoa have been used (See Tables 1 and 2), and we found that ICSI with a motile bull spermatozoon immobilized by tail scoring could improve fertilization rate and cleavage rate in cattle too [42, 43].

Although step by step improvements have been tried, there has been limited success in pregnancy and birth of offspring in all domestic animals (Table 1).

Problems in and Improvements for Practical Use of ICSI in Cattle

Technical problems

There are many reports that the fertilization rates

following ICSI by the conventional method were low in cattle. The overall length of bull spermatozoa is approximately 68 to 74 microns, the head is about 8 to 10 microns, and the widest portion of the head is about 4 to 5 microns [44]. Of course, the size of the sperm head is dependent on each individual bull, however, the size (widest portion) of a bull sperm head is larger than that of a human sperm head. As a result, the pore size of the injection pipette in bovine ICSI is larger than that used for human ICSI. Therefore, breakage of oocyte plasma membrane (oolemma) by aspiration results in remarkable damage. Also, the oolemma of bovine oocytes is elastic like those of mice, and it was difficult to puncture them by conventional ICSI. Kimura and Yanagimachi (1995) [8] showed that the use of a piezo micromanipulator (PRIM Tech., Japan) was effective for ICSI in the mouse. Also, in human ICSI, Huang, *et al.* (1996) [45] reported that it was posssible to achieve acceptable fertilization rates and pregnancy rates using a piezo maicromanipulator. Therefore, another option for ICSI technique is the use of a piezo maicromanipulator. Recently, Katayose *et al.* (1997) [46] showed that, in comparison with conventional ICSI, the rates of male pronuclear formation yield after ICSI using a piezo micromanipulator were significantly higher (81% vs 21%).

In addition to the difficulty of puncturing bovine oolemma, the observation and identification of a spermatozoon injected into cytoplasm of oocytes are extremely difficult, because bovine oocytes are opaque. Therefore, the details of the ICSI process cannot be observed. Bovine oocytes contain vacuoles and lipid droplets in their cytoplasm which prevent clear visualization. It is only possible to predict, therefore, the successful puncture of oolemma by their movement, which is probably insufficiently accurate. Centrifugation permits the removal of excess lipid for visualization of bovine oocytes [47]. The centrifugation of zygotes does not affect subsequent development and facilitates the visualization [48]. The confirmation of a spermatozoon injected into ooplasm must be effective for the increase of fertilization rate per injected oocytes. Although the effectiveness of centrifugation will have to be determined in future experiments, one potential problem is that some oocytes are activated by centrifugation and extrude the second polar body [47].

Polyvinylpyrrolidone (PVP) solution has been used for easy handling of spermatozoa in ICSI procedures. It not only prevents spermatozoa from adhering to the inner surface of glass injection pipette but it also allows good control of a spermatozoon in the injection pipette. It also facilitates immobilization of spermatozoa for the decline of sperm motility within PVP solution. But, PVP has a primary detrimental action on the plasma membrane of human spermatozoa [49]. Bars *et al.* [50] reported that some commercially-produced PVP solutions are toxic, causing failed embryo development after ICSI in mouse zygotes. Also, the presence of PVP in human oocytes caused some delay between sperm injection and the beginning of calcium osillations [51], or delay of sperm nucleus decondensation and oocyte activation [52]. Chromosomal abnormalities in pregnancies could be related to the injection of PVP into human oocytes [53]. Hinka *et al.* [54] found the 2PN rate after ICSI was significantly higher in a modified method of ICSI without the use of PVP than in conventional ICSI with the use of PVP. On the other hand, Motoishi *et al.* [55] reported that the injection of a small amount of PVP (about 2-3pl) was not detrimental to fertilization and embryo development of IVF bovine oocytes. Possibly, the injection volume is the important factor, however, the direct effect of PVP on early fertilization, oocyte cleavage and embryo development is still controversial in bovine ICSI.

Factors affecting decondensation and pronuclear formation of sperm nuclei after ICSI

The majority of bovine oocytes are prepared by in vitro maturation. The block of glutathione synthesis by L-buthionine- [S, R]-sulfoximine during in vitro maturation affects both pronuclear formation and apposition during IVF of bovine oocytes [56]. Glutathione is one of the important components for protamine disulfide bound reduction. After incorporation of the sperm head into the ooplasm, the sperm nuclear protamines are exchanged for the oocyte-derived DNA packing protein histones through nucleoplasmin [57]. After that, the decondensation of the sperm head starts. These results indicate that the quality of oocytes, e.g. failure or incompleteness of in vitro maturation of bovine oocytes resulting in poor quality oocytes, affects the success of ICSI. However, the interaction between the quality of bovine oocytes and the success of ICSI is unclear.

