Scientific Annals of Polish Society of Animal Production - Vol. 11 (2015), No 3, 55-66

Review article

Intra-cytoplasmic sperm injection (ICSI) as an alternative to standard *in vitro* fertilization in pigs*

Iwona Rajska

National Research Institute of Animal Production,
Department of Biotechnology of Animal Reproduction,
ul. Krakowska 1, 32-083 Balice

Injection of sperm into the oocyte cytoplasm (ICSI) is a method of assisted reproduction that has been used for *in vitro* production of embryos in animals and treatment of infertility in humans. ICSI makes it possible to eliminate polyspermy and to use sperm as vectors of exogenous DNA to obtain transgenic animals. It aids in the creation of biodiversity conservation programmes and in understanding mechanisms taking place during fertilization and early embryonic development. Fertilization by ICSI is performed using IVM, *in vivo* and cryopreserved oocytes and ejaculated or epididymal semen, fresh or preserved. Sperm injection is carried out using an inverted microscope equipped with a micromanipulator attached to the holding and injection pipettes. After injection, the oocytes are artificially activated and cultured *in vitro*. The embryos obtained are evaluated. Despite the potential of ICSI, there are still problems limiting its implementation for *in vitro* production of embryos on a commercial scale. Factors that may influence the efficiency of ICSI are oocyte and sperm quality and their preparation for fertilization, damage to the oocyte during injection, toxicity of compounds used to slow the sperm, and oocyte activation after injection. The number of embryos obtained as a result of ICSI fertilization is low and their quality is often reduced. Further studies are needed to optimize not only the fertilization technique, but also its individual stages, i.e. in vitro maturation of oocytes and sperm preparation.

KEY WORDS: ICSI / pig / oocyte / sperm

In vitro fertilization is one of the stages of in vitro production of embryos, which includes the collection and preparation of semen (capacitation), preparation of oocytes, and co-incubation of gametes.

***Study financed from IZ PIB statutory task no. 02-007.1 (2015)**

In pigs, in vitro fertilization by the standard method (IVF) results in polyspermic fertilization of over 50% of oocytes, which substantially reduces the efficiency of in vitro fertilization [31, 67]. Therefore methods are sought to reduce or eliminate polyspermy in this species.

A solution to the problem of polyspermic fertilization in pigs may be the use of a fertilization method involving the injection of just one spermatozoon into the cytoplasm (Intracytoplasmic Sperm Injection – ICSI) or under the zona pellucida of the mature oocyte (Subzonal Sperm Injection – SUZI).

Fertilization by injecting a spermatozoon into the oocyte of an echinoderm was first attempted by Hiramoto in 1962 [cited in 36, 68]. A subsequent experiment involved fertilization of an amphibian oocyte by Brun in 1974 [cited in 36]. This technique was first applied in mammals by Uehara and Yanagimachi in 1976 [64]. In subsequent years, offspring was obtained for the first time in cattle [20], and later in mice [1, 33], sheep [6], horses [9], monkeys [21], cats [19], rabbits [45], rats [22], hamsters [69] and goats [66]. Since the 1990s the ICSI technique has been used for in vitro fertilization in humans [58].

In pigs the ICSI method was first performed by Catt and Rhodes in 1995 [5], but it was only later experiments by Kolbe and Holtz [36, 37] and Martin [49] that enabled live offspring to be obtained in this manner.

The main advantage of fertilization by ICSI is the possibility of using semen with reduced sperm concentration or motility, sexed semen, cryopreserved semen, or semen collected *post mortem* [36, 46, 47, 51, 60, 65]. In pigs the ICSI method has mainly been used to eliminate polyspermy, which, as mentioned above, reduces the efficiency of in vitro fertilization [14, 16, 28, 34, 46, 57, 62, 65].

ICSI also enables the use of spermatozoa as vectors of exogenous DNA in order to obtain transgenic animals (Sperm-Mediated Gene Transfer – ICSI-SMGT) [4, 10, 11, 39, 40, 41, 59, 74]. It can also be useful in programmes for conserving biodiversity or protecting endangered species [43, 44, 47, 57]. It should also be emphasized that experiments using the ICSI method have revealed fundamental mechanisms taking place during fertilization and early embryonic development [8, 44, 46, 57, 71].

