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Pig in vitro fertilization: where are we and where do we go?

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Abstract

The pig is an important livestock animal. Biotechnological interest in this species has increased due to its use, among others, in the generation of transgenic animals for use in biomedicine based on its greater physiological proximity to the human species than other large domestic animals. This development has paralleled an improvement in Assisted Reproduction Techniques (ART) used for this species. However, the ability to generate animals from embryos produced entirely in vitro is still limited and a wide margin for improvement remains. Here we review the procedures, additives, and devices used during pig in vitro fertilization (IVF), focusing on the main points of each step that have offered the best results in terms of increased efficiency of the system. The lack of standardized protocols and consensus on the parameters to be assessed makes it difficult to compare results across different studies, but some conclusions are drawn from the literature. We anticipate that new physiological protocols will advance the field of swine IVF, including induction of prefertilization ZP hardening with oviductal fluid, sperm preparation by swim-up method, increased viscosity through the addition of inert molecules or reproductive biofluids, and the incorporation of 3D devices. Here we also reflect on the need to expand the variables on which the efficiency of pig IVF is based, providing new parameters that should be considered to supply more objective and quantitative assessment of IVF additives and protocols.

1. Introduction

The biomedical value of the porcine species as a large animal model to study human diseases is indisputable [1]. Moreover, in the application of genome editing to farmed animal species to increase their productivity and health (known as livestock 2.0), pigs will be a key species to meet future food demands [2]. The in vitro production of viable porcine embryos is feasible but its application on a large scale is not yet a reality, mainly due to inefficiencies of Assisted Reproduction Technologies (ART) in the porcine species relative to others. Developing more efficient and standardized protocols will be necessary to fully capitalize on the power of new methods such as the generation of genetically modified pigs [3]. The success of different ART procedures in pigs is variable. In vitro fertilization (IVF) is particularly hampered by the low rates of monospermic zygotes achieved after insemination, which leads to low success rate (ratio of monospermic zygotes/number of inseminated oocytes) not exceeding 45% (reviewed by [4]). Several reasons have been postulated to explain the high incidence of polyspermic fertilization, such as suboptimal oocyte maturation [5], excess acrosome reacted spermatozoa surrounding the oocyte at the time of fertilization [6] and the use of culture media and devices that fail to provide the precise microenvironment needed for fertilization (reviewed by [4]). Many different methods and protocols have been attempted to improve outcomes. However, to date there is no a single solution to this problem nor a generalized protocol that guarantees the success of porcine IVF with an output over 70%, as is achieved in cattle [7]. In addition, there is no consensus on what is (are) the variable(s) and end-points that should be evaluated to consider an IVF protocol more efficient than another.

A conventional IVF protocol comprises several steps and procedures that can be temporally grouped into three stages: before, during and after insemination. The first stage includes preparation of female gametes (i.e. partial removal of cumulus cells after in vitro maturation and induction of zona pellucida hardening), and preparation of male gametes (selection and capacitation of either ejaculated, epididymal, fresh or frozen-thawed sperm cells). The second stage includes the selection of IVF media for gamete coculture and its additives, together with the appropriate device and external conditions (gas, temperature and humidity). Finally, the parameters that dictate whether a system is optimal must be defined. The success of IVF will ultimately be influenced by the cumulative impact of all these factors. Here we review the most important milestones at each step, with a special emphasis on the factors that have increased the final efficiency of the system and those that need to be explored in the future.

2. Before insemination

In general terms, porcine cumulus-oocyte complexes (COCs) are matured in vitro for 42-44 h (the first 20 h in the presence of eCG, hCG and dibutyryl AMPc, followed by 20-22 h without hormones nor dibutyryl AMPc [8]), at 38.5 ºC, 5% CO_2 and 20% O_2 in a culture medium supplemented with follicular fluid (reviewed by [9]). Although this review does not focus on aspects related to the in vitro maturation of COCs, the culture conditions during this period are critical for the subsequent results of fertilization and embryonic development.

2.1 Oocyte pretreatment: prefertilization zona pellucida hardening

In the in vivo setting, ovulated COCs come in contact with oviductal secretions before being fertilized. These secretions induce changes at the zona pellucida (ZP) that affect further sperm-ZP binding and the role of the ZP in the control of polyspermy. Among these modifications is the hardening of the ZP, or an increase in its proteolytic resistance [10]. In vitro, the ZP hardening that occurs prior to fertilization has been directly associated with a reduction in polyspermic fertilization in pigs and cows and it was described as a novel mechanism in preventing polyspermy [11]. Oviductal glycoprotein 1 (OVGP1) was identified as one of the main factors in oviductal secretions responsible for hardening the ZP together with heparin, and probably other sulphated glycosaminoglycans (GAGs) [12]. The prefertilization hardening of the ZP is achieved by preincubating oocytes for a short time (from 30 to 60 min) with artificial agents, such as cross-linking reagents [11], or with natural components such as pure oviductal fluid (OF) obtained from gilts and sows [12]. OF from the preovulatory period functions better than that from the postovulatory period [13]. Incubation of oocytes in porcine OF prior to insemination increased the final efficiency and blastocyst rate to 40-45% [12, 14]. However, the addition of exogenous heparin together with OF during the incubation reduced the efficiency of monospermic zygote production [14]. The benefits of OF pretreatment might be due to the embryotrophic effect of OVGP1, and other proteins contained in the oviduct vesicles and exosomes, which are internalized during the incubation time and are later endocytosed by the blastomeres of preimplantational embryo [15, 16]. Pig oocytes use prefertilization ZP hardening as a means to control polyspermy but the concentration of the specific molecules responsible of the zona block is unknown and they are not commercially available. Moreover, since there are likely multiple factors in combination that contribute to the zona block, the preincubation of oocytes in OF from the preovulatory phase of the estrous cycle

represents the most efficient option to achieve full ZP maturation before coincubation with sperm.

