Oocytes recovered from cows treated with retinol become unviable as blastocysts produced *in vitro*

C Hidalgo, C Díez¹, P Duque¹, J M Prendes², A Rodríguez¹, F Goyache¹, I Fernández³, N Facal¹, S Ikeda¹, C Alonso-Montes¹ and E Gómez¹

Area de Selección y Reproducción Animal (SERIDA) and ¹Area de Genética y Reproducción Animal (SERIDA), Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA), Camino de los Claveles, 604 – Somio, Gijón 33203, Asturias, Spain, ²Cooperativa de Agricultores de Gijón, Carretera Carbonera s/n Gijón, Spain and ³Consejería de Medio Rural y Pesca, Navia, Asturias, Spain

Correspondence should be addressed to E Gómez; Email: egomez@serida.org

P Duque is now at Centro de Fertilización in Vitro de Asturias (CEFIVA), Alvarez Garaya, 12 33206, Gijón (Spain)

Abstract

Retinoids have been shown to enhance developmental competence of the oocyte in cattle, sheep and pigs. In this study we investigated whether exogenous retinol stimulates the bovine oocyte during its intrafollicular growth and the time limits of exposure to exogenous retinol. In addition, we also determined the efficiency of ovum pick-up techniques in combination with retinol treatment and the viability of embryos after transfer to recipients. In Experiment 1, heifers were injected with retinol or vehicle, and concentrations of retinol in the blood were analysed on Day 0 (prior to injection), Day 1 and, together with follicular fluid, Day 4. Blood retinol increased by Day 1 and cleared on Day 4, but retinol remained higher within the follicle. In Experiment 2, oocyte donors were injected weekly with retinol or vehicle four times during a twice-per-week cycle of eight recovery sessions (starting 4 days before the first session), followed by a second eight-session cycle without treatment. Oocytes recovered were fertilized and cultured *in vitro*. Retinol treatment yielded higher numbers of low-quality oocytes throughout, although retinol measured during cycles did not change. Total oocytes, and morulae and blastocyst rates, increased during the first five sessions following treatment with retinol. As previously shown with oocytes from slaughterhouse ovaries, retinoic acid stimulated blastocyst development. Following transfer to recipients, blastocysts from oocytes exposed to retinol were unable to establish pregnancy. Our study confirms the existence of an effect of retinol on the intrafollicular oocyte in the cow and provides evidence regarding the teratogenic effect of retinol.

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Introduction

Vitamin A (all-*trans* retinol; ROH) and its metabolites are regulators of cell 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 either continue proliferation or differentiate. In the cell there are several retinoid-binding proteins which play specific roles in regulating retinoid metabolism. While retinol-binding protein (RBP) is required for the intercellular transport of ROH, cellular retinol-binding protein (CRBP) is responsible for transporting intracellular ROH that may be either stored or metabolized to retinoic acid (RA), the most important retinoid metabolite required for vertebrate embryogenesis (Morris-Kay & Ward 1999). Bovine granulosa cells synthesize RBP and CRBP (Brown *et al.* 2003), and RBP mRNA has been detected in the

oocyte (Mohan *et al.* 2001). Regulation of intracellular RA concentrations is to a great extent controlled by cellular RA-binding proteins (CRABPs). In the pig, detection of CRABP I is cycle stage-dependent, and luteal cells in dioestrus, together with oviduct and uterus in oestrus, contain the protein (Schweigert & Siegling 2001). Rats have CRABP II in granulosa cells from mature follicles and luteal cells (Bucco *et al.* 1995, Wardlaw *et al.* 1997, Zheng *et al.* 1999).

In the live cell, biologically active retinoids mediate their effects by binding to two different kinds of nuclear receptors, namely, RA receptors (RARs) and retinoid X receptors (RXRs), each having three subtypes, namely α , β and γ . Upon entering the nucleus all-*trans* RA and 9-*cis* RA can activate RARs, whereas RXRs are activated only by 9-*cis*-RA (Mangelsdorf *et al.* 1994, Chambon 1996). Heterodimerization between RXR and RAR or other members of the steroid/thyroid hormone nuclear

receptor superfamily (Chambon 1996), can occur in response to ligand-mediated activation. Heterodimers are capable of binding to specific DNA sequences named RA response elements, to increase or decrease the expression of a gene. Several component of the RA signalling pathway such as RAR α and γ , RXR α and β , retinaldehyde dehydrogenase and peroxisome-proliferator activated receptor gamma, are expressed in bovine oocytes and embryos (Mohan *et al.* 2001, 2002) and in cumulus cells (Mohan *et al.* 2003). Immunostaining detected RAR α , and RAR β in blastocysts (Mohan *et al.* 2001, 2002) and RAR α and RXR β in cumulus cells (Mohan *et al.* 2003).

It has been reported that the developmental competence of the oocyte is enhanced by ROH treatment in the cow (Shaw *et al.* 1995), sheep (Eberhardt *et al.* 1999) and gilt (Whaley *et al.* 1997, 2000), as well as in rabbits that have high blood levels of vitamin A (Besenfelder *et al.* 1996). In studies involving cows (Shaw *et al.* 1995), sheep (Eberhardt *et al.* 1999) and gilts (Whaley *et al.* 1997, 2000) the effects of retinol on follicular development were monitored and recorded 4 days post-administration. The reason why these standards were set are not described and the limits (length and dosage) of ROH treatment are unknown. Furthermore, it would be interesting to determine whether the effects of exogenous ROH were exerted on the intrafollicular oocyte, the ovulated oocyte, the proper early embryo, or both the oocyte and the embryo.

The ability of blastocysts resulting from oocytes of domestic females treated with exogenous ROH to establish and maintain pregnancy is unknown. Development of these blastocysts after transfer to recipients is worth testing since disturbances in retinoid homeostasis have been linked to developmental abnormalities (reviewed by Zile 2001). The above questions can be tested in part by treating oocyte donor cows with ROH, as those oocytes aspirated from the follicle would develop free from the effects of circulating ROH when cultured *in vitro* up to the blastocyst stage. We have earlier reported that the presence of RA in *in vitro* maturation (IVM) medium enhanced oocyte maturation, embryo development (Duque *et al.* 2002a, Gómez *et al.* 2003, 2004, Hidalgo *et al.* 2003) and pregnancy rates (Hidalgo *et al.* 2003). Since ROH has no effect during IVM at physiological temperature (Duque *et al.* 2002b, Lawrence *et al.* 2004) it was necessary to perform an overexposure of the oocytes to retinoid by using RA. Therefore, our study is intended to analyse whether ROH acts in the cow as early as the oocyte intrafollicular growth period, to contribute to define limits and timing of the oocyte exposure to exogenous ROH, and to determine the efficiency and sustainability of Ovum-Pick Up (OPU) techniques based on ROH support in terms of oocyte and blastocyst yields and viability of embryos upon transfer to recipients.

Materials and Methods

Unless otherwise stated, all chemicals were purchased from Sigma and culture media were prepared with MilliQ-water type 1 (Waters, Madrid, Spain).

