Intracytoplasmic sperm injection in domestic and wild mammals

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Abstract

Intracytoplasmic sperm injection (ICSI) has become a useful technique for clinical applications in the horse-breeding industry. However, both ICSI blastocyst and offspring production continues to be limited for most farm and wild species. This article reviews technical differences of ICSI performance among species, possible biological and methodological reasons for the variable efficiency and potential strategies to improve the outcomes. One of the major applications of ICSI in animal production is the reproduction of high-value specimens. Unfortunately, some domestic species like the bovine show low rates of pronuclei formation after sperm injection, which led to the development of various artificial activation protocols and sperm pre-treatments that are discussed in this article. The impact of ICSI technique on equine breeding programs is considered in detail, since in contrast to other species, its use for elite horse reproduction has increased in recent years. ICSI has also been used to produce genetically modified animals; however, despite numerous attempts in several domestic species, only transgenic pigs have been consistently produced. Finally, the ICSI is a promising tool for genetic rescue of endangered and wild species. In conclusion, while ICSI has become a consistent ART for some species, it needs further development for others. The low results obtained for some domestic species, the high training needed and the equipment required have limited this technique to the production of elite specimens or for research purposes.

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General overview

ICSI is a micromanipulation technique that involves the injection of a single spermatozoon into the cytoplasm of a mature oocyte. The first report of pronuclei formation after ICSI in mammals was achieved in hamster gametes (Uehara & Yanagimachi 1976). In 1992, the first baby generated by sperm injection was born (Palermo et al. 1992), and it did not take long for ICSI to become an important technique for human-assisted reproduction worldwide (reviewed by Devroey & Van Steirteghem 2004). Following that attainment, the use of this technique extended to other species, including the cow (Goto et al. 1990), rabbit (Hosoi & Iritani 1993), mouse (Kimura & Yanagimachi 1995), sheep (Catt et al. 1996), horse (Cochran et al. 1998), domestic cat and wild felids (Pope et al. 1998), pig (Kolbe & Holtz 2000) and goat (Wang et al. 2003).

However, despite the efforts of several working groups around the world, the success of this technique has been limited in farm animals. The most extreme case is the cow, whose fertilization rates after ICSI are critically low (Chung et al. 2000, Devito et al. 2010, Arias et al. 2014). In sheep, although fertilization rates after ICSI can be improved by artificial activation treatments (Shirazi et al. 2011), development to blastocyst continues to be low. In regard to post-implantation development, the number of newborns produced by ICSI remains extremely low for these species (reviewed by Garcia-Rosello et al. 2009, Lopez-Saucedo et al. 2012).

In contrast to the situation for most domestic species, ICSI in horses has developed to a commercial level (Hinrichs 2005). In this species, embryo production by IVF continues to be a challenge, since it has not been possible to obtain repeatable results (Mugnier et al. 2009, reviewed by Leemans et al. 2016). For this reason, the combination of ovum pick-up (OPU) and ICSI followed by non-surgical embryo transfer to recipient mares is the current routine protocol for in vitro embryo production in horses. In recent years, surprising efficiency of this protocol ended in its inclusion in commercial breeding programs (Galli et al. 2014).

With regard to pigs, ICSI became an alternative fertilization technique for research purposes, since IVF produces high rates of polyspermy (reviewed by Coy & Romar 2002). Moreover, after the generation of the first transgenic piglet by sperm injection, ICSI gained importance as a new tool for inducing genetic
modifications in farm animals (Kurome et al. 2006, Garcia-Vazquez et al. 2010). However, the advent of CRISPR-Cas9 system for genetic engineering lead to the replacement of ICSI by regular or IVF zygotes for the generation of genetically modified animals (Hai et al. 2014, Whitworth et al. 2014, Proudfoot et al. 2015, Wang et al. 2015, Bevacqua et al. 2016).

In summary, the low efficiency of ICSI in domestic species, the high level of training needed, and the expensive equipment required has restricted this technology to the production of specimens of high commercial value or for research purposes. The great progress achieved by the development of this technique in some species, and the disappointing results observed in others emphasize the importance of re-examining the possible causes of such differences, encouraging the study of early fertilization events and considering new applications that have not been explored thoroughly yet.

The technique step by step

The ICSI procedure involves the use of complex equipment, including an inverted microscope coupled to a micromanipulation system. Basically, the micromanipulator converts macroscopic movements into microscopic ones, allowing the handling of gametes. It is equipped with two arms, one attached to a holding pipette and the other to an injection pipette that, in some cases, is connected to a piezo-driven system. The holding pipette attaches the oocyte, placing the first polar body (PB) in 6 or 12 clockwise position. The injection pipette, used for immobilizing and holding a single spermatozoon, will pass through the membrane of a metaphase II oocyte and deposit the sperm into the cytoplasm. Since the genetic material is expected to be next to the PB, it is kept far from the area of injection, in order to minimize the risk of chromosome or spindle damage.

