Review Article

Intracytoplasmic Sperm Injection in Livestock Species: An Update

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Contents

Intracytoplasmic sperm injection (ICSI) is a powerful technique in the field of assisted reproduction (ART) and provides exciting opportunities for studying the basic mechanisms of fertilization and early embryo development. Nevertheless, its application in agriculture and conservation biology has been greatly hampered by the low success rate reported for this method in respect of economically important species. Specifically, the rates of blastocyst formation and live newborn are greatly reduced when zygotes are generated by ICSI. Except for humans, ICSI remains a low efficiency technology in comparison with alternatives such as in vitro fertilization (IVF) and its application is less widespread. In this paper, we discuss the present status, applications and factors affecting ICSI in pigs and other species.

Origins and Applications of ICSI

Intracytoplasmic sperm injection (ICSI) consists of fertilizing a metaphase II (MII) oocyte by the direct injection of a single spermatozoon into the ooplasm with both the acrosome and sperm membrane intact. This technique was reported with success for the first time in hamsters 30 years ago (Uehara and Yanagimachi 1976). Thereafter in human assisted reproduction, Palermo and collaborators fine-tuned this technique in 1992 (Palermo et al. 1992); ICSI has been much utilized mainly in order to solve male infertility. This technique has been used throughout the world as a treatment of last resort for men with extremely severe oligo- and asthenozoospermia but also as a 'more efficient' treatment in less severe cases in which standard IVF could be envisaged (Tesarik 1996). It is even possible to use epididymal and testicular sperm with similar results (Nagy et al. 1995; Shulman et al. 1999; Vernaeve et al. 2003).

In laboratory or livestock species, ICSI is also used as a reproductive option to solve different problems but the focus is not about low male fertility. In fact, in livestock species, the most probable future of males with reproductive problems, unless they have very high value (i.e. endangered species or with high genetic merit), is the slaughterhouse. Intracytoplasmic sperm injection, then, could be used in livestock species in several areas such as biodiversity conservation, transgenic production or to solve fertilization problems in IVF systems. Despite recent progress, in pigs, in vitro embryo production from abattoir ovaries has been limited by a high incidence of polyspermy (reviewed by Coy and Romar 2002). So, ICSI is presented as one alternative way for in vitro production of monospermic zygotes. Moreover, recent findings show that heterologous ICSI could represent a powerful tool to study human sperm functionality using the pig as oocyte donor (Canovas et al. 2007).

The use of sex-sorted semen is becoming more and more popular in the recent years because of their potential application in animal production. With this type of semen samples, where very low numbers of spermatozoa are available and they are mostly of low motility, ICSI could be of great help (Iritani 1991; Rath et al. 1999; Probst and Rath 2003).

After oocyte cryopreservation, ICSI may be a suitable technique (Fujihira et al. 2004; Rho et al. 2004; Fabbri 2006) as cryopreserved oocytes present problems of polyspermy by the early extrusion of cortical granules (Vincent et al. 1990), and ICSI bypasses these events.

Some research groups have pursued an alternative procedure to pronuclear injection for producing transgenic animals using sperm as vector to introduce genes (Sperm-mediated gene transfer: SMGT; review by Lavitrano et al. 2006). The production of transgenic animals by ICSI–SMGT has been achieved in pigs (Lai et al. 2001), mice (Perry et al. 1999; Moreira et al. 2004) and rats (Hirabayashi et al. 2005). New or improved technologies in mammalian transgenesis, such as the use of lentiviral vectors (Whitelaw 2004) and the 'renaissance' of ICSI approaches have shown that generation of transgenic animals now can be made easier (Moreira et al. 2007). However, behind all these successful achievements there are many failed experiments where ICSI produced embryos that did not develop to term.