Routinely, in vitro matured porcine COCs are mechanically stripped of surrounding cumulus cells and the denuded oocytes are then inseminated. The effect of some molecules added to IVF media is opposite depending on whether cumulus-enclosed or cumulus-free oocytes are inseminated [17]. A recent study showed best IVF and embryo development results with cumulus-enclosed, rather than with cumulus-free oocytes, together with other factors such as sperm concentration [18]. Ex vivo analyses using digital video-microscopy showed that the external cumulus cells of ovulated bovine COCs attached to oviductal and cumulus cells detach slightly during migration of COCs down the oviduct to the fertilization site [19]. In pigs, this feature is simulated by partially denuding the mature COCs before submitting them to insemination [20].

2.2 Selection and capacitation of sperm cells

Sperm capacitation comprises a series of functional modifications on sperm cells that are necessary for sperm to fertilize an oocyte. These events include changes in the lipid membrane, loss of cholesterol, activation of the cAMP/PKA pathway, an increase in Ca^{2+} uptake and intracellular pH, hyperpolarisation of membrane potential, and protein phosphorylation (reviewed by [21]). Some well-known molecules involved in capacitation are bicarbonate and calcium, which are present in the majority of culture media used for sperm selection or IVF, and also cholesterol [21]. The techniques for sperm separation from seminal plasma, diluents or cryoprotectants should be quick, easy, low-cost and have the ability to select motile and morphologically normal sperm

with low production of reactive oxygen species (ROS) [22]. This may seem to be a straightforward and rapid part of the ART procedure in the laboratory (lasting 30 to 60 min, depending on the sperm treatment), but it has a key impact on further fertilization results [23, 24]. Moreover in mice, the stress suffered by the sperm cells is transmitted to the offspring in the form of metabolic and behavioural alterations [25].

The methods used for sperm preparation prior to IVF include a washing step, to remove diluents, media, cryoprotectants or decapacitating factors (from the seminal plasma or epididymal fluid), as well as the selection of motile and mature sperm (reviewed by [26]). In pigs, washing sperm by centrifugation in basic media containing capacitating factors, such as calcium and bicarbonate, and additional supplementation with more capacitating molecules (such as BSA and caffeine) (Table 1), induces increased and rapid contact of capacitated sperm with the female gametes. This is one reason why polyspermy is so prevalent in porcine IVF systems [27]. This approach of preparing boar sperm has been adapted from human ART procedures where it is necessary to achieve a high recovery rate with maximum fertilizing ability from azoospermic pathological semen samples. However, in the porcine species, the problem is the opposite, since samples come from highly selected boars with a maximal and proven fertilizing capacity. Since centrifugation can cause alterations in DNA and epigenetic marks [28-30], sperm preparation methods should be as physiological as possible. Moreover, in pigs, preparations that result in maximum fertilizing capacity are not necessary because of the propensity for polyspermy. Park et al. [31] decreased polyspermy by selecting boar sperm with a modified swim-up method using a 70 µm pore sized cell strainer. Nowadays, the traditional swim-up procedure is feasible with media specifically formulated for porcine species reaching a final IVF efficiency of

35.2% compared with 14.6% for Percoll [32], since motility and capacitation parameters are improved [33].

Future research should continue searching for and improving the methods to select motile and DNA-intact sperm for ART [34], as swim-up is not the only method that allows sperm to freely swim and be selected based on their motility. To this end, better results are achieved after insemination with sperm selected through viscous media [35, 36] by using three-dimensional (3D) microfluidic devices specifically designed for porcine sperm [37] and incorporating sperm chemo-attractants, thermotaxis [38] and bioactive components [39]. These options should be explored with the goal of developing a species specific protocol, instead of relying on the commonly used method of simply "washing and selecting by centrifugation in a highly capacitating medium".

3. During insemination

In vitro coculture of the male and female gametes requires adequate external environmental conditions (gases, temperature and humidity) as well as appropriate culture media and devices that ultimately facilitate the contact of both gametes and fertilization itself.

3.1 Fertilization culture media and additives

The classical media used for porcine IVF include modified Tyrode's albumin lactate pyruvate (TALP), modified Tris-buffered medium (mTBM), modified tissue culture medium 199 (mTCM-199) and porcine gamete medium (PGM) (reviewed by [4]). mTBM is the only medium without bicarbonate [40]. Except for TCM-199, the stock

media are not available commercially, so they are further modified in-house in different laboratories. Culture media share a common formulation containing various components including inorganic salts, nutrients, vitamins and growth factors [41], and they are supplemented for gamete coculture. The number and type of molecules added to these media are wide and varied. Defined media are those in which all of the chemical components are known, whereas in the semi-defined media there are one or more chemically undefined natural substances and in the undefined media the major components are mixtures of natural substances. Since the ideal concentration of each molecule necessary at the time of fertilization is unknown, in the laboratories the decision on choosing one medium over another is based on the type of semen employed (epididymal or ejaculated; fresh or frozen-thawed), the sperm capacitation method, the desired IVF culture medium (defined, semi-defined or undefined), the IVF device (4-well dishes, climbing-over-a-wall, microfluidic devices), the external conditions of gamete coculture (time, temperature and gas atmosphere), the volume of culture medium for gamete coculture (microdroplet or well), and the final objective of the experiment.

