Bovine Early Embryonic Development and Vitamin A

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Contents

Vitamin A and its derivatives, collectively termed as retinoids, have been paid attention in recent years because of their effects in bovine reproduction. However, the role of retinoids in the pre-implantatory period continues to be largely unexplored, in contrast to later stages of development. Retinoids control cell growth, differentiation and death through binding to specific nuclear receptors by retinoic acid and other active metabolites. This paper reviews how retinoids can influence early embryonic development in cattle through their influence on the follicle, the extrafollicular oocyte and the pre-implantatory embryo itself.

Uptake and Storage of Vitamin A

Nature only furnishes vitamin A (all-trans-retinol; ROH) as a result of enzymatic action on certain carotenoid precursors that takes place within the bodies of most vertebrates. Carotenoids are found in many plants and animals, but no animal is able to synthesize carotenoids. Of these, β-carotene (BC; provitamin A) makes by far the largest contribution to vitamin A activity in food-stuffs. Collectively, vitamin A and its derivatives are known as retinoids. Cows take up vitamin A as naturally contained BC in forages or as a diet supplementation with esterified ROH (NRC Nutrient requirements on Beef -2000- and Dairy -2001- cattle). The ROH metabolism has been reviewed by McLaren and Frigg (2001); http://www.sightandlife.org), and recently in cattle reproduction by Ikeda et al. (2005). Retynil esters are hydrolized in the intestinal mucosa to produce ROH, which is then absorbed by enterocytes. However, BC passes directly into the mucosal cells unchanged and gives two molecules of retinal (RAL) by symmetrical cleavage. Retinal is mostly reduced and esterified to retinyl ester, which is packaged into aggregates of lipids with fat-soluble vitamins and some apolipoproteins termed as chylomicrons. Chylomicrons pass into the lymph and subsequently into the blood, to be broken down in chylomicron remnants, which are mainly cleared by the liver. Cattle have from 70% to 90% of their total vitamin A stores in the liver, an organ crucial in retinoid storage and metabolism, but there exist important retinoid reserves stored in the fat tissue, as demonstrated in rats (Bonet et al. 2003). Hepatocytes take up most vitamin A from chylomicron remnants as retinyl esters. The produced ROH is transported where required by retinol binding proteins (RBP), forming a ternary complex bound to transthyretin in blood (In-genbleek and Young 1994). On its side, ROH in the adipocyte can be derived from hydrolysis of retinyl esters of chylomicrons (Blaner et al. 1994) or taken out from circulating ROH. There are no specific carrier proteins for carotenoids, which circulate in the cow bound to low-density lipoproteins (LDLs) (Schweigert et al. 1987; Schweigert and Eisele 1990) and enter cells bearing the LDL receptor.

Intracellular Metabolism of Retinoids

The members of the retinoid family play a fundamental role as the regulators of cell growth, embryonic morphogenesis and differentiation in many types of cell. Through a series of oxidative reactions, vitamin A generates the retinoic acid (RA) isomeres all-trans-RA (ATRA) and 9-cis-RA. The isomere ATRA is the most important ROH metabolite for vertebrate embryogenesis (Morriss-Kay and Ward 1999; Ross et al. 2000). Levels of RA are locally regulated by retinaldehyde dehydrogenases, which catalyse the last step of RA synthesis and by the cytochrome P450, 26 family of enzymes (CYP26), which degrade RA. In the live cell, RA binds to two different kinds of nuclear receptors, namely, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), each having subtypes α, β, and γ. The access of RA to the cell nucleus is controlled by cellular retinoic acid-binding proteins (CRABP) that protects against the RA excess. Upon entering the nucleus, ATRA 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 steroid/thyroid hormone nuclear receptors superfamily (Chambon 1996), can occur in response to ligand-mediated activation. Heterodimers are capable of binding to specific DNA sequences named retinoic acid response elements (RAREs), to increase or decrease the expression of a gene. Several components of the RA signalling pathway such as RAR α and γ, RXR α and β, retinaldehyde dehydrogenase (ALDH) and the RXR-heterodimer, peroxisome-proliferator activated receptor gamma (PPARγ), are expressed in bovine oocytes and embryos (Mohan et al. 2001,2002) and in cumulus cells (Mohan et al. 2003). Expression of RXR α, β and γ is higher in the oocyte and before major genome activation *in vitro*, while it strongly decreases (RXR α and β) or irregularly expresses (RXRγ) from the 8-cell to the blastocyst stage *in vitro* (Mamo et al. 2005). Within *in vivo* embryos, RXRγ show a pattern similar to that observed *in vitro* for RXR α and β (Mamo et al. 2005). The former authors also found that all RXR subtypes show increased expression in good quality oocytes and 2- and 4-cell embryos. Immunostaining detected RARα, and β in blastocysts (Mohan et al. 2001,2002) and RARα and RXRβ in cumulus cells (Mohan et al. 2003). Thecal, granulosa and luteal cells synthesize and secrete RBP (Brown et al. 2003). Therefore, several ways exist for retinoids to influence embryonic development.