Animal management

Asturiana de los Valles heifers aged 17–20 months were fed freely with home-made grass silage, mineral premix, and barley or oat straw along with 1–2 kg concentrate per day in order to maintain a body condition score of 2.5–3 (scale 1–5, where 1 emaciated and 5 obese). All experimental animals were housed, handled and used in accordance with Spanish national laws. Prior to an OPU session, each animal was restrained in a standing position in a chute and tranquillized with 2% xilacine (0.25 ml/kg live weight of Rompun; Bayer, Barcelona, Spain). Rectum and perineal regions were cleaned and disinfected prior to administering anaesthesia. Epidural anaesthesia was performed using 2% lidocaine (Laboratorios Ovejero, León, Spain). Subsequent to oocyte aspiration each donor cow received a vaginal wash with povidone-iodine (10% Betadine).

Follicle puncture

To recover oocytes by OPU, ovarian follicles were visualized with a 6.5 MHz convex echographic transducer (PB-06VE65/20BD; Sonovet, Kangwon-do, Korea) connected in parallel to an echo camera/ultrasound scanner SA 600 (Sonovet, Kangwon-do, Korea). The probe incorporated a guide through which a disposable puncture needle was introduced (V-OPAN-1760; Cook Veterinary Products, Queensland, Australia) and attached to Teflon tubing (internal and external diameter: 1.6 and 3.2 mm respectively). Tubing was connected to a 50 ml collection tube (Corning Inc., NY, USA) and the system was powered by a vacuum pump (V-MAR-5100; Cook Veterinary Products) providing pressure of 85 mmHg (equivalent to a water flow of 14 ml/min). The collection tube was kept at 37 °C in a heater (V-FTH-2012; Cook Veterinary Products). Before performing the procedure the system was rinsed with collection medium (CM) (TCM199; Invitrogen) with 25 mM Hepes, 0.4 g/l BSA Fraction V and 10 IU/ml heparin). The flow was periodically monitored and the system was rinsed with CM after the end of the session.

Collection of follicular fluid (FF) and blood, and retinol analysis

FF aspirated from single follicles were transferred into 1.5 ml Eppendorf tubes. After measuring the aspirate volume the tubes were centrifuged and the supernatant was frozen at –80 °C until further analysis. Following each collection, the aspiration circuit was extensively rinsed with physiological saline.

Blood samples were collected from coccygeal veins using vacutainer tubes containing no additives. Samples were centrifuged and the serum was stored at -80°C until further analysis. ROH concentration was determined by a quality-controlled HPLC method (Olmedilla *et al.* 1992). In brief, variable amounts of serum and FF (50–500 μl) were thawed and deproteinized with ethanol containing an internal standard (retinyl acetate). After vortexing for 45 s, analytes were extracted twice with hexane/methylene chloride (5:1 v/v), and the organic phases pooled, evaporated and reconstituted before injection into the HPLC equipment. Chromatographic analysis was carried out on Spheri-5-ODS (5 μm , 4.6×250 mm) (Applied Biosystems, San José, CA, USA), and isocratic elution was performed with acetonitrile/methylene with acetonitrile/ methylene chloride/methanol (ammonium acetate 0.025 M) (70:20:10) at a flow rate of 1.3 ml/min. Peak identification was confirmed by chromatographic behaviour and on-line spectra with a 996 Photodiode Array Detector (Waters Associates), compared with those obtained for standards and registered in the Millennium Data Station (Waters Associates). Retinyl acetate and ROH were detected at 325 nm. Using this protocol our quantification limit was 0.5 $\mu\text{g}/\text{dl}$.

Accuracy and precision of the analytical method used was evaluated periodically through participation in the Fat-Soluble Vitamins Quality Assurance Program conducted by the National Institute of Standards and Technology (NIST) (Gaithersburg, MD, USA). Accuracy was within 10% from assigned values, and within-day and between-day precision for evaluated analytes during the period of this study, assessed by blind duplicates distributed by NIST, were $<5\%$ and $<10\%$ respectively.

Oocyte search and classification

FF contents from each heifer were filtered (Emcon; Comextrade, Tarragona, Spain), rinsed with ovum culture medium + 5% fetal calf serum (FCS) and placed in a Petri dish. Intact cumulus-oocyte complexes (COCs) were picked using a stereomicroscope and washed four times in holding medium (HM) (TCM199 + 25 mM Hepes + 0.4 g/l BSA). COCs were then classified into four categories based on their morphological appearance at $\times 60$: (1) complete and compact cumulus, having at least four complete layers of clear cells; (2) complete but less compact cumulus, with at least four cell layers, or abnormally dark cells; (3) incomplete or expanded cumulus; and (4) denuded oocytes. Degenerated and broken oocytes were recorded but excluded from culture.

In vitro embryo production

For IVM, we used a basic maturation medium (MM) consisting of TCM199 + NaHCO_3 (2.2 g/l) + porcine follicle-stimulating hormone (FSH) (1 $\mu\text{g}/\text{ml}$) + luteinizing hormone (LH) (5 $\mu\text{g}/\text{ml}$) + 17β -oestradiol

(1 $\mu\text{g}/\text{ml}$) + poly vinyl alcohol (PVA) (0.5 mg/ml). After two washes in MM and a new wash in the medium assigned to each group, COCs were matured in 500 μl MM in four-well dishes (Nunc, Roskilde, Denmark) for 22–24 h at 39°C , in an atmosphere containing 5% CO_2 and saturated humidity. All categories of COCs described were used for IVM.

For *in vitro* fertilization (IVF), motile spermatozoa were separated by a swim-up procedure similar to that reported by Parrish *et al.* (1986). Frozen–thawed semen was layered into 1 ml sperm Tyrode–albumin–lactate–pyruvate (TALP) and incubated for 1 h. The upper 700 μl containing motile spermatozoa was centrifuged for 7 min at 100 g and the supernatant was aspirated leaving a pellet of approximately 100 μl in volume. Spermatozoa concentration was then determined using a haemocytometer. The COCs were washed twice in HM, twice in pre-equilibrated fertilization medium (Fert-TALP), and placed in four-well culture dishes containing Fert-TALP with heparin (10 $\mu\text{g}/\text{ml}$) (Calbiochem, La Jolla, CA, USA). Spermatozoa were then added at a concentration of 2×10^6 cells/ml in 500 μl of medium per well. IVF was accomplished by incubating oocytes and spermatozoa together for 18–20 h at 39°C in 5% CO_2 under high humidity.

For *in vitro* embryo culture, presumptive zygotes were gently passed back and forth through a small-gauge pipette to remove cumulus cells and sperm, washed three times in HM and twice in culture medium. Embryo culture was performed in modified synthetic oviduct fluid (285 mOsm, pH 7.2–7.3) containing amino acids, citrate and myo-inositol (Holm *et al.* 1999). FCS was added to the embryo culture media 42 h post-fertilization (PI). Droplets of culture medium (1–2 $\mu\text{l}/\text{embryo}$) were prepared in four-well dishes under mineral oil and equilibrated for 2 h before addition of zygotes. Incubations were carried out at 39°C in 5% CO_2 , 5% O_2 and 90% N_2 . Culture media were renewed at 66 h (Day 3) and 138 h (Day 6) PI, and embryonic development was recorded on Days 3, 6, 7 and 8.

