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Short communication

Selected steps of swine ICSI method to overcome seasonal effect and achieve acceptable early embryonic development, a preliminary study

I.A. Tsakmakidis, M. Tsantarliotou, I.A. Michos, A.G. Basioura, G. Tsousis, C.M. Boscos

Farm Animals Clinic, School of Veterinary Medicine, Aristotle University of Thessaloniki, St. Voutyra 11, 56427, Thessaloniki, Greece

Abstract

Porcine intra cytoplasmic sperm injection's (ICSI) efficacy by selected protocol steps was investigated. Three trials per year's period (hot, medium, cold) were carried out. Only large size follicles (6-8mm) were aspirated, brilliant cresyl blue (BCB) test was performed and only the BCB+ oocytes were *in vitro* maturated (40h) and involved to ICSI process. The presumptive embryos were *in vitro* cultured (15h). Raw boar semen and SpermCatch® as slowing medium were used. No differences were observed between periods regarding early embryonic development and maturation competence. ICSI achieves acceptable porcine early embryonic development rates under the investigated conditions.

Key words: BCB stain, pig oocytes, ICSI, boar sperm, pig embryos

Introduction

The objective of this study was to investigate whether a strict selection of aspirated pig oocytes in combination with as far as possible less pretreatment of used spermatozoa can support all over the year pig early embryonic development after intra cytoplasmic sperm injection (ICSI). Moreover, a natural alternative to polyvinylpyrrolidone (PVP) sperm slowing medium, which is used in human assisted reproduction (SpermCatch®), was used for the first time in the pig.

Materials and Methods

Modified Dulbecco's phosphate buffered saline (mDPBS), North Carolina State University 23 (NCSU-23) supplemented or not with BSA 0.4% and modified Tris-buffered medium (mTBM) supplemented with 2 mM caffeine and 0.2% BSA, were used as ovaries' transfer and washing, *in vitro* fertilization (IVF), maturation (IVM) and culture (IVC) medium, respectively. The experimental period of this study lasted all over a year. Oocytes and semen were retrieved from animals that were kept in Imathia region,

Correspondence to: I. A. Tsakmakidis, e-mail: iat@vet.auth.gr

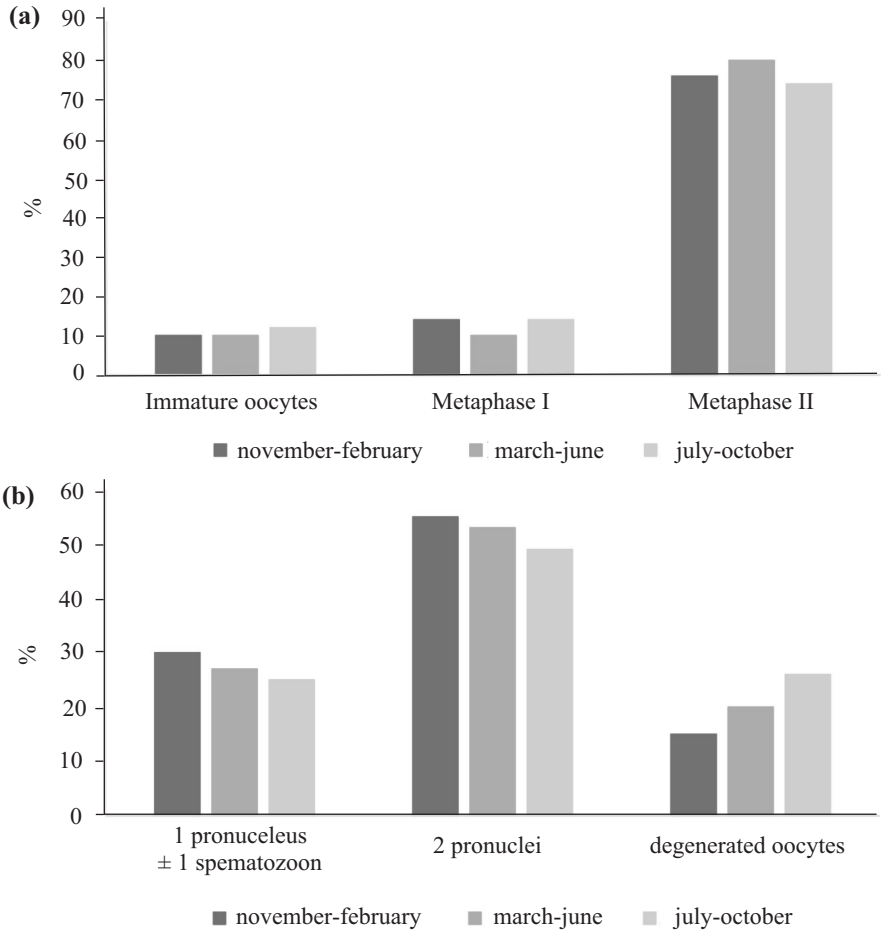

Fig. 1. (a) Evaluation of nuclear maturation competence of porcine oocytes by orcein stain (2%), per experimental period (size of aspirated follicles 6-8 mm, performance of BCB test before in vitro maturation, n=50 per period); (b) Estimation of fertilization and early embryo development per experimental period (n=100 per period).