Factors Affecting Developmental Ability of ICSI Produced Embryos

Sperm factors and early events at ICSI

Two main issues related to the sperm cell have been conceptualized to be affecting the developmental ability of ICSI-produced embryos, the sperm oocyte-activating factor and the introduction of sperm membranes into the ooplasm. Although the ICSI technique bypasses many of the normal sperm–oocyte interactions, most oocytes used for ICSI subsequently activate and become cleaved embryos. As long as the sperm nucleus has intact genetic integrity, ICSI can produce healthy offspring regardless of concentrations, morphology, and motility of spermatozoa (Yanagimachi 2005). Only one genomically non-damaged spermatozoon is needed to fertilize one oocyte. Two possible mechanisms exist

for oocyte activation (Fissore et al. 2002): these are the 'receptor theory' and the 'fusion/sperm factor theory'. Intracytoplasmic sperm injection supports the hypothesis that a sperm-associated product activates the Ca^{2+} releasing machinery of the oocyte from inside the oocyte cytoplasm, favouring an inside-out sequence of events to active Ca^{2+} release (Fissore et al. 2002). This finding suggests that signalling events, which are important for oocyte activation, could be triggered by sperm components in the absence of sperm–oocyte plasma membrane interactions, supporting the idea that a sperm factor is responsible for the initation of oocyte activation (Williams 2002). In fact, differences in sperm treatments prior to ICSI that may result in different amounts of damage to the sperm plasma membrane have been shown to affect the timing of onset of calcium oscillations, oocyte activation and pronuclear formation after ICSI (Yanagida et al. 1997; Kasai et al. 1999; Garcia-Rosello et al. 2006a; Morozumi et al. 2006).

Several candidate proteins have been proposed to be the sperm-borne oocyte-activating factor (SOAF, Williams 2002) that should induce calcium oscillations resembling those seen at fertilization, and it should be active when microinjected into oocytes. The strongest candidate for SOAF is phospholipase C *zeta* (Swann et al. 2004). At least part of SOAF is localized in the perinuclear theca in the post-acrosomal region (Kimura et al. 1998) and under the plasma membrane over the equatorial segment of the acrosome (Sutovsky et al. 2003).

During ICSI, as it was described previously, the sperm plasma membranes as well as the acrosome are introduced into the oocyte. This fact has been suggested to prevent the male pronucleus formation. In order to improve pronucleus formation following ICSI of bovine and porcine oocytes, sperms have often been pre-treated by various methods, including immobilizing sperm and damaging the sperm membrane by repeated freezing and thawing without cryoprotectant (Kolbe and Holtz 1999; Tian et al. 2006), treatment with Triton X-100 (an anionic detergent that induces membrane damage and dissolves nuclear proteins: Lee and Yang 2004; Tian et al. 2006), using dithiothreitol (DTT) to reduce disulfide bonds (Perreault et al. 1988; Rho et al. 1998a,b; Suttner et al. 2000), or progesterone (Katayama et al. 2002). These treatments focussed on to the strategy that removal of sperm membranes may improve male nuclear formation and make the sperm-borne oocyte-activating factor more easily available to the cytoplasm of the oocyte. However, it was recently reported that DTT pre-treatment (Szczygiel and Ward 2002), sperm freezing (Yong et al. 2005b) and Triton X-100 (Szczygiel and Ward 2002; Tian et al. 2006) did not improve the development of ICSI-derived porcine embryos.

Recent ICSI studies have demonstrated that increased damage to the sperm plasma membrane at immobilization with piezo pulses improved fertilization rates and decondensation of sperm chromatin due to the accelerated dissociation of subacrosomal sperm perinuclear theca after ICSI in pigs (Katayama et al. 2006). Yong et al. (2003) described a modified ICSI, whereby the injection of head membrane-damaged sperm using a 3–4 μm diameter injection pipette, without using an injector and polyvinyl pyrrolidone treatment, produced high fertilization rates compared with conventional ICSI (8–9 μm). In the modified ICSI, a fast-moving spermatozoon is aspirated either tail or head first and stuck via the head equatorial region onto the sharp tip of the injection pipette, during the injection procedure, the sperm membrane could be severely injured when the sperm penetrated through the *zona pellucida* and ooplasm membrane.

Several investigations have revealed that acrosomal contents could be potentially hazardous to the embryo development. A study in mice by Morozumi and Yanagimachi (2005) suggested that the success of ICSI could be improved by the removal of both sperm membranes and acrosome before the injection procedure. In the absence of sperm membranes, changes underlying oocyte-activation and embryo development take place much more rapidly and with far-reaching consequences, since oocyte cytoplasm was not exposed to hydrolyzing enzymes from the acrosome.

