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Chapter 5

# Reproductive technologies in swine

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## 5.1 General aspects of oocyte collection, evaluation, and in vitro maturation

Porcine cumulus-oocyte complexes (COCs) are commonly collected from ovaries of prepubertal gilts or cycling sows from crossbred commercial breeds, and a higher oocyte developmental competence has been reported in COCs from adult animals [1−3]. Nevertheless, COCs obtained from prepubertal animals can be successfully matured and fertilized in vitro, producing alive offspring after embryo transfer of 2−4-cell embryos [4]. Nowadays, the majority of in vitro studies use female gametes derived from prepubertal gilts since they represent the most common class of female pigs slaughtered in developed countries.

Ovaries are collected at the slaughterhouse immediately after sacrifice and transported to specialized laboratories in a thermoflask containing simple solutions (usually phosphate-buffered saline (PBS) or saline containing antibiotics at 38.5°C) within a short time from death. The transport of ovaries has received little attention and scarce research has been done in pigs on the effect of temperature, media, and time of ovaries transport on the progression of embryo development [5,6]. The use of organ preservation solutions used in human organ transplantation should be explored since the specific formulation is intended to prevent cell swelling, changes in pH and free radical formation, among others [7]. These emerging solutions [i.e., Solution de Conservation des Organes et des Tissus 15 (SCOT 15) [8]] might be of interest and effective in maintaining metabolic rate and functional ovarian cells, reducing the injury on COCs brought by ischemia, and thus improving further development. The temperature and the duration of the ovarian tissue transport to the laboratory are also important components in maintaining further oocyte viability, with temperatures and times ranging from 4°C to 39°C and 1 to 24 hours, respectively. As a general rule, as the storage temperature for the ovaries increases, the transportation time should be decreased (reviewed by Barberino et al. [9]). The use of new preservation solutions and standardized temperature/time ratio between labs for pig ovaries transport might help in implementing more efficient protocols.

Once in the lab, ovaries are subjected to several washes prior to recovering COCs, task commonly done by aspiration or slicing of ovarian preantral follicles 3−6 mm in diameter. The selection of suitable COCs for in vitro maturation (IVM) is done under stereomicroscope following morphological criteria such as presence of several compact layers of cumulus cells and appearance of dark and granulated cytoplasm. COCs with greater developmental competence can be selected with brilliant cresyl blue (BCB) staining, a simple test based on glucose-6-phosphate dehydrogenase (G6PDH) activity [10]. The use of this test yields controversial results since BCB-selected oocytes are more competent for nuclear and cytoplasmic maturation but did not improve cleavage and blastocyst formation [11]. Moreover, in porcine oocytes exposed to a BCB solution a specific pZP3 translocation from the zona pellucida to the cytoplasm has been reported [12], with the consequence of affecting further embryo development [10].

Once selected, COCs are subjected to IVM, which in porcine species lasts for 40−44 hours, with the objective of obtaining a female gamete competently matured both at the nuclear and cytoplasmic levels. Traditionally, the most commonly used maturation medium is tissue culture medium (TCM)-199 but other media have been already employed such as Waymouth MB 752/1, North Carolina State University (NCSU37 and NCSU23), modified Tyrode's solution and, more recently, porcine oocyte medium (reviewed by Redel et al. [13]). The incidence of in vitro matured COCs that develop to the blastocyst stage after in vitro fertilization (IVF) is higher when COCs are matured in a two-step system, where they are exposed to dibutyryl cyclic adenosine monophosphate for the first 20 hours of IVM [14].

