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9-*cis*-Retinoic Acid During In Vitro Maturation Improves Development of the Bovine Oocyte and Increases Midkine but Not IGF-I Expression in Cumulus-Granulosa Cells

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ABSTRACT The isomer 9-*cis* of retinoic acid (9-*cis*-RA) exerts a beneficial effect on bovine in vitro development when added to in vitro maturation (IVM) culture. In the present work, 9-*cis*-RA 5 nM was found to be stimulatory as opposed to 500 nM (toxic). Cumulus–oocyte complexes (COCs) were treated with the found physiological dose 9-*cis*-RA 5 nM, and the next determinations performed: (1) relative expression of midkine (MK) and IGF-I, by reverse transcriptase-polymerase chain reaction (RT-PCR), in cumulus-granulosa cells detached from oocytes; (2) cytoplasmic granular migration, by labeling of oocytes with fluoroscein isothiocyanate lectins; and (3) in vitro survival of blastocysts after vitrification and warming. Gene expression of MK was enhanced by 9-*cis*-RA, but not by 1% ethanol (vehicle). However, we did not detect IGF-I expression, both in dependence on or in the absence of 9-*cis*-RA acting on cumulus-granulosa cells. The ability of vitrified blastocysts to survive in vitro was not improved by 9-*cis*-RA. Nevertheless, since only blastocysts obtained from oocytes matured with serum survived, more factors should be considered when evaluating cryopreservation survival. The complete granular migration observed in oocytes matured with 9-*cis*-RA anticipates the gain in developmental competence of the oocyte, being MK probably involved in this beneficial effect. *Mol. Reprod. Dev. 66: 247–255, 2003.* © 2003 Wiley-Liss, Inc.

Key Words: embryo; ethanol; viability; cytoplasmic granule

INTRODUCTION

The retinoid are a large family of natural and synthetic compounds related to vitamin A (all-*trans*-retinol). The vitamin A derivative retinoic acid (RA) is the most relevant retinoid during vertebrate development (Morris-Kay and Ward, 1999), but retinol is essential for pregnancy maintenance in mammals. In the live cell, All-*trans*-RA binds to retinoic acid receptors (RAR; α, β, and γ) and its isomer 9-*cis* of retinoic acid (9-*cis*-RA) specifically binds to retinoid X receptors (RXR; α, β, and γ) (Mangelsdorf et al., 1994; Chambon, 1996). However, under in vitro conditions, 9-*cis*-RA activates both RXR and RAR. Information on RXRs and 9-*cis*-RA, the so-called second retinoid signaling pathway, is scarce and focused on later embryonic stages in the mouse (Mangelsdorf et al., 1992; Dolle et al., 1994). The RAR–RXR heterodimers are the functional units in transducing the retinoid pathway upon the ligand binding. Subsequently, the ligand–receptor complexes either activate or repress specific target genes by binding to specific response elements near the promoter region. Furthermore, the RXRs may either homodimerize or form heterodimers with the thyroid hormone receptor, the vitamin D receptor, and the peroxisome proliferator-activated receptor (Chambon, 1996). Information of 9-*cis*-RA and RXRs, the so-called second retinoid signaling pathway, is scant and centered on late embryonic development in the mouse (Mangelsdorf et al., 1992; Dolle et al., 1994). Recently, most of these RA receptors, including RAR α and γ, RXR α and β, retinaldehyde dehydrogenase and peroxisome-proliferator activated receptor gamma (PPARγ), were detected from the oocyte to the hatched blastocyst stage in vitro (Mohan et al., 2001, 2002) and in cumulus cells (Mohan, personal communication, 2002). These findings are consistent with the stimulation of oocyte development capacity by retinoid in cows (Shaw et al., 1995; Duque et al., 2002; Hidalgo et al., 2002), sheep (Eberhardt et al., 1999), gilts (Whaley et al., 1997, 2000), and rabbits that had higher blood levels of vitamin A (Besenfelder et al., 1996).

