Pregnancies and improved early embryonic development with bovine oocytes matured *in vitro* with 9-*cis*-retinoic acid

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Retinoids have an important role in cell growth, morphogenesis and differentiation. In the present study the developmental potential of bovine oocytes was examined after *in vitro* maturation in the presence of 9-*cis*-retinoic acid, a vitamin A metabolite, at 5 nmol l⁻¹ in chemically defined conditions. Experiments studied early *in vitro* development, blastocyst differential cell counts and the capacity of embryos to establish pregnancy after transfer to recipients. After *in vitro* fertilization and culture in simple medium, blastocyst development and hatching rates increased in oocytes matured with 9-*cis*-retinoic acid. Addition of ethanol (used as a solvent for 9-*cis*-retinoic acid) resulted in higher cell counts and proportions of cells in the inner mass of day 7 blastocysts. Day 8 blastocysts represented most differences observed in the number of

cells. In these embryos, ethanol and 9-*cis*-retinoic acid increased both the number of cells and proportions in the inner mass. However, while ethanol treatment reduced the number of cells in the trophectoderm, 9-*cis*-retinoic acid did not. The total number of cells was unaffected by treatment within 1 day, although untreated oocytes lead to day 8 blastocysts with reduced total cell counts. Once transferred to recipients, both fresh and vitrified-warmed blastocysts derived from oocytes matured with 9-*cis*-retinoic acid yielded more pregnancies at day 60. Modifications of retinoid metabolism affect development and trophectoderm differentiation, and *in vitro* maturation with 9-*cis*-retinoic acid increased the developmental competence of the oocyte.

Introduction

Vitamin A (all-*trans*-retinol) and its metabolites are regulators of cellular growth, embryonic morphogenesis and differentiation in many types of cell. These compounds, members of the retinoid family, play a fundamental role in controlling events during the cell cycle, affecting decisions to continue proliferation or to differentiate along a specific pathway. The all-*trans*-retinol metabolite retinoic acid is the most important retinoid for vertebrate embryogenesis (Morris-Kay and Ward, 1999), although maintenance of pregnancy in mammals is strongly dependent on all-*trans*-retinol. *In vivo*, all-*trans*-retinoic acid binds to retinoic acid receptors (RARs; α , β and γ), and 9-*cis*-retinoic acid binds specifically to retinoid X receptors (RXRs; α , β and γ) (Mangelsdorf *et al.*, 1994; Chambon, 1996); all-*trans*-retinoic acid is converted to 9-*cis*-retinoic acid and other isomers in a reversible way. However, isomerization does not occur *in vitro* and 9-*cis*-retinoic acid activates both RXRs and RARs. When activated, the RAR–RXR nuclear receptors heterodimerize and are capable of binding to specific DNA sequences named retinoic acid response elements (RAREs), to increase or decrease the expression of a gene.

In addition, the RXRs may form homodimers and other heterodimers with thyroid hormone receptor, vitamin D receptor and peroxisome proliferator-activated receptor (Chambon, 1996), which may explain the pleiotrophic effects of retinoids. Information of RXRs and 9-*cis*-retinoic acid, the so-called second retinoid signalling pathway, is scarce and focused on later embryonic stages in mice (Mangelsdorf *et al.*, 1992; Dolle *et al.*, 1994).

Recently, subtypes RAR α , RAR γ , RXR α , RXR β and retinaldehyde dehydrogenase-2 (RALDH2) have been detected in bovine embryos developed *in vitro*, from the oocyte to the hatched blastocyst stage (Mohan *et al.*, 2001, 2002). Immunostaining revealed the presence of proteins for RAR α and RAR β (Mohan *et al.*, 2001) and RXR β (Mohan *et al.*, 2002) in the trophectoderm and in the inner cell mass (ICM) from zona pellucida included and hatched blastocysts. Accordingly, evidence was given that the developmental capacity of the oocytes can be stimulated by retinoids. Thus, all-*trans*-retinol administration to donor animals improved embryonic quality in both superovulated cows (Shaw *et al.*, 1995) and sheep (Eberhardt *et al.*, 1999), and in non-superovulated gilts (Whaley *et al.*, 1997, 2000). In addition, more oocytes and embryos were obtained in response to superovulation from rabbits that had higher blood concentrations of vitamin A (Besenfelder *et al.*, 1996). Increased all-*trans*-retinol concentrations