Several authors have described in detail the methodology of ICSI technique (Yoshida & Perry 2007, Stein & Schultz 2012, Rader et al. 2016, Simopoulou et al. 2016). In the present section, we expose technical differences among species on the basis of our collective experience. These differences are evidenced during the ICSI procedure. After in vitro maturation (that will vary between 18 and 30 h depending on the species), cumulus cells are removed from COCs through enzymatic treatment followed by vortexing or gentle pipetting. For example, when manipulating horse or wild animal’s oocytes, vortexing is usually avoided to minimize the risk of losing or damaging them, given their high value.

Regarding sperm sample preparation frozen-thawed semen can be used, even for species with variable sperm freezability among individuals like horses (Hoffmann et al. 2011) and pigs (Casas et al. 2009). Depending on the quality of the sperm sample and the species used, methods for selection of motile spermatozoa like swim up or density gradient separation can be included on ICSI protocols (Gomez et al. 1997, Keskinolpe et al. 1997, Choi et al. 2002, Nakai et al. 2016a,b, Rader et al. 2016). It is interesting to note the possibility of optimizing the use of frozen semen straws when performing ICSI. Since only one spermatozoon per injected oocyte is needed, each straw can be sectioned into multiple ‘ICSI-cuts’. Up to ten ‘ICSI-cuts’ can be obtained from a single straw that can be thawed separately for its use in differ ICSI procedures (Rader et al. 2016). Additionally, some authors maximize the use of valuable straws not only by using this strategy, but also by diluting and refreezing sperm doses, for example, when a frozen semen store is limited or when expensive sex-sorted sperm straws are employed (Hamano et al. 1999, Rader et al. 2016, Canel et al. 2017).

After semen thawing and selection, a critical step is sperm immobilization. For this, the spermatoza must be placed in a polyvinylpyrrolidone (PVP) droplet, a solution of high viscosity that reduces sperm motility (Hyakutake et al. 2013). The slow movement of the sperm in PVP allows placing the injection pipette over the sperm tail and rolling it against the bottom of the ICSI dish to immobilize the sperm and easily take it into the ICSI pipette. The resulting damage of the sperm tail membrane not only makes sperm manipulation simpler (Kato & Nagao 2009), but also is thought to facilitate sperm head decondensation and oocyte activation, which are essential steps for early embryo development (Morozumi et al. 2006). The resistance of the sperm tail to be broken varies among species, being much higher for the bull, followed by the sheep, the pig and finally horses and domestic cats, whose sperm tails are easily broken. Regardless of motility, even when dead spermatozoa are used, sperm tail breakage is a step that should not be bypassed, since it was shown to improve sperm nucleus decondensation (Dozortsev et al. 1995).

With respect to the pipettes used, commercial or handmade models can be employed. Since sperm size varies among species, it must be taken into account that injection pipettes of different inner diameters should be used in each case. For example, we recommend the use of 9µm inner diameter pipettes for bull, ram (Fig. 1B) and pig sperm injection. On the other hand, commercial pipettes employed for human reproduction (7µm) can be used for horse (Fig. 1A) and cat sperm injection (Fig. 1C). Furthermore, the shape of the pipette will depend on the system used. While sharp pipettes with bevel and spike are employed for the conventional method or laser-assisted system (Smits et al. 2012a), but a blunt pipettes are used when a piezo-driven system is employed. The piezoelectric actuator couples to the micromanipulation system and attaches the injection pipette, driving its tip forward in a precise and fast movement. In this fashion, disruption of the sperm tail oolemma are performed mechanically rather than manually, rendering the procedure easier, and more
early embryo development. The complete activation of the oocyte implies the resumption of meiosis, the second PB (2PB) extrusion, the release of cortical granules and the formation of male and female pronuclei (PN) (reviewed by Alberio et al. 2001, Swann & Lai 2016). Oocyte activation occurs as the result of Ca\textsuperscript{2+} oscillations in the ooplasm (Stricker 1999), and it is widely accepted that the factor responsible for triggering these oscillations in mammals is a sperm-specific isoform of the phospholipase C, named PLC\textsubscript{ζ} (Saunders et al. 2002, Yoon & Fissore 2007). The currently accepted model is that PLC\textsubscript{ζ} enters the ooplasm after the fusion of both gametes and catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate, generating inositol 1,4,5-triphosphate (IP3) and diacylglycerol. IP3 binds to its receptor in the membrane of the endoplasmic reticulum and induces the release of Ca\textsuperscript{2+} to the cytosol. In this fashion, PLC\textsubscript{ζ} induces successive ooplasmic Ca\textsuperscript{2+} peaks (reviewed by Malcuit et al. 2006a). In mammals, such Ca\textsuperscript{2+} oscillations must be strictly regulated to induce a correct oocyte activation and normal embryo development (Rogers et al. 2006). Further in the cascade, Ca\textsuperscript{2+} peaks cause a decrease in the levels of maturation-promoting factor (MPF) and mitogen-activating protein kinase (MAPK), whose concentrations are at their maximum prior to fertilization, inducing oocyte activation (reviewed by Ducibella et al. 2002, Jones 2005).

