—Mini Review—

Production of Viable Porcine Embryos by In Vitro Fertilization (IVF) and Intracytoplasmic Sperm Injection (ICSI)

Kazuhiro Kikuchi[1], Michiko Nakai[1], Arata Shimada[2] and Naomi Kashiwazaki[2]*

[1]*Division of Animal Sciences, National Institute of Agrobiological Sciences, Tsukuba 305-8602, Japan*
[2]*Laboratory of Animal Reproduction, School of Veterinary Medicine, Azabu University, Sagamihara 229-8501, Japan*

Abstract: *In vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are highly specialized procedures used for artificial production of successive generations of domestic animals. However, in pigs, the efficacy of these procedures in generating viable embryos is still poor. We focus here on both IVF and ICSI in pigs in relation to the physiology of fertilization (morphological changes and nuclear remodeling). This knowledge should contribute to the development of advanced technologies for in vitro production of zygotes that have the potential for full-term development to the offspring stage after transfer to recipients.*

Key words: *Pig, Fertilization, Nuclear remodeling, IVF, ICSI*

Introduction

In vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are highly specialized procedures used for artificial production of successive generations of domestic animals. In humans, they are considered to be assisted reproductive technologies for producing healthy babies. In domesticated animals, they are expected to produce viable zygotes at low cost and low time input and are expected to contribute not only to the enhancement of animal industries, such as for meat and milk production, but also to establishment of new biotechnologies, such as advanced sperm conservation and gene transfer. However, in spite of the recent progress in these fields, the efficacy (survival ability and quality) of IVF and ICSI embryos still seems to be low. Under physiological conditions, capacitated spermatozoa and a matured oocyte encounter each other, the acrosome reaction of the sperm is then induced, and the reacted spermatozoa penetrate through the zona pellucida and proceed into the perivitelline space. After the fusion of both gamete membranes, the sperm nuclear contents are exposed to the ooplasm. The chromatin of the sperm nucleus is remodeled into a typical somatic state and participates in subsequent morphological chromosomal activities, such as decondensation of sperm nuclei and formation of the male pronucleus (MPN) to complete fertilization. Especially in ICSI, the process of fertilization is presumably unphysiological and may thus reduce the efficacy of embryo production because a whole spermatozoon with its acrosome and cell membranes is introduced directly into the ooplasm. Alternatively, the injected sperm heads may complete their chromatin remodeling in the same way as in natural sperm penetration. The relationship between these possibilities and the successful development of sperm-injected oocytes has not yet been investigated in detail. Here, we focus on both IVF and ICSI in pigs in terms of the physiology of fertilization. This basic knowledge will contribute to the development of advancing technologies for *in vitro* production of zygotes that have the potential for full-term development to the offspring stage after transfer to recipients. It will also help us to better understand the cellular events induced by ICSI.

Received: July 3, 2006
Accepted: July 10, 2006
*To whom correspondence should be addressed.
e-mail: kiku@nias.affrc.go.jp

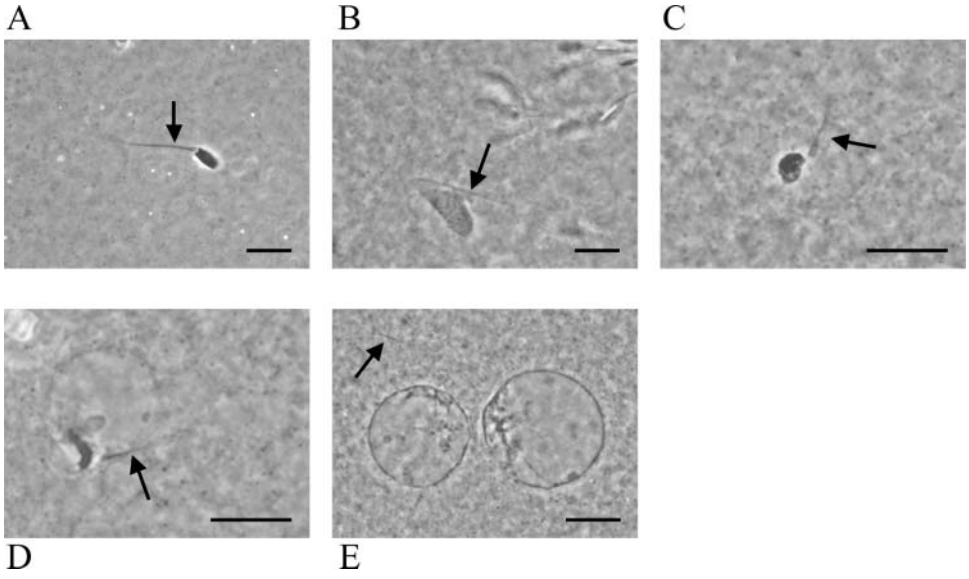

Fig. 1. Morphological changes in penetrating boar spermatozoa after IVF. A) Condensed spermatozoon just after membrane fusion 2 h after insemination; B) decondensing sperm head 4 h after insemination (upper right; spermatozoa with their original shapes that have not penetrated but are attached to the zona pellucida can be seen in different focal planes); C) recondensing sperm head 5 h after insemination; D) initiation of a male pronucleus (the chromatin mass from the recondensed sperm nucleus still exists, and formation of the pronuclear membrane is not complete); and E) well-developed male and female pronuclei. Arrows indicate sperm tails. Scale bars represent 10 μm.

Sperm Nuclear Remodeling during Porcine IVF

The penetrated sperm nucleus is remodeled from sperm-specific nuclear protein called protamines to somatic histones. This biochemical change or nuclear remodeling is considered to be accompanied by unique morphological changes that are required for successful fertilization. In summary, the cytological changes are as follows: after fusion of the acrosome-reacted sperm plasma membrane with the oocyte membrane, decondensation followed by recondensation into a mass of chromatin occurs, but the shape does not remain the same as the original. The recondensed nuclei have a fist shape instead of an oval spatulate shape. The nuclei then decondense again, resulting in MPN formation (typical porcine sperm heads after penetration are shown in Fig. 1). In the next section, we will focus on the sequences of morphological and molecular change in gamete chromatin during IVF in pigs.