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are characteristic of healthy, in contrast to atretic, follicles in sheep (Eberhardt *et al.*, 1999). In our laboratory donor cows injected with all-*trans*-retinol yield more oocytes (Hidalgo *et al.*, 2002), and oocytes pre-matured with 9-*cis* retinoic acid show increased development, cryo-preservation tolerance and quality of blastocysts (Duque *et al.*, 2002). The effects of all-*trans*-retinol are regulated by specific linkage to retinol-binding proteins (RBP). These chaperone elements are found in bovine oocytes (Mohan *et al.*, 2001), whereas the cellular RBP (CRBP) is detected in rat oocytes, nearby granulosa cells (Wardlaw *et al.*, 1997) and in human ovaries (Ong and Page, 1986). At the cell, all-*trans*-retinol is metabolized to retinoic acid, which binds to cellular retinoic acid-binding proteins (CRABP). All-*trans*-retinol accumulates in the cell forming more stable retinyl esters, with palmitate the usual combination for storage. Palmitate is the most abundant fatty acid in bovine (McEvoy *et al.*, 2000; Kim *et al.*, 2001), porcine and ovine (McEvoy *et al.*, 2000) oocytes. This evidence suggests that endogenous retinoid could have an effect on oocyte development and should be taken into account in experiments in which exogenous compounds are incorporated.

A site-restricted synthesis of retinoic acid from all-*trans*-retinol has been described (Morriss-Kay and Ward, 1999), but little is known about which cells produce retinoic acid and under what circumstances. Rat granulosa cells synthesize retinoic acid from all-*trans*-retinol (Zheng *et al.*, 1999), and the presence of RARs demonstrates that these cells are targets for retinoic acid in rats, humans and mice (Zhuang *et al.*, 1994). Retinoids could act to modify the transcriptional activity within the cumulus-oocyte complex (COC), exerting their effects directly on the oocyte, the adjacent cumulus-granulosa cells or both. As a consequence, *in vitro* maturation (IVM) and subsequent development might be influenced by retinoids. In the present study the effects of 9-*cis*-retinoic acid added during the bovine IVM on embryonic development were investigated, together with quality of blastocyst produced, measured as differential cell counts, and the ability to establish pregnancies once transferred into recipients.

Materials and Methods

All chemicals were purchased from Sigma (Madrid), unless otherwise indicated.

Oocyte recovery

Oocytes for *in vitro* experiments were obtained from bovine ovaries at an abattoir, whereas blastocysts transferred to recipients were obtained from healthy cows by ovum pick-up. Ovaries from Asturiana de los Valles cows were placed in NaCl (9 mg ml⁻¹) containing antibiotics (100 iu penicillin ml⁻¹ and 100 µg strepto-

mycin sulphate ml⁻¹) and maintained at 30–35°C until recovery of COCs. Ovaries were washed twice in distilled water and once in freshly prepared saline. COCs were aspirated from 2–7 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, Comex-trade, Tarragona) and rinsed three times with holding medium consisting of TCM199 (Invitrogen, Barcelona), 25 mmol Hepes l⁻¹ and 0.4 g BSA l⁻¹, supplemented with 2 iu heparin ml⁻¹.

