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Retinoid associated proteins in the ovary and oviduct and retinol's effect on oocyte maturation and embryonic development

Dawn M. Eberhardt

To the Graduate Council:

I am submitting herewith a dissertation written by Dawn M. Eberhardt entitled "Retinoid associated proteins in the ovary and oviduct and retinol's effect on oocyte maturation and embryonic development." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

James D. Godkin, Major Professor

We have read this dissertation and recommend its acceptance:

Judith M. Grizzle, Jeffrey A. MacCabe, F. Neal Shrick, Donald S. Torry

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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ABSTRACT

Vitamin A (retinol) and its natural metabolites, isoforms of retinoic acid, are collectively known as retinoids and are important signaling molecules in vertebrate development and differentiation. A growing body of evidence suggests retinoids play an important role in reproduction. Systemic and intercellular transport of retinol is mediated by retinol binding protein (RBP). The cellular binding proteins, cellular retinol binding proteins (CRBPs) and cellular retinoic acid binding proteins (CRABPs), participate in retinol storage and metabolism. The actions of retinoids are mediated through nuclear retinoic acid receptors which belong to the family of nuclear ligand inducible transcription factors. The goals of this study were to localize the retinoid binding proteins in the oviduct and ovary and examine the effect of retinoids on oocyte maturation/competence and early embryonic development. Retinol binding protein was immunolocalized to the luminal epithelia of oviductal mucosa of both the ampulla and isthmus. Retinol binding protein expression was considerably higher on day 1 than days 5 or 10 of the estrous cycle. Synthesis, secretion and gene expression of RBP was modulated by ovarian steroids. Within the ovary, RBP was localized

primarily in the thecal cells of non atretic follicles with some diffuse staining in the granulosa cells and stromal cells. Retinol binding protein staining was also present in the cytoplasm of oocytes from some antral but not pre-antral follicles. Cellular retinol binding protein was localized in thecal cells of non atretic follicles with diffuse staining in the stromal layer. Cellular retinol binding protein was observed in large cells of the corpus luteum while RBP was identified in both large and small luteal cells. Only CRABP exhibited intense staining in nuclei of oocytes from primordial follicles, but was not observed in oocytes or follicular cells of all other size follicles. Cellular retinoic acid binding protein was absent in all cells of the corpus luteum but was present in the stromal layer encapsulating it. In cattle, ovaries from normal cycling animals were analyzed for the presence of RBP and CRBP. Retinol binding protein mRNA was present in thecal but not granulosa cells of antral follicles. However, both thecal and granulosa cells synthesized RBP in vitro. No relative differences were observed in RBP mRNA concentrations or synthesis in luteal tissue between days 2, 6, 10 or 15. Retinol binding protein and CRBP were immunolocalized exclusively to large luteal cells. Experiments were conducted to identify effects of retinoid treatments on

superovulated ewes upon subsequent in vitro embryonic development. Ewes were treated with retinoids or vehicle on the first and last day of FSH treatment and embryos surgically recovered and cultured in vitro until occurrence of blastocyst formation and hatching. Treatment of ewes with retinol during superovulation resulted in a dramatic increase in both blastocyst formation and embryonic hatching in comparison with retinoic acid, 9 cis retinoic acid or vehicle. Retinol treatment also significantly improved the number of embryos that progressed through the 8-cell in vitro block. The final experiment was conducted to evaluate the effects of the retinoids, retinol (100 μM and 10 μM ROH) and retinoic acid (10 μM and 1 μM RA) on the maturation, fertilization and subsequent development of cattle oocytes/embryos in vitro. Addition of 100 μM ROH or 10 μM RA to either maturation and fertilization or embryonic culture media resulted in no blastocyst formation or reduced blastocyst formation. The presence of retinoids in the lower concentrations during oocyte maturation either had no effect or diminished blastocyst formation. Embryos cultured in the presence of 10 μM ROH or 1 μM RA, regardless of maturation and fertilization treatment, exhibited increased blastocyst formation in comparison with culture medium alone. Collectively, these experiments identify retinoid

binding proteins in the ovary and oviduct and provide evidence that retinoids may influence oocyte development/competence and early embryonic development. These results suggest that retinol has the potential to positively impact reproductive efficiency and assisted reproduction protocols in domestic animals.

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Part 1. Introduction and literature review.

INTRODUCTION

Retinol and its metabolites, all trans retinoic acid and 9 cis retinoid acid, are regulators of cellular growth, differentiation and development and are essential to reproduction in both males and females (1,2). Differentiation induced by retinoids in vitro has been shown to be accompanied by specific changes in expression of homeobox genes, growth factors and their receptors (reviewed in 1). The actions of retinoids are mediated by a transport protein, retinol binding protein (RBP); the cellular binding proteins, cellular retinol binding proteins 1 and 2 (CRBP and CRBP-2) and cellular retinoic acid binding proteins 1 and 2 (CRABP and CRABP-2); and the nuclear receptors, retinoic acid receptors (RAR) and retinoid X receptors (RXR). The goals of this study were to localize the retinoid binding proteins in the ovary and oviduct and examine retinol's effect on oocyte maturation/competence and early embryonic development. Specifically, the objectives were:

1. To identify RBP expression in the ovine oviduct and to determine its regulation by ovarian steroids.

2. To characterized cell and stage specific expression of retinoid binding proteins in the ovine/bovine ovary.
3. To assess the effect of retinoids on embryo number and quality in superovulated ewes.
4. To investigate the effects of retinoids on oocyte maturation/competence and subsequent development of bovine embryos.

LITERATURE REVIEW

Retinol (vitamin A alcohol) is the parent vitamin A molecule and its metabolites, derivatives and analogs are collectively known as retinoids. Vitamin A is provided in the diet as provitamin A carotinoids in plants or as retinyl esters in animal fat. These precursors undergo a series of metabolic conversions in the gut which results in retinol packaged as retinyl esters in chylomicrons which are secreted into the lymphatic system and eventually taken up by the liver (reviewed in 3). Vitamin A is essential for growth, maintenance of epithelial tissues, vision and reproduction (4). All-trans retinoic acid (RA) and its 9-cis isomer are believed to be the functional forms of vitamin A in growth, differentiation and development; whereas, retinal and its 11-

cis isomer function in the visual cycle (5). There appears to be a critical requirement of retinol in spermatogenesis and gestation which cannot be supported by RA (5,6).

In vitro, RA modulates differentiation and phenotype of many cell types including embryo carcinoma cells and embryonic stem cells (6). Differentiation induced by RA in vitro has been shown to be accompanied by specific changes in expression of homeobox genes, growth factors and their receptors. Moreover, retinoids modulate extracellular matrix formation by their influence on genes encoding glycoconjugates and proteases of the extracellular matrix (1).

The requirement for vitamin A (retinol) in normal embryonic and fetal growth has been established since the 1950's (7). Fetal retinol homeostasis is tightly regulated. Hypovitminanosis or hypervitaminosis can result in congenital defects and fetal loss (8). The presence of endogenous retinoid acid and distribution of retinoid binding proteins and nuclear receptors suggest that RA is a natural morphogen in vertebrate embryogensis (8-13). Some molecular cues involved in patterning during embryogenesis are governed by *Hox* homeobox genes (9). *Hox* homeotic genes were originally described in *Drosphila* and contain a conserved sequence that encodes a DNA binding homeodomain within the

proteins. They function by providing an ordered molecular system of positional values along the anteroposterior axis referred to as the *Hox* code (9,14,15). Retinoids are likely candidates for regulation/establishment of this *Hox* code (9).

While the requirement for vitamin A in normal fetal growth and development is well established, the mechanism of transplacental transport of retinol from maternal blood supply to the developing fetus is not completely understood. Recently, a model has been proposed for retinol delivery (19). Maternal retinol is delivered across the visceral yolk sac endoderm via a relay from maternal RBP to yolk sac RBP possibly through a RBP receptor. Cellular retinol binding protein is hypothesized to facilitate the endoderm intercellular transport of retinol (16). Several lines of evidence support this theory: localization of RBP and CRBP in the endoderm of the visceral yolk sac during early postimplantation embryonic development in both the rat and mouse (17,18); uptake of radiolabelled retinol by the visceral yolk sac of in vitro cultured rat embryos (16); and induction of stage specific embryonic RA deficiency by injection of antisense oligodeoxynucleotides for RBP into mouse embryos at the pre-somitic to 12 somite stage (19).

