Pesq. Vet. Bras. 36(Supl.1):58-64, junho 2016

In vitro fertilization of porcine oocytes is affected by spermatic coincubation time[1]

Guilherme Oberlender[2]*, Salvador Ruiz López[3], Aitor D. De Ondiz Sánchez[4], Luis A. Vieira[5], Mariane Barreto Pereira[2], Luany de Fátima Silva[2], Márcio G. Zangeronimo[6] and Luis D.S. Murgas[6]

ABSTRACT.- Oberlender G., Ruiz López S., De Ondiz Sánchez A.D., Vieira L.A., Pereira M.B., Silva L.F., Zangeronimo M.G. & Murgas L.D.S. 2016. ***In vitro* fertilization of porcine oocytes is affected by spermatic coincubation time.** *Pesquisa Veterinária Brasileira 36(Supl.1):58-64*. Instituto Federal de Educação, Ciência e Tecnologia do Sul de Minas, *Campus* Muzambinho, Estrada de Muzambinho Km 35, Bairro Morro Preto, Cx Postal 2, Muzambinho, MG 37890-000, Brazil. E-mail: guilherme.oberlender@muz.ifsuldeminas.edu.br

The aim was to study the effects of different gamete coincubation times on porcine *in vitro* fertilization (IVF), and to verify whether efficiency could be improved by reducing oocyte exposure time to spermatozoa during IVF. In groups of 50, a total of 508 immature cumulus-oocyte complexes (COCs) were matured in NCSU-37 medium. The COCs were cultured for 44 hours and then inseminated with *in natura* semen (2,000 spermatozoa/oocyte). The sperm and oocytes were coincubated according to the following treatments (*T*): *T1* = oocytes exposed to spermatozoa for one hour (173 oocytes), *T2* = oocytes exposed to spermatozoa for two hours (170 oocytes), and *T3* = oocytes exposed to spermatozoa for three hours (165 oocytes). After these coincubation periods, the oocytes were washed in fertilization medium (TALP medium) to remove spermatozoa not bound to the zona pellucida and cultured in another similar medium (containing no sperm). Eighteen to twenty hours after fertilization, the putative zygotes were stained in Hoechst-33342 to evaluate the IVF results. The penetration rate was higher ($P<0.05$) after two hours of coincubation time than it was for one or three hours. Furthermore, 68.60% of the ova coincubated with the spermatozoa for two hours were monospermic. The oocytes exposed to spermatozoa for one hour (*T1*) presented a higher ($P<0.01$) rate of polyspermy than those in *T2* and *T3*. Fertilization performance (%) did not differ ($P>0.05$) between oocytes exposed to spermatozoa for one (*T1*) and three hours (*T3*). However, optimum ($P=0.048$) results were obtained after two hours of coincubation, when the rate of fertilization performance was 50.16±8.52%. The number of penetrated sperm per oocyte, as well as male pronucleus formation, did not differ ($P>0.05$) between the treatments evaluated. Under these assay conditions, especially in relation to the sperm concentration used, gamete coincubation for a period of two hours appears to be optimal for monospermy and fertilization performance. Thus, it is the optimal time period for obtaining a large number of pig embryos capable of normal development.

INDEX TERMS: Coincubation time, fertilization performance, *in vitro* fertilization, oocyte, pig.

[1] Received on September 27, 2015.

Accepted for publication on April 13, 2016.

[2] Instituto Federal de Educação, Ciência e Tecnologia do Sul de Minas (IFSULDEMINAS), *Campus* Muzambinho, Estrada de Muzambinho Km 35, Bairro Morro Preto no. 2, Muzambinho, MG 37890-000, Brazil. *Corresponding author: guilherme.oberlender@muz.ifsuldeminas.edu.br

[3] Department of Physiology, Faculty of Veterinary Medicine, University of Murcia, Murcia 30071, Spain.

[4] Department of Animal Reproduction, Faculty of Veterinary Medicine, University of Zulia, Av. 16, Guajira, Ciudad Universitaria "Dr. Antonio Borjas Romero", Núcleo Agropecuario, Maracaibo, Venezuela.

[5] Programa de Pós-Graduação em Ciências Veterinárias (PPGCV), Universidade Estadual do Ceará (UECE), *Campus* Itaperi, Av. Paranjana 1700, Campus do Itaperi, Fortaleza, CE 60714-903, Brazil.

