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Original Article

Improvement of Normal Fertilization Rate and Embryo Development by Reduction of Sperm: Oocyte ratio during *In vitro* Fertilization in Pig

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Abstract

The present study was undertaken to determine the influence of sperm:oocyte ratio at fertilization *in vitro* using frozen boar semen on fertilization rates and subsequent embryo development and quality in pig. Cumulus-oocyte complexes (COCs) were collected from porcine ovaries and then matured *in vitro*. After 44 hours of culture, matured oocytes were fertilized for 6 hours with three different sperm:oocyte ratios (1000:1, 2000:1, and 4000:1). Presumptive zygotes were fixed at 18-20 hours post-fertilization and then examined for sperm penetration and monospermy rates. The developmental competence, in terms of cleavage and blastocyst rates, and the blastocyst quality were evaluated at 48 and 168 hours post-fertilization, respectively. Sperm penetration rate significantly increased when the oocytes were fertilized with 2000 (90.23±2.5%) and 4000 (93.46±3.7%) sperm: oocyte, compared with those fertilized with 1000 (74.08±1.2%) sperm:oocyte (p=0.005). The oocytes inseminated with 1000 sperm per oocyte had a significantly higher rate of monospermic zygotes (81.79±2.9%) than those inseminated with 2,000 and 4,000 sperm per oocyte (48.07±6.0 and 31.51±4.9%, respectively; p=0.001). The development of blastocysts increased significantly (p<0.05) in the group fertilized with 1,000 sperm:oocyte (29.02±1.8%) compared to those of 4,000 sperm:oocyte (14.00±3.0%). However, there was no significant difference in the mean number of blastocyst cells among the groups but blastocysts derived from 1000:1 oocyte/sperm ratio had the highest ICM cell numbers. Our results indicated that optimization of sperm:oocyte ratio at fertilization *in vitro* improved fertilization rates, in particular, the monospermic penetration rate and blastocyst rate.

Keywords: *in vitro* fertilization, pig, sperm:oocyte ratio

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บทคัดย่อ

การเพิ่มของอัตราการปฏิสนธิและการพัฒนาของตัวอ่อนโดยการลดอัตราส่วนจำนวนตัวอสุจิต่อโอโอไซต์ในระหว่างการปฏิสนธิภายนอกร่างกายในสุกร

ศศิธร พนโสภณกุล [1, 2] ธีรวัฒน์ ธาราศานิต [1] พนิดา ชนาภิวัฒน์ [1] มงคล เตชะกำพุ [1]*

