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Pre-incubation of porcine semen reduces the incidence of polyspermy on embryos derived from low quality oocytes

Pré-incubação dos espermatozoides suínos diminui polispermia e aumenta a produção embrionária em oócitos de baixa qualidade

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ABSTRACT

The main cause of low efficiency of in vitro *produced porcine embryos is the high polyspermic penetration rates at fertilization, which is aggravated in low quality oocytes. Experiment 1 evaluated the embryo development in high and low quality oocytes. Experiment 2 evaluated the embryo development and quality of low quality oocytes fertilized with sperm pre-incubated during 0h (control), 0.5h, 1h and 1.5h. Experiment 3 investigated fertilization and monospermic rates of the same groups of Experiment 2. Experiment 4 evaluated embryo development, cell density, fertilization and monospermic rates of high quality oocytes using semen pre incubated during the best time observed in the previous experiments. Cleavage and blastocyst rates were analyzed by chi-square test, and remaining data by ANOVA and Tukey test (P≤0.05). The cleavage (74.8 vs 51.7%) and blastocyst (33.7 vs 9.8%) rates were greater in oocytes of high versus low quality, with no differences in cell density. Fertilization rates (65.6 to 79.5%) were not influenced by pre-incubation time. However, semen pre-incubation during 1.5h increased monospermic penetration (53.3%) and cleavage rates (92.5%) in low quality oocytes. Blastocyst rate was improved with 1.5h of semen pre incubation; however they were still lower than that observed with high quality control oocytes. Ultimately, pre-incubation did not influence fertilization, monospermic penetration, embryo development rates, nor cell density in oocytes of high quality. Low-quality porcine oocytes resulted in better rates of embryo development if* in vitro *fertilized with sperm pre-incubated for 1.5 hour.*

Key words: *pig IVF, embryo IVP, monospermy, fertilization, oocyte.*

RESUMO

A principal causa da baixa eficiência na PIV de embriões suínos é a elevada taxa de polispermia, que é exacerbada em oócitos de baixa qualidade. O experimento 1 avaliou o desenvolvimento embrionário de oócitos de baixa e alta qualidade. O experimento 2 avaliou a qualidade e o desenvolvimento embrionário de oócitos de baixa qualidade fecundados com sêmen pré-incubado por 0h (controle), 0,5h, 1h e 1,5h. O experimento 3 investigou a fecundação e as taxas de monospermia dos mesmos grupos do experimento 2. O experimento 4 avaliou o desenvolvimento embrionário, a densidade celular, a fecundação e as taxas de monospermia de oócitos de alta qualidade, fecundados com sêmen pre-incubado com o melhor tempo observado nos experimentos anteriores. As taxas de clivagem e de blastocistos foram submetidas ao teste de Qui-quadrado e os demais dados submetidos à ANOVA e teste de Tukey (P≤0,05). As taxas de clivagem (74,8 vs 51,7%) e de blastocistos (33,7 vs 9,8%) foram superiores nos oócitos de alta qualidade, comparados aos de baixa qualidade, não havendo diferenças na quantidade de células embrionárias. As taxas de fecundação (65,6 vs 79,5%) não foram influenciadas pelo tempo de pré-incubação. Todavia, a pré-incubação do sêmen por 1,5h aumentou a penetração monospérmica (53,3%) e a taxa de clivagem (92,5%), nos oócitos de baixa qualidade. As taxas de blastocisto aumentaram com sêmen pré-incubado por 1,5h, que foram ainda inferiores às obtidas dos oócitos de alta qualidade do grupo controle. Finalmente, a pré-incubação do sêmen não influencia na fecundação, na penetração monospérmica, no desenvolvimento embrionário, nem na quantidade de células embrionárias com oócitos de alta qualidade. Oócitos suínos de baixa qualidade produzem melhores taxas de desenvolvimento embrionário se fecundados in vitro *com sêmen pré-incubado por 1,5 horas.*

Palavras-chave: *FIV suínos, embrião PIV, monospermia, fecundação, oócito.*

INTRODUCTION

The embryo IVP technique can be employed on different segments of assisted reproduction in humans and animals (DYCK et al., 2014), allowing a better understanding of

