

Retinoids during the *in vitro* transition from bovine morula to blastocyst

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BACKGROUND: The conversion of retinol (ROH) to retinoic acid (RA) is crucial during development but has been not studied during blastocyst formation. **METHODS AND RESULTS:** *In vitro*-produced bovine morulae were treated for 24 h with citral (which inhibits the synthesis of RA from ROH), citral + all trans retinoic acid (ATRA), ATRA or no additives. Citral interfered with blastocyst development, whereas exogenous RA had no effect. RA, however, reversed the effect of citral on development and stimulated cell proliferation. Neither citral nor RA changed the apoptotic index, but RA triggered an increase in the apoptotic frequency of the inner cell mass. Citral and RA reduced the necrotic index. Na/K-ATPase α 1-subunit mRNA concentrations (analysed by real-time PCR) increased after hatching and showed dependence on retinoid activity, but no evidence was found of any retinoid effect on p53 expression. Nevertheless, the p53 mRNA concentration increased in response to proliferation in hatched blastocysts. **CONCLUSION:** The preimplantation bovine embryo metabolizes endogenous ROH to RA, which participates in important cell processes. The true extent of the influence of RA is unknown, although the modulation of retinoid metabolism seems to be a means of increasing cell proliferation. This knowledge might be used to improve embryo quality and the efficiency of stem cell derivation.

Key words: apoptosis/cell culture/embryo development/embryo quality/gene expression

Introduction

In the living cell, the retinol (ROH) metabolite retinoic acid (RA) controls cell growth and differentiation by activating or silencing a large number of genes. The conversion of all-*trans* RA (ATRA) to 9-*cis*-retinoic acid (9-*cis*-RA) and other isomers (a reversible process) produces biologically active compounds that enter the nucleus and bind to specific receptors. The RA receptors (RARs) are activated by ATRA and 9-*cis*-RA, whereas retinoid X receptors (RXRs) are activated only by 9-*cis*-RA (Chambon, 1996). Both RARs and RXRs have α , β and γ subtypes. Heterodimerization between RXR and RAR, as well as with other members of the steroid/thyroid hormone nuclear receptor superfamily, can occur in response to ligand-mediated activation (Chambon, 1996). These heterodimers bind to specific DNA sequences, named RA-responsive elements, to increase or decrease gene expression. Transcripts of all retinoid subtypes of RXR and RAR (except for RAR β) are expressed during the preimplantation stages in bovine embryos produced *in vitro* (Mohan *et al.*, 2001, 2002; Mamo *et al.*, 2005). Other components of ROH metabolism, such as the RA-yielding enzyme, retinaldehyde dehydrogenase, and the peroxisome-proliferator-activated receptor gamma (PPAR γ), are also expressed in bovine oocytes, embryos produced *in vitro*

(Mohan *et al.*, 2002) and cumulus cells (Mohan *et al.*, 2003). Immunostaining techniques have shown RAR α , RAR γ -2 and RXR β in blastocysts (Mohan *et al.*, 2001, 2002) and RAR α and RXR β in cumulus cells (Mohan *et al.*, 2003).

The endogenous production of RA from ROH is essential throughout development. The disruption of retinoid homeostasis by exogenous ATRA or deprivation of RA has been associated with abnormalities during late development, because the normal relationship between cellular retinoid levels and the genetic developmental program is altered (Griffith and Zile, 2000; Roberts *et al.*, 2005). The experimental inhibition of RA production in null mutations of aldehyde dehydrogenase (ALDH) (Niederreither *et al.*, 1999) or its chemical inhibition using citral, a generic ALDH competitive antagonist (Griffith and Zile, 2000; Iwata *et al.*, 2004; Song *et al.*, 2004), is followed by developmental defects and death. The enzyme ALDH generates the majority of RA. Phenotypes produced by the inhibition of ALDH can be rescued by ATRA supplementation.

We have shown that *in vitro* maturation (IVM) of oocytes with 9-*cis*-RA improves blastocyst development and quality (Duque *et al.*, 2002; Hidalgo *et al.*, 2003, 2005; Gómez *et al.*, 2004) and that oocyte yields increase in donor cows injected

with ROH (Hidalgo *et al.*, 2005). High concentrations of ROH promote blastulation in bovine embryo cultures under atmospheric oxygen conditions (Lawrence *et al.*, 2004) and prevent heat stress during IVM (Livingston *et al.*, 2004). However, although oocytes matured *in vitro* with 9-cis-RA are viable and lead to pregnancy, oocytes from cows treated with ROH, after *in vitro* culture to the blastocyst stage, are non-viable upon transfer to recipients (Hidalgo *et al.*, 2005). In the mouse, the exposure of blastocysts to excess ATRA directly affects the inner cell mass (ICM) (Huang *et al.*, 2005), leading to adverse effects on development (Huang *et al.*, 2001, 2003). The cellular responses involved are apoptosis and inhibited proliferation (Huang *et al.*, 2003, 2005). In cattle, the role of retinoids during early embryonic development *in vitro* has not been studied.

Many cell types contain ROH that is esterified in the form of fatty acids (Bonet *et al.*, 2003). Bovine oocytes show high triglyceride levels (Ferguson and Leese, 1999; Leroy *et al.*, 2005), which increase in embryos during *in vitro* culture with serum (Ferguson and Leese, 1999; Leroy *et al.*, 2005) and remain relatively constant in embryos cultured in the absence of serum (Ferguson and Leese, 1999). Therefore, ROH derived from the oocyte might be stored in the bovine embryo and be made functionally active through the conversion to RA during blastocyst formation. The present work investigates the effects of exogenous and endogenous retinoids on blastocyst development, trophectoderm (TE) differentiation, apoptosis and necrosis, as well as the expression of the p53 (involved in growth arrest and apoptosis) and Na/K-ATPase α 1-subunit (the activity of which is essential for the differentiation of ICM and TE cells) genes.

