SRD Young Investigator Award

Factors Affecting Fertilization and Embryonic Development During Intracytoplasmic Sperm Injection in Pigs

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Abstract. In intracytoplasmic sperm injection (ICSI) technique, a sperm was injected into ooplasm directly using a glass pipette. The fertilization physiology in ICSI is considered quite different from that of the natural fertilization. The different mechanisms for fertilization may be the causes of various results in ICSI. In this paper, we focus on the state of sperm membranes, nuclear or DNA integrity during ICSI procedure and discuss the influence of these factors on fertilization and embryonic development. We also introduce some examples in application of ICSI for new technologies in pigs.

Key words: Embryonic development, Fertilization, ICSI, Pig

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During natural fertilization, sperm needs several steps; e.g., attachment to zona pellucida, penetration through zona pellucida with releasing of acrosomal enzymes from acrosomal caps, fusion of sperm membrane with oolemma, decondensation and recondensation of sperm nucleus after incorporation into ooplasm, and finally formation of male pronucleus [1]. Therefore, if sperm have no or faint motility, such sperm cannot reach and penetrate an oocyte resulting in a failure of fertilization. On the other hand, by intracytoplasmic sperm injection (ICSI), a whole spermatozoon or a sperm nucleus can be deposited directly into ooplasm by a glass pipette. Therefore, ICSI technique enables production of fertilized oocytes even if spermatozoa lack their competence for physiological fertilization.

As mentioned above, spermatozoa can be brought into ooplasm by ICSI without any physiological events. In other words, spermatozoa bring intact membrane and acrosomal enzymes into ooplasm. Furthermore, there causes a possibility that sperm with some abnormalities may participate in fertilization and further embryonic development resulting in offspring, because spermatozoa are injected into ooplasm by artificial procedure without physiological selection processes. In this paper, we discuss about sperm factors which may affect the completion of fertilization by ICSI in pigs as a model for large domestic animals. Furthermore, recently the combination of ICSI and some other techniques has been expected as novel procedure for conserving male genetic resource. For completion of this technology, ICSI is the most essential procedure. We introduced the recent results in our laboratory for these new approaches.

Carrying of Acrosomal Membrane and Their Enzymes Into Ooplasm

At the first step of fertilization, sperm have to travel through the cumulus cells and their matrix and then penetrate the zona pellucida. In natural fertilization, sperm release acrosomal enzymes from acrosome cap (acrosome reaction), move forward by a driving force of sperm tail. Therefore, after the penetration of zona pellucida, sperm without outer-acrosomal membrane and acrosomal enzymes reach to the oolemma. However, by ICSI, sperm do not lose those constructions and those with their complete form are injected into ooplasm. Therefore, it is important to discuss the effects of acrosome, acrosomal enzymes and sperm membranes on fertilization and embryonic development. It has been reported in mice that acrosomal enzymes may show harmful effects on fertilization (e.g. a deformation and lysis of oocytes) and also on embryonic development (e.g. decline in developmental ability to the blastocyst stage) [2]. Artificial induction of acrosome reaction before ICSI increases the rate of pronuclear formation in mice [3]. It has been also reported that damage of the sperm plasma membrane by squeezing its tail between the glass pipette and the bottom of the dish allows access by oocyte decondensing factors (e.g.; glutathione) to the sperm nucleus after ICSI in humans [4]. In contrast, ICSI with acrosome-reacted sperm have no effect on promotion of pronuclear formation in sheep [5]. The effect of existence of acrosome and their contents is variable depending of species. We evaluated in pigs the effect of the induction of acrosome reaction by calcium-ionophore prior to ICSI. We demonstrated that sperm without acrosomal membrane and enzymes did not affect the development of the blastocyst *in vitro* and the production of offsprings *in vivo* in pigs [6]. Therefore, we suggest that acrosomal membrane and enzymes have no negative effect on

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Table 1. Effects of treatment for induction of sperm nuclear decondensation and electrical stimulation to oocytes on fertilization and embryonic development after ICSI on pigs

Treatment	Stimulation	The rate of pronuclear formation	The rate of blastocyst formation	Mean no. of cells in blastocyst
T+D	+	52.2 ± 9.2 ^a	24.8 ± 4.8 ^a	24.8 ± 2.4
	–	14.2 ± 5.2 ^b	1.1 ± 1.0 ^b	14
Untreated	+	57.8 ± 1.6 ^a	27.1 ± 6.1 ^a	29.4 ± 2.3
	–	12.8 ± 12.0 ^b	4.1 ± 1.5 ^b	23.0 ± 3.6

Values different superscripts (a, b) within each column are significantly different; $P < 0.05$.

developmental ability of pig oocytes following ICSI.