การศึกษาครั้งนี้มีจุดประสงค์เพื่อศึกษาผลของอัตราส่วนจำนวนตัวอสุจิต่อโอโอไซต์ในช่วงการปฏิสนธิภายนอกร่างกายต่ออัตราการปฏิสนธิ ความสามารถในการพัฒนาและคุณภาพของตัวอ่อนที่ได้เก็บโอโอไซต์ที่ถูกหุ้มล้อมด้วยกลุ่มเซลล์คูมูลัสจากรังไข่สุกร และเลี้ยงในน้ำยาเลี้ยงนาน 44 ชั่วโมง แบ่งโอโอไซต์ที่มีการเจริญเต็มที่ออกเป็น 3 กลุ่มตามอัตราส่วนจำนวนตัวอสุจิต่อโอโอไซต์ที่ใช้ในการปฏิสนธิ คือ 1000:1 2000:1 และ 4000:1 นาน 6 ชั่วโมง และทำการเลี้ยงในน้ำยาเลี้ยงตัวอ่อน ตรึงตัวอ่อนบางส่วนที่ 18 ชั่วโมงหลังการปฏิสนธิ เพื่อประเมินอัตราการผ่านเข้าปฏิสนธิของตัวอสุจิ และการเข้าปฏิสนธิของตัวอสุจิหนึ่งตัวต่อโอโอไซต์ (Monospermic oocyte) จากนั้นประเมินความสามารถในการแบ่งตัวและเจริญเป็นระยะคลีเวทและระยะบลาสโตซิสหลังการปฏิสนธิที่ 48 และ 168 ชั่วโมง ตามลำดับ และคุณภาพของตัวอ่อนที่ผลิตได้ ผลการศึกษาพบว่าโอโอไซต์ที่ทำการปฏิสนธิด้วยจำนวนตัวอสุจิ 2000 และ 4000 ตัวต่อโอโอไซต์ มีอัตราการผ่านเข้าปฏิสนธิของตัวอสุจิมากกว่าโอโอไซต์ที่ทำการปฏิสนธิด้วยจำนวนตัวอสุจิ 1000 ตัวต่อโอโอไซต์อย่างมีนัยสำคัญ(p=0.005) และกลุ่มโอโอไซต์ที่ได้รับการปฏิสนธิด้วยจำนวนตัวอสุจิ 1000 ตัวต่อโอโอไซต์มีอัตราการเข้าปฏิสนธิของตัวอสุจิหนึ่งตัวต่อโอโอไซต์สูงกว่ากลุ่มการทดลองอื่นอย่างมีนัยสำคัญ (p=0.001) นอกจากนี้ยังพบว่ามีอัตราการเจริญของตัวอ่อนระยะบลาสโตซิสสูงกว่ากลุ่มของโอโอไซต์ที่ทำการปฏิสนธิด้วยจำนวนตัวอสุจิ 4000 ตัวต่อโอโอไซต์ (p<0.05) แต่ไม่พบว่ามีความแตกต่างของค่าเฉลี่ยจำนวนเซลล์ของตัวอ่อนระยะบลาสโตซิสในแต่ละกลุ่มทดลอง จากผลการศึกษาสรุปได้ว่าอัตราส่วนของจำนวนตัวอสุจิต่อโอโอไซต์ที่เหมาะสมในช่วงการปฏิสนธิภายนอกร่างกายสามารถเพิ่มอัตราการปฏิสนธิโดยเฉพาะอย่างยิ่งอัตราการเข้าปฏิสนธิของตัวอสุจิหนึ่งตัวต่อโอโอไซต์และอัตราการเจริญของตัวอ่อนระยะบลาสโตซิส

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Introduction

In vitro embryo production (IVP) techniques consisting of oocyte maturation, fertilization and embryo culture have been used to generate a large quantity of embryos for fundamental and applied biomedical research such as studying the developmental biology of embryos and also xenotransplantation. Until recently, embryonic stem (ES) cell, derived from porcine blastocyst, hold a tremendous interest for cell replacement therapy because it has been shown to be a better model for human diseases compared to other small laboratory animals (Ibrahim et al., 2006; Matsunari and Nagashima, 2009). It is therefore not surprising that researchers over the world have increasingly attempted to generate porcine embryonic stem cells although overall success has been markedly restricted to only porcine embryonic-like stem cells (Li et al., 2004; Brevini et al., 2010).

In vitro fertilization (IVF) technique has been considered as a suitable technique in producing blastocyts for ES cell establishment when compared with parthenogenetic activation (PA) technique (Panasophonkul et al., 2010). This non-invasive and cost-effective technique provides viable embryos similar to its *in vivo*-derived counterparts and also has advantages over *in vivo* embryos by the large-scale embryo production with less time consuming. However, multiple sperm penetration (polyspermy) is still a major problem of IVF in pig (McCauley et al., 2003; Kikuchi et al., 2004; Sherrer et al., 2004; Koo et al., 2005).

Previous studies have demonstrated a high correlation between the frequency of polyspermy and the absolute number of inseminated spermatozoa (i.e. sperm:oocyte ratio), rather than the number of spermatozoa per milliliter of IVF medium (Xu et al., 1996; Rath, 1992). IVF with excessive sperm numbers have elevated the frequency of polyspermic penetration (Abeydeera and Day, 1997; Marchal et al., 2002; Matas et al., 2003) that coincides with a low rate of embryo development *in vitro*, especially at the blastocyst stage (Machaty et al., 1998; Han et al., 1999).

Furthermore, this polyspermic penetration also decreases embryo quality in terms of total cell numbers and inner cell mass (ICM) ratio when compared with monospermic blastocysts (Giles and Foote, 1995; Funahashi and Day, 1997).