The Retinoids and the Follicle

The oocyte itself is under the retinoid influence within their intrafollicular growth, as the bovine follicular fluid (FF) contains ROH and BC (Chew et al. 1984; Schweigert and Zucker 1988; Haliloglu et al. 2002; Hidalgo et al. 2005; our unpublished results) in proportion to the respective blood contents (Chew et al. 1984; Haliloglu et al. 2002; Hidalgo et al. 2005; our unpublished results). Retinol and BC can diffuse freely between plasma and FF, but also bind to carrier molecules (Schweigert and Zucker 1988; Brown et al. 2003), which could explain in part the differences in concentration seen between both compartments. In cattle, large and medium size follicles contain higher ROH concentrations than follicles $\leqslant 3$ mm in diameter (unpublished results). Accordingly, expression of the CRABP II has been found in the rat, which is confined to granulosa cells from mature follicles and luteal cells (Bucco et al. 1995; Wardlaw et al. 1997; Zheng et al. 1999), but not found in other species. The late intrafollicular expression of CRABP II could mean that mature follicles need a more stringent RA regulation, which would be consistent with an increased RA synthesis in follicles bearing higher ROH contents.

It has been proposed in cattle that specifically reproductive effects of BC cannot be mimicked by supplemented vitamin A (reviewed in Ikeda et al. 2005). Although controversial, these authors suggest that exogenous BC can improve the conception rate in artificial insemination (AI) under certain stress or deprivation conditions (low BC diet, high milk production and heat stress). Further studies showed that cows with lower plasma BC had a reduced reproductive performance (Gossen and Hoedemaker 2005), and the exogenous use of BC was thus discouraged (Gossen et al. 2004). In superovulated cows, no clear benefits of BC on embryo production was found (Martínez-Bello et al. 2004), although cows with higher BC gave more transferable embryos (Goto et al. 1989, cited in Ikeda et al. 2005). It is difficult in these reports to distinguish the target of retinoids, as effects can be exerted on the intrafollicular oocyte, the ovulated oocyte, the proper early embryo or both the oocyte and the embryo. This also occurs when the developmental competence of the oocyte is enhanced by a 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). The effects of ROH on follicular development were usually monitored and recorded 4-day post-administration (Shaw et al. 1995; Whaley et al. 1997,2000; Eberhardt et al. 1999). The reasons why these standards were set are not described and the limits (length and dosage) of ROH treatment are unknown. Similarly, the ability of blastocysts resulting from oocytes of domestic females treated with exogenous ROH to establish and maintain pregnancy was so far unexplored. Development of these blastocysts after transfer to recipients is worth testing as disturbances in retinoid homeostasis have been linked to developmental abnormalities (reviewed by Zile 2001). The above challenges 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 tissular ROH when cultured *in vitro* up to the blastocyst stage. Recent work from our group (Hidalgo et al. 2005) analysed the effects of ROH directly on the oocyte during its intrafollicular growth, the time limits of exposure to exogenous ROH and the viability of these oocytes. Heifers injected once with 1×10^6 ROH IU showed blood ROH increased by Day 1 after the injection and clearance on Day 4, but ROH remained higher within the follicle, showing that monitoring of blood ROH is not indicative of the actual ROH status in reproductive tissues. Thereafter, oocyte donors were injected four times in a week with ROH or vehicle during a twice per week cycle of eight recovery sessions (starting 4 days before the first session), followed by a second 8-session cycle without treatment. The oocytes were retrieved from the cow by Ovum-Pick-Up and fertilized and cultured *in vitro*. Retinol treatment yielded higher numbers of low quality oocytes throughout, while total oocytes, morulae and blastocyst rates increased during the first five sessions of treatment with ROH. Following transfer to recipients, blastocysts from oocytes exposed to ROH were unable to establish pregnancy. As previously shown with oocytes from slaughterhouse ovaries (Hidalgo et al. 2003), the presence of 9-cis-RA during *in vitro* maturation (IVM) stimulated blastocyst development and led to pregnancies. The effect of ROH on the intrafollicular oocyte in the cow was confirmed and provides an early evidence regarding the teratogenic effect of ROH. The quality of bovine follicles, measured as degree of atresia, negatively correlates with ROH contents in the follicle, while estradiol concentration directly correlates with that of ROH (Schweigert and Zucker 1988). These are evidences of ROH regulation of final follicular development, which is dependent on gonadotropins at these stages.