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maturation, fertilization, sperm capacitation, embryo development and their regulatory mechanisms (COY & ROMAR, 2002). Since the first embryos obtained from *in vitro* fertilization (IVF) in pigs (MATTIOLI et al., 1989), the process experienced a great evolution. However, the porcine *in vitro* embryo development is still lower than the *in vivo* (KWAK et al., 2014). One of the determining factors for the low efficiency of porcine embryo IVP is the elevated rate of polyspermy (TOKESHI et al., 2007; FAUSTINI et al., 2010). Great variations have been observed in polyspermy rates of both fresh (SIRARD et al., 1993) and frozen semen (NAGAI et al., 1988). Polyspermy rates are directly related to the sperm number at the site of fertilization (FAUSTINI et al., 2010), and also with the oocyte quality (SUZUKI et al., 2003). It is believed that the oviduct microenvironment participates in polyspermic blocking during *in vivo* fertilization (LUÑO et al., 2013). The *in vivo* incidence of polyspermy is less than 10% (GIOIA et al., 2005). However, *in vitro* matured oocytes are known to show a lower cortical reaction, and an erroneous distribution of the granules around the oocyte. This failure of the IVC systems results in a poor release of cortical granules after sperm penetration, thus resulting in failure of the zona pellucida reaction, and consequently in polyspermy (FUNAHASHI et al., 2000). These ratios are even greater in low quality oocytes (SUZUKI et al., 2003). In porcine IVF procedures performed with slaughterhouse ovaries it is possible to obtain a great number of oocytes. However, after classification, approximately 10% of these oocytes are classified as of excellent quality (data not shown), demanding a great number of ovaries, especially when using adult gilts. Therefore, in assisted reproductive procedures (i.e. embryo IVP) where the goal is to preserve endangered pig breeds like Moura, Caruncho, Piau and others, the use of exclusively high/excellent quality oocytes might not be possible. Moreover, in procedures such as oocyte cryopreservation, the standard protocols per se negatively affect the gamete quality. Ultimately, the search for strategies that improve the number of blastocysts obtained out from a pool of low quality oocytes is very important.

MATERIALS AND METHODS

Experimental design

The first experiment was performed in order to assess embryo production from low versus high quality oocytes produced by a standard porcine IVF protocol. In experiment 1, the oocytes of high quality (n=163) and low quality grade (n=143) were matured, fertilized and cultured under identical conditions in order to assess the rates of embryonic development (5 replications). Aiming to improve the IVF efficiency using low quality oocytes, experiment 2 (n=1454) assessed cleavage and blastocyst rates, as well as cell density. Oocytes were *in vitro* matured and fertilized with sperm previously incubated during different periods: 0h (control), 0.5h, 1h and 1.5h. These results were compared to a positive control using oocytes of high quality (7 replications). In order to better understand the results obtained in the second experiment, the experiment 3 (n=619) aimed to assess fertilization and monospermy rates after IVF of low quality oocytes. To that, oocytes were *in vitro* matured and fertilized with sperm pre-incubated for different periods: 0h (control), 0.5h, 1.5h and 1h, being these results compared to a positive control of high quality oocytes (5 replications). The fourth experiment aimed to assess whether semen pre-incubation would improve the blastocyst rates for high quality oocytes. To that, experiment 4 evaluated the use of semen pre incubation for 1.5h (pointed in experiment 2) in oocytes of high quality. The embryo development, cell density, fertilization and monospermy rates (5 replications) were assessed.

Chemicals and reagents

Except where indicated, all chemicals used came from Sigma Chemical Co. (St. Louis, MO, USA).

Oocytes search and selection

Ovaries of peri-pubertal gilts obtained at abattoirs were kept in saline solution at 30 to 35°C, and transported to the laboratory within 5 hours. The cumulus oocyte complexes (COCs) were aspirated from 3 to 6mm diameter follicles, with the aid of a vacuum line at a rate of 15mL of water flow per minute, and stored in 15mL conic tubes. After 15 minutes, the sediment from each tube was deposited into a 90mm Petri dish containing centrifuged follicular fluid, to search the CCOs, under stereomicroscope. The COCs were classified as high or low quality, as follows: High quality CCOs had at least three layers of compact cumulus cells and homogeneous cytoplasm, whereas the low quality COCs were characterized by gaps in coverage of cumulus cells, as well as an heterogeneous cytoplasm due to the presence of vesicles or granules.