[6] Departamento de Medicina Veterinária (DMV), Universidade Federal de Lavras, *Campus* Universitário s/n, Cx. Postal 3037, Lavras, MG 37200-000, Brazil.

RESUMO.- [**A fertilização *in vitro* de ovócitos suínos é afetada pelo tempo de coincubação espermático**.] Esse estudo foi realizado para avaliar os efeitos de diferentes tempos de coincubação dos gametas sobre a fertilização *in vitro* (FIV) de suínos e se a eficiência dessa técnica poderia ser melhorada pela redução no período que os ovócitos são expostos aos espermatozoides durante a FIV. Um total de 508 (em grupos de 50) complexos cumulus-ovócito (COCs) imaturos foram maturados no meio NCSU-37. Os COCs foram cultivados por 40-44 horas e então inseminados com sêmen *in natura* (2.000 espermatozoides/ovócito). Os espermatozoides e ovócitos foram coincubados de acordo com os seguintes tratamentos (*T*): *T1* = ovócitos expostos aos espermatozoides por uma hora (173 ovócitos); *T2* = ovócitos expostos aos espermatozoides por duas horas (170 ovócitos) e *T3* = ovócitos expostos aos espermatozoides por três horas (165 ovócitos). Após esses períodos de coincubação, os ovócitos foram lavados em meio de fertilização (meio TALP) para remoção dos espermazoides não ligados a zona pelúcida e cultivados em outro mesmo meio (não contendo espermatozoides). Após 18-20 horas de fertilização, os prováveis zigotos foram corados com Hoechst-33342 para avaliação dos resultados da FIV. A taxa de penetração foi maior (P<0,05) após o tempo de coincubação de duas horas em comparação a uma e três horas. Além disso, 68,60% dos ovócitos coincubados com os espermatozoides por duas horas foram monospérmicos. Os ovócitos expostos aos espermatozoides por uma hora (*T1*) apresentaram elevada (P<0,01) taxa de polispermia em comparação com o *T2* e *T3*. A eficiência da fertilização (%) não diferiu (P>0,05) entre os ovócitos expostos aos espermatozoides por uma (*T1*) e três horas (*T3*). Entretanto, ótimos (P=0,048) resultados foram obtidos após duas horas de coincubação, quando a taxa da eficiência da fertilização foi 50,16 ± 8,52%. O número de espermatozoides penetrados por ovócito e a formação de pro-núcleo masculino não diferiu (P>0,05) entre os tratamentos avaliados. Sob as condições de ensaio realizadas, especialmente em relação à concentração espermática utilizada, a coincubação dos gametas por um período de duas horas parece ser ótima para as taxas de monospermia e eficiência da fertilização. Portanto, um tempo provavelmente ótimo para obter um elevado número de embriões suínos capazes de ter um desenvolvimento normal.

TERMOS DE INDEXAÇÃO: Eficiência da fertilização, fertilização *in vitro*, ovócito, suíno, tempo de coincubação.

INTRODUCTION

For many years, studies on *in vitro* fertilization (IVF) in pigs have been conducted as an important tool for reproduction (Cheng et al. 1986, Mattioli et al. 1989, Zhang et al. 2012, Ballester et al. 2014). However, the procedures currently used for *in vitro* maturation (IVM) and IVF for *in vitro* production (IVP) of porcine embryos frequently result in low rates of embryonic development (Grupen et al. 1997, Coy & Romar 2002).

Among the factors that affect the efficiency of this technique, we can highlight the high rates of polyspermy, which occur due to several factors, such as sperm concentration, the fertilization of immature and old oocytes, and, as a main factor, the spermatic coincubation period (Coy & Romar 2002, Wang et al. 2003, Oberlender et al. 2012, Romar et al. 2016). According to Coy et al. (1993), the penetration rates in IVF of porcine oocytes are high, but are also accompanied by high rates of polyspermic fertilization. Thus, polyspermy during IVF represents the main obstacle in the IVF of porcine embryos (Gil et al. 2004, Tokeshi et al. 2007, Oberlender et al. 2013b). According Coy & Romar (2002), while the rate of polyspermy can achieve rates ranging from 40% to 60%, the percentage of ova penetrated is close to 100%.