**Reproductive Technologies in Animals. DOI:** https://doi.org/10.1016/B978-0-12-817107-3.00005-9

In pigs the low efficiency in cytoplasmic maturation has been related to low rates of male pronuclear formation after fertilization, high rates of polyspermy, and low development to the blastocyst stage (reviewed by Romar et al. [15]). Over the years, these problems have been partially overcome by replacing fetal calf serum with porcine follicular fluid [16] and supplementing the IVM medium with cysteine [17,18]. Other additives with beneficial effects on oocyte's maturation are epidermal growth factor [19], beta-mercaptoethanol [20], and FLI [21], a combination of fibroblast growth factor 2, leukemia inhibitory factor, and insulin-like growth factor 1, which was recently reported to improve maturation of immature pig oocytes and increasing the efficiency of blastocyst production by twofold after IVF.

In the current porcine IVM systems, rate of metaphase II (nuclear maturation) and male pronuclear formation after IVF (one of the parameters directly correlated to oocyte's cytoplasmic maturation) are both over 90% [2,14,16,17,21]. However, the incidence of polyspermic penetration in pig IVF is greater than 40% of inseminated oocytes (reviewed by Romar et al. [22]).

More studies and improvements are still necessary in the IVM system in pigs to produce competent matured oocytes that will further develop to high-quality blastocysts capable of becoming live and healthy offspring.

## 5.2 General aspects of sperm assessment and preparation for assisted reproductive technology

Advanced sperm selection techniques are thought to improve the chance that structurally intact and mature sperm cells with high DNA integrity are selected for fertilization. Basically, the methods used for sperm preparation prior to assisted reproductive technology (ART) have been designed, on the one hand, to remove diluent media, cryoprotectants, or decapacitating factors (from the seminal plasma or epididymal fluid), and on the other hand, to select motile and mature sperm cells. However, depending on the method of sperm preparation, the outcome of ART is very different [23–25].

Basically four methods for sperm selection can be considered prior to ART in the porcine species: (1) dilution of semen and washing, (2) sperm migration: known as the direct swim-up method, (3) selective washing of semen (density gradient method: single or multiple layers), and (4) adherence method (glass wool, glass beads, or Sephadex columns) [26–28]. However, the latter are not practically used in pigs.

The first success in porcine IVF was achieved by Cheng [29] who used ejaculated spermatozoa that were washed and preincubated for 4–5 hours at 37°C or stored at 20°C for 16 hours, washed, and preincubated only at 37°C for 40 minutes. Today, washing procedures consist of two or three dilutions and washing in a saline solution supplemented with albumin (from 0.1% to 1%). Albumin promotes sperm capacitation acting as acceptor of cholesterol by removing it from the plasma membrane. A decreased cholesterol/phospholipid ratio consequently contributes to an increased membrane fluidity, which in turn promotes an increase of ion permeability (revised by López-Úbeda and Matás [30]).

The conventional swim-up procedure was originally described in human by Mahadevan and Baker in 1984 [31]. The methodology is based on the active movement of spermatozoa from the pellet generated after a centrifugation into an overlaying medium. This technique serves to select a very high population ($>90\%$) of motile sperm cells that are morphologically normal, with low DNA fragmentation and free of debris and other cells [32,33]. The swim-up method has also been modified by the use of a strainer as permeable barrier [34], using specific designed medium supplemented with natural additives such as oviductal fluid [35–37].

Increased quality sperm cells may also be selected based on their density differential. For this, they are centrifuged through a continuous or discontinuous density gradient of either colloidal or silanized colloidal silica [4,38]. Several studies have shown better performance of IVF using sperm cells selected with discontinuous density gradients than with washing procedures [23–25,39,40]. However, when spermatozoa came from the epididymis, the outcome was not affected by the method of sperm selection [24]. A *caveat* of this procedure, though, is that seminal plasma proteins and cholesterol can be removed from the sperm cell membrane [41], although this aspect does not seem to negatively affect IVF performance, as similar results were obtained when spermatozoa were centrifugated without a continuous gradient [42].