Retinoids and *In Vitro* Maturation

In bovine, we and other authors have studied the role of ROH of during IVM using a medium based on TCM199 (Gómez et al. 2002; Lawrence et al. 2004; Livingston et al. 2004). These conditions are probably no optimal, as TCM199 contains over 0.4 μM vitamin A acetate, which could mask in part the effects of ROH added for experimental purposes. In spite of these adverse conditions, Livingston et al. (2004) found that ROH 5 μM improved the blastocyst development within a subgroup of replicates whose controls exhibited lower efficiency in reaching the blastocyst stage, as well as tended to improve the blastocyst development when all replicates were analysed as a pool. The above research group demonstrated that ROH 5 μM during IVM prevents a reduction in blastocyst rates in heat-stressed oocytes (IVM performed at 41 °C) as compared with oocytes matured at physiological temperature (38 °C) (Lawrence et al. 2004). We found no effects with ROH 0.5 μM (Gómez et al. 2002). Collectively, the above data suggest that ROH can protect oocytes from certain perturbing conditions and stress, and that appropriate maturation conditions could contribute to clarify the effects of ROH on non-stressed oocytes.

The disruption of retinoid homeostasis by exogenous ATRA or deprivation of RA has been associated with late developmental abnormalities as the normal relationship between cellular retinoid levels and the genetic developmental programme is altered (Griffith and Zile 2000; Roberts et al. 2005). The enzyme ALDH generates the majority of RA. Experimental inhibition of RA production in null mutations of 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. Phenotypes produced by the inhibition of ALDH can be rescued by ATRA supplementation. The bovine COC contains an undetermined amount of ROH. A dose-response study with citral during IVM resulted in a gradual inhibition in development of the fertilized oocyte by increasing μM concentrations of citral (Table 1). Preliminary results point out that the inhibitory effect of citral in the COC can be reverted by RA (unpublished), such as the one which occurs in the early embryo (Rodríguez et al. 2006). These results are not unexpected, as a number of cell types contain ROH 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 stored in the oocyte might be made functionally active through the conversion to RA, both during IVM and during the blastocyst development, as will be described here.

