

In vitro maturation of porcine oocytes with retinoids improves embryonic development

C. Almiñana^A, M. A. Gil^A, C. Cuello^A, I. Caballero^A, J. Roca^A, J. M. Vazquez^A, E. Gomez^B and E. A. Martinez^{A,C}

^ADepartment of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, Campus de Espinardo, 30100 Murcia, Spain.

^BDepartment of Genetic and Animal Reproduction, SERIDA, Camino de los Claveles, 604, Somió 33203 Gijón, Spain.

^CCorresponding author. Email: emilio@um.es

Abstract. In the present study, the effects of retinoid metabolite administration during *in vitro* maturation (IVM) on oocyte maturation, parameters of *in vitro* fertilisation (IVF) and embryo development were examined. Varying concentrations of 9-*cis* retinoic acid (RA; 0, 5, 50 and 500 nM; Experiment 1) and all-*trans* retinol (ROH; 0, 125, 1250 and 12 500 nM; Experiment 2) were included in the maturation medium. Cumulus–oocyte complexes were matured *in vitro* and inseminated with frozen–thawed spermatozoa. Presumptive zygotes were cultured for 16 h to assess IVF parameters or for 7 days to assess embryo development and quality. In Experiment 1, the oocyte maturation rate to metaphase II was significantly decreased ($P < 0.001$), with values below 5%, in the presence of the highest concentration of RA (500 nM). However, 5 and 50 nM RA had no effect compared with control. Treatment with 5 nM RA improved the blastocyst development rate ($P < 0.001$). In Experiment 2, the oocyte maturation rate did not differ between 125 and 1250 nM ROH treatment groups and control. However, treatment with 12 500 nM ROH was deleterious because no matured oocytes were observed following the treatment. The penetration rate was lower in the group treated with 1250 nM ROH compared with the 125 nM ROH-treated and control groups, but the blastocyst formation rate did not differ among the three groups. In conclusion, 5 nM RA in the IVM medium significantly increased the blastocyst formation rate, suggesting that RA may play an important role during IVM.

Additional keywords: all-*trans* retinol, blastocyst formation, 9-*cis* retinoic acid, *in vitro* fertilisation, oocyte maturation.

Introduction

With growing interest in the generation of embryonic stem cells for the production of transgenic animals and the study of developmental gene regulation, there is an increasing reliance on the *in vitro* fertilisation (IVF) laboratory to maximise embryo viability and quality. However, the *in vitro* production of porcine embryos has been limited by low rates of development to the blastocyst stage (Abeydeera and Day 1997; Kikuchi *et al.* 2002) and their poor quality compared with blastocysts produced *in vivo* (Wang *et al.* 1999). The low quality of oocytes after *in vitro* maturation (IVM; Funahashi *et al.* 1997; Nagai 2001; Kikuchi *et al.* 2002), increased polyspermy after IVF (Niwa 1993; Abeydeera and Day 1997; Wang *et al.* 1997a; Abeydeera 2002; Gil *et al.* 2004, 2007; Almiñana *et al.* 2005, 2007a, 2007b) and poor developmental ability of embryos produced by IVM–IVF (Abeydeera 2001) are the main reasons for this limited performance, together with the unsuitability of *in vitro* culture systems (Kikuchi *et al.* 2002).

Successful nuclear maturation of oocytes *in vitro* is obtained with a high level of repeatability in most current porcine IVF systems. However, although a large proportion of oocytes

reaches metaphase II (MII) after IVM, the IVM environment may not support adequate cytoplasmic and molecular maturation (Sirard *et al.* 2006), which is required to prepare the oocyte for post-fertilisation events, allowing the oocyte to reach the blastocyst stage. Compared with *in vivo* maturation, IVM conditions are simple and materially limited, which can profoundly affect the maturation status of oocyte. For this reason, improved culture conditions are essential to obtain consistently successful and reliable oocyte maturation (both cytoplasmic and nuclear), leading to marked improvements in the efficiency of *in vitro* embryo production.

There is growing evidence for the essential role of retinol and its metabolites, namely all-*trans* retinol (ROH) and 9-*cis* retinoic acid (RA), in cell growth, differentiation and embryonic development under *in vivo* and *in vitro* conditions (Shaw *et al.* 1995; Hidalgo *et al.* 2003). Both ROH and RA are natural cellular metabolites of retinol. The ROH metabolite is converted to its isomer RA and other isomers in a reversible way. These metabolites enter the cell nucleus and are able to activate retinoic acid receptors (RAR), whereas retinoid X receptors (RXR) are activated only by RA (Mangelsdorf *et al.* 1992; Chambon 1996).

