—Mini Review—

Present Status and Prospects for Bovine Intracytoplasmic Sperm Injection with In Vitro-Matured Oocytes and Frozen Semen

Hitoshi Ushijima[1] and Takashi Nakane[1]*

[1]*Chiba Prefectural Livestock Experimental Station, Ichihara 290-0513, Japan*

Abstract: *In general, in vitro-matured oocytes and commercially available frozen semen are used for bovine intracytoplasmic sperm injection (ICSI), unlike ICSI in humans and experimental animals, which uses in vivo-matured oocytes and fresh semen. Bovine ICSI is also characterized by difficulties in pronuclear formation. Therefore, increasing in vitro development of produced ICSI embryos is considered to necessitate use of an artificial activation treatment after injection of motile sperm. However, because parthenogenetic embryos are found amongst produced ICSI embryos, it is necessary to establish a bovine ICSI protocol that leads to normal karyomorphism.*

Key words: *Intracytoplasmic sperm injection, In vitro-matured oocytes, Frozen semen, Artificial activation*

Introduction

Ever since establishment of the Honolulu method [1, 2], intracytoplasmic sperm injection (ICSI) has been shown to be practical in several animal species [3]. In general, the serial ICSI procedure of mechanical stimulus with an injection pipette, puncture of the oolemma, and injection of a spermatozoon results in oocyte activation and subsequent development at a high ratio. However, bovine *in vitro*-matured oocytes are not completely activated by the ICSI procedure such that additional activation by chemical or other stimuli is required [4–6]; however, even after inducement of artificial activation, incompletely decondensed sperm and asynchronized pronuclei are observed [7, 8].

In vivo matured oocytes and fresh semen are commonly used in most mammalian species, unlike bovine ICSI, which is nearly always characterized by the use of *in vitro*-matured oocytes and commercial frozen semen. This is perhaps the limiting factor for the especially low developmental potential of bovine ICSI embryos. When cooled or frozen-thawed human sperm are used in ICSI, developmental potential of the resultant embryos is lower than in embryos with fresh sperm [9]. In fact, the activation of *in vitro*-matured human oocytes after ICSI is also significantly lower than those matured *in vivo* [10]. And, even in relatively easy porcine ICSI, embryos obtained from frozen sperm and *in vitro* maturation (IVM) oocytes also confirmed the failure of male pronucleus formation [11]. The failure of pronuclear formation commonly found in the bovine is not specific to ICSI, as it also occurs in *in vitro* fertilization (IVF) [12, 13]. Therefore, we reviewed various factors herein that improve the productivity of bovine ICSI when using IVM oocytes and frozen semen.

Effects of IVM Oocyte Conditions for ICSI

Collection of cumulus oocytes complexes (COCs)

Larger numbers of oocytes with multiple cumulus layers can be obtained when COCs are collected from the ovaries of slaughtered bovine by the cutting method [14] and from *in vivo* ovaries by the transvaginal ovum pick-up method using various suction tools and pressures [15]. These methods also increase the subsequent maturation rate and developmental competence of the oocytes. However, IVM oocytes are more easily damaged by *in vitro* manipulation than *in vivo*-matured oocytes [16, 17]. Insufficient *in vitro* maturation conditions lead to a decrease in inositol 1,4,5-trisphosphate (IP3) and glutathione stored via cumulus cells such that a possibility exists that this adversely affects oocyte activation and male pronucleus formation [12, 18].