Embryo transfer (ET)

Day 7 blastocysts were selected for ET. Embryos transferred fresh included unexpanded and expanded blastocysts, while embryos transferred after vitrification and warming were predominantly expanded blastocysts. For vitrification, embryos were loaded into straws containing PBS (Invitrogen) with glycerol, ethylene glycol and FCS, adjacent to two fractions of galactose solution (Kaidi *et al.* 2001). Embryos were warmed for 5 s in air and 10 s in a water bath at 30°C . The contents of each straw were emptied into a Petri dish and embryos were mixed in the galactose solution by slight agitation. After 5 min, the embryos were transferred to another Petri dish containing PBS + 20% FCS for 5 min. Embryos were then allowed to re-expand in B2 INRA Ménézo (C.C.D., Paris, France) at 39°C in an incubator. Finally, re-expanded embryos were loaded into 0.25 ml straws

and non-surgically transferred into synchronized recipients, aged 15–18 months.

Experimental design

Experiment 1: ROH concentration in blood and FF after administration of ROH

We first studied short-term responses to a single ROH injection using 12 heifers. Oestrus was synchronized with progestagen (Crestar; Intervet, Salamanca, Spain) and then followed with an injection of prostaglandin F₂α (Estrumate; Shering-Plough, Kenilworth, New Jersey, USA) given on Day –2 of the subsequent cycle. Blood samples were first taken at the time of progestagen removal (Day 0), just prior to treatment administration. Animals were then injected with 1 × 10⁶ IU ROH (Fluka 95144; Madrid, Spain) (*n* = 6) or corn oil (vehicle; *n* = 6) on Day 0. Blood samples were taken again on Day +1 and Day +4, and contents of single follicles measuring between 3 and 10 mm in diameter were aspirated on Day +4, and the recovered FF volume was measured. Blood plasma and FF were analysed for ROH.

Experiment 2: Influence of exogenous ROH on oocyte production and development

This experiment was performed to investigate the effect of repeated OPU sessions following ROH administration. Oocyte donors (*n* = 26) were subjected to one cycle of eight OPU sessions without superovulation at alternating intervals of 3 and 4 days, as can be seen in Table 1. Animals were injected with 1 × 10⁶ IU ROH (*n* = 13) 4 days before the first session and at the end of the second, fourth and sixth sessions. Four days later, a second OPU cycle of eight sessions started without ROH stimulation. Control groups of heifers (*n* = 13) received corn oil (vehicle) during the first cycle and were not treated in the second

cycle. Blood plasma was analysed for ROH twice per week during the first cycle and once per week during the second cycle. Visible follicles were counted and aspirated. Recovered COCs were counted and classified as mentioned earlier. COCs obtained from similarly treated donors were pooled and assigned to each of two IVM groups that contained either 0 or 0.5 μM 9-*cis*-RA in a 2 × 2 factorial design, according to ROH-treated donors. Variables monitored were follicle counts, oocytes recovered, oocyte quality and oocytes selected for IVM; cleaved zygotes and embryos with more than eight cells on Day 3; and blastocyst development on Day 7 and Day 8. Cows were managed in groups of four and six animals, and the experiment was repeated five times. Cultures with no embryonic development in all groups were not considered for analysis. Three donors could not be used beyond the fourth session in the first cycle, and two more donors during the entire second cycle because of clinical problems.

Experiment 3: Influence of exogenous ROH on blastocyst viability after ET

Embryos transferred were produced from oocytes within the first six OPU sessions in all experimental groups from Experiment 2. Recipients received either one fresh embryo or two vitrified embryos transferred into the uterine horn adjacent to a corpus luteum. Pregnancy diagnosis was confirmed using plasma progesterone (P4) concentrations (>2.5 ng/ml) on Day 21 and ultrasonographic scanning on Day 35 and subsequently at Day 60.

Statistical analysis

Data from follicle numbers, oocyte production and embryo development 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, USA). 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. Secondly, those factors identified to be significant were used to fit a linear model using the procedure General Linear Models (GLM) (SAS). This procedure performs ANOVA 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. LSMs for monitored follicles, oocyte production and embryonic development, are expressed as absolute average values on a per donor and per treatment basis, although embryonic development data were as well referred to frequency percentages of oocytes in IVM. Significant interactions between ROH and RA were detected by means of CATMOD. GLM

Table 1 Recovery of oocytes and injection and sampling routine followed during Experiment 2.

Day	OPU cycle	Session	Routine
–4			I + B
0	ROH-stimulated	1	B
3		2	I + B
7		3	B
10		4	I + B
14		5	B
17		6	I + B
21		7	B
24		8	B
28	Non-ROH-stimulated	1	B
31		2	—
35		3	B
38		4	—
42		5	B
45		6	—
49		7	B
52		8	—

I: ROH or vehicle injection; B: blood sampling for ROH analysis.

was used to reject the null hypothesis (H_0 : $LSM_{(i)} = LSM_{(j)}$) for LSMs within interaction levels. Data for ROH concentrations were processed by GLM-ANOVA and Duncan's test. Pregnancy data were submitted to chi-square test of homogeneity for each stratum of treatment-day of pregnancy, and results refer to absolute frequencies.

Results

Experiment 1

In heifers not submitted to OPU (i.e. short-term responses), blood concentrations of ROH increased 24 h after ROH injection (Fig. 1), and this rise was cleared 4 days after injection. On Day +4, one single follicle was aspirated per cow ($n = 12$) and, interestingly, ROH concentration was higher in follicles in cows treated with ROH compared with controls. Stage of the oestrous cycle did not influence blood and FF ROH concentrations. The average FF volume recovered was $393 \pm 127 \mu\text{l}$ (range 80–950) and $351 \pm 127 \mu\text{l}$ (range 100–700) for ROH and oil-treated heifers respectively ($P > 0.8$), and the measured volume of FF correlated with ROH concentrations ($r = 0.70$, $P = 0.034$).

During the OPU cycles (Table 1), the samples were recovered at the end of each session in the first cycle (twice per week) and after the first, third, fifth and seventh sessions within the second cycle (once per week). Exogenous administration of ROH gave no differences ($P > 0.15$) between and within the treated first cycle and the untreated second cycle (Table 2).

Figure 1 Short-term changes in retinol concentrations in blood (diamonds) and ovarian follicle (squares) from cows injected with retinol (black) or vehicle (open). Error bars show $LSM \pm S.E.$ ($^{v,x,y,z}P < 0.01$)

Table 2 Average retinol blood concentrations in oocyte donor cows during the first cycle treated with retinol (weekly injected with 1×10^6 IU retinol or corn oil as vehicle during eight OPU sessions), and a second untreated cycle consisting of eight OPU sessions.

Cycle	Group	Cows (n)	Retinol ($\mu\text{g/dl}$)
1st (treated)	ROH	5	24.77 ± 1.55
2nd (untreated)	ROH		22.28 ± 1.52
1st (treated)	Oil	6	24.74 ± 1.83
2nd (untreated)	Oil		25.93 ± 1.70

Values are $LSM \pm S.E.$ ($P > 0.05$).

Experiment 2

Responses to ROH throughout cycles 1 and 2, in terms of follicles monitored and oocytes recovered are plotted in Fig. 2a and b. A gradual decrease in follicle numbers and oocytes recovered, although independent from treatment, was observed. Overall, the first cycle was more productive than the second cycle (Table 3), and ROH injected within the first cycle had no significant influence on subsequent oocyte production during the second cycle. More low-quality oocytes were harvested in the first cycle from donors treated with ROH.