The transport of retinol from its storage site in the liver to target tissues is accomplished by a specific

transport protein, retinol binding protein (RBP) (reviewed in 20). Nutritional retinol status strongly influences the secretion of RBP by the liver but not its biosynthesis. Depletion of liver retinol stores does not alter RBP mRNA levels but inhibits RBP secretion, resulting in accumulation of apo-RBP in the liver and a concomitant decline in serum RBP. Although the liver is the major site of plasma RBP synthesis, extrahepatic sites for RBP mRNA and/or RBP synthesis have been identified. These include the visceral yolk sac (21-23), preimplantation conceptuses (22-24), endometrium (25-28), placental membranes (22,23,29-31) and ovary (32).

In target cells, the function of vitamin A has been associated with the presence of two distinct cytoplasmic binding proteins, cellular retinol binding proteins 1 and 2 (CRBP and CRBP-2) and cellular retinoic acid binding proteins 1 and 2 (CRABP and CRABP-2) (reviewed in 33). Each has very specific binding properties for its endogenous ligand and are highly conserved proteins. Both CRBP and CRABP are widely distributed in body tissues but concentrations vary. CRBP 2 is found mainly in the small intestine and it is thought to be involved in retinol absorption. Cellular retinoic acid binding protein 2 is found primarily in the skin but has been recently identified in the rat ovary (34). Cellular retinol

binding protein and CRABP are involved in intracellular retinol homeostatsis (reviewed in 35 and figure 1-1). Cellular retinol binding protein has three major functions: retinol accumulation, stimulation of retinol mobilization from retinyl ester stores, and delivery of the retinol to dehydrogenase for conversion to retinal and eventually retinoic acid. The cellular transport/metabolism of retinoic acid is accomplished via the small, cytoplasmic proteins, CRABP-1 and 2. The actions of retinoic acid are mediated through two subgroups of nuclear receptors, retinoic acid receptors (RAR) and retinoid X receptors (RXR). Retinoic acid/receptor complexes cause gene repression or activation through association with specific response elements (retinoic acid response element or retinoid X response elements) found in the promoter regions of target genes.

To study the specific effects of retinoids, mice with targeted disruptions in cellular retinoic acid binding protein and retinoid receptors have been generated. Cellular retinoic acid binding protein null mice are essentially indistinguishable from their heterozygous or wild type littermates (36,37). Information from RAR and RXR knockout mice is hard to interpret due to a redundancy in these receptors. Members of the RAR family (types alpha, beta and gamma and their isoforms) are activated by most

physiologically occurring retinoids (all-trans RA, 9-cis RA, 4-oxo RA and 3,4-hihyro RA). In contrast, members of the RXR family (types alpha, beta, and gamma and their isoforms) are activated by 9-cis RA only. In addition to the multiplicity of receptors, ,the complexity of retinoid signaling is further increased by the formation of heterodimers between RARs and RXRs and RXRs with other nuclear receptors (reviewed in 38). Mutant mice containing a disruption of a single nuclear receptor isoform are essentially normal and identical to wild type mice (39). Deficiency in all isoforms of RAR alpha or RAR gamma results in symptoms of vitamin A deficiency including post-natal lethality, poor weight gain and male sterility (39,40). Mice with deficiencies in RAR beta are growth retarded, but fertile and have a normal longevity (41). Mice with multiple RXR disruptions are phenotypically normal (42). Disruption of combinations of receptors results in varying symptoms of vitamin A deficiency (reviewed in 39).

Early studies demonstrated that vitamin A deprivation had a dramatic negative impact on male and female reproduction (reviewed in 2). In the female, vitamin A deficiency can lead to decreased ovarian size, decreased ovarian steroid concentrations, abortion, and eventually reproductive senescence. Serum and ovarian progesterone

concentrations were decreased in vitamin A deprived rats regardless of supplementation with retinoic acid (43,44). Subsequent studies determined that steroidogenic enzyme activity was decreased even at mild stages of vitamin A deficiency (45,46). Complementing the studies which showed a negative effect of vitamin A deprivation on reproduction, a number of studies have indicated positive effects of retinoid supplementation when diets were adequate in vitamin A. A series of research reports from Germany in the late 1970's suggested that supplemental ß-carotene in vitamin A adequate diets improved reproductive performance in dairy cows (47,49). Several subsequent studies observed beneficial effects of ß-carotene supplementation on a wide variety of reproductive parameters (50-56). Factors such as breed and parity have been suggested to influence the response (57,58). An equally impressive number of studies found no effect of ß-carotene supplementation on reproductive performance, even in dairy cattle fed diets low in ß-carotene and failure to respond positively was not associated with breed or parity (59-65).

Several studies, performed in vitro, have indicated a role for retinoids in ovarian steroidogenesis. Retinoids have been shown to stimulate progesterone production in luteal cells from swine (66) and cattle (67). In addition,

retinoids stimulated progesterone production and enhanced steroid production in response to gonadotropins in rat granulosa cells (68,69). Retinol administration has also been shown to have biphasic actions in vivo. Retinol acetate was administered to rats at 50,000 I.U. or 80,000 I.U. daily for 10 days. The 50,000 I.U. dose stimulated ovarian steroidogenesis whereas the 80,000 I.U. dose inhibited steroidogenesis and caused a cessation of estrous cyclicity. A histochemical assay for 3-beta-ol-steroid dehydrogenase and alkaline phosphatase indicated that the effect of vitamin A was in the interstitial tissue, atretic corpora and the internal theca of follicles (69). A classical study performed in rats showed that the number of atretic follicles is highest in the vitamin A-deficient and lowest in the A-hypervitaminotic animals (70). This study also indicated that vitamin A may play a role in the formation and maintenance of the corpus luteum. A-hypervitaminotic rats showed an accelerated occlusion at the point of ovulation, increased vascularisation of the CL wall, and more rapid formation of the fibrous cavity surrounding the CL when compared to both control and A-deficient rats. The hyperemia associated with a mature CL occurs earlier and progresses longer in the A-hypervitaminotic animals in comparison with A-deficient animals (70). A more recent study (32) has also suggested

that vitamin A has a role in CL formation, specifically in granulosa cell remodeling. Cellular retinoic acid binding protein 2 was immunolocalized in the granulosa cells of healthy mature follicles and increased in intensity at the time of estrus. Cellular retinoic acid binding protein 2 expression was also seen in the early corpus lutea but decreased in intensity as the corpus lutea matured. The authors observed production of retinoic acid in the ovary localized exclusively in the granulosa cell layer but its functions are unknown.

Results from several studies indicate that retinoids may function in very early events associated with reproductive success including follicular development and ovum maturation. Schweigert and Zucker (70,71) determined that concentrations of retinol in bovine follicular fluid was a function of follicle quality and was highest in healthy follicles, lowest in atretic follicle and highly correlated with follicular fluid estradiol concentrations.

The most convincing evidence for a positive impact of retinoid supplementation on reproduction came from studies in litter bearing animals. Vitamin A or ß-carotene administration has been shown to increase embryo survival and in some cases litter weights in rats (73), rabbits (74), and swine (75-77).

In swine, a single injection of retinol palmitate given approximately 5 days prior to estrus increased litter size (75,76). In cattle, a single injection of retinol given with the first FSH injection, five to seven days prior to induced estrus, significantly improved the quality of embryos collected from superovulated cattle but had no effect on total number recovered (78). In a similar study, sheep were administered vitamin A in conjunction with PMSG during both the estrous and anestrous season. In both cases, the addition of vitamin A to the superovulation protocol resulted in a greater number of ovulations (79). In a subsequent study, vitamin A was administered with different doses of PMSG during the estrous season and the ovaries removed after ovulation. Vitamin A increased the total number of ovulations and decreased the number of tertiary atretic follicles (80).