The preparation and selection of spermatozoa for intracytoplasmic sperm injection (ICSI) require procedures beyond those described thus far (revised by Garcia-Rosello et al. [43]). For example, several approaches have been explored with the use of Percoll for selection of ejaculated and epididymal spermatozoa [44,45]. To disrupt their membranes, mechanical processes such as repeated freeze-thaw cycles or the use of compounds as Triton X-100 [46–49] as well as pretreatment with compounds such as lipase or hyaluronic acid have been employed [48,50]. Lastly, microfluidic systems have also been used for sperm selection [46].

## 5.3 In vitro fertilization and embryo culture

Cheng et al. (1986) successfully fertilized in vitro−ovulated oocytes and cultured the resulting embryos to the two- to four-cell stage. A total of 19 piglets were born after transfer of these embryos to recipient gilts [51]. The IVF system they described transformed the research conducted in this area, which had been plagued by a lack of success up until that time.

At the beginning of the porcine IVF development, there were several problems to solve. On the one hand, an insufficient maturation of the oocytes was achieved, and on the other hand the sperm preparation prior to fertilization was not perfected. Today, the main problem that remains unsolved is the high degree of polyspermy (reviewed by Romar et al. [15,22], Coy and Romar [52], and Dang-Nguyen et al. [53]). To solve this problem, a number of investigations have been performed with the goal of evaluating all the factors involved and needed in the fertilization process, leading to the establishment of different methods and protocols to improve the final outcome. Nevertheless, to date not a single solution to this problem is available nor a generalized protocol that guarantees a successful porcine IVF. Therefore nowadays the success rate (ratio of monospermic zygotes/number of inseminated oocytes) usually does not exceed 45% (reviewed by Romar et al. [15]).

The most frequently used source of oocytes for IVF comes from ovaries obtained from slaughterhouses. Subsequently, these are matured in vitro (described in previous paragraphs). However, in vivo−matured oocytes from gilts/sows collected by oviduct washing are also used [54,55]. COCs secrete various molecules, including progesterone, hyaluronic acid, and NO [56−58], and these molecules favor sperm capacitation and therefore fertilization [59,60]. However, the presence/absence of cumulus cells may have positive and/or negative effects on IVF parameters [61,62]. In this regard, some authors have shown that the effect of some molecules added to IVF media is opposite, depending on whether cumulus-enclosed or cumulus-free oocytes are inseminated [63].

With regard to sperm cells, epididymal and ejaculated spermatozoa have been compared in IVF system [24,64], together with fresh versus frozen/thawed samples, with no conclusive results [64,65]. This could be due to the high influence on IVF outcomes of other factors such as sperm concentration, sperm selection and preparation methodology, and IVF medium and additives [23,62,66].

IVF culture media share a common formulation containing various components including inorganic salts, nutrients, vitamins, and growth factors for gamete coculture (revised by Romar et al. [22]). The classical media used for porcine IVF include modified Tyrode's albumin lactate pyruvate, modified Tris-buffered medium, modified TCM-199 and porcine gamete medium (reviewed by Romar et al. [15,22]).

Under in vivo conditions, millions of spermatozoa are deposited in the female genital tract, but only a small subpopulation will reach the oviduct and site of fertilization. In in vitro systems, various methods have been devised to restrict the number of spermatozoa that will reach the oocyte within the insemination device, such as the climbing-over-a-wall method, biomimetic microchannel IVF system, straw IVF, modified swim-up method, and the microfluidic sperm sorter (revised by Grupen [67]). Each of these systems attempts to mimic the in vivo selection so that sperm cells have to overcome an obstacle while acquiring hypermotility.