The RAR–RXR heterodimers are the functional units transducing the retinoid signal at the gene level. The beneficial effects of ROH and RA have been demonstrated in bovine oocytes, especially on cytoplasmic competence after IVM (Duque *et al.* 2002; Gomez *et al.* 2003; S. Ikeda, unpubl. data) and embryonic development and quality (Duque *et al.* 2002; Gomez *et al.* 2003; Hidalgo *et al.* 2003; Livingston *et al.* 2004; Lima *et al.* 2006). Despite the beneficial effects of retinol, a suitable dose for the different retinoid metabolites has yet to be determined, because both excessive and inadequate exposure to retinol and its metabolites can be detrimental for mouse and bovine embryo development (Sporn and Roberts 1991; Huang *et al.* 2006). However, to the best of our knowledge, there have been no studies on the effects of different doses of retinol during IVM on the yield of porcine embryos.

The aim of the present study was to evaluate the effects of supplementation of the IVM medium with different concentrations of the retinoid metabolites on IVM and IVF parameters, as well as on *in vitro* embryo developmental quality.

Materials and methods

Culture media

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Madrid, Spain).

The medium used for the collection and washing of cumulus–oocyte complexes (COCs) was Dulbecco's phosphate-buffered saline (DPBS) supplemented with 4 mg mL⁻¹ bovine serum albumin (BSA; fraction V), 0.34 mM sodium pyruvate, 5.4 mM D-glucose and 70 µg mL⁻¹ kanamycin (mDPBS). The oocyte maturation medium was BSA-free North Carolina State University (NCSU)-23 (Petters and Wells 1993) supplemented with 10% (v/v) porcine follicular fluid (pFF), 0.1 mg mL⁻¹ cysteine and 10 ng mL⁻¹ epidermal growth factor (EGF). The basic medium used for fertilisation was essentially the same as that used by Abeydeera and Day (1997). The composition of this medium, designated as modified Tris-buffered medium (mTBM), was (in mM): NaCl 113.1; KCl 3; CaCl₂·2H₂O 7.5; Tris (crystallised free base) 20; glucose 11; sodium pyruvate 5. The mTBM was supplemented with 2 mM caffeine and 0.2% BSA (fraction V; A7888, initial fractionation by cold alcohol precipitation). The embryo culture medium was a sequential medium based on NCSU-23 supplemented with 0.4% BSA.

Recovery and IVM of COCs

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and were transported to the laboratory within 1 h after collection in 0.9% NaCl containing 70 µg mL⁻¹ kanamycin at 35°C. After the ovaries had been washed three times in NaCl solution, follicles 3–6 mm in diameter were aspirated using an 18-gauge needle connected to a 10-mL disposable syringe. Oocytes with a compact cumulus mass and a dark, evenly granulated cytoplasm were washed three times in maturation medium and matured in a four-well multidish (Nunc, Roskilde, Denmark) containing 150–200 COCs per well in 500 µL maturation medium supplemented with 10 IU mL⁻¹ equine chorionic gonadotrophin (eCG; Folligon; Intervet International, Boxmeer, The Netherlands) and 10 IU mL⁻¹ human

chorionic gonadotrophin (hCG; Veterin Corion; Divasa Farmavic, Barcelona, Spain) for 20–22 h and then for another 20–22 h in maturation medium without hormones. Oocyte maturation was performed under mineral oil at 39°C in an atmosphere of 5% CO₂ in air.

IVF

After maturation, COCs were denuded with 0.1% hyaluronidase in maturation medium by vortexing for 2 min at 1660 r.p.m. Oocytes were washed twice in maturation medium and three times in pre-equilibrated fertilisation medium. Groups of 50 denuded oocytes were then placed in 50-µL drops of fertilisation medium in a 35 × 10 mm Petri dish (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA) under mineral oil and held at 39°C in an atmosphere of 5% CO₂ in air for approximately 30 min until the addition of spermatozoa.