Keefer *et al.* (1986) [58] reported that the protamine disulfide bounds in bull sperm nuclei may be richer than those in hamster sperm nuclei, as a result of the delay of bull sperm decondensation in hamster oocytes after microinjection. Recently, Rho *et al.* (1998) [59] reported that pretreatment of sperm with dithiothreitol (DTT) could improve significantly the proportion of male pronuclear formation in bovine ICSI.

It is widely known that the decondensation of the sperm head is independent of oocyte activation [57].

Therefore, the failure of sperm injection into ooplasm would probably be the first reason for unfertilized oocytes. A high rate in male pronuclear formation by piezo-ICSI was reported by Katayose *et al.* (1997) [46] and Horiuchi *et al.* (1998) [42].

In human ICSI, immobilization by scoring a sperm tail increased the incidence of normal fertilization [60–63]. Physical damage of the sperm membrane may facilitate the effects of ooplasm factors on the sperm head [64], however, it is not clear whether the immobilization of a motile spermatozoon accelerates the interaction between the sperm head and the ooplasm.

Oocyte activation following ICSI and necessity of artificial oocyte activation

In normal fertilization, the membrane fusion between sperm and oocyte is essential for the incorporation of sperm into the cytoplasm of oocytes. The incorporation of the sperm head into the ooplasm results in the release from meiotic arrest. The second metaphase transferred into the G1/S phase. There are two hypotheses as to how sperm triggers the oocyte activation [65]. One is the transmembrane receptor mechanism, the other is a soluble sperm factors mechanism [66, 67]. In ICSI, the soluble sperm factors are the only oocyte activation factors except the physical stimulation by injection procedure.

In laboratory small animals, e.g., hamster and mouse, a sham injection, that is, picking of oolemma or aspiration of ooplasm, stimulated oocytes activation and caused parthenogenetic cleavage [68]. On the other hand, bovine matured oocytes have rarely been activated by sham injection [42].

Aged bovine oocytes could easily be activated by treatment of ethanol [69], electric stimulation and calcium ionophore (A23187) [70]. Age dependence of activation has been shown in cattle, and aging of bovine oocytes affects the response of oocyte activation. Unlike the aged bovine oocyte, activation of younger bovine oocytes (e.g., matured in vitro for 22–24 hr) could not be induced by ethanol, electric stimulation or A 23187 alone [69, 70]. However, activation of younger bovine oocytes could be induced by a combination of their parthenogenic agent and an inhibitor of protein synthesis (cycloheximide) [71, 72] or phosphorylation (6-dimethylaminopurine, 6-DMAP) [73–75]. It is difficult to activate younger bovine oocytes with a single activation stimulation alone [71, 72], nevertheless the younger bovine oocytes just after ICSI have been induced with a single activation stimulation alone according to many reports [4, 14–17, 19–22]. The insufficient stimulation or level of internal calcium leads from metaphase II to metaphase III in parthenogenetically activated mouse oocytes [76, 77]. Recently, Rho *et al.* (1998) [75] reported that delaying the addition of 6-DMAP for 3 h after ionomycin (used as the calcium ionophore) treatment greatly decreased the incidence of chromosomal abnormalities and produced a high proportion of activated oocytes with a single, haploid pronucleus. Furthermore, they showed that the efficiency of bovine ICSI could be improved by oocyte activation with this activation regimen [59].

Many investigators [4, 12, 14–16, 22] thought that induction of oocyte activation was essential for promoting male pronuclear formation in bovine ICSI. However, the improvement of the ICSI technique (from conventional ICSI to piezo-ICSI) has increased the rates of male pronuclear formation. In spite of live or dead spermatozoa, the second polar body was extruded after piezo-ICSI [46, 42], and with regard to the resumption of meiosis, bovine spermatozoa may possess the sperm associated oocyte activation factors (SOAF).

Factors affecting the cleavage of bovine oocytes after ICSI

In bovine ICSI, the main problem is a low cleavage rate. If you select the proper ICSI technique, the successful injection of bull spermatozoa allows sperm nuclei to decondense and transform into well-developed male pronuclei. Nonetheless, normal bipronuclear formation is not a guarantee of further first cleavage.

