Reproduction in Domestic Animals

Reprod Dom Anim **45** (Suppl. 2), 40–48 (2010); doi: 10.1111/j.1439-0531.2010.01623.x
ISSN 0936-6768

Advances in Swine *In Vitro* Embryo Production Technologies

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Contents

Recent advances in new technologies to produce cloned and genetically modified pigs involve manipulating oocytes and/or embryos *in vitro*. Although a great deal of progress has been made, the current IVM–IVF systems still result in major problems: a high rate of polyspermy; and a low development rate and low quality of blastocysts for *in vitro* compared with the *in vivo*–produced embryos. This study summarizes recent advancements in IVM–IVF–IVC porcine systems. Recent methods to select monospermic embryos are also discussed. Finally, achievements in vitrification and in somatic cell nuclear transfer are discussed.

Introduction

The *in vitro* production (IVP) of porcine embryos has been of particular interest to researchers for many years (reviewed in Abeydeera 2002; Wheeler et al. 2004). The availability of a large number of matured oocytes and embryos through *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) and embryo culture (IVC) techniques would be crucial for agricultural and biomedical purposes. Because of their physiological similarities to humans, pigs can be used as research biomodels and for creating genetically modified animals as potential donors of tissues and organs for xenotransplantation. These biotechnologies require mature oocytes and zygotes of good quality.

Currently, it is possible to exploit the large pool of fully grown oocytes present in medium-sized to large-sized follicles and mature them under proper conditions. The *in vitro*–matured oocytes can be penetrated by spermatozoa resulting in high rates of pronuclear formation and be converted into viable embryos with acceptable pregnancy rates and litter sizes after their transfer to recipient animals (Mattioli et al. 1989; Yoshida et al. 1993; Abeydeera et al. 1998a; Kikuchi et al. 1999; Yoshioka et al. 2003; Somfai et al. 2009). However, despite this extensive research, we are still faced with the unresolved problem of a high incidence of polyspermy resulting from IVF. In fact, the efficiency of IVF (percentage of monospermic oocytes from total inseminated) still remains at 30–50% in most laboratories (Abeydeera et al. 1998b; Funahashi et al. 2000; Suzuki et al. 2000; Gil et al. 2003, 2004). Furthermore, because polyspermic porcine embryos from IVF cleave and develop to the blastocyst stage at the same percentage as normal embryos (Han et al. 1999a,b), little information is obtained from monitoring blastocyst formation. Accordingly, it is necessary to visualize the formation of pronuclei by staining or cytogenetic examination by chromosomal karyotyping to actually assess the incidence of normal fertilization.

Several studies aiming to decrease polyspermy have investigated the efficiency of intracytoplasmic sperm injection (ICSI) for embryo production in pigs (Kolbe and Holtz 2000; Nakai et al. 2003). Unfortunately, the results remain lower than for IVF, and therefore, this technique is not used routinely. Recently, it was suggested that the centrifugation of presumptive zygotes to visualize pronuclei could be used to select monospermic embryos (Gil et al. 2008b; Somfai et al. 2008a).

In parallel with the advances in IVM–IVF–IVC technologies, important achievements have been made in somatic cell nuclear transfer (SCNT). Improvements in enucleation skills, electrofusion and activation are rapidly increasing the efficiency as well as creating the possibility to produce animals genetically modified according to specific goals (reviewed in Vajta et al. 2007). There has been a similar level of achievement in the cryopreservation of IVP embryos by vitrification (Men et al. 2005, 2006; Somfai et al. 2008b, 2009), enabling even the birth of the first cloned piglets after vitrification at the blastocyst stage (Li et al. 2006).

These reproductive technologies will be reviewed with emphasis on recent advancements and on our own research.

'Developments In IVM–IVF Systems'

The technology of pig IVM and IVF, and related problems, has been well reviewed (Day et al. 2000; Abeydeera 2002; Funahashi 2003; Gil et al. 2008a). Oocyte maturation includes nuclear as well as cytoplasmic maturation. According to Schoevers et al. (2005), these two processes must be considered interdependent. However, although nuclear maturation seems to be completely established during IVM, the maturation of the cytoplasm is still inappropriate. This is responsible, at least in part, for the frequent occurrence of polyspermy and the low developmental rates after IVF of IVM oocytes. The IVM conditions could cause incomplete movement of mitochondria to the inner cytoplasm of the oocytes and thus affect cytoplasmic maturation (Sun et al. 2001).