Boars and ejaculate variation appeared to influence the incidence of penetration, fertilization and embryo quality when fresh semen is used (Xu et al., 1996; Long et al., 1999). Suzuki et al., (2003) revealed that penetration and polyspermy rates were more variable among the breeds than among boars within a breed. In contrast to fresh semen, the use of frozen-thawed sperm from the same boar eliminates the variation between trials, providing much more reproducible data and the possibility of repeating the experiment (Funahashi and Nagai, 2001; Marchal et al., 2002; Gil et al., 2004). The present study was undertaken to determine the influence of sperm:oocyte ratio at fertilization *in vitro* on fertilization rate and subsequent embryo development and quality in order to produce a high number and quality of porcine blastocysts.

Materials and Methods

All chemicals used in this study were purchased from the Sigma-Aldrich (St. Louis, USA) unless otherwise stated.

Collection and culture of oocytes: The collection and *in vitro* maturation (IVM) of oocytes were performed as previous described (Panasophonkul et al., 2010). Briefly, cumulus oocyte complexes (COCs) were aspirated from antral follicles (∅ 3-8 mm). COCs with homogeneous cytoplasm surrounded with at least three uniform layers of compact cumulus cells were selected and cultured for *in vitro* maturation, groups of 30-50 COCs were cultured at 38.5°C in a humidified atmosphere with 5% CO_2 in air for 44 hours in a maturation medium that consisted of TCM199 with Earle's salts, 3.05 mM glucose, 26.2 mM sodium bicarbonate, 0.69 mM L-glutamine, 0.91 mM sodium pyruvate, 0.1 mM cysteamine, 10 ng/ml epidermal growth factor (EGF), 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) porcine follicular fluid (pFF).The IVM medium was supplemented with 10 IU/ml equine chorionic gonadotropin (Folligon®, eCG, Intervet-Schering-Plough Animal Health, Boxmeer, The Netherlands) and 10 IU/ml human chorionic gonadotropin (Chorulon®, hCG, Intervet-Schering-Plough Animal Health) for the first 22 hours. The oocytes were then additionally cultured for a further 22 hours in an absence of eCG and hCG.

In vitro fertilization: After 44 hours of *in vitro* maturation, expanded cumulus cells were partially removed and then washed three times with pre-equilibrated modified Tris-buffered medium (mTBM) (Abeydeera and Day, 1997) supplemented with 5 mM sodium pyruvate, 100 IU/ml penicillin and 100 µg/ml streptomycin (IVF medium). Groups of 30-50 oocytes were placed into a 4-well plate containing 500 µl of IVF medium and incubated for at least 30 hours before fertilization. Oocytes were coincubated with frozen-thawed spermatozoa for 6 hours at 38.5°C in a humidified atmosphere with 5% CO_2 in air.

Sperm preparation: The frozen semen from a single boar (Yorkshire) was thawed for 12 sec at 50°C. The viable and motile sperm were then selected using the Percoll gradient technique as described by Parrish et al. (1994). Briefly, the contents of semen were layered onto a discontinuous gradient of 45% (v/v) and 90% (v/v) Percoll in a 15 ml-conical tube (BD Falcon™, Spark, MD, USA) and then centrifuged at 26°C, 700 x g for 15 min. The sperm pellet at the bottom of the 90% Percoll fraction was slowly resuspended with 1 ml of IVF medium. After re-centrifugation, the sperm pellet was resuspended in IVF medium and the concentration of spermatozoa was calculated. The sperm motility was subjectively assayed at x100 magnification under a light microscope (TS1000 Nikon, Tokyo, Japan). Percoll-treated spermatozoa to be used for the experiment had more than a 90% progressive motility.

Embryo culture: After IVF, oocytes surrounded with cumulus cells were denuded and washed three times in an *in vitro* culture (IVC) medium (North Carolina State University (NCSU)-23; Petters and Wells, 1993), supplemented with 1% (v/v) non-essential amino acids (NEAA) and 4 mg/ml bovine serum albumin (BSA). Groups of 30-50 denuded oocytes were placed into a 4-well plate (Nunc, NY, USA) containing 500 µl of NCSU-23 and then covered with mineral oil. During the 5th to 7th days of embryo culture, the BSA in NCSU-23 was substituted with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco, Invitrogen USA). In all cases, embryos were cultured at 38.5°C in a humidified atmosphere with 5% CO_2 in air.

