Theriogenology 85 (2016) 125–134

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

In vitro fertilization in pigs: New molecules and protocols to consider in the forthcoming years

Raquel Romar [a], Hiroaki Funahashi [b], Pilar Coy [a,*]

[a] Department of Physiology, Faculty of Veterinary, University of Murcia, Campus Mare Nostrum, IMIB-Arrixaca, Murcia, Spain
[b] Department of Animal Science, Graduate School of Environmental and Life Science, Okayama University, Okayama, Japan

ARTICLE INFO

Article history:
Received 20 April 2015
Received in revised form 8 July 2015
Accepted 12 July 2015

Keywords:
IVF
Pig
Oviduct
Polyspermy

ABSTRACT

Assisted reproduction technology (ART) protocols are used in livestock for the improvement and preservation of their genetics and to enhance reproductive efficiency. In the case of pigs, the potential use of embryos for biomedicine is being followed with great interest by the scientific community. Owing to the physiological similarities with humans, embryos produced *in vitro* and many of those produced *in vivo* are used in research laboratories for the procurement of stem cells or the production of transgenic animals, sometimes with the purpose of using their organs for xenotransplantation. Several techniques are required for the production of an *in vitro*–derived embryo. These include *in vitro* oocyte maturation, sperm preparation, IVF, and further culture of the putative zygotes. Without doubt, among these technologies, IVF is still a critical limiting factor because of the well-known, but still unsolved, question of polyspermy. Despite the improvements made in the past decade, current IVF systems hardly reach 50% to 60% efficiency and any progression in porcine ARTs requires an unavoidable improvement in the monospermy rate. It is time, then, to learn from what happens under *in vivo* physiological conditions and to transfer this knowledge into ART. This review describes the latest advances in porcine IVF, from sperm preparation procedures to culture media supplements with special attention paid to molecules with a known or potential role in *in vivo* fertilization. Oviductal fluid is the natural medium in which fertilization takes place, and, in the near future, could become the definitive supplement for culture media, where it would help to solve many of the problems inherent in ARTs in swine and improve the quality of *in vitro*–derived porcine embryos.

1. Introduction

Owing to its relevance in the production of embryos for commercial purposes or for biomedical studies, pig IVF has been the focus of attention for research laboratories, and several review articles on this topic have been published over the years [1–5]. Citing just a few examples, experiments have been conducted into the role of different molecules included in the culture media [6,7], as well as the culture conditions themselves. Gamete coincubation times [8,9], sperm concentration [10,11], the source of spermatozoa [12–14], the source of oocytes [15,16], or the effect of co-culture with somatic cells [17–19] are all factors that influence embryo production. Overall, the main objective of these studies was to improve the frustratingly low success rates of pig IVF by reducing the consistently high levels of polyspermy.

More recently, specific studies have tried to recreate *in vitro* the ideal conditions for the concurrence of the physiological mechanisms that lead to fertilization. Molecular biology, microarray technologies [20], or, more recently, RNA sequencing [21] mean that it is now possible to determine the main genes that are upregulated or downregulated in the oviductal tissue at specific time points before and after the gametes encounter each other [22]. Similarly, liquid chromatography-tandem mass

* Corresponding author. Tel.: +34 868884789; fax: +34 868884147.
E-mail address: pcoy@um.es (P. Coy).

0093-691X/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2015.07.017

spectrometry (LC-MS/MS) [23] and techniques for relative isotope-coded affinity tag [24], or, in the near future, absolute selected reaction monitoring in tandem MS quantitation will provide knowledge of protein profiles and concentrations in the oviductal epithelium and fluid at the time of fertilization; these data could potentially be transferable to guide the composition of culture media. However, the extensive information that these tools will generate (when a significant number of studies in pig become available) is difficult to translate into useful laboratory protocols, so that the molecules or procedures of interest will need to be carefully chosen. Similarly, video microscopy and other imaging technologies would enable the visualization of sperm and oocytes in oviductal explants [25], or even *in vivo*, leading to a reinterpretation of the cell ratios at fertilization, the patterns of sperm movement, or the time interval for the release of spermatozoa from their oviductal epithelial cell attachments in the isthmus reservoir. Such data will also be useful for designing new protocols for sperm treatment before IVF.

The present review aims to collate the most recent information about porcine IVF and porcine oviductal molecular and microscopic physiology to help researchers obtain the best rates of *in vitro* porcine embryos.

2. Sperm preparation methods: Can we move toward more physiological protocols?

In general, fresh epididymal or ejaculated boar spermatozoa, in some cases after liquid preservation or cryopreservation, have been prepared for the IVF of porcine oocytes. Seminal plasma and/or extender contain components that function as decapacitation factors that must be removed before coincubation with oocytes. The fertilization medium, besides, contains chemicals that induce capacitation at suitable concentrations. However, sperm preparation methods seem to affect sperm capacitation status and penetrability *in vitro* [13]. Here, we discuss current methods for preparing spermatozoa intended for IVF.

2.1. Boar semen in the female reproductive tract

Under *in vivo* physiological conditions, epididymal spermatozoa are mixed with seminal plasma in the male reproductive tract just before ejaculation, the components of which play an active role in the transportation and survival of viable spermatozoa in the female reproductive tract [26]. After ejaculation, boar spermatozoa are already coated with a large amount of spermadhesins (AQN-1, AQN-2, AQN-3, AWN-1, and AWN-2), which are multifunctional proteins involved in boar sperm capacitation and gamete recognition [27]. Because most of these spermadhesins are removed from the surface of ejaculated spermatozoa during capacitation, a large subpopulation of boar spermadhesins are believed to function as decapacitation factors, whereas the remaining ones, which are tightly bound to the spermatozoa, may play a role as positive capacitation factors and/or in gamete recognition [27]. Porcine seminal plasma proteins I and II (PSP-I/PSP-II) have been reported to exert a decapacitation effect on highly extended boar spermatozoa [28]. The seminal plasma PSP-I/PSP-II spermadhesin, when present *in vitro*, blocks sperm–ZP binding [29]. Cholesterol is also known to be the predominant inhibitor of capacitation [30].

After artificial insemination, spermatozoa, seminal plasma, and semen extenders in the female reproductive tract all play roles in the induction of postmating uterine inflammation characterized by increased levels of cytokines, polymorphonuclear leukocytes, and mononuclear cells [31–34]. Seminal plasma suppresses polymorphonuclear leukocytes migration into the uterus after mating and enhances the rate of disappearance of uterine inflammation [35]. Moreover, contact between seminal plasma and the epithelium of the uterotubal junction is essential for the transduction of the local signals involved in the advancement of ovulation [36]. This means that part of the seminal plasma somehow must reach the uterotubal junction after insemination. There is also evidence that seminal factors influence ovarian function [31,37], the timing of ovulation, CL development, and progesterone synthesis [38]. Seminal plasma also stimulates the active transport of spermatozoa through the female reproductive tract [39] and increases the number of fertilized oocytes attaining the viable blastocyst stage [38]. As spermatozoa pass through the female reproductive tract from the cervix to the uterotubal junction, the seminal plasma may be reduced in volume by diffusion and backflow, and, consequently, spermatozoa may be separated from seminal plasma. The major proteome of boar seminal plasma and the association between specific seminal plasma proteins and semen parameters have been recently published opening the basis for determination of molecular markers of sperm function in the swine species [40].