Many studies have been conducted in an attempt to decrease the rate of polyspermy in IVF of porcine oocytes (Oberlender et al. 2013a, Ballester et al. 2014). *In vivo*, the incidence of polyspermy is less than 5% (Funahashi et al. 2000). Polyspermy increases when a high number of spermatozoa are deposited directly into the oviduct (Hunter 1973, 1971, Gil et al. 2004). Likewise, the reduction of the number of spermatozoa during *in vitro* coincubation with the oocytes increases monospermy rates. However, this is accompanied by a low penetration rate (Abeydeera & Day 1997, Gil et al. 2004).

Several different studies (Coy et al. 1993, Ocampo et al. 1994, Gil et al. 2004) have found that reducing the amount of time that oocytes are exposed to spermatozoa increases monospermy rates. Most current IVF systems use a three- to six-hour gamete coincubation period (Abeydeera & Day 1997, Funahashi et al. 1999, Wang et al. 1999, Abeydeera et al. 2000, Gil et al. 2003, Oberlender et al. 2013a, Nguyen et al. 2015), compared with the coincubation times used in the first IVF systems (Iritani et al. 1978). In other studies (Coy et al. 1993, Grupen & Nottle 2000, Gil et al. 2004), penetration rates were demonstrably improved when oocytes were exposed to spermatozoa for 10 minutes rather than three to six hours. Thus, the modification of gamete coincubation time during porcine IVF has been investigated to decrease the rate of polyspermy (Coy et al. 1993, Abeydeera & Day 1997).

Considering the above, our study was designed to evaluate the effects of different gamete coincubation times on porcine IVF and if the efficiency of this technique could be improved by a reduction (three hours to one) in the period of time that oocytes were exposed to spermatozoa during IVF.

MATERIALS AND METHODS

Place of investigation and location of animals. The study was performed in the Department of Physiology at Faculty of Veterinary Science, University of Murcia in Murcia, Spain. All biological materials (ovaries) were obtained from an abattoir (Matadero de "El Pozo Alimentación") in Alhama de Murcia, in the city of Murcia, Spain. Ovaries were collected from five- to six-month-old prepubertal Landrace × Large White crossbred gilts with an average weight of 90 to 100 kg.

Culture media and reagents. Unless otherwise indicated, all the chemicals and reagents used in this study were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain).

The basic medium for oocyte IVM was North Carolina State University-37 (NCSU-37) (Petters & Wells 1993) supplemented

with 0.57mм cysteine, 50μм β-mercaptoethanol, 5.0mg/L insulin, 1.0mм dibutyryl cAMP (dbAMPc), 10 IU/mL hCG (Chorulon®, Intervet International B.V., Boxmeer, Holland), 10 IU/mL eCG (Folligon®, Intervet International B.V., Boxmeer, Holland), and 10% (vol/vol) porcine follicular fluid (3-8mm in diameter) (Coy et al. 2008a, 2008b, Oberlender et al. 2013a).

The basic medium for IVF, designated as TALP medium (Tyrode's albumin lactate pyruvate), was the same as the one used by Rath et al. (1999); it was supplemented with 1.1mм sodium pyruvate and 0.3% fatty acid-free bovine serum albumin (BSA-FAF) (Coy et al. 2008a, 2008b).

All culture media employed were prepared using ultrapure water (Milli-Q, 18.2 мΩ cm-1; Direct-Q 5, Millipore, Darmstadt, Germany) and equilibrated in an incubator at 38.5°C under 5% CO2 and 100% humidity for three hours before use.

Oocyte collection and preparation. Ovaries from prepubertal gilts were collected immediately after slaughter and transported to the laboratory in a thermal container with 0.9% (wt/vol) NaCl solution containing 0.1% (wt/vol) of kanamycin sulfate at 38°C. The time elapsed from animal slaughter to oocyte recovery was ≤ 2 hours.

Following delivery to the laboratory as previously described, ovaries were washed twice in 0.04% (vol/vol) cetrimide solution and twice more in saline, both at 38°C. Cumulus–oocyte complexes (COCs) from follicles 3–8 mm in diameter were aspirated using an 18-ga needle attached to a 10-mL disposable syringe. Follicular contents obtained were stored in 15-mL sterile tubes, and COCs were allowed to sediment for 10–15 minutes under a warming plate at 38°C. Afterward, the supernatant was discarded and the pellets obtained were deposited into 90 × 15 mm petri dishes for the process of selecting COCs to be used for IVM.

