A new approach to sperm selection for *in vitro* fertilization in swine

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Introduction

Assisted reproductive technologies (ARTs) in swine are beginning to be highly relevant for commercial and biomedical purposes. Pig is appropriate as an experimental model due to its proximity to the human species in their anatomical, physiological and biochemical characteristics, representing an alternative to the use of other laboratory animals for the development of technologies such as xenotransplantation, transgenesis and cloning [1].

For producing embryos *in vitro*, three steps are required: *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and culture of zygotes until the desired stage before cryopreservation or transference [2]. While IVM protocols reach high percentages of success (above 90% of oocytes mature), IVF and embryo culture represent two technological challenges still unsolved. Specifically, efficiency of porcine IVF is low due to a high incidence of polyspermy [3], which results in the production of non-viable embryos. A possible cause of polyspermy *in vitro* is the absence of the role played by the female reproductive tract in reducing the number of sperm that arrive to the surface of the oocyte [4]. It is well known the role executed by epithelial cells of the uterotubal junction on arrival of sperm to the fertilization place [5] as well as by the oviductal fluid on the zona pellucida resistance to sperm penetration [6].

Certain authors suggest that an approach to physiological conditions could be successful in *in vitro* techniques [1]. Many studies have incorporated physiological elements such as oviductal cells [7] or oviductal fluid [6] to reduce polyspermy during IVF techniques; but in none of them it has been used a sperm selection method closer to *in vivo* conditions despite several studies show how the way of preparing the sperm can affect the health of progeny [8].

The quality of the sperm and the relation with its fertilization capacity is an important issue in animal production [9]. Contrary to oocytes that are more difficult to obtain, in an ejaculated or seminal doses there are millions of spermatozoa, being easier choosing those with high quality, thus avoiding abnormal spermatozoa during IVF techniques. In addition, during sperm preparation, it is necessary to remove the seminal plasma which could be harmful or not to sperm function [10]. Many studies have revealed that higher quality spermatozoa have special characteristics and ability to respond to female tract [11].

An approach of sperm selection methods to *in vivo* conditions for IVF techniques could be able to reduce polyspermy rates and even epigenetic changes that may affect offspring.

The aim of this study was to reduce the rate of polyspermy by adapting the method of sperm migration up or swim up, a sperm selection method widely used in other species such as human, to the swine model.

Material and methods

Culture Media, chemicals and reagents, unless otherwise indicated, were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain).
To set up the working conditions, a specific medium for sperm selection was formulated, modifying the one used in human protocols (Human Tubal Fluid Medium (HTFm, www.irvinesci.com). Later on, this medium was used in the designed protocol for swim-up without centrifugation, trying to avoid sperm dysfunction. On the other hand, the results of this method, assessed by the study of IVF, were compared with the results obtained by one of the most used methods in research laboratories for animal reproduction, the Percoll® density gradient technique.

Oocyte Collection and *In Vitro* Maturation
Ovaries from Landrance-Large-White gilts were transported to the laboratory in saline containing 100 µg/ml kanamycin sulfate at 38ºC, washed once in 0.04% cetrimide solution and twice in saline solution within 30 min of slaughter. Cumulus cell-oocyte complexes (COCs) were collected from antral follicles (3 to 6 mm in diameter), washed twice with Dulbecco's PBS supplemented with 1 mg/mL PVA and 0.005 mg/ mL red phenol, and washed twice more in maturation medium previously equilibrated for a minimum of 3h at 38.5ºC under 5% CO_2 in air. The maturation medium was NCSU-37 [3] supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP, 5 mg/mL insulin, 50 mM β-mercaptoethanol, 10 IU/mL equine chorionic gonadotropin (eCG; Folligon; Intervet International BV, Boxmeer, Holland), 10 IU/mL hCG (Veterin Corion; Divasa Farmavic, Barcelona, Spain), and 10% porcine follicular fluid (v/v).
Only COCs with complete and dense cumulus oophorus were used for the experiments. Groups of 50 COCs were cultured in 500 µl NCSU-37 supplemented with dibutyryl cAMP, eCG and hCGfor 22 hThen, oocytes were washed twice in NCSU-37 without dibutyryl cAMP, eCG, and hCG and cultured for an additional 20 to 22 h [12].

Sperm collection and preparation
Ejaculated spermatozoa from proven fertility boars (*Sus crofa domestica*) of 2-24 months old from an artificial insemination centre (CEFUSA, Pliego, Murcia, Spain) were collected by the gloved-hand method and immediately transported to the laboratory [13].
Samples of each ejaculates (500 µl) were pre-treated by centrifugation at 700g for 30 min in a 45-90 % (v/v) Percoll gradient (Pharmacia Uppsala, Sweden) [6]. Then, the resulting pellets were diluted in TALP medium (Tyrode's albumin lactate pyruvate, [14]) and centrifuged again at 700g for 10min. Resulting pellets of spermatozoa were diluted in 250 µl of TALP medium for insemination. For the swim up method, fractions from the same ejaculate samples (1000 µl of semen) were lay below of NaturARTs® PIG sperm swim-up medium (www.embryocloud.com, Patent ES 2532659B1) supplemented with 0,5% of bovine serum albumin (BSA) at the bottom of a conical tube at a 45º angle. After 20 minutes of incubation at 38.5ºC, 750 µl from the top of the tube were aspirated and diluted in 250 µl of TALP medium for insemination.