Decondensation of the Injected Sperm Nucleus

Sperm nucleus is condensed highly and stabilized by formation of disulfide bonds between protamines, which is the sperm-specific basic proteins [7–9]. For formation of male pronucleus, sperm nucleus has to decondense with reduction of those disulfide bonds by ooplasmic glutathione [10–13] and replacement of the protamines by somatic histones within an oocyte [14]. These events are considered to be essential for the completion of fertilization [15], but a failure of decondensation in sperm nucleus has been observed in many ICSI pig oocytes [16]. A similar problem has been reported in cattle [17, 18]. Additionally, the delay or asynchronous development of the male pronucleus has been considered as a negative factor for fertilization in cattle oocytes [19]. Therefore, the injection of sperm after treatment with disulfide-reducing agents, such as dithiothreitol (DTT) has been considered to be a benefit for fertilization by ICSI. As DTT is known to promote decondensation of the sperm chromatin only after the sperm plasma membrane has been made permeable [20], a combination of DTT and a detergent such as Triton-X has been used for this purpose [7]. However, simultaneous treatment of sperm with DTT and Triton-X induces extensive chromosomal breakage [21]. In cattle, the rates of cleavage, blastocyst formation [22] and male pronuclear formation [23] in ICSI oocytes are improved by DTT pretreatment of sperm. It was also reported that DTT treated cattle sperm decondensed and formed pronuclear more rapidly in an oocyte after the injection [24]. However, in pigs, the effects of DTT treatment seem to be different among each paper. It was reported that DTT treatment increased the rate of normal fertilization and blastocyst formation on the one hand [25] and have no significantly affect on fertilization and embryonic development on the other [26]. In our study [27], pretreated sperm with DTT shifted the timing of sperm decondensation forward, but pronucleus formation and development to the blastocyst stage were not improved (Table 1). Therefore, we consider that other factors besides a status of sperm nucleus affect fertilization and embryonic development in pig ICSI oocytes.

Responsibility of Oocyte Activation for Pronuclear Formation

Successful ICSI for *in vivo* matured oocytes without any artificial oocyte activation has been reported resulting in piglet

production [28, 29]. In case of using *in vitro* matured pig oocytes for ICSI, we think that the artificial treatment for induction of oocyte activation (resumption of meiosis, estrusion of a 2nd polar body, pronuclear formation and DNA replication) is one of the most important factors for fertilization and embryonic development. Because electrical stimulation promoted pronuclear formation and development to the blastocyst stage [30–32]. These positive effects did not depend on status of sperm membrane and nuclei [27] (Table 1). There are also some effective artificial stimulation protocols for pig oocytes such as CaCl_2 [33] and sonication [34]. Production of piglets derived from *in vitro* matured oocytes has been also reported after electrical stimulation [6]. However, these data suggests that the injected sperm cannot induce enough oocyte activation for embryonic development. Sperm has oocyte activation factor, phospholipase $\text{C}\zeta$ (PLC ζ) [35]. In natural fertilization, PLC ζ is considered to diffuse into ooplasm after the fusion of sperm with oocytes and the oocyte activation phenomena are triggered. As the cause of insufficient oocyte activation after ICSI in pigs has been unknown, the mechanism for oocyte activation is very interesting scientifically and the investigation may contribute for establishment of the technology. The fact that *in vivo* matured pig oocytes are able to develop to offspring without any artificial stimulation after the sperm injection [28, 29] may cause a possibility that the source of oocyte may be related to this discrepancy.

Sperm Chromosomal Normality

The ICSI procedure makes immotile spermatozoa possible for participating in fertilization. However, there is currently a debate about the risk of sperm with abnormalities achieving fertilization, because physiological selection process such as binding to the zona pellucid, acrosomal reaction and fusion to the ooplasm, are bypassed [36, 37]. It has been reported that DNA-fragmented spermatozoa had adverse affect on *in vitro* embryonic development [38], where we used the DNA-fragmented sperm caused significantly after freeze-drying. There also reported the impaired embryo implantation and fetus development after ICSI in mice [39, 40]. DNA fragmentation in human spermatozoa is one of the causes of failure of embryonic development and pregnancy [41]. Therefore, the presence of structurally intact DNA in the sperm is quite important for normal embryogenesis.

The integrity of the sperm chromatin structure is affected by various factors, including the extender type and storage temperature [42]. Sperm contain endogenous nuclease in the inactivated form.