Oocyte selection was performed with a stereoscopic microscope (Nikon). Only intact COCs obtained within two hours of slaughter (Matás et al. 1996) with a homogeneous cytoplasm an compact cumulus oophorus were used. Morphologically abnormal oocytes (brown-colored, shrunken, or granulated cytoplasm or indistinguishable cytoplasmic membranes) were excluded (Hong et al. 2004). After selection, the oocytes were washed twice in Dulbecco's phosphate buffer saline supplemented with 0.001% (wt/vol) polyvinyl alcohol and 0.0005% (wt/vol) phenol red as the pH indicator. Following this, the COCs were washed twice more in previously equilibrated NCSU-37 maturation medium.

Oocyte *in vitro* maturation (IVM). Groups of 50 COCs each were transferred into four-well culture multidishes and cultured in 500μL of the preequilibrated supplemented NCSU-37 medium for 20-22 hours at 38.5°C under 5% CO2 in air (Matás et al. 2003). After culture, the oocytes were washed twice with fresh IVM medium and transferred to fresh IVM medium without dibutyryl cAMP, eCG, or hCG; they were then cultured for an additional 20-22 hours (Funahashi & Day 1993, Park et al. 2009).

Semen collection and sperm preparation. Semen samples for IVF were collected using the gloved hand technique (Hancock & Howell 1959) from mature Pietrain boars of known fertility selected from the "Dalland Hybrid España, S.A." Artificial Insemination Center in Murcia, Spain. In each collection, a sperm-rich fraction was retained in a pre-warmed thermos (Larsen 1986, Gadea 2002), whereas the gel fraction was retained on a gauze tissue that covered the thermos opening.

Afterwards, the ejaculate was diluted 1:1 with isothermal Beltsville thawing solution (BTS) extender (MINITUB Abfüll- und Labortechnik GmbH & Co. KG, Tiefenbach, Germany) (Pursel & Johnson 1975) and immediately transported to the laboratory and protected from light. Only semen samples with normal physiological parameters suitable for use in artificial insemination were used for IVF.

For sperm preparation for IVF, sperm were separated and selected from extended semen by sedimentation/washing through a two-step (45% and 90% vol/vol) Percoll® gradient (Satake et al. 2006). For this purpose, 2mL of 45% Percoll® were layered on top of 2 mL of 90% Percoll® in a 15-mL conic centrifuge tube. Finally, 0.5 mL of diluted semen were added, with care taken to avoid mixing the solutions (Parrish et al. 1995).

The samples were then centrifuged at 800g for 30 minutes at 24°C. The supernatant layers were then removed by aspiration, after which the resultant sperm pellet was resuspended in 10mL of preequilibrated TALP medium (Rath et al. 1999) and washed by centrifugation at 800g for 10 minutes at 24°C (Matás et al. 2011). Finally, the pellet was resuspended and diluted in preequilibrated TALP medium to give a final adjusted concentration of 4×105 sperm/mL (Coy et al. 2010), as determined by a SpermaCue® photometer (MINITÜB Abfüll-und Labortechnik GmbH & Co. KG, Tiefenbach, Germany).

Oocyte *in vitro* fertilization (IVF). After IVM (40-44 hours of culture) and before insemination, oocytes were mechanically stripped of their enclosing cumulus cells by gentle aspiration with a pipette until they were completely denuded. The cumulus--free oocytes were then washed twice in TALP medium, at which point groups of 40–50 oocytes were transferred to each well of four-well culture multidishes containing 250μL of IVF medium (TALP), which had been previously equilibrated at 38.5°C under 5% CO2. This process was carried out simultaneously with the first semen centrifugation using a Percoll® gradient.