The developmental competence of the oocyte can be improved both during IVM and during an induced meiotic arrest. The purpose of inhibiting meiosis *in vitro* is to allow for stable mRNA and protein accumulation in the cytoplasm. This culture period, also termed as pre-maturation, presents a window of opportunity for transcription factors such as RA to act. Treatment with 9-cis-RA 5 nM during meiotic inhibition alone was more effective than treatment during IVM alone or during both periods (Duque et al. 2002). The so-treated oocytes showed partial cytoplasmic granular migration prior to IVM, and complete granular migration after IVM, resembling that observed in oocytes matured *in vivo*. On the other hand, blastocyst development rates as well as survival rates of cryopreserved blastocysts were improved when the oocyte received 9-cis-RA during pre-maturation. The benefits of this treatment extended to total cell numbers. Conversely, a retinoid overexposure (i.e. 9-cis-RA both during pre-maturation and IVM), was detrimental for the oocyte in all the above analysed parameters.

In a later study during a non-inhibited, conventional IVM (Gómez et al. 2003), the presence of 9-cis-RA 5 nM gave a completely migrated cortical granule and improved the blastocyst development, while 500 nM was toxic. The ability of vitrified blastocysts to survive *in vitro* was not improved by 9-cis-RA. Nevertheless, as only blastocysts obtained from oocytes matured with serum survived, more factors should be considered when evaluating cryopreservation survival. The complete granular migration observed in oocytes matured with 9-cis-RA anticipates a gain in the developmental competence of the oocyte. Contrary to controls, blastocysts obtained from oocytes matured *in vitro* in chemically defined conditions with 9-cis-RA led to pregnancies (Hidalgo et al. 2003).

Retinoids and Embryonic Development

Few studies have analysed the direct influence of retinoids on embryonic development on mammalian species both *in vitro* and *in vivo*. Researches on retinoids have classically focused on late development stages, probably because the striking, wide spectrum of malformations in the offspring of humans (Guillonneau and Jacqz-Aigrain 1997), rodents (Shenefelt 1972; Kessel and Gruss 1991), chickens (Thaller and Eichele 1987) and Xenopus (Durston et al. 1989; Sive et al. 1990), exert considerable attraction for the researchers. Alterations include defects in neural tube and central nervous system, skeleton, cleft palate, ear and multiple craniofacial malformations, heart, thymus, urogenital system, limb and digit reduction or duplication. Normal development requires retinoids, but deficiency or excess at the improper stage or time can be detrimental to the developing embryo (Sporn and Roberts 1991).

Eberhardt et al. (1999) injected ROH to donor ewes at the time of FSH administration and recovered embryos both at the 1- to 4-cell stage and at the morula to blastocyst stage. In both stages, embryos recovered from ROH-treated donors formed blastocysts and hatched at higher rates, although ovulation and fertilization rate were unmodified. As reported above, it is

Table 1. Presence of citral during *in vitro* maturation of bovine oocytes

Citral	N	R	Cleaved	Blastocysts (%) Day 7	Day 8
0 μM	267	7	85.4 ± 4.7	21.5 ± 2.8[a]	21.5 ± 2.6[a]
10 μM	204	6	67.3 ± 5.2	17.1 ± 3.1	19.0 ± 3.0
50 μM	205	6	72.0 ± 5.2	17.3 ± 3.1	17.3 ± 3.0
200 μM	270	7	75.3 ± 4.7	9.5 ± 2.8[b]	10.2 ± 2.6[b]

One-way ANOVA; LSM ± SEM.
Different superscripts a, b express significant differences ($p < 0.02$).
Maturation medium consisted of Synthetic Oviduct Fluid (SOF) with 0.5 μg/ml Polyvynil-alcohol, and FSH, luteinizing hormone and E2. Oocytes were fertilized *in vitro* with frozen/thawed semen and cultured in SOF, amino-acids, citrate and myo-inositol, with 10% FCS up to Day 8 under 5% CO_2 and 5% O_2.
N, Cumulus-oocyte complexes cultured; R, replicates.