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 holding medium and twice in maturation medium, which consisted of bicarbonate-buffered TCM199, 1 µg FSHp ml⁻¹, 5 µg LH ml⁻¹, 1 µg oestradiol ml⁻¹ and 0.5 mg polyvinyl alcohol ml⁻¹. 9-*cis*-Retinoic acid was dissolved in ethanol, divided into aliquots and stored at -80°C in the dark. Treatments within maturation were: (1) 5 nmol 9-*cis*-retinoic acid l⁻¹; (2) 1% ethanol (vehicle); (3) no additives (negative control); and (4) 10% fetal calf serum (FCS; positive control). Maturation was performed by culturing approximately 50 COCs in 500 µl of maturation medium in four-well dishes at 39°C in 5% CO₂ in air and high humidity for 22 to 24 h.

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-Tyrodé's albumin lactate pyruvate (TALP). After 1 h of incubation, approximately 700 µl of the upper layer of supernatant containing the motile spermatozoa was removed. The spermatozoa were then centrifuged for 7 min at 200 g and the supernatant was aspirated to leave a pellet of approximately 100 µl. Spermatozoa concentration was determined with a haemocytometer. The COCs were washed twice in holding medium and placed in four-well culture dishes containing pre-equilibrated fertilization medium (Fert-TALP) with 10 µg heparin ml⁻¹ (Calbiochem, La Jolla, CA). Spermatozoa were then added at 2 × 10⁶ cells ml⁻¹ in 500 µl of medium per well containing ≤100 COCs. IVF was accomplished by incubating oocytes and sperm cells together for 18–20 h at 39°C in 5% CO₂ and high humidity. As a control for oocyte activation induced by ethanol, some COCs matured in 1% ethanol and negative control were cultured in Fert-TALP with heparin but without spermatozoa.

In vitro culture

Presumptive zygotes were vortexed for 2 min to separate cumulus cells and washed three times in holding medium and once in synthetic oviduct fluid (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 5% FCS was added at 66 h after fertilization. Droplets of culture medium (1–2 μ l per embryo) were prepared under mineral oil in four-well dishes and equilibrated in an incubator for 2 h before addition of zygotes. Culture was carried out at 39°C, 5% CO₂, 5% O₂ and 90% N₂. Culture media were renewed at 66 h (day 3) and 138 h (day 6) after fertilization, and embryonic development was evaluated on days 3, 6, 7 and 8.

Differential cell counting in blastocysts

Cells were counted in fully expanded and hatched blastocysts at day 7 and day 8 in each experimental group. These embryos were selected, fixed and stained for differential cell counts as described by Van Soom *et al.* (1996). Briefly, expanded embryos were incubated in PBS + 5% pronase for 1 min and in acid Tyrode's solution for 1 min to remove the zona pellucida. Subsequently, expanded and hatched blastocysts were incubated in trinitrobenzenesulphonic acid and rabbit anti-serum (antiDNP-BSA) solution. Thereafter, the blastocysts were incubated in guinea-pig complement serum for 30 min at 39°C and subsequently washed in TCM199 Hepes + 10 μ g propidium iodide ml⁻¹. Samples were fixed in ethanol and incubated in bisbenzimidazole (Hoechst 33342 in 10 μ l ethanol ml⁻¹). Finally, after mounting on a glass slide the samples were evaluated under a fluorescence microscope at $\times 400$ with an excitation filter of 330–385 nm and barrier filter of 420 nm. Cells from the trophectoderm fluoresce red whereas the ICM appears blue.

Embryo transfer

Blastocysts at day 7 from the groups of COCs matured with 9-*cis*-retinoic acid and no additives (negative control) were selected for embryo transfer. Embryos transferred fresh were blastocysts and expanded blastocysts, while embryos transferred after vitrification and warming were expanded blastocysts. For vitrification, embryos were loaded into straws in PBS (Invitrogen, Barcelona) with glycerol, ethylene glycol and FCS, adjacent to two fractions of galactose solution (Kaidi *et al.*, 2000). Embryos were warmed for 5 s in air and 10 s in a water bath at 30°C. The content of the straws was transferred to a Petri dish and embryos were mixed in the galactose solution by slight agitation. After 5 min, embryos were transferred to PBS + 20% FCS

for 5 min. Embryos were allowed to re-expand in B2 INRA Ménézo (C.C.D., Paris) at 39°C in an incubator. Finally, re-expanded embryos were loaded into 0.25 ml straws and non-surgically transferred into synchronized Asturiana de los Valles heifers, aged 15–18 months. Each animal received either one fresh embryo or two vitrified embryos in the uterine horn adjacent to a corpus luteum. Pregnancy diagnosis was established by analysing plasma progesterone concentration (> 2.5 ng ml⁻¹) on day 21 and ultrasonography on day 35 and subsequent confirmation at day 60.