IVF involves fertilization in an artificial environment. In this regard, temperature, pH, gases, and humidity must be maintained closer to what physiologically typical of the oviductal environment. Consequently, performing these procedures using precise temperatures and gas compositions that best mimic those found in reproductive organs would be a major step toward improving in vitro conditions for IVF. The pH in the porcine oviductal ampulla is close to 8.0 during the periovulatory phase [68]; however, IVF is usually done at a pH closer to neutrality (7.4) [69]. Modification in the percent of $CO_2$ in the incubator so that the pH was 8 induced a marked improvement in IVF results [60]. With regard to the atmosphere surrounding the gametes, the conventional gas composition is $\sim$20% $O_2$ (i.e., air) and 5% $CO_2$. It has been shown though that by lowering the oxygen tension (7%), measured in vivo in the pig reproductive organs, the IVF output is increased [36]. A similar approach was followed with the temperature, and the use of 37°C for IVF, as measured in the oviduct, demonstrated to produce a higher efficiency than the pig body temperature (38.5°C) conventionally used, increasing monospermy rates, and blastocyst yield [70].

The in vitro culture of porcine zygotes to the blastocyst stage still remains very challenging since the proportion of success, after years of research, barely reaches 30%−40% today [62]. A comprehensive and thorough account of the different culture media on porcine preimplantation embryo has been recently published [13], and it is strongly suggested to read that review for further information pertaining to this topic. Briefly, the most common embryo culture media used in the porcine species are the North Carolina State University (NCSU-23 medium [71]), the porcine zygote medium [72], and the Beltsville Embryo Culture Medium 73], with their different modifications. As not all of them are commercially available, they need to be prepared in the laboratories. This fact may create variations in the batches if

quality controls are not employed; hence the claims about the consistently high quality of the chemically defined media may be called into question. There is a tendency to use these kinds of media as it permits to pinpoint the impact of specific components on embryo development. However, these modifications to culture media have turned them "antinatural" as they no longer mimic the in vivo situation. Indeed, the reproductive fluids soaking the lumen of the oviduct and uterus have a chemical composition extremely complex, and the molecules included in the chemical definition of the media prepared in the lab represent a poor facsimile of a few components found in these environments. Artificial media are in fact made of electrolytes, amino acids, and some carbohydrates, together with antibiotics and, occasionally a protein source such as serum albumin. Even this last molecule is often being replaced by polyvinyl alcohol (PVA) arguing that chemical variations in serum albumin can have an impact on embryo development [74]. The net result in the use of defined media is to force the embryos to grow in a considerably stressful environment because, in nature, they on the contrary would develop in a milieu containing, to say the least, a considerable proportion of glycosaminoglycans, several hundreds of different proteins, and hormones [75–77].

The lipidomic variations in the endometrium can also affect the success of implantation after embryo transfer, making the picture even more complicated [78]. The increasing evidence supporting the idea that oviduct and uterine fluids play a key role in the "developmental origins of health and disease" concept in different species represents the best wake-up call for researchers to start re-thinking about the future of embryo culture systems. It is time to decide if the final goal, in the pig embryo production systems, consists of getting the maximum number of animals or the healthiest individuals [35,79–81].

## 5.4 Tissue culture and isolation of germline

Germinal cells are in charge of transmitting, mainly, genetic information from one generation to the next to ensure species conservation. Germline specification begins in the preimplantation embryo with the formation of the bipotential primordial germ cells (PGCs), which will result, after a highly orchestrated regulatory process, in male or female gametes. The final maturation stage will give haploid gametes, and by fusion of the female and the male counterparts, a new fertilization occurs and with it the development of a new organism.

PGC isolation is challenging in all mammalian species due to the low number of cells from which they originate and the position within the embryo. Isolation of PGC has been unsuccessful for decades in pigs, due to a lack of knowledge about the germline specification pathways and specific germline markers. Recently, Kobayashi et al. (2017) reported that PGC specification in pigs [82] starts from the posterior preprimitive-streak competent epiblast. Sequential upregulation of SOX17 and BLIMP1 seems critical in this process, similar to what has been observed in human PGC specification.