In large domestic animals, oocytes within the ovary remain arrested at the diplotene stage of prophase of the first meiotic division (81). Meiosis resumes in response to the LH surge, leading to ovulation (82). Removal of oocytes from their follicles and culture in vitro results in spontaneous resumption and completion of the first meiotic division, a process categorized as nuclear maturation (83). It is generally accepted that substance(s) in follicular

fluid, produced by follicular cells, inhibit nuclear maturation. Although a universal mammalian oocyte maturation inhibitor has not been clearly identified, numerous compounds have been shown to inhibit or transiently inhibit oocyte maturation. These include cyclic AMP and compounds which affect its metabolism or signal transduction pathway, calcium, calmodulin, steroids, hypophyseal hormones, purine bases, putative polypeptide inhibitors and intracellular communication between cumulus cells and the oocyte via small cytoplasmic projections (reviewed in 84). The second process involved in oocyte maturation is referred to as cytoplasmic maturation and entails changes necessary to prepare for egg activation, fertilization and preimplantation development. Both of these processes are required for monospermic fertilization, processing of sperm and embryonic development to blastocyst (85). No apparent differences are observed between in vivo and in vitro matured oocytes at the level of nuclear maturation, fertilization and cleavage. However, developmental capacity, as measured by blastocyst formation, is diminished in in vitro versus in vivo matured oocytes (86). Immature oocytes are capable of resuming meiosis (i.e. nuclear maturation) in the absence of serum and gonadotropins (87), but subsequent embryonic development

(i.e. cytoplasmic maturation) is improved with the addition of serum and gonadotropins (88-90).

Cytoplasmic maturation requires species specific protein synthesis that culminates in the activation of mitosis promoting factor (MPF), a universal complex essential for the G2/M phase progression in all cells (91). Mitosis promoting factor activity slightly proceeds germinal vesicle breakdown (GVBD) (92-95) and is involved in phosphorylation of many of the proteins responsible for nuclear envelop oscillation, chromatin condensation and microtubule reorganization (96-98). Mitosis promoting factor consist of 2 subunits, cyclin B and p34/cdc2 (99,100). Cyclin B binds to p34/cdc2 at the S and G2 phase (101) and this complex becomes phosphorylated on Thr-161, Tyr-15, and Thr-14 of p34/cdc2 (100-103). This compound is referred to as pre-MPF and is biologically inactive. Activation of MPF is induced by dephosphorylation at Thr-15 and Thr-14 by cdc25 and the cell begins the G2/M phase transition (100).

Although MPF is present in all oocytes, regulation of its activity is species specific. In species such as *Xenopus*, starfish, clams and mice, no protein synthesis is required just prior to GVBD. Mitosis promoting factor is maintained in an inactive form (pre-MPF) by phosphorylation,

and dephosphorylation is required for initiation of GVBD (104-110). Protein synthesis is required for activation of MPF and progression of GVBD in domestic animals such as pigs (111) and cattle (112). In cattle, cyclin B is synthesized just prior to GVBD and is thought to be the critical step in resumption of meiosis (113).

Oocyte maturation and embryonic culture conditions affect subsequent embryonic development to blastocyst. There are two critical transitions in production of in vitro derived embryos, the switch from maternal to embryonic genome control (zygotic gene activation) and the development from morula to blastocyst (reviewed in 114 and 115). Many approaches have been undertaken to improve the in vitro development of embryos such as co-culture with somatic cells (116-118), the use of conditioned medium (119-121) the addition of growth factors to medium (reviewed in 122) and supplementation of medium with serum (123,124).

There exists a growing body of evidence that retinoids influence follicular development and oocyte maturation/competence. As mentioned previously, bovine follicular fluid concentrations of retinol are associated with follicular quality (71,72). Retinol has been shown to inhibit GV breakdown in *Spisula* oocytes (122) and potentiate the inhibitory effects of forskolin on GV breakdown in

denuded mouse oocytes (123). Retinol injection prior to ovulation has been reported to increase litter size in swine (75,76), ovulation rate in sheep (79,80) and embryo quality in cattle and sheep (78,79).

The oviduct is another possible site for the positive effect of retinol on embryo quality. The oviduct is a steroid responsive organ that undergoes cyclic changes in luminal epithelial cell morphology and secretory patterns (127-129). The oviduct also provides the environment for gamete transportation, fertilization, and early embryonic development. The establishment of pregnancy is controlled by complex interactions between the embryo and the mother that begin in the estrogen dominated oviduct (reviewed in 130). Estrogen dependent proteins have been identified in sheep (128,131), cow (132), pig (133), hamster (134), human (135,136) and baboon (137,138). Some of these proteins are influenced by the presence of sperm cells (139) and others are found associated with the oocyte (127,140). Co-culture of oviductal cells with sperm (141) and with embryos (142-144) has been beneficial to early ovine embryonic development in vitro.

Spermatozoa produce both superoxide anion and hydrogen peroxide free radicals during normal motility. Production of these free radicals has been suggested to play a role in

mediating sperm-zona interactions at fertilization (145,146). However, free radicals have been implicated in the loss of motility of sperm cells (146-148) and in impaired development of preimplantation embryos in vitro (149-150). In vitro matured and fertilized pre-implantation ovine embryos co-cultured with oviductal cells in an atmosphere of 5% CO_2 in air had a significantly higher survival rate (to blastocyst) rate than those cultured without oviductal cells. When the oxygen level was decreased to 5%, this trend was reversed (151). This suggests the oviductal cells perform an antioxidant activity. The secretion of RBP into the lumen of the oviduct could be to recruit retinol, a known antioxidant (152).

Production of oviductal RBP is not unique to the sheep. Retinol binding protein or its mRNA has been identified in equine and porcine oviducts (153,154). In addition, uterine production of RBP has been shown to be responsive to steroids (155,156). Although the physiological role of vitamin A in early pregnancy is unknown at the present time, significant evidence exists to imply a relationship with gamete interaction and early pregnancy.

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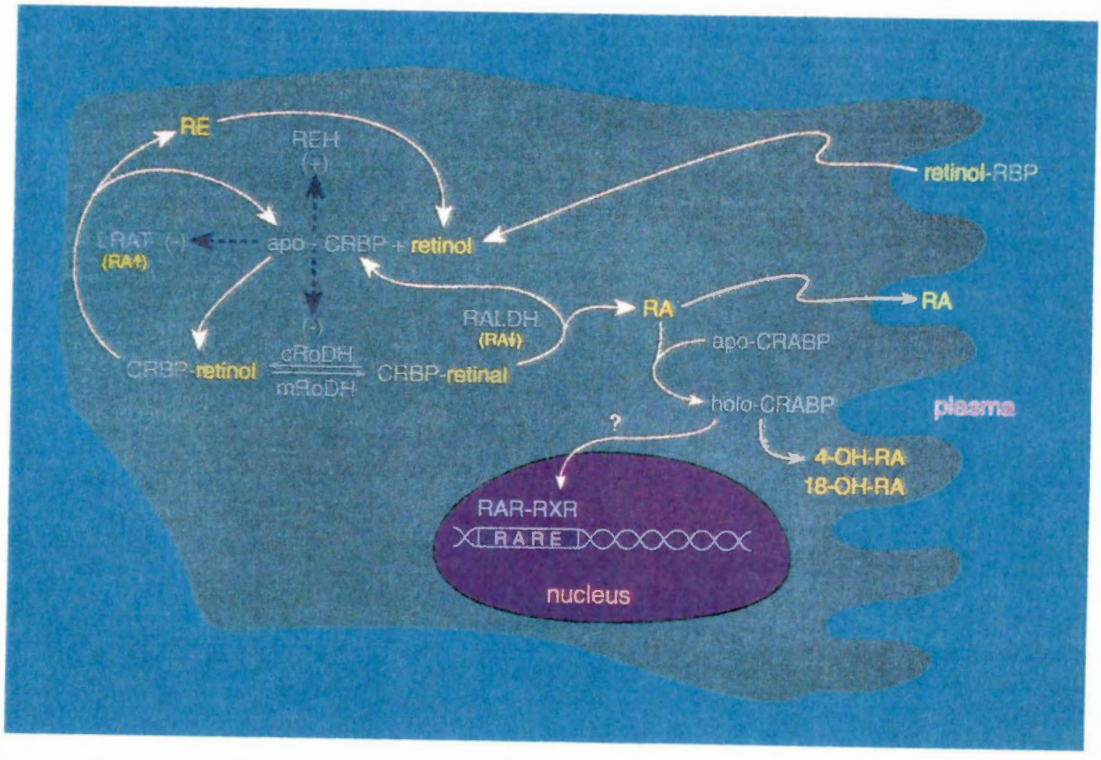

Figure 1-1 Working model of retinol and retinoic acid metabolism showing the interactions with and effects of retinoid-binding proteins on retinoid specific enzymes (reproduced from 35).