Intracellular glutathione (GSH) content and the ability of cytoplasm to decondense the sperm nucleus or to induce male pronucleus formation have been used as indicators of cytoplasmic maturation. The addition of cysteine, cysteamine, epidermal growth factor, glutamine, beta-mercaptoethanol, 9-cis retinoic acid and hormones improves cytoplasmic maturation and subsequent development of pig oocytes (Grupen et al. 1995; Abeydeera et al. 1998b, 2000; Day et al. 2000; Almiñana et al. 2008b). In general, most of these chemicals are included in the more common maturation media:

NCSU23, NCSU37 and TCM199. Although only chemically defined media should be used to standardize the investigations between different laboratories, most of them supplement the maturation medium with porcine follicular fluid (pFF). The beneficial effect of the addition of pFF as the only protein supplement has been demonstrated in several studies (Yoshida et al. 1992; Algriany et al. 2004). Recently, Takemoto et al. (2004) found that pFF is critical in protecting oocytes from oxidative stress. However, the exact role of pFF in the maturation medium remains unclear. Furthermore, successful piglet production has been reported using oocytes matured in a chemically defined system (Yoshioka et al. 2008; Mito et al. 2009), even by making it gonadotropin-free (Akaki et al. 2009).

Another critical point of the maturation culture is the selection of the oocytes. Immature oocytes are generally recovered from ovaries of slaughtered animals, which results in a mixing of oocytes at different growth phases. Results obtained by Marchal et al. (2002b) show that oocytes from large follicles (more than 5 mm in diameter) have more ability to develop into embryos than oocytes from small follicles (< 3 mm in diameter). However, in terms of efficiency, the diameter of follicles is difficult to control. Furthermore, after aspiration of the follicles, the oocytes are commonly selected using various criteria such as their morphology, including the numbers of cumulus cell layers and evaluation of the granulation of the cytoplasm. Those morphological evaluations are subjective, and categorization standards vary among investigators. As an alternative, exposure of the oocytes to brilliant cresyl blue (BCB) has been suggested to select developmentally competent oocytes, according to differing colour (Roca et al. 1998; Wongsrikeao et al. 2006; Ishizaki et al. 2009). The BCB test permits assessment of the intracellular activity of glucose-6-phosphate dehydrogenase (G6PD), an enzyme synthesized in growing oocytes, but with decreased activity in oocytes that have finished their growth phase. BCB is a blue compound giving a blue colour to the cytoplasm of oocytes that have finished their growth, because G6PD activity decreases. Nevertheless, we have been unable to find significant differences for penetration, monospermic and blastocyst formation between oocytes selected by the BCB test or by morphological criteria (Almiñana et al. 2008d).

With current IVM protocols, high rates of mature oocytes (75–85%) are achieved after 40–44 h (Hong and Lee 2007; Almiñana et al. 2008b; Yoshioka et al. 2008; Yuan and Krisher 2010), in terms of oocytes arrested at the metaphase II (MII) stage with the first polar body extruded. Thereafter, the mature oocyte can be directly manipulated for SCNT or cultured with spermatozoa to obtain *in vitro* embryos. During IVF, sperm penetration as well as polyspermic fertilization begins at 2 h post insemination with frozen-thawed spermatozoa (Marchal et al. 2002a) and/or with fresh semen (Martinez et al. 1996). Sperm penetration quickly induces the resumption of meiosis and the cortical reaction that blocks further penetration of surplus spermatozoa (polyspermy). It has been reported that the high level of polyspermy in pig oocytes inseminated *in vitro* is not attributable to delayed or incomplete cortical granule exocytosis but more likely to a delayed zona reaction and/or multiple sperm entry (Wang et al. 1998). Typically in standard IVF systems, a large number of spermatozoa are present at the site of fertilization, often resulting in high number of spermatozoa penetrating the oocyte simultaneously. Therefore, suboptimal IVF conditions are one of the reasons for polyspermic penetration.