Materials and methods

In vitro embryo culture

Bovine cumulus-oocyte complexes were aspirated from 3–8 mm follicles collected from ovaries removed at the slaughterhouse. Those with a compact cumulus investment and homogeneous ooplasm were rinsed thrice with HEPES-buffered TCM199 (Invitrogen, Barcelona, Spain) containing 0.4 mg/ml of bovine serum albumin (BSA) (Sigma, Madrid, Spain) and twice with maturation medium [TCM199, 10% fetal calf serum (FCS), 1 mg/ml of porcine FSH (F2293 Sigma), 5 mg/ml of sheep LH (L5269 Sigma) and 1 mg/ml of 17 β -estradiol (E8875 Sigma)]. Groups of 40–50 oocytes were allowed to mature *in vitro* in the above conditions (39°C, 5% CO₂ in humidified air) in four-well culture dishes (Nunc; Biocen, Barcelona, Spain). After 24 h, all oocytes were fertilized with frozen semen, as described in Hidalgo *et al.* (2003). Oocytes and spermatozoa were co-cultured (day 0) for 22–24 h (39°C, 5% CO₂ in humidified air). The remaining cumulus cells were detached using a vortex and zygotes cultured in droplets of B2 medium (INRA-Menezo, Paris, France) with Vero cell (European Collection Animal Cell Culture) monolayers (39°C, 5% CO₂ in humidified air) under mineral oil until 139 h after insemination (day 6). On day 6, mostly morulae (with no more than 8% showing incipient cavitation) were equally distributed among the experimental groups and cultured for 2 days in modified synthetic oviduct fluid (mSOF) containing amino acids, citrate, myo-inositol (Holm *et al.*, 1999) and 5% FCS, at 39°C with 5% CO₂/O₂ in humidified air in four-well dishes (Biocen). Embryonic development was recorded as being at the early-, mid-, expanded- or hatched-blastocyst stage.

Differential cell counts

Blastocysts were fixed and stained for differential cell counting as described by Thouas *et al.* (2001). Blastocysts enclosed in the zona pellucida were incubated in 500 μ l of BSA-free TCM199 HEPES (Invitrogen) with 1% Triton-X-100 and 100 μ g/ml of propidium iodide for 30 s. Samples were then fixed in 500 μ l of ethanol with 25 μ g/ml of bisbenzimidazole (Hoechst 33342) and stored overnight at 4°C. Fixed and stained blastocysts were transferred directly into a glycerol microdrop on a glass microscope slide. Cell counts were made using digital images obtained with an inverted microscope equipped with a UV-excitation filter at 330–385 nm and a barrier filter at 420 nm. TE cells were identified by their red fluorescence; ICM cells appeared blue.

Apoptosis and necrosis

The blastocysts were fixed in 4% (w/v) phosphate-buffered saline (PBS)-buffered paraformaldehyde and permeabilized with PBS containing 0.5% (v/v) Triton-X-100 and stored overnight in PBS containing 10 mg/ml of BSA. After washing with PBS containing 0.1 mg/ml of polyvinyl alcohol (PVA), the blastocysts were incubated for 1 h at 37°C in 30 μ l droplets of TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labelling (TUNEL) reaction mixture (In Situ Cell Death Detection Kit; Roche, Penzberg, Germany) under paraffin oil in darkness. Negative controls contained no TdT. A positive control was included by treating samples with 100 U/ml of DNaseI for 1 h at 37°C. After the TUNEL reaction, the nuclei were stained with 10 μ g/ml of bisbenzimidazole diluted in PBS-PVA for 10 min. The blastocysts were washed in PBS with 0.1 mg/ml of PVA, mounted on a glass slide with droplets of glycerol containing 2.5% (w/v) 1,4-diazabicyclo[2.2.2]octane, flattened with a coverslip and examined under an Olympus IX50 fluorescence microscope. The wide-band filters for blue and UV excitation were used for the detection of TUNEL and bisbenzimidazole staining, respectively.

Cells were regarded apoptotic when they showed both TUNEL labelling and apoptotic morphology. The criteria for apoptotic morphology were any of the following traits (Gjorret *et al.*, 2003): (i) marginal chromatin [i.e. condensed chromatin aggregated in granular masses around the nuclear membrane giving the nucleus a crescent, half-moon (Figure 1A) or lobed appearance (Figure 1B)]; (ii) pycnotic appearance [i.e. condensed chromatin shrunk into a single dense, homogeneously stained mass with a roughly spherical or ovoid shape (Figure 1C)]; (iii) multiple fragments, consisting of condensed chromatin with variable volumes of multiple, densely stained masses (Figure 1D) or (iv) apoptotic bodies, which are multiple fragments scattered within the intercellular spaces, eventually producing membrane-bound structures (Figure 1E). These nuclei were generally TUNEL positive (Figure 1a–e). Nuclei that showed unclear or fluffy edges and swelling (Figure 1F) or fragmentation into numerous fluffy elements plus swelling (Figure 1G) were considered necrotic. These nuclei were often TUNEL positive (Figure 1f and g). Clusters of nuclear fragments confined in an area comparable with or smaller than the volume of a normal nucleus were regarded as originating from a single nucleus and were therefore counted as such. Apoptotic bodies isolated within intercellular spaces were considered to have originated from a single nucleus and then dispersed; thus, two to five isolated apoptotic bodies were counted as one apoptotic nucleus.