Assessment of sperm penetration: Of 940 IVM oocytes inseminated with three different sperm: oocyte ratios, 243 oocytes were fixed and stained at 18-20 hours after culture to assess fertilization parameters. Presumptive zygotes were washed twice in phosphate buffered saline (PBS) supplemented with 0.1% (w/v) BSA and then were fixed in 4% (w/v) paraformaldehyde at room temperature for 15 min.

To assess the nuclear status of presumptive zygotes, they were first stained with 1 µg/ml fluorescent DNA labeling (4′,6′ Diamidino-2-phenylindole dihydrochloride: DAPI) for 15 min. The fluorescently labeled zygotes were mounted on a glass microscope slide in a 2 µl droplet of anti-fade medium (Vectashield™, Vector Lab, CA, USA) to retard photobleaching, and then examined under an epifluorescent microscope (BX51 Olympus, Japan) at x 200 and x 400 magnifications .

Sperm penetration of porcine oocytes was classified by the presence of at least one male pronucleus that had formed in the ooplasm. Monospermic fertilization typified by the presence of only one male pronucleus, while multiple pronuclei (more than 2) indicated polyspermic fertilization.

Differential staining of blastocysts: Differential staining of the inner cell mass (ICM) and the trophectoderm (TE) cells was performed as our

previous description (Panasophonkul et al., 2010). Fluorescently labeled embryos were mounted onto a glass microscopic slide in a droplet of anti-fade medium (Vectashield™, CA, USA) and sealed with a coverslip. The embryos were examined and counted for ICM and TE cell numbers using an epifluorescent microscope. The ICM was classified as a group of embryonic cells that was stained with only Hoechst 33342 (blue), while the TE cells were positive to both Hoechst 33342 and propidium iodide.

Experimental design: In order to examine the effect of sperm numbers on the percentage of normal fertilization (monospermic fertilization), the *in vitro* matured oocytes were fertilized with three different sperm:oocyte ratios (1000:1, 2000:1, and 4000:1). The frozen-thawed sperm used in this experiment were obtained from a single boar and the sperm quality following Percoll gradient treatment by means of progressive motility was presumed to be equal in all replicates. The presumptive embryos were either fixed at 18-20 hours post-IVF for examination of pronuclear formation or further cultured for 7 days in order to determine their developmental competence (cleavage and blastocyst formation rates) and embryo quality (ICM, TE and ICM: total cells ratio).

Statistical analysis: Data expressed by mean ± standard error of the mean (SEM) was pooled from at least 4 independent replicates. Fertilization parameters, developmental competence and quality of IVF embryos among the experimental groups were compared by ANOVA and protected least significant different (LSD) statistical tests. Statistical analysis was carried out with SPSS version 13.0 software (SPSS Inc., Chicago, IL). $P<0.05$ was considered statistically significant.

Results

Effect of sperm:oocyte ratios on fertilization parameters

There was a significant effect of sperm:oocyte ratio on penetration rate, monospermy, and the efficiency of fertilization (Table 1). Our results showed that sperm penetration rate significantly increased when the oocytes were fertilized with 2000 (90.23±2.5%) and 4000 (93.46±3.7%) sperm: oocyte, compared with those fertilized with 1000 (74.08±1.2%) sperm:oocyte ($p=0.005$). However, the increase in sperm penetration rate was associated with a high rate of polyspermy (multiple pronucleus) since the oocytes inseminated with 1000 sperm per oocyte had a significantly higher rate of monospermic zygotes (81.79±2.9%) than those inseminated with 2,000 and 4,000 sperm per oocyte (48.07±6.0 and 31.51±4.9%, respectively; $p=0.001$, Fig. 1). The efficiency of fertilization also significantly increased when 1000 spermatozoa per oocyte were used, compared to those 4000 sperm per oocyte (60.64±2.7 versus 29.36±4.8%; $p=0.002$). However, the fertilization efficiency was not significantly different between 2000 and 4000 sperm:oocyte groups.

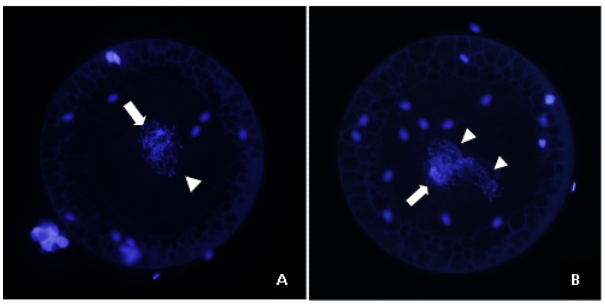

Figure 1 Pronuclear formation of fertilized oocytes at 18-20 hours post-IVF. A) Monospermic fertilization typified by the presence of only one male (arrowhead) and one female pronucleus (arrow) while polyspermic fertilization; B) presents more than one of male pronucleus (arrowheads). (x200).