The birth of pigs has been reported from zygotes produced in different culture media such as TCM-199 [42], TALP [43, 44], TBM [45-47] and PGM [48]. Considering that live piglets have been obtained from different IVF media and systems, it is necessary to focus on the specific additives that markedly increase IVF efficiency in pigs. A thorough review of the different molecules that have been used during gamete coculture in different laboratories offers some clear conclusions. As single molecules, sildenafil, relaxin, casein phosphopeptides and methylcellulose are the additives with best results. However, the highest improvement is achieved when the IVF medium is supplemented

with 1% OF. In the future, the exosomes and microvesicles contained in the OF [49] could be explored as new additives. Detailed information about the increased efficiency achieved with the different additives, fertilization media and sperm selection methods, is presented in Table 1. Considering that live piglets have been obtained from so many different IVF media and systems, it is necessary to focus on in vivo parameters such as fertility rate, number of piglets born, growing parameters, health status and productivity of animals born.

3.2 Fertilization devices and environmental conditions

Supplementing the fertilization medium with a single molecule is not the catch-all solution to improving the efficiency of IVF in pigs. A better approach is to turn to more physiologic options for supporting the maturation, fertilization and development of the early embryos. This will enable improved production of zygotes and embryos that will contribute to healthier and more-productive livestock [2].

The recreation of the oviductal environment is currently possible through the use of 3D culture systems. In 2000, Funahashi and Nagai [50] introduced the climbing-over-a-wall (COW) method by inseminating oocytes in the inner cap of a Falcon tube whereas the sperm were deposited in the outer ditch of the chamber. With this simple, ingenious, and cost-effective method, the efficiency increased from 10.2 % (standard method: 50-µl droplet covered by oil) to 24.2 % monospermic fertilization using the COW method. Since during the periovulatory phase, the pH in the porcine oviductal ampulla is close to 8.0 [51], Soriano et al. [52] improved the COW system by conditioning the medium with the natural secretions contained within the oviduct at the time of fertilization

(cumulus-oocyte secretions, porcine OF and follicular fluid) together with a pH=8. This new IVF system reduced polyspermy and increased the final efficiency compared to traditional insemination systems in 4-well dishes, a medium lacking natural secretions and at pH=7.4.

The 3D systems have evolved very quickly and more sophisticated methods have emerged, such as the use of tissue-engineering which phenocopy complex native organs, instead of conventional reductionist culture systems [53]. In the near future we will undoubtedly witness the development of artificial reproductive organs as new tools to be used in the IVF laboratory. Thus, the recreation of a 3D environment in which sperm and oocytes meet, built on a scaffold where the cellular interactions are maintained and supported by a multifunctional microdevice including a microfluidic system that allows the continuous or pulsatile perfusion of reproductive fluids, will likely support monospermic pig zygotes with full developmental ability and correct epigenetic marks. This is already a partial reality for the bovine species, where a culture system has been developed for the formation of an in vivo-like oviduct tissue substitute from primary oviduct epithelial cells [54]. While its efficiency in supporting blastocyst formation was lower than optimized, there is a wide margin for improvement in conventional in vitro embryo production procedures, for instance by including natural reproductive fluids in the system. Similarly, a 3D oviduct-on-a-chip model has been described in this species and, according to the authors, it supports in vivo-like zygote genetic reprogramming [39]. This is a promising approach that would need to be confirmed for pigs in the future.

The use of new additives and devices for pig IVF must be accompanied by a review of the environmental conditions in which both gametes are cocultured, with regard to pH, as well as temperature and gas atmosphere, since both parameters are easily adjustable in the incubator. The most widespread coculture conditions for porcine gametes are 38.5 to 39 ºC under 5% CO_2, 20% O_2 and 75% N_2. In vivo, the average temperature in the oviductal ampulla ranges from 37.8 ºC (in mated pigs) to 38.2 ºC (in unmated pigs). The isthmus is 0.4 ºC to 0.7 ºC cooler than the ampulla, but only by 0.1 ºC in animals that have recently ovulated [55]. The in vitro coculture of gametes is routinely performed at temperatures about 1º C higher than physiological ones. This may have an impact on capacitation and heat stress in sperm cells. Such fine adjustments of temperature during IVF in pigs warrants additional research.

As for gases, in vivo measurements of O_2 tension using minimally invasive methods within the oviduct and uterus of animals at different stages of the estrous cycle revealed higher O_2 in gilts compared to sows (10.0% *vs.* 7.6%) [20]. When the physiological O_2 tension in the female reproductive tract was mimicked in the laboratory, and gametes and embryos were cultured under 5% CO_2, 7% O_2 and 88% N_2, the final efficiency increased compared to traditional conditions (38.5 °C, 5% CO_2, 20% O_2 and 75% N_2) as did embryo development and quality [20].