The effects of artificial oocyte activation on cleavage and in vitro development of bovine oocytes after ICSI are summarized in Fig. 1. When a motile bull spermatozoon immobilized by a tail-scoring was injected into bovine oocytes, the treatment of artificial oocyte activation (7% ethanol for 5 min) increased the development rate to more than two-cell stage in bovine oocytes with a second polar body after ICSI (74% vs 33%). Also, the blastocyst rate was increased by treatment with 7% ethanol for 5 min (29% vs 14%). Therefore, it is thought that the low cleavage rate of ICSI bovine oocytes without additional activation stimuli is caused by insufficient stimulation of oocyte activation for embryo development, not resumption of meiosis and extrusion of the second polar body. Stice and Robl (1990) [78] reported that the activity of a soluble factor from bull spermatozoa in oocyte activation was lower than that of rabbit spermatozoa.

Irrespective of the addition of oocyte activation, the injection of a motile (live) bull spermatozoon into the ooplasm after immobilization yields more cleaved embryos than the injection of a dead (killed by repeated

freezing and thawing) bull spermatozoon. The use of dead bull spermatozoa caused the remarkable decrease in cleavage rates in bovine ICSI [43]. There are many reports [4, 12, 15, 17, 24] on low cleavage rates in ICSI with dead bull spermatozoa.

High rates of embryo development by ICSI with dead spermatozoa or isolated sperm nuclei were reported in rodents, for example mice [8, 79] and hamsters (Yamauchi and Horiuchi, unpublished). Ohsako *et al.* [80] found genomic DNAs of spermatozoa are very resistant to freezing and thawing treatment compared with somatic cells (liver, kidney and testis) in golden hamster. In terms of cytoskeletal organization and dynamics during normal fertilization, the differences between rodents and other mammals are shown in Table 3. In cattle, a radical microtuble aster is formed in association with the incorporated sperm head [82]. These microtubles are necessary for pronuclear migration. The sperm aster splits to form two asters, which serve as the poles for the first mitotic spindle. The sperm tail remains associated with one the poles. In cattle, the sperm centrosome after incorporation into the ooplasm has an important role in the migration of the male pronucleus, the apposition of bipronuclei and the first miosis. On the other hand, in rodents, centrosomal inheritance appears to be primarily maternal. Cytoplasmic asters are found in unfertilized oocytes, and these asters are necessary for pronuclear migration.

Introducting a defective centriole or multiple centrioles into an oocyte may result in abnormal fertilization and embryonic development in human [83, 84]. Therefore, the abnormalities of the sperm centriole in dead bull spermatozoa killed by repeated frozen-thawing must be one of the reasons for the low rate of cleavage. However, the behavior of sperm asters of dead spermatozoa after ICSI is not clear.

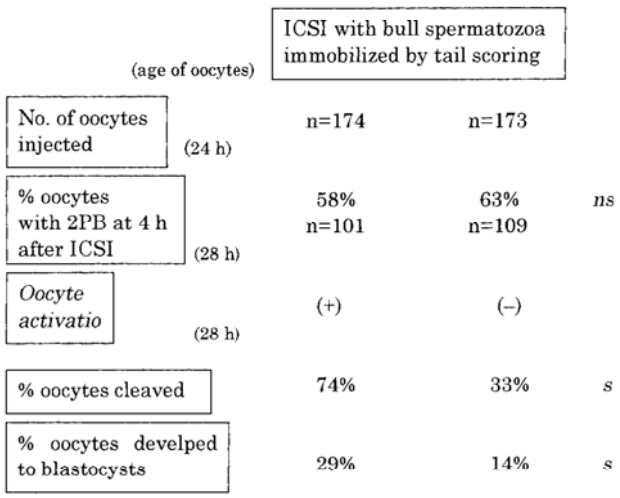

Fig. 1. Summary of the extrusion of 2 polar body (PB) and in vitro development of bovine oocytes after ICSI using a piezo micromanipulator [42]. *ns*=no significant (P>0.05), *s*=significant (P<0.05).