TE and ICM cells were enumerated separately. The allocation of cells to the TE or ICM was based on their position in the embryos and the relative size of their nuclei. Cells within the ICM appear as a mass within the embryo and have smaller nuclei than those seen in TE cells. The ratios of apoptotic and necrotic cells to total cells were termed the apoptotic index (AI) and necrotic index (NI),

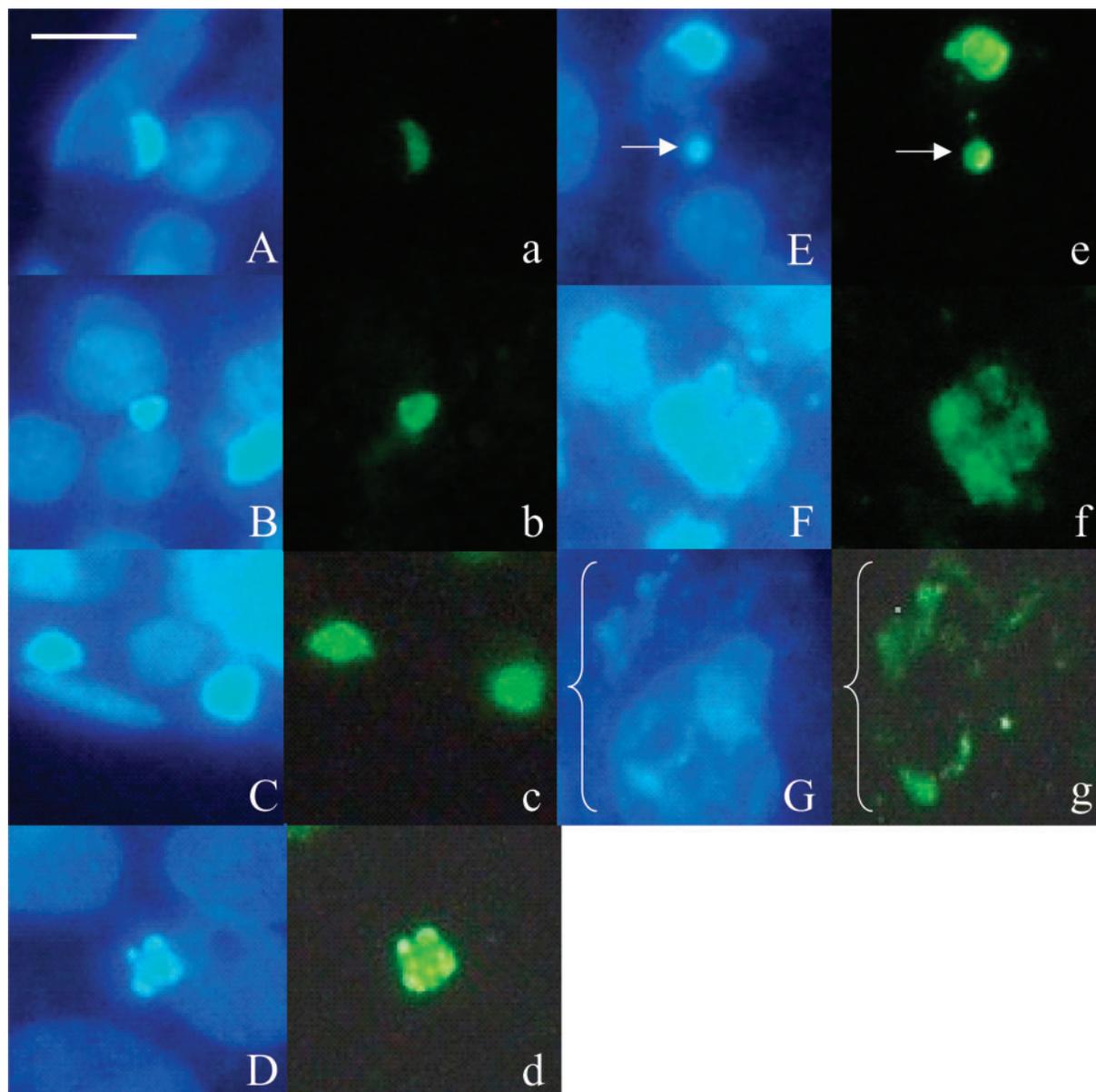


Figure 1. Bisbenzamide (A–G) staining and TdT-mediated dUTP nick-end labelling (TUNEL) (a–g) images of apoptotic and necrotic nuclei in bovine blastocysts. Apoptotic nuclei were characterized by either (A) crescent to half moon-like chromatin, (B) lobed chromatin, (C) pycnotic chromatin, (D) multiple fragments or (E) apoptotic bodies (arrow). Necrotic nuclei were characterized by unclear or fluffy edges with either swelling (F) or fragmenting into numerous fluffy elements (brace) (G). These nuclei were often TUNEL positive (a–g). Scale bar represents 10 μ m.

respectively. The relative frequencies (RFs) of apoptotic cells within the ICM and TE were calculated as:

$$\frac{\text{AI of the ICM}}{\text{AI of the TE}}$$

RNA extraction

Balanced pools of 8–12 expanded and 5–10 hatched blastocysts were stored in RNA-Later (Ambion, Austin, TX, USA), kept overnight at -4°C and then stored at -80°C . Isolation of mRNA was performed using the Dynabeads mRNA Direct KIT (Dynal, Oslo, Norway), following the manufacturer's instructions. Samples were lysed in 180 μ l of lysis/binding buffer (Dynal). Hybridization was performed with 100 μ l of Dynabeads oligo (dT)25 for 10 min, and poly(A) RNA–bead complexes were separated from the binding buffer and rinsed in buffer

A and B (Dynal), using a magnetic separator. Poly(A) RNA was finally eluted in 180 μ l of 10 mM Tris–HCL.