Part 2. Steroid Regulation of Retinol-Binding Protein in the Ovine Oviduct.

ABSTRACT

Two studies were conducted to identify RBP expression in the ovine oviduct and to determine the role of ovarian steroids in its regulation. Ewes were salpincectomized on Days 1, 5, or 10 of their respective estrous cycles and oviducts homogenized for RNA analysis, fixed for immunocytochemistry (ICC), or cultured for 24 hours for protein analysis. Immunocytochemistry localized RBP to the epithelium of all oviducts. Synthesis of RBP was demonstrated by immunoprecipitation of radiolabelled RBP from the medium of oviductal explant cultures. Conditioned media from oviductal explant tissue harvested on Day 1 contained significantly more RBP than from tissue collected on Days 5 or 10. Slot blot analysis demonstrated that steady state RBP mRNA levels were significantly higher on Day 1 than Days 5 or 10. In the second experiment, ovariectomized ewes were treated with estradiol 17-β (E2), progesterone (P4), E2+P4, or vehicle (C) and oviducts were analyzed as above. Progesterone alone or in combination with E2, significantly reduced steady state RBP mRNA levels compared to E2 treated animals. Oviductal explants from E2 and E2+P4 treated animals released 5 fold more RBP into the medium than C and P4 treatments as determined by ELISA. Retiol binding protein

synthesis of metabolically labeled RBP was increased by E2 and E2+P4 treatments. This study demonstrates that progesterone applied on an estradiol background negatively regulates RBP gene expression in the oviduct; whereas, estradiol appears to stimulate RBP synthesis and secretion.

INTRODUCTION

The mammalian oviduct is a steroid responsive organ that provides the environment for gamete transportation, fertilization, and early embryonic development. The establishment of pregnancy is controlled by complex interactions between the embryo and the mother that begin in the estrogen dominated oviduct (reviewed in 1). Cyclic changes in proteins synthesized and secreted by the ovine oviduct have been described (2,3,4). Estrogen dependent proteins have been identified in sheep (3,5), cattle (6), pigs (7), hamsters (8), humans (9,10) and baboons (11,12). Some of these proteins are influenced by the presence of sperm cells (13) and others are found associated with the oocyte (2,14). Co-culture of oviductal cells with sperm (15) or with embryos (16,17,18) has been beneficial to fertilization and early ovine embryonic development in vitro.

Retinol is essential to reproduction in both males and

females (reviewed in 19). Deficiencies in vitamin A lead to decreased ovarian size, reproductive senescence, abortion and congenital fetal malformation. The transport of retinol is accomplished by a specific transport protein, retinol-binding protein (RBP). Retinol binding protein or its mRNA has been identified in equine and porcine oviducts (20,21) and has been shown to be steroid responsive in uterine tissues (22,23). The objective of this study was to identify RBP in ovine oviducts and determine the role of ovarian steroids in regulating its expression.

MATERIALS AND METHODS

Materials

Silastic tubing and Bouin fixation fluid were purchased from Baxter Scientific Products (McGaw Park, IL). Silicone sealant was purchased from Dow Corning Corp. (Midland, MI). Cesium trifluoroacetate (CsTFA) was obtained from Pharmacia Biotech, Inc (Uppsala, Sweden). Formamide and Random Prime™ labeling kits were purchased from United States Biochemicals (Cleveland, OH). S & S Nytran membrane was purchased from Schleicher and Schuell, Inc (Keene, NH). Alpha ^{P32}-dCTP and

^{3}H-leucine were obtained from ICN Pharmaceuticals, Inc (Costa Mesa, CA). Microtiter plates (96 well) were obtained from Corning, Inc (Corning, NY). Goat anti-rabbit IgG alkaline phosphatase conjugate was purchased from Bio-Rad Laboratories (Hercules, CA). The p-nitrophenylphosphate (p-NPP) substrate was obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD) and prepared according to manufacturers directions. Histogen^{R} Peroxide anti-Peroxidase Immunostaining Sytem was purchased from BioGenex Laboratories (San Ramon, CA). Microprobe system including slide holder assemby, 30-well Isolon reagent isolators and ProbeOn^{TM} Microscope slides were purchased from Fisher Scientific (Pittsburgh, PA). All other reagents were purchased from Sigma Chemical Corp. (St. Louis, MO).

Animals

For the first study, 13 sexually mature crossbred ewes that had exhibited at least three normal 16 day estrous cycles were salpincectomized via mid ventral laparotomy on Day 1 (n=5), Day 5 (n=4) or Day 10 (n=4) (Day 0=estrus) of their subsequent estrous cycle. The oviducts were prepared for RNA analysis, immunocytochemistry, or organ culture as described below.

In the second study, 25 sexually mature crossbred ewes that had exhibited at least 2 normal estrous cycles were ovariectomized via mid ventral laparotomy. Ovariectomies were performed at least 45 days prior to the experiment to allow sufficient time for endogenous ovarian steroids to be cleared from the circulation. Animals assigned to the control group (n=5) received no exogenous steroids. Steroid treatments were administered according to the method of Homanics and Silvia (24) with a modification of the steroid pretreatment. Steroids that were injected were dissolved in corn oil. Briefly, 20 animals underwent nine days of progesterone treatments (12 mg/day i.m.) and 2 days of estradiol 17β treatment (3.5 ug, 7.0 ug, 14.0 ug, 7.0 ug and 3.5 ug intramuscularly sequentially, at 8 hour intervals) to induce behavioral estrus. The day following the last injection was designated Day 0 and animals were checked for estrus with vasectomized rams. All ewes showed estrous behavior and were randomly allotted to one of three treatments: 1) P4, 2) E2, or 3) E2+P4. On the day following estrus, ewes assigned to groups 1 and 3 received 12 mg i.m. injections of progesterone once daily. The ewes assigned to treatments 2 and 3 received subcutaneous silastic implants (3 cm long, 3.35 mm inner diameter and 4.65 mm outer diameter) containing estradiol 17β (25). The control treatment group

received only vehicle(n=5). Ewes were bled via jugular venipuncture on days 6, 10 and 13 of treatment. Blood was collected in chilled tubes containing 200µl of sodium citrate (0.025% solution) and immediately placed on ice. Blood samples were centrifuged for 20 minutes at 2500 X g. Plasma was recovered and stored at -20°C until radioimmunoassays were performed. Three animals were removed from the study due to loss of estradiol implant; 2 from E2 and 1 from E2+P4 treatments. Therefore 5 animals were utilized in the E2 and CONTROL groups and 6 animals in the E2+P4 and P4 groups. After 13-15 days of treatment, ewes were salpincectomized and oviducts treated as in experiment one. All procedures were approved by The University of Tennessee Institutional Animal Care and Use Committee (IACUC).