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In the last decade, a number of genes have been studied in an effort to explain relevant traits during embryonic development and differentiation. Midkine (MK) is a RA inducible, growth and differentiation factor that binds to heparin and to oversulfated chondroitin sulfate (Muramatsu, 2000, 2002), being involved in epithelial–mesenchymal interactions (Muramatsu, 2002; Sumi et al., 2002). MK and pleiotrophin are 55% homologous and belong to a newly evolving family of neurotrophic and developmentally regulated molecules. The peptide was isolated in bovine follicular fluid (Ohyama et al., 1994), and the gene product in rat granulosa cells in dependence on gonadotropins (Karino et al., 1995; Minegishi et al., 1996). However, as opposed to gonadotropins, addition of RA does not increase cAMP levels in a granulosa cell culture system (Minegishi et al., 1996, 2000), which means that the MK-inducing mechanism of FSH is not the same as RA. The MK signaling system is generally assumed to include PI3 kinase, and downstream MAP kinase (reviewed by Muramatsu, 2002). The MK receptor is thought to be a molecular complex containing proteoglycans including protein-tyrosine phosphatase ζ (PTPζ) (Maeda et al., 1999) and members of the low-density lipoprotein receptor-related protein (LRP) family (Muramatsu et al., 2000). Syndecan-1, -3, and -4 proteins bind strongly to MK (Mitsiadis et al., 1995; Kojima et al., 1996; Nakanishi et al., 1997). The mature peptide of 13×10^3 Da is highly conserved among species and exhibits functional similarity to members of the fibroblast growth factor family (Kurtz et al., 1995). The bovine gene product has been recently cloned (Ikeda et al., 2000a), and resulting recombinant MK enhanced development to the blastocyst stage of bovine oocytes, being its effect mainly mediated by cumulus-granulosa cells during in vitro maturation (IVM) (Ikeda et al., 2000a,b).

In addition to MK, insulin-like growth factor-I (IGF-I) is a potential mediator of RA. After retinoid treatment, IGF-I increased in follicles of gilts (Whaley et al., 2000), in rat testes (Bartlett et al., 1990) and osteoblasts (Gabbitas and Canalis, 1997), and in mouse embryonic tissue (Qin et al., 2002). Transcripts for IGF-I receptor were noted in the bovine oocyte (Yoshida et al., 1998; Yaseen et al., 2001). Accordingly, the presence of ligand was beneficial to IVM both in cumulus enclosed (Harper and Brackett, 1992; Lorenzo et al., 1994; Pawshe et al., 1998; Sirotkin et al., 1998) and in denuded bovine oocytes (Pawshe et al., 1998; Sirotkin et al., 1998). This suggests that the effect of IGF-I is not necessarily mediated by secretions of surrounding cumulus cells, although IGF-I increases responsiveness of granulosa cells to FSH (Monget and Bondy, 2000). In contrast to IGF-I, RA decreases expression of the FSH receptor (Minegishi et al., 1996, 2000). Nevertheless, the detailed pattern of mRNA expression and production of IGF-I in bovine follicles remains controversial. Cattle granulosa cells were reported to produce IGF-I in vitro (Spicer et al., 1993; Bao et al., 1995; Spicer and Chamberlain, 1998, 2000; Sirotkin et al., 1998), but one study concluded that IGF-I is not produced by these cells (Gutierrez et al., 1997). In the same way, IGF-I mRNA was detected in bovine granulosa cells (Spicer et al., 1993; Spicer and Echternkamp, 1995; Yuan et al., 1998; Schams et al., 1999), although other works report null (Armstrong et al., 2000) or weak IGF-expression in only 17% of the follicles evaluated (Perks et al., 1999). Upon in vitro culture, IGF-I mRNA is detected in very low amounts in granulosa cells (Armstrong et al., 2000; Spicer and Chamberlain, 2000). To our knowledge, studies on IGF-expression in follicles are abundant in mural granulosa cells and scarce in cumulus cells, which are more important for embryonic development in vitro.

Since RA acts on cells to establish or change the pattern of gene activity, this retinoid could influence cytoplasmic maturation and subsequent capacity of the oocyte to progress in development. Apart of the oocyte itself, the influence of the RA could be exerted through cumulus-granulosa cells. In the present work, we aim to determine whether the effect of RA during IVM, as a promoter of bovine embryo development in vitro, might be explained in part via expression of *MK* and *IGF-I* genes. Thus, we firstly made a study to find out the range into which 9-*cis*-RA during IVM is able to sustain in vitro embryonic development up to the blastocyst stage. Subsequently, cytoplasmic granular migration in oocytes, levels of expression of *MK* and *IGF-I* genes in cumulus-granulosa cells, and blastocyst viability, measured as survival to cryopreservation, were analyzed in the presence of a selected concentration of 9-*cis*-RA.

MATERIALS AND METHODS

Oocyte Recovery

Ovaries from slaughtered Asturiana de los Valles cows were placed in NaCl solution (9 mg ml^{-1}) containing antibiotics (penicillin, 100 UI ml^{-1} and streptomycin sulfate, 100 μg ml^{-1}) and maintained at 30–35°C until recovery of cumulus–oocyte complexes (COCs). Ovaries were washed twice in distilled water and once in freshly prepared saline. COCs were aspirated from 2- to 7-mm visible follicles through an 18-gauge needle connected to a syringe and recovered into a 50-ml Corning tube. Follicular fluid and COCs were placed in an ovum concentrator (Em-Con, Comextrade, Tarragona, Spain) and rinsed three times with holding medium (HM) (TCM199-Gibco, Barcelona, Spain + 25 mM HEPES + BSA 0.4 g L^{-1}) supplemented with 2 UI ml^{-1} of heparin.