The conditions used for the fertilization of *in vitro*–matured porcine oocytes are constantly being studied and re-invented to reduce the rate of polyspermy *in vitro* by attempting to simulate the oviductal microenvironment in the Petri dish. Co-culture of spermatozoa or *in vitro*–matured oocytes with oviduct cells, pig oviduct-specific secretory glycoprotein (pOSP), pig follicular fluid (pFF), oviductal cell monolayers or pig oviduct epithelial cells (POECs) before and during IVF has been evaluated (Nagai and Moor 1990; Funahashi and Day 1993; Vatzias and Hagen 1999; Bureau et al. 2000; Kouba et al. 2000; McCauley et al. 2003;. These conditions have resulted in significant improvements in the rates of monospermy, but they are still not optimal. Moreover, such co-culture involves undefined factors. The tendency now is to use defined or semi-defined culture media in conjunction with the use of frozen-thawed spermatozoa, which provides much more reproducible and repetitive data than fresh semen because of the individual effect of the boar (Martinez et al. 1993; Suzuki et al. 2005). Recent research has concentrated on modifying the co-incubation time from 5 h to 10 min, maintaining the oocytes with the zona-bound spermatozoa in a fresh IVF medium (containing no spermatozoa) for an additional 5 h to reduce the exposure of oocytes to an excessive number of sperm cells. This modification of the IVF conditions has yielded high penetration and blastocyst development rates (Grupen and Nottle 2000; Funahashi and Romar 2004; Gil et al. 2004). Based on our experience, we can confirm that sperm bound to the zona pellucida within the first 2–10 min of gamete co-incubation is able to penetrate a high number of oocytes (Gil et al. 2004; Almiñana et al. 2008c). However, there are a number of considerations when using a reduced co-incubation period as a strategy to increase the efficiency of IVF. First, there is an evident boar effect on the fertilizing ability of spermatozoa during short co-incubation (reviewed in Gil et al. 2008a), and secondly, we have observed that the effectiveness of a reduced co-incubation time in decreasing polyspermy is dependent on the sperm:oocyte ratio (Gil et al. 2007). For a 10-min co-incubation, IVF efficiency was best when combined with high sperm:oocyte ratios (36–38% for 1500 and 1000 sperm:oocyte, respectively). While for long co-incubations, a low sperm:oocyte ratio (37% for 500 sperm:oocyte) was better.

Other studies have attempted to modify the equipment used for IVF to regulate the number of penetrable spermatozoa near the oocytes. The climbing over a wall (COW) method (Funahashi and Nagai 2000), biomimetic microchannel IVF system (Wheeler et al. 2004), straw IVF (Li et al. 2003) or the modified swim-up method (Park et al. 2009) has been proposed as ways to separate gametes and ensure that only motile spermatozoa gain

access to the oocytes, mimicking the physical conditions of fertilization *in vivo*. These alternative IVF techniques could moderate the frequency of polyspermy but still not eliminate it completely. In that sense, we have combined the straw IVF system suggested by Li et al. (2003) with a 10-min co-incubation time, leading to increases in monospermy, fertilization efficiency and blastocyst quality compared to a microdrop-IVF system, independent of the sperm:oocyte ratio (Almiñana et al. 2008a). This potentially conveys a great advantage over traditional IVF protocols, because it allows for the use of a wide range of sperm:oocyte ratios to achieve high levels of monospermic fertilization.