Morphological changes in oocyte and sperm chromatin

After initiation of co-incubation of IVM oocytes and frozen-thawed epididymal spermatozoa [1–4] in pigs, the first evidence of sperm penetration into the ooplasm is seen at 2 h (Fig. 2A). At this time, the chromatin of the oocytes is still at metaphase-II (Fig. 3A), and none of the penetrated sperm heads have decondensed (Fig. 3B). The rate of penetration of oocytes by sperm (Fig. 2A) and the number of sperm heads per oocyte (Fig. 2B) increase with the time elapsed after insemination. The penetrated sperm heads begin to decondense 3 h after insemination, recondense from 5 h after insemination, and finally reach full development into MPNs by 8 h after insemination (Fig. 3B). The detailed morphological changes that occur in the penetrated boar sperm nuclei are shown in Fig. 1. Simultaneously, meiosis of the matured oocytes progresses from the metaphase-II to telophase-II stage with increasing time elapsed after insemination. Finally, the oocyte produces a female pronucleus after extrusion of the second polar body (Fig. 3A). In our system, we conducted co-culture of gametes for only 3 h to reduce the chance of gamete encounter and thus help to prevent polyspermy. However, the results of IVF could be dramatically different if the conditions for IVF were different, for example, in terms of the medium used and the time allowed for insemination. The morphological changes in the penetrating sperm nuclei have not been

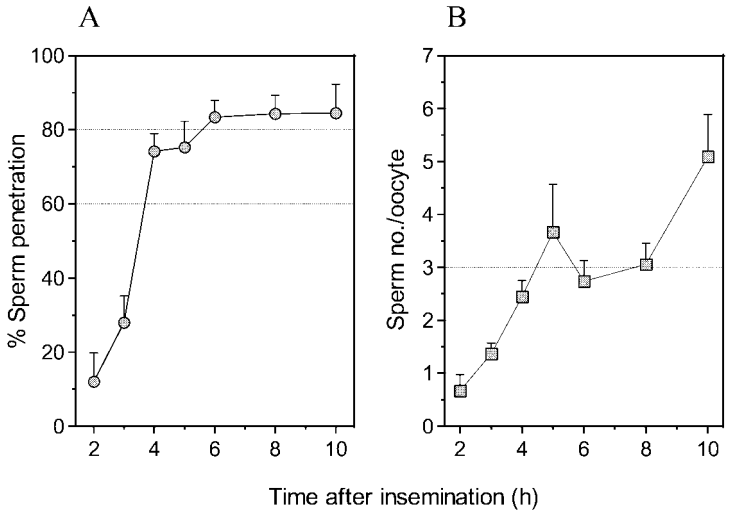

Fig. 2. Rate of sperm penetration and number of spermatozoa per oocyte. Porcine oocytes matured *in vitro* were inseminated and fixed in whole-mount preparations 2, 3, 4, 5, 6, 8, or 10 h after insemination. The oocytes were stained with 1% (w/v) aceto-orcein and examined by phase-contrast microscopy. Means ± SEM are presented.

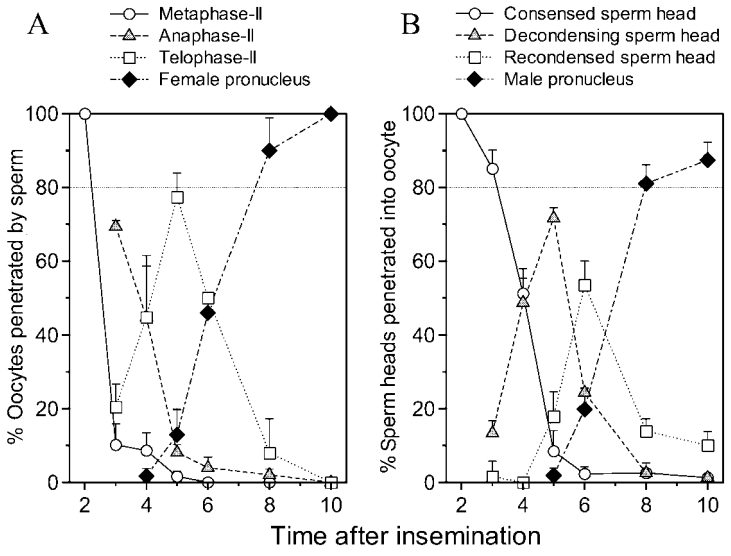

Fig. 3. Oocyte nuclear status (A) and morphological changes in penetrating sperm nuclei of pig oocytes fertilized *in vitro* (B). Sperm penetration was initiated 2 h after insemination; however, the oocyte chromatin was arrested at the metaphase-II stage. Oocytes were activated and their nuclear status proceeded to the anaphase-II stage at 3 h after insemination. After reaching the telophase-II stage, the female pronucleus began to develop. Female pronucleus formation with a second polar body occurred before 10 h after insemination in all oocytes penetrated by sperm. At 2 h after insemination, all of the spermatozoa were condensed. Decondensing nuclei began to be observed after 3 h and male pronuclei after 5 h. Male pronuclear formation occurred in about 80% of the penetrated sperm nuclei. Means ± SEM are presented.