In some species, in which spermatozoa are ejaculated into the vagina, ovulatory cervical mucus is a candidate for the removal of cholesterol and glycerophospholipids from the sperm plasma membrane, acting as a “sperm membrane scrubber” [41]. However, in pigs, in which semen is ejaculated into the cervix entering the uterus, the detailed mechanism of how seminal plasma is eliminated probably differs from the way in which it occurs in other species. Seminal plasma is somehow separated from spermatozoa, and cholesterol may be partially removed from the sperm plasma membrane. This is probably as a result of high uterine sterol sulfatase activity, promoting an increase in membrane fluidity [41]. After reaching the uterotubal junction, carbohydrate-mediated binding with the epithelium traps the spermatozoa. Although carbohydrate-binding proteins (AQN-1) of uncapacitated spermatozoa can bind to the exposed high-mannose type N-glycans of oviductal membrane glycoproteins (LAMP-1/2 and others), the coating proteins dissociate from the surface, exposing proteins of the multimeric receptors (AWN, AQN-3, P47, and others) in capacitated cells, allowing binding to the ZP through the recognition of a set of neutral complex N-glycans [42]. Cholesterol appears to be further removed from the sperm plasma membrane to increase membrane fluidity, which is a prerequisite for subsequent membrane fusion, i.e., acrosome reaction by albumin and high-density lipoprotein in the oviduct fluid [43] after scramblase activation *via* a bicarbonate adenylate cyclase protein kinase A signaling pathway [44].

2.2. Current methods for preparing spermatozoa for IVF

Before the induction of sperm capacitation for IVF, boar spermatozoa have conventionally been washed to separate them from seminal plasma and extender by simple centrifugation [12,45–48]. Boar spermatozoa appear to resist a high *g*-force (2400 × *g*) for a relatively short centrifugation time (3 minutes) [49]. Recently, boar spermatozoa have increasingly been treated by Percoll gradient centrifugation [50–54] because this procedure results in higher *in vitro* penetration rates [55–57] and increases cleavage [58] and blastocyst formation rates [59] after IVF. Percoll treatment is also recommended for porcine intracytoplasmic sperm injection with poor-quality fresh semen [60]. Although the swim-up procedure has been successfully used to isolate a highly motile sperm population [61,62] and to reduce polyspermy during IVF of porcine oocytes [63], there are few articles on this topic and more research is necessary to assess the efficiency of this method. Single-layer centrifugation processing (500 × *g* for 20 minutes) of boar ejaculates using the pig-specific colloid Androcoll-P has also been reported to improve the quality and fertilizing ability of cryopreserved boar sperm [64,65], whereas sperm survival after colloid centrifugation varies according to the part of the sperm-rich fraction used [66]. The single-layer centrifugation of spermatozoa is known to remove porcine seminal plasma proteins PSPI and PSPII but only partially removes cholesterol [67]. Furthermore, a double-processing technique consisting of single-layer centrifugation with Androcoll-P followed by a swim-up procedure has been reported to remove more than 99% of the virus, porcine circovirus type 2, without any effects on sperm quality [68]. Therefore, centrifugation in Percoll gradient or single-layer of colloid seems to be a suitable way for separating the motile spermatozoa from boar semen. Furthermore, because sperm selection in a microfluidic chamber, named SpermSorter, results in a relatively high normal penetration rate, this method may be a better way to separate the motile and penetrable spermatozoa from a sperm suspension [69]. However, the limited number of studies investigating the survivability of sex-sorted, frozen-thawed boar sperm has produced promising *in vitro* results but poor *in vivo* outcomes [70].

2.3. Preparation of cryopreserved boar spermatozoa for IVF

As mentioned previously, some researchers have used frozen-thawed epididymal [12,71,72] or ejaculated [64,73] boar spermatozoa for IVF in standard or chemically defined media [74]. Single-layer centrifugation processing of boar ejaculates using colloid such as Androcoll-P [64] or centrifugation with an iodixanol cushion at the bottom of the tube [75] also appears to improve the quality and fertilizing ability of frozen-thawed boar spermatozoa. The rates of cryosurvival and IVF also seem to be improved when boar spermatozoa are frozen in the presence of seminal plasma from ejaculates from “good freezer” boars [76], whereas seminal plasma supplementation is known to be beneficial during thawing but detrimental during freezing [77]. Furthermore, to improve the quality and function of postthaw boar spermatozoa, antioxidant supplements [78], such as glutathione [79], cysteine/rosemary [80], epigallocatechin gallate (a major polyphenol in green tea) [81], alpha-tocopherol [82], catalase/superoxide dismutase [83], and N-acetyl-l-cysteine [84] have been used, whereas *in vitro* fertility of Percoll-separated spermatozoa varied among boars and between sperm samples *in vitro* [85].

3. Culture media composition: The influence of specific molecules with an *in vivo* known biological function on porcine IVF results

It is not easy to highlight only one among all of the culture media that have been used in the recent decades for IVF in pigs. The choice of the medium is made together with the device and system used for IVF, the final objective of the experiment, and the background of the research group involved. However, once selected, the basal fertilization medium is always supplemented with different molecules to improve the final results. The selection of these molecules will maintain the nature of the medium as chemically defined or undefined.

Among the basal media used for porcine IVF, we can mention modified Tris-buffered medium (mTBM) [48,86], tissue culture medium 199, and Tyrode’s albumin lactate pyruvate (TALP) [87]. More recently, porcine gamete medium has been used for IVF in a given system together with specific culture media for the IVM of oocytes (porcine oocyte medium) and embryo culture (porcine zygote medium) [88,89]. The main differences among the media include the concentrations of glucose, bicarbonate, caffeine, and calcium, as shown in Table 1.

As regards additives, many specific molecules have been used as supplements to IVF media and the reason for using one molecule or another has not always been on the basis of whether they play a potential role during *in vivo* fertilization or have been described as components of the oviductal fluid (OF). Among the classical supplements used in porcine IVF, methylxanthines such as caffeine and theophylline are considered inhibitors of the cyclic nucleotide phosphodiesterase, resulting in an increase in intracellular cAMP [90]. Both caffeine and theophylline are used to induce sperm capacitation, but caffeine stimulates both the capacitation and spontaneous acrosome reaction of boar spermatozoa [91,92], resulting in the induction of polyspermic penetration of porcine oocytes [93]. Meanwhile, theophylline stimulates the ability of spermatozoa to penetrate *in vitro*–matured porcine oocytes but is not accompanied by polyspermy [89]. Although caffeine is useful for inducing sperm capacitation, its use during the whole gamete coincubation period (usually 6–8 hours) should be carefully considered. A transient coincubation IVF system, in which denuded oocytes are cocultured with spermatozoa in medium containing caffeine for 5 to 30 minutes and then in caffeine-free medium, reduces the incidence of polyspermic penetration by 40% [6]. Despite these results, most researchers use caffeine as the main inducer of sperm capacitation and, furthermore, for long coincubation times (18 hours). This molecule, rather than theophylline or other natural inducers, still tends to be used in current IVF systems.

Table 1
Composition of basal IVF media used in the pig.