Reverse transcription

Reverse transcription was achieved using the first-strand complementary DNA (cDNA) synthesis kit for RT–PCR, (AMV; Roche, Barcelona, Spain) with oligo d(T)25, following the manufacturer's instructions. The tubes were then incubated at 25°C for 10 min to allow annealing. The RNA was subsequently reverse-transcribed at 42°C for 60 min, followed by 5 min incubation at 99°C to denature the enzyme. The samples were then cooled at 4°C .

Real-time PCR

Quantitative PCR was performed with an i-Cycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). For the amplification

of p53 and ATPase α 1-subunit, the bovine-specific primers described by Favetta *et al.* (2004) and Wrenzycki *et al.* (2003), respectively, were used. The reaction mixture consisted of 1 μ l of cDNA and 0.2 μ M (p53 gene) or 0.5 μ M (ATPase gene) of each sense and antisense primer (Table I). SYBR Green PCR Supermix (\times 2) (Bio-Rad) was used as a double-stranded DNA-specific fluorescent dye. The final reaction volume was 20 μ l. Assays were performed in triplicate. The RT-PCR protocol included an initial step of 95°C (3 min), followed by 45 cycles of 30 s at 95°C for DNA denaturation, 1 min for primers annealing (see Table I for temperatures) and 1 min at 72°C for primer extension. Fluorescence data were acquired at 72°C. Melting-curve analysis to confirm product specificity was performed immediately after amplification, following 1 min denaturation at 95°C, 1 min annealing at 65°C and 60 cycles of 0.5°C increments (10 s each), beginning at 65°C, while monitoring fluorescence. The melting temperature was always >80°C for both p53 and the ATPase α 1-subunit. Product identity was confirmed by electrophoresis using an ethidium bromide-stained 2% agarose gel in \times 1 Tris/borate/EDTA buffer. The standard curve was made with cDNA prepared as above and serially diluted. The amplification efficiency and correlation coefficient were higher than 0.90 and 0.98, respectively. Quantification was performed by normalizing data with the total number of cells (Bustin, 2002) per treatment and replicate, as determined by differential cell counts.

Experimental design

Citral was used to inhibit RA production from ROH, and exogenous ATRA was used to reverse the effects of citral inhibition. Treatments of cultures, in 400 μ l of medium, were as follows: (i) citral 150 μ M; (ii) ATRA 0.7 μ M; (iii) citral 150 μ M + ATRA 0.7 μ M and (iv) no additives (negative control). Citral and ATRA were dissolved in dimethylsulphoxide (DMSO). The concentration of the vehicle in the culture was adjusted to 0.1% in all groups. After 24 h of treatment (day 7), blastocyst development was recorded, and embryos were allowed to recover for 30 h (day 8) in 50 μ l droplets of mSOF under mineral oil. Droplets of 50- μ l culture medium under mineral oil were not used during the treatment period because of the presence of lipophilic DMSO as a solvent for ATRA and citral. As both the number of cells and their activity, pumping fluid to the blastocoele, could influence blastocyst size, the

diameter of day 8 fully expanded blastocysts of very good/excellent morphological quality was measured with an eyepiece micrometer connected to an inverted microscope. Day 8 fully expanded and hatched blastocysts, which underwent differential cell counts, were evaluated for apoptosis and necrosis and analysed for the expression of the p53 and Na/K-ATPase (α 1-subunit) genes. Cells were counted in 231 blastocysts (154 expanded and 77 hatched; 19 replicates), apoptosis was analysed in 103 blastocysts (62 expanded and 41 hatched; seven replicates) and gene expression was determined in 266 blastocysts (155 expanded; four replicates and 111 hatched; three replicates, with one more replicate of hatched ATRA and controls).

Statistical analysis

The data were analysed in two steps. First, the factors with significant influence were identified by categorical data modelling (CATMOD), using SAS Version 8.2 software (1999; SAS Institute, Cary, NC, USA). CATMOD fits linear models to response–frequency functions. Treatment, replicate and the blastocyst stage at which cell counts were made were found to have a significant influence on the dependent variables. Second, those factors identified as significant were used to produce a linear model, using the general linear models procedure (GLM; SAS software). This procedure performs the analysis of variance for unbalanced data. GLM was used to estimate the least square means for each fixed effect having a significant *F* value. Duncan's multiple-range test was used to compare the raw means calculated for the main effects. Development and apoptosis/necrosis data were transformed to frequency percentages. Blastocyst cell counts were handled as absolute values, whereas gene expression was expressed as arbitrary units.