Fertilization efficiency is also affected by the type of culture medium and by the components added during IVF. The more common formulations include modified Tris-buffered medium (mTBM), Tissue Culture Medium 199 (TCM199), modified Tyrode's medium (mTALP) and modified Whitten's medium (mWM). Most of these IVF media are supplemented with caffeine to enhance sperm motility and to induce capacitation before insemination and/or during coculture with oocytes. However, the spontaneous acrosome reaction induced by caffeine may be related to the high incidence of polyspermic fertilization in IVF (Funahashi and Nagai 2001). Funahashi et al. (2000) significantly reduced polyspermic penetration by replacing caffeine with other capacitating compounds such as adenosine and fertilization-promoting peptide (FPP) in the fertilization medium (87% vs 21% and 25%, respectively). Similar to these compounds, hyaluronic acid (HA) appears to induce sperm capacitation without the subsequent spontaneous acrosome reaction and decreases polyspermy without any significant effect on penetration rate (Suzuki et al. 2000). However, when the combined effects of caffeine, HA and adenosine were evaluated as additives to the fertilization medium, a clear boar influence was observed when frozen-thawed spermatozoa from two boars were evaluated in our laboratory (Almiñana et al. 2005). Only caffeine in the IVF medium had a significant effect on fertilization efficiency, but there were significant differences between sires. For one boar, the efficiency of fertilization was higher in caffeine-free treatments, reaching values between 42% and 53% ($p < 0.001$), while for another boar, it was higher ($p < 0.001$) when caffeine was present (36–42%) than when it was absent (26–30%). Accordingly, caffeine may be a necessary supplement for some boars, but not for others. Recently, Miao et al. (2009) reported positive effects, and they propose caffeine as a stabilizing compound for microtubules and centrosomes in MII oocytes to overcome oocyte ageing in IVF procedures.

As mentioned earlier, to date, no IVF system has been able to mimic the *in vivo* situation. Furthermore, a single IVF protocol may not be optimal for different batches of frozen-thawed spermatozoa. This implies that before using a batch of frozen-thawed semen for IVF, it is imperative to empirically define the optimal IVF conditions to obtain maximum efficiency. Therefore, the number of spermatozoa per oocyte and the presence/absence of caffeine need to be established for each individual batch of semen (reviewed in Gil et al. 2008a).

Selection of Monospermic Zygotes

Considering the fact that the protocol to block polyspermy has not yet been established for porcine IVF, a good method for isolating monospermic zygotes should be of a great importance. Generally, the pronuclei of pig oocytes cannot be visualized in the dark cytoplasm without nuclear staining because of the high amount of lipid droplets. Ten years ago, Han et al. (1999a) proposed an alternative method to classify IVF oocytes into two (2PN) and poly-pronuclear (PPN) zygotes. That selection method involved the centrifugation of the presumptive zygotes, approximately 10–16 h after insemination, at 10 000–15 000 $\times g$ for 10–20 min to isolate the lipids of the cytoplasm and permit the visualization of the pronuclei. This method has been used by different researchers with accuracies of 94.6% (Han et al. 1999a,b), 75–83% (Somfai et al. 2008a) and 85.2% in our laboratory (Gil et al. 2008b) for presumptive zygotes classified as 2PN zygotes. Furthermore, embryo development to the blastocyst stage is not affected by the high force of centrifugation. In fact, experiments performed in our laboratory indicate that in comparison with PPN or not selected zygotes, higher blastocyst formation is obtained after the IVC of 2PN zygotes (53.3%, 33.4% and 80.7%, for PPN, not selected and 2PN, respectively). Based on these results, we can now suggest that the centrifugation of presumptive zygotes could be used to select monospermic blastocyst.

Currently, one experiment is underway to determine the ability of 2PN zygotes to develop to term after their transfer to recipients by laparoscopy. Laparoscopy is a less invasive technique than laparotomy for the transfer of embryos into the oviduct. This procedure has routinely been applied to commercial inseminations of sheep and goats (Evans and Maxwell 1987). Recently in our laboratory, we have adapted the laparoscopy procedure in pigs for intra-oviductal insemination of sex-sorted sperm (Vazquez et al. 2006; Garcia et al. 2007) and for embryo transfer, which must be carried out on anaesthetized animals. We have transferred presumptive zygotes into twenty gilts with spontaneous oestrus and with ovulation confirmed at the time of the laparoscopy. The promising results obtained confirm the use of the centrifugation method as a selection system for *in vitro* embryos. In addition, the development of a minimum invasive embryo transfer technique may eventually result in a simpler procedure for establishment of pregnancies with cloned embryos as well.

'Somatic Cell Nuclear Transfer'

Since the famous Dolly was born (Wilmut et al. 1997), somatic cell nuclear transfer (SCNT) has become the primary technique for the production of transgenic domestic animals. In pigs, the main application for SCNT is to create additional models of human diseases and/or to provide organs for transplantation. The first piglets produced by SCNT were achieved with both *in vivo*–matured (Onishi et al. 2000; Polajaeva et al. 2000) and *in vitro*–matured oocytes (Betthauser et al. 2000). Since then, the number of cloned piglets produced

has continuously increased but still with a very low overall efficiency (reviewed in Vajta et al. 2007). Unfortunately, some of the offspring resulting from SCNT present abnormal phenotypes resulting from epigenetic modification (Rideout et al. 2001; Prather et al. 2003).