For IVF, 100 µL thawed semen was washed three times by centrifugation at 1900g for 3 min in mDPBS. The resulting pellet was resuspended in fertilisation medium and, after appropriate dilution, 50 µL of this sperm suspension was added to a 50-µL drop of fertilisation medium containing the oocytes. The spermatozoa : oocyte ratio was 1000 : 1. Gametes were cocultured at 39°C in a humidified atmosphere of 5% CO₂ in air for 6 h.

In vitro culture

Presumptive zygotes were removed from the fertilisation medium and washed three times in pre-equilibrated embryo culture medium. Subsequently, zygotes were transferred to a four-well multidish (50 zygotes per well), with each well containing 500 µL of the same medium under mineral oil, and cultured at 39°C in 5% CO₂ in air either for 16 h to assess fertilisation parameters or for 7 days to assess embryo development. Presumptive zygotes were cultured for the first 2 days (Day 0 = day of fertilisation) in glucose-free NCSU-23 supplemented with 0.33 mM pyruvate and 4.5 mM lactate. Following 48 h culture, all presumptive zygotes were removed and cultured subsequently in fresh NCSU-23 medium containing 5.5 mM glucose until Day 7.

Assessment of maturation, sperm penetration and embryo development

To evaluate maturation and fertilisation parameters, oocytes and presumptive zygotes were mounted on slides, fixed in a solution of acetic acid : ethanol (1 : 3) for 48–72 h at room temperature, stained with 1% lacmoid in 45% (v/v) acetic acid and examined under a phase-contrast microscope at a magnification of ×400. The maturation rate was assessed at 44 h of IVM. Oocytes with chromosomes at MII and an extruded polar body were considered mature. Fertilisation parameters were evaluated 16–18 h after insemination. Oocytes were considered penetrated when they contained one or more swollen sperm heads and/or male pronuclei, with their corresponding sperm tails, and two polar bodies. Degenerated oocytes and oocytes with a broken oolemma or abnormal cytoplasmic appearance were not counted. The fertilisation parameters evaluated were penetration (number of oocytes penetrated/total oocytes matured), monospermy (number of oocytes containing only one male pronucleus/total penetrated), number of spermatozoa per oocyte (number of

spermatozoa in penetrated oocytes) and the efficiency of fertilisation (number of monospermic oocytes/total inseminated).

To examine the ability of embryos to develop to the blastocyst stage *in vitro*, presumptive zygotes were cultured for 7 days. Cleavage rate (number of embryos cleaved/total cultured) on Day 2 and blastocyst formation (number of blastocyst/total cultured) on Day 7 were evaluated under a stereomicroscope. An embryo that had cleaved to the two-cell stage or beyond was counted as cleaved and an embryo with a clear blastocele was defined as a blastocyst. The total cell number, as an indicator of embryo quality, was evaluated by mounting each blastocyst on a slide in 4 μ L glycerol : DPBS (3 : 1) containing 10 μ g mL⁻¹ Hoechst-33342, followed by examination using fluorescence microscopy.

Experimental design

Experiment 1: effect of RA during IVM on parameters of IVM and IVF parameters, as well as in vitro embryo development

In an attempt to improve *in vitro* oocyte maturation and *in vitro* embryo development, various concentrations of RA (0, 5, 50 and 500 nM) were added to the maturation medium. The concentrations of RA were chosen on the basis of those used in previous reports in other species in order to establish a suitable dose for porcine IVM. A total of 2161 IVM oocytes, from five replicates, were used in this experiment.

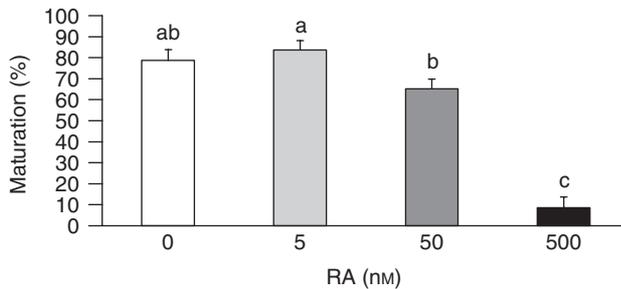


Fig. 1. Effect of 9-*cis* retinoic acid (RA) on the *in vitro* maturation (IVM) rate of porcine oocytes. Oocytes with a compact cumulus mass and a dark, evenly granulated cytoplasm were washed three times and matured in maturation medium supplemented with 0, 5, 50 or 500 nM RA. Maturation rates were assessed after 44 h of IVM. Oocytes with chromosomes at metaphase II and an extruded polar body were considered mature. Different superscript letters represent significant differences (at least $P < 0.05$).