Fig. 1. Ectopic pig testicular xenograft in nude mice. A: Histological observation of testis from a 5-day old piglet; the testis does not have differentiated seminiferous tubule but seminiferous cords and contain only gonocytes/spermatogonia in them. B: Overview of host nude mice 125 days after xenografting. Testis tissues (red arrows) are the locations of grafting under the back skin. C: Histological observation of testis 158 days after xenografting; Spermatozoa were detected on this photograph. Appearance of spermatocytes and spermatids was observed on day 60 and day 90, respectively.

The fragmentation of sperm chromosomal DNA is caused by the nuclease activation [43]. The endogenous sperm nucleases are released from plasma membrane-damaged spermatozoa [44] and activated with divalent cations such as Mg^{2+} and Ca^{2+} [45]. Furthermore, it has been reported that incubation of spermatozoa with endogenous nuclease released from sperm leads to an increase in the proportion of DNA fragmented spermatozoa [39]. In our study, supplementation with EGTA, a chelating agent for the cations, in the buffer can inhibit the sperm DNA fragmentation and improved embryonic development after ICSI [38]. However, it also has been reported that sperm endogenous nucleases could be activated without divalent cations and the nuclease activity might be present even under physiological conditions [46]. We need further investigation about the correlation between activity of sperm endogenous nucleases and condition to use sperm with intact DNA for ICSI.

Utilization of ICSI

The usefulness of ICSI technique is enhanced in combination with other biotechnological methods. The followings are some examples in application of ICSI for new technologies in pigs, which have been studied recently in our laboratory.

ICSI with sperm grown in testicular tissues xenografted into immunodeficient mice

Recently, ectopic xenografting of testis tissue has been developed as novel tool for conserving and allowing sperm production from immature male animals. Although xenogeneic spermatogenesis after transplantation of donor germ cells to recipient testes has been established in experimental animals (mouse [47]; rat [48]; hamster [49]), transplantation of germ cells from large animal donors resulted in arrested spermatogenesis within host testes (pig, cattle, equine [50]). It seems that the ectopic xenogeneic spermatogenesis after germ cells transplantation depends on the phylogenetic distance between donor and recipient species [51]. Therefore, in large domestic animals, testicular xenografting into under skin of back of immunodeficient animals such as nude mice or SKID mice has been conducted. To date, sperm have been obtained from testicular grafts of several species after ectopic

xenotransplantation (pig [52–54]; goat [52]; cat [55]; and rhesus monkey [56]). In our study [57], we obtained testicular tissues from 5 days old piglet (Fig. 1-A) and xenografted under back skin of nude mice. Beyond day 120 after xenografting, tissues grown in the host mice (Fig. 1-B) and we confirmed complete spermatogenesis by histological observation (Fig. 1-C). Interestingly, xenogeneic sperm show motility [58]. Unfortunately, as the motility of xenogeneic sperm was too faint to use for *in vitro* fertilization, ICSI should be applied to produce zygotes. Honaramooz *et al.* [52] reported for the first time that male pronuclear formation after injection of xenogeneic pig and goat sperm to mouse oocytes; however, the developmental competence of the ICSI oocytes had not been described yet. In the later years, embryonic development to the blastocyst stage has been confirmed in monkey [56] and pigs [58, 59]. Our research group has been finally succeeded in producing of piglets using sperm obtained from ectopic testicular xenografts [60]. This report proves for the first time that oocytes fertilized with a sperm from ectopic xenografts have developmental ability to viable offspring in large domestic animals. It is expected now the application of ectopic xenografting of testis tissue to various wild and domestic animals as assistant reproductive technology in the future.

ICSI with freeze-dried sperm

Freeze-drying has been studied to establish new technique for sperm preservation substitute for cryopreservation in liquid nitrogen at $-196^{\circ}C$. It has been expected that the application of freeze-drying technology for sperm preservation should enable sperm storage at ambient temperatures or at $4^{\circ}C$ without liquid nitrogen. As freeze-dried spermatozoa are not survived and lose their motility after rehydration [44], ICSI is required for successful fertilization or embryo development. To date, viable offspring have been produced by ICSI of freeze-dried spermatozoa in mice [44, 61–63], rabbits [64], and rats [65]. On the other hand, in large domestic animals, it has been confirmed that oocytes resulting from ICSI with freeze-dried spermatozoa have developed to the blastocyst stage (pig [38, 66]; cattle [67]) and have grown to day 39 fetuses after zygote transfer (pig [38]). Although we need further attempts to precede freeze-dried sperm for practical use in domestic

animals, combination of ICSI and freeze-drying technique will contribute for preservation of male genetic resources.

Conclusion

ICSI technique has developed rapidly and has been regarded as important in reproductive field. On the other hand, it is true that safety aspects of ICSI should be discussed. We need to study further for the establishment of ICSI technique as safety and certain reproductive tool in the future.

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