In Vitro Maturation

Only oocytes enclosed in a compact cumulus with evenly granulated cytoplasm were selected for maturation. The COCs were washed three times in HM and twice in maturation medium, which consisted of TCM199, porcine FSH (1 μg ml^{-1}), LH (5 μg ml^{-1}), 17β-estradiol (1 μg ml^{-1}), and polyvinyl alcohol (PVA) (0.5 mg ml^{-1}). Maturation was performed by culturing approximately 50 COCs in 500 μl of maturation medium in 4-well dishes at 39°C in 5% CO_2 in air and high

humidity for 22–24 hr. For its use in IVM, 9-*cis*-RA was solved in ethanol, aliquoted, and stored at −80°C in the darkness.

Gene Expression in Cumulus-Granulosa Cells

COCs were selected according to the above criteria. Cumulus-granulosa cells were detached from groups of ten oocytes using 0.05% hyaluronydase and repeated pipeting. To avoid possible individual differences, groups of ten cumulus cells masses from the same treatment were pooled to carry out RNA isolation following the recommended Trizol reagent protocol (Invitrogen Corp., Carlsbad, CA). Samples were treated with DNAse-I and total RNA diluted in diethyl pyrocarbonate treated H_2O. Relative amounts of MK and IGF-I mRNA were determined by a semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay as a proportion of the housekeeping gene *β-actin* (Bustin, 2000).

Two pairs of primers were used to analyze IGF-I expression (see Table 1), one of which previously showed to detect IGF-I expression in bovine oocytes (Lonergan et al., 2000). Each primer within the couple was located in different exons in order to distinguish between DNA and RNA amplification through size of the amplicons. RT-PCR was carried out using the Superscript One-Step RT-PCR with Platinum Taq (Invitrogen) protocol, in a GeneAmp PCR System 9700 Thermal Cycler (P.E. Applied Biosystems, Foster City, CA). Suitable RT-PCR protocols were tested to find the lowest number of cycles ensuring enough RNA amplification as to be detected in ethidium bromide stained agarose gel. Finally, β-actin RT-PCR protocol consisted of 37 cycles, while MK and IGF-I protocols consisted of 40 cycles. The RT-PCR amplifications were preceded by a cDNA synthesis and pre-denaturation at 50°C for 30 min and 95°C for 5 min, followed by the corresponding number of cycles each. These cycles were made at 94°C for 15 sec, 55°C for 30 sec, and 72°C for 60 sec, including for each gene 200 ng a sample of total RNA from human liver (Ambion®) as a positive control. Twenty microliters of the RT-PCR products were loaded into an agarose 2% gel in TBE buffer containing ethidium bromide, and were separated by horizontal electrophoresis. Three RT-PCR replicates were obtained from each total RNA sample. To confirm the identity of MK, its RT-PCR product was cut with the diagnostic restriction enzyme HpaII (Amersham Biosciences, Freiburg, Germany). RT-PCR products from each gene within a sample were loaded into the same agarose gel, visualized under ultraviolet light and digitally photographed. Images were analyzed using the software Photo-capt® (version 99.03, 1999, Ecogen S.R.L., Barcelona, Spain). The intensity of bands was measured and values of amplification were calculated as a proportion of the amount of β-actin amplified in each group.

Labeling of oocytes with fluoroscein isothiocyanate (FITC)-lectins and CG and chromosomal staining.

After removal of surrounding cumulus cells with a narrow glass pipette, zona pellucida was removed with 0.1% pronase. Zona-free oocytes were washed three times, fixed with 2% paraformaldehyde in PBS for at least 12 hr in a 35-mm dish at 5°C, and washed four times in blocking solution (PBS containing sodium azide and 100 mM glycine). Oocytes in blocking solution were incubated in 10 μg/ml FITC-labeled *Lens Culinaris* agglutinin (LCA, FL-1041, Vector Labs, Inc., Burlingame, CA) for 15 min in the dark. Chromatin was stained with 10 μg/ml propidium iodide for 5 min. After staining, oocytes were washed and mounted between a coverslip and a glass slide supported by silicone (Lorenzo et al., 1994) with antifade mounting medium (Vectashield; Vector Labs, Inc.), and the coverslip was sealed with nail polish. Samples were examined using laser-scanning confocal microscopy. Laser-scanning confocal microscopy was performed using a Bio-Rad MRC 1024 ES equipped with a Krypton ion laser for the simultaneous excitation of fluorescein for CG and propidium iodide for DNA (488, laser line and 680 DF 32, respectively). Oocytes serving as controls were treated using the same procedure and were incubated in blocking solution without (FITC)-labeled LCA. Images were recorded digitally and archived on an erasable magnetic optical disk. Typical examples of the CG distribution in the oocytes are shown in the figures.