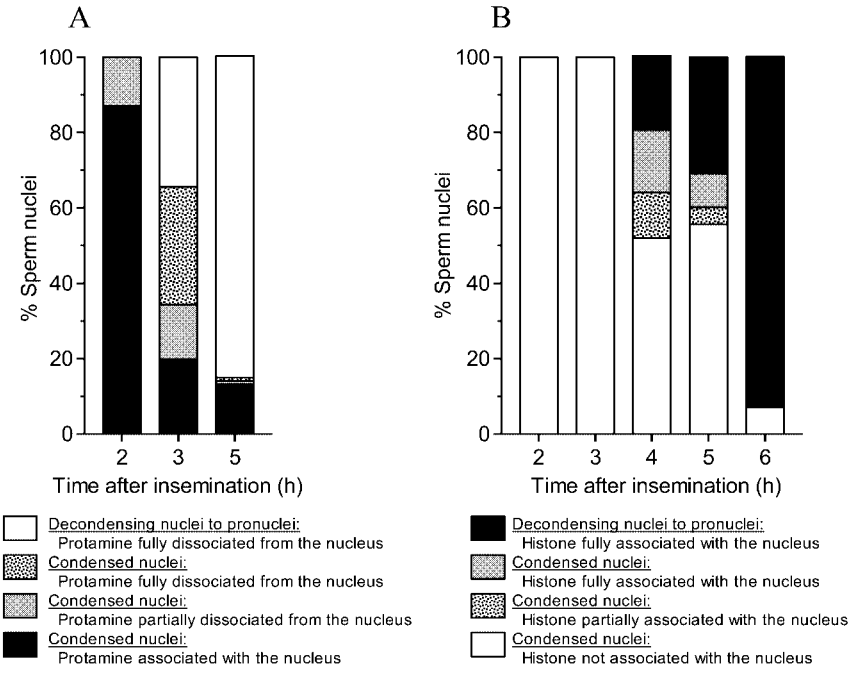

Fig. 4. Nuclear remodeling of boar sperm nuclei during *in vitro* fertilization. A) Protamine dissociation from the nucleus and B) histone association with the nucleus. Two hours after insemination, protamine dissociation was initiated from the penetrating sperm nuclei, which were still condensed. Four hours after insemination, histone association with the nuclei, which were also condensed, was observed. Recondensed nuclei could not be distinguished from condensed ones.

reported in detail in any mammal, with the sole exception of mice [5]. The results of that study and our findings suggest that the length of time from the initiation of anaphase or telophase in oocytes to MPN formation of the penetrated sperm nuclei is similar in the two species (mouse, 160 min; pig, 2–3 h).

Protamine dissociation and histone association of penetrated sperm heads

As spermatogenesis or sperm maturation proceeds, the sperm nuclear DNA becomes packed tightly with strongly basic DNA-binding proteins, which are termed protamines or sperm-specific nuclear proteins. Mammalian sperm protamines, which contain cysteine, differ from those of other species, such as fish, amphibians, and birds [6, 7]. The fundamental structure of sperm chromatin is not completely understood; however, it has been shown that mammalian sperm protamines have many disulfide (S–S) bonds. The formation of these S–S linkages makes sperm nuclei tight, or compact, and resistant to detergents [8, 9], trypsin [10], and micrococcal nuclease [11]. It has been suggested that chromosomal remodeling after penetration of sperm into the ooplasm requires two steps, reduction of S–S to S–H and then, replacement of protamines by somatic histones [12]. Recently, it has been reported that the ability for S–S reduction in the ooplasm depends on the concentration of free thiol glutathione [7, 13] resulting from cytoplasmic maturation [14, 15].

In our laboratory, we used immunohistochemical methods to evaluate the correlation between morphological changes in the penetrating sperm nuclei and the dynamics of protamine dissociation from, or histone association into, sperm nuclei during IVF of porcine oocytes. We purified protamines from porcine sperm nuclei and developed an antiserum against them. After affinity purification, we used the antiserum to perform immunohistochemical analysis of paraffin sections of the inseminated oocytes [16]. The proportion of sperm nuclei in a condensed state that reacted with the antiserum was 87% for sperm nuclei that had penetrated at 2 h post-insemination, and this decreased to 20% and 13% for penetration at 3 h and 5 h post-insemination, respectively (Fig. 4A). During the whole insemination period, none of the decondensing

sperm nuclei or MPNs reacted with the antiserum. These results indicate that protamine is dissociated from boar sperm nuclei before decondensation during IVF.

Next, we used immunohistochemical analysis with monoclonal anti-histone H1 antibody (Leinco Technologies, Inc., St. Louis, MO, USA) [17] to evaluate the replacement of nuclear protein by histone in boar spermatozoa during IVF. Immunohistochemistry of serial sections of oocytes that had been matured and co-cultured with boar spermatozoa for 2 to 6 h was carried out. At 2 to 3 h after insemination, penetrating sperm nuclei in the condensed state were not immunoreactive (Fig. 4B). At 4 to 5 h after insemination, some of the condensed sperm nuclei were immunoreactive in part or the whole area of the nucleus, and all of the decondensing nuclei and MPNs were immunoreactive. At 6 h after insemination, the decondensing sperm nuclei and well-developed MPNs were immunoreactive.

These results suggest that remodeling of sperm nuclear protein from protamines to histone H1 is initiated at the time of sperm penetration and before the onset of decondensation and MPN formation in pigs. However, some studies of mammalian fertilized oocytes have concluded that removal of protamine during fertilization occurs as the sperm nucleus decondenses [18]. Furthermore, protamine loss has been reported to occur as histones appear in the well-developed MPN of mice [19]. Until now, the reasons for this discrepancy in the timing of protamine disappearance among species had not been clarified. Spermatozoa from many species, including boars, contain only a single type of protamine, called protamine 1 [20]. However, two types of protamine, protamine 1 and 2, have been reported in mice [21]. This variety in protamines may mean that protamines are remodeled in mice through a pathway different from that in pigs and other mammals. A recent study of somatic cell transfer into mature porcine oocytes suggests that nuclear remodeling, including one of the core histones (histone H3), phosphorylation, and acetylation may be important for reprogramming of cloned embryos [22]. However, precise information on the core histone modification during remodeling of penetrated sperm nuclei is not available in relation to fertilization of mammalian oocytes, although information is available for sea urchin [23] and *Xenopus* [24] oocytes.

Unsolved problems in porcine IVF

In pigs, successful IVF of *in vitro*-matured oocytes from unfrozen [25] and frozen-thawed [26] spermatozoa has been reported previously. Since then, polyspermic fertilization has been the most important and unsolved problem in porcine IVF [27]. Polyspermy can also be observed in *in vivo* fertilization of *in vivo*-matured oocytes under physiological [28] and experimental conditions when an excessive number of capacitated sperm are present at the fertilization site [29]; however, the incidence of polyspermy is much higher in IVF (65%) than in *in vivo* fertilization (30–40%) [30]. The molecular mechanism for preventing polyspermy is not understood clearly [31]. Furthermore, the data from Han *et al.* [32] and our (Somfai *et al.*, unpublished observations) laboratory suggests the possibility of embryonic development even after polyspermy, although the ploidy of polyspermic embryos is determined at the zygote stage before the first cell division in accordance with the pronuclear location [33]. Information on this phenomenon is limited, and therefore generation of porcine embryos capable of normal development to the piglet stage is open to further discussion.