Results

Blastocyst development

We selected 3078 morulae, starting from 9271 cultured oocytes (33.2%). Morulae whose endogenous ROH metabolism was blocked by a 24-h treatment with citral showed reduced development at all analysed stages (Table II). The effect of citral was reversed by exogenous ATRA, and ATRA alone showed

Table I. Details of primers used for quantitative real-time PCR

Gene	Sequence (5'–3')	Fragment size (bp)	Annealing temperature (°C)	Accession number
p53	CTCAGTCCTCTGCCATACTA GGATCCAGGATAAGGTGAGC	364	55	U74486
ATPase α 1-subunit	ACCTGTTGGGCATCCGAGAGAC AGGGGAAGGCACAGAACCACCA	336	64.3	NM-012504

The first (above) primer described in each gene is the sense (5'–3'), and the second one is the antisense.

Table II. Blastocyst development rates of day 6 bovine embryos *in vitro* after a 24-h challenge culture (day 7) in modified synthetic oviduct fluid (mSOF) containing citral, all-*trans* retinoic acid (ATRA), citral + ATRA or no additives (negative control), and a subsequent 24-h recovery period (day 8) in mSOF medium without additives

Treatment	N	Treatment period (% day 7 blastocysts)			Recovery period (% day 8 blastocysts)			
		Early	Medium	Expanded	Early	Medium	Expanded	Hatched
ATRA	783	60.4 \pm 1.6 ^x	38.9 \pm 1.5 ^x	18.4 \pm 1.0 ^a	53.5 \pm 1.6 ^x	46.7 \pm 1.6 ^x	40.6 \pm 1.5 ^x	12.6 \pm 1.2 ^a
Citral + ATRA	745	61.6 \pm 1.7 ^x	42.4 \pm 1.6 ^x	19.2 \pm 1.1 ^a	54.1 \pm 1.7 ^x	47.1 \pm 1.7 ^x	38.5 \pm 1.5 ^x	10.1 \pm 1.2
No additives	747	60.5 \pm 1.6 ^x	39.7 \pm 1.5 ^x	17.3 \pm 1.1	54.6 \pm 1.7 ^x	48.1 \pm 1.6 ^x	41.0 \pm 1.5 ^x	10.0 \pm 1.2
Citral	803	52.3 \pm 1.6 ^y	33.4 \pm 1.5 ^y	14.7 \pm 1.1 ^b	47.2 \pm 1.7 ^y	39.9 \pm 1.6 ^y	32.3 \pm 1.5 ^y	8.4 \pm 1.2 ^b

N, number of day 6 embryos.

Data are least square mean \pm SE percentages from cultured morulae (36 replicates).

Different superscript letters express significant differences: ^{x,y,z}*P* < 0.01; ^{a,b}*P* < 0.03.

increased hatching rates as compared with citral. However, treatment with ATRA alone had no effect on blastulation. Expanded blastocysts pretreated with citral + ATRA were larger ($176.2 \pm 4.5 \mu\text{m}$) than the controls ($165.3 \pm 3.7 \mu\text{m}$) and citral ($161.7 \pm 4.1 \mu\text{m}$) group ($P < 0.03$), whereas embryos pretreated with ATRA showed intermediate values ($171.3 \pm 3.6 \mu\text{m}$).

Day 8 blastocysts—differential cell counts

Manipulating retinoid metabolism profoundly affected the cell counts and the distribution of cells within the blastocysts. Figure 2 shows the cell-count patterns for the TE (Figure 2a) and ICM