In Vitro Fertilization

Sperm separation was carried out using a swim-up procedure similar to that reported by Parrish et al. (1986). Briefly, semen from one frozen straw of a single bull was thawed in a water bath and added to a polystyrene tube containing 1 ml of pre-equilibrated Sperm-TALP. After 1 hr of incubation, approximately 700 μl of the upper layer of supernatant containing the motile sperm was removed. The sperm were centrifuged for 7 min at 200*g* and the supernatant aspirated to leave a pellet of approximately 100 μl in volume. Sperm concentration was determined with a haemocytometer.

TABLE 1. Forward and Reverse Primers and Amplicon Expected Sizes Used to Assess Gene Expression in Bovine Cumulus Granulose Cells

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size (pb)
β-Actin	GACTACCTCATGAAGATCCTC	CGGATGTCCACGTCACACTTC	299
Midkine (MK)	CAAGGACTGCGGCGTGGGATTC	CTGGGCATTGTATCGCGCCTTC	199
IGF-I	TCTCCAGTTCGTGTGCGGAGAC	TCGACTCCCTCTACTTGTGTTC	255
IGF-I[a]	AAGATGCCCATCACATCCTCC TCG	TTCTGAGCCTTGGGCATGTCGG	334

[a]Primers used by Lonergan et al. (2000) to amplify IGF-I in bovine oocytes.

The COCs were washed twice in HM and placed in 4-well culture dishes containing pre-equilibrated fertilization medium (Fert-TALP) with heparin (10 μg ml^{-1}, Calbiochem, La Jolla, CA). Spermatozoa were then added at a concentration of 2×10^6 cells ml^{-1} in 500 μl of medium per well containing 100 COCs for maximum. In vitro fertilization (IVF) was accomplished by incubating oocytes and sperm cells together for 18–20 hr at 39°C in 5% CO_2 and high humidity.

In Vitro Culture

Presumptive zygotes were vortexed for 2 min to separate cumulus cells and washed three times in HM and once in SOF before being transferred to droplets. Embryo culture was performed in SOF containing amino-acids, citrate, and myo-inositol (Holm et al., 1999) (285 mOsm, pH 7.2–7.3), to which 10% FCS was added at 42 h post-fertilization (PI). Droplets of culture medium (1–2 μl/embryo) were prepared in 4-well dishes under mineral oil and equilibrated in incubator for 2 hr before addition of zygotes. Culture was carried out at 39°C, 5% CO_2, 5% O_2, and 90% N_2. Culture media were renewed at 66 hr (day 3) and 138 hr (day 6) PI, and embryonic development was evaluated on days 3, 6, 7 and 8.

Embryo Vitrification and In Vitro Survival

Very good quality (grade 1; Lindner and Wrigth, 1983), day 7 expanded blastocysts were vitrified as described by Kaidi et al. (2001). Embryos were kept in 10% glycerol in ovum culture medium (OCM) with 20% FCS for 5 min. Then, blastocysts were incubated in 20% glycerol + 10% ethylene glycol in OCM with 20% FCS. Finally, the samples were washed in 25% glycerol + 25% ethylene glycol in OCM with 20% FCS. Embryos were loaded into 0.25-ml straws between two columns of a 0.85 M galactose solution in OCM and separated from the embryos by air bubbles. The straws were placed in liquid nitrogen vapors for 2 min before being plunged into liquid nitrogen.

The vitrified embryos were warmed by 5 sec in air and 10 sec in a water bath at 30°C. The content of the straws was emptied in a Petri dish and embryos were mixed in the galactose solution by slight agitation. After 5 min, embryos were transferred to OCM with 20% FCS during 5 min. Warmed embryos were washed in B2 medium (INRA) + 5% FCS and co-cultured in 4-well dishes (500 μl) on a confluent monolayer of Vero cells at 39°C, 5% CO_2 in air and high humidity.

Experimental Design

Experiment 1. The effect of a physiological dosage of 9-*cis*-RA during IVM was investigated in an in vitro embryo culture system. Groups of COCs were allotted to the next groups: (1) 9-*cis*-RA 5 nM; (2) 9-*cis*-RA 50 nM; (3) 9-*cis*-RA 500 nM; and (4) 9-*cis*-RA 0 nM (negative control). Oocytes were fertilized and cultured in vitro as described above. The effect of 1% ethanol (vehicle for 9-*cis*-RA) in same maturation conditions and presence of 9-*cis*-RA has been evaluated elsewhere for in vitro embryo development (Hidalgo et al., 2003).

Experiment 2. The expression of *MK* and *IGF-I* genes within cumulus-granulosa cells during IVM was analyzed by semi-quantitative analysis. Cumulus-granulosa cells were detached from COCs: (1) immature; and matured with (2) 9-*cis*-RA 5 nM (selected as a physiological dose resulting from Experiment 1); (3) 9-*cis*-RA 0 nM; and (4) 1% ethanol (vehicle).