ICSI and Recent Advances

ICSI is a technique being established in many mammals to generate live offspring when their spermatozoa lack motility, resulting in infertility. Since the 1990s, there have been reports of successful production of live offspring after ICSI using both *in vivo*-matured (human [34] and mouse [35–37]) and IVM (cattle [38], ram [39], and rabbit [40]) oocytes. These reports confirm the cytoplasmic ability for fertilization and development to term after ICSI in both *in vivo*-matured and IVM oocytes in mammals. Boar spermatozoa are capable of being frozen and stored as genetic resources, but they sometimes show great loss of motility, or immotility, after thawing, depending upon the individual from which the spermatozoa was collected [41]. For such spermatozoa, ICSI is the optimum procedure for producing the next generation of pigs. The first successful production of piglets after ICSI was reported using *in vivo*-matured oocytes [42, 43]. Several years later, the use of porcine IVM oocytes for ICSI resulted in living piglets [44–47]. However, the rate of development to the piglet stage has remained low, and it is therefore important to seek possibilities for effectively generating embryos after ICSI that have the ability to develop to term. In the next section, we will focus on the differences between ICSI and physiological fertilization and discuss the attempts to overcome these situations.

Table 1. Fertilization and embryonic development by sperm after treatment for acrosome reaction

Report	Treatment for acrosome reaction	Sperm without an acrosome (%)	Male pronuclear formation[a] (%)	Blastocyst[b] %	Blastocyst[b] Average cell no.
Katayama *et al.* [50]	1 mg/ml progesterone for 10 min	61.4[#]*	48.5*	–	–
	Control	5.6[#]	25.7	–	–
Nakai *et al.* [44]	10 μM calcium ionophore for 120 min	78.6[$]*	–	8.6	25.6
	Control	58.4[$]	–	4.0	22.7

[a]Oocytes were examined at 12 h post-insemination. [b]Embryos were examined on the sixth day post-insemination. [#]Acrosome statuses were examined under transmission electron microscope. [$]Acrosome statuses were examined and under light microscope after triple-staining. *The data of the treated group was significantly higher than that of the control ($P<0.05$).

Acrosome introduced into the ooplasm

In many cases during ICSI, the spermatozoa with their complete structures are injected into ooplasm. The acrosome, with its content, outer membrane, and cell membrane, is still present around the sperm nucleus in the ooplasm, even after injection. In mice, both the membrane [48] and acrosomal content [49] may induce failure of completion of fertilization and development. Previous reports using porcine oocytes have suggested that removal of the acrosomal membrane causes delay or failure of MPN formation [50], but it does not affect embryonic development to the blastocyst stage [44] (Table 1). Katayama *et al.* [50] reported that MPN formation after injection with acrosome reaction-induced spermatozoa was 48.5%, but without treatment, it was 25.7%. Nakai *et al.* [44] reported that the rates of blastocyst formation were not significantly different between the two groups. The rates for oocytes injected with acrosome reaction-induced sperm heads and for those injected with control sperm heads were 8.6% and 4.0%, respectively. The mean number of cells in the blastocysts did not differ significantly between the two groups (25.6 and 22.7, respectively). Although the methods for induction of acrosome reaction differed between the two papers (progesterone and calcium ionophore A23187, respectively), there seems to be a certain mechanism supporting embryonic development after incorporation of either acrosome- or membrane-intact spermatozoa. In fact, the acrosome appears to disintegrate in the ooplasm even when the acrosome reaction does not take place before injection [51].

Induced decondensation of sperm nuclei and oocyte activation

In many injected porcine oocytes, the female pronucleus forms but most of the sperm heads do not decondense [52]. As stated above, this phenomenon has a close relationship to the sperm chromatin remodeling that accompanies morphological change. The injected sperm nuclei must be remodeled into the typical somatic state and must then participate in subsequent chromosomal activities, such as nuclear decondensation and formation of an MPN. Remodeling of sperm nuclei requires the reduction of S–S bonds, a process regulated by ooplasmic glutathione [7, 13, 14, 53], and the replacement of protamines by histones [12]. These events are considered essential for the completion of fertilization in pigs [16, 17]. If it were possible to regulate the S–S status of sperm nuclei before injection, the relationship between S–S reduction and sperm decondensation or pronucleus formation would become clearer and the efficacy of porcine ICSI might be improved. On the other hand, oocyte activation is also an event fundamental to embryonic development in ICSI. In mice [36, 54], hamsters [55], humans [56], and rabbits [57], ICSI alone is sufficient to activate oocytes for embryonic development. However, artificial oocyte activation in pigs is considered essential for successful ICSI; additional electrical stimulation after ICSI results in better embryonic development than injection alone [58]. It has also been reported that oocyte activation by electrical stimulation enhances both normal fertilization after injection of round spermatids [59] and the development of oocytes to the blastocyst stage after ICSI with frozen-thawed sperm [60]. However, the precise effect of oocyte stimulation on embryonic development, in relation to sperm decondensation has not yet been clarified. We conducted the following experiments to evaluate the effect of oocyte stimulation in relation to the induced decondensation of sperm heads before injection.

Spermatozoa were divided into two groups; one group was pretreated with 1% Triton X-100 (TX-100) and 5 mM dithiothreitol (DTT) (T+D group) and the other group was left untreated. They were then injected into *in vitro* matured oocytes [61]. Similarly, electrical stimulation (1.5 kV/cm, 20-μs DC pulse) was either