For species like the human, mouse, horse and domestic cat, the sole injection of the sperm into the oocyte is enough to trigger oocyte activation, sustaining development to blastocyst, and even to term (Palermo et al. 1992, Cochran et al. 1998, Gomez et al. 2000, Kimura & Yanagimachi 1995). In human and mouse oocytes subjected to sperm injection, Ca\textsuperscript{2+} oscillations similar to those of IVF embryos have been observed (Tesarik et al. 1994, Sato et al. 1999, Markoulaki et al. 2007). In horses and domestic cats, although there are no reports comparing oscillation patterns of ICSI vs IVF embryos, empirical data suggest that the sole sperm injection stimulus would be enough to induce embryo development, since most ICSI embryos are able to cleave, and some of them reach the blastocyst stage (Pope et al. 1998, Bedford et al. 2003, Tharasnit et al. 2012, Moro et al. 2014). Indeed, 43–74% of horse oocytes formed pronuclei after sole sperm injection (Dell’Aquila et al. 2001, Tremoleda et al. 2003, Lewis et al. 2016). For this reason, it is not necessary to employ additional protocols to induce oocyte activation and subsequent embryo development. Nevertheless, it is not the case for some large domestic species, whose development to blastocyst and even more to term is extremely low (reviewed by Horiuchi & Numabe 1999).

Developmental rates of cow ICSI embryos are much lower compared to those produced by IVF (Goto et al. 1990, Chung et al. 2000). More than 90% of oocytes are unable to perform Ca\textsuperscript{2+} oscillations after sperm injection.
(Malcuit et al. 2006a), resulting in an incomplete inactivation of MPF (Fujinami et al. 2004). Therefore, most ICSI cow embryos show inconsistencies in sperm decondensation and pronuclei formation (Rho et al. 1998a, Chung et al. 2000, Malcuit et al. 2006b, Arias et al. 2015), making ICSI success in a complex challenge. It is under discussion if such inconsistencies are due to the inability of bull sperm to induce the complete oocyte activation or to the poor response of cow ooplasm to injection stimulus, which provoke an incorrect sperm head decondensation (Aguila et al. 2017). For this reason, several oocyte activation protocols and sperm pretreatments have been developed, in order to improve releasing and/or activation of PLCζ and the complete inactivation of MPF and MAPK (see below). Although, there are a few exceptions, there is a general consensus about the need to artificially activate ICSI embryos to generate blastocysts in the cow (Rho et al. 1998a, Chung et al. 2000, Fujinami et al. 2004, Oikawa et al. 2005, Bevacqua et al. 2010).

After ICSI in sheep, development to blastocyst is low compared to IVF (Gomez et al. 1998a,b). In contrast to bovine ICSI, it is not due to failed pronuclei formation, but to the arrest of most ICSI embryos at the 8- to 16-cells stage (Gomez et al. 1998a). In our hands, cleavage rates increase from 53 to 85% after chemical activation, but no differences were observed at later stages (Pereyra Bonnet et al. 2008).

For pig embryos, although the need of artificial activation after ICSI continues to be controversial (Kikuchi et al. 2002, Yong et al. 2006, Li et al. 2013), several authors improved blastocyst rates through the employment of activation treatments (Lee et al. 2003, Nakai et al. 2003, 2006, Probst & Rath 2003). Indeed, it was recently reported that 50% of pig oocytes do not show Ca^{2+} oscillations after ICSI (Nakai et al. 2016a). However, in this particular species, the main problem is the absence of in vitro maturation and culture systems specially designed for pig oocyte and embryo requirements, that led to poor-quality blastocysts and low rates of in vitro and in vivo development (Garcia-Rosello et al. 2009, Li et al. 2013, Nakai et al. 2016b). In this sense, domestic cat ICSI embryos are in a similar situation. Although most injected oocytes are activated by sperm injection stimulus, only a few are capable of reaching the blastocyst stage. In this species, the problem probably lies on inadequate maturation, since developmental competence of ICSI and IVF embryos was shown to be lower for in vitro-matured oocytes compared to their in vivo counterparts (Gomez et al. 2000).