Component (mM)	mTBM	mTALP	mTCM-199	PGM	PGMtac4
NaCl	113.10	114.06	116.35	108.00	108.00
KCl	3.00	3.20	5.36	10.00	10.00
KH_2PO_4	—	—	—	0.35	0.35
$MgCl_2•6H_2O$	—	0.50	—	—	—
$MgSO_4$	—	—	0.81	—	—
$MgSO_4•7H_2O$	—	—	—	0.40	0.40
Na-lactate 60% syrup, (mL/L)	—	1.85	—	—	—
NaH_2PO_4	—	0.35	1.01	—	—
Glucose	11.00	5.00	3.05	1.00	—
$NaHCO_3$	—	25.07	26.19	25.07	25.00
Caffeine	1.00	2.00	5.00	—	—
Ca-lactate$•5H_2O$	—	—		—	—
Ca-$(lactate)_2$	—	—	2.92	—	—
Ca-$(Lactate)_2•5H_2O$	—	—	—	2.50	4.00
Tris	20.00	—		—	—
Na-pyruvate	5.00	0.11	0.91	0.20	0.20
$CaCl_2•2H_2O$	7.50	4.70	1.80	—	—
Sorbitol	—	—	12.00	—	—
Polyvinyl alcohol (mg/mL)	—	1.00	—	3.00	3.00
Theophylline	—		—	—	2.50
Adenosine (uM)	—		—	—	1.00
Cysteine (uM)	—		—	—	0.25
Gentamicin (mg/mL)	—		—	0.05	0.01
Penicillin G/streptomycin		—	0.17/0.07	—	—
Amikacin sulfate (mg/mL)	—	0.10	—	—	—
BSA (mg/mL)	1.00	3.00	4.00	—	—

mTBM: formulation from Abeydeera and Day [48]. Formulation in the study by Abeydeera and Day [86] contains 10 mM of $CaCl_2$ instead of 7.5 mM of $CaCl_2•2H_2O$.
mTALP: formulation from [87].
mTCM-199: partial listing of components of TCM-199 with Earle's salts and L-glutamine (cat. no. M-5017; Sigma). There are different supplementations of TCM. The table shows the formulation from [6].
PGM: formulation from Funahashi and Romar [89].
PGMtac4: formulation from Yoshioka et al. [89].
Abbreviations: mTALP: modified Tyrode's albumin lactate pyruvate; mTBM, modified Tris-buffered medium; mTCM-199, modified tissue culture medium-199; PGM, porcine gamete medium; PGMtac, porcine gamete medium theophylline–adenosine–cysteine.

Current examples of additives with a known *in vivo* biological function are glycosidases, serine proteases, growth factors, amino acids, and proteins. Fertilization is a carbohydrate-mediated process, and glycosidases catalyze the hydrolytic cleavage of terminal sugar residues from the glycan portion of glycoproteins and glycolipids; in the pig, it is known that the OF shows variable glycosidase activity within the estrous cycle [94,95]. Taking all these facts into consideration, the supplementation of IVF medium with these enzymes to modulate the sperm–egg interactions and reduce the incidence of polyspermy seems a logical approach. In a recent study, Romero-Aguirregomezcorta et al. [96] supplemented modified TALP (mTALP) medium with exogenous α-fucosidase on the basis of the detected concentrations in porcine OF around the time of *in vivo* fertilization. Gamete coincubation with 0.169-U α-L-fucosidase increased the percentage of penetration by 30%, doubled the number of spermatozoa bound to the ZP, and thus decreased monospermy by 50%. The opposite result might be expected but this is a clear example where mimicking *in vitro* the *in vivo* conditions does not simply involve "adding a single molecule" to the culture medium.

It is well documented that the serine proteases or proteins with serine protease activity released during the cortical reaction are responsible for protease-mediated reactions that contribute toward the block to polyspermy in the hamster [97], mouse [98] and other mammals [99]. The role of serine proteases during fertilization has been explored by adding inactive forms [100] and inhibitors [101]. In the first case, it was shown that plasminogen contributes to the regulation of sperm entry into the oocyte, not by inducing a ZP hardening or a decrease in sperm functionality but by detaching more than 50% of sperm bound to the ZP *via* releasing of the active enzyme plasmin [102]. The results showed that the oocyte has the necessary machinery to activate the plasminogen added to the IVF medium to plasmin. It also decreased the penetration rate and the mean number of spermatozoa and increased the monospermy rate [100]. The supplementation of media with serine proteases inhibitors [101] is more recent and, although inhibitors differ in the way they reduce the fertilization rate, the results show that 100-μM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride and 5-μM soybean trypsin inhibitor from *Glycine max* could be used in future IVF studies without compromising sperm quality.

Growth factors and amino acids are included in most culture media for both oocyte maturation and embryonic culture, but their addition as supplements to fertilization media is not so common. Lysophosphatidic acid (LPA), which is a member of the phospholipid autacoid family, is present in follicular fluid and has been recently used to supplement mTBM for pig IVF [103]. Lysophosphatidic acid has also been shown to exhibit growth factor-like and

hormone-like activities in a wide range of animal cells, and the addition of 10-μM LPA for 6 hours to the fertilization medium increased the proportion of eggs penetrated by spermatozoa by 10% and the monospermy rate by 5%. However, the mechanism by which LPA reduces the frequency of polyspermy remains unclear. Regarding amino acids, Tareq et al. [104] have recently studied the effects on IVF carried out for 6 hours when mTALP is supplemented with various combinations of dipeptides. The addition of 2-mM L-alanyl-L-glutamine and 2-mM L-glycyl-L-glutamine significantly improved fertilization by 10% and monospermy by 30% compared with oocytes fertilized in mTALP without dipeptides. The observed improvement in fertilization, in maturation and embryonic development, would be due to the reduction of the level of accumulated ammonia measured in the culture media. Although amino acid supplementation of the culture medium is a standard protocol in porcine embryo culture, its use as an additive in the IVF medium is much less common. However, amino acids, together with other molecules, act as antioxidants that scavenge free radicals and can be considered as a supplement to alleviate glutathione depletion during oxidative stress. As in the case of other supplements, the choice of antioxidant depends on the type of medium being used for fertilization. Thus, complex culture media such as tissue culture medium 199 are originally rich in amino acids and vitamins, whereas simple culture media such as TALP, TBM, and porcine gamete medium do not contain amino acids in their original formulation. Thus, the supplementation of mTALP with different concentrations of vitamin E and selenium in the form of sodium selenite and seleno-L-methionine improves fertilization results [105] as does the addition of N-acetyl-L-cysteine to mTBM [84].

Classically, culture media for pig IVF have been supplemented with proteins of different origin, either fetal bovine serum or BSA (Table 1), except when it is preferable to maintain the culture medium as chemically defined and the use of purified proteins is simply an alternative. One of the specific proteins with a known biological function is the oviductal protein OVGP1 (reviewed by [106]), the effects of which on the oocyte include increased sperm penetration, increased fertilization rates, and decreased polyspermy. OVGP1 purified by a heparin–agarose affinity column and obtained from oviducts of gilts in estrous [7] or oviduct culture medium [107] has been used to incubate pig gametes before fertilization or to directly supplement mTBM medium at concentrations ranging from 0 to 100 μg/mL. In both studies, exposure to OVGP1 before and during fertilization had beneficial effects. Supplementation of mTBM with 50 or 100 μg/mL reduced the incidence of polyspermy by 40%, reduced the number of bound sperm, and increased the postcleavage development to blastocyst. Other proteins with interest are a subset of 70-kDa oviductal surface proteins that bound to spermatozoa, one of which is the heat shock 70-kDa protein 8 (HSPA8 previously known as HSPA10). This protein maintains the *in vitro* survival of mammalian spermatozoa [108], and a 15-minute incubation of boar spermatozoa with a recombinant form of HSPA8 rapidly promotes the viability of uncapacitated spermatozoa and enhances IVF performance [109].

Summarizing, the type of IVF medium used and particular modifications of the same can reduce the incidence of polyspermy. Supplementing the IVF medium with molecules that can enhance the results of fertilization, and at the appropriate concentration, can be a daunting task. The methodology needs to be reassessed and move toward supplements that provide all the beneficial and necessary molecules. The porcine OF obtained at the periovulatory moment has already been used with good results and could become in the near future the definitive supplement for pig IVF media especially considering that it is already commercially available (NaturARTs by EmbryoCloud, University of Murcia, Spain).