Experiment 2: effect of ROH during IVM on parameters of IVM and IVF parameters, as well as in vitro embryo development

This experiment evaluated the effect of ROH on porcine IVM and *in vitro* embryo production. Various concentrations of ROH (0, 125, 1250 and 12 500 nM) were tested. The concentrations of ROH were chosen in the same way as concentrations of RA in Experiment 1. A total of 2141 IVM oocytes from five replicates were used in this experiment.

Statistical analysis

All data editing and statistical analyses were performed by SPSS, version 11.5 (SPSS, Chicago, IL, USA). Data from all replicates were assessed by analysis of variance using the MIXED procedure according to a statistical model including the fixed effect of the concentration of RA (Experiment 1) and ROH (Experiment 2) and the random effect of replicate in each experiment. Maturation, penetration and monospermic rates, efficiency data and blastocyst formation were modelled according to the binomial model of parameters, as described by Fisz (1980), before analysis. When analysis of variance showed a significant effect, values were compared using the Bonferroni test. The threshold for significance was set at $P < 0.05$. Results are expressed as the least-squares mean \pm s.e.m.

Results

Experiment 1: effect of RA during IVM on parameters of IVM and IVF, as well as in vitro embryo development

Oocyte maturation rates were significantly decreased ($P < 0.001$), with values below 5%, when the highest concentration of RA (500 nM) was used (Fig. 1). At 5 and 50 nM, RA had no effect on *in vitro* oocyte maturation compared with control. However, maturation rates were higher for 5 compared with 50 nM RA ($P < 0.05$).

Penetration rate, monospermy rate and the mean number of spermatozoa per oocyte were not influenced by RA treatment (Table 1). There were no differences in the efficiency of IVF between the control and 5 nM RA-treated groups, but efficiency decreased for 50 ($P < 0.05$) and 500 nM RA ($P < 0.001$) compared with control. The low efficiency of IVF for the highest concentration of RA (0.7 ± 3.8) was due to the low maturation rate (2.0 ± 2.7).

Table 1. Effect of 9-*cis* retinoic acid (RA) during *in vitro* maturation on *in vitro* fertilisation parameters of porcine oocytes

^{a,b,c}Different superscripts within the same column represent significant differences ($P \leq 0.05$). Penetration, number of oocytes penetrated/total oocytes matured; monospermy, number of oocytes containing only one sperm head or one male pronuclei/total penetrated; EO (number of spermatozoa per oocyte), number of spermatozoa in penetrated oocytes; efficiency, number of monospermic oocytes/total inseminated

RA (nM)	No. oocytes	Penetration (%)	Monospermy (%)	EO	Efficiency (%)
0	179	91.4 \pm 3.5	54.0 \pm 5.7	1.84 \pm 0.19	45.4 \pm 3.4 ^a
5	206	84.6 \pm 3.4	53.2 \pm 5.5	1.80 \pm 0.18	41.8 \pm 3.2 ^{ab}
50	164	87.2 \pm 3.7	39.9 \pm 5.9	1.93 \pm 0.19	30.7 \pm 3.5 ^b
500	144	96.6 \pm 23.4	55.2 \pm 35.3	2.89 \pm 0.80	0.7 \pm 3.8 ^c