These results lead us to conclude that a successful porcine IVF system (Figure 1) should start by inducing the prefertilization ZP hardening by incubating oocytes in OF and selecting motile sperm by a method that does not involve centrifugation, but includes a viscous medium. Gametes should be cocultured in a 3D physical support, under low oxygen tension (~7% O_2) and a temperature ranging from ~37.0 ºC to 38.0 ºC. The

281 medium should be viscous and slightly basic (pH around 7.8 to 8) and supplemented
282 with oviductal secretions.
283
284 **4. After insemination**
285
286 *4.1 What parameters do we need to assess?*
287
288 Final efficiency and embryo quality are critical performance indicators of an IVF
289 system. However, in contrast to other species such as humans (Istanbul [56] and Vienna
290 [57] consensus) and bovine, in pigs there is no consensus about the parameters that
291 should be assayed to determine IVF success and embryo quality [58, 59]. One factor
292 that hinders evaluation is the high lipid content in porcine oocytes/embryos relative to
293 their mouse and human counterparts. This dark appearance makes it difficult to evaluate
294 morphological parameters, which are easily assessed in other species. As a result,
295 invasive techniques, that require fixing and staining, although not desirable, are
296 acceptable to assess the IVF output and developmental potential of the
297 preimplantational pig embryo. In addition, some of the latest non-invasive techniques,
298 such as morphokinetic analysis or metabolic measurements, could also be applied.
299 However, the lack of standardized parameters for pig IVF assessment and a systematic
300 embryo quality classification, makes it difficult to compare and extrapolate conclusions
301 across studies given the diversity of parameters used to assess porcine IVF and embryo
302 production (Supplementary Table 1). Monospermy rate is a commonly evaluated and
303 reported metric but, as shown in Table 1, an increase in this parameter does not
304 necessarily imply improved efficiency, a higher blastocyst rate, or healthy offspring.
305

4.1.1 Morphological embryo evaluation in swine is a post-IVF parameter that should be considered to assess the efficiency of the system, assuming that embryo culture conditions (out of the scope of this review) such as temperature, oxygen concentration, culture media and additives will affect final early embryo development. Embryo development in pigs has some peculiarities that need to be considered. Firstly, cleavage rate is not a good predictor of blastocyst formation because polyspermy occurs frequently, and polyspermic embryos can reach the blastocyst stage [60]. Secondly, the blastocyst rate is not a fully accurate measure of embryo viability since mixoploid embryos from polyspermic zygotes can develop to term [60]. Pronucleus formation is another marker that can be assessed, based on the desirability of two pronuclei at zygote stage. In addition to their number, the location, size, and distribution of pronuclei are also indicative of further zygote development [60]. In fact, an increase in the distance between the two pronuclei has been related to a failure of alignment of parental spindles and the appearance of binucleated blastomeres [61]. The number and distribution of nucleoli within the pronuclei are also useful indicators of developmental potential in human zygotes [56]. In pigs, enucleolated oocytes do not reach the blastocyst stage whereas nucleolar injection rescues development [62]. Impaired nucleologenesis has been associated with ART in pigs, in contrast to cows where there is no apparent impact [63]. Nucleoli are not visible under light microscopy in porcine embryos, and labeling or centrifugation is required [64, 65]. In the latter case, lipids can be displaced to one pole of the blastomeres helping in visualization of pronuclei. However, this procedure promotes nucleolar fusion within the same pronucleus, thus impairing the evaluation of the number and distribution of nucleoli [66]. Optical coherence microscopy (OCM) is a promising alternative for high-resolution intracellular time-lapse imaging, which circumvents the requirement for labeling and fluorescence photodamage [67]. The size

and distribution of pronuclei and nucleoli are visible in scans, so this system may be a useful tool for embryo selection although further studies are necessary to verify its effectiveness in predicting embryo development to term.

4.1.2 Number of blastomeres and Inner Cell Mass (ICM)/Trophoectoderm (TE) ratio. In vitro derived blastocysts show a reduced number of cells per blastocyst compared with in vivo ones, suggesting delayed development and compromised developmental potential. Nonetheless, total cell number is not the ideal predictor of pregnancy potential, at least under some circumstances [68, 69]. The ICM/TE ratio has been used as a predictive marker of embryo quality under the premise that a minimum number of ICM cells, from which the embryo develops, is required to achieve a successful pregnancy after embryo transfer [70]. There are differences in the ICM/TE ratio among studies, but in vivo derived embryos show a tendency to have a higher number of ICM cells than in vitro derived ones (Supplementary Table 2). Moreover, even some contradictory data exist in humans, where preferential allocation of cells to the TE has been proposed as indicative of chromosomal abnormalities [71], thus reinforcing the idea that a low ICM/TE rate would indicate low blastocyst quality.

4.1.3 Transcriptomic and epigenetic signature. Transcriptomic and epigenetic profiling techniques are useful to reveal differences between in vivo and in vitro embryos which show a reduced ability to develop to term. Discovering the transcripts and epigenetic marks responsible for this decreased competence, and designing culture conditions that recapitulate in vivo profiles, is the aim of many studies in the porcine species [32, 69, 72-74]. Classically, gene expression of a few apoptotic, pluripotency, and cell cycle regulation factors has been used as an embryo quality marker. However, imprinted

genes, reprogramming factors, DNA repair and other genes are also critical for embryo development. Whole genome analysis and emergent metabolomic analysis have improved the understanding of in vitro porcine embryogenesis, but there are no conclusive data as yet to establish a standardized panel of quality markers.

4.1.4 Morphokinetic analysis. Time-lapse monitoring allows continuous observation of developmental progress, without disturbing culture conditions [75], and its use has risen in humans during the last two decades. This technology has not yet been established in livestock species [76], but it could be useful to assess embryo quality in a rapid and automated manner. The procedure cannot be applied in the same way in porcine species as it has in humans, due to the high lipid content of pig zygotes and embryos, several parameters could be evaluated to predict the formation of blastocysts, such as the development kinetics and cleavage pattern [77, 78].