Table 1 Effect of sperm:oocyte ratios on fertilization parameters during IVF of pig oocytes matured *in vitro*

Ratio	No. of inseminated oocytes	Percentage of oocytes (mean±SEM)		% Efficiency** (mean±SEM)
		Penetrated	Monospermic*	
1,000:1	79	74.08±1.2^{a}	81.79±2.9^{a}	60.64±2.7^{a}
2,000:1	79	90.23±2.5^{b}	48.07±6.0^{b}	43.96±6.9^{a,b}
4,000:1	85	93.46±3.7^{b}	31.51±4.9^{c}	29.36±4.8^{b}

*Percentage of the number of monospermic oocytes/total of penetrated oocytes; ** percentage of the number of monospermic oocytes/total of inseminated oocytes.
$^{a, b, c}$ within a column, different superscripts denote values that differ significantly ($p<0.05$ at least).

Table 2 Effect of different sperm:oocyte ratios on the cell numbers of blastocysts

Sperm:oocyte ratio	No. of blastocysts	No. of cells (mean±SEM)			ICM : Total cell (%mean±SEM)
		ICM	TE	Total	
1000:1	14	10.6±0.6	40.5±2.2	51.1±2.7	20.7±0.8
2000:1	10	9.6±0.7	38.5±2.4	48.1±2.9	19.9±0.7
4000:1	10	9.2±0.6	39.1±2.8	48.3±3.2	19.2±0.8

ICM: inner cell mass and TE: trophectoderm

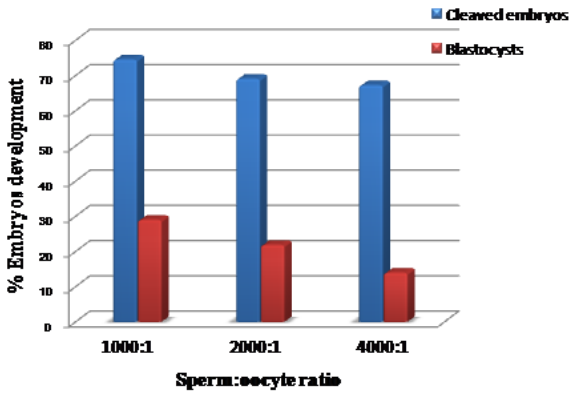

Figure 2 Effect of sperm:oocyte ratios on developmental competence of embryos at cleavage and blastocyst stages after IVF

Development and quality of IVF embryos after fertilization with different sperm:oocytes ratios

Cleavage and blastocyst development was observed at 48 and 168 hour after IVF, respectively. As shown in Fig 2, the sperm:oocyte ratio during IVF did not affect the cleavage rate, while the blastocyst rate increased significantly ($p<0.05$) in the group fertilized with 1,000 sperm:oocyte (29.02±1.8%) compared to those 4,000 sperm:oocyte (14.00±3.0%). Moreover, we examined the quality of blastocysts in terms of the mean number of cells including ICM and TE. Although there was no statistical difference in the mean number of blastocyst cells ($p>0.05$) among the experimental groups, the blastocysts derived from oocytes inseminated with 1000 sperm:oocyte had a higher tendency of ICM cell numbers than thoseobtained from the higher sperm:oocyte ratios (Table 2).

Discussion

In this study, we demonstrate that sperm: oocyte ratio at *in vitro* fertilization affects the efficiency and quality of embryos. A decresing sperm:oocyte reduced the percentage of polyspermy and also improved the developmental competence. This work is correlated with previous studies reporting that excessive numbers of spermatozoa at the time of fertilization increase the percentage of polyspermic penetration (Rath, 1992; Xu et al., 1996; Gil et al., 2007), thereby impairing embryo development.