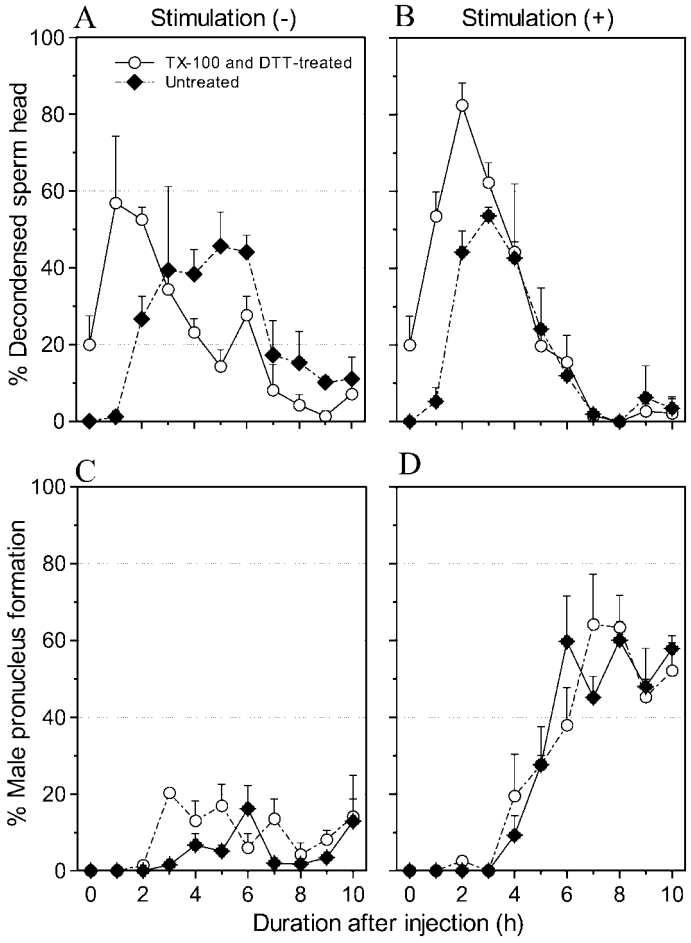

Fig. 5. Time-dependent changes in the rates of decondensation (A, B) and pronucleus formation (C, D) of boar sperm heads every 1 h after injection into ooplasm. Heads were untreated or pretreated with 1% Triton X-100 (TX-100) and 5 mM dithiothreitol (DTT). Electrical stimulation (1.5 kV/cm, 20-μs DC pulse) was applied to the oocytes 1 h after injection [B, D; stimulation (+)] or was not applied [A, C; stimulation (–)]. Mean percentages ± SEM are presented.

applied (stimulated group) or not applied (unstimulated group) to oocytes 1 h after injection. Morphological changes in the sperm nuclei were evaluated at hourly intervals until 10 h post-injection. Unstimulated oocytes injected with untreated spermatozoa showed a delayed peak in the rate of nuclear decondensation (39.4% to 44.1%, 3–6 h after injection) compared with oocytes injected with T+D-treated spermatozoa (57.0% and 52.6%, 1 and 2 h, respectively) (Fig. 5A). However, in stimulated oocytes, the peak rate in the untreated group occurred earlier (42.6–44.1%, 2–4 h) and was at the same time as that observed in the T+D-treated group (82.5%, 2 h) (Fig. 5B). The rate of MPN formation peaked 6 h after stimulation (40% to 60%) after injected oocytes were stimulated with an electrical pulse, irrespective of whether or not the spermatozoa had been pretreated (Fig. 5D). In unstimulated oocytes, the rate of MPN formation did not increase and stayed at the basal level (less than 20%) throughout the culture period, regardless of the sperm treatment. Thus, T+D treatment of spermatozoa did not affect completion of fertilization (Fig. 5C). We evaluated the effects of sperm treatment with T+D and electrical stimulation on the rate of blastocyst formation and the mean number of

cells per blastocyst. Oocytes stimulated after injection with either T+D-treated or untreated spermatozoa showed significantly higher percentages of blastocyst formation (24.8% and 27.1%, respectively) than unstimulated oocytes (1.1% and 4.1% for T+D-treated and untreated, respectively; $P<0.01$). The rate of blastocyst formation did not differ between the T+D-treated and untreated groups. The mean number of cells per blastocyst did not differ among any of the groups (14.0–29.4 cells). These results suggest that pretreatment of sperm with TX-100 and DTT shifted the timing of sperm nuclear decondensation forward. However, pronucleus formation and development to the blastocyst stage *in vitro* were not improved by sperm treatment. Thus, electrical stimulation of injected oocytes, but not chemical pretreatment of sperm, enhances *in vitro* development to the blastocyst stage in pigs.

Chromatin remodeling after ICSI

No detailed observations of chromatin remodeling from protamines to histones in injected sperm heads have previously been reported elsewhere or in our laboratories. DTT treatment of sperm before injection into the ooplasm induces decondensation in the ooplasm after injection [61]. Completion of replacement of nuclear proteins may be affected by the degree of decondensation, which also seems to be an important factor for the success of fertilization in ICSI.

Applications of ICSI in pigs

The utilization of immature sperm cells in ICSI has been suggested. In mice, round spermatids [62–64], secondary spermatocytes [65], and primary spermatocytes [66–68] can be used for successful offspring production. In pigs, round spermatids have been injected [59, 69] and embryos at the blastocyst stage have been generated [70], but, to our knowledge, no one has been successful at piglet production after injection of immature sperm cells, including round spermatids.

ICSI enables the use of new methods for conservation of spermatozoa; one of them is freeze-drying. Viable offspring have been produced by ICSI of freeze-dried spermatozoa in mice [71–74], rabbits [75], and rats [76]. However, in pigs [77] and cattle [78], it has only been reported that oocytes resulting from ICSI with freeze-dried spermatozoa have developed to the blastocyst stage. Recently, fertilization by ICSI with freeze-dried and rehydrated boar sperm and transfer of the putative zygotes to recipients in our laboratory have resulted in fetal development to day 39 of gestation [79]. This suggests the possibility of successful piglet production from freeze-dried spermatozoa.

ICSI is now considered to be a useful procedure, not only for producing live offspring from non-motile sperm cells, but also for generating transgenic animals through ICSI-mediated gene transfer. Reports of successful transgenic offspring production by this method have accumulated in mice [80] and pigs [46, 47, 81]. This method is expected to have advantages over conventional methods, such as pronuclear injection [60].

Conclusion

Recent progress in porcine IVF and ICSI has opened up the possibility of benefits to pork production and biomedical sciences; however, there are still problems with the efficacy of embryo and piglet production. Normalization during fertilization, at the time when new life begins, is still needed for these technologies, and for this reason, further investigations are still necessary.

Acknowledgements

The authors acknowledge to Drs. I. Domeki, M. Namano, K. Akama, T. Nagai, J. H. Kaneko, J. Noguchi, Y. Nakazawa, K. Ohnuma, M. Ozawa, and N. Maedomari for performing experiments and discussion. The authors also thank Mss. T. Aoki, E. Yamauchi, and C. Terui for their technical assistance.