Experiment 3. Oocytes matured in vitro in the presence of 9-*cis*-RA 5 nM, 9-*cis*-RA 0 nM, and immature were labeled with FITC-lectins for CG migration and chromosomal staining.

Experiment 4. Blastocysts were vitrified, warmed, and cultured in vitro as an indirect test for viability. Blastocysts derived from oocytes matured in the presence of: (1) 9-*cis*-RA 5 nM; (2) 9-*cis*-RA 0 nM; (3) 1% ethanol; and (4) 10% FCS (introduced as a positive control for in vitro survival).

Statistical Analysis

Data from embryo development and blastocyst survival were considered as categorical variables, and therefore, analyzed previously by PROC CATMOD of SAS (SAS Version 8.2, 1999; SAS Institute, Inc., Cary, NC). As a second step, those effects showing a significant influence on the analyzed variables (treatment and replicate) were used to fit a linear model using PROC GLM of SAS. Values are expressed as frequency percentages of matured oocytes (embryo development), vitrified and warmed blastocysts (in vitro survival after vitrification), and proportions of the expression level of the constitutive control gene, *β-actin* amplified in each group (expression level of *MK* and *IGF-I* genes in cumulus-granulosa cells). For gene expression in cumulus-granulosa cells, the model included treatment and number of samples of CG as fixed effects. Least square means and their correspondent standard errors were estimated for all fixed effects showing a significant *F* value. Duncan's multiple-range test was performed on these main-effect means.

RESULTS

Experiment 1

As shown in Table 2, a concentration of RA 500 nM during IVM was toxic, while comparable stimulatory embryo development rates were obtained in the 5–50 nM range. Cleavage rates (data not shown in table) were negatively affected by RA 500 nM (10.6 ± 6.9), but did not differ between other treatments (values comprised between 80.4 ± 4.3 and 89.0 ± 2.6).

Experiment 2

Although an amplicon of the expected size was found in liver RNA controls, RT-PCR failed to detect IGF-I expression in cumulus-granulosa cells regardless the primers used. However, as seen in Table 3, expression of MK did not increase during maturation and showed to be dependent on 9-*cis*-RA but not on ethanol (Fig. 1).

TABLE 2. Effect of 9-*cis*-Retinoic Acid (RA) on Embryonic Development During In Vitro Maturation (IVM) of Bovine Oocyte–Cumulus Complexes (COCs)

					Blastocysts			
Treatment	N	R	5–8 Cells	Morulae	Day 6	Day 7	Day 8	Expanded
RA 5 nM	185	5	58.9 ± 5.9^{a}	50.7 ± 3.5^{a}	17.8 ± 2.1^{a}	35.0 ± 2.9^{a}	36.5 ± 3.4^{a}	18.7 ± 1.7^{a}
RA 50 nM	130	4	50.8 ± 8.0^{a}	51.4 ± 4.7^{a}	14.8 ± 2.8^{a}	$28.5 \pm 3.9^{a,b}$	$32.7 \pm 4.5^{a,b}$	19.2 ± 2.3^{a}
RA 500 nM	62	3	3.7 ± 3.2^{b}	2.9 ± 2.7^{b}	0.0^{b}	0.0^{c}	0.0^{c}	0.0^{b}
RA 0 nM	201	6	56.2 ± 6.0^{a}	44.1 ± 3.5^{a}	12.0 ± 2.1^{a}	20.8 ± 2.9^{b}	23.9 ± 3.4^{b}	10.5 ± 1.7^{c}

N, number of matured oocytes; R, number of replicates per group.
Data are least square means percentages of matured oocytes ± SE. Different superscripts show significant differences: $^{a,b,c}(P < 0.05)$.

Vitrification

Upon warming (2 hr), embryos in all groups re-expanded at high rates, but only blastocysts matured with serum survived and hatched from 48 to 72 hr (Table 4). Blastocysts from oocytes matured without additives tended to re-expand at higher rates.

Cytoplasmic Maturation Study

Twenty one oocytes were examined for cytoplasmic granules (CG) localization and migration. The pattern in immature oocytes (n = 10) were CG distributed only in the central medullar zone and nucleus at GV stage (Fig. 2a). All oocytes matured in the absence of RA (n = 6) showed CG located between the oolemma and the medullar zone, as a sign of incomplete cytoplasmic maturation (Fig. 2b). The presence of RA during maturation allowed oocytes (four out of five) to show completely migrated CG and to form clusters lining the oolemma, with the M-II nuclear stage (Fig. 2c). The remainder RA matured oocyte showed GC located between the oolemma and the medullar zone and M-I nuclear stage.