Identification and isolation of spermatogonial stem cells (SSCs) could be useful for achieving genetic gain or restore fertility, and genetic modification of SSCs combined with germ cell transplantation provides the possibility to produce transgenic animals. In the last years, the concept of surrogate sires has emerged as an alternative for breeding in livestock production, including the swine industry. This is based on the regenerative capacity of SSCs, which are isolated from selected boars, and transferred into male recipients with germline ablation to produce mature spermatozoa with the donor haplotype [83]. Reestablishment of fertility using this technology was demonstrated more than 25 years ago in mice. In that experiment, offspring were produced from infertile males with transplanted SSCs [84]. However, the scarcity of SSCs in the testes requires in vitro expansion of SSCs to obtain enough material for cell transplantation, which has limited progress in pigs. More promising results in swine have been recently reported by Zhang et al. (2017) who showed long-term maintenance of porcine neonatal testis cells with similar morphology to cattle and mouse spermatogonia and expression of conserved, but no specific, markers of undifferentiated spermatogonia (GFRA1, LIN28, and PLZF) [85]. However, they did not show SSC regenerative capacity after cell transplantation. Few studies have reported SSC transplantation in domestic pigs [86–88] and unequivocal evidence for SSC regeneration in recipient males have not been provided. Even with these advances, several challenges remain unsolved, such as identification of specific markers to distinguish SSCs from progenitor spermatogonia that lack regenerative capacity.

Another challenge already surpassed was the development of effective strategies for complete germline ablation in recipient males. Knockout (KO) of NANOS2, a male germline specific factor, in pigs has been reported recently, resulting in male specific germline ablation with normal physiology and histology of the seminiferous tubules [89]. Considering that an increased efficiency in livestock animal production is required to cover the unprecedented demand that is predicted for next decades, the use of surrogate sires is proposed as a valuable breeding tool in swine production, with some utility even in natural breeding.

Despite the fact that mammals are programmed to require male and female gametes to generate an embryo, because both genomes are not equivalent, recent technology has allowed to obtain new individuals without the necessity of both gametes.

## 5.5 Nuclear transfer, micromanipulation, and embryo biopsy

Somatic cell nuclear transfer (SCNT) provides the possibility to obtain embryos using enucleated oocytes and a somatic cell [90]. It is almost two decades since the first reports on cloned pigs using SCNT and adult cells were reported [91,92]. SCNT has been used to produce transgenic pigs for generation of humanized organs for xenotransplantation [93] or the development of models to investigate human diseases [94,95]. More recently, the interest of using pigs for xenotransplants has been rekindled after the publication showing genome-wide inactivation of porcine endogenous retroviruses by the use of CRISPR/Cas9 technology [96].

Unfortunately, the efficiency of SCNT in pigs remains poor, with only 1%−3% of transferred embryos developing to term [97,98]. Among the biggest challenges for cloning pigs are: (1) the ineffective in vitro embryo culture system, which is required to culture reconstructed embryos before transfer to surrogate sows and (2) the necessity for at least four good embryos for maternal recognition of pregnancy in sows. During the past few years, porcine embryo culture systems have been significantly improved by us and others (reviewed by Romar et al. [22]). This progress, in combination with different strategies to promote cell donor reprogramming [99] or XIST nullification in the donor cells [97], could result in enhanced cloning efficiency in the near future.

## 5.6 Molecular application to embryo identification, selection, and sex determination

In pigs embryo selection is critical in order to increase embryo production efficiency, especially when using a nonoptimized in vitro culture system. Even though noninvasive embryonic selection criteria based on morphokinetics still remain prevalent over other markers, a wide range of transcriptomic, epigenetic, and metabolomic markers have been developed during the last decade. Gene expression analysis of a few pluripotency, embryonic, and/or cell cycle regulation factors has been extensively used for embryo quality assessment with limited utility. Recently, transcriptomic and epigenetic profiling of single embryos has become affordable cutting-edge technologies. This fact has allowed comparison between in vivo and in vitro−produced embryos and permitted the identification of differentially expressed genes that could be used as quality markers to help in improving embryo culture and embryo selection. For example, our group [35] recently reported whole-genome DNA methylation and transcriptome analyses in single pig blastocyst, findings that will undoubtedly shed light on the mechanisms underlying the abnormalities observed in ART-derived offspring.