Statistical analyses

Data from embryo development and blastocyst differential cell counts were analysed in two steps. First, since these values can be represented by contingency tables, those factors significantly affecting the variables were identified by means of the procedure Categorical Data Modelling (CATMOD; SAS Version 8.2, 1999; SAS Institute Inc., Cary, NC). CATMOD extends analysis from continuous data to categorical data, fitting linear models to functions of response frequencies. Treatment and replicate were found to have significant influence on dependent variables. Second, factors identified as significant were used to fit a linear model using the General Linear Models (GLM; SAS) procedure. This procedure performs analysis of variance for unbalanced data. GLM was used to estimate least-square means (LSM) for each fixed effect having a significant *F* value. Duncan's multiple-range test was carried out on raw means calculated for the main effects on embryo development. Development data are referred to frequency percentages of oocytes in IVM, while blastocyst cell counts are absolute values. When blastocyst cell counts in day 7 and day 8 were pooled for analysis, a significant interaction of day $\times$ treatment was detected by means of CATMOD. GLM was used to reject the null hypothesis (H_0 : $LSM_{(i)} = LSM_{(j)}$) for LSMs within interaction levels. Pregnancy data were submitted to chi-square test of homogeneity for each stratum of treatment day of pregnancy, and results are referred to absolute frequencies.

Results

Embryo development

As shown in Table 1, 9-*cis*-retinoic acid supported morulae, blastocyst and hatching rates comparable to those of embryos derived from oocytes matured in the presence of serum. Ethanol, which was used as a solvent for retinoids, showed a slight stimulatory effect. Serum promoted the appearance of blastocysts on day 6, earlier than in other groups, but retinoic acid and ethanol enhanced blastocyst developmental kinetics too. Proportions of embryos at the cleavage stage (data not

Table 1. Effect of the presence of 9-*cis*-retinoic acid during *in vitro* maturation of bovine cumulus-oocyte complexes upon subsequent embryonic development

Treatment	n	5–8 cells	Morulae	Percentage of blastocysts				
				Day 6	Day 7	Day 8	Expanded	Hatched
9- <i>cis</i> -Retinoic acid	202	54.0 ± 4.8 ^a	53.1 ± 3.0 ^x	15.3 ± 2.8 ^a	40.2 ± 2.9 ^{xa}	40.4 ± 3.4 ^{ab}	20.7 ± 3.6	17.2 ± 1.3 ^x
Ethanol	210	59.4 ± 3.6	41.0 ± 2.3 ^y	13.6 ± 2.1 ^a	29.8 ± 2.2 ^b	32.1 ± 2.5 ^{bc}	18.9 ± 3.6	10.3 ± 1.3 ^a
Control	172	49.8 ± 4.9 ^a	41.7 ± 3.0 ^y	8.5 ± 2.8 ^{bx}	21.6 ± 2.9 ^y	24.0 ± 6.6 ^{cx}	11.6 ± 3.5 ^b	3.1 ± 1.2 ^{by}
Fetal calf serum	162	65.7 ± 4.9 ^b	57.9 ± 3.1 ^x	24.6 ± 2.8 ^{cy}	42.6 ± 3.0 ^{xa}	43.8 ± 3.5 ^{ay}	27.8 ± 4.6 ^a	19.9 ± 1.7 ^x

Data are percentages of matured oocytes, expressed as least-square means ± SE (six replicates).

ANOVA and Duncan's test. Values with different superscripts are significantly different: ^{a,b,c}(*P* < 0.05); ^{x,y,z}(*P* < 0.01).