Oocyte maturation

All steps of embryo *in vitro* production were performed in NUNC 4-well dishes (Nunclon, Roskilde, Denmark). For maturation, 40 to 50 COCs were placed into wells containing 400µL of TCM-199 medium supplemented with 26.19mM Sodium Bicarbonate, 25% gilts Follicular Fluid, 0.1mg mL^{-1} L-cysteine, 10ng mL^{-1} Epidermal Growth Factor, 2.19mM Sodium Pyruvate, 3.05mM D-glucose, 0.5mg mL^{-1} Luteinizing Hormone (LH) (Lutropin, Bioniche), 0.01UI mL^{-1} Follicle Stimulating Hormone (FSH) (Folltropin, Bioniche), 1mM dibutirilAMPc (dbcAMP), 100IU mL^{-1} Penicillin G and 0.1mg mL^{-1} Streptomycin Sulfate. The COCs remained in this medium for 22 hours at 38.8°C, in an atmosphere of 5% CO_2 and saturated humidity. Afterwards, oocytes were transferred to a second maturation medium, similar to the above but without LH, FSH, and dbcAMP, where they remained for another 18 to 22 hours under the same culture conditions.

Sperm Collection and Selection

Freshly ejaculated semen was obtained from a boar previously tested for IVF, and proven to produce IVF embryos in a regular rate. The ejaculate was mixed with BTS (Beltsville Thawing Solution) extender containing 41.5g mL^{-1} D-glucose, 5.9g mL^{-1} Sodium Citrate (Merck), 0.5g mL^{-1} Sodium Bicarbonate, 0.8g mL^{-1} EDTA, 0.3g mL^{-1} Potassium Chloride and 1mg mL^{-1} Gentamicin Sulfate. Semen was diluted 1:3 in BTS and maintained in a temperature of 15 to 17°C, being gently mixed every six hours, until IVF. Each ejaculate was obtained and processed for IVF within maximum 18 hours after collection. Prior to use, semen was incubated at 38.8°C for 10 minutes and the sperm selection for IVF was then performed by centrifuging (13.100 x g / two minutes) 150µL of semen through discontinuous Percoll gradient (45 and 90%, respectively). The selected sperm cells were then mixed in 1000µL of m-TBM consisting of 113.1mM Sodium Chloride, 3.0mM Potassium Chloride, 7.5mM Calcium Chloride, 20mM Tris (Merck), 11mM D-glucose, 5mM Sodium Pyruvate, supplemented with 2mg mL^{-1} Bovine Serum Albumin (BSA) and centrifuged again for 30 seconds at 11.400 x g. Semen was then diluted in 5mL m-TBM, and sperm concentration was determined with the aid of a Neubauer chamber.

Pre-incubation

Spermatozoa were transferred to 4 well dishes with 400µL of m-TBM supplemented with 0.4mg mL^{-1} caffeine and 2mg mL^{-1} BSA in a concentration of 62,500 spermatozoa mL^{-1}. According to the experimental groups, sperm samples were pre-incubated in the fertilization medium for different periods: 0 hour (control), 0.5, 1 or 1.5h at 38.8°C, under a 5% CO_2 atmosphere and saturated humidity. The semen of all groups came from the same ejaculate.

In vitro fertilization (IVF)

The matured COCs were mechanically denuded in TCM-199 added with 2.19mM Sodium Pyruvate, 100IU mL^{-1} Penicillin G, 0.1mg mL^{-1} Streptomycin Sulfate, and 20% Fetal Bovine Serum (FBS) (Nutricell - SP). Just after, the oocytes were transferred to a dish containing the pre-incubated spermatozoa, remaining for 3 hours in co-incubation.

IVF assessment

After IVF, the sperm adhered to zona pellucida were removed by successive pipetting. Twelve hours after fertilization, approximately one third part of the structures were fixed in absolute Ethanol (Labsynth, Brazil) supplemented with 0.01mg mL^{-1} Bisbenzimide Trihydrochloride H-33342. The fixed and stained structures were placed between slide and cover slip and exposed to UV light for visualization and counting of male and female pronuclei as well as first and second polar bodies. Structures were classified as non-fertilized (absence of sperm and /or male pronucleus); correctly fertilized (presence of two polar bodies and two (male and female) pronuclei); or improperly fertilized (presence of polyspermy, of more than two pronuclei).

In vitro culture

After IVF excessive sperm were removed and presumptive zygotes were washed and cultured in PZM-3 (Porcine Zygote Medium 3**)** (YOSHIOKA et al., 2002) during four days (D0=fertilization) at 38.8°C under an atmosphere of 5% CO_2 and saturated humidity. On fourth day of culture 10% FCS was added to the medium. Cleavage and embryo development rates were assessed at day two (cleavage) and day seven (blastocysts) of culture.