Problems for Practical Use of ICSI in Pig, Horse and Sheep

There are a few reports on ICSI in pig [20, 24], horse [25–30] and sheep [20, 31, 32]. The same as ICSI in cattle, cleavage rates were low in these domestic animals. The ICSI technique has been applied in horse, because of low fertilization rates by in vitro fertilization [40]. In pig, the remarkable progress of in vitro maturation, in vitro fertilization and in vitro culture system have been reported [85, 86]. In the near future, the rapid progress of ICSI in pig can be expected. At the very least, the basic information of fertilization and oocyte activation etc., needs to be recorded for the success of ICSI in these animals.

Conclusion

At the present, there are several problems regarding the practical use of ICSI on cattle and other domestic animals. For resolution of these problems, a lot of studies have been performed. The use of piezo-ICSI has facilitated the puncturing of bovine oolemma and the

Table 3. Differences of cytosketal organization and dynamics during fertilization between domestic animals and other mammals

	Cow	Pig	Sheep	Human	Mouse	Hamster
Cytoaster	No	No	No	No	Yes	Yes
Sperm aster	Yes	Yes	Yes	Yes	No	No
Sperm tail at miotic pole	Yes	Yes	Yes	Yes	No	No
Centriols at 1st mitosis	Yes	Yes	Yes	Yes	No	No

Citated from Navara *et al.* (1995) [81].

injection of bull spermatozoa, and the injection of an immobilized spermatozoon by tail scoring has proved useful in cattle, as in humans. Additional activation stimuli are required for cleavage and embryo development in bovine ICSI, but not for male pronuclear formation. The ICSI technique for producing bovine blastocysts is being improved continuously. In future, the developmental competence of ICSI bovine embryos into offspring must be confirmed on a large scale. Furthermore, we need to clarify the reason why ICSI with dead bull spermatozoa lead to lower embryo development rates.