RNA isolation and analysis

Oviducts were obtained surgically and transported to the laboratory on ice. The broad ligament, infundibulum, and any excess uterine tissue were removed. A 5-7 mm section was removed from the end of the ampulla and isthmus and reserved for ICC. The remaining tissue was minced and homogenized in 4M guanidine isothiocyanate (4°C) (26). Total RNA isolation was performed according to the procedure described by Dore'

et al (27). Briefly, supernatant was layered on CsTFA (prepared following the manufacturer's instructions to a specific gravity of 1.51) for isopycnic gradient ultracentrifugation. The RNA pellet was resuspended in a solution of 30 mM sodium citrate, 0.1% (w/v) sodium dodecyl sulfate, and 1% (v/v) β-mercaptoethanol. Ethanol precipitation was performed to remove any residual CsTFA. The RNA pellet was dissolved in a solution of 40 mM morpholinopropane sulfate (MOPS), 10 mM sodium acetate, 1 mM EDTA as described by (26) with the modifications 0.1% (w/v) SDS and 1% (v/v) β-mercaptoethanol (27), quantified by absorbance at 260 nm and stored at -100°C.

A DNA probe specific for RBP, designated bcRBP-700 and isolated from a bovine conceptus cDNA library, was used for slot blot analysis (28). The probe DNA was prepared and random prime labelled with α^{32}P-dCTP to specific activities of 2.0×10^9 cpm/ug DNA as described by Liu et al (28). Total RNA (10 ug) was electrophoresed in 1.5% (w/v) agarose MOPS/formaldehyde gels (26) and stained with 0.2 ug/ml ethidium bromide to confirm its integrity. Intact RNA (10 ug) was then loaded onto nylon membranes using a Minifold slot blot apparatus following the procedure recommended by the manufacturer (Schleicher and Schuell, Keene, NH). RNA was cross linked to membranes by UV irradiation (0.12 Joules,

UVC 1000; Hoeffer, San Francisco, CA). Membranes were prehybridized, hybridized, and washed as described previously (28). Washed, hybridized membranes were exposed to Kodak X-Omat AR film for 96 hours at -100°C. The membrane was stripped of the RBP probe by boiling in 0.1% SDS and rehybridized with rat β-actin (specfic activity 1-1.5 x 10^9 cpm/ug DNA) to correct for loading inaccuracies as described by Dore' et al. (27). Signal intensities from each exposure were analyzed by integration using an LKB Ultrascan Laser Scanning Densitometer (LKB Produkter AB, Bromma, Sweden) and Gelscan XL version 2.0 software package (Pharmacia LKB Biotechnology, Uppsala, Sweden). β-actin signals were used to correct for loading inaccuracy and allow RBP area to be used to quantify relative RBP expression from each day of the estrous cycle or hormone treatment group.

In vitro culture of oviductal explants

Oviducts were removed, transported to the lab in 0.9% NaCl at 30-35°C and excess tissue removed as above. Each oviduct was then split into four approximately equal sections and cultured as described by Godkin et al. (29). Briefly, oviducts were divided into isthmus or ampullar sections,

minced to approximately 1 mm^3 and explants placed in 5 ml of leucine deficient, high glucose Dulbeccos minimum essential medium containing 50 uCi of L-[^{3}H] leucine. Tissues were cultured for 24 hours at 37°C on a rocking platform in a gaseous atmosphere of 45% O_2, 50% N_2 and 5% CO_2. Cultures were terminated by centrifugation at 12,000 x g for 15 minutes and the supernatant stored at -80°C.

Immunoprecipitation

Aliquots of dialyzed medium from oviductal cultures were lyophilized and samples resuspended in water such that 200,000 cpm of nondialyzable radioactivity was present in 750 ul aliquots. Aliquots were incubated with 5 ul of anti-RBP or normal rabbit serum overnight at 4°C. The immunprecipitation was performed as described by Lifsy et al (30) and analyzed by one dimensional PAGE (31). Fluorography was performed according to the procedure of Chamberlain (32). Signal intensities of 5 autoradiographs, each representing 1 animal from each treatment (6 animals per autoradiograph), were analyzed by integration using LKB Ultrascan Laser Densitometer and GSXL version software and expressed as AU/mm2 (AU=arbitrary optical density units).

Immunocytochemistry

Ampullar and isthmus sections from each ewe were immersion fixed in Bouin fixation fluid for 4 - 6 hours and changed into 70% ethanol twice daily until the yellow color had dissipated. Tissues were dehydrated, embedded in paraffin and ICC was performed according to Lui and Godkin (33). Briefly, tissue was sectioned at 4μm and adhered on ProbeOn slides. Anti-RBP serum , diluted (1:200) in phosphate buffered saline containing 1% normal goat serum and 1% bovine serum albumin, was applied to sections and incubated at 37°C for 2 hours. Controls were treated with RBP-adsorbed anti-RBP (1:200 dilution) and incubated under the same conditions. Binding of anti-RBP was detected according to instructions for the Histogen^{R} Peroxide anti-Peroxidase Immunostaining kit. Visualization of the specific interaction between the secondary and primary antiserum was achieved using 3,3'-diaminobenzidine staining for 3 minutes. The ICC procedure was performed on oviducts (ampulla and isthmus section) such that 1 animal from each treatment was represented in each analysis. In each replication, all slides were processed in the microprobe slide assembly so that all tissue sections received identical treatment. Similar conditions were applied to each replication and a

representative sample is shown.

ELISA

Explant media from the second experiment was analyzed for RBP by ELISA according to the procedure of MacKenzie et al (34). Briefly, microtiter plates were coated with purified serum RBP (35). Samples and standards (ranging from 0.1-10 ng/well) were mixed with bovine placental RBP antibody (35) and incubated overnight at 4°C. The sample-antibody mixture was then transferred to the antigen coated plates and allowed to incubate for 2 hours in a humidified chamber at 30°C. Plates were then washed (Dynatech Ultrawash II Microplate Washer, Chantilly VA) with a solution of PBS with 0.05 (v/v) Tween-20. Goat anti-Rabbit IgG alkaline phosphatase conjugate (diluted 1:3000) was added to each well and the plate was then incubated for 2 hours at 30°C. The plate was washed as before and p-NPP phosphatase substrate was added to the wells. Plates were read (absorbance 405 nm, BioTek Automated Microplate Reader, Winooski VT) every 15 minutes until the 100% antibody wells (antigen coated wells with anti-RBP antibody only) reached and optical density of 0.8-0.9. The reaction was then stopped with 5% Na_2EDTA and the plate read to calculate the results. The intra-assay

variation was <5% and the inter-assay variation was <10%.

Radioimmunoassays

Circulating estradiol 17β concentrations were determined in plasma samples by a validated RIA according to the method of Moura and Erickson (36). Circulating progesterone levels were determined by a RIA validated by Seals et al (37). All samples were run in a single assay and intraassay coefficient of variation for estradiol 17β and progesterone was 9% and 4%, respectively. Minimum sensitivity per tube was 1.5 pg/mL (estradiol 17β) and 0.05 ng/mL (progesterone).

Statistical Analyses

Data were analyzed using SAS (SAS Institute Inc, SAS Circle, Cary, NC). Analysis of variance was performed using the General Linear Models Procedure (PROC GLM) to detect differences due to days (experiment 1) and differences due to hormone treatments (experiment 2). Differences among days/hormone treatments were tested utilizing protected least significant difference (38).

RESULTS

RBP mRNA Expression

Temporal changes in RBP mRNA expression in oviductal tissue prepared from cyclic animals were determined by quantitative slot blot analysis. Mean areas for each day were normalized to β-actin levels and plotted (figure 2-1A). RBP mRNA expression on Day 1 of the cycle was dramatically higher ($p<0.01$) than on either Day 5 or Day 10. There were no significant differences ($p>0.95$) in RBP mRNA expression between oviducts collected on Days 5 and 10.

Relative changes in RBP mRNA expression in oviductal tissue from ewes treated with steroid hormones were also determined by quantitative slot blot analysis and the normalized plots are illustrated in figure 2-1B. Retinol binding protein mRNA expression was not different between ovariectomized controls and E2 treated animals ($p>0.10$). Progesterone treatment significantly decreased RBP mRNA expression levels when compared to the E2 but not control treated animals ($p<0.05$). The E2+P4 treatment significantly reduced RBP mRNA expression levels when compared to control or E2 treatments ($p<0.05$), but was not significantly

different ($p>0.15$) from P4 treated animals.