5. Conclusions

Currently there is no ideal system of porcine IVF. Figure 1 summarizes the global changes that must be faced in the coming years in order to find a consensus on the way to evaluate the improvements that are introduced. There is a wide margin for improvement of ART in the porcine species, and it can certainly be achieved if we approach the problems from a global perspective, instead of focusing on a single stage of each process.

Competing Interests

The authors declare that there are no conflicts of interest.

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780 Figure 1. Proposal of a porcine in vitro fertilization system in pigs to improve efficiency

781 and parameters to be assessed in future research.

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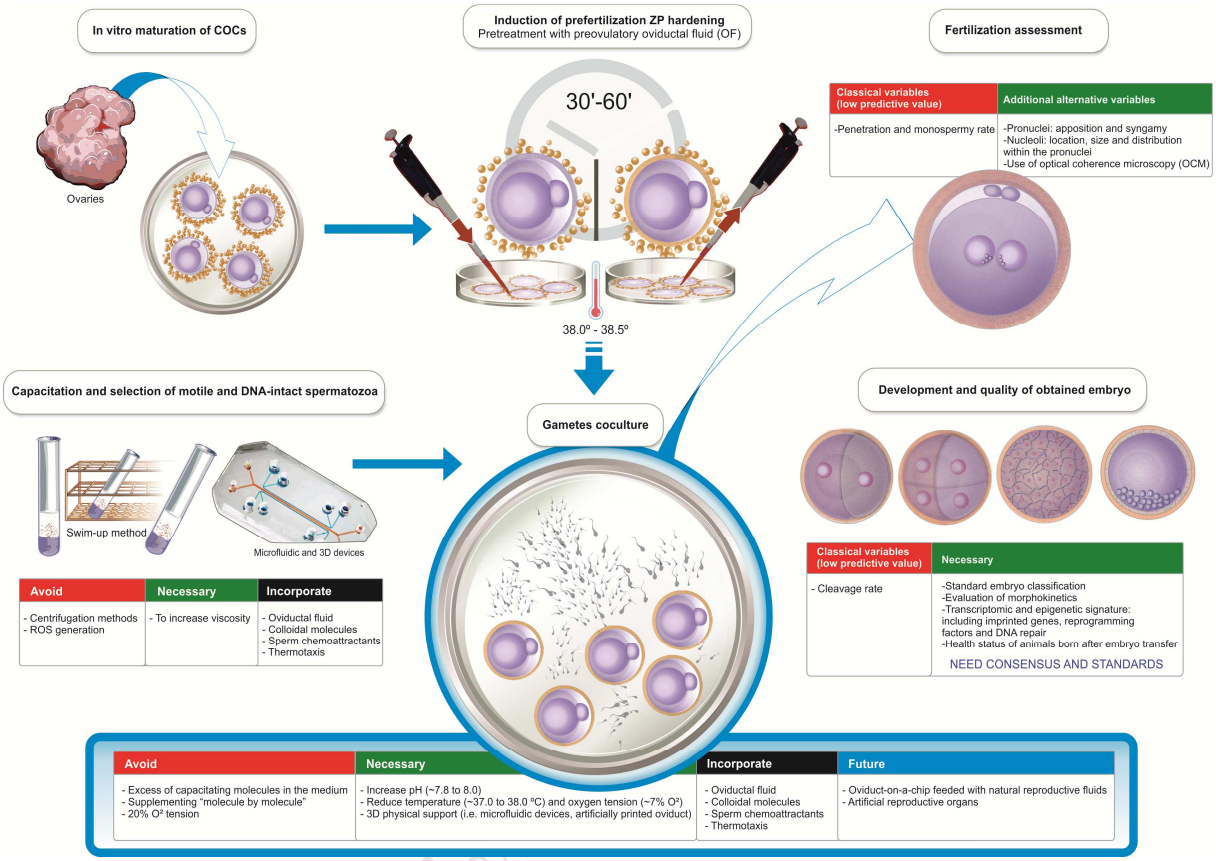

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787 **Table 1**. *In vitro* fertilization (IVF) efficiency after insemination of pig oocytes in medium supplement with (experimental group) or without
788 (control group) different molecule(s). Standard external conditions (Std.) during IVF refer to 39.0 ºC, 5 % CO_2 and 20% O_2. Only the parameter
789 that does not follow the standard is indicated. Efficiency in each study has been calculated (penetration rate x monospermy rate/100) to facilitate
790 the comparison across the different references (Ref.). Efficiency increase and relative increment of efficiency are calculated as indicated in the
791 table. Unless it is indicated (*), all the studies are referred to IVF performed with ZP-enclosed *in vitro* matured porcine cumulus oocytes
792 complexes collected from gilts and denuded before insemination. Some specific details have been omitted to simplify the table content, so
793 information depicted here does not fully reflect each study.
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Ref.	Molecule added to gamete coculture medium (IVF medium)	Type of semen used/ Preparation method	IVF medium employed/ External conditions	Efficiency experimental group (%) (A)	Efficiency control group (%) (B)	Efficiency increase (A-B) (%)	Relative increment of efficiency (A-B/B)*100
	Antibodies						
[79]*	Anti-Izumo (0.5 µg/mL)*	Frozen-thawed epididymal spermatozoa Preincubation in TCM199 (15 min, pH 7.8)	Pig FM-caffeine/Std.	70.0[a]	90.0[a]	-20.0[a]	-22.2[a]
[80]	Anti-porcine ZP4 protein (KLH-peptide) (10% v/v test serum)	Freshly ejaculated semen One wash (800 g, 5 min) in PBS-PVA	TCM199-caffeine-FCS/No mentioned	23.9[a]	75.5[a]	-51.6[a]	-68.3[a]