In the current study, frozen-thawed semen from a single donor was treated with Percoll gradient density in order to minimize boar-to-boar and sperm quality variations during the experiments. Percoll treatment has been reported to improve sperm viability and quality in bovines (Somfai et al., 2002; Mendes et al., 2003) and porcines (Jeon and Yang, 2001; Matas et al., 2003). When IVF was carried out with different sperm: oocyte ratios for 6 hours, our results showed that the rates of sperm penetration were significantly enhanced by increasing the ratios of sperm number from 2000 to 4000 per oocyte, compared with the 1000 sperms per oocyte group. Although we were not able to decrease the number of inseminated sperm to lower than 1000 sperm per oocytes, our results showed that the monospermic penetration rate (81.79%) was significantly high when partial cumulus-enclosed oocytes were co-incubated with 1000 sperm per oocyte. These results were similar to previous reports indicating that a relative reduction of spermatozoa numbers during *in vitro* fertilization results in greater monospermic penetration rates (Xu et al., 1996; Abeydeera and Day, 1997; Gil et al., 2004). Rath (1992) revealed a high correlation between polyspermy and the absolute number of spermatozoa and oocytes but not between the polyspermy rate and sperm concentration per milliliter. Our results obtained a higher incidence of monospermy (82%) when 1000 sperm per oocyte were used for IVF compared with 50-60% from other studies. The observed differences might be caused by many factors such as sperm quality (Popwell and Flowers, 2004; Gadea, 2005) and the inter- and intra-boar variability (Suzuki et al., 2003).

In addition, suboptimal oocyte maturation and IVF systems caused by culture medium and/or sperm incubation time have also been demonstrated to involve polyspermic penetration (reviewed by Funahashi, 2003). Until recently, the mechanism for the prevention of multiple sperm entry has not been well understood. It has been demonstrated that normal distribution of intracellular organelles of mitochondria and cortical granules (CG) during IVM played an important role in preventing polyspermy (Cran and Cheng, 1986; Grupen et al., 1997). More specifically, the CG contents released into the peri-vitelline space (PVS) of the fertilized oocyte induce hardening of the zona pellucida (ZP), thereby preventing polyspermy. In this respect, a delay and incomplete exocytosis of CG due principally to poor cytoplasmic maturation causes an improper pattern of cortical distribution and exocytosis (Wang et al., 1997). In addition, the forming of narrowed PVS in *in vitro* matured oocytes caused by culture medium may also interfere with the distribution of CG contents and could also delay the zona block (Funahashi et al., 1994).

In the present study, we designed to use partial cumulus-enclosed oocytes instead of cumulus-denuded oocytes because the presence of cumulus cells surrounding the oocyte during the IVF has been demonstrated to be beneficial to a normal fertilization rate in several species (Vanderhyden and Armstrong, 1989; Kikuchi et al., 1993; Wang and Niwa, 1995; Zhang et al., 1995) principally by secreting the substances that promote penetration and acrosome reaction of sperm. Cumulus cells also provide a bidirectional microenvironment for attracting, trapping and the selection of morphologically normal and hyperactive spermatozoa during IVF (Lavy et al., 1988; Tanghe et al., 2002).

In this study, we did not found any difference in the cleavage rates among the three different sperm:oocyte ratios. However, the number of cleaved embryos that developed to blastocyst stage was significantly higher in oocytes fertilized with the lowest sperm:oocyte (1000:1) ratio than those

fertilized with greater sperm:oocyte (4000:1) ratio (29 and 14%, respectively). These observations were similar to a study demonstrating that fewer than 20% of inseminated oocytes developed to blastocyst stage when a high sperm concentration was used (Koo et al., 2005). However, there was no significantly difference in embryo quality among experimental groups although the blastocysts obtained from low sperm number insemination tended slightly to increase the ICM cell numbers which was different from a report indicating the retard development of the ICM in polyploidy embryos (Han et al., 1999). This indicates that other factors may also influence ICM segregation and self-renewal. It is also likely that embryos have intrinsic ability to control the optimal number of ICM probably via programmed cell death (Hardy et al., 2003) and gene regulating pathways (Marikawa and Alarcón, 2009).

In conclusion, our findings demonstrate that optimization of sperm: oocyte ratio during *in vitro* fertilization improves fertilization rates and, in particular, monospermic penetration and the quality of blastocysts. Nevertheless, other factors involved in the IVM-IVF system should be considered to increase the success rates of porcine IVF.

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