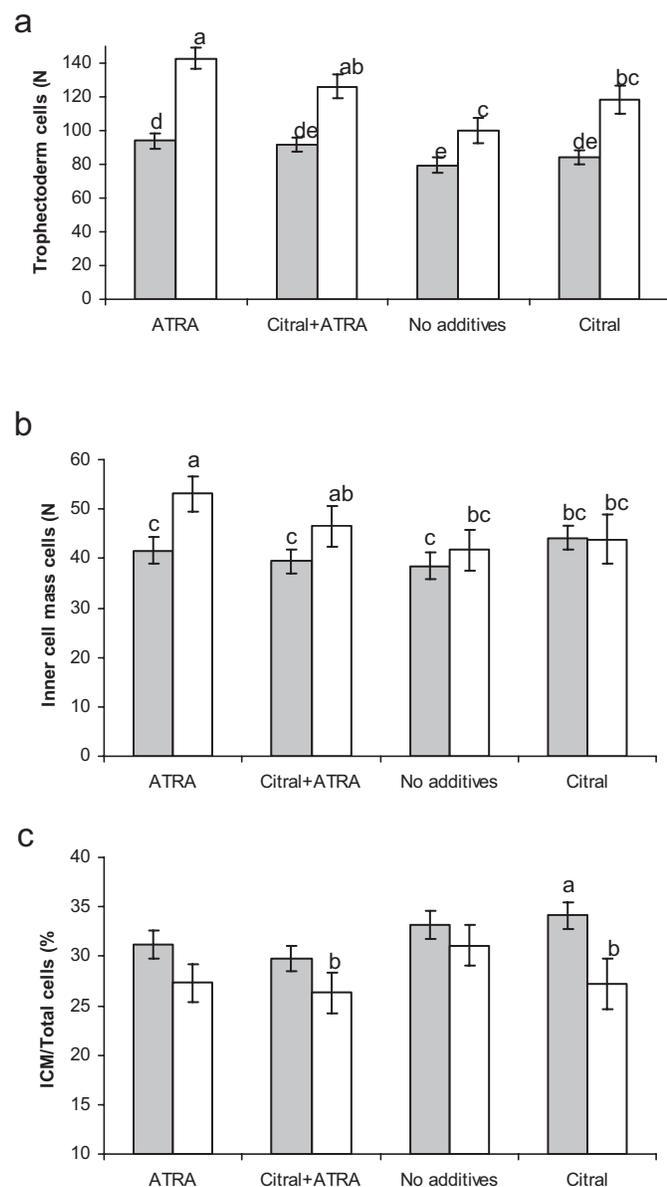


Figure 2. Number of cells contained in the trophectoderm and inner cell mass, and percentage of inner cell mass/total cells from day 8 expanded (grey bars) and hatched (open bars) blastocysts derived from day 6 morulae *in vitro*. Day 6 morulae were cultured for 24 h in modified synthetic oviduct fluid (mSOF) with all-*trans* retinoic acid (ATRA), citral, citral + ATRA or no additives and allowed to recover in mSOF without additives for 24 h. Different superscript letters express significant differences: a, b, c, d ($P < 0.05$).

(Figure 2b), and the ICM/total cell ratios (Figure 2c). The experimental groups are arranged in accordance with their presumed retinoid activity (i.e. from ATRA to citral-treated, representing the highest and lowest retinoid activities, respectively). Within the ICM and TE, the cell numbers were enhanced by ATRA, the proliferative pattern being similar between expanded and hatched blastocysts. However, the magnitude of the effect was much higher in the hatched blastocysts, as a 41% increase in total cell numbers was seen in ATRA-treated embryos compared to the untreated controls (196.3 ± 8.1 compared to 139.0 ± 11.3 , respectively; $P < 0.01$; not shown in Figures). Proliferation in the TE was greater in retinoid excess (i.e. in the presence of ATRA) and did not differ from controls in retinoid deficiency (i.e. when endogenous RA synthesis was inhibited with citral). Interestingly, differences between expanded and hatched blastocysts with respect to proliferation in the ICM increased with presumed retinoid activity. No increase was seen, however, in culture with citral. Expanded blastocysts pre-exposed to citral gave the highest ICM/total cell ratio (34.1 ± 1.4), significantly different ($P < 0.05$) to that for hatched counterparts cultured with citral (27.2 ± 2.1) or citral + ATRA (26.3 ± 2.1).

Apoptosis and necrosis in day 8 blastocysts

No interactions were detected between treatment and blastocyst stage. The data for this experiment are therefore presented as cumulative values (Table III). None of the treatments affected the AI, but ATRA reduced the NI in ICM and TE (and therefore in total cells), and citral tended ($P = 0.074$; not listed in table) to reduce NI in total cells. However, the higher RF of apoptosis in the ICM compared to the TE observed in these blastocysts indicates an effect of ATRA on the distribution of apoptosis, which is opposite to that seen with citral.

Gene expression

The expression of the Na/K-ATPase $\alpha 1$ -subunit was greatest in hatched embryos pretreated with citral + ATRA, but also increased in the citral and control groups (Figure 3a). In expanded blastocysts, the ATRA and citral treatments induced the highest expression. The relative abundance of p53 (Figure 3b) increased similarly in hatched blastocysts belonging to groups in which the retinoid pathway was affected (i.e. the ATRA-, citral + ATRA- and citral-treated groups). The effect observed did not appear to depend on the predicted retinoid activity. The expression of p53 in each of these three groups was different to that seen in the untreated controls. No differences were seen in p53 expression between expanded blastocysts under any treatment conditions ($P > 0.10$).

Discussion

The present work provides evidence that active retinoids are endogenous to bovine morulae produced *in vitro* and that these embryos are responsive to RA. The transition from the morula to the hatched-blastocyst stage was primarily affected by the deprivation of ROH metabolism. All stages analysed during development showed reduced blastocyst rates in the presence of citral. The effects of citral on development were reverted by

Table III. Apoptotic and necrotic index (AI and NI, respectively) in the inner cell mass (ICM), trophectoderm (TE) and total cells, plus relative frequency (RF) of apoptotic cells (AI of ICM/AI of TE) in day 8 blastocysts

Treatment	N	ICM		TE		Total		RF
		AI	NI	AI	NI	AI	NI	
ATRA	28	20.17 ± 1.27	1.50 ± 0.46 ^a	5.86 ± 0.70	2.08 ± 0.42 ^a	10.23 ± 0.63	1.83 ± 0.33 ^a	6.19 ± 0.69 ^a
Citral + ATRA	27	19.24 ± 1.30	2.23 ± 0.47	6.18 ± 0.72	2.37 ± 0.43	10.17 ± 0.65	2.24 ± 0.34 ^a	4.76 ± 0.71
No additives	24	22.43 ± 1.39	3.41 ± 0.51 ^b	6.53 ± 0.77	3.49 ± 0.46 ^b	11.62 ± 0.69	3.48 ± 0.36 ^b	4.51 ± 0.76
Citral	23	19.95 ± 1.44	2.17 ± 0.53	6.29 ± 0.80	2.48 ± 0.48	10.95 ± 0.72	2.37 ± 0.37	3.88 ± 0.79 ^b

Day 6 morulae were cultured *in vitro* for 24 h in modified synthetic oviduct fluid (mSOF) with or without additives, followed by a 24-h recovery period without additive.

Significant differences are expressed by different superscript letters: ^{a,b} ($P < 0.05$).

Data are least square mean ± SE (embryos taken from seven replicates).