Received: May 17, 2006
Accepted: June 19, 2006
*To whom correspondence should be addressed.
e-mail: h.ushjm@ma.pref.chiba.lg.jp

Countermeasures against the special measures law concerning bovine spongiform encephalopathy (BSE)

When follicular oocytes collected from slaughtered bovine ovaries are used experimentally, it is known that the storage temperature for ovaries and storage period until COCs are suctioned from the ovaries affect the viability of COCs [19]. For example, the frequency of oocytes with heterogeneous cytoplasts and an uneven distribution of lipids during maturation culture increases when ovaries and COCs are preserved at room temperature for extended periods [20]. The productivity of the resulting blastocysts also decrease if oocytes that have been kept for extended periods of time are used for IVF or as recipient cytoplast for nucleus transfer [21]. Recently, due to the law for prevention of BSE transmission, modifications were necessary to procedures for collecting of ovaries using *in vitro* production (IVP) systems. Due to a change in the storage conditions for COCs to comply with the law, it is now possible to quickly collect COCs from ovaries at slaughterhouses, and this allows for maturation culture during storage. These modifications have realized viability equivalent to that of the present conventional IVP systems [20].

Relationship between maturation time and activation rate of oocytes

Most oocytes from bovine ovarian follicles with diameters of 2–8 mm progress to the second metaphase stage 16 h after *in vitro* maturation culture [22]. However, these oocytes are not effectively activated by artificial stimulation [23–25]. As *in vitro* maturation causes disturbances in the nuclei, cytoplasm, and cumulus cell maturation that would normally occur simultaneously *in vivo* [26, 27], this leads to a lag in cytoplasmic maturation that may cause a low response to artificial activation. On the other hand, oocytes 28 h after maturation culture are easily activated, although the developmental competence of the obtained embryos is low [28]. Hence, a period of 24–27 h after maturation culture is probably the optimum activation timing of non-aged oocytes. Essentially, extended time after *in vitro* oocyte maturation adversely affects pronuclear formation and subsequent embryo developmental competence of IVF and ICSI embryos [10, 28]. Therefore, advancement of bovine ICSI requires the following improvements: (1) an activation method for oocytes immediately after maturation and (2) *in vitro* maturation of oocytes with synchronized nuclei and cytoplast maturation.

Effects of Injected Sperm on Pronuclear Formation

Selection of injected sperm

Following the production of the first ICSI-derived calves by Goto *et al.* [4], further use of immobilized sperm for ICSI has been reported, such as sexed sperm heads [29], dead sperm [4], and freeze-dried sperm [30]. In humans and mice, however, sperm immotility and time-dependence after sperm immobilization treatment leads to an increase in DNA fragmentation of the sperm head [31], lower chromosomal normality, and decreased developmental competence in ICSI embryos [32, 33]. Moreover, the developmental competence of ICSI bovine embryos using motile sperm is higher than that of dead sperm. Additional artificial activation also increased developmental competence of ICSI embryos in many reports [4–6, 34]. A protocol involving motile sperm injection followed by oocyte activation should therefore improve the productivity of bovine ICSI.

Correspondence with male pronuclear formation

It is known that delayed male pronuclear formation and asynchronous pronuclei occur in bovine ICSI oocytes at a high frequency even in artificial activation-induced oocytes [7]. Accordingly, improvement of male pronuclear formation after ICSI is considered to be a key factor for increasing the productivity of bovine ICSI using frozen-thawed semen. In general, male pronucleus formation is not required for new protein synthesis in the bovine zygote [26]; therefore, two strategies can be considered for increasing male pronuclear formation. Namely, one dependent on the intrinsic factors of sperm and the other is dependent on artificially assisting pronuclear formation. Both are described below.

Wei and Fukui [35] promoted bovine ICSI using lower polyvinylpyrrolidone (PVP) concentrations in the medium such that the sperm head would be more readily modified into oocyte cytoplasm. As a result, ICSI embryos were found to have a high frequency of male pronuclear formation, reaching the blastocyst stage without exogenous oocyte activation. We also know that injection of a fresh human or equine spermatozoon [9, 36] and injection of a sperm factor into the cytoplasmic extracts of fresh semen [37, 38] lead to bovine oocyte activation and normal pronuclear formation at a high frequency without artificial activation [34]. Furthermore, use of tail score [39], swim-up [36], and Percoll density gradient [40] to select frozen bovine sperm with high motility results in normal pronuclear

formation at a high frequency without artificial activation, which suggests male pronuclear formation can be improved by evaluating the quality of injected sperm.