**Two strategies for improving pronuclei formation**

As discussed earlier, cow oocytes are not effectively activated by sperm after ICSI. In contrast to other animal groups, the great economical interest of this species has led to the development of in vitro production systems highly adapted to cow embryo requirements. However, male pronucleus formation after ICSI continues to fail (Wei & Fukui 1999, Suttner et al. 2000, Sekhavati et al. 2012). For these reasons, in the present section, we will mainly refer to bovine outcomes for describing the approaches assessed in order to improve pronuclei formation. Two types of strategies have been implemented: one focuses on the oocyte, by the use of exogenous activation treatments to induce early embryo development, and the other focuses on damaging the sperm membrane through the use of various pretreatments in order to emulate, as much as possible, regular fertilization events.

Regarding activation protocols, they usually include a chemical stimulus to increase Ca^{2+} concentrations in the injected oocytes. Examples are ionomycin (Rho et al. 1998a) or Ca^{2+} ionophore A23187 (Kolbe & Holtz 2000). In the case of pig embryos, an electrical stimulus is more widely employed to induce activation (Lee et al. 2003, Lee & Yang 2004, Matsuura et al. 2014). These treatments induce a single Ca^{2+} peak in the oocyte, causing a temporary inactivation of MPF that leads to the release from the meiotic arrest. However, it is not enough to induce pronuclei formation, since the inactive state of this factor needs to be maintained to allow complete activation (Kubiak et al. 1993, Susko-Parrish et al. 1994).

For this reason, activation protocols for domestic species combine the use of physical or chemical Ca^{2+} release inducers with an inhibitor of MPF and/or MAPK activities. The most widely used compounds are cycloheximide (CHX), a general inhibitor of protein synthesis (Baliga et al. 1969) and 6-dimethylaminopurine (6-DMAP), a protein kinase phosphorylation inhibitor (Szöllösi et al. 1993). Both treatments are capable of giving rise to acceptable blastocyst rates in the cow (Suttner et al. 2000, Oikawa et al. 2005, Bevacqua et al. 2010), which explains their extensive use for in vitro studies. However, only one newborn has been produced with 6-DMAP (Oikawa et al. 2005), while no births with CHX have been reported. Therefore, more specific activation treatments have been proposed, such as the use of a Ca^{2+} ionophore followed by dehydroecdysone (Vichera et al. 2010), roscovitine (Fernandes et al. 2014) and anisomycin (Arias et al. 2016), which were able to produce blastocysts. An important methodological aspect to consider prior to performing ICSI is how the activating agents affect 2PB extrusion. For example, the use of 6-DMAP immediately after Ca^{2+} ionophore inhibits 2PB extrusion (Rho et al. 1998b), a mistake commonly observed even in current ICSI studies. For this reason, the activation protocol for ICSI embryos must include a window of 3 h between Ca^{2+} ionophore and incubation with 6-DMAP (Ock et al. 2003). In contrast, when dehydroecdysone, roscovitine, CHX or anisomycin are used immediately after Ca^{2+} ionophore, 2PB extrusion occurs in most of the oocytes treated (Canel et al. 2010,
Arias et al. 2016, Suva et al. 2016). Additionally, the choice of an activation treatment must not be only based on its capability to produce blastocysts, since it is not necessarily related to the ability to sustain development to term. It is reflected by the outcomes produced by ethanol treatment, which is less efficient than others to produce blastocysts (Bevacqua et al. 2010), but has shown the higher birth rates worldwide. In fact, most calves produced by ICSI to date were activated with ethanol (Table 1). Moreover, the use of 6-DMAP has been shown to produce high numbers of ICSI and parthenogenetic embryos with chromosomal abnormalities (De La Fuente & King 1998, Rho et al. 1998b, Ross et al. 2008, Canel et al. 2010), explaining in part the frequent pregnancy loss.

Although there are no studies in domestic species, alterations in Ca$^{2+}$ signaling pathways during the activation of murine oocytes have been reported to affect not only early embryonic development, but also gene expression during embryo genome activation and, at the blastocyst stage, implantation and even development to term (Ozil & Huneau 2001, Ozil et al. 2006, Rogers et al. 2006). Therefore, it is essential to develop activation treatments that better mimic what occurs after regular fertilization. In this sense, Ross et al. (2008) proposed an interesting approach. These authors performed the intracytoplasmic injection of $PLC_{3}$-cRNA into cow oocytes and were able to induce sperm-like Ca$^{2+}$ oscillation patterns, resulting in rates of parthenogenetic development similar to those produced with ionomycin followed by CHX or DMAP. Additionally, embryos activated with $PLC_{3}$-cRNA showed lower levels of aneuploidy, which did not differ from those of IVF embryos. Although the use of cRNA is more complex than the routine protocols, these results are promising for their application on ICSI assays.