DISCUSSION

The presence of 9-*cis*-RA 5 nM during IVM improved blastocyst formation, while 9-*cis*-RA 500 nM was toxic. The supply of retinoid to the embryos must be ensured within a physiological range, since both excess and deficiency of retinoid cause abundant teratogenic defects due to its pleiotrophic activity. These detrimental effects could be related to a well documented retinoid imbalance associated with developmental abnormality (see Morris-Kay and Ward, 1999 for review). Cellular retinoic acid binding protein (CRABP) protects against RA excess and limits the transcriptional potential of RA (Morris-Kay and Ward, 1999). Although CRABP has not been investigated in bovine, the existence of CRABP II in rats is restricted to granulosa cells from mature follicles and luteal cells (Bucco et al., 1995; Wardlaw et al., 1997; Zheng et al., 1999). Therefore, protection against RA in cumulus-granulosa cells surrounding immature bovine oocytes, such as those used in the present experiments, could be reduced. This could explain in part the observed toxicity exerted by 9-*cis*-RA 500 nM.

The retinol concentration in human serum is 1–2 μM, and fetal bovine serum contains less than 20 nM (see Lane et al., 1999). Recently we obtained improved day 60 pregnancy rates after transfer to recipients of blastocysts derived from oocytes matured with 9-*cis*-RA (Hidalgo et al., 2003). This retinoid compound improved blastocyst development and quality (i.e., increased inner cell mass/trophectoderm cell proportions) during permissive maturation (Hidalgo et al., 2003), as well as during a roscovitine-inhibited prematuration period (Duque et al., 2002). Superior blastocyst rates were obtained by Ikeda with all-*trans*-RA 1 μM (personal communication), an isomer reported to be 25 times less potent that 9-*cis*-RA (Thaller et al., 1993). However, blastocyst production decreased by overexposure of bovine oocytes to retinoid (Duque et al., 2002), a detrimental effect analogous to suggested with oocytes matured with RA recovered from cows treated with retinol (Hidalgo et al., 2002). The developmentally stimulating effects of RA are consistent with an increased proportion of blastocysts recovered from donors treated with retinol found by other authors (Shaw et al., 1995; Whaley et al., 1997, 2000; Eberhardt et al., 1999). Both RA and ROH could act via cumulus-granulosa cells, directly on the oocyte or both, in an autocrine and/or paracrine manner. Although the present work provides evidence for responsiveness to RA in granulosa cells and within the COC as a whole, the immature oocyte exhibits retinoid receptors (Mohan et al., 2001, 2002) and retinol-binding proteins (RBP) (Mohan et al., 2001).

TABLE 3. Relative MK Expression Levels in Cumulus-Granulosa Cells Detached From COCs Immature, and In Vitro Matured in the Presence of 9-*cis*-RA 5 nM, Ethanol (Vehicle) or no Additives

			Relative abundance
Treatment	N	R	MK/actin (%)
9-*cis*-RA 5 nM	12	5	82.8 ± 4.9^{x}
9-*cis*-RA 0 nM	17	6	70.5 ± 4.0^{y}
Ethanol 1% (vehicle)	13	5	67.9 ± 4.7^{y}
Immature	17	6	63.3 ± 4.0^{y}

Data are least square means ± SEM. Different superscripts show significant differences: $^{x,y}(P < 0.01)$. Experiments were conducted as PCR numbers (N) run on a number of repeated cumulus-granulosa cell samples from groups of (R).

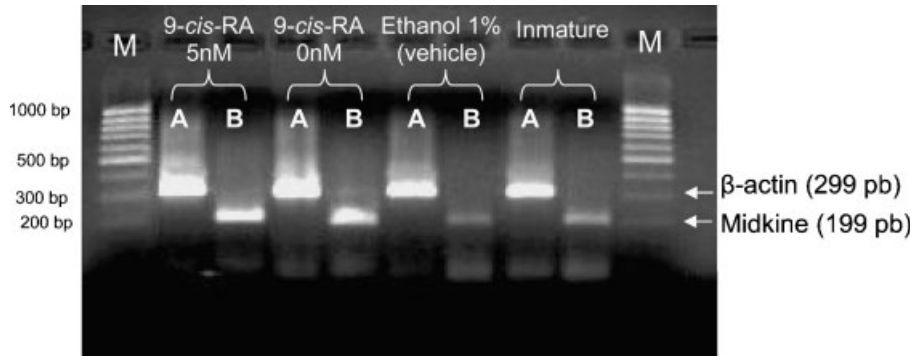

Fig. 1. Detection of β-actin and midkine (MK) bovine transcripts by reverse transcriptase-polymerase chain reaction (RT-PCR) in 2% agarose gel with 100 ng of DNA-marker (100 bp ladder, **line M**). **Line A** shows β-actin (299 bp) and **line B** shows MK (199 bp) within each treatment. Experimental groups correspond to cumulus-granulosa cells adjacent to oocytes immature or matured in the presence of 9-*cis* of retinoic acid (9-*cis*-RA) 0.5 nM, 9-*cis*-RA 0 nM, and 1% ethanol.