Table 2. Number of cells after *in vitro* production of day 7 and day 8 expanded blastocysts derived from cumulus-oocyte complexes matured in medium containing 9-*cis*-retinoic acid, ethanol, no additives (control) and fetal calf serum

Treatment	Number of cells						Percentage (ICM:total)
	Day	n	ICM	TE	Total		
<i>In vitro</i> maturation							
9- <i>cis</i> -Retinoic acid	7	13	36.0 ± 5.7 ^{ax}	69.7 ± 5.6 ^{axy}	105.7 ± 8.2		33.7 ± 3.8 ^{ax}
Ethanol	7	17	50.3 ± 4.9 ^{ya}	69.0 ± 4.9 ^{axy}	119.4 ± 7.1 ^{xa}		40.7 ± 3.3 ^{axa}
Control	7	16	44.6 ± 4.9 ^{xb}	73.2 ± 4.9 ^{xy}	117.9 ± 7.1 ^{xa}		36.3 ± 3.3 ^{ax}
Fetal calf serum	7	13	49.2 ± 5.5 ^{ab}	66.7 ± 5.4 ^{xy}	115.9 ± 7.9 ^{ab}		43.2 ± 3.6 ^{xa}
9- <i>cis</i> -Retinoic acid	8	20	39.2 ± 4.3 ^{ax}	55.9 ± 4.3 ^{xza}	95.1 ± 6.2 ^c		41.9 ± 2.9 ^{axa}
Ethanol	8	12	66.2 ± 5.7 ^{Byc}	41.5 ± 5.6 ^{Bzb}	107.7 ± 8.2		59.7 ± 3.8 ^{By}
Control	8	20	30.7 ± 4.4 ^{axd}	62.8 ± 4.3 ^{xy}	92.3 ± 6.0 ^{yc}		31.5 ± 2.9 ^{axb}
Fetal calf serum	8	9	49.6 ± 6.6 ^{ab}	69.3 ± 6.5 ^{xy}	119.0 ± 9.4 ^a		41.1 ± 3.3 ^x

Data are least-square means ± SE. Blastocysts counted were from four (fetal calf serum day 8) to nine (control day 8) replicates per group. Values with different superscripts are significantly different within a variable: ^{a,b,c,d}(*P* < 0.05); ^{x,y,z}(*P* < 0.01); ^{α,β}(*P* < 0.001).

ICM: inner cell mass; TE: trophectoderm.

shown in tables) did not differ among groups (values were between 80.5 ± 3.5, for negative control, and 85.9 ± 3.5, for serum). Ethanol did not promote activation of oocytes (one replicate; five oocytes out of 45 cleaved in the unfertilized ethanol group, and six out of 45 cleaved in the unfertilized untreated controls; no oocyte developed beyond the 5- to 8-cell stage).

Differential cell counts

A total of 120 blastocysts were counted out of 168 samples processed for differential staining. Addition of ethanol during IVM increased values in ICM from day 7 and day 8 blastocysts (Table 2). Besides the ICM increases, addition of ethanol led to day 8 blastocysts showing a marked reduction in trophectoderm, leading to embryos with the highest ICM proportions. Increases in ICM proportions were noted as well with day 8 blastocysts derived from oocytes matured with 9-*cis*-retinoic acid and FCS. In contrast to day 8 embryos, differences between treatments were not outstanding in day 7 embryos. The total number of cells tended to decrease in day 8 embryos from all groups except the FCS treated group.

Table 3. Pregnancy rates from fresh (one) or vitrified (two) bovine blastocysts transferred to recipients

Treatment (oocyte/blastocyst)	Recipients (embryos transferred)	Recipients diagnosed as pregnant		
		Day 21	Day 35	Day 60
9- <i>cis</i> -Retinoic acid	9 (12)	5	5	4 ^a
Fresh	6 (6)	4	4	3
Frozen	3 (6)	1	1	1
Untreated	6 (9)	4	1	0 ^b
Fresh	3 (3)	3	0	0
Frozen	3 (6)	1	1	0

Embryos were derived from oocytes matured in medium containing 9-*cis*-retinoic acid or no additives (untreated).