The technique of SCNT involves the removal of the nuclear material of matured oocytes, injection of the donor cell, the activation of the reconstructed oocytes and finally the culture *in vitro* and/or the transfer to a recipient. Briefly, oocytes are usually fluorescently stained with bisbenzimide dye (Hoechst 33342) to visualize the position of the nuclear material and the polar body. Enucleation is usually performed by aspirating both the first polar body and adjacent cytoplasm under an inverted microscope equipped with ultraviolet (UV) light. Recently, we have observed that staining for 10–12 min with Hoechst 33342 and visualization under UV light for 30 s significantly decreased the development of IVM oocytes to embryos after their insemination *in vitro*. The cleavage and blastocyst formation rates were 49.8–7.6% and 77.7–30.9% for stained/UV irradiated or not stained (control), respectively (Maside C, Gil MA, Martinez EA, unpublished data). These findings agree with those of Smith (1993). This author demonstrated that Hoechst 33342 staining should not be used for UV exposure periods of more than 30 s in the bovine, suggesting some cytoplasmic effect owing to damage to the transcriptional ability of mitochondria. Experiments are underway to evaluate the effects of UV irradiation on the intracellular components of IVM porcine oocytes stained with Hoechst. Instead of enucleation based on fluorescent staining, some researches remove the nuclear material orientated only by the position of the polar body, pushing out the first polar body and adjacent cytoplasm containing presumably the metaphase-II chromosomes (Lee et al. 2003a). An interesting enucleation method was reported by Yin et al. (2002). They obtained offspring after chemically assisted enucleation using demecolcine incubation and the subsequent removal of a small extrusion containing the chromatin.

Once the nuclear material of the oocytes has been removed, the donor cell can be transferred to the enucleated oocyte by whole injection (Lee et al. 2003b), injection into the perivitelline space (Lee et al. 2008), by electrofusion of the donor and cytoplast (Polajaeva et al. 2000) or by piezo-actuated microinjection of isolated donor nuclei (Onishi et al. 2000). Following cell injection, oocyte–cell couples must be fused and/or artificially activated to initiate embryonic development. Usually, activation is induced by electrical stimulus, using a short and high voltage direct current pulse. In the literature, there are different activation strategies, voltages, cases of simultaneous fusion/activation by electrical stimulus or delayed activation. However, because the same results are not always observed between laboratories, it is difficult to establish a standard fusion–activation protocol.

After fusion–activation, the reconstructed oocytes can be cultured *in vitro* and/or transferred into a recipient. Pregnancy has already been achieved in many laboratories from different countries. Furthermore, the number of pregnant recipients after embryo transfer of 50–100 or sometimes even more embryos on the day of SCNT may be high, even 100% (reviewed in Vajta et al. 2007). Recently, we have achieved the first cloned domestic animal produced in Spain by SCNT. In brief, SCNT embryos (110–135 embryos/recipient), produced by enucleation and injection of fibroblasts isolated from foetuses on Day 30 of gestation, were transferred by mid-ventral laparotomy to three recipients gilts in spontaneous oestrus. Two of them became pregnant and one farrowed three live and one stillborn cloned piglets. Two cloned piglets died during the first week of life from gastrointestinal disease. The third piglet, named Kaka, is now a healthy 6-month-old boar.

Interestingly, successful cloned piglet production has been reported after co-transfer of parthernotes (Kawarasaki et al. 2009) and hormonal injection after the transfer (Lee et al. 2008) to enhance and maintain the signal of pregnancy.

More research is needed on technical innovations to simplify the micromanipulations involved in SCNT to make the procedure easier and increase the speed of work. Zona-free nuclear transfer with mechanical aspiration of the metaphase plate (Lagutina et al. 2007) or micromanipulator-free handmade cloning (Vajta et al. 2005) has resulted in high *in vitro* developmental rates to blastocyst formation of good quality embryos. The absence of the zona pellucida makes enucleation easier and significantly increases cell fusion success. However, it requires prolongation of *in vitro* embryo culture up to the morula or blastocyst stages.