For fertilization, 250μL of the sperm suspension were added to each well containing oocytes at a final ratio of 2,000 sperm per oocyte (1×105 sperm/well) (Malo et al. 2010). Afterward, the sperm and oocytes were coincubated at 38.5°C in 5% CO2 in air according to the following treatments (*T*): treatment 1 (*T1*) = oocytes were exposed to spermatozoa for one hour, treatment 2 (*T2*) = oocytes were exposed to spermatozoa for two hours, and treatment 3 (*T3*) = oocytes were exposed to spermatozoa for three hours. After these coincubation periods, adherent spermatozoa and cumulus mass were removed from the zona pellucida by pipetting (Mattioli et al. 1989); the oocytes were then washed twice in previously equilibrated fresh TALP medium and cultured for 18-20 hours until fixation.

Assessment of IVM and IVF. Eighteen to twenty hours after fertilization, the putative zygotes of the three different experimental groups were fixed in 0.5% glutaraldehyde in phosphate buffer saline (PBS). They were then stained in 1% Hoechst-33342 in PBS for 30 minutes, washed in PBS, and mounted on glass slides and examined under an epifluorescence microscope (Leica DMLS) at ×200 and ×400 magnification and 495nm wavelength ultraviolet filter for evidence of maturation and sperm penetration.

Oocyte IVM rate, percentage of degenerated oocytes, penetration rate, monospermy rate, fertilization performance, number of penetrated sperm per oocyte, and pronuclear formation rate were assessed (Algriany et al. 2004, Coy et al. 2010, Malo et al. 2010, Oberlender et al. 2013a). The IVM rate was evaluated as the percentage of oocytes with a nuclear morphology corresponding to metaphases II (MII), which was considered "mature" (Bijttebier et al. 2008). Degenerate oocytes were discarded and the proportion of MII oocytes was calculated from the nondegenerate oocytes. The penetration rate was assessed as the percentage of mature oocytes penetrated by one or more sperm. The monospermy rate was evaluated as the percentage of oocytes with two pronuclei or with one pronucleus together with one decondensed sperm head; fertilization performance was the percentage of monospermic oocytes with two pronuclei in relation to the total number of fertilized oocytes. The rate of pronucleus formation was defined as the percentage of monospermic oocytes that showed both a male and a female pronucleus.

Experimental design. To assess the effects of different gamete coincubation times on porcine IVF, a randomized block design (RBD) with three treatments (oocytes exposed to spermatozoa for one hour - *T1*, two hours - *T2*, and three hours - *T3*) was used. The blocks consisted of fertilization days. A total of three replicates per treatment were performed, and each experimental plot was represented by 50 oocytes.

Statistical analysis. Data are presented as mean ± standard deviation (SD). All variables obtained were modeled according to the binomial model of parameters (Coy et al. 2008b, 2010, Romar et al. 2012, Oberlender et al. 2013a). A normality test (Shapiro-Wilk) was performed and data were analyzed by analysis of variance (ANOVA). When ANOVA results were significant, the IVM rate and IVF data at different spermatic coincubation times (one hour - *T1*, two hours - *T2*, and three hours - *T3*) were compared using Tukey's test.

For variables that were not normally distributed (percentage of degenerated oocytes, rate of male pronucleus formation, and number of penetrated sperm per oocyte), an arcsine transformation was performed to achieve a normal distribution (Coy et al. 2008a, Oberlender et al. 2013a). A significance level of 5% was considered to indicate a statistically meaningful difference. All statistical analyses were performed using the statistical package IBM® SPSS for Windows, version 20.0[7].

RESULTS

A total of 508 oocytes were collected and examined from prepubertal gilts. From these, 173 were in *T1* (sperm and oocytes coincubated for one hour), 170 in *T2* (sperm and oocytes coincubated for two hours), and 165 in *T3* (sperm and oocytes coincubated for three hours).

Regardless of the treatment used, the oocyte maturation rate was 88.59±9.84%. The maturation rate was higher (P=0.023) after coincubation time for 1 and 3 hours than it was for 2 hours (Fig.1). Out of all evaluated oocytes, 24.78% reached the stage of metaphase II, but were not penetrated; no differences were found (P=0.330) between the groups for this variable (Table 1). The percentage of degenerated oocytes was higher (P=0.023) in the ova of *T2* oocytes (those exposed to spermatozoa for 2 hours) with mean of 17.65% (n=30 degenerated oocytes of the total examined), compared to 8.67% in *T1* oocytes (n=15 of 173) and 8.48% in *T3* oocytes (n=14 of 165), which did not differ from each other (P>0.05) (Fig.2).