Embryo quality assessment

Blastocysts at day seven of culture were fixed in absolute Ethanol (Labsynth, Brazil) supplemented with 0.01mg mL^{-1} Bisbenzimide Trihydrochloride H-33342 for at least 15min. The fixed and stained structures were placed in a 2µL glycerol drop between slide and cover slip and exposed to UV light for visualization. The stained

nuclei of the embryo cells were counted as an estimative of embryo cell density.

Statistical analysis

Data were analyzed using the JMP software version 5, 2002 (SAS INSTITUTE INC. CARY, NC, USA). Cleavage and blastocyst development were analyzed by the chi-square test. All remaining data, including fertilization and monospermic penetration rates, IVF efficiency and blastocysts total cell number were analyzed by ANOVA and compared through the Tukey test. For fertilization and monospermic penetration rates as well as for and IVF efficiency, data were normalized by the square root of the value divided by 100. Percentages of development to the blastocyst stage were based on the number of oocytes cultured. The fertilization rates and IVF efficiency were based in the total of matured oocytes. Monospermic penetration rates were calculated with basis on the total of fertilized oocytes. Level of significance for all analyses was 5%.

RESULTS

In the experiment 1, oocytes of high quality presented greater cleavage (74.8%) and blastocyst (33.7%) rates ($P<0.05$) than their low quality counterparts (51.7 and 9.8%, respectively). However, no differences were observed in the embryo cell density, with 66.7± 3.7 for the high quality versus (68.5±7.1) for the low quality oocytes.

In the second experiment, among the low-quality oocytes, all pre-incubation periods (0.5h, 1h and 1.5h) resulted in an increase in the cleavage rate, in comparison with the absence of pre-incubation. The greatest cleavage rate (92.5%) among all the groups was observed with 1.5h pre-incubation time (Table 1). Regarding the embryo development to the blastocyst stage, it was observed a significant improvement with a longer (1.5h) incubation period, when low quality oocytes were used. However, it did not reach the rates observed with high quality oocytes in the control group (Table 1).

In the Experiment 3, although the fertilization rate was similar among all groups ($P>0.05$), the rate of monospermy in the group pre incubated for 1.5h was the highest ($P<0.05$) amongst all groups (Table 2). The highest monospermy rate resulted in an increase of IVF efficiency, which was similar to the oocytes with high quality (control group).

In the experiment 4, the pre-incubation of spermatozoa did not affect ($P\geq0.05$) the cleavage (76.1 vs. 75.3%) and blastocyst (30.5 vs. 24.7%) rates for oocytes of high quality, nor influenced the cell density in embryos (66.7±3.5 vs 72.0±3.7). Furthermore, it has also been shown that semen pre-incubation does not affect the fertilization rates (64.9 vs. 75.3%) or monospermic penetration rates (44.3% vs 48.4/n=179) in fertilization of high quality porcine oocytes.

DISCUSSION

Although high polyspermic penetration rates are well documented in pigs (FUNAHASHI 2003; SUZUKI et al., 2003), some interesting interactions of oocyte quality and the sire have been reported in cattle (OHLWEILER et al., 2013), where depending on the technique used to artificially fertilize the oocyte, the embryo development rate was affected or not by the oocyte quality. Unfortunately, oocytes of low quality are predominant in gilt ovaries. In this study, among all oocytes classified for the experiments, the ones of excellent quality were as low as around 10% of the total. This low efficiency was confirmed in the experiment 1 of this study, which resulted in a blastocyst yield of 33.7% with high quality oocytes vs. 9.8% for the ones of low quality. It has been already established that low quality oocytes have poor maturation and deficient cortical reaction at the time of fertilization, which leads to a lower embryo production (SUZUKI et al., 2003). NAGAI et al. (1996) suggested that to develop practical IVM-IVF protocols in pigs it is necessary to stablishe reliable methods to synchronize the fertilizing capacity of sperm and fertility of the oocytes. So, as alternative to obtain an efficient IVP of porcine embryo is to search for protocol adaptations so that good rates of embryo development could be achieved, even when using low quality oocytes. A key factor for the low efficiency of IVP porcine embryos is the high rate of polyspermy (TOKESHI et al., 2007), which is directly associated to the amount of sperm at the site of fertilization (FAUSTINI et al., 2010), and also the oocytes quality (SUZUKI et al., 2003). When several spermatozoa penetrate the oocyte, the DNA de-condensation is difficult, probably due to intense competition for factors involved in the process of pronucleus formation (COY et al., 1993b). Thus, reduction in the polyspermy rates is highly desirable in the porcine IVP. To overcome the obstacle posed by pig oocytes of low quality, we proposed to adjust the sperm preparation according to the oocyte quality grade. Therefore, by using different semen pre-incubation periods prior to