[81]	UBA1 inhibitor (PYR41) (0.5 µM)	Freshly ejaculated semen Two washes (800 g, 5 min) in PBS-PVA	mTBM-caffeine-BSA/38.5º C	56	42.7	13.3	31.1
	Aminoacids, antioxidants and hormones						
[82]	Monosodium glutamate (1 mM)	Freshly ejaculated semen Two washes (900 g, 2 min) in PBS-BSA	BO-FCS-caffeine/38.0 ºC	39.0	29.2	9.8	33.5
[83]	Epigallocatechin-3-gallate (10 µg/mL)	Freshly ejaculated semen Two washes (734 g, 5 min) in PBS-BSA	BO-FCS-caffeine/7 % O_2	51.2	50.6	0.6	1.19
[84]	*Quercus* extract (QR2E) (10 µg/mL)	Freshly ejaculated semen Two washes (900 g, 2 min) in PBS-BSA	BO-FCS-caffeine/ Std.	9.1	28.0	-18.9	-67.5
[13]	E2+P4 (100 pg/mL E2 and 2.5 ng/mL P4)	Freshly ejaculated semen Percoll gradient 45/90% (740 g, 30 min) followed by wash in TALP (740 g, 10 min)	mTALP-caffeine-BSA/38.5º C	21.12	13.1	8.0	61.2
[85]	Relaxin (20 ng/mL)	Freshly ejaculated semen Two washes (900 g, 5 min) in mTALP followed by 1 h swim-up	mTALP/Std.	15.6	4.5	11.1	246.6
	Glycosaminoglycans						

[17]	Chondroitin sulfate A (100 µg/mL)	Frozen/thawed ejaculated semen Three washes (1000 g, 4 min) in DPBS-BSA	mTBM-caffeine-BSA/Std.	55.2	37.6	17.6	46.8
[86]	Heparin (5 µg/mL)	Frozen/thawed ejaculated semen Three washes (550 g, 5 min) in TCM199B	TCM199B-caffeine/Std.	34.0	45.5	-11.5	-25.2
[87]	Heparin (5 µg/mL)	Freshly ejaculated semen Three washes in 0.9%NaCl solution-BSA followed by 90 min preincubation in mTBM	mTBM-caffeine-BSA/Std.	26.8	15.6	11.2	71.8
[14]	Heparin (10 µg/mL)	Frozen/thawed ejaculated semen Percoll gradient 45/90% (700 g, 30 min) followed by one wash in mTBM (100 g, 10 min)	mTBM-caffeine-BSA/38.8 ºC	15.5	17.5	-2.0	-11.4
[88]	Hyaluronan (0.5 mg/mL)	Frozen/thawed ejaculated semen One wash (600 g, 8 min) in WS-PVA	mTyrodes-BSA-caffeine/Std.	34.3	31.9	2.4	7.5
[89]	Hyaluronan (0.5 mg/mL)	Frozen/thawed ejaculated semen Three washes (1900 g, 3 min) in mDPBS-BSA	mTBM-caffeine-BSA/Std.	41.8	36.6	5.2	14.2
	Glycosidases and lectins						

[90]	α-L-Fucosidase (0.169 IU)	Freshly ejaculated semen Percoll gradient 45/90% (750 g, 30 min) followed by wash in TALP (700 g, 10 min)	mTALP-caffeine-BSA/38.5º C	13.9	20.0	-6.1	-30.5
[91]	Frutalin (0.6 µg/mL)	Freshly ejaculated semen Three washes (1900 g, 3 min) in DPBS-BSA	mTBM-caffeine-BSA/38.5º C	47.1	23.9	23.2	97.1
	Macromolecules to increase viscosity						
[36]	Methylcellulose (1% w/v)	Freshly ejaculated semen Two washes (600 g, 3 min followed by 1200 g, 3 min) in Tyrode's medium	mTALP-caffeine-BSA/38.5º C	71.4	34.1	37.3	109.38
	Other enzymes						
[92]	Apyrase (0.1 mg/mL)	Frozen/thawed ejaculated semen Percoll gradient 60/400% (1500 g, 30 min) followed by two washes in PBS-PVA (700 g, 10 min)	mTALP-caffeine-BSA/38.5º C	21.4	33.9	-12.5	-36.9
[93]	Plasmin (75 µg/mL)	Freshly ejaculated semen Percoll gradient 45/90% (750 g, 30 min) followed by wash in TALP (700 g, 10 min).	mTALP-caffeine-BSA/38.5º C	38.8	0	38.0	100

	Phosphodiesterase inhibitors						
[94]	Adenosine (10 µM)	Frozen/thawed ejaculated semen Three washes (1000 g, 4 min) in DPBS-BSA	mBO/Std.	56.2	40.6	15.6	38.4
[95]	Adenosine (10 µM)	Frozen/thawed ejaculated semen Three washes (1000 g, 4 min) in DPBS-BSA	mBO/38.5º C	54.5	43.0	11.5	26.7
[94]	Caffeine (1 mM)	Frozen/thawed ejaculated semen Three washes (1000 g, 4 min) in DPBS-BSA	mBO/Std.	12.3	40.6	-28.3	-69.7
[95]	Caffeine (1 mM)	Frozen/thawed ejaculated semen Three washes (1000 g, 4 min) in DPBS-BSA	mBO/38.5º C	7.9	43.0	-35.1	-81.6
[6]	Caffeine (5 mM)	Fresh refrigerated ejaculated semen Three washes (750 g, 3 min) in TL-HEPES-PVA followed by 90 min preincubation with 10 µM adenosine	mTCM-199-BSA/Std.	38.7	22.5	16.2	72.0
[96]	Sildenafil	Fresh refrigerated ejaculated semen Three washes (750 g, 3 min) in mTL-HEPES-PVA	Adenosine- and Theophylline-free PGM-tac4/Std.	35.3	7.4	27.9	377.0
[48]	Theophylline (2.5 mM)	Frozen/thawed ejaculated semen	PGM/5% O_2	40.7	21.7	19.0	87.6