References

1) Kikuchi, K., Naito, K., Dean, F.P., Izaike, Y. and Toyoda, Y. (1995): Histone H1 kinase activity during in vitro fertilization of pig follicular oocytes matured in vitro. Theriogenology, 43, 523–532.
2) Kikuchi, K., Nagai, T., Kashiwazaki, N., Ikeda, H., Noguchi, J., Shimada, A., Soloy, E. and Kaneko, H. (1998): Cryopreservation and ensuing in vitro fertilization ability of boar spermatozoa from epididymides stored at 4°C. Theriogenology, 50, 615–623.
3) Kikuchi, K., Kashiwazaki, N., Noguchi, J., Shimada, A., Takahashi, R., Hirabayashi, M., Shino, M., Ueda, M. and Kaneko, H. (1999): Developmental competence, after transfer to recipients, of porcine oocytes matured, fertilized, and cultured in vitro. Biol. Reprod., 60, 336–340.
4) Kikuchi, K., Onishi, A., Kashiwazaki, N., Iwamoto, M., Noguchi, J., Kaneko, H., Akita, T. and Nagai, T. (2002): Successful piglet production after transfer of blastocysts produced by a modified in vitro system. Biol. Reprod., 66, 1033–1041.

5) Adenot, P.G., Szollosi, M.S., Geze, M., Renard, J.P. and Debey, P. (1991): Dynamics of paternal chromatin changes in live one-cell mouse embryo after natural fertilization. Mol. Reprod. Dev., 28, 23–34.
6) Poccia, D. (1986): Remodeling of nucleoproteins during gametogenesis, fertilization, and early development. Int. Rev. Cytol., 105, 1–65.
7) Zirkin, B.R., Perreault, S.D. and Naish, S.J. (1989): Formation and function of the male pronucleus during mammalian fertilization. In: The Molecular Biology of Fertilization, pp. 91–114, Academic Press, San Diego.
8) Calvin, H.I. and Bedford, J.M. (1971): Formation of disulphide bonds in the nucleus and accessory structures of mammalian spermatozoa during maturation in the epididymis. J. Reprod. Fertil. Suppl., 13, 65–75.
9) Bedford, J.M. and Calvin, H.I. (1974): The occurrence and possible functional significance of -S-S- crosslinks in sperm heads, with particular reference to eutherian mammals. J. Exp. Zool., 188, 137–156.
10) Meistrich, M.L., Reid, B.O. and Barcellona, W.J. (1976): Changes in sperm nuclei during spermatogenesis and epididymal maturation. Exp. Cell Res., 99, 72–78.
11) Tobita, T., Tanaka, H., Tanaka, K., Tanaka, T., Kojima, S. and Nakano, M. (1984): Characteristic of cysteinyl sulfhydryls and nuclease sensitivity of boar sperm nuclei during epididymal maturation. Bioch. International, 9, 161–168.
12) Yanagimachi, R. (1994): Mammalian fertilization. In: The Physiology of Reproduction, 2nd ed., pp. 189–317, Raven Press Ltd., New York.
13) Perreault, S.D., Barbee, R.R. and Slott, V.L. (1988): Importance of glutathione in the acquisition and maintenance of sperm nuclear decondensing activity in maturing hamster oocytes. Dev. Biol., 125, 181–186.
14) Yoshida, M. (1993): Role of glutathione in the maturation and fertilization of pig oocytes in vitro. Mol. Reprod. Dev., 35, 76–81.
15) Yoshida, M., Ishigaki, K., Nagai, T., Chikyu, M. and Pursel, V.G. (1993): Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. Biol. Reprod., 49, 89–94.
16) Shimada, A., Kikuchi, K., Noguchi, J., Akama, K., Nakano, M. and Kaneko, H. (2000): Protamine dissociation before decondensation of sperm nuclei during in vitro fertilization of pig oocytes. J. Reprod. Fertil., 120, 247–256.
17) Nakazawa, Y., Shimada, A., Noguchi, J., Domeki, I., Kaneko, H. and Kikuchi, K. (2002): Replacement of nuclear protein by histone in pig sperm nuclei during in vitro fertilization. Reproduction, 124, 565–572.
18) Perreault, S.D. (1992): Chromatin remodeling in mammalian zygotes. Mutation Res., 296, 43–55.
19) Nonchev, S. and Tsanev, R. (1990): Protamine-histone replacement and DNA replication in the male mouse pronucleus. Mol. Reprod. Dev., 25, 72–76.
20) Balhorn, R. (1989): Mammalian protamines: structure and molecular interactions. In: Molecular Biology of Chromosome Function (Adolph, K.W., ed.), pp. 366–395, Springer Verlag, New York.
21) Bellve, A.R., McKay, D.J., Renaux, B.S. and Dixon, G.H. (1988): Purification and characterization of mouse protamines P1 and P2: amino acid sequence of P2. Biochemistry, 27, 2890–2897.
22) Bui, H.T., Van Thuan, N., Wakayama, T. and Miyano, T. (2006): Chromatin remodeling in somatic cells injected into mature pig oocytes. Reproduction, 131, 1037–1049.
23) Oliver, M.I., Concha, C., Gutierrez, S., Bustos, A., Montecino, M., Puchi, M. and Imschenetzky M. (2002): Remodeling of sperm chromatin after fertilization involves nucleosomes formed by sperm histones H2A and H2B and two CS histone variants. J. Cell Biochem., 85, 851–859.
24) Arnan, C., Saperas, N., Prieto, C., Chiva, M. and Ausio, J. (2003): Interaction of nucleoplasmin with core histones. J. Biol. Chem., 278, 31319–31324.
25) Iritani, A., Niwa, K. and Imai, H. (1978): Sperm penetration in vitro of pig follicular oocytes matured in culture. J. Reprod. Fertil., 54, 379–383.
26) Nagai, T., Takahashi, T., Masuda, H., Shioya, Y., Kuwayama, M., Fukushima, M., Iwasaki, S. and Hanada, A. (1988): In-vitro fertilization of pig oocytes by frozen boar spermatozoa. J. Reprod. Fertil., 84, 585–591.
27) Nagai, T., Funahashi, H., Yoshioka, K. and Kikuchi, K. (2006): Up date of in vitro production of porcine embryos. Front. Biosci., 11, 2565–2573.
28) Hunter, R.H.F. (1967): Polyspermic fertilization in pigs during the luteal phase of the estrous cycle. J. Exp. Zool., 65, 451–459.
29) Hunter, R.H.F. (1973): Polyspermic fertilization in pigs after tubal deposition of excessive numbers of spermatozoa. J. Exp. Zool., 183, 57–63.
30) Xia, P., Wang, Z., Yang, Z., Tan, J. and Qin, P. (2001): Ultrastructural study of polyspermy during early embryo development in pigs, observed by scanning electron microscope and transmission electron microscope. Cell Tissue Res., 303, 271–275.
31) Sun, Q.Y. and Nagai, T. (2003): Molecular mechanisms underlying pig oocyte maturation and fertilization. J. Reprod. Dev., 49, 347–359.
32) Han, Y.M., Abeydeera, L.R., Kim, J.H., Moon, H.B., Cabot, R.A., Day, B.N. and Prather, R.S. (1999): Growth retardation of inner cell mass cells in polyspermic porcine embryos produced in vitro. Biol Reprod., 60, 1110–1113.
33) Han, Y.M., Wang, W.H., Abeydeera, L.R., Petersen, A.L., Kim, J.H., Murphy, C., Day, B.N. and Prather, R.S. (1999): Pronuclear location before the first cell division determines ploidy of polyspermic pig embryos. Biol. Reprod., 61, 1340–1346.
34) 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.
35) Ahmadi, A., Ng, S.C., Liow, S.L., Ali, J., Bongso, A., and Ratnam, S.S. (1995): Intracytoplasmic sperm injection of mouse oocytes with 5 mM Ca2+ at different intervals. Hum. Reprod., 10, 431–435.