4. The whole OF as an undefined source of molecules with a potential to improve IVF results in pig

Despite the interest shown in deciphering the role of one specific molecule in fertilization and in using this molecule as an additive during sperm–oocyte coincubation, it is clear, from a practical point of view, that this is not solely a matter of one or a number of factors affecting the process. For this reason, studies have been developed into using OF as a supplement for the culture medium.

Almost 20 years ago, it was shown that the addition of OF to the fertilization medium decreased sperm–ZP binding and penetration [110]. The proportions of OF, surgically collected, varied from 0.1% to 10%, and the authors concluded that OF might affect fertilization by reducing sperm penetration ability (triggering acrosome reaction) rather than affecting oocyte condition [110,111]. However, they also showed that preincubation of oocytes in 30% OF without the further presence of the fluid in the fertilization medium increased by several minutes the resistance of the ZP to proteolytic digestion and decreased polyspermy after IVF, without affecting penetration rates. The authors further suggested that some oviductal glycoproteins could enter the perivitelline space and facilitate a more efficient cortical reaction to prevent polyspermy.

Subsequent studies have shown that the specific molecule responsible for increased ZP resistance to proteolysis is the oviductal protein OVGP1 and that its effect is reversible, depending on the presence or absence of heparin in the IVF medium [112]. It is also known that the whole undiluted OF strongly affect pig ZP, increasing its resistance to digestion by up to several hours after a 30-minute period of incubation and that this effect is independent of the presence of spermatozoa or of the cortical granules [112]. Incubation also decreases polyspermy, without reducing penetration, up to 10 fold compared with oocytes without preincubation in OF [112]. This beneficial effect of OF strongly depends on the phase of the estrous cycle when it is collected, i.e., it depends on the estradiol/progesterone ratios (Fig. 1). So, when the oocytes are incubated in OF collected just before ovulation (high estradiol concentrations) from animals with large preovulatory follicles, the ZP digestion time in pronase solution increases significantly compared with oocytes incubated in OF collected just after ovulation (higher progesterone concentrations) from animals with recent ovulation points. Similarly, the percentage of monospermy increases [73]. However, the OF does not affect the

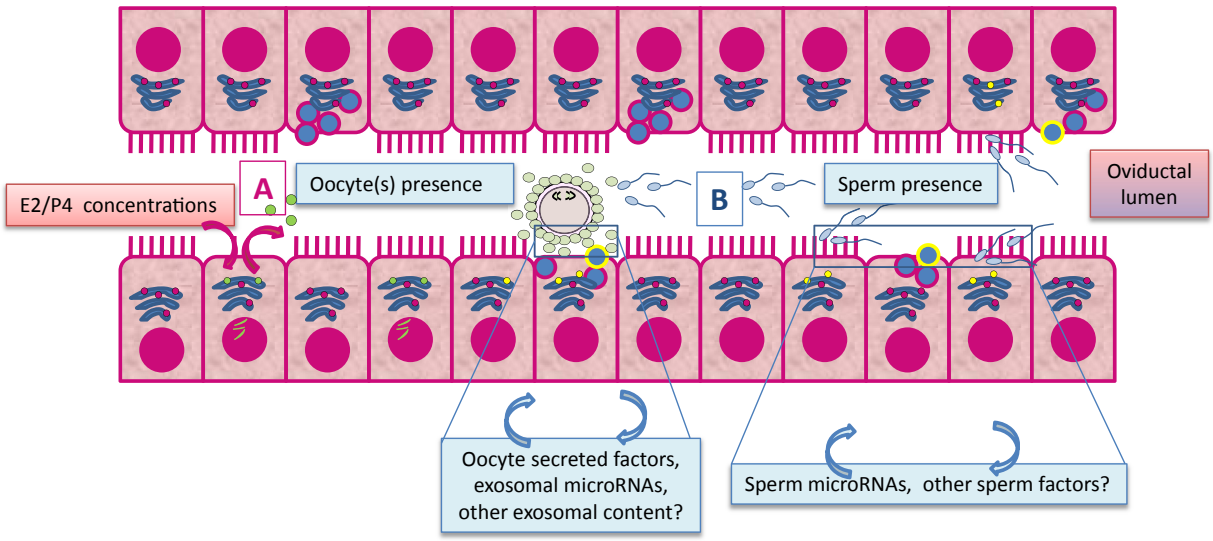

Fig. 1. Diagrammatic representation of some of the factors responsible for the changes in oviductal fluid composition. (A) Changes in steroid ratios (E2, estradiol and P4, progesterone) affect the gene expression in the epithelium and, consequently, the protein profile of the oviductal fluid. (B) Changes in concentrations of oviductal proteins have been detected depending on the presence of gametes, although the specific mechanisms by which they act on the oviductal cells have not been described. Exosomal content, particularly microRNA, sperm microRNAs, or unknown oocyte-secreted factors can be the way by which gametes and oviduct communicate.

penetration ability of frozen or fresh boar spermatozoa equally. When the oocytes are preincubated in preovulatory OF but fertilized with frozen–thawed spermatozoa from the same boar, the percentage of monospermy decreases and penetration is greatly enhanced, although the ZP is still highly resistant to protease digestion. This means that the OF increases the ability of frozen–thawed boar spermatozoa to penetrate the oocyte, whereas the opposite effect is observed with fresh spermatozoa [73]. Obviously, the mechanisms involved in the final capacitation and hyperactive motility pattern in both types of sperm under similar *in vitro* conditions must be different. Not only this, but also in fresh semen samples, it was shown that subpopulations of boar spermatozoa responded differentially to OF suggesting that the oviduct plays a significant role in the process of sperm selection [113–115].

All these effects of OF also have consequences for early embryo development and gene expression [116], so we hypothesize that the epigenetic marks that are first erased in zygotes and during initial first cleavage stages in the oviduct, and which are later reestablished during the genome-wide reprogramming of methylation in embryos, could be influenced by oviductal factors. If this is the case it would be a good idea to include in the IVF culture medium all the oviductal molecules showing a potentially favorable role in the production of a healthy (epigenetically normal) embryo. But the question is which molecules?

Attempts have already been made to identify the specific oviductal proteins involved in the mechanism of ZP resistance to protease digestion and the regulation of polyspermy [117]. Among them, and apart from OVGP1, other components of the OF fraction responsible for the ZP effect include different chaperones participating in the correct folding of the proteins, such as members of the protein disulphide and heat shock protein families [20,118]. However, the OF proteome does not only depend on the estrous cycle phase when it is produced [119]. In the pig, a body of research shows that there are oviductal proteins secreted in response to the presence of oocytes or spermatozoa in the oviduct. For example, Georgiou et al. [23] found that at least 19 oviductal proteins are regulated by the presence of spermatozoa and four more by the presence of oocytes, whereas one protein was commonly regulated by both sperm and oocytes (Fig. 1). Most of these proteins were molecular chaperones, regulators of protein folding and stability, or antioxidant and free radical–scavenging proteins. Surgically approaching the oviducts in living animals rather than using *ex vivo* oviducts, these results were confirmed in a further experiment [24] in which OVGP1, particularly, was found to be upregulated (more than threefold change) by the presence of spermatozoa in the female reproductive tract. The mechanisms by which gametes can alter the oviductal proteome are still not clear, but the involvement of cell-to-cell communication mediated by exosomes (containing microRNAs), by undiscovered factors secreted by the oocyte, or by the sperm microRNAs themselves [120] should be explored (Fig. 1).