Moreover, it has been demonstrated by ICSI that it is possible to obtain successful fertilization in the absence of a male-derived centrosome. Kim et al. (1998) reported normal pronuclear formation following the injection of porcine sperm or isolated sperm heads into porcine oocytes. Functional microtubules for complete fertilization were organized solely from maternal stores. Furthermore, male pronuclear formation also occurred even following the injection of sperm from other species into porcine oocytes (Kim et al. 1999; Canovas et al. 2007), suggesting that these processes are not species-specific.

Oocyte factors affecting the development of ICSI-produced zygotes

Many of the events that prepare the female gamete for fertilization and make it capable of supporting the initiation of embryonic development take place during oocyte maturation (Gioia et al. 2005). After sperm penetration, the sperm undergoes chromatin decondensation, nuclear swelling and pronuclear formation. During this sperm chromatin structure reorganization, and particularly during protamine–histone replacement, profound DNA epigenetic modifications take place. In the same mature ooplasm and at the same time, the maternal genome is also modified and prepared for integration with the paternal genome. The ability of the oocyte to carry out the correct genome remodelling after fertilization therefore represents a solid opportunity to characterize oocyte maturation (Gioia et al. 2005). In general, this characterization takes place on *in vitro* matured oocytes since their production is less costly and time-consuming than that of *in vivo*-matured oocytes, which are less readily available.

However, several ICSI studies conducted on pigs suggested incomplete terminal differentiation of the oocyte as the main cause for the low capacity of *in vitro*-matured oocytes developed after fertilization (Probst and Rath 2003; Garcia-Rosello et al. 2006b). In order to solve these *in vitro* maturation problems, some strategies have been developed. Chemically defined media have been developed to contribute to an

understanding of the basic molecular mechanism of embryonic development. The majority of these studies were related to IVF embryo production and mainly undertaken using chemically defined medium (Abeydeera et al. 1998; Watson et al. 2000).

Concerning porcine ICSI, embryos derived from oocytes matured in a defined medium based on TCM199-containing cysteine and EGF developed up to the blastocyst stage but at a lower efficacy than in a serum supplemented medium (Kishida et al. 2004). Later, the same authors (Kobayashi et al. 2006), in a similar study and with higher blastocyst rates, demonstrated that the addition of cysteine or β-mercaptoethanol to a defined maturation medium can enhance blastocyst formation after ICSI, to a level similar to that achieved by adding porcine follicular fluid. Besides the protein source, other undefined biological factors commonly used are hormonal supplements such as gonadotropins. For example, a recent study substituted a hormonal supplement based on eCG and hCG in a defined medium by recombinant human FSH/LH on maturation medium without differences in ICSI blastocyst formation (Silvestre et al. 2007).

Different meiotic inhibitors which maintain the oocyte at the germinal vesicle stage trying to mimic the internal *in vivo* conditions of the follicle, and increase the cytoplasmic maturation period, have been used. Roscovitine is one of the most effective metaphase promoting factor (MPF) inhibitors with fewer detrimental effects and successful birth of piglets after its use has been reported (Coy et al. 2005). Franz et al. (2003) worked with mare oocytes that had been pre-matured in roscovitine and fertilized by ICSI. They demonstrated that roscovitine is capable of increasing the developmental capacity when cumulus-enclosed oocytes are used, and a higher cleavage rate was also obtained. Recently, we compared the use of oocytes pre-cultured in roscovitine and fertilized by ICSI and did not observe any differences with the non pre-maturated group (Garcia-Rosello et al. 2006b).