References

1) Uehara, T. and Yanagimachi, R. (1976): Microsurgical injection of spermatozoa into hamster eggs with subsequent transformation of sperm nuclei into male pronuclei. Bio. Reprod., 15, 467–470.
2) Uehara, T. and Yanagimachi, R. (1977): Behavior of nuclei of testicular, caupt and cauda epididymal spermatozoa injected into hamster eggs. Bio. Reprod., 16, 315–321.
3) Hosoi, Y., Miyake, M., Utsumi, K. and Iritani, A. (1988): Development of rabbit oocytes after microinjection of spermatozoon. In: Proc. 11th Int. Congr. Anim. Reprod. AI; 3, 331–333 (abstr.).
4) Goto, K., Kinoshita, A., Takuma, Y. and Ogawa, K. (1990): Fertilization of bovine oocytes by the injection of immobilized, killed spermatozoa. Vet. Rec., 24, 517–520.
5) Palermo, G., Joris, H., Devroey, P. and Van Steirteghem, A.C. (1992): Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet, 340, 17–18.
6) Van Steirteghem, A.C., Nagy, Z., Joris, H., Liu, J., Staessen, C., Smitz, J., Wisanto, A. and Devroey, P. (1993): High fertilization and implantation rates after intracytoplasmic sperm injection. Hum. Reprod., 8, 1061–1066.
7) Van Steirteghem, A.C., Liu, J., Joris, H., Nagy, Z., Jassenswillen, C., Tournaye, H., Derde, M.P., Van Assche, E. and Devroey, P. (1993): Higher success rate by intracytoplasmic sperm injection than by subzonaal insemination. Report of a second series of 300 consecutive treatment cycles. Hum. Reprod., 8, 1055–1060.
8) Kimura, Y. and Yanagimachi, R. (1995): Intracytoplasmic sperm injection in the mouse. Biol. Reprod., 52, 709–720.
9) Ahmadi, A., Ng, S.C., Liow, S.L., Ali, J., Bongso, A. and Ratnam, S.S. (1995): Intracytoplasmic injection of mouse oocytes with 5 mM Ca at different intervals. Hum. Reprod., 10, 431–435.
10) Lacham-Kaplan, O. and Trounson, A. (1995): Intracytoplasmic sperm injection in mice: increased fertilization and development to term after induction of the acrosome reaction. Hum. Reprod., 10, 2642–2649.
11) Goto, K. and Yanagita, K. (1995): Normality of calves obtained by intracytoplasmic sperm injection. Hum. Reprod., 10, 1554.
12) Westhusin, M.E., Anderson, J.G., Harms, P.G., Kraemer, D.C. (1984): Microinjection of spermatozoa into bovine eggs. Theriogenology, 21, 274–275.
13) Kameyama, K., Hamano, K., Sugawara, S. and Masaki, J. (1985): Freeze-dried sperm head injection into bovine eggs matured in vitro. J. Mamm. Ova Res., 2, 25–26.
14) Younis, A.I., Keefer, C.L. and Bracket, B.G. (1989): Fertilization of bovine oocytes by sperm injection. Theriogenology, 31, 276 (abstr.).
15) Keefer, C.L., Younis, A.I., Brackett, B.G. (1990): Cleavage development of bovine oocytes fertilized by sperm injection. Mol. Reprod. Dev., 25, 281–285.
16) Heuwieser, W., Yang, X., Jiang, S. and Foote, R.H. (1992): A comparison between in vitro fertilization and microinjection of immobilized spermatozoa from bulls producing spermatozoa with defects. Mol. Reprod. Dev., 33, 489–491.
17) Li, X., Iwasaki, S. and Nakahara, T. (1993): Investigation of various conditions in microfertilization of bovine oocytes and subsequent changes in nuclei and development to embryos. J. Reprod. Dev., 39, j49–55 (Jpn).
18) Pavasuthipaisit, K., Kitiyanant, Y. and Tocharus, C. (1994): Embryonic development of bovine oocytes fertilized by sperm microfertilization: Comparison between subzonal and ooplasmic injection. Theriogenology, 41, 270 (abstr.).
19) Iwasaki, S. and Li, X. (1994): Experimental production of triploid bovine embryos by microinjection of two sperm and their development. J. Reprod. Dev., 40, 317–322.
20) Catt, J.W. and Rhodes, S.L. (1995): Comparative intracytoplasmic sperm injection (ICSI) in human and domestic species. Reprod. Fertil. Dev., 7, 161–166.
21) Qian, X.Q., Inagaki, H., Sasada, H. and Sugawara, S. (1996): Decondensation and male pronuclear formation in bovine oocytes after microinjection of bovine sperm pretreated with disulfide bound reducing agents. J. Mamm. Ova Res., 13, 118–121.
22) Chen, S.H. and Seidel, G.E.J. (1997): Effects of oocyte activation and treatment of spermatozoa on embryonic development following intracytoplasmic sperm injection in cattle. Theriogenology, 48, 1265–1273.
23) Gordon, A.C. and Carroll, M.J. (1997): Factors affecting the outcome of intracytoplasmic sperm injection (ICSI) of bovine oocytes matured in vitro. J. Reprod. Fertil. Abstr. Series, 19, 28–29.
24) Hosoi, Y., Torii, R., Saeki, K., Matsumoto, K. and Iritani, A. (1998): Microinjection of domestic and zoo animal spermatozoa into oocytes and their develop-