Immunolocalization of RBP

RBP was immunolocalized to the luminal epithelia of oviductal mucosa with diffuse staining in stroma and muscularis in both the ampulla and isthmus of all animals. Only isthmus sections are shown for brevity. Intense RBP staining was noted in the epithelium on Day 5 of the cycle; it was somewhat decreased on Day 10 and noticeably diminished on Day 1 (figure 2-2). In hormone treated animals, ICC analysis revealed intense RBP staining in the oviductal epithelium of control and P4 treated animals; whereas, E2 treatments alone or in combination with P4 resulted in diminished staining (figure 2-2).

Oviductal RBP synthesis in vitro

Oviductal tissues from both experiments were incubated for 24 hours in DMEM containing L-[^{3}H] leucine. Immunocomplex precipitation with anti-bpRBP serum, followed by 1D-PAGE and fluorography, was used to show oviductal RBP synthesis. No differences in patterns of RBP synthesis were observed between ampulla and isthmus tissue. Ampulla is

shown in the cycling ewe fluorograph and isthmus is shown in the steroid treated ewe fluorograph (figure 2-3).

Relative changes in metabolically labeled RBP production by oviductal tissue collected on three different days of the estrous cycle are illustrated in figure 2-4A. Retinol binding protein production by oviducts collected on Day 1 was strikingly higher ($p<0.05$) than production on either Day 5 or Day 10. However, no differences in oviductal RBP production were noted between Day 5 and Day 10 of the cycle ($p>0.60$).

Temporal changes in metabolically labeled RBP production by oviductal tissue collected from ovariectomized, steroid treated ewes were quantified and are shown in figure 2-4B. Retinol binding protein production by oviducts collected from ewes treated with E2 alone or in combination with P4 was dramatically higher ($p<0.05$) than that produced by P4 treated or control animals. No differences were noted between E2 and E2+P4 treatments or between P4 treated and control animals ($p>0.75$).

ELISA

Total RBP in the medium conditioned by oviductal explants from steroid treated ewes was determined by ELISA

and expressed as ng of RBP per mg of oviductal tissue (figure 2-5). No differences in patterns of concentrations were observed between ampulla and isthmus tissue so samples were combined in final analysis. Estradiol treatment increased RBP production 5 fold over P4 or control treatments ($p<0.05$). The E2+P4 treatment also significantly increased RBP production over P4 and control treatments ($p<0.07$ and $p<0.05$, respectively). There were no differences between either the E2 and E2+P4 treatments ($p>0.40$) or P4 and control treatments ($p>0.50$).

Plasma Steroid Concentrations

Concentrations of exogenous steroids in the peripheral circulation were determined by radioimmunoassay. The range of progesterone (in P4 treated animals) was 1-3 ng/ml which is within the range of normal luteal phase production. Estradiol 17β treated animals had greater than 10 pg/ml of estradiol which is above the normal cyclic estradiol production (25). The reason for this is unknown since the same size and type of silastic implants were used as those used by Homanics and Silvia (24) and steroid treatments were identical, except for a 9 day progesterone pretreatment period instead of a 6 day pretreatment period.

DISCUSSION

Results of this study demonstrate that RBP expression by the oviduct is strongly influenced by the ovarian steroids, estradiol and progesterone. Oviductal RBP mRNA expression was significantly greater on Day 1 than on Days 5 or 10 of the estrous cycle. Likewise, synthesis and release of RBP by oviductal explants, prepared from ewes on Days 1, 5 and 10 of the cycle, reflected the pattern of RBP gene expression. The synthetic and secretory capacity of the oviduct is recognized to vary during the cycle and early pregnancy in response to the ovarian steroids. Production of oviductal fluid, total protein and a 92 kD oviduct specific glycoprotein (3,4,39) peaks at or shortly after estrus (Days 0-2) in response to high estrogen levels then declines in response to increasing progesterone concentrations. In addition, Murray (5) reported structural alterations in the oviductal secretory epithelium during the cycle that correspond to changes in protein production and steroid levels.

Results from the steroid hormone replacement study provide insight into the mechanisms regulating RBP production. Analyses of steady state RBP mRNA expression levels as well as RBP synthesis and release by oviductal

explants from ovariectomized ewes demonstrate that RBP is constitutively expressed in the absence of ovarian steroids. Progesterone, administered on a background of estradiol, appears to negatively regulate RBP gene expression, whereas estradiol stimulates RBP synthesis and secretion. We point out that although progesterone diminished RBP mRNA steady state levels compared to controls, this decrease was not statistically significant, whereas the progesterone plus estradiol treatment significantly reduced RBP mRNA expression compared to controls or estradiol treatments. These results may be explained by the regulation of progesterone receptors by the ovarian steroids. Spencer and Bazer (40) showed that treatment of ewes with progesterone for 12 days resulted in down-regulation of progesterone receptor expression in the uterus. On the other hand, treatment with progesterone and estradiol increased progesterone receptor expression by about two fold compared to progesterone treated animals. In our study, down-regulation of the progesterone receptor in progesterone treated animals could account for RBP mRNA levels being similar to controls. Conversely, in ewes treated with progesterone plus estradiol, it is likely that progesterone receptors were up-regulated allowing progesterone to exert negative control over RBP mRNA

expression. Steady state levels of RBP mRNA were highest in estradiol treated ewes and this level of expression was significantly different from progesterone or progesterone plus estradiol treated animals but not controls. Oviductal explants from estradiol treated animals synthesized and released significantly more RBP than explants from control or progesterone treated animals. It is possible that estradiol treatment stimulated both transcription and mRNA degradation. This would explain the dramatic effects of estradiol on RBP synthesis and release but only modest effects on steady state RBP mRNA levels. Unfortunately, we can not confirm this suggestion since RNA turnover studies were not performed.

Results from the immunocytochemical analysis of oviduct tissues support the in vitro data which demonstrated that estradiol stimulates RBP secretion. Oviducts from all animals under estradiol influence (ie., intact ewes on Day 1, and ovariectomized ewes that received estradiol alone or in combination with progesterone) exhibited diminished immunostaining of RBP in the secretory epithelium compared to ovariectomized control, progesterone treated ewes and luteal phase intact animals. Apparently, in the absence of estradiol stimulation, the oviductal epithelium accumulates RBP.

Uterine production of RBP is also regulated, in part, by ovarian steroids. It has been shown in sheep (4,23,41) and cattle (23,34) that RBP mRNA levels are elevated in endometrium during proestrus and estrus. Conversely, RBP concentrations in uterine flushings from cattle are low and declining at these times (34). In addition, estradiol was observed to stimulate RBP mRNA expression in bovine uterine endometrial explants and epithelials cells (42). Hence, it appears that estradiol stimulates RBP transcription, synthesis and secretion by the oviduct (present study); whereas, it may stimulate only RBP transcription in the uterus and may be inhibitory to secretion. In both the oviduct (present study) and uterus (34), rising progesterone levels appear to negatively regulate RBP and the mRNA that encodes it during late metestrus and early diestrus but in the uterus RBP secretion and mRNA levels rise from mid to late diestrus, possibly due to progesterone receptor down regulation (34). Conversely, in the oviduct, RBP expression remains depressed thoughout diestrus. Differential regulation of RBP expression by the oviduct and uterus may be explained by the fact that the degenerative morphological alteration of the oviductal secretory epithelium initiates as progesterone levels begin to rise about 4 days after estrus (43) which include dedifferentiation of secretory

apparatus and reduced numbers of secretory granules. Similar changes were observed in ovariectomized ewes that received exogenous progesterone on an estradiol background (5).