Overall embryonic development within both OPU cycles is showed in Table 4. While no differences could be observed between cleavage and the five to eight cell stage (not shown in Table), embryos matured with RA showed higher developmental kinetics on Day 3, as more embryos developed to the 8–16 cell stage. During the first cycle, RA increased the formation of morula but did not increase blastocyst formation rates. However, cumulatively, RA did increase blastocyst rates.

When oocyte production was studied on a cumulative session basis, we found that significant responses to ROH were obtained in sessions one to five within the first cycle (Table 5). Anticipating overall results, COCs yields within the ROH group accounted for higher numbers of low-quality recovered COCs (categories 3 and 4), as well as for higher follicle numbers. Interestingly, more morulae and blastocysts developed from oocytes collected during sessions one to five from ROH-treated animals (Table 6). On the other hand, embryonic development observed with oocytes obtained from control animals treated with RA was even better than those observed with ROH-treated animals. Differences were not observed later than session five since oocytes recovered in subsequent sessions during both the first and second cycle did not respond to RA.

Experiment 3

Embryos derived from cows treated with ROH were unable to establish a viable pregnancy (Table 7). As shown by low P4 levels on Day 21, development of transferred blastocysts was most probably disrupted before implantation. Pregnancy rates were independent of whether fresh or vitrified embryos were transferred.

Discussion

In the live animal, ROH is transported in the blood bound to RBP and depending on the requirement is delivered to the target tissues for metabolism to RA. Therefore, the influence of ROH on follicular and oocyte growth could have been exerted through its conversion to RA following its uptake from the plasma. The RA thus formed in the follicular cells can exert its action by binding to and activating specific RA receptors. In the present study, cows injected with ROH developed follicles containing high

Figure 2 (a) Follicles monitored during two oocyte recovery cycles (retinol stimulated and unstimulated) in donor cows treated with retinol (■) or vehicle (□). (b) Oocytes recovered during two cycles (retinol stimulated and unstimulated) in donor cows treated with retinol (■) or vehicle (□). (ROH data, black regression line; Control data, grey regression line).

Table 3 Monitored follicles and oocyte production during the 1st and 2nd cycle of eight sessions from oocyte donor cows treated with ROH or corn oil and a 2nd cycle of eight sessions without treatment.

Cycle	Group	Cows (n)	S	N	Follicles	Average COCs			
						HQ	LQ	Cultured	Total
1st (treated)	ROH	13	89	517	9.52 ^a	2.68 ^a	2.90 ^a	5.59 ^a	5.92 ^a
1st (treated)	Oil	13	91	458	8.56 ^a	2.61 ^a	2.16 ^b	4.77 ^a	5.15 ^a
2nd (untreated)	ROH	10	63	199	5.87 ^b	1.75 ^b	1.35 ^c	2.98 ^b	3.20 ^b
2nd (untreated)	Oil	11	72	225	5.38 ^b	1.51 ^b	1.22 ^c	2.87 ^b	3.07 ^b

^{a,b,c}($P < 0.03$). S = number of sessions. N = total oocytes recovered. HQ: COCs quality 1 and 2; LQ: COCs quality 3 and 4.

Table 4 Developmental competence of oocytes obtained during the first OPU cycle consisting of eight sessions from donor cows treated with ROH or corn oil (vehicle, controls) and during the 2nd cycle consisting of eight sessions without treatment following *in vitro* maturation with or without 9-*cis* retinoic acid (RA).

OPU cycle	ROH (cow)	RA (IVM)	Day 3 (%)		Day 6 morulae (%)	Blastocysts (%)	
			Cleaved	8–16 cells		Day 7	Day 8
1	Oil	–	64.5 ± 5.2	3.7 ± 2.1	12.9 ± 4.0 ^a	7.3 ± 2.6	8.9 ± 2.8
1	Oil	+	62.9 ± 5.2	8.6 ± 2.1	31.1 ± 4.0 ^b	13.3 ± 2.6	15.1 ± 2.8
1	+	–	56.2 ± 5.2	2.5 ± 2.1 ^a	22.4 ± 4.0	10.7 ± 2.6	10.8 ± 2.8
1	+	+	63.7 ± 5.2	4.4 ± 2.1	22.1 ± 4.0	10.5 ± 2.6	11.8 ± 2.8
2	–	–	70.4 ± 6.7	3.4 ± 2.6	25.7 ± 5.0	6.9 ± 3.6	8.1 ± 3.9
2	–	+	64.1 ± 6.8	10.3 ± 2.7 ^b	20.3 ± 5.0 ^a	13.3 ± 3.6	15.0 ± 3.9
2	+	–	71.5 ± 7.0	3.3 ± 2.8	24.2 ± 5.1	9.5 ± 3.6	11.9 ± 3.9
2	+	+	70.3 ± 7.1	3.0 ± 2.8 ^a	27.6 ± 5.3	9.6 ± 3.6	10.5 ± 3.9
Cumulative							
1 + 2	–	–	66.0 ± 4.2	3.5 ± 1.6 ^x	—	7.2 ± 2.1 ^a	8.7 ± 2.3 ^a
1 + 2	–	RA	64.9 ± 4.2	9.3 ± 1.7 ^y	—	13.3 ± 2.1 ^b	15.1 ± 2.3 ^b
1 + 2	ROH	–	61.3 ± 4.3	2.8 ± 1.7 ^x	—	10.4 ± 2.1	11.9 ± 2.3
1 + 2	ROH	RA	63.1 ± 4.3	3.8 ± 1.7 ^x	—	10.3 ± 2.1	10.7 ± 2.3

^{a,b} $P = 0.055$; ^{x,y} $P < 0.01$.

Table 5 Follicles monitored, COC recovery rate, their numbers and quality in oocyte donor cows treated with ROH or vehicle during sessions 1 to 5 within the 1st ROH-stimulated cycle.

Group	Sessions 1–5			Follicles (n/session)	Recovered COCs (%)	COCs (n)			
	Cows	OPU	COCs			HQ	LQ	Cultured	Total
ROH	13	53	350	10.3 ± 0.6 ^a	63.0 ± 4.4	2.6 ± 0.3	3.4 ± 0.2 ^x	6.1 ± 0.4 ^a	6.5 ± 0.5 ^a
Control	13	55	270	8.3 ± 0.6 ^b	57.0 ± 4.4	2.4 ± 0.3	2.4 ± 0.2 ^y	4.8 ± 0.4 ^b	5.1 ± 0.5 ^b

Different superscripts differ significantly: ^{a,b} $P < 0.03$; ^{x,y} $P < 0.01$. Recovered COCs are a percentage of number of follicles observed. OPU: number of OPU sessions. Values refer to a session average; HQ: COCs quality 1 and 2; LQ: COCs quality 3 and 4.

Table 6 Embryonic development of oocytes obtained during sessions 1 to 5 from cows treated with all-*trans* retinol (ROH) or corn oil (vehicle) and matured with or without 9-*cis* retinoic acid (RA).