Fertilization by the ICSI method in pigs is carried out using ejaculated semen [3, 14, 15, 24, 28, 43, 61] or epididymal semen [36, 55, 60, 72]. The semen is used immediately after collection and preliminary dilution (fresh semen) [37], or after being preserved in a liquid state [32, 49], frozen [15, 16, 25, 34, 44], or in some cases freeze-dried [26, 38, 51, 56]. The ICSI method also enables the use of sperm obtained from the gonads of sexually immature young boars transplanted into animals of another species, such as mice [54].

Boar sperm for ICSI must be appropriately prepared. Fresh semen and semen preserved in liquid form are washed 2-3 times to remove the remains of the extender [15, 16, 32]. Cryopreserved sperm are thawed in a water bath, and freeze-dried sperm are dissolved in deionized distilled water. Then the sperm are centrifuged in a Percoll gradient (45%/90%) [16] or incubated at 39°C for 30 minutes (swim-up technique), in order to obtain a fraction containing live sperm [25, 34], and suspended in PBS-BSA (bovine serum albumin) solution [51] or a medium with PVP (polyvinylpyrrolidone) to slow their movement [38]. The

sperm prepared in this manner are incubated at 38°C for no longer than 1-2 hours before the injection. At the same time the viability of the sperm cells is assessed. For this purpose a semen sample is incubated with fluorescent dyes, such as SYBR-14 and PI (propidium iodide) to identify dead and live sperm [44] or with the dyes FITC-PNA (fluorescein isothiocyanate-peanut agglutinin) and PI, enabling identification of sperm with an intact or damaged acrosome [16].

Pig oocytes to be fertilized in vitro by ICSI are obtained from sows or gilts after slaughter [25, 32, 34, 46, 62]. The immature oocytes are matured in vitro in a medium with a defined composition, at 39°C, 5% CO_2 atmosphere and maximum humidity. The oocyte culture is carried out in two stages, for 42 to 50 hours [8, 16, 38, 42, 44, 52, 61, 72]. Cumulus cells are removed from the mature oocytes with the enzyme hyaluronidase, followed by evaluation of the appearance of the cytoplasm and the presence of the first polar body [46, 49, 61, 62]. An additional criterion of maturity is assessment of the stage of the oocyte. For this purpose the oocytes are fixed and then stained with orcein [24, 34, 35] or Hoechst 33342 [15].

ICSI can also be used to fertilize oocytes matured in vivo [36, 49, 60] or cryopreserved oocytes [12, 48, 61]. Thus far attempts at in vitro fertilization of cryopreserved pig oocytes, either by IVF or ICSI, have not succeeded in producing offspring. This has been accomplished using vitrified oocytes transferred to inseminated recipients and fertilization in in vivo conditions [13].

The final step of ICSI is injection of the spermatozoon into the oocyte. This is done in an inverted microscope with micromanipulation devices. A Petri dish with one drop of a mixture of diluted semen and PBS-PVP solution and one drop of medium, e.g. NCSU-23, TLH-PVA or PBS with 10% FCS, is placed on the microscope stage heated to 38.5°C, and 10-18 oocytes intended for microinjection, without cumulus cells, are injected [7, 24, 25, 34]. Another variant is the formation of single drops of medium, into which the oocytes are introduced singly or in pairs (10-16) [14, 16, 48, 49, 60]. The drops are covered with heated mineral oil. Then the sperm cell is injected into the oocyte with a commercially available glass injection pipette [49, 60] or one prepared in laboratory conditions [38, 71]. A holding pipette is used to immobilize the oocytes so that the first polar body is at the 12 or 6 o'clock position. This position of the oocyte allows the injection to be performed without damaging its meiotic spindle [28, 36, 48]. After a spermatozoon with normal morphology has been identified, the end of the injection pipette is used to immobilize it by breaking the midpiece or tail, and together with a small quantity of medium the spermatozoon is aspirated head-first or tail-first into the pipette [15, 16, 36, 44, 46, 49]. Then the injection needle is used to manually or piezoelectrically break through the zona pellucida at the 3 o'clock position, a small amount of the oocyte's cytoplasm is aspirated into the injection pipette, and the contents of the injection pipette (the sperm together with the cytoplasm and a small volume of medium) are released into the oocyte [3, 28, 32, 36, 53, 55, 60, 62]. The injection pipette is gently removed immediately after the injection and the oocyte is released from the holding pipette, in order to minimize intracellular pressure on the oocyte [32, 72]. The oocyte can be injected with a motile spermatozoon [24, 32, 34, 35, 46, 62], a dead spermatozoon or one with damaged motor

apparatus [16], an isolated sperm head [32, 38, 44, 55], a spermatid [42], or a sperm extract [50].