In summary, results from these studies demonstrate that RBP and the mRNA that encodes it are produced by the epithelium of the ampulla and isthmus of the ovine oviduct. In addition, RBP is expressed throughout the estrous cycle and in the absence of ovarian steroids. These finding are in contrast to the expression of the well characterized 92 kD oviduct specific glycoprotein (2,3,4) which has been shown to be estradiol dependent and expressed only in the ampullary end of the oviduct (5). The ovarian steroids modulate RBP synthesis, secretion and gene expression. Production of RBP by the oviduct is not limited to the ovine species. The protein has been identified in the equine oviduct on Days 1 and 4 and the mRNA has been identified in the Day 0 porcine oviduct (20,21). The high level of RBP expression at the time when the ovum and early embryo are present in the oviduct may indicate an important role for retinol in early development.

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Figure 2-1. Relative concentrations of oviductal RBP mRNA expression of cyclic (A) and ovariectomized, steroid treated (B) ewes. RNA values are means and measured in arbitrary optical density units (AU). Error bars represent standard deviation of the mean. Data points with different letter superscripts are significantly different ($p<0.05$).

Figure 2-2. Immunocytochemical localization of retinol-binding protein in the isthmus of cyclic and ovariectomized, steroid treated ewes. Note strongest staining in luminal epithelia of oviductal mucosa from (a) control and (c) progesterone treatments and, (g) Day 5, and (h) Day 10 of the cycle. Also, notice slightly diminished staining in (d) estrogen + progesterone and greatly diminished staining in (b) estrogen and (e) Day 1. Note lack of staining in (f) which is a control slide of Day 1 isthmus. Counter stain is Mayer's Hemotoxylin. Magnification × 168. See text-section *materials and methods* for treatment definition.

Figure 2-3. Production of RBP in vitro by explants from oviducts collected from cycling or ovariectomized, steroid treated ewes. Metabolically labeled RBP (arrow) was immunoprecipitated from culture medium, separated by 1D PAGE and identified by fluorography. Lanes 1, 2 and 3 are from

oviductal tissue collected on Days 1, 5 and 10, respectively; lanes 4, 5, 6 and 7 are from tissue from control, E2, P4 and E2+P4 treated animals. Lanes designated (a) represent precipitation with anti-RBP and (b) represent precipitation with normal rabbit serum.

Figure 2-4. Relative concentrations of metabolically labeled oviductal RBP from cyclic (A) and ovariectomized, steroid treated (B) ewes. Retinol binding protein expression is mean value of samples and is measured in arbitrary optical density units (AU). Error bars represent standard deviation of the mean. Data points with different letter superscripts are significantly different ($p<0.05$).

Figure 2-5. Retinol binding protein production by oviductal explant cultures from ovariectomized, steroid treated ewes expressed as ng protein/mg tissue. Error bars represent standard deviation of the mean. Data points with different letter superscripts are significantly different ($p<0.05$).

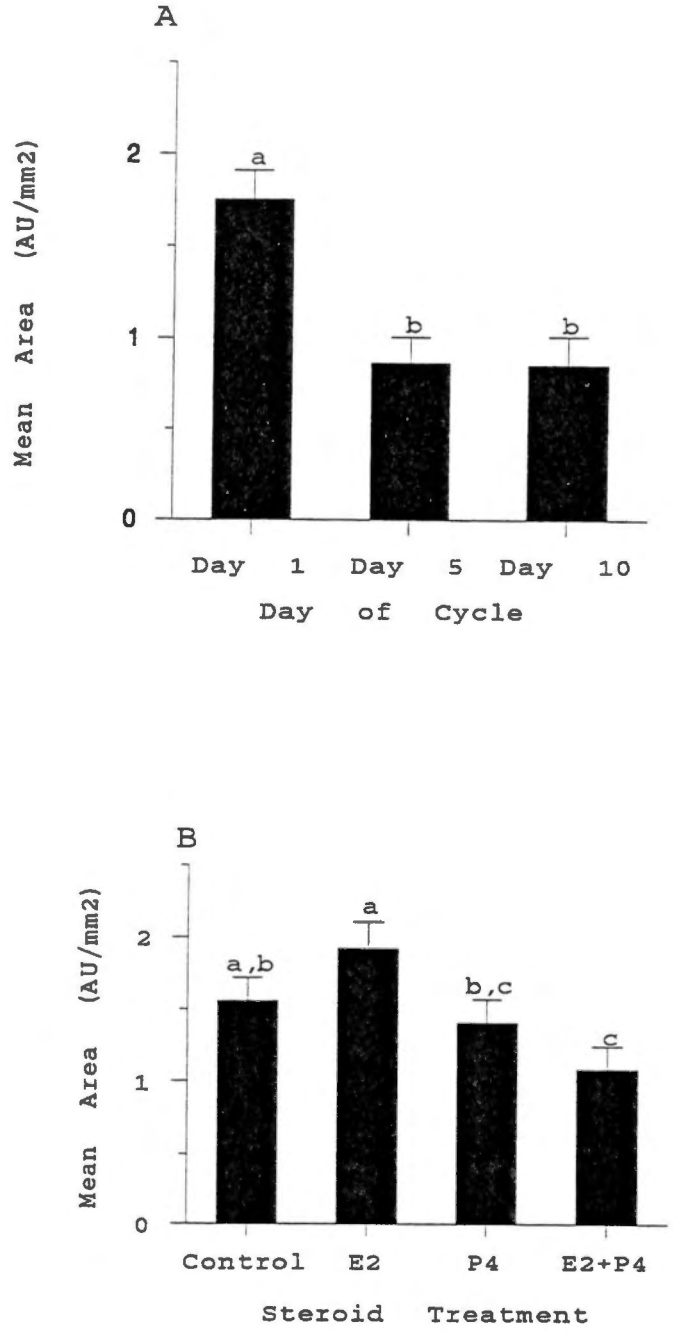

Figure 2-1. Relative concentrations of oviductal RBP mRNA expression of cyclic and ovariectomized, steroid treated ewes.