During meiotic arrest, the nuclear status and morphology of MII oocytes do not change. However, cytoplasmic changes occur when the arrest period is prolonged (Chian et al. 1992). For example, Kikuchi et al. (2000) observed an increased likelihood of parthenogenetic activation by aged oocytes that could be attributed in part to the gradual decrease of MPF activity in pig oocytes during prolonged culture. Recently, Bai et al. (2006) performed an experimental design reconstructing oocytes with MII chromosome spindle and cytoplasm from aged and fresh oocytes by the use of nuclear transfer. They showed that in *in vitro* aged oocytes, loss of developmental potential after parthenogenetic activation and IVF was mainly due to cytoplasmic deficiencies rather than nuclear deficiencies. The age of oocyte can affect embryo viability after ICSI; delayed injection may lead to rabbit oocyte aging and failure of male pronucleus formation, thus reducing the fertilization rate (Zheng et al. 2004) or lower rabbit embryo development after somatic cell nuclear transfer (13 h vs 17 h after ovulation induction treatment; Cervera and Garcia-Ximenez 2003). Duration of *in vitro* maturation process could affect

fertilization rates after ICSI (Garcia-Rosello et al. 2006b) and further embryo development. In this sense, porcine nuclear transfer experiments revealed that a greater development to blastocyst was obtained with oocytes matured for 40 h than for either 38 or 42 h (Holker et al. 2005).

Finally, some studies have been undertaken to determine the relationship between follicle diameter and the competence of the oocyte to develop up to the blastocyst stage. Jimenez-Macedo et al. (2006) demonstrated a positive correlation between oocyte diameter and embryo development in goat ICSI.

ICSI technical factors and further embryo development

A critical component of oocyte activation is the resumption of meiosis II that occurs during the time that the sperm chromatin is decondensing in the oocyte cytoplasm; this resumption marks re-entry of the oocyte into the cell cycle. The cell cycle is controlled by a balance of the activities of kinases and phosphatases that modulate the activity of cellular proteins. Prior to fertilization, the meiotic cell cycle of the mammalian oocyte is arrested at MII because of the presence of active MPF. Oocyte activation includes a large number of well defined morphological and biochemical endpoints, some of which occur within seconds or minutes of sperm–oocyte plasma membrane interaction, and some that occur over the course of several hours (Yanagimachi 1994; Schultz and Kopf 1995). One of the earliest events of oocyte activation is an increase in the level of intracellular calcium. During fertilization, mammalian oocytes of all the species studied to date exhibit a series of intracellular calcium ($[Ca^{2+}]i$) elevations which are responsible for triggering the activation of MII oocytes (Miyazaki et al. 1993). The primary role of the fertilization calcium signal is to down-regulate the activity of the cell cycle regulatory MPF that leads to exit from the metaphase arrest (Lorca et al. 1993). On the other hand, parthenogenetic activation can be induced through the elevation of cytoplasmic free calcium by several methods (see review by Machaty et al. 1999). Even, spontaneous activation by *in vitro* oocyte ageing can be performed (Kikuchi et al. 1995). In several species such as human (Tesarik et al. 1994; Catt and Rhodes 1995), rabbit (Keefer 1989), hamster (Uehara and Yanagimachi 1976) and mice (Kimura and Yanagimachi 1995), the injection procedure itself is apparently sufficient to activate the oocyte, as the sperm nucleus can undergo decondensation and formation of a pronucleus when injected into the oocytes. However, this technique has failed to produce physiological rates of embryonic or foetal development in the absence of exogenous activation stimulus in some domestic species such as bovine. A great variety of procedures have been used to induce artificial activation after ICSI such as electrical current (Lee et al. 2003; Lee and Yang 2004), ethanol (Horiuchi et al. 2002), calcium ionophore A23187 (Kolbe and Holtz 1999; Lee et al. 2003), $CaCl_2$ (Probst and Rath 2003), ionomycin (Rho et al. 1998a,b; Suttner et al. 2000), inositol-1-4-5-triphosphate (Garcia-Rosello et al. 2006a), or a combination of protein synthesis inhibitors (Lee et al. 2003). In cattle, the

success of ICSI depends on proper activation of oocyte (Keefer et al. 1990; Rho et al. 1998a,b). In fact, Malcuit et al. (2006) observed that less than 10% of all bovine oocytes subjected to monospermic ICSI showed any $[Ca^{2+}]i$ responses, and in general some of the signalling mechanisms that led to the activation of the phosphoinositide pathway and generation of oscillations during natural fertilization are not replicated by ICSI. In pigs, conclusions about the need for exogenous activation stimuli to improve embryo development are controversial (Table 1). There is no clarity as yet about what is the best of activation procedures. Moreover, the process of oocyte injection without sperm (sham) while ICSI is being performed is often sufficient to activate the pig oocytes, developing until blastocyst stage (Yong et al. 2005a).