In vitro fertilization

The medium used for IVF was TALP previously equilibrated at 38.5ºC, plenty humidity and 5% CO2. COCs cultured for a total of 44h in maturation medium were washed three times with TALP medium [14]. Groups of 50 denuded oocytes (cumulus cells removed by pipetting) were preincubated in oviductal fluid of the late follicular phase of the estrous cycle NaturARTs® PIG OF-LF (www.embryocloud.com, patent ES 2532659B1) for 30 to 60 minutes and transferred into each well of a 4-well multidish containing 250 µl TALP medium. After sperm selection by swim up or density gradient methods, the cell concentration was calculated and adjusted at 2 x 10^4 sperm/ml. A 250 µl dilution of each sperm sample was added to the wells containing the oocytes. The gametes remained in coculture for 18 h. Four replicates were performed including the two experimental groups. Then, putative zygotes were washed and gently pipetted to remove excess of spermatozoa weakly attached. Afterwards, they were fixed with 0.5% glutaraldehyde in PBS, stained with 1% Hoechst 33342 in PBS, washed in PBS and mounted on glass slides. Finally, the putative zygotes were examined under an epifluorescence microscope at x400 (Leica® DMR, USA).

Statistical Analysis of IVF data

Data are shown as mean ± SEM and all percentages were modelled according to the binomial model of variables and arcsin transformation to achieve normal distribution. The variables in all the experiments were analyzed by one-way ANOVA. A P value < 0.05 was taken to denote statistical significance.

Results

The results of the two sperm selection methods are shown in Table 1.The swim up method increased the efficiency of IVF technique in about 15% compared to the density gradient procedure. Penetration rates and monospermy rates were different for swim up and gradient-centrifugation methods (Table 1). The swim up method also decreased the mean number of sperm per penetrated oocyte (Spz/oo, 2.13 ± 0.12 vs. 8.42 ± 0.71) and the number of sperm bound to the zona pellucida (Spz/ZP, 7.24 ± 0.52 vs. 17.36 ± 2.33) (Table 1)

Table 1. Values (mean ± SEM) for the studied variables after IVF with two sperm selection methods (Swim-up and Percoll).

Sperm selection method	N	Penetration (%)	Monospermy (%)	Spz/oo	Spz/zp	Efficiency (%)
Swim-up	180	69.6± 3.5a	42.7± 4.6a	2.1 ± 0.1a	7.2 ± 0.5a	29.7± 0.2a
Percoll	105	84.3± 3.6b	17.4± 4.1b	8.4 ± 0.7b	17.3± 2.3b	14.7 ± 0.1b

Different letters (a, b) indicate statistical differences (p < 0.05)

Discussion

The results of this study indicate that the swim up procedure for sperm selection increases the efficiency of IVF techniques compared with conventional methods in swine. While the Percoll® density gradient involves centrifugation and therefore loss of some membrane glycoproteins from seminal plasma [15], swim up does not involve this physical process. It

was demonstrated that glycoproteins covering the sperm membrane are masking other proteins involved in fertilization [16], so that, with this last technique, these proteins could be placed in the sperm membrane for longer, avoiding a precipitous capacitation.

Despite swim up could be capacitating the sperm due to the presence of bicarbonate, albumin and calcium in the medium, the differences in the IVF data indicate that this capacitation should be lower than with density gradient methods, which also include these three components in the selection medium. Therefore, a possible explanation for these results would be that conventional procedures induce capacitation at a higher rate because proteins involved in sperm-oocyte interaction are exposed after centrifugation and not because of differences in the media composition.

Difference in the membrane status among the subpopulation of sperm selected by each method may be translated in sperm differential capacitation state. For this reason, another explanation for our results could be that, with swim up, sperm would be able to be capacitated in waves, as under *in vivo* conditions, therefore oocyte could deploy its blocking system efficiently reducing the incidence of polyspermic penetration [17]. However, Percoll® density gradient would induce capacitation in most spermatozoa simultaneously and the oocyte could not defend from a massive attack.

Few studies have reported the efficiency of the use of swim up procedure in swine. However, our data, in which a higher IVF efficiency with swim up method is achieved, coincide with those obtained by Park et al. (2009) [18]

Consequently, the data obtained in this study and those reported by Park et al. (2009) with the swim up method demonstrated that this methodology is highly recommended for its use in IVF techniques in swine.

In conclusion, the efficiency for the production of embryos *in vitro* with this new sperm selection method is higher than with density gradient technique due to the reduction of the polyspermy rates. Therefore, this method can be pointed out as an alternative to the use of conventional methods involving centrifugation.

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