difficult to distinguish the ROH influence on the oocyte or the embryonic development when donors and not the oocyte or the embryo are treated with retinoid. Just recently studies dealing with effects of retinoid on blastocyst development have been accomplished in mice. Huang et al. (2001,2003,2005,2006) developed a series of experiments with the aim to demonstrate that the early developmental stages in the mouse were also susceptible to the retinoid damage. These researchers worked with low (0.1 μM) and high (10 μM) RA dosages, omitting concentrations of RA that could be considered as physiological (0.3–1 μM). These experiments are particularly useful when looking for beneficial effects of retinoids, as embryos exposed to RA develop characteristic dose-dependent defects. In cultured mice blastocysts ATRA 10 μM exerts anti-proliferative and proapoptotic effects, which can result in subsequent retardation of post-implantation development (Huang et al. 2001,2003,2006). These effects were mainly targeted to the inner cell mass (ICM; Huang et al. 2005). However, in cattle embryos produced entirely *in vitro*, blastocyst development rates improve in simple medium under atmospheric conditions, in contrast to embryos cultured under low oxygen conditions (Livingston et al. 2004). This study demonstrated that anti-oxidant defense mechanisms can be affected by retinoids during embryonic development. Recently, we investigated a part of the above effects using an *in vitro* model to analyse the effect of exogenous and endogenous retinoids on blastocyst development (Rodríguez et al. 2006). We examined the morula to hatched blastocyst transition starting from Day-6 morula produced *in vitro*. After a 24 h treatment, ATRA 0.7 μM increased the proliferation both in the ICM and trophectoderm (TE), without affecting the apoptosis rate, but reducing necrosis and maintaining the ICM/TE cell ratio. In the mammalian blastocyst, the ICM has a higher apoptotic ratio than the TE, which has been suggested to occur naturally to eliminate non-committed cells (Gjorret et al. 2003; Davidson et al. 2004). An evidence of increased apoptotic activity in the ICM can be observed in Fig. 1a,b. Our results in cattle are in contrast to the detrimental effects of ATRA 10 μM in mice (Huang et al. 2001,2003,2006). Differences could depend on 5% serum and 0.7 μM RA we used (Rodríguez et al. 2006), while the above mouse studies were performed in the absence of serum or BSA. It was reported that release of ATRA is physiological and slower in culture with serum proteins, as is its uptake by cells (Klaassen et al. 1999). The slow release of ATRA from proteins was confirmed in morulae cultured with very high BSA concentrations ($\cong$20 mg/ml), where physiological doses of ATRA had no effect on development and cell counts (unpublished results). A hatched blastocyst cultured with ATRA and BSA, fixed and stained for differential cell counts (Thouas et al. 2001), can be seen in Fig. 2.

The endogenous conversion of RA from ROH is essential throughout the development, although 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).
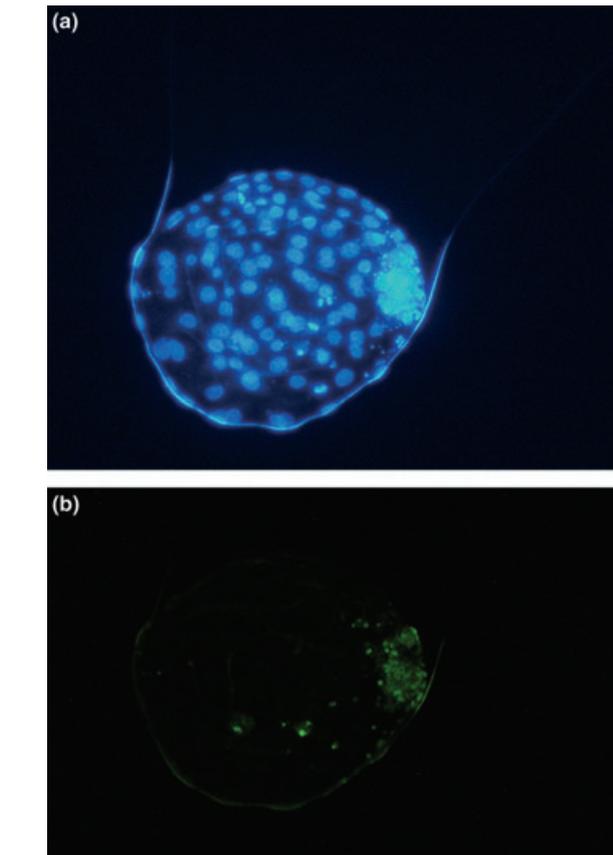