MK gene was selected to be analyzed in the present study upon the basis of its responsiveness to retinoic and its stimulating effect on oocyte development. The *MK* gene product could be an intermediary in the above reported developmental phenomena (Griffith and Zile, 2000). In rat granulosa cells, 0.3 μM RA resulted in a two fold increase in MK mRNA levels (Minegishi et al., 1996), and 1 μM RA altered LH receptor mRNA expression (Hattori et al., 2000). The embryotrophic effects of MK reported by Ikeda et al. (2000a,b) reproduce in part the effect of RA within the present work. The inducing effect of RA on *MK* gene expression is in contrast with the reduced transcriptional activity existing during IVM. However, in the beginning of this period, some weak ^{3}H-uridine labeling of the GV area (Pavlok et al., 2000) was seen together with incomplete inactivation of hnRNA synthesis (Hyttel et al., 1997). Although limited in time, this remnant transcription would contribute to explain the beneficial effect of RA.

Ethanol did not affect *MK* gene expression in maturing cumulus-granulosa cells. The absence of short-term effects of ethanol is consistent with normal nuclear maturation and pronucleus formation after incubation of COCs with 1 and 2% ethanol for 1 day (Avery and Greve, 2000). At same time, we recently have found that 1% ethanol does not promote oocyte activation (Hidalgo et al., 2003). However, in contrast with the present results, 1% ethanol reduced blastocyst development in maturation medium containing BSA and epidermal growth factor (Avery and Greve, 2000). In addition, a severe inhibition of trophectodermal differentiation was observed with 1% ethanol during IVM (Hidalgo et al., 2003).

Despite expression of IGF-I has been reported in bovine oocytes (Lonergan et al., 2000), the absence of both proper and RA-induced expression of IGF-I in cumulus-granulosa cells is consistent with works reporting null (Armstrong et al., 2000) or scarce (Perks et al., 1999) expression of this growth factor in the bovine antral follicle. It should be considered that the source of granulosa cells used in all IGF-I production and/or expression studies cited in the present work was not those cells surrounding the oocyte, which represents an important difference to the biological materials used in our experiments. In this support, functional differences associated to IGF-I between mural granulosa and cumulus cells have been described in cattle (Ingman et al., 2000; Li et al., 2000), being the oocyte itself responsible in part for this different phenotype (Li et al., 2000). Interestingly, growth hormone (GH) was found to

TABLE 4. Post-Warming Development in Medium B2 With Vero Cells of Vitrified Day 7 Bovine Expanded Blastocysts Derived From COCs Matured in Defined Media Containing 9-*cis*-RA, Ethanol (Vehicle), Serum (FCS) as a Positive Control or no Additives

		Re-expansion rate		Hatching rate	
Treatments	N	2 hr	48 hr	48 hr	72 hr
RA 5 nM	23	76.5 ± 8.1	1.9 ± 5.1^{a}	0.0^{x}	1.9 ± 1.6^{x}
RA 0 nM	22	70.4 ± 8.1	16.2 ± 5.0^{a}	0.0^{x}	0.0^{x}
Ethanol	20	65.1 ± 9.7	5.3 ± 6.1^{a}	1.8 ± 2.1^{x}	3.8 ± 1.9^{x}
FCS	30	63.0 ± 9.7	35.5 ± 6.1^{b}	25.5 ± 2.1^{y}	24.9 ± 1.9^{y}

Data from four replicates were least square mean percentages of warmed and cultured blastocysts ± SE. ANOVA and Duncan's test. Different superscripts show significant differences: $^{a,b}(P < 0.05)$; $^{x,y}(P < 0.01)$.