Greece. According to the temperature data (National Meteorological Service) in this region (40º36'52.9"N, 22º22'08.1"E), the year was divided in three periods/seasons: a) cold (November-February), mean 6.42±2.12ºC, b) mild (March-June), mean 16.75±1.73ºC and c) hot (July-October), mean 21.77±4.56ºC. Three repetitions of ICSI per seasonal period (beginning, middle, end of each season) were homogeneously carried out. Only the large size follicles (6-8 mm) of slaughtered prepubertal gilts, which represented less than 10% of the total number on an ovary, were aspirated. Two hundred fifty (250) morphologically selected cumulus-oocyte complexes (COCs) per period were used for BCB test (Roca et al. 1998). Only the BCB+ COCs were *in vitro* matured for 40 h (Almiñana et al. 2005). After *in vitro* maturation, fifty (50) COCs per experimental period were used randomly to obtain an indication about nuclear maturation competence by orcein 2% staining in acetic acid (w/v). Semen of the same adult crossbred boar of proven fertility and low DNA fragmentation rate (<0.5%), was used in the total of the experiments. Before ICSI, raw extended semen was centrifuged twice by a density gradient medium, was resuspended in mDPBS and a sperm aliquot was further *in vitro* incubated in a natural alternative to PVP medium (SpermCatch®, Nidacon, Sweden) for 30 min (38.5ºC, 5% CO_2, maximum humidity). The remained BCB+ matured denuded oocytes were evaluated stereoscopically and one hundred (100) of them per period were selected for ICSI, depending on showing homogenous cytoplasm and extrusion of the first polar body. The inseminated oocytes were *in vitro* cultured for 15 h to assess pronuclei (PN) formation. After IVC, the presumptive embryos stained with 2% orcein in acetic acid (w/v) and pronuclei formation was assessed microscopically (x1000). Statistical analysis was conducted with SAS V9.3, with χ^2 test performance and significant level at $p<0.05$.

Results and Discussion

Last three years survey data of routine pig IVM (aspirated follicles 3-6 mm, no BCB test) performed

in our laboratory (n=5758 oocytes), showed a significantly lower maturation rate during the hot (56%) compared to the cold (72.7%) and mild (67%) seasons ($p<0.05$). In this study, no significant differences ($p>0.05$) in oocytes' nuclear maturation competence were found between the groups (Fig. 1a). Probably, our results were supported because of the aspiration of large size follicles (6-8 mm). Although follicles of 3-6mm are usually used, it has been reported that *in vitro* developmental competence of oocytes relates with increased oocyte and follicle diameter in pigs. Moreover, BCB test was applied in combination with the selection of large size follicles to enforce maturation potential, achieving maturation rates 74-80%, all over the year. That was also reflected in early embryonic development (Fig. 1b), where no significant differences ($p>0.05$) were observed among periods. The percentage of porcine embryos with apoptotic cells is higher between 5-cell and morula stages compared with the 4-cell or before stages. Therefore, this study recorded the pronuclear status, as a first step, to evaluate the applied protocol's efficiency. Our results of two PN formation (49-55%) are similar with those of García-Roselló et al. (2006a) (55.4-59.7%). Semen variation, sperm morphological abnormalities and poor sperm chromatin integrity have negative effect on the ICSI's outcome (Lasiene et al. 2013). The selection of one boar and of an alternative to PVP, probably supported the results of the present study. SpermCatch®, which was demonstrated sufficient in human *in vitro* embryo production (IVP), was used for the first time to reduce boar spermatozoa's motility. Even though, ICSI methodology is far from standardized, the injection of the whole spermatozoon, a minimum sperm pretreatment and the use of fresh semen without DNA fragmentation are preferable (García-Roselló et al. 2006b). These factors were followed, deriving comparable results with García-Roselló et al. (2006b) who achieved 54% of two PN formation after ICSI with boar fresh semen. The findings of our study indicate, that swine ICSI based on a strict selection of oocytes (large size of aspirated follicles, morphological criteria, BCB test) combined with as far as possible less pretreatment of fresh sperm, can be an efficient method to achieve acceptable early porcine embryos rates throughout the year. Additionally, SpermCatch® can be used to swine IVP. Further embryo development to the blastocyst stage remains to be investigated.

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