Treatment		Sessions 1–5			Morulae (Day 6)		Blastocysts (Day 7)		Blastocysts (Day 8)	
Cow	IVM	D	OPU	COC	N	%	N	%	N	%
Oil	–	13	28	128	0.61 ± 0.3 ^x	7.9 ± 4.4 ^x	0.28 ± 0.2 ^a	2.9 ± 2.8 ^{ax}	0.36 ± 0.3	4.7 ± 3.0 ^{ax}
Oil	RA		27	142	2.05 ± 0.2 ^y	38.2 ± 4.4 ^{xy}	1.10 ± 0.2 ^b	20.3 ± 2.8 ^{by}	1.11 ± 0.3	22.0 ± 3.0 ^{by}
ROH	–	13	26	169	2.08 ± 0.3 ^y	24.8 ± 4.4 ^{by}	1.01 ± 0.2	12.3 ± 2.8 ^c	1.12 ± 0.3	13.1 ± 3.0 ^c
ROH	RA		27	181	2.00 ± 0.2 ^y	24.7 ± 4.4 ^{by}	0.95 ± 0.2	11.6 ± 2.8 ^c	1.05 ± 0.3	11.6 ± 3.0 ^c

D: number of donors; OPU: number of OPU sessions; COC: number of COCs cultured. Data are either average embryos obtained per treatment and session (N: corrected by COCs cultured within the same cow treatment), or proportions of matured oocytes (%). Different superscripts in the same column differ significantly: ^{a,b,c}*P* < 0.05; ^{x,y}*P* < 0.01; ^{α,β}*P* < 0.001.

Table 7 Pregnancy rates after the transfer to heifers of fresh (one embryo/recipient) or vitrified (V) (two embryos/recipient) bovine blastocysts produced *in vitro* using oocytes recovered from donor cows treated with all-*trans* retinol (ROH) or corn oil (vehicle; negative controls (–), and matured in defined medium TCM199–PVA and hormones) with (+) or without (–) 9-*cis*-retinoic acid (RA).

Cow	IVM	Embryos transferred		ETs (n)	Pregnancy (%)		
		Fresh	Vitrified		Day 21	Day 35	Day 60
ROH	–	5	12	11	0 ^a (0/11)	0 ^a	0*
ROH	RA	1	6	4	0 ^a (0/4)	0 ^a	0*
–	–	4	6	7	43 (3/7) (1V)	14 (1/7) (1V)	0*
–	RA	8	6	11	45 ^b (5/11) (1V)	45 ^b (5/11) (1V)	36** (4/11) (1V)
Cumulative							
ROH	–	6	18	15	0 ^x (0/15)	0 ^x	—
–	–	12	12	18	44 ^y (8/18)	33 ^y (6/18)	—

Pregnancy diagnosis: Day 21, P4 concentrations >4 ng/ml + no oestrus; Days 35 and 60, ultrasound scanning; different superscripts within columns statistically differ: ^{x,y}*P* < 0.02; ^{a,b}*P* < 0.05; ^{*,**}*P* = 0.07.

A proportion of control embryos transferred and their pregnancy rates were published in Hidalgo *et al.* (2003) reporting that RA during IVM increases pregnancy rates. These data are used in the present experiments merely as positive controls; they are not treated as results and do not form part of the discussion by themselves.

FF ROH concentrations while maintaining normal concentrations in the blood. This suggests that the follicle is able to incorporate ROH in less than 4 days following exogenous administration. This increase substantiates the administration of ROH at least 4 days before an effect is expected. Concentrations of ROH in FF appeared to be lower in our control group than has been reported by others for untreated animals (Chew *et al.* 1984, Schweigert *et al.* 1986, Haliloglu *et al.* 2002). However, the FF ROH concentrations in the treated group fell within the above reported range for untreated animals (Chew *et al.* 1984, Schweigert *et al.* 1986, Haliloglu *et al.* 2002). In addition, mean ROH blood concentrations in our study, in both ROH-treated and controls, are in agreement with previous reports (Chew *et al.* 1984, Schweigert *et al.* 1986, Frye *et al.* 1991, Velasquez-Pereira *et al.* 1998, Haliloglu *et al.* 2002, Katamoto *et al.* 2003). During all OPU cycles, ROH values did not differ between treatments and between cycles, providing evidence that blood ROH regulatory mechanisms were not influenced by repeated ROH injections. It is possible that supplementary ROH was either stored or compartmentalized (FF). Using Holstein cattle, Haliloglu *et al.* (2002) found differences in ROH concentrations between blood and FF only during the oestrous phase of the cycle and no differences were observed during other phases of the oestrous cycle. In

agreement with the findings of Schweigert *et al.* (1986), the concentration of ROH in the blood was higher than in FF, especially towards the beginning of dioestrus. Contrary to the findings of Haliloglu *et al.* (2002), we detected a positive correlation between FF volume and ROH as reported by others (Schweigert *et al.* 1986, Schweigert & Zucker 1988). The ability to take up ROH possibly indicates that the follicles were healthy and viable as non-atretic large follicles during the oestrous phase have the highest concentrations of ROH (Schweigert & Zucker 1988). Unlike in the pig, in the present study, the volume of FF recovered was not influenced by retinoid treatment as reported in pigs (Whaley *et al.* 2000).

Gonadotrophins such as FSH either alone (Bousquet *et al.* 1999, Goodhand *et al.* 1999, Sirard *et al.* 1999) or in combination with LH (Blondin *et al.* 2002, see a review by Merton *et al.* 2003) and pregnant mare serum gonadotrophin (Pieterse *et al.* 1991) have been used to improve the outcome of OPU in adult donor cows. Oocytes from superovulated cows are developmentally more competent than their non-stimulated counterparts (Faber *et al.* 2003, Merton *et al.* 2003), and specific changes in superovulatory treatment can greatly increase developmental competence of the oocyte (Blondin *et al.* 2002). However, due to high variability among gonadotrophin batches and being an animal product we considered ROH as a potential

alternative or complementary treatment to hormonal stimulation. In addition, OPU performed at 3- or 4-day intervals is a scheme that can be used for up to 4 consecutive months (Petyim *et al.* 2003) and has been shown to yield more oocytes per session and embryos from each oocyte batch collected at 3-day intervals (Merton *et al.* 2003), although we found no significant differences between same intervals (data not shown). Our hypothesis is further supported by the fact that retinoid treatment has been previously shown to increase litter size in the gilt (Brief & Chew 1983, Coffey & Britt 1993) and rabbit (Besenfelder *et al.* 1996), and improve embryonic quality without modifying ovulation rates (Shaw *et al.* 1995) in superovulated cows (Shaw *et al.* 1995) and ewes (Eberhardt *et al.* 1999).