ment. Zygote, 6, S65–66.
25) Meintjes, M., Graff, K.J., Paccamonti, D., Elilts, B.E., Cochran,R., Sullivan, M., Fall, H. and Godke, R.A. (1996): In vitro development and embryo transfer of sperm-injected oocytes derived from pregnant mare. Theriogenology, 5, 304 (abstr.).
26) Squires, E.L., Wilson, J.M., Kato, H. and Blaszczyk, A. (1996): A pregnancy after intracytoplasmic sperm injection into equine oocytes matured in vitro. Theriogenology, 45, 306 (abstr.).
27) Dell'Aquila, M.E., Cho, Y.S., Minoia, P., Traina, V., Fusco, S., Lacalandra, G.M. and Maritato, F. (1997): Intracytoplasmic sperm injection (ICSI) verse conventional IVF on abattoir-derived and in vitro-matured equine oocytes matured equine oocytes. Theriogenology, 47, 1139–1156.
28) Dell'Aquilia, M.E., Cho, Y.S., Minoia, P., Traina, V., Lacalandra, G.M. and Maritato, F. (1997): Effects of follicular fluid supplement of in vitro maturation medium on the fertilization and development of equine oocytes after in vitro fertilization or intracytoplasmic sperm injection. Hum. Reprod., 12, 2766–2772.
29) Gondahl, C., Hansen, T.H., Hossaini, A., Heinze, I. and Greve T. (1997): Intracytoplasmic sperm injection of in vitro-matured equine oocytes. Biol. Reprod., 57, 1495–1501.
30) Kato, H., Seidel, G.E., Squires, E.L. and Wilson J.M. (1997): Treatment of equine oocytes with A23187 after intracytoplasmic sperm injection. Equine Vet. J., Suppl., 25, 51–53.
31) Gomez, M.C., Catt, J.W., Gillian, L., Evanse, G. and Maxwell, W.M. (1997): Effect of culture, incubation and acrosome reaction of fresh and frozen-thawed ram spermatozoa for in vitro fertilization and intracytoplasmic sperm injection. Reprod. Fertil. Dev., 9, 665–673.
32) Gomez, M.C., Catt, J.W., Evans, G. and Maxwell, W.M. (1998): Sheep oocyte activation after intracytoplasmic sperm injection. Reprod. Fertil. Dev., 10, 197–205.
33) Van Steirteghem A., Nagy P., Janssenswillen C., Verheyen, G., Camus, M., Tournaye, H., Devroey, P.. (1998): Results of intracytoplasmic sperm injection with ejaculated, fresh and frozen-thawed epididymal and testicular spermatozoa. In: Current Theory and Practice of ICSI, Hum. Reprod. 13 Suppl. (Devroey, P., Tarlatzis, B.C. and Van Steirteghem, A., eds.), pp. 134–142, Oxford University Press, Oxford.
34) Yanagimachi, R. (1998): Intracytoplasmic sperm injection experiment using the mouse as a model. In: Current Theory and Practice of ICSI, Hum. Reprod. 13 Suppl. (Devroey, P., Tarlatzis, B.C. and Van Steirteghem, A., eds.), pp. 87–98, Oxford University Press, Oxford.
35) Keefer, C.L. (1990): New techniques for assisted fertilization. Theriogenology, 33, 101–112.
36) Iritani, A. (1991): Micromanipulation of gametes for in vitro assisted fertilization. Mol. Reprod. Dev., 28, 199–207.
37) Goto, K. (1993): Bovine microfertilization and embryo transfer. Mol. Reprod. Dev., 36, 288–290.
38) Goto, K. (1993): Microinsemination: In vitro fertilization by micromanipulation of gamate. Anim. Sci. Technol., 64, 758–766, (Jpn).
39) Goto, K. (1997): Current status and future of micromanipulation-assisted fertilization in animals and human. J. Reprod. Dev., 42, 107–119.
40) Hinricks, K. (1998): Production of embryos by assisted reproduction in the horse. Theriogenology, 49, 13–21.
41) Gordon, I. (1994): Culturing the eary embryo. In: Laboratory Production of Cattle Embryos, pp. 227–292, CAB International, Wallingford.
42) Horiuchi, T., Yamauchi, Y., Emuta, C., Takahashi, K., Numabe, T., Oikawa, T., Katayose, H. and Yanagida, K. (1998): Effect of artificial activation on pronuclear formation and in vitro development of bovine oocytes after ICSI of a tail-scored motile bull spermatozoon. J. Mamm. Ova Res. 15, S33 (abstr.) (Jpn).
43) Horiuchi, T., Emuta, C., Yamauchi, Y., Oikawa, T. and Numabe, T. (1999): Intracytoplasmic sperm injection (ICSI) with bull spermatozoa immobilized by tail scoring. Theriogenology, 51, 357 (abstr.).
44) Salisbury, G.W., Van Demark, N.L. and Lodge, J.R. (1978): Morphology and motility of spermatozoa. In: Physiology of Reproduction and Artificial Insemination of Cattle, pp.286–328, W.H. Freeman & Co., San Francisco.
45) Huang, T., Kimura, Y. and Yanagimachi, R. (1996): The use of piezo micromanipulation for intracytoplasmic sperm injection of human oocytes. J. Assist. Reprod., 13, 320–328.
46) Katayose, H., Maeda, Y., Kimura, Y., Suzuki, K., Yanagida, K., Satou, A., Shinoko, T. and Kawahara, T. (1997): Successful ICSI by using piezo micromanipulator in bovine oocytes. J. Mamm. Ova Res., 14, S30 (abstr.).
47) Tatham, B.G., Sathananthan, A.H., Dharnmawardena, V., Munesinghe, D.Y., Lewis, I. and Trounson, A.O. (1996): Centrifugation of bovine oocytes for nuclear micromanipuration and sperm microinjection. Hum. Reprod., 11, 1499–1503.
48) Wall, R.J. and Hawk, H.W. (1988): Development of centrifuged cow zygotes cultured in rabbit oviducts. J. Reprod. Fertil., 82, 673–680.
49) Strehler, E., Baccetti, B., Sterzik, K., Capitani, S., Collodel, G., De Santo, M., Gambera, L. and Piomboni, P. (1998): Detrimental effects of polyvinylpyrrolidone on the ultrastructure of spermatozoa (Notulae seminologicae 13). Hum. Reprod., 13, 120–123.
50) Bras, M., Dumoulin, J.C.M., Pieters, M.H.E.C., Michiels, A.H.J.C., Geraedts, J.P.M. and Evers, J.L.H. (1994): The use of a mouse zygote quality control system for training purposea and toxicity determination