Altogether, the above findings seem to indicate that OF is a very complex and variable fluid, whose reliable and accurate synthesis in the laboratory is practically impossible. The direct consequence of this assumption would be the recognition that IVF will never be able to produce porcine embryos with the genetic, epigenetic, and, in general terms, physiological characteristics of a naturally produced embryo. We hypothesize that the establishment of biobanks of oviductal (and follicular or uterine) fluids as additives for culture media, for use in pig or other mammalian species, would contribute to significantly improving the success rate of the ARTs, not only by increasing the proportion of

fertilized oocytes after IVF but also by increasing their ability to develop healthy and successfully. Although this idea may appear far-fetched, the technology to store OF samples from animals, classified according to the phase of the estrous cycle when they are recovered and processed to assess their sanitary quality as well as their biological activity, is already available (Patent ES 2532659 A1) and the results after initial testing are promising. Similarly to the accepted use of porcine follicular fluid as a common additive in IVM media (which shows high rates of success), the future use of IVF media with different proportions of OF included is envisioned as a solution for the current problems affecting the technique in pigs.

5. Conclusions

Some studies have modified the equipment used for IVF to regulate the number of penetrating spermatozoa in the vicinity of the oocytes, resulting in a reduction of polyspermy; i.e., the climbing-over-a-wall method [121], biomimetic microchannel IVF system (microfluidic culture system) [122,123], or straw IVF [124]. These methods have been proposed as ways to separate spermatozoa and mature oocytes and to ensure that only motile spermatozoa gain access to the oocytes, mimicking the physical conditions of fertilization *in vivo*. More recently, a rolling culture–based system has been assessed by rotating (1 rpm for 6 hours at 38.5 °C) a tube containing both gametes [125]. The results showed a 50% increase the monospermy rate and a 10% increase in the blastocyst formation rate. These devices, together with appropriate sperm preparation and suitable OF-containing IVF media, may offer a more optimistic perspective for the *in vitro* production of porcine embryos and lead to an increased use of this species not only in biomedicine but also in the trade of frozen embryos worldwide. Specifically, the sperm preparation protocols need to be standardized to permit the reproducibility in different laboratories. A possible way for future research could be focused on the development of procedures avoiding centrifugation and selecting the motile spermatozoa by making them swim up through media with a composition closer to the OF. In the same way, IVF media including recombinant proteins such as OVGP1 or purified OF fractions should be developed and tested bearing always the pig's physiological environment in the reproductive tract as the model to mimic.

As noted throughout this review, in the past decades, the ARTs have been improved with great advances although problems around swine IVF are still an obstacle for obtaining large-scale viable embryos. It is time to use different strategies from those used until now to solve this problem. The key may be in the use of oviductal and uterine secretions to correct the suboptimal conditions during IVF and/or embryo culture similarly wherein the follicular fluid is used as a supplement in the IVM medium.

Acknowledgments

The Spanish Ministry of Economy and Competitiveness and the European Commission (FEDER/ERDF) supported the research of Raquel Romar and Pilar Coy (AGL2012-40180-C03-01). The authors thank Dr William V. Holt for his scientific advice and for help with the English language.

Competing Interests

The authors declare that there are no conflicts of interest.

References

[1] Nagai T, Funahashi H, Yoshioka K, Kikuchi K. Update of in vitro production of porcine embryos. Front Biosci 2006;11:2565–73.
[2] Grupen CG. The evolution of porcine embryo in vitro production. Theriogenology 2014;81:24–37.
[3] Funahashi H. Polyspermic penetration in porcine IVM-IVF systems. Reprod Fertil Dev 2003;15:167–77.
[4] Coy P, Romar R. In vitro production of pig embryos: a point of view. Reprod Fertil Dev 2002;14:275–86.
[5] Piedrahita JA, Olby N. Perspectives on transgenic livestock in agriculture and biomedicine: an update. Reprod Fertil Dev 2011; 23:56–63.
[6] Funahashi H, Romar R. Reduction of the incidence of polyspermic penetration into porcine oocytes by pretreatment of fresh spermatozoa with adenosine and a transient co-incubation of the gametes with caffeine. Reproduction 2004;128:789–800.
[7] Kouba A, Abeydeera L, Alvarez I, Day B, Buhi W. Effects of the porcine oviduct-specific glycoprotein on fertilization, polyspermy, and embryonic development in vitro. Biol Reprod 2000;63:242–50.
[8] Coy P, Martínez E, Ruiz S, Vázquez J, Roca J, Matas C, et al. In vitro fertilization of pig oocytes after different coincubation intervals. Theriogenology 1993;39:1201–8.
[9] Gil MA, Almiñana C, Cuello C, Parrilla I, Roca J, Vazquez JM, et al. Brief coincubation of gametes in porcine in vitro fertilization: role of sperm:oocyte ratio and post-coincubation medium. Theriogenology 2007;67:620–6.
[10] Coy P, Martínez E, Ruiz S, Vázquez J, Roca J, Matas C. Sperm concentration influences fertilization and male pronuclear formation in vitro in pigs. Theriogenology 1993;40:539–46.
[11] Rath D. Experiments to improve in vitro fertilization techniques for in vivo-matured porcine oocytes. Theriogenology 1992;37:885–96.
[12] Nagai T, Takahashi T, Masuda H, Shioya Y, Kuwayama M, Fukushima M, et al. In-vitro fertilization of pig oocytes by frozen boar spermatozoa. J Reprod Fertil 1988;84:585–91.
[13] Matás C, Sansegundo M, Ruiz S, García-Vázquez FA, Gadea J, Romar R, et al. Sperm treatment affects capacitation parameters and penetration ability of ejaculated and epididymal boar spermatozoa. Theriogenology 2010;74:1327–40.
[14] Rath D, Niemann H. In vitro fertilization of porcine oocytes with fresh and frozen-thawed ejaculated or frozen-thawed epididymal semen obtained from identical boars. Theriogenology 1997;47: 785–93.
[15] Wang WH, Abeydeera LR, Prather RS, Day BN. Morphologic comparison of ovulated and in vitro-matured porcine oocytes, with particular reference to polyspermy after in vitro fertilization. Mol Reprod Dev 1998;49:308–16.
[16] Marchal R, Feugang JM, Perreau C, Venturi E, Terqui M, Mermillod P. Meiotic and developmental competence of prepubertal and adult swine oocytes. Theriogenology 2001;56:17–29.
[17] Nagai T, Moor RM. Effect of oviduct cells on the incidence of polyspermy in pig eggs fertilized in vitro. Mol Reprod Dev 1990;26:377–82.
[18] Romar R, Coy P, Ruiz S, Gadea J, Rath D. Effects of oviductal and cumulus cells on in vitro fertilization and embryo development of porcine oocytes fertilized with epididymal spermatozoa. Theriogenology 2003;59:975–86.
[19] Romar R, Coy P, Campos I, Gadea J, Matás C, Ruiz S. Effect of co-culture of porcine sperm and oocytes with porcine oviductal epithelial cells on in vitro fertilization. Anim Reprod Sci 2001;68:85–98.
[20] Almiñana C, Heath PR, Wilkinson S, Sanchez-Osorio J, Cuello C, Parrilla I, et al. Early developing pig embryos mediate their own environment in the maternal tract. PLoS One 2012;7:e33625.
[21] Sironen A, Fischer D, Laiho A, Gyenesei A, Vilkki J. A recent L1 insertion within SPEF2 gene is associated with changes in PRLR expression in sow reproductive organs. Anim Genet 2014;45:500–7.
[22] Yeste M, Holt WV, Bonet S, Rodríguez-Gil JE, Lloyd RE. Viable and morphologically normal boar spermatozoa alter the expression of