For optimizing developmental competence of porcine embryos *in vitro*, it is important to improve the culture environment. In relation to the zygote-embryo culture, two media have been used for porcine embryos; chemically defined medium such as the *porcine zygote medium* (PZM)-4 (Yoshioka et al. 2002) and semi-defined (since they are supplemented with bovine serum albumin (BSA) and/or serum) as the (PZM)-3 and NCSU. The developmental ability of embryos derived from IVC using chemically defined media is usually low compared with those of embryos cultured in media containing BSA or foetal bovine serum. Production of piglets with PZM-4 has nevertheless been described (Yoshioka et al. 2002). Embryonic development *in vitro* is affected by several factors, such as co-culture with somatic cells (Smith et al., 1992), supplementation with antioxidants (Kitagawa et al. 2004), growth factors (Grupen et al. 1997), and oxygen tension (Karja et al. 2004; Booth et al. 2005). Moreover, another important factor is embryo density during culture (Lane and Gardner 1992), which influences the interaction of embryonic factors in the microenvironment. The embryonic factors enhance the development of embryos through the autocrine–paracrine action. For *in vitro* culture of porcine zygotes or embryos, microdrops of various medium volumes (10 to 100 μl; Yoshioka et al. 2002; Kim et al. 1993), four-well system (Garcia-Rosello et al. 2006a,b; Silvestre et al. 2007) and well of the well (WOW) system (Taka et al. 2005), have been used. The

WOW system is created at the bottom of a four-well dish and provides a more constant and suitable microenvironment for embryos. The open condition of this system may also provide an appropriate method of nutrition and dilution of metabolized toxic products. It seemed that the WOW system may be a useful IVC method for blastocyst development of IVM porcine oocytes following ICSI when a semi-defined medium such as modified (PZM)-3 or NCSU-23 is used (Kamiya et al. 2006).

Current Achievements by ICSI in Livestock Animals

Since the 1990s, there have been reports of the successful production of live offsprings after ICSI using both *in vivo*-matured and *in vitro* -matured oocytes (see Tables 1 and 3). Few studies conducted with IVM-ICSI and embryo transfer in pigs have been performed and these showed that the number of piglets obtained was very low (Kolbe and Holtz 2000; Martin 2000; Lai et al. 2001; Nakai et al. 2003; Probst and Rath 2003; Katayama et al. 2006). Only in one of these studies (Nakai et al. 2003), three piglets were born from IVM oocytes injected with acrosome-reacted sperm heads and electrical activation. Success rate seemed to be increased when *in vivo*-matured oocytes were employed (Probst and Rath 2003), reaching 6–7 piglets per litter. These were the first piglets born from ICSI with sorted spermatozoa. More recently, Katayama et al. (2006) reported three deliveries with an average litter size of 4 (see Table 1). The study of Katayama also showed that the porcine oocytes matured in a chemically defined medium had the ability for full-term development after piezo-ICSI without additional treatments for oocyte activation.

Pig embryo transfer was performed quirurgically and the stage/number of embryos transferred to female recipients are quite different among laboratories; 16–32 embryos of 2–4–8 cells (Kolbe and Holtz 2000; Lai et al. 2001; Katayama et al. 2006), 55–100 zygotes (Nakai et al. 2003; Probst and Rath 2003).

As shown Table 2, pig oocytes resumed meiosis after ICSI in rates around 70% with or without exogenous activation; electrical pulses (Lai et al. 2001; Nakai et al.