Fig. 1. Bisbenzimide (a) staining and TUNEL (b) images within a bovine blastocyst. Apoptotic nuclei display peculiar shape and TUNEL positive reaction, being more abundant in the inner cell mass region
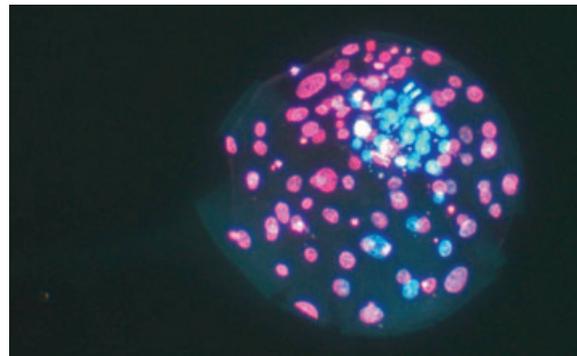

Fig. 2. Hatched blastocyst fixed and stained for differential cell counting as described by Thouas et al. (2001). Blastocysts were incubated in medium with Triton ×-100 and propidium iodide, and then fixed in ethanol with bisbenzimide (Hoechst 33342). Trophectoderm cells were identified by their red fluorescence; inner cell mass appears blue

These levels are functionally active, and a higher ROH contents in the TE is consistent with tissues undergoing active differentiation (Schweigert et al. 2002). Endogenous retinoids in the embryonic cells can derive from the culture environment, the originating oocyte or both. In co-cultures, RA can be secreted by cultured cells (Parrow et al. 1998; Dirami et al. 2003) from internal

ROH stores. When endogenous RA synthesis in Day 6 bovine morulae was inhibited with citral 150 μM, blastocyst development decreased at all stages analysed, being the effect of citral reversed by ATRA 0.7 μM in a specific manner, as ATRA 0.7 μM alone had no effect (Rodríguez et al. 2006). From their ROH stores, bovine embryonic cells produce active retinoid metabolites that certainly appear to regulate development, differentiation and cell death at early stages, although the true dimension of the retinoid stock is still unknown. These results are in support of a role for ATRA as the only ligand clearly necessary for retinoid receptor signalling (Mic et al. 2003). However, although more than physiological doses of ATRA are needed to detect cellular concentrations of 9-cis-RA (Ulven et al. 2001; Mic et al. 2003), isomerization of ATRA to 9-cis-RA can occur in culture (Klaassen et al. 1999). As information on RXRs and 9-cis-RA is scarce and focused on later embryonic stages in the mouse (Mangelsdorf et al. 1992; Dolle et al. 1994; Romert et al. 1998), we wished to analyse the so-called second retinoid pathway. However, the unspecific binding of 9-cis-RA to both RAR and RXR receptors made it difficult to study RXR transactivation, so we used a specific synthetic RXR agonist [LG100268 (LG); a gift of Ligand Laboratories, MI, USA] to study the blastocyst development and differentiation in a preliminary report (Gómez et al. 2006). Subsequent experiments (unpublished) point out that Day-6 morulae cultured for 48 h with low μM LG concentration show improved blastocyst development, hatching and diameter at full expansion, while cell counts remain unaffected. However, high LG concentration had no effect on blastocyst development and diameter, but reduces TE, increases ICM and alters the ICM/total cells ratio. These studies with a RXR specific agonist will be replicated at earlier embryonic stages, where expression of RXRs is dramatically higher than in morulae and blastocysts (Mamo et al. 2005).