On the other hand, stores of glutathione in oocytes are necessary for the reduction of disulfide bonds in sperm nuclei and male pronuclear formation. The amount of stored glutathione in the cytoplasm, however, was low in oocytes that underwent *in vitro* maturation in unsuitable culture conditions [18], i.e., depletion of glutathione in the oocytes blocks decondensation of the male pronucleus during fertilization [13, 41]. Dithiothreitol (DTT) is an agent that promotes decondensation of sperm nuclei and assists pronuclear formation in glutathione-dependent oocytes. The efficiency of male pronuclear formation of bovine ICSI oocytes and subsequent embryo development improves when sperm is treated with DTT before ICSI [40, 42].

Effects of Artificial Activation on ICSI Embryos

Artificial activation agents for ICSI oocytes

Calcium ionophore A23187 [4, 24], ethanol [3, 7, 29], electric stimulus [43], ionomycin (IA) [8, 40, 42, 44], and IP3 [45, 46] have been used to induce activation of *in vitro*-matured bovine oocytes. In actuality, a multiple activation method is needed to induce activation of oocytes 24–27 h after maturation culture [47] because a single artificial stimulation is not sufficient to inactivate maturation promoting factors in the oocytes [48]. One such activation treatment is the combination of IA and dimethylaminopurine (DMAP), which increases the cleavage and blastocyst rates of bovine ICSI embryos [8, 30, 42, 44].

If activation is induced in bovine oocytes at the second metaphase stage, the karyomorphology of the activated nuclei is dependent on the type of activation treatment [34]. In brief, activation by ethanol, IP3, or sperm extracts leads to haploid female pronucleus formation with 2nd polar body extrusion occurring as normal pronuclear formation. On the other hand, activation by IA or electrical stimuli tends to inhibit the release of the 2nd polar body. Of particular interest is the use of DMAP culture, which results in increased activation of oocytes without 2nd polar body extrusion [34].

Developmental ability of ICSI embryos induced with artificial activation

Here, IVF and ICSI were performed using sperm suspensions derived from frozen/thawed semen of progeny-tested supersires. The karyomorphology of ICSI embryos 18 h after activation is shown in Fig. 1, while Fig. 2 shows *in vitro* development. The rate of normal nuclei in IVF embryos was 49% and that for *in vitro* development to the blastocyst stage was 15%. In contrast, the rates for ICSI embryos activated with IP3 were 64% and 30%, respectively, significantly higher rates than those of IVF ($P<0.05$) embryos and non-activated ICSI embryos ($P<0.01$). ICSI embryos activated by IP3 and cultured for 7 d led to the birth of a normal offspring after transfer to a surrogate recipient [34]. These results suggest IP3 is a feasible activation treatment for ICSI oocytes.

The normality of ICSI embryos activated using IA treatment followed by DMAP treatment was confirmed by chromosomal [42] and PCR [44] tests. However, the results showed that these blastocysts contained parthenogenetic embryos. Also, in our previous study [34], a high frequency of polar body extrusion inhibition and abnormal karyomorphism was observed in ICSI embryos treated with IA and then DMAP. Oikawa *et al.* [49] demonstrated that full-term development of blastocysts obtained from IA + DMAP-treated ICSI embryos was extremely low, suggesting that a high proportion of the blastocysts produced using this method are parthenogenetic embryos.

To become a practical method for producing offspring, the number of parthenogenetic embryos should be minimized. Rho *et al.* [42] reported a reduction in ICSI embryos with chromosomal abnormalities using a 3-h interval between oocyte activation and DMAP treatment, i.e., the 2nd polar body of the ICSI embryo could be extruded using this interval. Unlike DMAP, cycloheximide does not block either extrusion of the 2nd polar body or male pronuclear formation [44, 50]. A cycloheximide treatment may therefore be more effective [40], although further investigation is required to confirm the significance of these findings.