in an ICSI programme. Hum. Reprod., 9, suppl.4, 23–24 (abstr.).
51) Dozortsev, D., De Sutter, P., Rybouchkin, A. and Dhont, M. (1995): Oocyte activation and ICSI., Assist. Reprod. Rev., 5, 32–39.
52) Dozortsev, D., Rybouchkin, A., De Sutter, P. and Dhont, M. (1995): Sperm plasma membrane damage prior to intracytoplasmic sperm injection: a necessary condition for sperm nucleus decondation., Hum. Reprod., 11, 2792–2796.
53) Feichtinger, W., Obruca, A. and Brunner, M. (1995): Sex chromosomal abnormalities and intracytoplasmic injection., Lancet, 346, 1566.
54) Hinka, D., Herman, M., Vesela, J., Hredzak, R., Horvath, S. and Pacin, J. (1998): A modified method of intracytoplasmic sperm injection without the use of polyvinylpyrrolidone., Hum. Reprod., 13, 1922–1927.
55) Motoishi, M., Goto, K., Tomita, K., Ookutsu, S. and Nakanishi, Y. (1996): Examination of the safety of intracytoplasmic injection procedures by using bovine zygotes. Hum. Reprod., 11, 618–620.
56) Sutovsky, P. and Schatten, G. (1997): Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronuclear apposition during fertilization. Biol. Reprod., 56, 1503–1512.
57) Yanagimachi, R. (1994): Mammalian fertilization. In: The Physiology of Reproduction. (Knobil, E. and Neill, J.D., eds.), pp. 189–317, Raven Press, New York.
58) Keefer, C.L., Brackett, B.G. and Perreault, S.D. (1986): Behavior of bull sperm nuclei following microinjection into hamster oocytes. Biol. Reprod., 34 (Suppl. No. 1), 54 (abstr.).
59) Rho, G.J., Kawarsky, S., Johnson, W.H., Kochhar, K. and Betteridge K.J. (1998): Sperm and oocyte treatments to improve the formation of male and female pronuclei and subsequent development following intracytoplasmic sperm injection into bovine oocytes. Biol. Reprod., 59, 918–924.
60) Catt, J. and O'Neill, C. (1995): Manipulation of sperm before intracytoplasmic sperm injection improves fertilization rates. Fertil. Steril., 64, 1210–1212.
61) Fishel, S., Lisi, F., Rinaidi, L., Green, S., Hunter, A., Dowell, K. and Thornton, S. (1995): Systematic examination of immobilizing spermatozoa before intracytoplasmic sperm injection in human. Hum. Reprod., 10, 497–450.
62) Gerris, J., Mongelschots, K., Van Royen, E., Joostens, M., Eestermans, W. and Ryckaert, G. (1995): ICSI and severe male factor infertility: breaking the sperm tail prior to injection. Hum. Reprod., 10, 484–504.
63) Van de Bergh, M., Bertrand, E., Biramane, J. and Englert, Y. (1995): Importance of breaking a spermatozoon's tail before intracytoplasmic injection: a prospective randomized trial. Hum. Reprod., 10, 2819–2820.
64) Konnai, K., Yanagida, K., Katayose, H., Yazawa, H., Kimura, Y., Yamada, H., Hashimoto, S., Akutsu, H. and Sato, A. (1998) The study of the effect of sperm immobilization on sperm plasma membrane and the occurrence of Ca^{2+} osillation in ICSI., J. Mamm. Ova Res. 15, S32 (abstr.) (Jpn).
65) Wilding, M. and Dale, B. (1997): Sperm factor: What is it and what does it do? Mol. Hum. Reprod., 3, 269–273.
66) Swann, K. (1990): A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. Development, 110, 1295–1302.
67) Swann, K. (1996): Soluble sperm factors and Ca^{2+} release in egg at fertilization. Rev. Reprod., 1, 33–39.
68) Uehara, T. and Yanagimachi, R. (1977): Activation of hamster eggs by pricking. J. Exp. Zool., 199, 269–274.
69) Nagai, T. (1987): Parthenogenetic activation of cattle follicular oocytes in vitro with ethanol. Gamete Res., 16, 243–249.
70) Ware, C., Barnes, F., Maiki-Lauria, M. and First, N.L. (1989): Age dependence of bovine oocyte activation. Gamete Res., 22, 265–275.
71) Yang, X., Presicce, G.A., Moraghan, L., Jiang, S. and Foote, R.H. (1994): Synergistic effect of ethanol and cyclohexinide on activation of freshly matured bovine oocytes. Theriogenology, 41, 395–403.
72) Presicce, G.A. and Yang, X.N. (1994): Parthenogenetic development of bovine oocytes matured in vitro for 24 hr and activated by ethanol and cycloheximide. Mol. Reprod. Dev., 38, 380–385.
73) Susko-Parrish, J.L., Leibfried-Rutledge, M.L., Northy, D.L., Schutzkus, V. and First, N.L. (1994): Inhibition of protein kinases after an induced calcium transient cause transition of bovine oocytes to embryonic cycles without meiotic completion. Dev. Biol., 166, 729–739.
74) Liu, L., Ju, J.C.and Yang, X. (1998): Parthenogenetic development and protein pattarns of newly matured bovine oocytes after chemical activation. Mol. Reprod. Dev., 49, 298–307.
75) Rho, G.J., Wu, B., Kawarsky, S., Leibo, S.P. and Betteridge, K.J. (1998): Activation regiments to prepare bovine oocytes for intracytoplasmic sperm injection. Mol. Reprod. Dev., 50, 485–492.
76) Kubiak, J.Z. (1989): Mouse oocytes gradually develop the capacity for activation during the metaphase II arrest. Dev. Biol., 136, 537–545.
77) Vincent, C., Cheek, T.R. and Johnson, M.H. (1992): Cell cycle progression of parthenogenetically activated mouse oocytes to interphase is dependent on the level of internal calcium. J. Cell Sci., 103, 389–396.
78) Stice. S. and Robl, J. (1990): Activation of mammalian oocytes by a factor obtaied from rabbit sperm. Mol. Reprod. Dev., 25, 272–280.
79) Kuretake, S., Kimura, Y., Hoshi, K. and Yanagimachi, R. (1996): Fertilization and development of mouse oocytes injected with isolated sperm head. Biol. Reprod.,