heat-shock protein genes in oviductal epithelial cells during co-culture in vitro. Mol Reprod Dev 2014;81:805–19.
[23] Georgiou AS, Sostaric E, Wong CH, Snijders AP, Wright PC, Moore HD, et al. Gametes alter the oviductal secretory proteome. Mol Cell Proteomics 2005;4:1785–96.
[24] Georgiou AS, Snijders AP, Sostaric E, Aflatoonian R, Vazquez JL, Vazquez JM, et al. Modulation of the oviductal environment by gametes. J Proteome Res 2007;6:4656–66.
[25] Kolle S, Dubielzig S, Reese S, Wehrend A, Konig P, Kummer W. Ciliary transport, gamete interaction, and effects of the early embryo on the oviduct: ex vivo analyses using a new digital video-microscopic system in the cow. Biol Reprod 2009;81:267–74.
[26] Troedsson MH, Desvousges A, Alghamdi AS, Dahms B, Dow CA, Hayna J, et al. Components in seminal plasma regulating sperm transport and elimination. Anim Reprod Sci 2005;89:171–86.
[27] Dostalova Z, Calvete JJ, Sanz L, Topfer-Petersen E. Quantitation of boar spermadhesins in accessory sex gland fluids and on the surface of epididymal, ejaculated and capacitated spermatozoa. Biochim Biophys Acta 1994;1200:48–54.
[28] Caballero I, Vazquez JM, Mayor GM, Alminana C, Calvete JJ, Sanz L, et al. PSP-I/PSP-II spermadhesin exert a decapacitation effect on highly extended boar spermatozoa. Int J Androl 2009;32:505–13.
[29] Caballero I, Vazquez JM, Rodriguez-Martinez H, Gill MA, Calvete JJ, Sanz L, et al. Influence of seminal plasma PSP-I/PSP-II spermadhesin on pig gamete interaction. Zygote 2005;13:11–6.
[30] Cross NL. Effect of cholesterol and other sterols on human sperm acrosomal responsiveness. Mol Reprod Dev 1996;45:212–7.
[31] Katila T. Post-mating inflammatory responses of the uterus. Reprod Domest Anim 2012;47:31–41.
[32] Rodriguez-Martinez H, Saravia F, Wallgren M, Martinez EA, Sanz L, Roca J, et al. Spermadhesin PSP-I/PSP-II heterodimer induces migration of polymorphonuclear neutrophils into the uterine cavity of the sow. J Reprod Immunol 2010;84:57–65.
[33] Bischof RJ, Lee CS, Brandon MR, Meeusen E. Inflammatory response in the pig uterus induced by seminal plasma. J Reprod Immunol 1994;26:131–46.
[34] Woelders H, Matthijs A. Phagocytosis of boar spermatozoa in vitro and in vivo. Reproduction 2001;58:113–27.
[35] Rozeboom KJ, Troedsson MH, Molitor TW, Crabo BG. The effect of spermatozoa and seminal plasma on leukocyte migration into the uterus of gilts. J Anim Sci 1999;77:2201–6.
[36] Waberski D, Kremer H, Borchardt Neto G, Jungblut PW, Kallweit E, Weitze KF. Studies on a local effect of boar seminal plasma on ovulation time in gilts. Zentralbl Veterinarmed A 1999;46:431–8.
[37] Waberski D, Dohring A, Ardon F, Ritter N, Zerbe H, Schuberth HJ, et al. Physiological routes from intra-uterine seminal contents to advancement of ovulation. Acta Vet Scand 2006;48:13.
[38] Robertson SA. Seminal fluid signaling in the female reproductive tract: lessons from rodents and pigs. J Anim Sci 2007;85:e36–44.
[39] Dittrich R, Henning J, Maltaris T, Hoffmann I, Oppelt PG, Cupisti S, et al. Extracorporeal perfusion of the swine uterus: effect of human seminal plasma. Andrologia 2012;44:543–9.
[40] González-Cadavid V, Martins JA, Moreno FB, Andrade TS, Santos AC, Monteiro-Moreira AC, et al. Seminal plasma proteins of adult boars and correlations with sperm parameters. Theriogenology 2014;82:697–707.
[41] De Jonge C. Biological basis for human capacitation. Hum Reprod Update 2005;11:205–14.
[42] Topfer-Petersen E, Ekhlasi-Hundrieser M, Tsolova M, Topfer-Petersen E, Ekhlasi-Hundrieser M, Tsolova M. Glycobiology of fertilization in the pig. Int J Dev Biol 2008;52:717–36.
[43] Martinez P, Morros A. Membrane lipid dynamics during human sperm capacitation. Front Biosci 1996;1:d103–17.
[44] Witte TS, Schafer-Somi S. Involvement of cholesterol, calcium and progesterone in the induction of capacitation and acrosome reaction of mammalian spermatozoa. Anim Reprod Sci 2007;102:181–93.
[45] Cheng WTK, Polge C, Moor RM. In vitro fertilization of pig and sheep oocytes. Theriogenology 1986;25:146.
[46] Mattioli M, Bacci ML, Galeati G, Seren E. Developmental competence of pig oocytes matured and fertilized *in vitro*. Theriogenology 1989;31:1201–7.
[47] Funahashi H, Cantley TC, Day BN. Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following in vitro fertilization. Biol Reprod 1997;57:49–53.
[48] Abeydeera LR, Day BN. Fertilization and subsequent development in vitro of pig oocytes inseminated in a modified Tris-buffered medium with frozen-thawed ejaculated spermatozoa. Biol Reprod 1997;57:729–34.
[49] Carvajal G, Cuello C, Ruiz M, Vazquez JM, Martinez EA, Roca J. Effects of centrifugation before freezing on boar sperm cryosurvival. J Androl 2004;25:389–96.
[50] Yeste M, Lloyd RE, Badia E, Briz M, Bonet S, Holt WV. Direct contact between boar spermatozoa and porcine oviductal epithelial cell (OEC) cultures is needed for optimal sperm survival in vitro. Anim Reprod Sci 2009;113:263–78.
[51] Guthrie HD, Welch GR. Use of fluorescence-activated flow cytometry to determine membrane lipid peroxidation during hypothermic liquid storage and freeze-thawing of viable boar sperm loaded with 4, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid. J Anim Sci 2007;85:1402–11.
[52] Petrunkina AM, Simon K, Gunzel-Apel AR, Topfer-Petersen E. Regulation of capacitation of canine spermatozoa during co-culture with heterologous oviductal epithelial cells. Reprod Domest Anim 2003;38:455–63.
[53] Horan R, Powell R, McQuaid S, Gannon F, Houghton JA. Association of foreign DNA with porcine spermatozoa. Arch Androl 1991;26:83–92.
[54] de Vries AC, Colenbrander B. Isolation and characterization of boar spermatozoa with and without a cytoplasmic droplet. Int J Biochem 1990;22:519–24.
[55] Matas C, Vieira L, Garcia-Vazquez FA, Aviles-Lopez K, Lopez-Ubeda R, Carvajal JA, et al. Effects of centrifugation through three different discontinuous Percoll gradients on boar sperm function. Anim Reprod Sci 2011;127:62–72.
[56] Caballero I, Vazquez JM, Gil MA, Calvete JJ, Roca J, Sanz L, et al. Does seminal plasma PSP-I/PSP-II spermadhesin modulate the ability of boar spermatozoa to penetrate homologous oocytes in vitro? J Androl 2004;25:1004–12.
[57] Matas C, Coy P, Romar R, Marco M, Gadea J, Ruiz S. Effect of sperm preparation method on in vitro fertilization in pigs. Reproduction 2003;125:133–41.
[58] Grant SA, Long SE, Parkinson TJ. Fertilizability and structural properties of boar spermatozoa prepared by Percoll gradient centrifugation. J Reprod Fertil 1994;100:477–83.
[59] Jeong BS, Yang X. Cysteine, glutathione, and Percoll treatments improve porcine oocyte maturation and fertilization in vitro. Mol Reprod Dev 2001;59:330–5.
[60] Garcia-Rosello E, Matas C, Canovas S, Moreira PN, Gadea J, Coy P. Influence of sperm pretreatment on the efficiency of intracytoplasmic sperm injection in pigs. J Androl 2006;27:268–75.
[61] Clarke RN, Johnson LA. Effect of liquid storage and cryopreservation of boar spermatozoa on acrosomal integrity and the penetration of zona-free hamster ova in vitro. Gamete Res 1987;16:193–204.
[62] Holt WV, Hernandez M, Warrell L, Satake N. The long and the short of sperm selection *in vitro* and *in vivo*: swim-up techniques select for the longer and faster swimming mammalian sperm. J Evol Biol 2010;23:598–608.
[63] Park CH, Lee SG, Choi DH, Lee CK. A modified swim-up method reduces polyspermy during in vitro fertilization of porcine oocytes. Anim Reprod Sci 2009;115:169–81.
[64] Martinez-Alborcia MJ, Morrell JM, Gil MA, Barranco I, Maside C, Alkmin DV, et al. Suitability and effectiveness of single layer centrifugation using Androcoll-P in the cryopreservation protocol for boar spermatozoa. Anim Reprod Sci 2013;140:173–9.
[65] Sjunnesson YC, Morrell JM, Gonzalez R. Single layer centrifugation-selected boar spermatozoa are capable of fertilization in vitro. Acta Vet Scand 2013;55:20.
[66] Morrell JM, Saravia F, van Wienen M, Rodriguez-Martinez H, Wallgren M. Sperm survival following colloid centrifugation varies according to the part of the sperm-rich fraction used. Soc Reprod Fertil Suppl 2009;66:85–6.
[67] Kruse R, Dutta PC, Morrell JM. Colloid centrifugation removes seminal plasma and cholesterol from boar spermatozoa. Reprod Fertil Dev 2011;23:858–65.
[68] Blomqvist G, Persson M, Wallgren M, Wallgren P, Morrell JM. Removal of virus from boar semen spiked with porcine circovirus type 2. Anim Reprod Sci 2011;126:108–14.
[69] Sano H, Matsuura K, Naruse K, Funahashi H. Application of a microfluidic sperm sorter to the in-vitro fertilization of porcine oocytes reduced the incidence of polyspermic penetration. Theriogenology 2010;74:863–70.
[70] Bathgate R. Functional integrity of sex-sorted, frozen-thawed boar sperm and its potential for artificial insemination. Theriogenology 2008;70:1234–41.
[71] Umeyama K, Honda K, Matsunari H, Nakano K, Hidaka T, Sekiguchi K, et al. Production of diabetic offspring using