On the other hand, Horiuchi and Numabe [3] found that about 60% of ICSI embryos injected with motile sperm extruded a 2nd polar body 4 h after ICSI. When these embryos were selected and then activated artificially, they not only developed into blastocysts *in vitro* at a high frequency, but offspring were produced from them with a high pregnancy rate [49]. This method is therefore considered to be a feasible way to gather normal fertilized embryos with high viability from ICSI embryos at an early stage of fertilization.

Utility of ICSI for Practical Field Applications

Although bovine ICSI, as an assisted reproductive

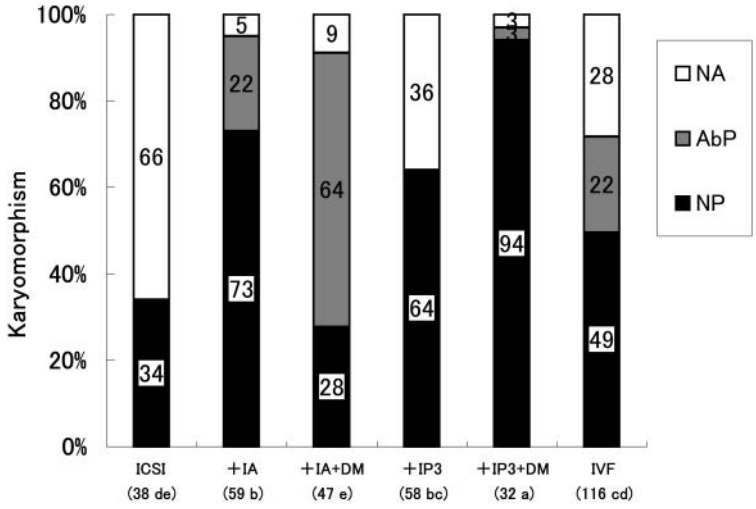

Fig. 1. Karyomorphism of bovine ICSI embryos activated by various methods. The closed, gray, and open bars, respectively, indicate the frequency of embryos with 1st and 2nd polar bodies and normal pronuclei consisting of a pair of male and female pronuclei (NP); the frequency of abnormal pronuclei, not including category NP but including, for example, embryos without 2nd PB extrusion, embryos with a condensed sperm head, and embryos with multiple female pronuclei (AbP); and the frequency of non-activated nuclei without any pronuclei in the embryos (NA). Values in parentheses indicate the numbers of embryos examined. Values inside the bar graphs indicate the percentages of the number of embryos used. Values in parentheses with different superscripts are significantly different ($P<0.01$).

technology, expands the possibilities for the cattle industry, *in vitro* development to the blastocyst stage of ICSI embryos (20–30%) is still lower compared to the usual results from IVF [40]. However, bovine embryo production based on conventional IVF systems [51] is known to be heavily influenced by individual differences in bull semen [52], and thus use of IVF with commercial frozen semen does not produce acceptable results (Table 1). This makes IVF the bottleneck for producing embryos in IVP systems for practical field applications. Bovine ICSI can bypass critical *in vitro* fertilization events including acrosome reaction, capacitation, and penetration of the zona pellucida, and it may very well overcome problems due to bull variations in bovine IVF, such as sperm motility, sperm concentration, and sensitivity to capacitation agents. Horiuchi and Numabe [3] point toward the benefits of bovine ICSI in producing embryos using different types of valuable semen that have an insufficient sperm concentration to adjust to conventional IVF conditions. Here, valuable frozen semen was used for embryo production, and subsequent *in vitro* development of the ICSI embryos was higher than that using conventional IVF, indicating that ICSI can improve *in vitro* productivity of bovine embryo production in field trials.