Values with different superscripts are significantly different: ^{a,b}(*P* = 0.056).

Embryo transfer

Embryos matured in medium containing 9-*cis*-retinoic acid, both fresh and vitrified or warmed, were viable after transfer to recipients (Table 3), and were capable of establishing pregnancies. This result is in contrast to

embryos matured without retinoids, with no pregnancies detected at day 60.

Discussion

The supply of retinoids to the embryo must be ensured within a physiological range, which makes the choice of dosage for retinoids important, as both excess and deficiency of retinoids cause abundant teratogenic defects due to pleiotrophic activity (for review, see Morriss-Kay and Ward, 1999). A marked response in development and trophectodermal differentiation was obtained by overexposure of bovine oocytes to retinoids (Duque *et al.*, 2002). Retinoic acid at a concentration of 5 nmol l⁻¹ affected embryonic development within the experiments reported here. Comparable results have been obtained with 5 nmol 9-*cis*-retinoic acid l⁻¹ when meiosis is inhibited by roscovitine (Duque *et al.*, 2002), and by Ikeda and co-workers using 1 µmol all-*trans*-retinoic acid l⁻¹ (personal communication). The 9-*cis*-retinoic acid isomer used in our experiments has been reported to be 25-fold more potent than the all-*trans* form (Thaller *et al.*, 1993).

Ethanol can decrease retinoic acid synthesis in competition with all-*trans*-retinol to inhibit alcohol dehydrogenase (E.C. 1.1.1.1; ADH) (Duester, 1991; Duester *et al.*, 1991; Leo and Lieber, 1999; Molotkov and Duester, 2002). Therefore, ethanol was chosen as a solvent in the present study to outline the effects of exogenous retinoic acid, downstream of the inhibitory point, and to dissociate them from endogenous metabolism of retinoids. Chronic ethanol treatment increases retinoic acid degradation by ethanol-inducible CYP2E1, a P450 which metabolizes retinoic acid to more polar metabolites *in vitro* (Liu *et al.*, 2001). This process can take days or weeks of treatment to occur, a situation which does not correspond to our experimental conditions. The same above reported effect was seen in acute ethanol treatment *in vivo* in mice, although the effect of ethanol on retinoic acid synthesis was fourfold greater than on retinoic acid degradation (Molotkov and Duester, 2002).

The presence of 9-*cis*-retinoic acid, and to a lesser extent ethanol, during IVM improved blastocyst formation and hatching. The developmentally stimulating effects of retinoic acid are consistent with an increased proportion of blastocysts recovered from donors treated with retinol as reported by other authors (Shaw *et al.*, 1995; Whaley *et al.*, 1997, 2000; Eberhardt *et al.*, 1999). Both retinoic acid and all-*trans*-retinol could act via cumulus-granulosa cells, directly on the oocyte or in both ways, in an autocrine or paracrine manner. Retinoid receptors (Mohan *et al.*, 2001, 2002), RBP (Mohan *et al.*, 2001) and RALDH2 have been detected in immature oocytes. In contrast to the results of the present study, with PVA as a macromolecular source, Avery and Greve (2000) found that 1% ethanol decreases

development of bovine COCs matured with BSA and epidermal growth factor. Ethanol is used to induce oocyte activation at concentrations of about 7% and short periods (Fukui *et al.*, 1992; Pressice and Yang, 1994a,b). However, 1% and 2% ethanol have been shown not to affect nuclear maturation and pronucleus formation after incubation for 1 day (Avery and Greve, 2000). These results are consistent with the findings in the present study, since ethanol did not promote oocyte activation. Although serum promoted the earlier appearance of blastocysts (day 6), blastocyst yields on days 7 and 8 were comparable between FCS and 9-*cis*-retinoic acid. The role of serum during maturation as a stimulatory agent for blastocyst development is contradictory, being described as either beneficial (Ectors *et al.*, 1992; Carolan *et al.*, 1998; Cho *et al.*, 2001) or unnecessary (Takagi *et al.*, 1991; Chung and Seidel, 1994). Rzedidlo *et al.* (2001) showed that the presence of serum during the maturation interval affects gene expression pattern in matured bovine oocytes. In a recent study, premature blastulation did not occur when serum was present during IVM and IVF. Instead, a stimulatory effect was noted in cycles before the morula stage with serum during IVM and IVF (Holm *et al.*, 2002).