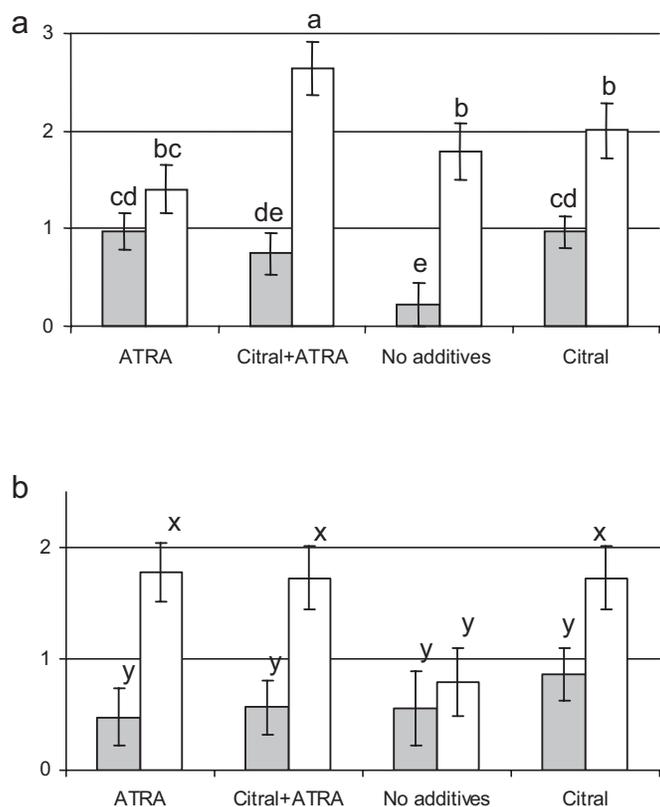


Figure 3. Quantitative gene expression (arbitrary units) for Na/K-ATPase $\alpha 1$ -subunit (a) and p53 (b) in day 8 expanded (grey bars) and hatched (open bars) blastocysts. Different superscript letters express significant differences x, y, z ($P < 0.01$); a, b, c, d, e ($P < 0.05$).

ATRA in a specific manner, because (i) blastocyst rates in the reversion group (citral + ATRA) were the same as for the controls with unaltered retinoid metabolism and (ii) ATRA alone had no effect.

The ROH and RA concentrations in the bovine blastocyst are unknown. However, the porcine blastocyst at day 10 contains RA (36.2 nM in the whole embryo and 35.4 nM in the TE) and ROH (48.9 nM in the whole embryo and 122.3 nM in the TE) (Parrow *et al.*, 1998). These levels are functionally active, and a higher ROH content in the TE is consistent with tissues undergoing active differentiation (Schweigert *et al.*, 2002). Culture media require concentrations several times

higher to produce the above intracellular concentrations, especially in cultures with serum or BSA (Klaassen *et al.*, 1999). We added 5% FCS to the cultures after day 6 because it maintains the ATRA concentration (the physiological release of ATRA from the serum proteins is slower, as is its uptake by cells) (Klaassen *et al.*, 1999). The ROH concentration we detected in 5% FCS (16 nM; high-performance liquid chromatography analysis; Hidalgo *et al.*, 2005) was consistent with that described by Lane *et al.* (1999), who found 25 nM ROH and <1 nM RA in 5% FCS. This RA concentration is negligible compared to the 0.7 μ M ATRA used in the present work. Concentrations of ATRA between 0.3 and 1 μ M are usual in the literature dealing with reproductive cells (Minegishi *et al.*, 1996; Hattori *et al.*, 2000), and ATRA 1 μ M during IVM improved blastocyst development (S.Ikeda, unpublished data). We used 150 μ M citral here, based on a dose-dependent inhibition of *in vitro* development by citral during bovine IVM (unpublished data).

In our work, blastocysts pretreated with ATRA showed strong cell proliferation in the TE and ICM at the hatched stage and moderate proliferation in the TE at the expanded stage. Exogenous RA led to the greatest differences in ICM and TE cell counts between expanded and hatched blastocysts. However, ICM counts in expanded and hatched blastocysts pretreated with citral were the same, although these hatched blastocysts showed higher TE counts (reflected in altered ICM/total cell ratios). The effects of ATRA on apoptosis distribution were opposite to those of citral, indicating that apoptosis regulation in the blastocyst is influenced by retinoid. In cultured mouse blastocysts, Huang *et al.* (2003) found that anti-proliferative and proapoptotic effects of 10 μ M ATRA were mainly targeted to the ICM (Huang *et al.*, 2005). These studies lacked protein in the medium, and their high ATRA concentrations might explain the contrast between our results and the detrimental effects of 10 μ M ATRA in mice (Huang *et al.*, 2003). In addition, there are important species-specific differences, because RAR β -mRNA expression has been shown in mice (Wu *et al.*, 1992) but not in bovine blastocysts (Mohan *et al.*, 2001). This article and other studies reported that ICM has a higher apoptotic rate than the TE in mammalian blastocysts (Gjorret *et al.*, 2003; Davidson *et al.*, 2004), suggesting that apoptosis occurs naturally to eliminate non-committed ICM cells. A new concept of necrosis includes secondary necrosis, which occurs when apoptotic bodies are not quickly removed

by phagocytes or neighbouring cells, and classic oncotic necrosis (Levin *et al.*, 1999). The reduced NI in blastocysts caused by ATRA suggests an inhibitory effect of RA on apoptosis that falls outside the classical apoptotic traits.