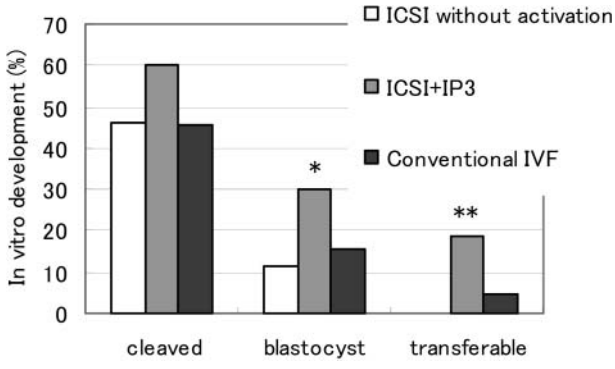

Fig. 2. *In vitro* development of ICSI embryos without activation (open bar) ICSI+IP3 injected embryos (grey bar), and conventional IVF embryos (solid bar). Development to the blastocyst stage ($P<0.05$) and to transferable embryos ($P<0.01$) of ICSI+IP3 was significantly different from the other two groups.

Table 1. Field trial results of *in vitro* development of IVM/IVF embryos produced from commercially-available frozen semen

Frozen semen	IVP tested	No. of embryos cultured	*In vitro* development to the (%)		
			2-cell stage	blastocyst stage	transferable stage
Holstein	Unknown	205	92 (45)[b]	42 (20)[b]	16 (8)[b]
Japanese Black	Unknown	583	286 (49)[b]	93 (16)[b]	32 (5)[b]
Practical used*	Already	406	350 (86)[a]	127 (31)[a]	69 (17)[a]

*The semen was confirmed beforehand to be suitable for conventional IVF. Values within columns with different superscripts are significantly different. [a, b]$P<0.01$.

Conclusions

ICSI could clearly improve the system of *in vitro* production of bovine embryos. The developmental competence of bovine ICSI embryos is affected by oocytes under *in vitro*-matured conditions, sperm motility, and additional artificial stimulation for oocyte activation. However, use of artificial activation, which is useful for effectively producing ICSI embryos, also has a drawback of producing parthenogenetic embryos. This therefore requires the establishment of ICSI conditions that enable formation of male/female haploid pronuclei at a high frequency.

Acknowledgements

I extend sincere thanks to Dr. H. Nagashima (Meiji University) for valuable direction and to Dr. A. Ogura (RIKEN Tsukuba Institute) for his valuable advice on intracytoplasmic injection using a Piezo micromanipulator. I would also like to thank the staff at the Chiba Prefectural Meat Hygienic Laboratory East Inspectorate, Chiba-ken Meat Public Co., and Toyo Meat Public Co. for providing ovaries.