During the first oocyte recovery sessions ROH increased the total number of monitored follicles and aspirated oocytes. However, most of the oocytes recovered were of low quality. In the present study we found that oocyte donors were more responsive to ROH following the first three doses, giving us some indication regarding the optimal length of ROH treatment. Oocytes from cows treated with ROH showed reduced morulae and blastocysts formation rates, analogous to that seen in an earlier study with oocytes continuously stimulated with RA during IVM in the presence of a meiotic inhibitor (Duque *et al.* 2002). It has been described that resistance to RA, a phenomenon involving a multifactorial aetiology that is not very well understood, develops rapidly (Marill *et al.* 2003) and could be involved in decreased late responses observed in our cows and oocytes. Higher transition rates by the eight cell stage were observed with *in vivo* embryos from ewes treated with ROH (Eberhardt *et al.* 1999), but in our study we did not observe this effect with ROH. Nevertheless, the ROH metabolite RA gave more embryos produced by OPU at the 8–16 cell stage on Day 3. This effect was not observed in our previous experiments with slaughterhouse oocytes, so it probably depends on heterogeneous quality within cultured oocytes and/or the OPU procedure itself. The oocyte transcriptional machinery and protein synthesis can be altered by RA stimulation through activating specific genes such as midline (Gómez *et al.* 2003). The recombinant peptide product of midline exerts beneficial effects on embryonic development when added during IVM (Ikeda *et al.* 2000a,b), although it is also an intermediary candidate in the induction of developmental alterations (Griffith & Zile 2000). The stimulation by RA at IVM triggers a more physiological cytoplasmic granule migration (Duque *et al.* 2002) and increases oocyte poly-adenylation, suggesting superior mRNA quality and increased processing (Gómez *et al.* 2004). This coincides with unaltered expression levels of cyclins B1 and H1, specific genes regulating the cell cycle, and less oxidative damage (Gómez *et al.* 2004). Altogether, the above findings correspond to more developmentally competent oocytes (Duque *et al.* 2002, Hidalgo *et al.* 2003). The ovarian response to FSH and LH can also be modulated by RA. In response to this

retinoid, stimulation of steroidogenesis (Bugavandoss & Midgley 1988, Graves-Hoagland *et al.* 1988), and restricted expression of FSH receptor (FSHR) in rat granulosa cells has been reported (Minegishi *et al.* 1996, 2000), which could affect oocyte growth and in turn the outcome of OPU in the cow (Argov *et al.* 2004). In the porcine, the effects are controversial, as exposure of immature granulosa cells to RA inhibited expression of LH receptor (LHR) by downregulation of c-Fos mRNA (Hattori *et al.* 2000). In addition, feeding gilts with ROH did not affect P4 secretion by granulosa cells with or without LH stimulation (Whaley *et al.* 2000). In the bovine, decreased levels of FSHR and LHR in cumulus-granulosa cells have been shown to be associated with low-quality oocytes (Calder *et al.* 2003). In the presence of RA, the growth hormone (GH) receptor (GHR) gene increases transcription (Ohlsson *et al.* 1993, Sloomweg *et al.* 1996). The transcript of the GHR was demonstrated in oocytes, follicular cells and the corpus luteum of the bovine ovary (Kolle *et al.* 1998), and GH was found to stimulate the bovine oocyte during IVM via cumulus cells (Izadyar *et al.* 1997, Kolle *et al.* 2003).

In the present study, development of blastocysts from cows that received multiple ROH injections was disrupted before implantation. This is in contrast to pregnancies obtained when RA was present during IVM (Hidalgo *et al.* 2003), as these embryos were able to survive beyond the expected time for completion of organogenesis (Day 42) (Hubbert 1974). Vitamin A deficiency and excess can adversely affect the development of the vertebrate embryo as almost every organ or tissue can be affected if there is an imbalance in RA homeostasis during certain critical stages of development (reviewed by Zile 2001). Adequate metabolic systems must inactivate unused RA in order to maintain a stringent retinoid balance, as additional RA synthesis by the embryo is essential for post-implantation development (Niederreither *et al.* 1999). Most studies on retinoids have focused on later stages of embryonic development, although exposure of zygotes to RA during early embryonic development yields anomalies during pre-gastrulation and organogenesis (Rutledge 1997). Recently, RA treatment during the pre-implantation period in the mouse (Huang *et al.* 2001, 2003) was shown to cause developmental retardation, inhibition of cell proliferation and induction of apoptosis. In previous reports, development of blastocysts to term was not analysed (Shaw *et al.* 1995, Whaley *et al.* 1997, 2000, Eberhardt *et al.* 1999), and the precise stage (oocyte, embryo or both) during which retinoids exerted their effects was not determined. Our results involving oocytes isolated by OPU following ROH treatment and subsequently cultured *in vitro* demonstrate that ROH can influence development by acting on the oocyte during its intrafollicular growth. In the future, it would be interesting to find out the outcome had the oocyte following ROH treatment been fertilized *in vivo* and the embryo recovered at specific stages during pre-implantation development, as done in earlier studies (Shaw *et al.* 1995, Besenfelder *et al.* 1996, Whaley *et al.* 1997, 2000, Eberhardt *et al.* 1999).

It is interesting to note that in the present study, the development of embryos following transfer was negatively influenced by ROH treatment of the oocyte donor cow while RA treatment of the oocyte during IVM in our earlier studies promoted oocyte developmental competence. The animal cell contains all the components required to bind and regulate the release of retinoids, from β -carotene up to the nuclear effector RA. It is possible that this system could have failed since repeated ROH administration did not produce long-term increases in blood ROH concentrations above physiological levels. Instead, excess ROH could have been compartmentalized and stored (i.e. the follicle). Serum-derived follicular ROH can bind to specific protein in granulosa cells (Brown *et al.* 2003) and the oocyte (Mohan *et al.* 2001). After entry into the cell, ROH can be metabolized to RA, which can then be bound by specific CRABPs. As previously demonstrated in rats (Bucco *et al.* 1995, Wardlaw *et al.* 1997, Zheng *et al.* 1999), intracellular proteins that regulate RA availability may not be expressed in immature follicles; as a result the availability of RA in the follicle could have gone unchecked. On the contrary, under *in vitro* conditions, by providing a carefully calculated dose, it is possible to ensure that an optimum concentration of RA is made available to the COCs during *in vitro* culture.

The present study confirms the existence of an effect of ROH on the bovine oocyte during intrafollicular growth. As a single dose, injected ROH increases ROH concentration in blood by 24 h. By 4 days after injection, blood ROH clearance was observed, but interestingly ROH remained higher within the follicle. Weekly repeated ROH injections enabled the recovery of higher numbers of low-quality oocytes by OPU throughout the cycles studied. Furthermore, total oocytes recovered as well as morulae and blastocyst formation rates increased during the first five sessions in cows treated with ROH. However, ROH levels measured during OPU cycles did not change, suggesting the non-existence of ROH downregulation. On the other hand, as previously demonstrated using oocytes from slaughterhouse ovaries, RA stimulated blastocyst development (Duque *et al.* 2002, Hidalgo *et al.* 2003). Accordingly, under the RA influence, higher proportions of embryos overcame the developmental block on Day 3 of culture. Once transferred to recipients, blastocysts derived from oocytes from cows treated with ROH were unable to set pregnancy, showing an early sign of teratogenic effect of ROH. Exposure to ROH and RA should be avoided in candidate mothers as early as possible prior to the fertilization time.