57, 789–795.
80) Ohsako, S., Nagano, R., Sugimoto, Y. and Goto, K. (1997): Comparison of the nuclear DNA stability against freezing-thawing and high temperature treatment between spermatozoa and somatic cells. J. Vet. Med. Sci., 59, 1085–1088.
81) Navara, C.S., Wu, G-J., Simerly, C. and Schatten, G. (1995): Mammalian model systems for exploring cytoskeletal dynamics during fertilization. In: Cytoskeletal Mechanisms during Animal Development. (Capco, D.G., ed.), pp.321–342, Academic Press, San Diego.
82) Navara, C.S., First, N.L. and Schatten, G. (1994): Microtuble organization in the cow during fertilization, polyspermy, pathenogenesis and nuclear transfer: The role of the sperm aster. Dev. Biol., 162, 29–40.
83) Asch, R., Simerly, C., Ord, V.A. and Schatten, G. (1995): The stages at which human fertilization arrest: microtuble and chromosome configurations in inseminated oocytes which failed to complete fertilization and development in human. Hum. Reprod., 10, 1897–1906.
84) Navara, C.S., First, N.L. and Schatten, G. (1996): Phenotypic variations among paternal centrosomes expressed within the zygote as disparate microtuble lengths and centrosome activity and developmental success. Proc. Natl. Acad. Sci., 93, 5384–5388.
85) Funahashi, H. and Day, B.N. (1997): Advances in in vitro production of pig embryos. J. Reprod. Fertil. Suppl., 52, 271–283.
86) Prather, R.S. and Day, B.N. (1998): Practical considerations for the in vitro production of pig embryos. Theriogenology, 49, 23–32.