The second strategy widely employed to facilitate male pronucleus formation is to treat the sperm previous to ICSI, in order to emulate as much as possible how gametes interact in a regular fertilization event. Before in vivo fertilization, mammalian sperm cells undergo two physiological events in the female tract: capacitation, that confers the spermatozoon its ability to interact with the oocyte, and acrosome reaction, which consists on the exocytosis of the acrosome content. As a result, the sperm suffer a massive loss of membranes, and after penetrating the zona pellucida, it fuses to the oolemma (Yanagimachi 1994). After fusion, the remaining inner acrosomal membrane is also disrupted, allowing the direct interaction of both sperm and oocyte cytoplasm and the release of $PLC_{3}$, which triggers oocyte activation (Malcuit et al. 2006b, Roldan 2006). In contrast, these events are bypassed when performing ICSI, since an intact spermatozoon is directly injected in the cytoplasm of a mature oocyte. Therefore, the complex structure of sperm membranes maintains the sperm nucleus and the ooplasm separated (Sutovsky & Schatten 2000, Morozumi et al. 2006, Roldan 2006), which might be a possible cause of the reduced developmental competence of the resulting embryos (Morozumi et al. 2006, Yanagimachi 2011, Aguila et al. 2017).

### Table 1

Reported live born offspring after ICSI in bovine, sheep, goat, pig and horse.

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>Live born</th>
<th>Observations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>Ethanol</td>
<td>10</td>
<td>Cell sorted sperm heads</td>
<td>Hamano et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>Piezo</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Piezo/ethanol</td>
<td>9 (ethanol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Piezo/6-DMAP</td>
<td>1 (Io + 6-DMAP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Piezo/ethanol</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Piezo/dithiothreitol</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>Io</td>
<td>1</td>
<td>Cell sorted</td>
<td>Catt &amp; Rhodes (1995)</td>
</tr>
<tr>
<td></td>
<td>CaCl$_2$ activated</td>
<td>13</td>
<td>Cell sorted sperm</td>
<td>Gomez et al. (1998a,b)</td>
</tr>
<tr>
<td></td>
<td>Electrical</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Electrical. Piezo</td>
<td>62 (tg 8)</td>
<td>BAC</td>
<td>Watanabe et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Electrical. Piezo</td>
<td>6 (tg 2)</td>
<td></td>
<td>Matsunari et al. (2014)</td>
</tr>
<tr>
<td>Goat</td>
<td>Piezo</td>
<td>2</td>
<td>Fresh sperm</td>
<td>Wang et al. (2003)</td>
</tr>
<tr>
<td>Pig</td>
<td>Io</td>
<td>3</td>
<td>Fresh sperm in vivo matured/centrifuged</td>
<td>Martin (2000)</td>
</tr>
<tr>
<td></td>
<td>CaCl$_2$ activated</td>
<td>13</td>
<td>Cell sorted sperm</td>
<td>Koilbe &amp; Holtz (2000)</td>
</tr>
<tr>
<td></td>
<td>Electrical</td>
<td>3</td>
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<tr>
<td></td>
<td>Electrical. Piezo</td>
<td>62 (tg 8)</td>
<td>BAC</td>
<td>Watanabe et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Electrical. Piezo</td>
<td>6 (tg 2)</td>
<td></td>
<td>Matsunari et al. (2014)</td>
</tr>
<tr>
<td>Horse</td>
<td>–</td>
<td>2</td>
<td>Euthanasia or after death for oocyte donor</td>
<td>Hinrichs et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Piezo</td>
<td>7</td>
<td></td>
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<tr>
<td></td>
<td>Piezo</td>
<td>2</td>
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<tr>
<td></td>
<td>Piezo</td>
<td>10</td>
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Io, Ca-ionophore; Tg, transgenic.
As outlined earlier, several sperm treatments prior to ICSI were designed to remove or modified the sperm membranes for facilitating sperm decondensation and pronuclear formation. Among chemical agents, the most widely used for these purposes is dithiothreitol (DTT). Although some studies have reported that DTT increases blastocyst rates (Rho et al. 1998a, Wei & Fukui 1999, Oikawa 2013, Okawa 2016) and has led to the birth of a viable calf (Galli et al. 2003), no positive effect has been observed in other studies (Suttner et al. 2000, Arias et al. 2014). Moreover, severe damage on sperm DNA was observed after DTT treatment, which ultimately could affect the quality of ICSI embryos (Sekhavati et al. 2012). In recent years, many membrane-disrupting agents were tested, such as Triton X-100 (Lee & Yang 2004), sodium hydroxide (Arias et al. 2014), dithiobutylamine (Suttirojpattana et al. 2016), lyssolecithin (Morozumi et al. 2006, Zambrano et al. 2017) and methyl-β-cyclodextrin (Arias et al. 2017). Some of these treatments raised blastocyst rates, but male pronucleus formation was not improved in all cases. Furthermore, some of them were reported to induce a decrease of PLCζ (Zambrano et al. 2016) and their effects on development to term have not been evaluated. The location of PLCζ in the sperm is an important issue to be taken into account for ICSI protocol design, since it varies among species. For example, it was found in the equatorial area of bull sperm (Yoon & Fissore 2007), in the post-acrosomal region and the tail of pig sperm (Nakai et al. 2011) and in the acrosomal and equatorial regions of the stallion sperm head as well as in the principal piece of the flagellum (Bedford-Guas et al. 2011). Such differences can be a source of variability in the response of sperm to pre-treatments, including the use of the piezo drill.