References

1) Kimura, Y. and Yanagimachi, R. (1995): Intracytoplasmic sperm injection in the mouse. Biol. Reprod., 52, 709–720.
2) Yanagida, K., Katayose, H., Yazawa, H., Kimura, Y., Konnai, K. and Sato, A. (1998): The usefulness of a piezo-micromanipulator in intracytoplasmic sperm injection in humans. Hum. Reprod., 14, 448–453.
3) Horiuchi, T. and Numabe T. (1999): Intracytoplasmic sperm injection (ICSI) in cattle and other domestic animals: Problems and improvements in practical use. J. Mamm. Ova. Res., 16, 1–9.
4) Goto, K., Kinoshita, Y., Takuma, Y. and Ogawa, K. (1990): Fertilization of bovine oocytes by the injection of immobilized, killed spermatozoa. Vet. Rec., 127, 517–520.
5) Horiuchi, T., Emuta, C., Yamauchi, Y., Oikawa, T., Numabe, T. and Yanagimachi, R. (2002): Birth of normal calves after intracytoplasmic sperm injection of bovine oocytes: a methodological approach. Theriogenology, 57, 1013–1024.
6) Fujinami, N., Hosoi, Y., Kato, H., Matsumoto, K., Saeki, K. and Iritani, A. (2004): Activation with ethanol improves embryo development of ICSI-derived oocytes by regulation of kinetics of MPF activity. J. Reprod. Dev., 50, 171–178.
7) Li, X., Hamano, K., Qian, X.Q., Funauchi, K., Furudate, M. and Minato, Y. (1999): Oocyte activation and parthenogenetic development of bovine oocytes following intracytoplasmic sperm injection. Zygote, 7, 233–237.
8) Chung, J.T., Keefer, C.L. and Downey, B.R. (2000): Activation of bovine oocytes following intracytoplasmic sperm injection (ICSI). Theriogenology, 53, 1273–1284.
9) Nakamura, S., Terada, Y., Horiuchi, T., Emuta, C., Murakami, T., Yaegashi, N. and Okamura, K. (2001): Human sperm aster formation and pronuclear decondensation in bovine eggs following intracytoplasmic sperm injection using a Piezo-driven pipette: a novel assay for human sperm centrosomal function. Biol. Reprod., 65, 1359–1363.
10) Balakier, H., Sojecki, A., Motamedi, G. and Librach, C. (2004): Time-dependent capability of human oocytes for activation and pronuclear formation during metaphase II arrest. Hum. Reprod., 19, 982–987.
11) Lee, J.W., Tian, X.C. and Yang, X. (2003): Failure of male pronucleus formation is the major cause of lack of fertilization and embryo development in pig oocytes subjected to intracytoplasmic sperm injection. Biol. Reprod., 68, 1341–1347.
12) Chian, R.C., Niwa, K. and Sirard, M.A. (1994): Effects of cumulus cells on male pronuclear formation and subsequent early development of bovine oocytes in vitro. Theriogenology, 41, 1499–1508.
13) Sirard, M.A., Richard, F., Blondin, P. and Robert, C. (2006): Contribution of the oocyte to embryo quality. Theriogenology, 65, 126–136.
14) Hamano, S. and Kuwayama, M. (1993): In vitro fertilization and development of bovine oocytes recovered from the ovaries of individual donors: a comparison between the cutting and aspiration method. Theriogenology, 39, 703–712.
15) Konishi, M., Aoyagi, Y., Takedomi, T., Itakura, H., Itoh, T.