In Vitro Embryo Culture

In Vitro embryo culture is an important procedure for improving the developmental competence of *in vitro* embryos produced by IVM–IVF as well as by SCNT. Furthermore, Yamanaka et al. (2009) have demonstrated that pig SCNT embryos are more easily affected by culture environment compared with IVF embryos. Therefore, the developmental competence of SCNT embryos would be further enhanced by developing more appropriate culture conditions (Yamanaka et al. 2009). For IVC of pigs, several media have been developed and used. However, the IVC media reported by Petters and Wells (1993), the North Caroline State University (NCSU-23 and NCSU-37), are the most successful media for the culture of embryos after IVF or SCNT and currently widely used by different researchers. Nevertheless, these media are still not entirely appropriate for two reasons: first because they are supplemented with BSA throughout the whole culture period (semi-defined media) and secondly because they are still suboptimal for embryonic development. Porcine embryos cultured *in vitro* have lesser developmental competence than embryos cultured *in vivo*. Kikuchi (2004) demonstrated that IVM–IVF oocytes have higher potential for developing to the blastocyst stage, equal to that of *in vivo*–matured oocytes, when they are cultured in oviducts of synchronized recipients, than when they are cultured *in vitro* (mean cell number 181.5 and 38.4 per blastocyst when cultured *in vivo* and *in vitro*, respectively). Culture of embryos in modified NCSU 23 or NCSU 37 without glucose, but supplemented with

low levels of lactate and pyruvate for the first 48–72 h and followed by culture in IVC medium with glucose, improved blastocyst development and total cell number compared with those cultured for the whole period in the presence of glucose (Abeydeera 2002; Kikuchi et al. 2002). It seems that *in vitro*–derived 2–4 cell embryos utilized less glucose than morulae and blastocysts (Gandhi et al. 2001), where it plays a major role during compaction and blastocyst formation.

Recently, Yoshioka et al. (2008) developed a chemically defined medium (porcine zygote medium: PZM5) for *in vitro* culture of zygotes based on the composition of pig oviduct fluid. They also established a chemically defined system for IVM, IVF and IVC, which is not only useful for analysing the physical action of substances, but also for decrease the variability between laboratories and thereby increasing the reproducibility of results. For the culture with PZM medium, embryo development was optimized in an atmosphere of 5% CO_2; 5% O_2; 90% N_2 compared to 5% CO_2 in air (Yoshioka et al. 2002, 2008).

Reducing oxygen tension during *in vitro* culture from 20% to 5–7% could be also an important factor for *in vitro* development to blastocysts. However, controversial reports have been published. Some authors suggest that low (5–7%) O_2 concentration is helpful to *in vitro* development of embryos (Karja et al. 2004; Kitagawa et al. 2004); others, for example Machaty et al. (1998), reported that developmental rates and total cell numbers of blastocyst were higher in embryos cultured in an atmosphere of 5% CO_2 in air than under 5% CO_2; 5% O_2; 90% N_2, while others, such as Ock et al. (2005), reported no differences under low and high oxygen concentrations in cleavage rate, blastocyst formation, total cell number and incidence of apoptosis. Booth et al. (2005) proposed that the effect of oxygen tension on embryonic development is dependent on embryo type. Similarly, Abeydeera (2002) suggested the use of a sequential culture environment: 20% O_2 up to the morula stage and 5% O_2 for later stages. Further studies are required to better understand the exact role of oxygen tension during *in vitro* culture on the development of porcine oocytes.