A more physiological approach was the use of reduced glutathione, an endogenous disulfide bond reducer that in combination with heparin induces in vitro decondensation of spermatozoa of several species (Reyes et al. 1989, 1996, Sanchez-Vazquez et al. 1996, 1998, Delgado et al. 2001). Sperm treatment with heparin and glutathione prior to ICSI was shown to facilitate sperm decondensation without damaging DNA and to improve embryo development and blastocyst quality after ICSI in the cow (Sekhavati et al. 2012, Canal et al. 2017). However, since no offspring were reported, these results should be treated with caution.

Finally, the mechanical damage induced by piezo drill on the sperm deserves special attention. During the ICSI procedure, the sperm tail is intentionally damaged before injection, since it was shown to be a critical step for ultimate success (Wei & Fukui 1999). Some researchers consider that it would cause the release of PLCζ and other factors within the oocyte cytoplasm after injection, giving rise to the activation cascade (Yanagida et al. 2001, Morozumi et al. 2006). It is well known that the sole sperm injection stimulus into the cytoplasm of a mature oocyte is sufficient to activate embryo development in horses (Dell’Aquila et al. 2001, Tremoleda et al. 2003, Lewis et al. 2016). It might be due to the great capability of the equine isoform of PLCζ to generate Ca2+ peaks, combined with the use of piezo drill, which allows the release of PLCζ in the ooplasm. Ca2+ peaks induced by equine isoform of PLCζ were shown to begin earlier and to have a higher frequency than those observed in other species studied (Sato et al. 2013). Therefore, the localization and strength of equine PLCζ may explain, at least in part, the major repeatability achieved for ICSI in horses by the use of piezo drill (Galli et al. 2002, Choi et al. 2003), in contrast to other domestic species, specially the bovine (Katayose et al. 1999, Horiiuch et al. 2002, Wang et al. 2003, Devito et al. 2010). Nonetheless, it is important to highlight that most new born calves produced by ICSI have been subjected to piezo drill, in combination with an activation treatment (Table 1).

Coincident with the observations of Wei and Fukui (1999), our experience led us to hypothesize that a big part of the variability of ICSI outcomes in domestic species is due to the different responses of males to sperm pre-treatments. Thus, greater knowledge of the mechanisms governing the early events of fertilization is needed to determine the exact combination of sperm pre-treatments and activation protocols required for successful ICSI in domestic species, particularly in the bovine.

ICSI-mediated gene transfer (ICSI-MGT)