Table 1. Results of ICSI embryo transfer in pigs

References	Oocytes	IVM medium	Exogenous activation	Sperm	IVC	No. embryo transfer stage	No. piglets
Martin 2000	*In vivo*	–	NO	Fresh	NCSU-23	22	3
Kolbe and Holtz 2000	*In vivo*	–	Ca-ionophore	Fresh	NCSU-23	32 embryos 2–4 cells	1
Lai et al. 2001	IVM	TCM-199	Electrical	Frozen-thawed	NCSU-23	16 embryos > 8 cells	1 dead
Probst and Rath 2003	*In vivo*	–	CaCl₂	Sex sorted		85.3 zygotes	13/(4 recipients)
Nakai et al. 2003	IVM	NCSU	Electrical	Acrosome-reacted head	IVC-PyrLac IVC-glu	55–100 zygotes	3
Yong et al. 2006	IVM	–	NO	Transgenic frozen sperm			1
Katayama et al. 2006	IVM	TCM-199	NO	Fresh	NCSU-23	28 4–8cells	6 4 2

Table 2. Results of activation, pronuclear formation, cleavage, blastocyst formation and number of cells per blastocyst in porcine ICSI

References	IVM medium	Activation %	Fertilization %	Cleavage %	Blastocyst %	Cells/blast
Martin 2000	*In vivo*	81	–	69	38[a]	24.7
Kolbe and Holtz 2000		–	–	26	–	
Lai et al. 2001	TCM-199	–	64.8	60[b]	30[a]	23.5
		–	28.5	63.3[b]	4.6[a]	18.0
Probst and Rath 2003	NCSU	70.4	49.9	–	–	–
		45.9	33.2	–	–	–
Wu et al. 2001	NCSU	–	64	51	21[b]	31.3
Katayama et al. 2002	TCM-199	78	64	–	–	–
Lee et al. 2003	NCSU	100	67.4	61.6	20.9	24–31
Yong et al. 2003	TCM-199	–	46.7	60.6	17.5[a]	32.8
Kishida et al. 2004	TCM-199		56	58.3	23.3[c]	41.4
Garcia-Rosello et al. 2006a	NCSU	54	54	55	10[c]	20.9
Garcia-Rosello et al. 2006b	Ros-NCSU	79.8	72	–	–	–
Kobayashi et al. 2006	TCM-199	–		56.3–72.8	15.6–36.7[c]	41.6–52.0
Silvestre et al. 2007	TCM-199	–	–	69.0–69.4	6.7–9.1 a	19.8–20.6

[a]In relation to cleaved oocytes.
[b]In relation to fertilizing oocytes.
[c]In relation to MII oocytes.

Table 3. Results of activation, pronuclear formation, cleavage, blastocyst formation and live born in bovine, caprine, ovine and equine ICSI

Species	Oocyte	Activation	Sperm	PN (%)	Cleavage (%)	Blastocyst (%)	Live born	References
Bovine	IVM	Piezo	Frozen-thawed	85	72	ND	ND	Katayose et al. 1999
	IVM	Piezo	fresh	86.3	71.8	22.7	Yes	Wei and Fukui 2002
	IVM	Piezo	Frozen-thawed	ND	13	4	ND	Fujinami et al. 2004
	IVM	Piezo + ethanol	Frozen-thawed	ND	51	14	ND	Fujinami et al. 2004
	IVM	Ionomycin + 6-DMAP	liophilized	74	63.3	29.6	ND	Keskintepe et al. 2002
	IVM + frozen- thawed	Ionomycin + CHX	Fresh	54.8	70	16.3	ND	Rho et al. 2004
			Frozen-thawed	51.1	52.2	9.8	ND	
	IVM	Ionomycin + 6-DMAP	Frozen-thawed	ND	83.9	40.1	1/11 9.1%	Oikawa et al. 2005
	IVM	Ethanol	Frozen-thawed	ND	75.6	29.4	9/19 47.4%	Oikawa et al. 2005
	IVM	Ethanol	Fresh	ND	72	20	5/10 50%	Horiuchi et al. 2002
	IVM	Ionomycin + CHX	Frozen-thawed	88.6	59.7	6.1	ND	Galli et al. 2003
	IVM	NO	Frozen-thawed	88.9	58.5	7.3	1/11 9.1%	Galli et al. 2003
	IVM	Ionomycin	Frozen-thawed	ND	50.7	5.8	ND	Galli et al. 2003
	IVM	Ionomycin + 6-DMAP	Frozen-thawed	ND	83	25	ND	Li et al. 2004
Ovine	IVM	NO	Fresh	ND	42.5	8.5	2/38 5.2%	Gomez et al. 1998
	IVM	Ionomycin + 6-DMAP	Frozen-thawed	ND	71.8	7	ND	Gou et al. 2002
Caprine	IVM	NO	Frozen-thawed	ND	67	18	ND	Keskintepe et al. 1997
	IVM	Piezo	Fresh	ND	89	35	0/6 0%	Wang et al. 2003
	IVM	Ionomycin + 6-DMAP	Fresh	40	73.4	5.2 morulae	ND	Jimenez-Macedo et al. 2005
	IVM	NO	Fresh	40.9	66.9–75.0	8.3–11.7	ND	Jimenez-Macedo et al. 2006
Equine	IVM	NO	Frozen-thawed	43.5	8.7	ND	ND	Dell'aquila et al. 1997
	IVM	NO	ND	ND	63	ND	2/4 50%	Cochran et al. 1998
	IVM	NO	Frozen-thawed	ND	63	30	ND	Li et al. 2001
	IVM	Piezo	Fresh	71	72	ND	ND	Choi et al. 2002
			Frozen-thawed	60	55	ND	ND	
	IVM	NO	Frozen-thawed	ND	75	27	ND	Choi et al. 2006