[7] SPSS Statistics 20.0, IBM® Corp. Released. SPSS® Statistics for Windows. Version 20.0, Release 20.0.0. Armonk, New York: IBM Corp., 2011.

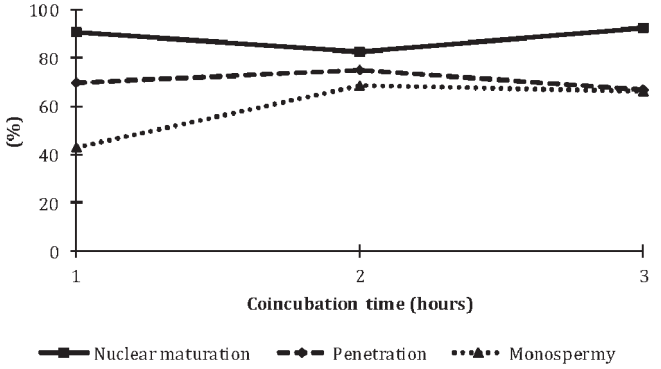

Fig.1. Evolution of nuclear maturation, penetration and monospermy rate in oocytes coincubated with spermatozoa for 1, 2, and 3 hours.

Table 1. Values (mean ± SD – %) of maturation rate and porcine oocytes in metaphase II after 1, 2 and 3 hours of coincubation with spermatozoa

Coincubation time (hours)	Maturation (n)[α]	Metaphase II (n)
1	91.01 ± 7.38[ab] (160)	25.05 ± 10.45 (14)
2	82.49 ± 11.56[b](156)	20.42 ± 9.26 (14)
3	92.49 ± 7.29[a] (160)	28.88 ± 18.95 (5)
Mean	88.59 ± 9.84	24.78 ± 13.74
P value	0.023*	0.330[NS]

[α] (n) =number of oocytes evaluated. [a,b] Different letters in the same column denote significant differences by Tukey test (*P<0.05). [NS] Non significant.

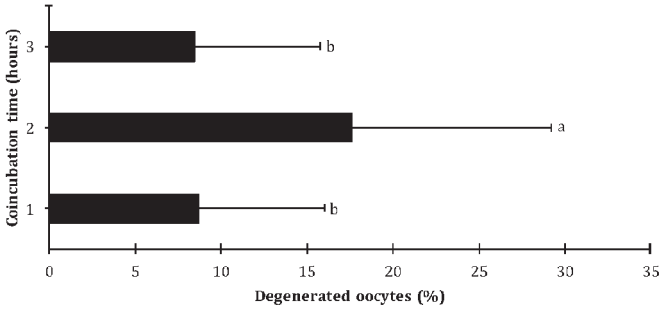

Fig.2. Percentage of degenerated oocytes after 1, 2, and 3 hours of coincubation with spermatozoa. [a,b] Different letters denote significant differences according to Tukey's test (P=0.023).

The effects of different coincubation time on porcine oocytes IVF are shown in Table 2 and Figure 1. The penetration rate was higher (P<0.05) after two hours of coincubation time (75.20±10.42%) versus 1 or 3 hours (69.78 ± 11.45% and 66.57±22.68%, respectively). Furthermore, 68.60% of the ova coincubated with the spermatozoa for 2 hours were monospermic. The percentage of monospermically fertilized ova after 1 and 3 hours of coincubation was 42.67% and 66.35%, respectively (Fig.1). Despite having a lower rate (P<0.05) of sperm penetration, the oocytes exposed to spermatozoa for 3 hours presented a similar rate of monospermic fertilization after 1 hour of incubation.

The percentage of polyspermy ranged from 29.49 to 57.33% in all three groups. The oocytes exposed to spermatozoa for 1 hour (*T1*) presented a higher (P<0.01) rate of polyspermy than those in *T2* and *T3*.

Fertilization performance (%) did not differ (P>0.05) between oocytes exposed to spermatozoa for 1 (*T1*) or 3 hours (*T3*). However, the ova coincubated with the spermatozoa for 2 hours (*T2*) presented the best (P=0.048) rate of fertilization performance (50.16 ± 8.52%) (Fig.3).