Imprinted genes, which show parental-specific methylation, play a key role in fetal and placental development and they have been associated to ART-derived defects (review by Canovas et al. [100]). Expression and DNA methylation of imprinted genes (such as H19, IGF2R, XIST, IGF2, NNAT, PEG10, or MEST) are also molecular markers of embryo viability. Similarly, expression of DNA methyltransferases, involved in de novo (DNMT3a/b) and maintenance (DNMT1) DNA methylation, together with TET enzymes responsible for the modification of methyl DNA, are also useful molecular marker. However, the inclusion of these markers in reliable and easy-to-use kits for embryo selection before transfer remains challenging.

On the other hand, metabolomic markers provide the possibility to select embryos based on the change in the levels of specific nutrients and metabolites and the consumption of key nutrients from the culture media [101,102]. In humans, early studies reported correlation between metabolomic profiles and implantation and pregnancy rates. However, randomized controlled trials using the same metabolomics-based viability index did not result in increased pregnancy rates compared with the routine morphology selection (reviewed by Krisher et al. [102]). In pigs, Krisher et al. (2015) reported some metabolic characteristics of the in vitro−derived porcine embryos, such as the dependency on glutamine, but there is still a lack of metabolic markers for embryo quality in pigs [101]. Moreover, embryo selection using metabolic analysis requires single-embryo culture, which is not a routine practice in porcine in vitro embryo culture.

In addition to embryo quality, preselection by sex is desired to manipulate the expected male and female ratio (50/50) in the offspring. In pigs, it could be useful to reduce the male ratio and avoid the unpleasant specific male boar taint, the peculiar male taste given to the muscles by the male hormones, which is rejected by consumers [103]. It would entail economic benefit by a faster female growth and the avoidance of male castration. Nonetheless, even though boar sexed semen is available through fluorescence-activated sperm sorting, its use is not a routine practice in commercial farms, even after more than 15 years from the first successful report using porcine sexed semen and

nonsurgical insemination [104]. The susceptibility of sperm cells to the sorting procedure, the high number of sperm required for insemination in pigs (even with the development of an intrauterine insemination device), and the slow speed of the sex-sorting process (which affects the costs and availability of samples) are limitations to the commercial application of sexed semen in the swine industry [105]. Another alternative is the selection of the embryo by karyotyping or gene expression analysis to identify chromosome Y specific factors. However, the use of embryo transfer in pigs remains dramatically low, which seriously limits its use.

## 5.7 Transgenesis/genetically modified animals

Genome engineering in animals has become a reality for the scientific community since 1980, when transgenic mice were obtained by recombinant plasmid pronuclear microinjection [106]. A few years later, in 1985 the first transgenic pigs were produced by two different research groups by microinjection into the pronuclei with a random insertion of foreign DNA [107,108]. In the next two decades, other techniques were implemented to generate transgenic pigs with the use of retrovirus [109] and lentivirus as vectors [110], sperm-mediated gene transfer by artificial insemination [111,112], and ICSI [113,114]. All these techniques had limitations and produced only insertion of new genes in the pig genome [only knock in (KI)] in a random and not targeted manner and with low efficiency. A new situation was opened with the use of somatic nuclear transfer (NT) technology [91,92,115], giving thus the possibility to first make the modification in the somatic cells and then use the modified cells as donors for NT. SCNT was used to produce KI [116,117] and KO pigs [118,119]. One decade later, the use of transcription activator-like effector nuclease and zinc finger nuclease genome editing [120,121] opened the possibility of genome edition. Later, CRISPR-Cas9 gene editing made the rapid and economic generation of KO/KI pigs feasible [122].