The technique of ICSI-MGT is based on the fact that transgenes may spontaneously attach to the external sperm membrane, and then be passively transported into the cytoplasm of a mature oocyte when the spermatozoon is introduced by ICSI. In this way, integration of transgenes is possible during early stages of pronuclei formation (Perry et al. 1999). Some benefits associated with ICSI-MGT are that it avoids the epigenetic failures induced by SCNT (Rideout et al. 2001) and the high rates of oocyte lysis provoked by pronucleus microinjection. In our laboratory, cow, sheep, horse, pig and domestic cat GFP (green fluorescent protein) expressing embryos have been produced by ICSI-MGT (Pereyra Bonnet et al. 2008, 2011). Moreover, by the use of improved activation treatments, rates of GFP-expressing cow blastocysts exceeded 80% after ICSI-MGT (Bevacqua et al. 2010). Although several researchers reported experiments of ICSI-MGT in the cow (Canet et al. 2017), ewe (Gou et al. 2002), goat (Shadanloo et al. 2009), horse (Zaniboni et al. 2013) and monkeys (Chan et al. 2000), most of them only observed cytoplasmic expression of the transgenes, without giving evidence of their integration into the genome, or the birth of transgenic live animals. In contrast, several transgenic pigs have been successfully generated using this technology (Kurome et al. 2006, Yong et al. 2006, Wei & Fukui 1999).
Umeyama et al. 2012, Matsunari et al. 2014). In conclusion, ICSI-MGT in farm animal has only produced repeatable results in pigs, wherein several transgenic offspring have resulted. However, interest in producing transgenic animals by ICSI-MGT has decreased with the advent of new tools of gene edition (CRISPR-Cas9 and TALEN system), which are technically simpler (Hai et al. 2014, Whitworth et al. 2014, Proudfoot et al. 2015, Wang et al. 2015, Bevacqua et al. 2016). In our laboratory, an alternative application of ICSI-MGT has been proposed. The high lipid content of cow oocytes impedes the visualization of pronuclei after fertilization. Since ICSI in cattle must be followed by artificial activation, ICSI embryos (which are products of proper sperm decondensation) need to be distinguished from those produced merely by artificial activation. To do this, sperm can be subjected to a brief incubation with pCX-EGFP before ICSI. This plasmid contains the gene coding for green fluorescent protein (GFP) under the control of a promoter that is constitutively expressed at early stages of embryo development (Ikawa et al. 1995). Such expression can be simply detected by observation of embryos under UV light at day 4 of in vitro culture. A previous report from our group (Bevacqua et al. 2010) showed that all GFP-expressing embryos had successfully undergone pronuclei formation. By contrast, more than 50–100% of embryos without GFP expression showed a condensed sperm head inside them, depending on the activation treatment employed. These results reflect a strong association between pCX-EGFP expression and sperm head decondensation after ICSI. Therefore, the joint injection of pCX-EGFP with sperm, and the subsequent evaluation of GFP expression can be used as an indicator of efficient sperm decondensation, as was done by Canel et al. (2017). In addition, this method might be easily adapted to other domestic species whose oocytes present similar or even greater lipid content, since sperm–plasmid incubation previous to ICSI also produces GFP-expressing embryos (Pereyra Bonnet et al. 2008).

**Clinical applications for ICSI: a success in horses**

Breeding selection in horses is usually based on their sporting performance, beauty or body conformation, rather than reproductive abilities, as is the case for other farm animals. Consequently, subfertility and infertility problems are unintentionally conserved in donor mares and stallions. Several conditions like chronic uterine diseases, endometritis, cervical lacerations and other serious physical injuries in the female reproductive tract frequently reduce or restrict the chances of mares to conceive a pregnancy (Foss et al. 2013, Rader et al. 2016). As well, some stallions that show good performance are sub-fertile or their sperm supplies are limited, since they are castrated before showing valuable genetic characteristics or die unexpectedly. For these reasons, the advent of new reproductive technologies inevitably led to the inclusion of an in vitro embryo production system in horse reproductive programs. In particular, the ICSI technique gained importance in this species since a consistent IVF protocol has not been yet developed, possibly due to an incomplete capacitation of the stallion spermatozoa, that apparently disables them to penetrate the zona pellucida in vitro (Leemans et al. 2016). Unfortunately, in spite of numerous attempts to make conventional IVF successful for horses, outcomes continue to be disappointing.

After the first report of a pregnancy derived from an in vitro-matured oocyte fertilized using ICSI (Squires et al. 1996), the encouraging outcome was followed by a period of variable results. The introduction of the piezo drill and modifications in the culture media allowed an improvement in cleavage rates and repeatability of ICSI protocols (Choi et al. 2002, 2004, Galli et al. 2002). However, reported blastocyst rates still vary from 0 to 42% depending on mare age, follicle stage, oocyte quality and fertility of the stallion (Tremoleda et al. 2003, Hinrichs et al. 2012, 2013, Foss et al. 2013, Choi et al. 2016, Rader et al. 2016). With the use of these technologies, pregnancy rates after embryo transfer are usually high, varying from 50 to 80% (Hinrichs 2013, Galli et al. 2014).

Additional advantages for ICSI are seen in horses. For example, oocytes can be placed at room temperature in commercial embryo-holding media for 18–24h, so in vitro maturation can be delayed allowing a flexible work schedule and simplifying the transport of immature oocytes from the farm to the laboratory (Choi et al. 2006, Foss et al. 2013, Martino et al. 2014, Carnevale 2016, Dini et al. 2016). Although the equine is a seasonal species (long day breeders), ICSI can be performed at any time of the year and at any stage of the reproductive cycle, as long as there are follicles present in the ovaries. It avoids interference with training or sporting activities of the donor mares, which is critical for a species of commercial interest. Additionally, in contrast to other species like pigs, horse ICSI embryos can be successfully cryopreserved for later transfer (Galli et al. 2002, Hinrichs 2013).