Following the injection the oocytes with the spermatozoon are activated in a BTX Electro Cell Manipulator [7, 43, 55], incubated in a medium with calcium ions [8, 60], or immediately transferred to a culture medium [15, 34, 49, 70]. Activation can also be carried out before or during the injection, by introducing a small amount of calcium ion solution into the oocyte together with the spermatozoa [60].

In vitro culture of potential pig zygotes obtained by ICSI is usually carried out in NCSU-23 medium [8, 25, 60, 63, 71, 73]. The embryos are cultured to the blastocyst stage in drops of medium under mineral oil or in Nunc 4-well dishes, at 39°C, 5% CO_2 concentration in the air and maximum humidity. During the in vitro culture the rate of development and morphological state of the embryos is assessed every 24 hours. The blastocysts obtained are evaluated. Usually the degree of DNA fragmentation of the embryonic cells is determined, e.g. by the TUNEL method [46, 48], as well as the total number of cell nuclei of the embryo, e.g. by DAPI, Hoechst 33342 or Giemsa staining [12, 14, 15, 34, 35, 46, 48], and a chromosome analysis is performed [7, 32, 42, 68, 72].

The final assessment of the quality of pig embryos obtained following in vitro fertilization is their ability to fully develop in vivo, i.e. following transfer to synchronized recipients. Then the evaluation includes the percentage of pregnant sows and the number of piglets born live and dead in relation to the number of transferred embryos [17, 28, 37, 39, 49, 55, 60, 70, 72].

The potential of ICSI in pigs has thus far been exploited only to a small degree. Problems remain which limit the implementation of this technique to in vitro production of embryos on a commercial scale [16]. Factors that may influence the efficiency of this fertilization method include the quality of oocytes used for maturation and fertilization [73], the quality and preparation of sperm [3, 14, 16, 28, 38, 44, 51, 71, 72], damage to the oocyte during the injection procedure [30], toxicity of compounds used to slow sperm movement [34], and activation of the oocytes after injection [8, 14, 15, 30, 43, 44, 50, 52, 72].

The main source of oocytes for in vitro culture and then in vitro fertilization by both IVF and ICSI is ovaries obtained from slaughtered animals. These are mainly sexually immature individuals in which ovulation and luteal bodies are not observed on the ovaries. Oocytes from such animals have lower developmental capacity, which reduces the efficiency of their maturation and fertilization and of embryonic development in vitro conditions [73].

One of the reasons for the low efficiency of ICSI is disturbances in decondensation of the nuclear chromatin of the spermatozoon and in the formation of the male pronucleus [14, 34, 43, 57, 60]. Spermatozoa to be injected into oocytes require appropriate preparation. In in vitro conditions the spermatozoon is capacitated so that it will be capable of fertilizing the oocyte. A spermatozoon injected into the oocyte during injection by ICSI does not undergo an acrosome reaction, retaining certain elements, such as hydrolytic enzymes and a nearly intact cell membrane and perinuclear envelope, which in physiological conditions, as well as during IVF, are removed during penetration of the zona pellucida of the oocyte. The presence of these elements may be one of the reasons for the

lack of decondensation of the nuclear DNA of the spermatozoon and the low percentage of formation of the male pronucleus [14, 28]. For this reason, before the spermatozoon is injected into the oocyte, procedures are carried out which lead to partial damage to the cytoplasmic membrane. Examples include freezing without protective substances [27], the addition of progesterone, which can induce an acrosome reaction [27], or the addition of chemical compounds that dissolve cell membranes, such as Triton X-100 [14, 44], or break disulphide bonds, e.g. dithiothreitol (DTT) [7, 70], the addition of glutathione (GSH) [7], or mechanical damage to the sperm cell membrane [71]. The cytoplasmic membrane of the spermatozoon can also be damaged by exposing the sperm to the effects of temperature or ultrasound. For this reason spermatozoa from semen that has been freeze-dried [26, 38, 51], sonicated [38] or stored for a long period at 4-24°C [3] have been used for injection.