		Percoll gradient 45/90% (700 g, 20 min) followed by one wash in PGM (500 g, 5 min)					
	Proteins, peptides and protein inhibitors						
[97]	Casein phosphopeptides (1 mg/mL)	Frozen/thawed epididymal spermatozoa One wash (500 g, 5 min) in DPBS-BSA	BO-10 mM caffeine/Std.	100[a]	46.0[a]	54.0[a]	117.4[a]
[98]	Casein phosphopeptides (1 mg/mL)	Freshly ejaculated semen One wash (500 g, 3 min) in TCM199-BSA	BO-5 mM caffeine/37º C	27.3	25.3	2.0	7.9
[99]	CaMKII inhibitor (KN-93) (50 µM)	Fresh refrigerated ejaculated semen One wash (700 g, 5 min) in mTBM-BSA	mTBM-caffeine-BSA/Std.	25.0[a]	82.8[a]	-57.8[a]	-69.8[a]
[94]	Fertilization Promoting Peptide (pGlu-Glu-ProNH_2) (100 nM)	Frozen/thawed ejaculated semen Three washes (1000 g, 4 min) in DPBS-BSA	mBO/Std.	56.6	40.6	16.0	39.4
[100]	Osteopontin (0.1 µg/mL)	Frozen/thawed ejaculated semen Two washes (1900 g, 4 min) in DPBS-BSA	mTBM-caffeine-BSA/38.5º C	46.7	32.3	14.4	44.6
[101]	Oviductin (purified porcine OVGP1) (10 µg/ml)	Frozen/thawed ejaculated semen Three washes (1900 g, 4 min) in DPBS-BSA	mTBM-caffeine-BSA/Std.	44.6	28.8	15.8	54.9

[93]	Plasminogen (75 µg/mL)	Freshly ejaculated semen Percoll gradient 45/90% (750 g, 30 min) followed by wash in TALP (700 g, 10 min).	mTALP-caffeine-BSA/38.5º C	28.9	18.0	10.9	60.6
[102]	PSP-I/PSP-II heterodimer (1.5 mg/mL)	Frozen/thawed ejaculated semen Two (400 g, 4 min) in DPBS-PVA	mTBM-caffeine-BSA/Std.	46.0[a]	98.0[a]	-52.0[a]	-53.1[a]
	Reproductive biofluids						
[103]	Pure pOF (1% v/v)	Freshly ejaculated semen Three washes in 0.9%NaCl solution-BSA followed by 90 min preincubation in mTBM	mTBM-caffeine-BSA/Std.	18.2	15.6	2.6	16.7
[13]	Pure pOF (1% v/v)	Freshly ejaculated semen Percoll gradient 45/90% (740 g, 30 min) followed by wash in TALP (740 g, 10 min)	mTALP-caffeine-BSA/38.5º C	29.6	3.2	26.4	825.0
[32]	Pure pOF (1% v/v)	Fresh refrigerated ejaculated semen Swim-up in NaturARTs PIG sperm swim-up	mTALP-caffeine-BSA/38.5º C	52.3	31.7	20.6	65.0
[104]	Porcine seminal plasma (10% v/v)	Frozen/thawed ejaculated semen	Pig FM-caffeine/Std.	0	8.4	-8.4	-100.0

		One wash (600 g, 8 min) in WS-PVA					
[104]	Porcine seminal plasma (10% v/v)	Fresh refrigerated ejaculated semen One wash (600 g, 8 min) in WS-PVA	Pig FM-caffeine/Std.	0	36.7	-36.7	-100.0

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796 CaMKII (calmodulin-dependent protein kinase II); DPBS (Dulbecco's phosphate buffer saline); E2 (oestradiol), FCS (fetal calf serum); mBO
797 (modified Brackett and Oliphant solution); mTBM (modified trish buffered medium); P4 (progesterone); PGM (porcine gamete medium); Pig-
798 FM (pig fertilization medium); pOF (porcine oviductal fluid); PSP (seminal plasma spermadhesins); PVA (polyvinyl alcohol); PZM (porcine
799 zygote medium); UBA1 (ubiquitin-activating enzyme); WS-PVA (saline solution supplemented with PVA).

800 [a]The study does not show monospermy rate, so efficiency was not calculated and only penetration rates are shown for each group.

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Supplementary Table 1. Fertilization and embryo quality parameters assessed after pig *in vitro* fertilization (IVF). hpi (hours postinsemination); ICM/TE (rate inner cell mass/trophectoderm in blastocysts); MPN (male pronucleus); ROS (reactive oxygen species); Spz/oocyte (mean number of spermatozoa per penetrated oocyte); Spz/ZP (spermatozoa bound to zona pellucida). Grey columns indicate the most frequently studied parameters.