In 2014, it was reported that the CRISPR/Cas9 system could be used to produce genetically engineered pigs by intracytoplasmic injection of zygotes [123,124] and SCNT [124,125]. Electroporation has also been used to lead the entry of CRISPR/Cas9 into oocytes and early embryos [126]. This last technique requires less sophisticated equipment and highly trained specialized personnel than microinjection or NT methodology, making more feasible its implementation. In the past few years an exponential number of studies have tried to optimize the factors that affect the efficiency of this methodology in pigs and in diverse areas and applications [127–133]. A new area of gene editing is dawning with the development of new methodologies associated with the use of base editors for multiple genes [134] or conditional and tissue-specific expression of mutations using Cre/loxP site-specific recombination systems [135,136].

Applications of transgenic and gene editing in pig animal production are associated with improvement in the carcass composition [137] and meat quality [138,139], lactational performance [140,141] and reduction of manure phosphorus contamination [142,143]. However, the most interesting application in the pig industry is related to the increase in the resistance to important diseases such as porcine reproductive and respiratory syndrome [144,145], coronavirus [146], classical swine fever virus [147], and foot and mouth disease [148].

In addition, there are applications of these technologies in biomedicine. Generation of KO and KI mice by targeting genes in embryonic stem (ES) cells revolutionized biomedicine by making it possible to study gene function in a living mammal [149,150]. However, mice are far from an ideal model for many human pathophysiological processes. Pigs are much more like humans in size, diet, physiology, and drug uptake/metabolism [151,152]. However, their usefulness as a biomedical model has been limited by a failure to isolate ES cells for generating KO/knocking versions of this species. The application of CRISPR/Cas technology opened a new era for the generation of pig models of human diseases [95,133,153,154]. Furthermore, gene-edited pigs hold promise for the development of humanized organs that may be used for xenotransplantation [155–158] as well as for the production of recombinant proteins in the modified animals for biopharmaceutical and nutrition industries [159,160].

## 5.8 Sperm, oocyte, and embryo cryopreservation

Semen cryopreservation greatly facilitates the distribution of male desirable genes over distant places and without time restraints, and this aspect alone could determine a rapid increase in swine industry productivity [161]. However, the application of frozen semen in commercial farms is limited to genetically selected animals, for long-distance transport or to preserve specific valuable individuals or breed/lines [162,163].

The fertilizing ability of frozen-thawed samples has increased in recent years, with farrowing rates well over 80% in experimental field trials [164–166]. The improvement in the results has been related to the selection of good freezing boars [167], changes in freezing procedures, and use of additives in the extenders [164,168], ovulatory synchrony with

insemination [169], and by the use of intrauterine and postcervical insemination (revised by Knox [162], Yeste et al. [163], and Roca et al. [170]).

One other alternative to preserve material from a male could be the cryopreservation of testicular tissue that later could be xenografted into immunodeficient nude mice [171] or transferred to "recipient testis" by germ cell transplantation [86]. The implantation of testicular tissue in the nude mouse model leads to spermatogenesis and the recovery of normal spermatozoa with fertilizing capacity by ICSI, and to the production of live piglets after embryo transfer into recipients [172,173].

Porcine oocytes and embryos are very sensitive to cold shock, mainly related to the high content of lipids in the cytoplasm, and for this reason making it difficult to cryopreserve by using conventional freezing or vitrification protocols (reviewed by Zhang et al. [174], Men et al. [175], and Kikuchi et al. [176]). Nevertheless, some studies have reported embryo development and birth of piglets derived from cryopreserved oocytes [50,177] and embryos [178,179], indicating that it is feasible to use this technology.

Whole ovarian tissue [180] or fragments of ovarian cortex [181] could be cryopreserved and later implanted by xenografting in an immunodeficient nude mice [182], although the efficiency is limited [183]. Another alternative explored for fertility preservation is the xenotransplantation of the whole ovary into irradiated ovariectomized nude rats [184]. Ovarian autotransplantation in pigs is under study as a clinical method for the preservation of fertility and hormonal function [185].

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