Maturation with ethanol altered the differentiation process in the bovine blastocyst, with a higher proportion of cells allocated to the ICM pool. Ethanol and 9-*cis*-retinoic acid did not modify total cell numbers in day 7 and day 8 blastocysts, and their influence was strictly reduced to the trophectoderm and ICM distribution. Remarkably, the differences observed are attributable to day 8 blastocysts rather than to day 7 blastocysts, which suggests that these treatments affect maternal regulation of the trophectoderm differentiation process and are dependent on the intrinsic quality of the oocyte. This can be inferred assuming that blastocysts that develop faster derive from more competent oocytes and are more viable after transfer (Hasler *et al.*, 1995) and after cryopreservation (Dinnyes *et al.*, 1999), than blastocysts developing more slowly. Oocytes becoming late expanding day 8 blastocysts would be more sensitive to the treatments used in the present study. Once the retinoid pathway is affected in the COC (i.e. treatments with 9-*cis*-retinoic acid and ethanol), the ICM:total cell rate increases in day 8 with regard to day 7 blastocysts. In the case of ethanol, the increase was marked and accompanied by an increase in the number of cells in ICM. This is contrary to oocytes matured without additives and with FCS, in which their ICM:total cells did not vary between day 7 and day 8 blastocysts. Generally, the total number of cells in embryos at blastulation decreases in a time-dependent manner in embryos produced *in vitro* (Yoshioka *et al.*, 1997; Van Soom *et al.*, 1997; de la Fuente and King, 1997; Mori *et al.*, 2002), such as occurs in ICM (Van Soom *et al.*, 1997; de la Fuente and King, 1997). *In vivo*, embryos exhibit a comparable reduction in both parameters, but

ICM:total cells remain similar between day 8 and day 9 embryos (de la Fuente and King, 1997).

The above cited developmental promoting side-effects of ethanol would mask its inhibitory effect on retinoic acid synthesis in the COC. However, side-effects would not affect cell distribution in the blastocyst. Some of the observed effects of the retinoid metabolite on trophectoderm and ICM values are in fact an attenuation of the effects exerted by its ethanol solvent. In addition, having observed the magnitude of the effects of ethanol on the number of cells in ICM compared with that of untreated oocytes, there is evidence that endogenous retinoids exert a role in oocyte maturation. Thus, and although not generally opposed to each other, the effect of ethanol leads to a preponderance of ICM, whereas 9-*cis*-retinoic acid gives rise to more cells in the trophectoderm in day 8 blastocysts.