Responses to Retinoids at the Gene Level

Retinoids act to induce or silence the expression of hundreds of genes, and little is known of the normal chain of events within an intact tissue. In cattle oocyte and embryonic development not many authors have been able to provide a physiological explanation of the observed effects of retinoids (Ikeda et al. 2005), and the number of genes analysed is scarce.

Granulosa and cumulus cells express retinoid responsive genes, and retinoids undoubtedly influence the oocyte growth by acting on the surrounding cells. Gonadotropins regulate the differentiation status of granulosa cells and controls follicle development. Granulosa cells express gonadotropin receptors FSH-receptor (FSH-R) and LH-receptor (LH-R), and RA suppress the expression of FSH-R (Minegishi et al. 1996,2000a; Xing and Sairam 2002) and LH-R (Bagavandoss and Midgley 1988; Hattori et al. 2000; Minegishi et al. 2000b) induced by FSH in vitro.

The peroxisome-promoted activated receptor (PPAR) family of nuclear receptors, which consists of α, β and γ isoforms, exerts a major role in the regulation of lipid and glucose metabolism (Uauy et al. 2000). The PPAR

family are well-established heterodimeric partners for RXR in vitro, and granulosa cells express PPARα (Rodríguez et al. 2004). When activated by specific ligands (such as certain short-chain fatty acids), the PPARs triggers synthesis of enzymes related to lipid metabolism. Mohan et al. (2002) detected mRNA encoding for PPARγ in all stages of pre-attachment development in vitro, from the 2-cell stage to the hatched blastocyst. Transcripts were also detected in the oocytes, suggesting a likely role during IVM and fertilization. Expression of PPARγ during pre-attachment development and PPARα in granulosa cells would indicate the presence of a heterodimeric partner for RXRs and highlight the complexity of the retinoid signalling pathway in the bovine embryo. Furthermore, events of lipid breakdown are coincident with blastocyst formation (i.e. TE differentiation) in mammalian embryos, and an increase of RA activity is characteristic of tissues undergoing active differentiation (Schweigert et al. 2002). Therefore, RA synthesis could request ROH freed from their esterified fatty acids, and PPARs could be involved in these mechanisms.

The recombinant product of the midkine gene has been found to enhance development to the blastocyst stage of bovine oocytes, its effect being mainly mediated by cumulus-granulosa cells during IVM (Ikeda et al. 2000a,b). Midkine has been isolated in bovine FF (Ohyama et al. 1994), and in rat granulosa cells in dependence on gonadotropins (Karino et al. 1995; Minegishi et al. 1996). As simultaneous addition of FSH and RA to a granulosa cell culture did not significantly change the accumulation of midkine mRNA observed with either FSH or RA alone (Minegishi et al. 1996), this effect could be associated with the suppression of FSH-R by RA (Minegishi et al. 1996,2000a). Midkine suppresses apoptosis in the bovine cumulus cells during IVM (Ikeda et al. 2005), and gene expression of midkine is enhanced by 9-cis-RA in these cells (Gómez et al. 2003). Therefore, reducing apoptosis in cumulus cells during IVM, which may positively correlate with the cytoplasmic maturation degree of the oocytes (Ikeda et al. 2003), is a possible mechanism of RA in cumulus cells via activation of midkine.

Retinoids can also modify gene expression by nontranscriptional procedures. Upon a 9-cis-RA treatment, 3′ polyadenylation [poly-(A)] increases after meiotic arrest and decreased when these oocytes matured in vitro (Gómez et al. 2003). During oocyte growth, large amounts of mRNA are synthesized and polyadenylated in the nucleus in an essentially co-transcriptional procedure (Neugebaeuer 2002). Polyadenylation tails have been shown to regulate both translation and mRNA stability. The amount of poly-A in the oocyte is associated with a gain in the quality of mRNA stored in the ooplasm (Lonergan et al. 2003), and defective developmental competence is accompanied by abnormal poly-A levels (Brevini et al. 2002).