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References

- Argov N, Arav A & Sklan D 2004 Number of oocytes obtained from cows by OPU in early, but no late lactation increased with plasma insulin and estradiol concentrations and expression of mRNA of the FSH receptor in granulosa cells. *Theriogenology* **61** 947–962.
- Besenfelder UL, Solti J, Seregi M, Müller M & Brem G 1996 Different roles for β -carotene and vitamin A in the reproduction of rabbits. *Theriogenology* **45** 1583–1591.
- Blondin P, Bousquet D, Twagiramungu H, Barnes F & Sirard MA 2002 Manipulation of follicular development to produce developmentally competent bovine oocytes. *Biology of Reproduction* **66** 38–43.
- Bousquet D, Twagiramungu H, Morin N, Brisson C, Carboneau G & Durocher J 1999 *In vitro* embryo production in the cow: an effective alternative to the conventional embryo production approach. *Theriogenology* **51** 59–70.
- Brief S & Chew BP 1983 Effects of vitamin A and β -carotene on reproductive performance in gilts. *Journal of Animal Science* **60** 998–1004.
- Brown JA, Eberhardt DM, Schrick FN, Roberts MP & Godkin JD 2003 Expression of retinol-binding protein and cellular retinol-binding protein in the bovine ovary. *Molecular Reproduction and Development* **64** 261–269.
- Bucco RA, Melner MH, Gordon DS, Leers-Sucheta S & Ong DE 1995 Inducible expression of cellular retinoic acid-binding protein II in rat ovary: gonadotropin regulation during luteal development. *Endocrinology* **136** 2730–2740.
- Bugavandoss P & Midgley AR 1988 Biphasic action of retinoids on gonadotropin receptor induction in rat granulosa cells *in vitro*. *Life Sciences* **43** 1607–1614.
- Calder MD, Caveney AN, Smith LC & Watson AJ 2003 Responsiveness of bovine cumulus-oocyte-complexes (COC) to porcine and recombinant human FSH, and the effect of COC quality on gonadotropin receptor and Cx43 marker gene mRNAs during maturation *in vitro*. *Reproductive Biology and Endocrinology* **1** 14.
- Chambon P 1996 A decade of molecular biology of retinoic acid receptors. *FASEB Journal* **10** 940–954.
- Chew BP, Holpuch DM & O'Fallon JV 1984 Vitamin A and beta-carotene in bovine and porcine plasma, liver, corpora lutea, and follicular fluid. *Journal of Dairy Science* **67** 1316–1322.
- Coffey MT & Britt JH 1993 Enhancement of sow reproductive performance by β -carotene or vitamin A. *Journal of Animal Science* **71** 1198–1202.
- Duque P, Díez C, Royo L, Lorenzo PL, Carneiro G, Hidalgo CO *et al.* 2002 Enhancement of developmental capacity of meiotically inhibited bovine oocytes by retinoic acid. *Human Reproduction* **17** 2706–2714.
- Eberhardt DM, Will WA & Godkin JD 1999 Retinol administration to superovulated ewes improves *in vitro* embryonic viability. *Biology of Reproduction* **60** 1483–1487.
- Faber DC, Molina JA, Ohlrichs CL, Vander Zwaag DF & Ferré LB 2003 Commercialization of animal biotechnology. *Theriogenology* **59** 125–138.
- Frye TM, Williams SN & Graham TW 1991 Vitamin deficiencies in cattle. *Veterinary Clinics of North America. Food Animal Practice* **7** 217–275.
- Gómez E, Royo LJ, Duque P, Carneiro G, Hidalgo C, Goyache F *et al.* 2003 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. *Molecular Reproduction and Development* **66** 247–255.