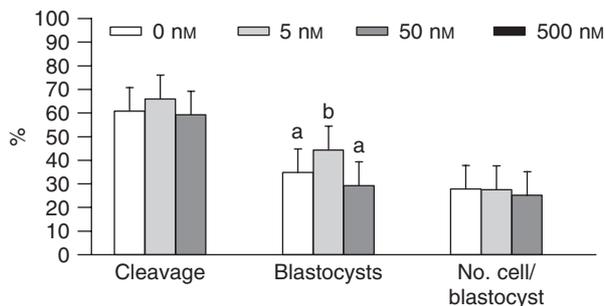


Fig. 2. Effect of 9-*cis* retinoic acid (RA) during *in vitro* maturation on the *in vitro* development of porcine embryos. Oocytes were matured in maturation medium supplemented with 0, 5, 50 or 500 nM RA for 44 h and were fertilised with frozen-thawed spermatozoa (1000 sperm per oocyte). After 6 h coincubation, presumptive zygotes from all groups were cultured in NCSU-23 medium supplemented with 0.4% bovine serum albumin for 7 days to assess embryo development. No embryo cleavage and blastocyst formation were observed when oocytes were exposed to the highest concentration of RA (500 nM) during IVM. Different superscript letters within the same variable represent significant differences (at least $P < 0.05$). % Cleavage, percentage of embryos cleaved/total oocytes inseminated; % Blastocysts, percentage of blastocysts/total oocytes inseminated. The total cell number was evaluated by mounting each blastocyst on slides in 4 μ L glycerol : Dulbecco's phosphate-buffered saline (3 : 1) containing 10 μ g mL⁻¹ Hoechst-33342, followed by examination with fluorescence microscopy.

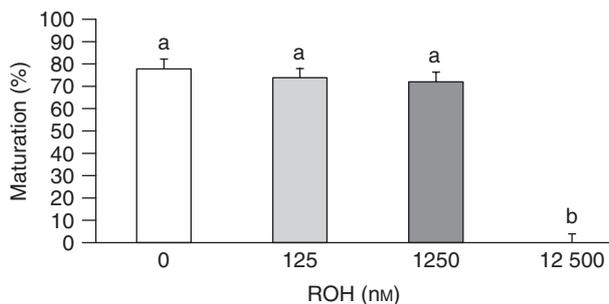


Fig. 3. Effect of all-*trans*-retinoic (ROH) during *in vitro* maturation (IVM) on eht maturation rates of porcine oocytes. Oocytes with a compact cumulus mass and a dark, evenly granulated cytoplasm were washed three times and matured in maturation medium supplemented 0, 125, 1250 or 12 500 nM ROH. Maturation rates were assessed before fertilisation after 44 h of IVM. Oocytes with chromosomes at metaphase II and an extruded polar body were considered mature. Different superscript letters represent significant differences ($P < 0.001$).

Data on embryo development are presented in Fig. 2. There were no differences in cleavage rate between the control and 5 and 50 nM RA-treated groups, but the rate of blastocyst formation was increased significantly in the 5 nM RA-treated group ($P < 0.001$). The total cell number per blastocyst did not differ among treatments and ranged from 25.14 ± 1.52 to 27.82 ± 1.44 . No embryo cleavage and blastocyst formation were observed when oocytes were exposed to the highest concentration of RA (500 nM) during IVM.

Experiment 2: effect of ROH during IVM on parameters of IVM and IVF parameters, as well as *in vitro* embryo development

In this experiment, maturation rates did not differ between the control and 125 and 1250 nM ROH-treated groups, but 12 500 nM ROH was deleterious (Fig. 3). No oocytes matured following treatment with 12 500 nM ROH, so it was not possible to evaluate IVF parameters and embryo development.

The penetration rate was significantly lower for 1250 compared with 125 nM ROH and control, but the monospermy rate, the mean number of spermatozoa per oocyte and the efficiency of IVF were not affected by ROH treatment (Table 2).

The addition of 125 nM ROH resulted in a significant increase ($P < 0.001$) in the percentage of cleaved embryos (Fig. 4). However, the blastocyst formation rate and total cell number were not affected by ROH treatment, apart from a decrease in the percentage of blastocysts after maturation of oocytes in the presence of 1250 nM ROH (Fig. 4).

Discussion

Although retinol metabolites have been recommended as important components of IVM media to improve cytoplasmic maturation and embryo development, both beneficial and detrimental effects of the retinol metabolites have been described for bovine embryo development *in vitro* (Duque *et al.* 2002; Gomez *et al.* 2003; Hidalgo *et al.* 2003; Lima *et al.* 2006). To the best of our knowledge, there are no reports on the effects of retinoids on the developmental ability of porcine oocytes.