Significant differences were not found (P>0.05) between the treatments evaluated for either the number of penetrated sperm per oocyte or the male pronucleus formation. The average number of penetrated sperm per oocyte and the mean of male pronucleus formation regardless of the treatment were 1.75±0.70 and 91.79±11.72%, respectively.

DISCUSSION

The reduction of coincubation time from 3 to 2 hours resulted in an efficient increase in sperm penetration, mono-

Table 2. Data (mean ± SD) of IVF after 1, 2 and 3 hours of oocytes exposed to spermatozoa

Variable analyzed	Coincubation time (hours)			Mean	CV (%)	P value
	1 (n)[α]	2 (n)	3 (n)			
Penetration (%)	69.78 ± 11.45[b](146)	75.20 ± 10.42[a] (142)	66.57 ± 22.68[b] (155)	70.54 ± 15.92	21.47	0.043*
Monospermy (%)	42.67 ± 15.93[b] (102)	68.60 ± 17.44[a] (108)	66.35 ± 24.49[a] (99)	59.67 ± 22.46	27.53	<0.01#
Polyspermy (%)	57.33 ± 15.93[a] (44)	31.41 ± 17.44[b] (34)	29.49 ± 25.68[b] (56)	38.90 ± 23.40	36.17	<0.01#
Performance (%)	30.36 ± 9.52[b] (102)	50.16 ± 8.52[a] (108)	42.45 ± 16.68[b] (99)	33.00 ± 12.93	29.14	0.048*
NPSPO[β]	1.93 ± 0.35	1.48 ± 0.25	1.85 ± 1.10	1.75 ± 0.70	37.50	0.248[NS]
MPF (%)[γ]	96.88 ± 7.05 (99)	92.08 ± 9.32 (103)	86.83 ± 15.48 (89)	91.79 ± 11.72	9.36	0.119[NS]

[α] (n) =number of oocytes evaluated; [β] NPSPO = Number of penetrated sperm per oocyte; [γ] MPF = Male pronucleus formation (%); [a,b] Different letters in the same row, in each fertilization result, indicate significant differences by Tukey test (*P<0.05 and #P<0.01); [NS] Non significant.

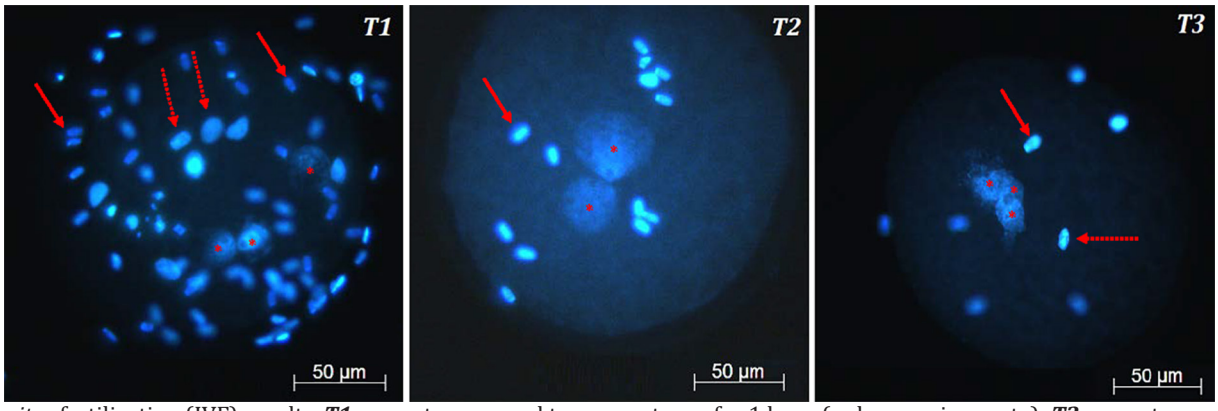

Fig.3. *In vitro* fertilization (IVF) results. ***T1*** = oocytes exposed to spermatozoa for 1 hour (polyspermic oocyte); ***T2*** = oocytes exposed to spermatozoa for 2 hours (monospermic oocyte), and ***T3*** = oocytes exposed to spermatozoa for three hours (polyspermic oocyte). Dashed arrow = decondensed sperm head; straight arrow = sperm adhered to the zona pellucida and * = pronucleus formation.