and Yazawa, S. (1996): Production and transfer of IVF embryos from individual inhibin-immunized cows by ultrasound-guided transvaginal follicular aspiration. J. Vet. Med. Sci., 58, 893–896.
16) Leibfried, M.L., Critser, E.S., Eyestone, W.H., Northey, D.L. and First, N.L. (1987): Development potential of bovine oocytes matured in vitro or in vivo. Biol. Reprod., 36, 376–383.
17) Pivko, J., Landa, V., Kubovicova, E., Supova, A., Grafenau, P., Makarevic, A., Riha, L. and Zibrin, M. (2003): Comparative morphometry of precompacted bovine embryos produced in vivo or in vitro after parthenogenetic activation and intracytoplasmic sperm injection (ICSI): ultrastructural analysis. Zygote, 11, 207–217.
18) Geshi, M., Youngs, C.R. and Nagai, T. (1999): Addition of Cysteamine to a serum-free maturation medium enhances in vitro development of IVM-IVF bovine oocytes. J. Mamm. Ova. Res., 16, 135–140.
19) Abe, S., Hasegawa, K. and Kawahira, M. (1991): Effect of short-term preservation of ovaries at 38 C on the development of bovine follicular oocytes matured and fertilized in vitro. J. Mamm. Ova. Res., 8, 155–163 (in Japanese).
20) Shino, M., Sakamoto, K., Takanawa, S., Naito, R., Naito, M., Yamashita, H., Nakane, T., Ohtani, S., Kashiwazaki, N. and Ushijima, H. (2004): In vitro development of bovine embryos obtained from follicular oocytes stored at slaughterhouse after inspection confirmed no possibility of BSE. Jpn. J. Anim. Hygiene, 30, 111–116 (in Japanese).
21) Ushijima, H., Sakane, J., Ozawa, H., Saito, I., Eto, T. and Ogawa, S. (1995): Effect of donor embryonic stage on the development of nuclear transplanted bovine embryos. Bllet. Chiba Pref. Exp. Stn., 19, 1–6 (in Japanese).
22) Shioya, Y. (1992): Bovine in vitro fertilization. Sustainable Livestock Production, 46, 455–458 (in Japanese).
23) Nagai, T. (1987): Partheogenetic activation of cattle follicular oocytes in vitro with ethanol. Gamete Res., 16, 243–249.
24) Ware, C.B., Barns, F.L., Maiki-Lairila, M. and First N.L. (1989): Age dependence of bovine oocyte activation. Gamete Res., 22, 265–275.
25) Akagi, S., Adachi, N., Matsukawa, K., Kubo, M. and Takahashi, S. (2003): Developmental potential of bovine nuclear transfer embryos and postnatal survival rate of cloned calves produced by two different timings of fusion and activation. Mol. Reprod. Dev., 66, 264–272.
26) Sato, E. (1991): Regulation of maturation and death of bovine oocytes. Nihon Chikusan Gakkaiho, 62, 978–989 (in Japanese).
27) Kikuchi, K., Naito, K., Noguchi, J., Shimada, A., Kaneko, H., Yamashita, M., Aoki, F., Tojo, H. and Toyoda, Y. (2000): Maturation/M-phase promoting factor: a regulator of aging in porcine oocytes. Biol. Reprod., 63, 715–722.
28) Long, C.R., Damiani, P., Pinto-Correia, C., MacLean, R.A., Duby, R.T. and Robl, J.M. (1994): Morphology and subsequent development in culture of bovine oocytes matured in vitro under various conditions of fertilization. J. Reprod. Fertil., 102, 361–369.
29) Hamano, K., Li, X., Qian, X., Funauchi, K., Furudate, M. and Minato, Y. (1999): Gender preselection in cattle with intracytoplasmically injected, flow cytometrically sorted sperm heads. Biol. Reprod., 60, 1194–1197.
30) Keskintepe, L., Pacholczyk, G., Machnicka, A., Norris, K., Curuk, M.A., Khan, I. and Brackett, B.G.(2002): Bovine blastocyst development from oocytes injected with freeze-dried spermatozoa. Biol. Reprod., 67, 409–415.
31) Kaneko, S. (2003): Observation of DNA fragmentation in human sperm and their exclusion from ejaculated human sperm. J. Reprod. Engineer., 6, 290–295 (in Japanese).
32) Hoshi, K., Yanagida, K., Yazawa, H., Katayose, H. and Sato, A. (1995): Intracytoplasmic sperm injection using immobilized or motile human spermatozoon. Fertil. Steril., 63, 1241–1245.
33) Kimura, Y., Katayose, H., Yazawa, H., Hayashi, S., Yanagida, K., Sato, A., Tateno, H. and Yanagimachi, R. (1999): Effect of time and environment after immobilization on the sperm chromosomes. J. Mamm. Ova. Res., 16, 64 (abstract in Japanese).
34) Ushijima, H., Kosaki, T., Ishimura, Y., Sakamoto, K., Ishikawa, T. and Nagashima, H. (2006): Effects of activation condition on producing bovine intracytoplasmic sperm injection embryos used with in vitro matured oocytes. J. Reprod. Engineer., 9 (in press).
35) Wei, H. and Fukui, Y. (2000): Technical improvement in intracytoplasmic sperm injection (ICSI) in cattle. J. Reprod. Dev., 46, 403–407.
36) Choi, Y.H., Love, C.C., Love, L.B., Varner, D.D., Brinsko, S. and Hinrichs, K. (2002): Developmental competence in vivo and in vitro of in vitro-matured equine oocytes fertilized by intracytoplasmic sperm injection with fresh or frozen-thawed spermatozoa. Reprod., 123, 455–465.
37) Swann, K. (1990): A cytosonic sperm factor stimulates repetitive calcium releases and mimics fertilization in hamster eggs. Development, 110, 1295–1302.
38) Okitsu, O., Yamano, S. and Aono, T. (2001): Activation of bovine oocytes matured in vitro by injection of bovine and human spermatozoa or their cytosolic fractions. Zygote, 9, 89–95.
39) Wei, H. and Fukui, Y. (2002): Births of calves derived from embryos produced by intracytoplasmic sperm injection without exogenous oocyte activation. Zygote, 10, 149–153.
40) Galli, C., Vassiliev, I., Lagutina, I., Galli, A. and Lazzari, G. (2003): Bovine embryo development following ICSI: effect of activation, sperm capacitation and pre-treatment with dithiothreitol. Theriogenology, 60, 1467–1480.
41) 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.
42) Rho, G.J., Wu, B., Kawarsky, S., Leibo, S.P. and Betteridge, K.J. (1998): Activation regimens to prepare