Table 1 - Cleavage and blastocyst rates and cellular density obtained after embryonic *in vitro* production from porcine oocytes of high and low quality grade, without pre-incubation (0h) or with different periods of sperm pre-incubation (Experiment 2).

Group	Total of structures	----------Cleavage----------		----------Blastocyst----------		Cell number
		n	%	n	%	
High 0h	265	210	79.2[b]	67	25.3[a]	66.3 ± 3.5[a]
Low 0h	361	157	43.5[d]	27	7.5[c]	68.5 ± 7.1[a]
Low 0.5h	346	243	70.2[c]	27	7.8[c]	68.8 ± 7.7[a]
Low 1h	386	283	73.3[bc]	30	7.8[c]	58.7 ± 6.9[a]
Low 1.5h	361	334	92.5[a]	54	15.0[b]	71.3 ± 4.9[a]

Different letters in the same column differ statistically (P≤0.05).

IVF, a positive effect was observed. The semen pre-incubated during 1.5h resulted in an increase for cleavage and blastocyst rates. Previous reports had already shown that a reduced number of available adequately selected/pre-capacitated sperm during *in vitro* fertilization results in lower polyspermy rate in pigs (FUNAHASHI & NAGAI 2000). This is probably due to the better recognition of sperm penetration by oocytes, when sperm are properly prepared. We showed an increase in monospermic penetration rates with semen pre incubation during 1.5 hours (experiment 3), without changing in the fertilization rates (Table 2). This shows that the sperm pre incubation does not affect the oocyte penetration rates, but probably does improve the sperm cell recognition by the oocyte, thus triggering the blockage to polyspermy. Even showing better monospermic penetration rates than the high quality oocytes, the IVF of low quality oocytes using pre-incubation of semen for 1.5h did not reach similar embryo development. Conversely, it was demonstrated that high quality oocytes used in IVF were not influenced by semen pre-incubation. This occurs because the oocytes of better quality are more efficient in blocking polyspermy, in comparison to their low quality counterparts (JACKOWSKA et al., 2009). This reinforces that pre incubation does not affect the viability of sperm cells, but only facilitates low quality oocytes recognition for sperm penetration. It should be noted that within the different experiments, no effect has been observed in embryo cell density, evidencing that the blastocyst quality remains the same, despite semen being pre-incubated or not. This shows that using sperm pre incubation, low quality oocytes were allowed to produce a larger amount of embryos, but without affecting their quality. However, there is clear evidence that pre-incubation of sperm cells during 1.5h before IVF procedures is a good strategy to improve embryo developing rates out of a pool of low quality porcine oocytes.

CONCLUSIONS

For low quality oocytes, the blastocyst rates are improved after semen pre-incubation for 1.5h. Overall, semen pre-incubation for 1.5h improved monospermy in low quality oocytes, even

Table 2 - Fertilization, monospermic index and efficiency of IVF in oocytes of low and high quality, fertilized with semen without pre-incubation (0h) or with pre-incubation (Experiment 3).

Group	Total of structures	----------Fertilized----------		---------Monospermic---------		IVF efficiency
		n	%	n	%	%
High 0h	70	47	67.1[a]	18	38.3[b]	25.7[ab]
Low 0h	131	86	65.6[a]	27	31.4[b]	20.6[b]
Low 0.5h	150	118	78.7[a]	46	39.0[b]	30.7[ab]
Low 1h	136	98	72.1[a]	27	27.6[b]	19.9[b]
Low 1.5h	132	105	79.5[a]	56	53.3[a]	42.4[a]

Different letters in the same column differ statistically (P≤0.05).

in comparison to high quality oocytes fertilized with not pre-incubated semen. Nonetheless, blastocyst rates for oocytes of high quality are not influenced by semen pre incubation during porcine IVF procedures.

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