spermic fertilizations, and, most importantly, fertilization performance. On the other hand, the increase in coincubation time reduced the number of polyspermic fertilizations. The percentage of penetration remained at about 70%, and monospermy was considerably higher with 2 and 3 hours of gamete coincubations. In the short coincubation time (1 hour), the rate of monospermic fertilization was low, even with a penetration rate similar to the long incubation time (3 hours). The number of penetrated sperm per oocyte and the male pronucleus formation after 1, 2, and 3 hours remained constant, with average values of 1.48 to 1.93 and 86.83 to 96.88%, respectively. With fewer than two hours of coincubation, monospermy decreased significantly (P<0.01), affecting fertilization performance. Thus, we demonstrated that penetration, monospermy, and fertilization performance have a positive relation with spermatic coincubation time.

The modification of gamete coincubation time during porcine IVF to decrease the rate of polyspermy *in vitro* has been previously investigated (Cheng et al. 1986, Mattioli et al. 1989, Coy et al. 1993, Abeydeera & Day 1997, Gil et al. 2004, 2007, lmiñana et al. 2005).

According to Coy et al. (1993), the longer the coincubation time, the greater the number of collisions between spermatozoa and zona pellucida; thus, the greater the risk that polyspermy will occur. However, this was not observed in our study, as we obtained a higher rate of polyspermy when the oocytes were exposed to spermatozoa for one hour.

The penetration and monospermy rates obtained in this study differed from the data presented by Coy et al. (1993). For all data, our study showed an average higher than those obtained by the authors mentioned above. This difference can be explained by the fact that, in our study, we used oocytes obtained from the ovaries of prepubertal gilts after slaughter. In the study of these authors, the oocytes were recovered from the oviducts of prepubertal gilts. Furthermore, their medium of sperm capacitation and IVF was different from what was used in our study.

Regarding the percentage of degenerated oocytes at the end of the IVM period, the results of our study were in agreement with other experiments reporting averages ranging from 8% to 23% (Illera et al. 1998, Suzuki et al. 2003, Kim et al. 2010, Oberlender et al. 2013a).

In the present study, regardless of the time of coincubation (oocytes exposed to spermatozoa), maturation rates were within the range reported by various studies (Kikuchi et al. 2009, Zhang et al. 2012, and Oberlender et al. 2013a); during IVM, approximately 10% to 30% of oocytes did not reach metaphase II of the second meiotic division. In our study, approximately 7.51% to 17.57% of IVM failure was due to oocyte degeneration.

The numbers of penetrated sperm per oocyte obtained in our study were similar to those observed by Matás et al. (2011), Romar et al. (2012), and Oberlender et al. (2013a); in those studies, gamete coincubation was performed for one to three hours. Conversely, Coy et al. (2008b) obtained an average of 5.2 to 12.7 penetrated sperm per oocyte, values higher than those found in our study (1.75±0.70). This result may have been due to time that the oocytes were exposed to spermatozoa, which was 4 hours in their study.

Regarding male pronucleus formation, results from our study were in agreement with those of Romar et al. (2012) and Oberlender et al. (2013a), who observed that male pronucleus formation was in excess of 85% of oocytes by 18 hours after fertilization. In our study, the rate of male pronucleus formation ranged from 86.83% to 96.88%.

CONCLUSIONS

Under these assay conditions, especially in relation to the sperm concentration used, gamete coincubation for a period of two hours appears to be optimal for monospermy and fertilization performance. Thus, this is the optimal time period for obtaining a large number of pig embryos capable of normal development.

In addition, as described by other researchers, the examination of other parameters, such as medium volume, sperm concentration, and physiological environment, may further improve the efficiency of porcine IVF.

Acknowledgements.- To the staff of the slaughterhouse "ElPozo Alimentación", Alhama de Murcia, Murcia, Spain for supplying the biological samples. This work was supported by Federal Institute of Education, Science and Technology of South of Minas Gerais (IFSuldeMinas, Muzambinho Campus ("Instituto Federal de Educação, Ciência e Tecnologia do Sul de Minas Gerais (IFSuldeMinas, Campus Muzambinho") and in part by FAPEMIG (Research Support Foundation of the State of Minas Gerais), CNPq (National Council for Scientific and Technological Development) and CAPES (Coordination of Improvement of Higher Education Personnel), Brazil.

Conflict of interest statement.- The authors have no competing interests.

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