Previous studies have shown that preparation of sperm for ICSI affects fertilization outcomes and embryonic development [16]. Frozen or freeze-dried sperm may have damaged nuclear chromatin and centrioles, resulting in abnormal decondensation and preventing transformation into the male pronucleus [16, 51]. In this case chemical agents are sought which could stabilize and protect the sperm DNA from damage incurred during freezing or lyophilization [26, 51]. At the same time, the use of chemical compounds such as Triton X-100 or DTT and damage to the oocyte arising during the injection procedure, which is not a physiological phenomenon, may have a harmful impact on the further development of oocytes following ICSI and reduce the efficiency of in vitro fertilization [30]. Moreover, the effect of some chemical compounds used, such as PVP, should not be overlooked, as following the injection of cytoplasm to the oocyte they may have a harmful effect [34].

Oocyte activation was not originally applied in pigs [37, 49, 68, 70], but later research [8, 39] showed that it has a beneficial effect, manifested as improved efficiency of fertilization by ICSI. Moreover, activation in an electric field in combination with a high concentration of calcium ions has been shown to increase the percentage of pig oocytes dividing parthenogenetically [60]. In contrast, in mice [33, 59], hamsters [23] and rabbits [29] additional oocyte activation is not required after fertilization by ICSI [43, 52].

The consequences of abnormal decondensation of the nuclear chromatin of the sperm cell and a delay in the formation of the male pronucleus, mentioned above, lead to a reduction in the developmental potential of embryos [34, 46]. This is manifested as a slower development rate, a decrease in the number of blastocysts obtained [57], and reduced blastocyst quality [14, 46, 51, 57, 63].

It should be emphasized that some authors, such as Yong et al. [71] and Li et al. [46], obtained a lower percentage of blastocysts (2% and 20%, respectively, in the two studies) as a result of in vitro fertilization by ICSI as compared to the percentage of embryos obtained at this stage following IVF (from 27% to 35%, respectively). In contrast, in studies by Mandryk [48] and Wu et al. [68], more blastocysts were obtained by ICSI than by IVF (36 and 30 blastocysts in ICSI vs. 12 and 8 in IVF). In pig embryos obtained by ICSI a slower development rate has been observed in comparison with the IVF [46, 57].

However, no significant differences in the number of apoptotic cells have been noted in blastocysts obtained following fertilization by the two methods [46, 48]. On the other hand, smaller numbers of cells have been observed in blastocysts obtained by ICSI in comparison with IVF [46, 48, 57]. In contrast, research by Wu et al. [68], Yong et al. [71] and Yoo et al. [72] showed no significant differences in the number of cells in blastocysts obtained by the two methods.

It is believed that in vitro fertilization, irrespective of the method used, can significantly affect the transcriptome of the embryo in the pre-implantation period, particularly genes responsible for basic cell functions and metabolism [2, 18]. One example is bovine embryos obtained by ICSI, in which a high level of expression of the gene encoding the Bax protein (a protein with pro-apoptotic activity) was observed, but this was not correlated with the number of apoptotic cells in the embryo. In contrast, pig embryos obtained by ICSI fertilization have shown a lower level of expression of pro-apoptotic genes, while the level of expression of genes counteracting apoptosis was higher than in embryos obtained by IVF [46].

To sum up, injection of a spermatozoon into the cytoplasm of the oocyte is one method of assisted reproduction which may be an alternative to standard in vitro fertilization in pigs. It mainly finds application in in vitro production of embryos, e.g. for breeding purposes, owing to the possibility of using sexed semen. Additional benefits associated with this method of in vitro fertilization are the use of spermatozoa as vectors in creating transgenic animals and in conservation programmes for endangered species. It should also be noted that the number of embryos resulting from the use of the ICSI method is low and their quality is often reduced. Therefore further research is necessary to optimize not only the fertilization technique itself but also its individual stages, including in vitro maturation of oocytes, semen preparation, and in vitro culture of the embryos obtained.

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