Genes involved in blastocyst activity and cell proliferation and apoptosis can be affected by RA. The composition and accumulation of fluid in the blastocoel is regulated by Na/K-ATPase. In hatched blastocysts, Na/K-ATPase α1-subunit expression is influenced by inhibition of endogenous retinoid synthesis and by

exogenous ATRA (Rodríguez et al. 2006). Similar changes were also observed in the expression of the p53 gene (Rodríguez et al. 2006), whose encoded protein becomes stabilized in response to stress or proliferative signals, causing cells to undergo either cell cycle arrest or apoptosis (Vousden and Lu 2002). However, 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 with proliferation being a consequence of retinoid treatment.

Other Mechanisms for Retinoids in Bovine Reproduction

The effect of BC contained in the follicle has been recently suggested to act as an antioxidant on the oocyte (Ikeda et al. 2005). This proposal has been yet unexplored in bovine, but it is based on increasing evidence of reactive oxygen species compromising cytoplasmic maturation (Hashimoto et al. 2000; Cetica et al. 2001; Ali et al. 2003; Iwata et al. 2003; Dalvit et al. 2005a,b). The protective effect of BC is probably independent on retinoid receptors, in contrast with a role for RA in the generation of oxidative stress in some cell types (Castro-Obregon and Covarrubias 1996; Mantymaa et al. 2000; Ahlemeyer et al. 2001). Nevertheless, we did not find signs of oxidative damage because of treatment with 9-cis-RA in meiotically inhibited and/or in vitro matured bovine oocytes (Gómez et al. 2004). In these oocytes the expression of genes coding for Mn-superoxide dismutase and glucose-6-phosphate dehydrogenase, enzymes involved in neutralization response against oxidative damage, did not change with 9-cis-RA.

In the cow, cyclooxygenase-2 (cox-2) is present in granulosa and cumulus cells and increases at periovulation time (Liu et al. 2002; Nuttinck et al. 2002). Inhibition of cox-2 during the pre-ovulatory period blocks ovulation (De Silva and Reeves 1985; Algire et al. 1992). As ATRA and 9-cis-RA suppress cox-2 transcription (Yang and Frucht 2001; Subbaramaiah et al. 2002) these molecules may contribute to reduce prostaglandin synthesis and thus enhancing cytoplasmic maturation (Ikeda et al. 2005). These authors also suggested a possible role of RA in regulating cytoplasmic maturation via the nitric oxide (NO)/NO synthase (NOS) system, based on inhibition of inducible NOS expression by RA in cumulus-granulosa cells through RARα (Sirsjo et al. 2000). RA. In porcine oocytes, RA inhibits expression of endothelial NOS and NO synthesis (Hattori et al. 2002).

As derived from its effects on blastocyst development kinetics and proliferation, it is likely that retinoids can affect sex (as related to embryonic development kinetics) and ploidy (as dependent on increased proliferation; ploidy and proliferation: 5734 entries in pubmed). Through induction of Stra8 gene expression in embryonic mice ovaries, RA is responsible for meiotic initiation in female while it precludes its occurrence in embryonic testes until after birth (Koubova et al. 2006). Whether Stra8 gene is involved in sex and retinoid dependent mechanisms in mitotic initiation after embryonic genome activation is unknown. The above are interesting topics for future research.

Conclusion

Findings reported in this review corroborate some essential traits observed with retinoids in somatic cells: The supply of retinoid to the embryos must be ensured within a physiological range, both in length and in concentration, as both excess and deficiency of retinoid cause abundant teratogenic defects because of its pleiotrophic activity. On the other hand, manipulation of retinoid metabolism can lead to in vitro embryos with improved ICM and high ICM/total cell rates, which is a major objective in reproductive and stem cells technology. In agreement with Ikeda et al. (2005), it is possible that requirements of vitamin A are different in both conventional reproduction and in vitro procedures, which requires further clarification as long-term developmental competence of the oocyte can be directly involved.

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