	Variables assessed after IVF					**Variables assessed for EMBRYO QUALITY**																
Reference	**Cleavage rate**	**Penetration rate**	**Spz/oocyte**	**Monospermy and polyspermy rates**	**MPN formation**	**Spz/ZP**	**Morphokinetic parameters**	**Blastocyst formation rate**	**Blastocyst grade**	**Blastocyst hatching**	**Total number cells/blastocyst**	**ICM/TE ratio**	**Apoptosis**	**Embryo fragmentation**	**Blastocyst diameter**	**Gene expression**	**Epigenetic profile**	**Pregnancy after embryo transfer**	**Mitochondrial activity**	**ROS level**	**Nucleolar morphology**	**Metabolism**
[42]	X	X	X	X				X		X								X				
[47]	X	X	X	X	X			X			X							X				
[45]	X	X		X				X			X							X				
[105]	X	X		X				X			X					X					X	
[63]	X			X				X													X	
[89]		X	X	X																		
[77]	X						X	X								X						
[106]		X		X																		

[64]	X	X		X				X			X							X					
[14]	X	X	X	X		X		X			X												
[69]								X			X	X	X			X			X				
[107]	X	X						X			X	X	X										
[91]		X	X	X																			
[108]								X			X							X					
[20]	X	X	X	X		X		X		X	X												
[36]		X	X	X	X	X																	
[109]	X	X	X	X				X	X		X					X							
[12]		X	X	X		X																	
[110]	X	X	X	X	X													X					
[111]	X	X		X				X		X	X	X								X			
[112]	X	X		X				X			X												
[73]	X							X			X		X					X	X			X	
[32]	X	X	X	X		X		X		X	X					X	X						
[113]	X	*In vivo* collection 24hpi							X		X	X	X		X				X	X			
[114]		Electrical activation						X		X	X	X		X	X	X							

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Supplementary Table 2. Total cell number and Inner Cell Mass (ICM)/Trophectoderm (TE) ratio in porcine blastocysts derived in vivo and in vitro. The protocol used to stain the embryos is indicated. IVC (in vitro culture); IVF (in vitro fertilization); IVM (in vitro maturation); KSOM (potassium simplex optimized medium); NCSU (North Carolina State University); PZM (porcine zygote medium); MU2 (Missouri University medium 2 consisting on PZM3 supplemented with 1.69 mM arginine and 5μM PS48); AA (aminoacids); PVA (Polyvinyl alcohol); BSA (Bovine Serum Albumin); PI (propidium iodide); ND (not determined); NA (not applicable).

Reference	**Age blastocyst (days)**	**Origin of embryo**	**Total cell number**	**ICM:TE ratio**	**Culture media, main additives, and external conditions (gas concentration*, temperature)**	**Methods used to stain TE**
[115]	5-8 d	*In vivo*	ND	0.22 (early blastocyst)	N.A.	Anti-pig serum+PI
			ND	0.25 (expand blastocyst)	N.A.	
			ND	0.17 (hatched blastocyst)	N.A.	
[70]	4-5 d	*In vivo*	47	0.33 (early blastocyst)	N.A.	Anti-pig serum+PI
			75	0.28 (blastocyst)	N.A.	
[116]	6 d	*In vivo*	57	0.5	N.A.	Anti-pig serum+PI
		1 or 2 cell collected *in vivo* followed by IVC	30.5	0.22	NCSU-23, BSA, 20% O_2, 39ºC	
			21.4	0.18	KSOM, AA, 20% O_2, 39ºC	
[117]	8 d	1 or 2cell collected *in vivo* followed by IVC	54.9	0.40	NCSU-23, BSA, 20% O_2, 38.7ºC	Anti-pig serum+PI
			52.1	0.47	NCSU-23, BSA, 5% O2, 38.7ºC	
			77.9	0.47	PZM-3, BSA, 20% O2, 38.7ºC	
			92.4	0.58	PZM-3, BSA, 5% O_2, 38.7ºC	
	6 d	*In vivo*	57.6	0.45	N.A.	

		IVM/IVF/IVC	23.2	0.14	NCSU-23, BSA, 20% O_2, 38.7ºC	Anti-pig serum+PI
			41.1	0.40	PZM-3, BSA, 5% O_2, 38.7ºC	
			38.4	0.40	PZM-4, PVA, 5% O_2, 38.7ºC	
[118]	6 d	*In vivo*	120	0.41	N.A.	Triton+PI
[113]	6-7 d	1 cell collected *in vivo* followed by IVC	30	0.23 (expand blastocyst)	NCSU-23, BSA, 20% O_2, 39.0ºC	DAPI**
[114]	6 d	IVM/electrical activation/IVC	22-37	0.20-0.22	PZM-3, BSA, cytochalasin, 20% O_2, 38.5ºC	Anti-pig serum+PI
	7 d			0.18-0.21		
	8 d			0.14-0.17		
[69]	6 d	IVM/IVF/IVC	65	0.41	PZM-3, BSA, arginine-glycine; 5% O_2, 38.5ºC	Anti-pig serum+PI
[107]	6 d	IVM/IVF/IVC	30	0.32-0.36	PZM-3, PVA, 20% O_2, 38.5ºC	Anti-pig serum+PI
[32]	7.5 d	*In vivo*	87	N.D.	N.A.	N.D.
		IVM/IVF/IVC	82	N.D.	NCSU-23, oviductal and uterine fluids, 20% O_2, 38.5ºC	
		IVM/IVF/	50	N.D.	NCSU-23, BSA, 20% O_2, 38.5ºC	
[73]	6 d	IVM/IVF/IVC	45	N.D.	MU2, 20% O_2, 38.5ºC	N.D.

*In all studies CO_2 concentration was set at 5%.

**ICM is not specifically stained but ICM cell number is calculated based on ICM location.

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