



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



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### 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.

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### 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

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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 multi-functional 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 ( $2400 \times g$ ) 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 ( $500 \times g$  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 cocubation period (usually 6–8 hours) should be carefully considered. A transient cocubation 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 cocubation 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 <sub>2</sub> PO <sub>4</sub>	—	—	—	0.35	0.35
MgCl <sub>2</sub> •6H <sub>2</sub> O	—	0.50	—	—	—
MgSO <sub>4</sub>	—	—	0.81	—	—
MgSO <sub>4</sub> •7H <sub>2</sub> O	—	—	—	0.40	0.40
Na-lactate 60% syrup, (mL/L)	—	1.85	—	—	—
NaH <sub>2</sub> PO <sub>4</sub>	—	0.35	1.01	—	—
Glucose	11.00	5.00	3.05	1.00	—
NaHCO <sub>3</sub>	—	25.07	26.19	25.07	25.00
Caffeine	1.00	2.00	5.00	—	—
Ca-lactate•5H <sub>2</sub> O	—	—	—	—	—
Ca-(lactate) <sub>2</sub>	—	—	2.92	—	—
Ca-(Lactate) <sub>2</sub> •5H <sub>2</sub> O	—	—	—	2.50	4.00
Tris	20.00	—	—	—	—
Na-pyruvate	5.00	0.11	0.91	0.20	0.20
CaCl <sub>2</sub> •2H <sub>2</sub> O	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<sub>2</sub> instead of 7.5 mM of CaCl<sub>2</sub>•2H<sub>2</sub>O.

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-Aguirregomez et al. [96] supplemented modified TALP (mTALP) medium with exogenous  $\alpha$ -fucosidase on the basis of the detected concentrations in porcine OF around the time of *in vivo* fertilization. Gamete coinoculation with 0.169-U  $\alpha$ -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- $\mu$ M 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride and 5- $\mu$ M 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- $\mu$ M 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  $\mu$ g/mL. In both studies, exposure to OVGP1 before and during fertilization had beneficial effects. Supplementation of mTBM with 50 or 100  $\mu$ g/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].

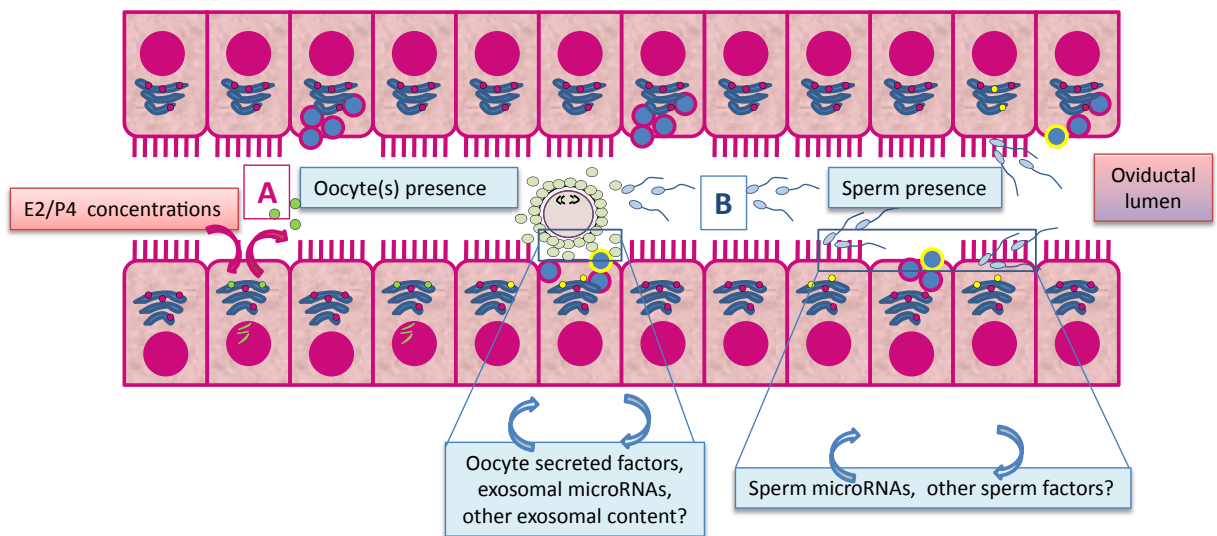
Summarizing, the type of IVF medium used and particular modifications of the same can reduce the incidence of polyspermy. Supplementing the IVF medium with molecules that can enhance the results of fertilization, and at the appropriate concentration, can be a daunting task. The methodology needs to be 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 OVGPI 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.

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