Ethanol exhibited different behaviour including its stimulating effect on embryo development and its influence on blastocyst differentiation. To our knowledge, the content of endogenous retinoids in the bovine oocyte or granulosa cells are unknown, but 1% (175 mmol l⁻¹) ethanol should inhibit the ADH activity considerably. In fact, 100 mmol ethanol l⁻¹, but not 10 mmol ethanol l⁻¹, led to a significant decrease in retinoic acid detection in cultured mouse embryos at day 7.5 of age (Deltour *et al.*, 1996), and 17.5 mmol ethanol l⁻¹ enhanced the expression of the proto-oncogene *c-Myc* (Leach *et al.*, 1999). This promoter of cell proliferation is required for mouse blastocyst development and has been detected in elongating bovine blastocysts (Tetens *et al.*, 2000). In contrast, retinoic acid represses *c-Myc* expression in a variety of cell types (Sejersen *et al.*, 1986; Falasca *et al.*, 1998; Tinel *et al.*, 1999; Dimberg *et al.*, 2002; Sarkar and Sharma, 2002). The embryo developmental pattern brought about by retinoic acid in the present study reproduces in part the effects reported by Ikeda *et al.* (2000a,b) using recombinant peptide midkine, a product from a retinoic acid-inducible gene. Other retinoic acid-responsive genes in the bovine embryo are known to be associated with blastocyst formation (leukaemia inhibitory factor, LIF and its receptor LIFr; laminin B1; cytokeratin). Therefore, inhibition of retinoic acid synthesis during IVM would not be used by ethanol to influence development, but alternative mechanisms such as enhancement in the production of cAMP from ATP via stimulation of adenylate cyclase activity (Rabbani *et al.*, 1999). Altered cellular content of cAMP could account for some of the major effects of ethanol (for review, see Klemm, 1998), whereas the addition of retinoic acid was shown not to affect the cAMP content of a granulosa cell culture system (Minegishi *et al.*, 1996, 2000). Maintenance of an optimal intracellular concentration of cAMP before and during IVM ensures a high developmental competence of oocytes to the blastocyst stage and does not alter the number of cells in the blastocyst (Luciano *et al.*, 1999).

It is assumed that a minimal number of ICM cells are required to obtain a pregnancy, and excessive allocation of cells to the TE might lead to pregnancy abnormalities (Van Soom *et al.*, 1996; Leese *et al.*, 1998). Transfer of *in vitro* cultured bovine embryos gives rise to placentas with increased mass and reduced caruncular area (Farin *et al.*, 2001), together with hydroallantois and polyhydroamnios (van Wagtenonk-de Leeuw *et al.*, 2000). The large offspring syndrome, characterized by abnormally large animals showing a number of congenital defects, can be explained in part by placental abnormality (Bertolini and Anderson, 2002). In embryos produced *in vitro* there is no difference in amounts of methylation among embryos with high (4.5) or low (0.6) ICM:trophectoderm ratio (Kang *et al.*, 2002), a range similar to the number of cells in the present study (data not shown). This indicates that the wide variation in the ICM:trophectoderm ratio is not necessarily a reflection of epigenetic alteration. It may not be desirable to use culture systems containing ethanol to produce embryos with a large number of cells in the ICM, as ethanol may increase the proportion of cells in the ICM by interfering with important metabolic pathways. In this respect, the apparent better quality of embryos cultured in B2 medium (faster cleavage, earlier blastocyst formation) was not reflected in a reliable number of inner cells of blastocysts (Van Soom *et al.*, 1996), and pregnancy rates were not diminished with regard to embryos cultured in TCM199 (Hasler *et al.*, 1995). Pregnancies and calves born are the best indicator of embryonic viability, and the addition of retinoic acid produced embryos that were viable beyond the expected time for completion of organogenesis (day 42; Hubbert, 1974), in contrast to untreated oocytes. In our laboratory, fresh embryos matured in medium containing serum usually experienced 40% pregnancy rates as diagnosed at day 60, while no success was attained in the present study in chemically defined conditions before incorporation of 9-*cis*-retinoic acid to IVM medium. Taken together, the results of the present study indicate that the selected concentration of 9-*cis*-retinoic acid during IVM probably conditions superior oocyte viability. Subsequent fetal and perinatal development will require careful analysis of calves for normality.

Addition of retinoic acid during IVM improved early development of the bovine oocyte and led to pregnancies upon transfer of day 7 blastocysts. Day 8 blastocysts from oocytes treated with retinoic acid showed improved quality as judged by their ICM values. Ethanol stimulates embryo development but leads to a marked increase in the number of cells in the ICM; whether this observation implies that these blastocysts exhibit compromised viability is worth investigating. The use of retinoids during *in vitro* maturation is compatible with late pregnancy and, within a physiological dose range and precise conditions, could be used to improve developmental competence of the bovine oocyte.

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