36) Kimura, Y. and Yanagimachi, R. (1995): Intracytoplasmic sperm injection in the mouse. Biol. Reprod., 52, 709–720.
37) 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.
38) Goto, K., Kinoshita, A., Takuma, Y. and Ogawa, K. (1991): Birth of calves after the transfer of oocytes fertilized by sperm injection. Theriogenology, 35, 205.
39) Catt, S.L., Catt, J.W., Gomez, M.C., Maxwell, W.M. and Evans, G. (1996): Birth of a male lamb derived from an in vitro matured oocyte fertilised by intracytoplasmic injection of a single presumptive male sperm. Vet. Rec., 139, 494–495.
40) Li, G.P., Chen, D.Y., Lian, L., Sun, Q.Y., Wang, M.K., Liu, J.L., Li, J.S. and Han, Z.M. (2001): Viable rabbits derived from reconstructed oocytes by germinal vesicle transfer after intracytoplasmic sperm injection (ICSI). Mol. Reprod. Dev., 58, 180–185.
41) Woelders, H., Matthijs, A., and Den Besten, M. (1996): Boar variation in 'freezability' of the semen. Proceedings III Conference on Boar Semen Preservation. Reprod. Dom. Anim., 31, 153–159.
42) Kolbe, T. and Holtz, W. (2000) Birth of a piglet derived from an oocyte fertilized by intracytoplasmic sperm injection (ICSI). Anim. Reprod. Sci., 64, 97–101.
43) Martin, M.J. (2000): Development of in vivo-matured porcine oocytes following intracytoplasmic sperm injection. Biol. Reprod., 63, 109–112.
44) Nakai, M., Kashiwazaski, N., Takizawa, A., Hayashi, Y., Nakatsukasa, E., Fuchimoto, D., Noguchi, J., Kaneko, H., Shino, M. and Kikuchi, K. (2003): Viable piglets generated from porcine oocytes matured in vitro and fertilized by intracytoplasmic sperm head injection. Biol. Reprod., 68, 1003–1008.
45) Probst, S. and Rath, D. (2003): Production of piglets using intracytoplasmic sperm injection (ICSI) with flowcytometrically sorted boar semen and artificially activated oocytes. Theriogenology, 59, 961–973.
46) Kurome, M., Ueda, H., Tomii, R., Naruse, K. and Nagashima, H. (2006): Production of transgenic-clone pigs by the combination of ICSI-mediated gene transfer with somatic cell nuclear transfer. Transgenic Res., 15, 229–240.
47) Yong, H.Y., Hao, Y., Lai, L., Li, R., Murphy, C.N., Rieke, A., Wax, D., Samuel, M. and Prather, R.S. (2006): Production of a transgenic piglet by a sperm injection technique in which no chemical or physical treatments were used for oocytes or sperm. Mol. Reprod. Dev., 73, 595–599.
48) Kasai, T., Hoshi, K. and Yanagimachi, R. (1999): Effect of sperm immobilisation and demembranation on the oocyte activation rate in the mouse. Zygote, 7, 187–193.
49) Morozumi, K. and Yanagimachi, R. (2005): Incorporation of the acrosome into the oocyte during intracytoplasmic sperm injection could be potentially hazardous to embryo development. Proc. Natl. Acad. Sci. USA., 102, 14209–14214.
50) Katayama, M., Miyano, T., Miyake, M. and Kato, S. (2002): Progesterone treatment of boar spermatozoa improves male pronuclear formation after intracytoplasmic sperm injection into porcine oocytes. Zygote, 10, 95–104.
51) Katayama, M., Koshida, M. and Miyake, M. (2002): Fate of the acrosome in ooplasm in pigs after IVF and ICSI. Hum. Reprod., 17, 2657–2664.
52) Kren, R., Kikuchi, K., Nakai, M., Miyano, T., Ogushi, S., Nagai, T., Suzuki, S., Fulka, J. and Fulka, J.Jr. (2003): Intracytoplasmic Sperm Injection in the Pig: Where is the Problem? J. Reprod. Dev., 49, 271–273.
53) Katagiri, C. and Ohsumi, K. (1994): Remodeling of sperm chromatin induced in egg extracts of amphibians. Int. J. Dev. Biol., 38, 209–216.
54) Kuretake, S., Kimura, Y., Hoshi, K. and Yanagimachi, R. (1996): Fertilization and development of mouse oocytes injected with isolated sperm heads. Biol. Reprod., 55, 789–795.
55) Hoshi, K., Yanagida, K. and Sato, A. (1992): Pretreatment of hamster oocytes with Ca2+ ionophore of facilitate fertilization by ooplasmic micro-injection. Hum. Reprod., 7, 871–875.
56) Tesarik, J. and Sousa, M. (1995): More than 90% fertilization rates after intracytoplasmic sperm injection and artificial induction of oocyte activation with calcium ionophore. Fertil. Steril., 63, 343–349.
57) Keefer, C.L. (1989): Fertilization by sperm injection in the rabbit. Gamete Res., 22, 59–69.
58) 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.
59) Kim, N.H., Shin, J.S., Kim. C,, Jun, S.H., Lee, H.T. and Chung, K.S. (1999): Fertilization and in vitro development of porcine oocytes following intracytoplasmic injection of round spermatid or round spermatid nuclei. Theriogenology, 51, 1441–1449.
60) Nagashima, H., Fujimura, T., Takahagi, Y., Kurome, M., Wako, N., Ochiai, T., Esaki, R., Kano, K., Saito, S., Okabe, M. and Murakami, H. (2003): Development of efficient strategies for the production of genetically modified pigs. Theriogenology, 59, 95–106.
61) Nakai, M., Kashiwazaki, N., Takizawa, A., Maedomari, N., Ozawa, M., Noguchi, J., Kaneko, H., Shino, M. and Kikuchi, K. (2006): Morphological changes in boar sperm nuclei with reduced disulfide bonds in electrostimulated porcine oocytes. Reproduction, 131, 603–611.
62) Ogura, A., Matsuda, J. and Yanagimachi, R. (1994): Birth of normal young after electrofusion of mouse oocytes with round spermatids. Proc. Natl. Acad. Sci. USA., 91, 7460–7462.
63) Kimura, Y. and Yanagimachi, R. (1995): Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. Development, 121, 2397–2405.