Another interesting alternative for the use of ICSI is when an unfortunate event like accident or illness results in the death or euthanasia of a valuable mare. The application of this technique might offer the chance of getting offspring by recovery of oocytes within 7h of death (Ribeiro et al. 2008, Carnevale 2016). The subsequent ICSI performance using sperm from a desired stallion can result in valuable embryos that can be transferred to a recipient mare or cryopreserved for future transfer (Carnevale et al. 2003, Hinrichs et al. 2012).

Nowadays, the horse remains at the forefront of the ICSI technique over other domestic species, showing an exponential increase in the use of this technology...
in the last two decades. Viable embryos are routinely produced from donor mares in a consistent and repeatable manner, by the combination of transvaginal aspiration followed by ICSI and in vitro culture of embryos to the blastocyst stage. Moreover, embryos are obtained using small pieces of frozen semen straws or even from lyophilized sperm (Choi et al. 2011). Currently, perspectives are focused on the conservation of female gametes by vitrification. Blastocyst rates after vitrification of immature oocytes have reached values of 10% (Siqueira Canesin et al. 2017), showing great potential for female genetic preservation, that might also give rise to new alternatives for genetic rescue of wild equids (Smits et al. 2012b). However, more research is needed to improve its efficiency and also to unveil the mysteries of the IVF technique in horses. Such discovery would mean a resounding improvement for the horse industry.

A promising technique for endangered and nontraditional species

Small populations of endangered species have a lack of genetic diversity increasing the chance of inbreeding and homozygosis (Roldan et al. 2006). This reduces the adaptation capacity and increases the risk of inherited diseases, congenital defects and decreases fertility (Comizzoli et al. 2000). In many cases, these animals have reduced sperm quality, which limits regular reproduction or the use of ARTs like AI or IVF (Howard et al. 1993, Koester et al. 2015), ICSI can be used in cases. Additionally, it is possible to produce offspring from gametes of deceased animals (Fernandez-Gonzalez et al. 2015) or improve the fertility of poor sperm (Choi et al. 2016) or oocyte quality (Jimenez-Macedo et al. 2007, Catala et al. 2012, Ohlweiler et al. 2013). This technique allows the selection of morphologically normal spermatozoa, even from samples containing a large proportion of teratozoospermic sperm (Penfold et al. 2003), which are frequently observed in zoo inbred species. In camelids, where sperm freezing and thawing protocols are not efficient, ICSI might be an option to achieve in vitro-produced embryo (Sansinena et al. 2007, Conde et al. 2008).

Concerning to felids, ICSI in the domestic cat has been a valuable model to develop this technology for their wild counterparts (Moro et al. 2014). Some researchers have reported that artificial activation is necessary to restart the oocyte cell cycle after ICSI (Boglìolo et al. 2001, Comizzoli et al. 2006), while others observed embryo development without the need of any type of activation treatment (Pope et al. 1998, Penfold et al. 2003, Moro et al. 2014). This difference may be correlated with the concentration of PLCζ, which was shown to vary among males (Villaverde et al. 2013). Since the first cat was produced by ICSI using fresh semen and in vivo-matured oocytes (Pope et al. 1998, Gomez et al. 2000), kittens have also been produced with the use of frozen semen (Gomez et al. 2003, Tharasani et al. 2012). However, there are few reports of blastocysts production by in vitro maturation of oocytes by ICSI in wild felids.

In wild or endangered species, oocytes are a limiting factor. Thus, interspecific ICSI can be used to evaluate the fertilizing capability of spermatozoa from exotic species using in vitro-matured oocytes from domestic animals. In our laboratory, good rates of ICSI blastocysts were produced after injecting cheetah and leopard spermatozoa into domestic cat oocytes, without any activation treatment (Moro et al. 2014). Also Kaneko et al. (2014) used mouse oocytes to evaluate freeze-dried sperm samples from the chimpanzee, giraffe, jaguar, weasel and the long-haired rat.

Finally, ICSI allows the reproduction of wild animals that are separated by space (natural habitat and zoos) and time (cryobanking), even when sperm are poorly cryopreserved or in low number.

Final considerations

Nowadays, the low repeatability and the high complexity of ICSI technique in domestic species have restricted this technology to the production of elite horses. It is expected that the use of ICSI would contribute to preserve the genetic diversity of endangered mammals, especially for those species that are closely related to domestic ones, for which ICSI has shown promising results. Currently, the ICSI technique is an unlimited source of information regarding the fertilization process. It offers a great potential for clarifying mechanistic differences among mammalian species, with high impact perspectives in both basic and applied research fields.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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