Too much or too little exposure to retinoids, at the wrong stage or at the wrong time, can adversely affect embryo development (Sporn and Roberts 1991; Huang *et al.* 2006; Rodríguez *et al.* 2007). In the present study, the highest concentrations of both metabolites (500 nM RA and 12 500 nM ROH) were cytotoxic because they prevented oocytes from achieving nuclear maturation. This detrimental effect could be related to a retinoid imbalance that has been shown to be highly correlated with developmental abnormalities (Morriss-Kay and Ward 1999). In support of this hypothesis, high concentrations of retinoids during IVM, such as 500 nM RA (Gomez *et al.* 2003) and 100 μ M ROH (Livingston *et al.* 2004), have been shown to be detrimental for bovine oocytes. In contrast with these findings, Lima *et al.* (2006) reported that the addition of 500 nM RA to bovine IVM medium enhanced blastocyst formation. A possible explanation for these discrepancies could be the different IVM conditions used by these authors, especially the different hormone supplementation regimens. Thus, Gomez *et al.* (2003) suggested that the detrimental effect of overexposure to retinoids, or its neutral effect observed during IVM, may depend on an interaction between retinoids and follicle-stimulating hormone (FSH). Moreover, it has been demonstrated that retinol decreases FSH-induced expression of luteinising hormone receptor in porcine granulosa cells (Hattori *et al.* 2000).

Taking into consideration the importance of an accurate dose, in the present study we examined the effects of a range of retinoid concentrations during IVM. The results showed no effect of 5 and 50 nM RA on *in vitro* nuclear oocyte maturation. However, in the presence of 5 nM RA there was a tendency ($P = 0.18$) for

Table 2. Effect of all-trans-retinoic (ROH) during *in vitro* maturation on *in vitro* fertilisation parameters of porcine oocytes

^{a,b}Different superscripts within the same column represent significant differences ($P \leq 0.05$). Penetration, number of oocytes penetrated/total oocytes matured; monospermy, number of oocytes containing only one sperm head or one male pronuclei/total penetrated; EO (number of spermatozoa per oocyte), number of spermatozoa in penetrated oocytes; efficiency, number of monospermic oocytes/total inseminated

ROH (nM)	No. oocytes	Penetration (%)	Monospermy (%)	EO	Efficiency (%)
0	165	89.2 ± 2.8 ^a	50.2 ± 4.8	1.72 ± 0.12	42.5 ± 3.4
125	169	85.7 ± 2.9 ^a	47.2 ± 4.9	1.78 ± 0.12	38.8 ± 3.5
1250	170	72.2 ± 3.0 ^b	52.8 ± 5.4	1.87 ± 0.13	34.3 ± 3.4
12 500	164				

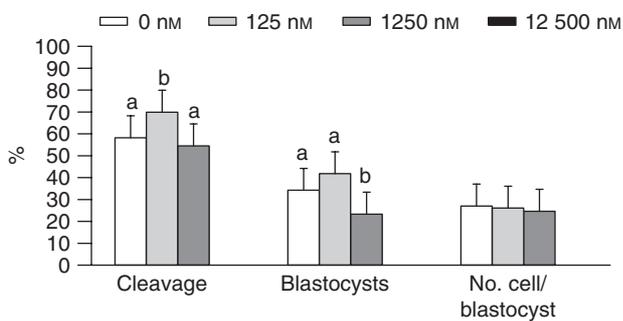


Fig. 4. Effect of all-trans-retinoic (ROH) during *in vitro* maturation on the *in vitro* development of porcine embryos. Oocytes were matured in maturation medium supplemented with 0, 125, 1250 or 12 500 nM ROH for 44 h and were fertilised with frozen-thawed spermatozoa (1000 sperm per oocyte). After 6 h coincubation, presumptive zygotes from all groups were cultured in NCSU-23 medium supplemented with 0.4% bovine serum albumin for 7 days to assess embryo development. No embryo cleavage and blastocyst formation were observed when oocytes were exposed to the highest concentration of ROH (12 500 nM) during IVM. Different superscript letters within each variable represent significant differences (at least $P < 0.05$). % Cleavage, percentage of embryos cleaved/total oocytes inseminated; % Blastocysts, percentage of blastocysts/total oocytes inseminated. The total cell number was evaluated by mounting each blastocyst on slides in 4 μ L glycerol:Dulbecco's phosphate-buffered saline (3:1) containing 10 μ g mL⁻¹ Hoechst-33342, followed by examination with fluorescence microscopy.

an improvement in maturation rates compared with control and the rate was significantly better following 50 nM RA treatment. When ROH was tested during IVM, maturation rates did not differ between the control and 125 and 1250 nM ROH-treated groups.