bovine oocytes for intracytoplasmic sperm injection. Mol. Reprod. Dev., 50, 485–492.
43) Hwang, S., Lee, E., Yoon, J., Yoon, B.K., Lee, J.H. and Choi, D. (2000): Effects of electric stimulation on bovine oocyte activation and embryo development in intracytoplasmic sperm injection procedure. J. Assis. Reprod. Genet., 17, 310–314.
44) Suttner, R., Zakhartchenko, V., Stojkovic, P., Muller, S., Alberio, R., Medjugorac, I., Brem, G., Wolf, E. and Stojkovic, M. (2000): Intracytoplasmic sperm injection in bovine: effects of oocyte activation, sperm pretreatment and injection technique. Theriogenology, 54, 935–948.
45) Fissore, R.A., Dobrinsky, J.R., Balise, J.J., Duby, R.T. and Robl, J.M. (1992): Patterns of intracellular Ca2+ concentrations in fertilized bovine eggs. Biol. Reprod., 47, 960–969.
46) Ushijima, H., Ishida, K. and Nagashima, H. (2002): Bovine nucleus transplantation by intracytoplasmic injection. J. Reprod. Dev., 48, 619–626.
47) Susko-Parrish, J.L., Leibfried-Rutledge, M.L., Northey, D.L., Schutzkus, V. and First, N.L. (1994): Inhibition of protein kinases after an induced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion. Dev. Biol., 166, 729–739.
48) Hashimoto, N., Watanabe, N., Furuta, Y., Tatemoto, H., Sagata, N., Yokoyama, M., Okazaki, K., Nagayoshi, M., Takeda, N. and Ikawa, Y. (1994): Parthenogenetic activation of oocytes in c-mos-deficient mice. Nature, 370, 68–71.
49) Oikawa, T., Takada, N., Kikuchi, T., Numabe, T., Takenaka, M. and Horiuchi, T. (2005): Evaluation of activation treatments for blastocyst production and birth of viable calves following bovine intracytoplasmic sperm injection. Anim. Reprod. Sci., 86, 187–194.
50) Chian, R.C. and Sirard, M.A. (1996): Protein synthesis is not required for male pronuclear formation in bovine zygotes. Zygote, 4, 41–48.
51) Parrish, J.J., Susko-Parrish, J.L., Leibfried-Rutledge, M.L., Critser, E.S., Eyestone, W.H. and First, N.L. (1986): Bovine in vitro fertilization with frozen-thawed semen. Theriogenology, 25, 591–600.
52) Saeki, K., Nagao, Y., Hoshi, M. and Nagai, M. (1995): Effect of heparin, sperm concentration and bull variation on in vitro fertilization of bovine oocytes in a protein-free medium. Theriogenology, 43, 751–759.