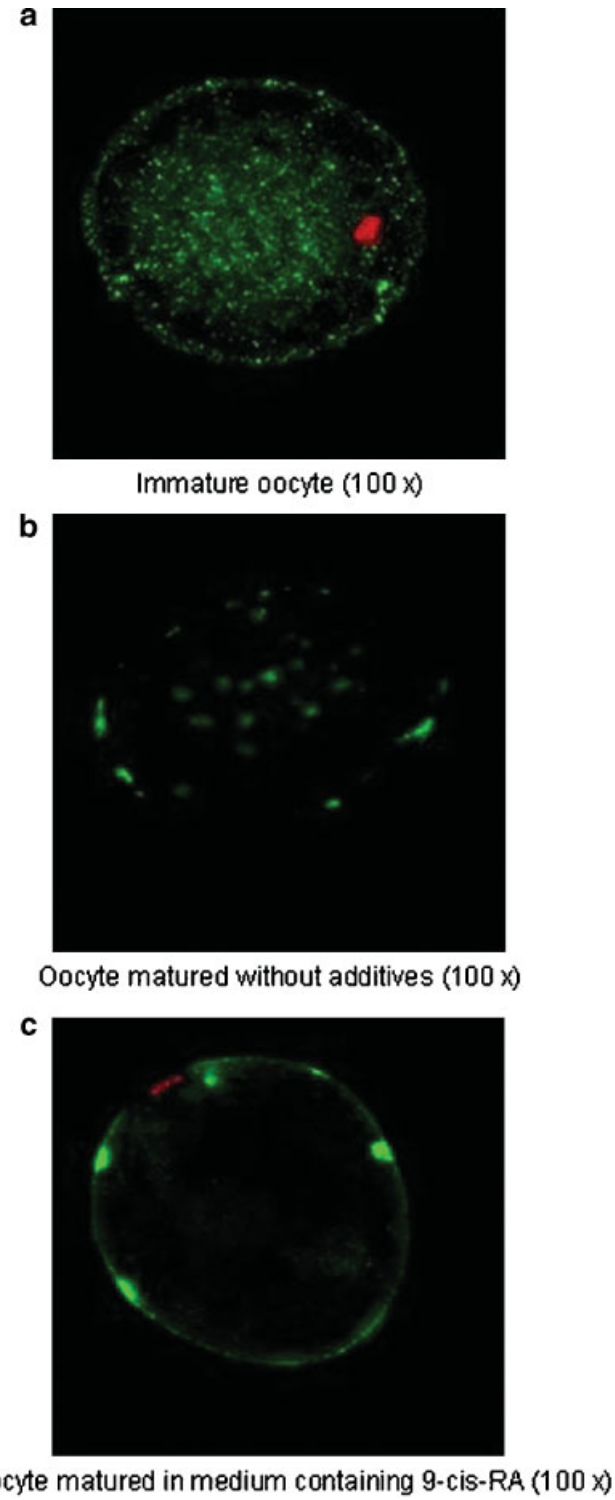

Fig. 2. Confocal microscopic images of bovine oocytes after staining with fluorescein isothiocyanate (FITC)-LCA and propidium iodide showing the pattern of cortical granules distribution (**a**, **b**, and **c**; green) and chromatin configuration (a, metaphase I, and c, metaphase II; red). Images were taken at equatorial section of each oocyte. The bar represents 25 μm.

stimulate embryonic development via cumulus cells, but its effect is not mediated by IGF-I (Izadyar et al., 1997), as opposed to the GH mechanism in other tissues. The case of RA in the present work can bear similar explanation, and collectively these data suggest that there is little or no IGF-I transcriptional activity in cattle cumulus cells. In this respect, most of the IGF-I present in follicular fluid has been suggested to be derived from the circulation (Perks et al., 1999).

Cortical granule migration is a common phenomenon in mammalian oocytes during maturation both in vivo and in vitro (Yanagimachi, 1994; Wang et al., 1997). This migration is associated with a gain in developmental competence by the oocyte (Hosoe and Shioya, 1997). Cortical granules are distributed in the cytoplasm of immature oocytes at the GV stage. As maturation resumes once the oocyte is freed from its follicle, the granules migrate to the cortex and occupy the area just beneath the oolemma. At same time the nucleus enters M-II stage. In contrast to untreated oocytes, we observed that 9-*cis*-RA induced complete GC migration and clusters lining the oolemma, which suggests a beneficial role for 9-*cis*-RA in the oocyte developmental competence. This effect, reported as well in meiotically inhibited oocytes (Duque et al., 2002), corresponds to an improved embryonic development in our study.

Re-expansion and hatching in somatic cell co-cultures after cryopreservation is a common test for embryonic viability. In the present work, embryos matured with RA survived at 4 hr post-warming at higher rates than the other groups. Twenty-four hours after warming, embryos matured without serum did not survive in any group, and only embryos matured with serum hatched in co-culture with Vero cells. Our results strongly contrast with those found by Cho et al. (2001) which obtained higher hatching rates after thawing with embryos derived from oocytes matured without serum. However, Dochi et al. (2001) observed improved hatching rate in frozen/thawed embryos derived from serum-free maturation medium.

Although higher concentrations of 9-*cis*-RA can be toxic for the oocyte development in vitro, in the present work, we have provided evidence that 9-*cis*-RA 5 nM, a physiological concentration, is able to stimulate expression of MK. The gene expression detected was unaffected by ethanol, although ethanol at concentration higher than 5% could be inhibitory. The presence of 9-*cis*-RA during IVM stimulated embryonic development, such as the recombinant product of *MK* gene does. However, IGF-I expression was undetected, both in dependence on or in the absence of a 9-*cis*-RA treatment acting on cumulus-granulosa cells. The ability of vitrified blastocysts to survive in vitro was not improved by 9-*cis*-RA, although the fact that only blastocysts, which were oocytes matured with serum would survive, suggests that more factors should be considered when evaluating cryopreservation survival. The complete granular migration in the cytoplasm of oocytes matured with 9-*cis*-RA anticipates the observed gain in developmental competence of the oocyte.

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