Live born % (no. Live born/ no. embryos transferred). Piezo-driving system.
IVM, *In-vitro* matured; 6-DMAP, 6-dimethylaminopurine; CHX, Cycloheximide; ND, not determined.

2003), Ca-ionophore treatment (Kolbe and Holtz 2000) or CaCl$_2$ injection (Probst and Rath 2003). Rates of fertilization (two pronuclear formations) by ICSI in pigs varied around 60%. However, blastocyst formation was reduced drastically when IVM oocytes were used.

The success of ICSI depends on the protocol used for each species. In sheep, rabbit, goat and horse, conventional ICSI seems sufficient to activate the oocyte for further embryonic development. However, very few works were performed in these species. In cattle, the majority of studies demonstrate that artificial activation of bovine oocytes after conventional ICSI improved the fertilization rates (Table 3). Several different strategies of artificial activation in bovine have been tested. Among them, the ionomycin plus 6-DMAP protocol

seems to be a consistent procedure for bovine oocyte activation after ICSI. Also, ICSI with a piezo-driven needle, to break a hole into the oolemma, is an alternative option to chemical activation. As a side effect, good developmental rates were obtained both in cows (Katayose et al. 1999; Wei and Fukui 2002) and in goats (Wang et al. 2003) when the mechanical pulses were used to induce activation of oocytes. Birth of calves was obtained without additional exogenous activation, but the piezo- driving system was needed (Wei and Fukui 2002). Fertilization rates in cattle ranged from 50 to 80%, which are higher than those obtained in pigs. Embryo development to the blastocyst stage ranged from 6.1% to 40.1% (see Table 3), however, in small ruminants blastocyst rates varied from 11.7% to 35%. The lower rates of blastocyst formation in goats could be explained by the failure of pre-pubertal goat oocytes to undergo correct cytoplasmic maturation and consequently correct fertilization (Jimenez-Macedo et al. 2005). Concerning equine species, the initial trials using ICSI started in 1997 (see Table 3) with the first injections being performed in IVM oocytes and using frozen-thawed semen. In 1998, Cochran et al. obtained the first foals with IVM oocytes. Rates of blastocyst formation were around 30%. Pregnancies and foals have resulted from the transfer of ICSI-produced zygotes and embryos to the oviducts of recipient mares: however, the efficiency of the technique is still low (Cochran et al. 1998). One of the main problems in equine seems to be related to embryo-culture systems which are unable to support optimal embryonic development.

Conclusions

Intracytoplasmic sperm injection in livestock animals can be a valuable research tool to investigate fundamental aspects about oocyte and sperm interaction during fertilization, especially in relation to oocyte activation and male pronuclear formation at the very early embryo developmental stages. Experimental studies in model species are necessary to identify underlying problems or limitations in techniques and mechanisms before putting them into practice in humans.

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