Vitrification of *In Vitro* Embryos

The traditional cryopreservation of pig embryos by slow freezing has been ineffective because of their sensitivity to chilling injury. At present, vitrification is regarded as the best alternative to traditional slow freezing procedures. Vitrification is an ice-free cryopreservation method that involves the treatment of embryos with high concentrations of cryoprotectants combined with very rapid cooling achieved by direct plunging in liquid nitrogen (Rall and Fahy 1985). In an effort to increase the cryotolerance of embryos, Nagashima et al. (1994) reported that delipation, which removes cytoplasmic lipid droplets from embryos, remarkably improves embryo viability following cryopreservation. Compared with slow freezing, the vitrification has resulted in high embryo survival, usually evaluated by the ability of the embryo to re-expand the blastocoele and to hatch after culture *in vitro* (Dobrinsky and Johnson 1994; Vajta et al. 1997; Berthelot et al. 2003; Cuello et al. 2004, 2008; Sanchez-Osorio et al. 2008). Piglets have been born after transfer of pre-treated (delipidated) or untreated vitrified/warmed *in vivo* embryos in several laboratories (Kobayashi et al. 1998; Berthelot et al. 2000; Cameron et al. 2000; Dobrinsky et al. 2000; Cuello et al. 2005).

Over the last few years, important improvements have been carried out from a practical point of view. As in other species, the conventional warming method has been simplified from three steps to one step (direct warming) (Cuello et al. 2004; Sanchez-Osorio et al. 2008). Considerable improvement has been obtained with the use of chemically defined media for embryo vitrification and warming solutions, improving the reliability and reproducibility of results and reducing the risk of disease transmission (Sanchez-Osorio et al. 2010).

In regard to IVP embryos, the cryopreservation of embryos produced by IVM–IVF–IVC had been considered more difficult than that of *in vivo* embryos, especially because of their low developmental potential, the high rates of polyspermy and the abnormally high oxidative stress occurred during IVP compared with *in vivo* conditions. Nevertheless, successful vitrification of IVP embryos has been reported recently (Esaki et al. 2004; Men et al. 2005, 2006; Nagashima et al. 2007) in combination with different vitrification procedures and different stages of embryonic development (Table 1). Open pulled straws (OPS; with a cooling rate approximately eightfold higher than that of 0.25 ml straws;

Table 1. *In vitro* embryo survival after vitrification of IVP embryos

References	Vitrification procedure	Developmental stage	Treatment	Survival rate (%)
Esaki et al. 2004	OPS	Parthenogenetic Blastocyst	–	41.2[a]
			Invasive Delipation	83.3[b]
		Parthenogenetic Morulae	–	8.6[a]
			Non-invasive delipation	82.5[b]
			Invasive delipation	82.1[b]
		Parthenogenetic 4 Cells	–	9.7[a]
			Invasive delipation	36.0[b]
Men et al. 2006	OPS	Blastocyst	–	37.1[a]
			Chemical delipation	71.2[b]
Nagashima et al. 2007	Cryotop	4 Cells	Invasive delipation	40.0
Somfai et al. 2009	SSV	2 PN Zygotes	–	11–18
Cuello et al. 2010	SOPS	Blastocyst	–	34.1[a]
			Chemical delipation	44.0[b]

Within a study, a, b indicate significant differences ($p < 0.05$).

Vajta et al. 1997), superfine open pulled straws (SOPS; Isachenko et al. 2003) and more recently solid surface vitrification (SSV; Somfai et al. 2007) have been successfully used with *in vitro* embryos. With SSV, a high proportion of *in vitro*–produced zygotes have been successfully cryopreserved at the pronuclear stage without delipation (Somfai et al. 2009). An important achievement has been also the birth of the first cloned piglets after vitrification at the blastocyst stage (Li et al. 2006). From these studies, it is obvious that vitrification protocols can be used successfully with IVP and SCNT embryos.

Conclusions

Despite the progress in the IVP of pig embryos, the methodologies involved with IVM–IVF–IVC need to be improved to overcome the polyspermy as well as to enhance oocyte maturation and embryo development. The increase in overall efficiency of these grounding technologies combined with advances in somatic cell nuclear transfer and freezing procedures will further allow studies on pig cloning and the establishment of a gene bank of genetically modified pigs.

Acknowledgements

The authors thank Emeritus Professor W.M.C. Maxwell for the helpful discussion and critical reading of the manuscript. This study was supported by the Seneca foundation of Murcia (GERM 04543/07), MICINN (AGL2009-12091) and CDTI (2008/0268).

Conflict of Interest

None of the authors have any conflict of interest to declare.

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Submitted: 4 Feb 2010; Accepted: 26 Mar 2010

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