The composition and accumulation of fluid in the blastocoele is regulated by Na/K-ATPase. In hatched blastocysts, Na/K-ATPase α 1-subunit expression was highest with the citral + ATRA treatment, whereas groups with greater and lower than predicted retinoid activity had comparable expression levels. This type of response is not unusual when working with retinoids (Micallef *et al.*, 2005). The expression of α 1-subunit of the Na/K-ATPase in renal cells depends on hypertonicity (Ferrer-Martínez *et al.*, 1996; Takayama *et al.*, 1999; Capasso *et al.*, 2001) and does not always correlate with increased α 1-subunit protein concentrations (Ferrer-Martínez *et al.*, 1996). In expanded blastocysts, therefore, α 1-subunit activity can be affected by the treatment and the internal hydrostatic pressure (a function of the number of cells pumping Na⁺ and water and of the blastocyst diameter). Combinations of these variables might explain the increased zona-shedding with the ATRA treatment and may influence gene expression, masking the direct effects of treatments in fully expanded blastocysts. Mouse embryos with targeted disruption in the Na/K-ATPase α 1-subunit progress through blastulation, but they do not expand and eventually die (Barcroft *et al.*, 2004). The latter authors indicate that, in the absence of the α 1-isoform, the expression of multiple isoforms of α - and β -subunits by the blastocyst maintains cavitation. Embryonic Na/K-ATPase activity increases when a nascent blastocoele cavity forms and then decreases to baseline levels at full expansion (Houghton *et al.*, 2003). The present control embryos showed the expression of the Na/K-ATPase α 1-subunit to return to high levels after hatching. The α -subunit has been reported to be retinoid-responsive in somatic cells (rat: Alam and Alam, 1983; Chin *et al.*, 1998; Berdanier *et al.*, 2001; human: Gilmore-Hebert *et al.*, 1989) but also to show high variability in its expression from one tissue to another, probably because of cells bearing different combinations of the RAR and RXR subtypes (Chambon, 1996).

Increased p53 expression in hatched blastocysts occurred in those groups whose retinoid activity was affected by ATRA, citral and ATRA + citral. In response to stress or proliferative signals, the p53 protein becomes stabilized, causing cells to undergo either cell-cycle arrest or apoptosis (Vousden and Lu, 2002). The regulatory effects of p53 in bovine heat-stressed blastocysts require that the protein localizes to the nucleus, whereas undisturbed embryos show only a cytoplasmic localization (Matwee *et al.*, 2000, 2001). In the present work, the TUNEL-positive morphology analysis showed that there was no p53-induced apoptosis. Similarly, p53 protein was not involved in development arrest during the early cleavage stages (Favetta *et al.*, 2004). The post-hatching behaviour of the p53 gene might not be directly retinoid dependent but rather a cell-cycle arrest response to increased proliferation signals in groups showing the highest cell counts (i.e. ATRA, citral and ATRA + citral). The expression of RAR β , a receptor with which p53 often appears to be related (Suzui *et al.*, 2004; Youssef *et al.*, 2004), might be induced by RA, as in mouse embryos (Huang *et al.*, 2006). It is likely that RAR β activation

is a signal for RA to suppress proliferation (Lee *et al.*, 1995), and a silenced RAR β in the bovine embryo (Mohan *et al.*, 2001) may be necessary to allow proliferation. Increased hydrostatic pressure in the expanded blastocyst may limit cell proliferation, as it occurs in cells subject to high atmospheric pressure (Iizuka *et al.*, 2004; Vouyouka *et al.*, 2004) or in conditions of acute hypertonicity (Dmitrieva and Burg, 2005). Osmotic stress transiently arrests the cell cycle and, when the NaCl level is too high, triggers apoptosis with participation of p53. Once the zona pellucida is broken down, pressure would decrease and cells would continue to proliferate according to program, hence the minor effects of proliferation-inducing agents, such as ATRA, in fully expanded as compared with hatched blastocysts. The expression of p53 would increase after blastocyst hatching in treatments that led to increased proliferation.

Our findings help explain some mechanisms of the hatching process *in vitro*, probably a culture artefact (Gonzales *et al.*, 2001). The modulation of retinoid metabolism may be a way to overcome the classically reduced cell numbers of *in vitro*-cultured embryos. Furthermore, combinations that enhance the ICM cells at the expense of other lineages can increase the efficiency of stem cell derivation (Buehr and Smith, 2003). In fully expanded blastocysts, the zona pellucida might represent a physical constraint to procedures aimed at stimulating cell proliferation, but cells may show their proliferative ability at hatching. Proliferation induced by 0.7 μ M ATRA regulates the distribution of apoptosis but does not increase the apoptotic rate, nor reduces necrosis, and maintains the ICM/TE cell ratio. Increases in Na/K-ATPase α 1-subunit mRNA concentration after hatching seem to depend, in part, on retinoid activity, whereas there is no clear evidence of a retinoid effect on p53 expression. Nevertheless, p53 could play a role in regulating proliferation events in blastocysts. This article also shows that embryonic cells produce active-retinoid metabolites from stored ROH. The true dimension of the retinoid stock is unknown, but these compounds certainly appear to regulate development, differentiation and cell death at early stages.

Acknowledgements

The authors thank Fernando García Ruiz for excellent technical assistance. This study was funded by Spanish Ministry of Science and Technology (Project No. AGL2002-01175). Shuntaro Ikeda was supported by MEXT, KAKENHI (15-4780).

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Submitted on December 22, 2005; resubmitted on February 13, 2006, March 7, 2006; accepted on March 14, 2006