64) Sasagawa, I. and Yanagimachi, R. (1996): Comparison of methods for activating mouse oocytes for spermatid nucleus transfer. Zygote, 4, 269–274.
65) Kimura, Y. and Yanagimachi, R. (1995): Development of normal mice from oocytes injected with secondary spermatocyte nuclei. Biol. Reprod., 53, 855–862.
66) Kimura, Y., Tateno, H., Handel, M.A. and Yanagimachi, R. (1998): Factors affecting meiotic and developmental competence of primary spermatocyte nuclei injected into mouse oocytes. Biol. Reprod., 59, 871–877.
67) Ogura, A., Suzuki, O., Tanemura, K., Mochida, K., Kobayashi, Y. and Matsuda, J. (1998): Development of normal mice from metaphase I oocytes fertilized with primary spermatocytes. Proc. Natl. Acad. Sci. USA., 95, 5611–5615.
68) Sasagawa, I., Kuretake, S., Eppig, J.J. and Yanagimachi, R. (1998): Mouse primary spermatocytes can complete two meiotic divisions within the oocyte cytoplasm. Biol. Reprod., 58, 248–254.
69) Lee, J.W., Kim, N.H., Lee, H.T. and Chung, K.S. (1998): Microtubule and chromatin organization during the first cell-cycle following intracytoplasmic injection of round spermatid into porcine oocytes. Mol. Reprod. Dev., 50, 221–228.
70) Choi, J.Y., Lee, E.Y., Cheong, H.T., Yoon, B.K., Bae, D.S. and Choi, D.S. (2004): Effects of activation timing on the fertilization rate and early embryo development in porcine ROSI procedure. J. Assist. Reprod. Genet., 21, 329–334.
71) Wakayama, T. and Yanagimachi, R. (1998): Development of normal mice from oocytes injected with freeze-dried spermatozoa. Nat. Biotechnol., 16, 639–641.
72) Kusakabe, H., Szczygiel, M.A., Whittingham, D.G. and Yanagimachi, R. (2001): Maintenance of genetic integrity in frozen and freeze-dried mouse spermatozoa. Proc. Natl. Acad. Sci. USA., 98, 13501–13506.
73) Kaneko, T., Whittingham, D.G. and Yanagimachi, R. (2003): Effect of pH value of freeze-drying solution on the chromosome integrity and development ability of mouse spermatozoa. Biol. Reprod., 68, 136–139.
74) Kaneko, T., Whittingham, D.G., Overstreet, J.W. and Yanagimachi, R. (2003): Tolerance of the mouse sperm nuclei to freeze-drying depends on their disulphide status. Biol. Reprod., 69, 1859–1862.
75) Liu, J.L., Kusakabe, H., Chang, C.C., Suzuki, H., Schmidt, D.W., Julian, M., Pfeffer. R., Bormann, C.L., Tian X.C., Yanagimachi, R. and Yang, X. (2004): Freeze-dried sperm fertilization leads to full-term development in rabbits. Biol. Reprod., 70, 1776–1781.
76) Hirabayashi, M., Kato, M., Ito, J. and Hochi, S. (2005): Viable rat offspring derived from oocytes intracytoplasmically injected with freeze-dried sperm heads. Zygote, 13, 79–85.
77) Kwon, I.K., Park, K.E. and Niwa, K. (2004): Activation, pronuclear formation, and development in vitro of pig oocytes following intracytoplasmic injection of freeze-dried spermatozoa. Biol. Reprod., 71, 1430–1436.
78) Keskintepe, L., Pacholczyk, G., Machinicka, 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.
79) Nakai, M., Kashiwazaki, N., Takizawa, A., Maedomari, N., Ozawa, O., Noguchi, J., Kaneko, H., Shino, M. and Kikuchi, K. (2006): Effects of chelating agents during freeze-drying of boar spermatozoa on DNA fragmentation and on developmental ability in vitro and in vivo after intracytoplasmic sperm head injection. Zygote, (in press).
80) Perry, A.C., Wakayama, T., Kishikawa, H., Kasai, T., Okabe, M., Toyoda, Y. and Yanagimachi, R. (1999): Mammalian transgenesis by intracytoplasmic sperm injection. Science, 284, 1180–1183.
81) Lai, L., Sun, Q., Wu, G., Murphy, C.N., Kuhholzer, B., Park, K.W., Bonk, A.J., Day, B.N. and Prather, R.S. (2001): Development of porcine embryos and offspring after intracytoplasmic sperm injection with liposome transfected or non-transfected sperm into in vitro matured oocytes. Zygote, 9, 339–346.