cryopreserved epididymal sperm by in vitro fertilization and intrafallopian insemination techniques in transgenic pigs. J Reprod Dev 2013;59:599–603.
[72] Ikeda H, Kikuchi K, Noguchi J, Takeda H, Shimada A, Mizokami T, et al. Effect of preincubation of cryopreserved porcine epididymal sperm. Theriogenology 2002;57:1309–18.
[73] Ballester L, Romero-Aguirregomezcorta J, Soriano-Ubeda C, Matas C, Romar R, Coy P. Timing of oviductal fluid collection, steroid concentrations, and sperm preservation method affect porcine in vitro fertilization efficiency. Fertil Steril 2014;102:1762–8.
[74] Suzuki C, Yoshioka K, Itoh S, Kawarasaki T, Kikuchi K. In vitro fertilization and subsequent development of porcine oocytes using cryopreserved and liquid-stored spermatozoa from various boars. Theriogenology 2005;64:1287–96.
[75] Matas C, Decuadro G, Martinez-Miro S, Gadea J. Evaluation of a cushioned method for centrifugation and processing for freezing boar semen. Theriogenology 2007;67:1087–91.
[76] Hernandez M, Roca J, Calvete JJ, Sanz L, Muino-Blanco T, Cebrian-Perez JA, et al. Cryosurvival and in vitro fertilizing capacity post-thaw is improved when boar spermatozoa are frozen in the presence of seminal plasma from good freezer boars. J Androl 2007;28:689–97.
[77] Okazaki T, Shimada M. New strategies of boar sperm cryopreservation: development of novel freezing and thawing methods with a focus on the roles of seminal plasma. Anim Sci J 2012;83:623–9.
[78] Zhang W, Yi K, Chen C, Hou X, Zhou X. Application of antioxidants and centrifugation for cryopreservation of boar spermatozoa. Anim Reprod Sci 2012;132:123–8.
[79] Gadea J, Selles E, Marco MA, Coy P, Matas C, Romar R, et al. Decrease in glutathione content in boar sperm after cryopreservation. Effect of the addition of reduced glutathione to the freezing and thawing extenders. Theriogenology 2004;62:690–701.
[80] Malo C, Gil L, Gonzalez N, Martinez F, Cano R, de Blas I, et al. Antioxidant supplementation improves boar sperm characteristics and fertility after cryopreservation: comparison between cysteine and rosemary (Rosmarinus officinalis). Cryobiology 2010;61:142–7.
[81] Kaedei Y, Naito M, Naoi H, Sato Y, Taniguchi M, Tanihara F, et al. Effects of (-)-epigallocatechin gallate on the motility and penetrability of frozen-thawed boar spermatozoa incubated in the fertilization medium. Reprod Domest Anim 2012;47:880–6.
[82] Satorre MM, Breininger E, Beconi MT. Cryopreservation with alpha-tocopherol and Sephadex filtration improved the quality of boar sperm. Theriogenology 2012;78:1548–56.
[83] Roca J, Rodriguez MJ, Gil MA, Carvajal G, Garcia EM, Cuello C, et al. Survival and in vitro fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. J Androl 2005;26:15–24.
[84] Whitaker BD, Casey SJ, Taupier R. N-acetyl-l-cysteine supplementation improves boar spermatozoa characteristics and subsequent fertilization and embryonic development. Reprod Domest Anim 2012;47:263–8.
[85] Suzuki K, Nagai T. In vitro fertility and motility characteristics of frozen-thawed boar epididymal spermatozoa separated by Percoll. Theriogenology 2003;60:1481–94.
[86] Abeydeera LR, Day BN. In vitro penetration of pig oocytes in a modified Tris-buffered medium: effect of BSA, caffeine and calcium. Theriogenology 1997;48:537–44.
[87] Rath D, Long CR, Dobrinsky JR, Welch GR, Schreier LL, Johnson LA. In vitro production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer. J Anim Sci 1999;77:3346–52.
[88] Yoshioka K, Suzuki C, Tanaka A, Anas IM, Iwamura S. Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biol Reprod 2002;66:112–9.
[89] Yoshioka K, Suzuki C, Itoh S, Kikuchi K, Iwamura S, Rodriguez-Martinez H. Production of piglets derived from in vitro-produced blastocysts fertilized and cultured in chemically defined media: effects of theophylline, adenosine, and cysteine during in vitro fertilization. Biol Reprod 2003;69:2092–9.
[90] Casillas ER, Hoskins DD. Activation of monkey spermatozoal adenyl cyclase by thyroxine and triiodothyronine. Biochem Biophys Res Commun 1970;40:255–62.
[91] Funahashi H, Asano A, Fujiwara T, Nagai T, Niwa K, Fraser LR. Both fertilization promoting peptide and adenosine stimulate capacitation but inhibit spontaneous acrosome loss in ejaculated boar spermatozoa in vitro. Mol Reprod Dev 2000;55:117–24.
[92] Funahashi H, Fujiwara T, Nagai T. Modulation of the function of boar spermatozoa via adenosine and fertilization promoting peptide receptors reduce the incidence of polyspermic penetration into porcine oocytes. Biol Reprod 2000;63:1157–63.
[93] Funahashi H, Nagai T. Regulation of in vitro penetration of frozen-thawed boar spermatozoa by caffeine and adenosine. Mol Reprod Dev 2001;58:424–31.
[94] Carrasco LC, Romar R, Aviles M, Gadea J, Coy P. Determination of glycosidase activity in porcine oviductal fluid at the different phases of the estrous cycle. Reproduction 2008;136:833.
[95] Carrasco LC, Coy P, Aviles M, Gadea J, Romar R. Glycosidase determination in bovine oviductal fluid at the follicular and luteal phases of the oestrous cycle. Reprod Fertil Dev 2008;20:808–17.
[96] Romero-Aguirregomezcorta J, Matás C, Coy P. α-L-fucosidase enhances capacitation-associated events in porcine spermatozoa. Vet J 2015;203:109–14.
[97] Cherr GN, Drobnis EZ, Katz DF. Localization of cortical granule constituents before and after exocytosis in the hamster egg. J Exp Zool 1988;246:81–93.
[98] Hoodbhoy T, Talbot P. Mammalian cortical granules: contents, fate, and function. Mol Reprod Dev 1994;39:439–48.
[99] Wong JL, Wessel GM. Defending the zygote: search for the ancestral animal block to polyspermy. Curr Top Dev Biol 2006;72:1–151.
[100] Grullón LA, Gadea J, Mondéjar I, Matás C, Romar R, Coy P. How is plasminogen/plasmin system contributing to regulate sperm entry into the oocyte? Reprod Sci 2013;20:1075–82.
[101] Beek J, Maes D, Nauwynck H, Piepers S, Van Soom A. A critical assessment of the effect of serine protease inhibitors on porcine fertilization and quality parameters of porcine spermatozoa in vitro. Reprod Biol 2015;15:9–19.
[102] Coy P, Jiménez-Movilla M, García-Vázquez FA, Mondéjar I, Grullón L, Romar R. Oocytes use plasminogen-plasmin system to remove supernumerary spermatozoa. Hum Reprod 2012;27:1985–93.
[103] Zhang JY, Jiang Y, Lin T, Kang JW, Lee JE, Jin DI. Lysophosphatidic acid improves porcine oocyte maturation and embryo development in vitro. Mol Reprod Dev 2015;82:66–77.
[104] Tareq KM, Akter QS, Tsujii H, Khandoker MA, Choi I. Effect of dipeptides on in vitro maturation, fertilization and subsequent embryonic development of porcine oocytes. Asian-Australas J Anim Sci 2013;26:501–8.
[105] Tareq KM, Akter QS, Khandoker MA, Tsujii H. Selenium and vitamin E improve the in vitro maturation, fertilization and culture to blastocyst of porcine oocytes. J Reprod Dev 2012;58:621–8.
[106] Buhi WC, Alvarez IM. Identification, characterization and localization of three proteins expressed by the porcine oviduct. Theriogenology 2003;60:225–38.
[107] McCauley TC, Buhi WC, Wu GM, Mao J, Caamano JN, Didion BA, et al. Oviduct-specific glycoprotein modulates sperm-zona binding and improves efficiency of porcine fertilization in vitro. Biol Reprod 2003;69:828–34.
[108] Elliott RM, Lloyd RE, Fazeli A, Sostaric E, Georgiou AS, Satake N, et al. Effects of HSPA8, an evolutionarily conserved oviductal protein, on boar and bull spermatozoa. Reproduction 2009;137:191–203.
[109] Moein-Vaziri N, Phillips I, Smith S, Alminana C, Maside C, Gil MA, et al. Heat shock protein A8 restores sperm membrane integrity by increasing plasma membrane fluidity. Reproduction 2014;147:719–32.
[110] Kim N, Funahashi H, Abeydeera L, Moon S, Prather R, Day B. Effects of oviductal fluid on sperm penetration and cortical granule exocytosis during fertilization of pig oocytes in vitro. J Reprod Fertil 1996;107:79–86.
[111] Kim NH, Day BN, Lim JG, Lee HT, Chung KS. Effects of oviductal fluid and heparin on fertility and characteristics of porcine spermatozoa. Zygote 1997;5:61–5.
[112] Coy P, Cánovas S, Mondéjar I, Saavedra MD, Romar R, Grullón L, et al. Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during fertilization and contribute to the control of polyspermy. Proc Natl Acad Sci U S A 2008;105:15809–14.
[113] Coy P, Lloyd R, Romar R, Satake N, Matas C, Gadea J, et al. Effects of porcine pre-ovulatory oviductal fluid on boar sperm function. Theriogenology 2010;74:632–42.
[114] Holt WV, Fazeli A. Do sperm possess a molecular passport? Mechanistic insights into sperm selection in the female reproductive tract. Mol Hum Reprod 2015;21:491–501.
[115] Holt WV, Fazeli A. The oviduct as a complex mediator of mammalian sperm function and selection. Mol Reprod Dev 2010;77:934–43.