These results are in contrast with those obtained in bovine oocytes, where retinoids were reported to improve nuclear maturation (E. B. Bortolotto, unpubl. data). There could be species-specific differences in the susceptibility to retinoids, with varied requirements during IVM in order to protect oocytes from detrimental effects on the one hand and from the epigenetic influences of retinol on the other, as has been reported by several authors (e.g. Gomez *et al.* 2003; Huang *et al.* 2006). The different concentrations of retinoids used between studies could also have been responsible for these discrepancies, given that the

effects of these compounds are dependent on their concentration (Gomez *et al.* 2003; Livingston *et al.* 2004; Lima *et al.* 2006).

In the present study, a significant increase in blastocyst development was obtained after treatment of oocytes with 5 nM RA. However, a neutral or detrimental effect was observed when 50 nM RA or 125 and 1250 nM ROH were used. The better developmental competence achieved by oocytes, and consequently increases in blastocyst formation, after exposure to 5 nM RA during IVM could be related to more complete granular migration in the matured oocyte cytoplasm induced by RA, as suggested to occur in bovine oocytes (Gomez *et al.* 2003). This migration provides a block to polyspermy once migrated cortical granules (CGs) have been released (Wang *et al.* 1997b; Nagano *et al.* 1999). It is well known that CGs play an important role in the block to polyspermy in mammalian oocytes (for a review, see Yanagimachi 1994). Polyspermy is one of the main unresolved problems of porcine IVF that greatly limits the production of useful pig embryos. Based on the hypothesis that the benefits of RA would be reflected in improved granular migration, an increase in monospermy rates should have been obtained in the present study. However, the proportion of monospermic oocytes was not affected by RA treatment. Moreover, exposure of oocytes to RA did not affect the penetration rate and the mean number of spermatozoa per oocyte. This indicates that the improved developmental competence of oocytes after RA treatment was not associated with a reduction in polyspermy, at least under our experimental conditions.

A possible explanation for the increased blastocyst development after 5 nM RA treatment is protection from oxidative damage, which is a major cause of *in vitro* embryonic wastage (Guerin *et al.* 2001). Previous studies have shown that retinoids participate in a biological anti-oxidant network and have been implicated as important regulators of redox signalling pathways (Olson 1993; Imam *et al.* 2001; Ikeda *et al.* 2005). Furthermore, it has been reported that retinoids protect against oxidative damage by maintaining adequate endogenous competency and levels of anti-oxidants that are essential for oocyte maturation, fertilisation and embryonic development (Guerin *et al.* 2001). However, this beneficial effect was not reflected in embryo cleavage or embryo quality in the present study, in contrast with bovine embryos, where increased cell numbers and proportions of cells allocated to the inner cell mass have been reported (Duque *et al.* 2002). These differences may be dependent on the

amount of triglyceride stored in the pig oocyte, higher than that in bovine (McEvoy *et al.* 2000; Genicot *et al.* 2005). Sterification to fatty acids, particularly palmitate, is the preferential form for retinol storage in cells and palmitate is the most abundant fatty acid in oocytes of domestic species, including the pig (McEvoy *et al.* 2000; Kim *et al.* 2001). Therefore, it is feasible that the pig oocyte contains larger amounts of retinol than the bovine oocyte. Because retinol could be activated through conversion to retinoic acid, such as detected in bovine oocytes and embryos (Gómez *et al.* 2006; Rodríguez *et al.* 2006), this could lead to differences in endogenous retinoid activity. Interference of exogenous compounds with endogenous retinoid may explain differences between species.

When ROH treatment was evaluated, the penetration rate was significantly lower for 1250 than 125 nM ROH or control. The monospermy rate and the mean number of spermatozoa per oocyte were not affected by ROH treatment. Although treatment of oocytes with 125 nM ROH resulted in better embryo cleavage compared with control, this enhancement was not reflected in blastocyst development. Moreover, the number of cells per blastocyst was not influenced by any of the ROH treatments. These results are in disagreement with those obtained in bovine IVF, where it has been reported that 5000 nM ROH during IVM increases embryonic development to the blastocyst stage (Livingston *et al.* 2004). It may be that different concentrations of ROH to those used in the present study are required during porcine IVM to achieve improved embryo development.

In conclusion, the present study showed that RA, at a concentration of 5 nM, in the IVM medium significantly increased blastocyst formation. These data suggest that inclusion of RA in IVM media used for current IVF protocols could improve the yield of porcine IVP embryos. Additional studies are needed to elucidate the possible mechanisms by which RA influences the developmental capacity of porcine oocytes.

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