- Gómez E, Rodríguez A, Goyache F, Díez C, Jose Royo L, Moreira PN *et al.* 2004 Retinoid-dependent mRNA expression and poly-(A) contents in bovine oocytes meiotically arrested and/or matured *in vitro*. *Molecular Reproduction and Development* **69** 101–108.
- Goodhand KL, Watt RG, Staines ME, Hutchinson JSM & Broadbent PJ 1999 *In vivo* oocyte recovery and *in vitro* embryo production from bovine donors aspirated at different frequencies or following FSH treatment. *Theriogenology* **51** 951–961.
- Graves-Hoagland RL, Hoagland TA & Woody CO 1988 Effect of β -carotene and vitamin A on progesterone production by bovine luteal cells. *Journal of Dairy Science* **71** 1058–1062.
- Griffith M & Zile MH 2000 Retinoic acid, midkine and defects of secondary neurulation. *Teratology* **62** 123–133.
- Haliloglu S, Baspinar N, Serpek B, Erdem H & Bulut Z 2002 Vitamin A and beta-carotene levels in plasma, corpus luteum and follicular fluid of cyclic and pregnant cattle. *Reproduction in Domestic Animals* **37** 96–99.
- Hattori M, Takesue K, Nishida N, Kato Y & Fujihara N 2000 Inhibitory effect of retinoic acid on the development of immature porcine granulosa cells to mature cells. *Journal of Molecular Endocrinology* **25** 53–61.
- Hidalgo CO, Díez C, Duque P, Facal N & Gómez E 2003 Pregnancies and improved early embryonic development in bovine oocytes matured *in vitro* with 9-*cis*-retinoic acid. *Reproduction* **125** 409–416.
- Holm P, Booth PJ, Schmidt MH, Greve T & Callesen H 1999 High bovine blastocyst development in a static *in vitro* production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum proteins. *Theriogenology* **52** 683–700.
- Huang FJ, Wu TC & Tsai MY 2001 Effect of retinoic acid on implantation and post-implantation development of mouse embryos *in vitro*. *Human Reproduction* **16** 2171–2176.
- Huang FJ, Shen CC, Chang SY, Wu TC & Hsuw YD 2003 Retinoic acid decreases the viability of mouse blastocysts *in vitro*. *Human Reproduction* **18** 130–136.
- Hubbert WT 1974 Factors affecting survival of the bovine fetus and neonate. *Theriogenology* **1** 15–34.
- Ikeda S, Ichihara-Tanaka K, Azuma T, Muramatsu T & Yamada M 2000a Effects of midkine during *in vitro* maturation of bovine oocytes on subsequent developmental competence. *Biology of Reproduction* **63** 1067–1074.
- Ikeda S, Nishikimi A, Ichihara-Tanaka K, Muramatsu T & Yamada M 2000b cDNA cloning of bovine midkine and production of the recombinant protein, which affects *in vitro* maturation of bovine oocytes. *Molecular Reproduction and Development* **57** 99–107.
- Izadyar F, Van Tol HT, Colenbrander B & Bevers MM 1997 Stimulatory effect of growth hormone on *in vitro* maturation of bovine oocytes is exerted through cumulus cells and not mediated by IGF-I. *Molecular Reproduction and Development* **47** 175–180.
- Kaidi S, Bernard S, Lambert P, Massip A, Dessy F & Donnay I 2001 Effect of conventional controlled-rate freezing and vitrification on morphology and metabolism of bovine blastocysts produced *in vitro*. *Biology of Reproduction* **65** 1127–1134.
- Katamoto H, Yamada Y, Nishizaki S & Hashimoto T 2003 Seasonal changes in serum vitamin A, vitamin E and beta-carotene concentrations in Japanese Black breeding cattle in Hyogo prefecture. *Journal of Veterinary Medical Science* **65** 1001–1002.
- Kolle S, Sinowatz F, Boie G & Lincoln D 1998 Developmental changes in the expression of the growth hormone receptor messenger ribonucleic acid and protein in the bovine ovary. *Biology of Reproduction* **59** 836–842.
- Kolle S, Stojkovic M, Boie G, Wolf E & Sinowatz F 2003 Growth hormone-related effects on apoptosis, mitosis, and expression of connexin 43 in bovine *in vitro* maturation cumulus-oocyte complexes. *Biology of Reproduction* **68** 1584–1589.
- Lawrence JL, Payton RR, Godkin JD, Saxton AM, Schrick FN & Edwards JL 2004 Retinol improves development of bovine oocytes compromised by heat stress during maturation. *Journal of Dairy Science* **87** 2449–2454.
- Mangelsdorf DJ, Umeson K & Evans RM 1994 Cellular biology and biochemistry of the retinoids. In *The Retinoids – Biology, Chemistry and Medicine*, pp 319–349. Eds MB Sporn, AB Roberts & DS Goodman. New York: Raven Press.
- Marill J, Idres N, Capron CC, Nguyen E & Chabot GG 2003 Retinoic acid metabolism and mechanism of action: a review. *Current Drug Metabolism* **4** 1–10.
- Merton JS, de Roos APW, Mullaart E, de Ruigh L, Kaal L, Vos PLAM *et al.* 2003 Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. *Theriogenology* **59** 651–674.
- Minegishi T, Karino S, Tano M, Ibuki Y & Miyamoto K 1996 Regulation of midkine messenger ribonucleic acid levels in cultured rat granulosa cells. *Biochemical and Biophysical Research Communications* **229** 799–805.
- Minegishi T, Hirakawa T, Kishi H, Abe K, Tano M, Abe Y *et al.* 2000 The mechanisms of retinoic acid-induced regulation on the follicle-stimulating hormone receptor in rat granulosa cells. *Biochimica et Biophysica Acta* **1495** 203–211.
- Mohan M, Malayer JR, Geisert RD & Morgan GL 2001 Expression of retinol-binding protein messenger RNA and retinoic acid receptors in preattachment bovine embryos. *Molecular Reproduction and Development* **60** 289–296.
- Mohan M, Malayer JR, Geisert RD & Morgan GL 2002 Expression patterns of retinoid X receptors, retinaldehyde dehydrogenase, and peroxisome proliferator activated receptor gamma in bovine preattachment embryos. *Reproductive Biology and Endocrinology* **66** 692–700.
- Mohan M, Thirumalapura NR & Malayer J 2003 Bovine cumulus-granulosa cells contain biologically active retinoid receptors that can respond to retinoic acid. *Reproductive Biology and Endocrinology* **1** 104.
- Morris-Kay GM & Ward SJ 1999 Retinoids and embryonic development. *International Review of Cytology* **188** 73–133.
- Niederreither K, Subbarayan V, Dolle P & Chambon P 1999 Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nature Genetics* **21** 346–347.
- Ohlsson C, Lovstedt K, Holmes PV, Nilsson A, Carlsson L & Tornell J 1993 Embryonic stem cells express growth hormone receptors: regulation by retinoic acid. *Endocrinology* **133** 2897–2903.
- Olmedilla B, Granado F, Blanco I & Rojas E 1992 Determination of nine carotenoids, retinol, retinyl palmitate and alpha-tocopherol in control human serum, using two internal standards. *Food Chemistry* **45** 205–213.
- Parrish JJ, Susko-Parrish JL, Leibfried-Ruthledge ML, Critser ES, Eyestone WH & First NL 1986 Bovine *in vitro* fertilization with frozen-thawed semen. *Theriogenology* **25** 591–600.
- Petyim S, Bage R, Hallap T, Bergqvist AS, Rodriguez-Martinez H & Larsson B 2003 Two different schemes of twice-weekly ovum pick-up in dairy heifers: effect on oocyte recovery and ovarian function. *Theriogenology* **60** 175–188.
- Pieterse MC, Vos PLAM, Kruip Th AM, Wurth YA, Van Beneden Th H, Willamse AH *et al.* 1991 Transvaginal ultrasound guided follicular aspiration of bovine oocytes. *Theriogenology* **35** 19–24.
- Rutledge JC 1997 Developmental toxicity induced during early stages of mammalian embryogenesis. *Mutation Research* **396** 113–127.
- Schweigert FJ & Siegling C 2001 Immunolocalization of retinol-binding protein, cellular retinoic acid-binding protein I and retinoid X receptor beta in the porcine reproductive tract during the oestrous cycle. *Reproduction, Fertility, and Development* **13** 421–426.
- Schweigert FJ & Zucker H 1988 Concentrations of vitamin A, beta-carotene and vitamin E in individual bovine follicles of different quality. *Journal of Reproduction and Fertility* **82** 575–579.
- Schweigert FJ, Lutterbach A, Rambeck WA & Zucker H 1986 Vitamin A- and beta-carotene concentrations in bovine follicular

- fluid in relationship to follicle size. *Journal of Veterinary Medicine A, Physiology, Pathology, Clinical Medicine* **33** 360–364.
- Shaw DW, Farin PW, Washburn SP & Britt JH** 1995 Effect of retinol palmitate on ovulation rate and embryo quality in superovulated cattle. *Theriogenology* **44** 51–58.
- Sirard MA, Picard L, Dery M, Coenen K & Blondin P** 1999 The time interval between FSH administration and ovarian aspiration influences the development of cattle oocytes. *Theriogenology* **51** 699–708.
- Slootweg MC, Salles JP, Ohlsson C, de Vries CP, Engelbregt MJ & Netelenbos JC** 1996 Growth hormone binds to a single high affinity receptor site on mouse osteoblasts: modulation by retinoic acid and cell differentiation. *Journal of Endocrinology* **150** 465–472.
- Velasquez-Pereira J, McDowell LR, Risco CA, Prichard D, Martin FG, Calhoun MC et al.** 1998 Effects on performance, tissue integrity, and metabolism of vitamin E supplementation for beef heifers fed a diet that contains gossypol. *Journal of Animal Science* **76** 2871–2884.
- Wardlaw SA, Bucco RA, Zheng WL & Ong DE** 1997 Variable expression of cellular retinol- and cellular retinoic acid-binding proteins in the rat uterus and ovary during the estrous cycle. *Biology of Reproduction* **56** 125–132.
- Whaley SI, Hedgpeth VS & Britt JH** 1997 Evidence that injection of vitamin A before mating may improve embryo survival in gilts fed normal or high-energy diets. *Journal of Animal Science* **75** 1071–1077.
- Whaley SL, Hedgpeth VS, Farin CE, Martus NS, Jayes FC & Britt JH** 2000 Influence of vitamin A injection before mating on oocyte development, follicular hormones, and ovulation in gilts fed high-energy diets. *Journal of Animal Science* **78** 1598–1607.
- Zheng WL, Bucco RA, Sierra-Rievera E, Osteen KG, Melner MH & Ong DE** 1999 Synthesis of retinoic acid by rat ovarian cells that express cellular retinoic acid-binding protein-II. *Biology of Reproduction* **60** 110–114.
- Zile MH** 2001 Function of vitamin A in vertebrate embryonic development. *Journal of Nutrition* **131** 705–708.

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