[116] Lloyd R, Romar R, Matás C, Gutiérrez-Adán A, Holt W, Coy P. Effects of oviductal fluid on the development, quality, and gene expression of porcine blastocysts produced in vitro. Reproduction 2009; 137:679–87.
[117] Mondéjar I, Martínez-Martínez I, Avilés M, Coy P. Identification of potential oviductal factors responsible for zona pellucida hardening and monospermy during fertilization in mammals. Biol Reprod 2013;89:67.
[118] Coy P, Yanagimachi R. Common and species-specific roles of oviductal proteins in mammalian fertilization and embryo development. Bioscience. doi:10.1093/biosci/biv119; 2015.
[119] Seytanoglu A, Georgiou AS, Sostaric E, Watson PF, Holt WV, Fazeli A. Oviductal cell proteome alterations during the reproductive cycle in pigs. J Proteome Res 2008;7:2825–33.
[120] Salas-Huetos A, Blanco J, Vidal F, Mercader JM, Garrido N, Anton E. New insights into the expression profile and function of micro-ribonucleic acid in human spermatozoa. Fertil Steril 2014;102: 213–22.
[121] Funahashi H, Nagai T. Sperm selection by a climbing-over-a-wall IVF method reduces the incidence of polyspermic penetration of porcine oocytes. J Reprod Dev 2000;46:319–24.
[122] Clark SG, Haubert K, Beebe DJ, Ferguson CE, Wheeler MB. Reduction of polyspermic penetration using biomimetic microfluidic technology during in vitro fertilization. Lab A Chip 2005;5:1229–32.
[123] Wheeler MB, Clark SG, Beebe DJ. Developments in in vitro technologies for swine embryo production. Reprod Fertil Dev 2004;16:15–25.
[124] Li YH, Ma W, Li M, Hou Y, Jiao LH, Wang WH. Reduced polyspermic penetration in porcine oocytes inseminated in a new in vitro fertilization (IVF) system: straw IVF. Biol Reprod 2003;69:1580–5.
[125] Kitaji H, Ookutsu S, Sato M, Miyoshi K. A new rolling culture-based in vitro fertilization system capable of reducing polyspermy in porcine oocytes. Anim Sci J 2015;86:494–8.