a
b
c
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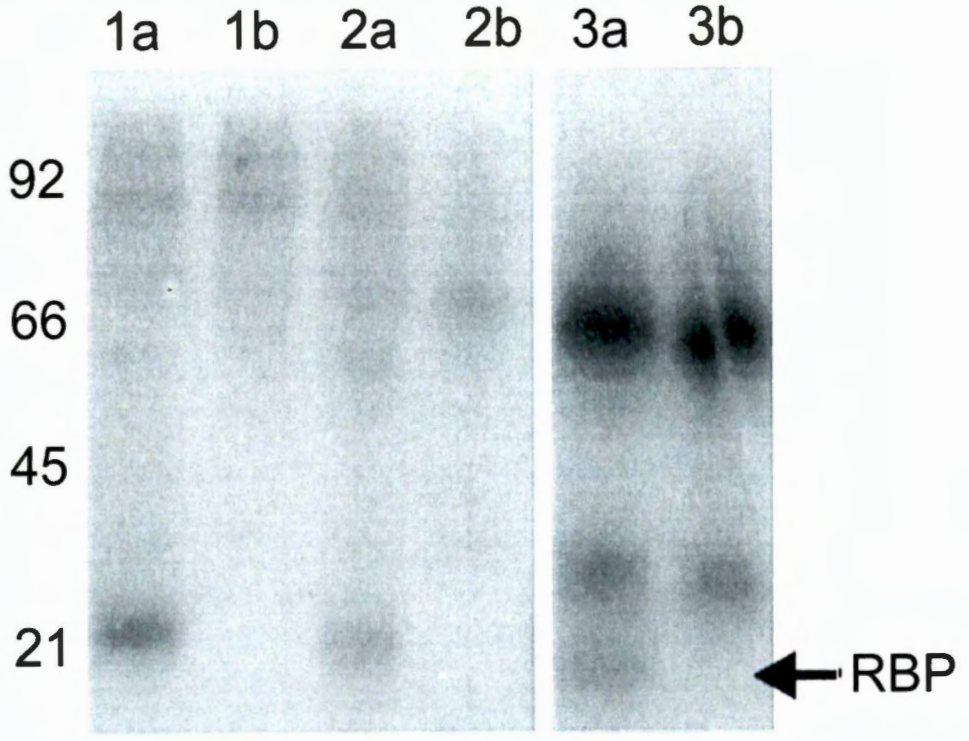
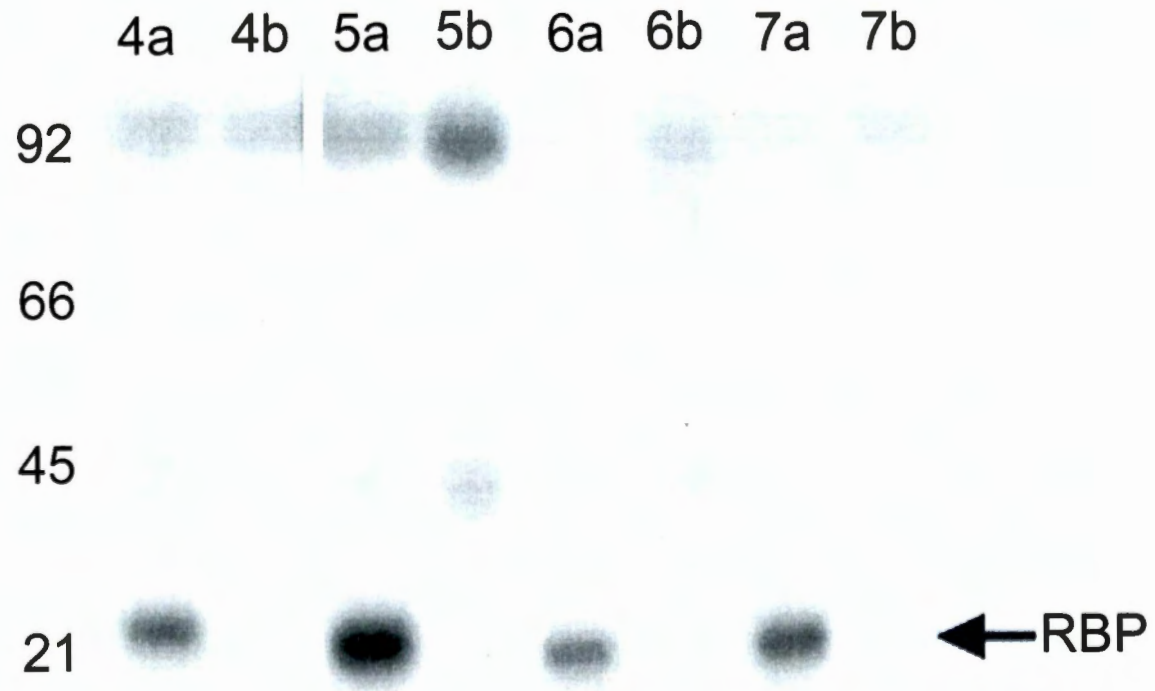

1a
1b
2a
2b
3a
3b
92
66
45
21
RBP


4a
4b
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92
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21
RBP

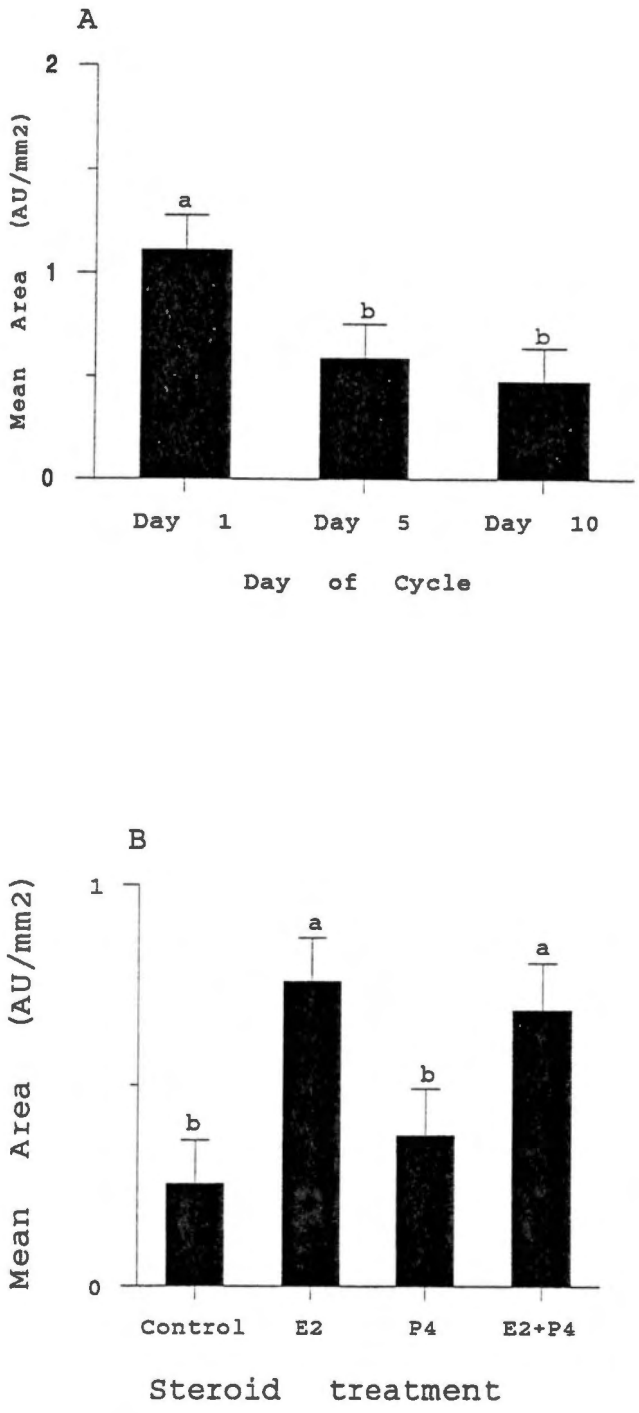

Figure 2-4. Relative concentrations of metabolically labeled oviductal RBP from cyclic and ovariectomized, steroid treated ewes.

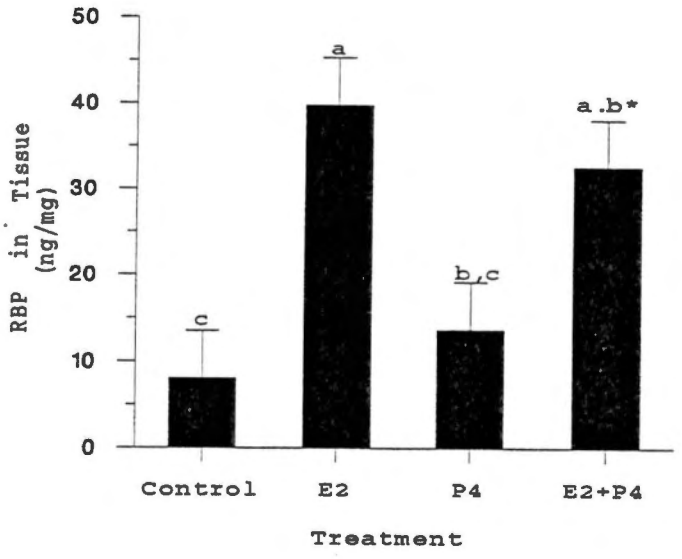

Figure 2-5. Retinol binding protein production by oviductal explant cultures from ovariectomized, steroid treated ewes expressed as ng protein/mg tissue.

Part 3. Immunolocalization of Retinoid-Binding Proteins in the Ovine Ovary.

ABSTRACT

Ovaries from normal cycling adult ewes were analyzed for the presence of retinol-binding protein (RBP), cellular retinol-binding protein-1 (CRBP) and cellular retinoic acid binding protein (CRABP). Retinol binding protein and CRBP were immunolocalized in the thecal cell layers of non atretic antral follicles. Retinol binding protein also exhibited some diffuse staining in the granulosa cells and stromal cells of these follicles; whereas, CRBP showed some staining in the stroma but not in the granulosa cell layers. Retinol binding protein was also immunolocalized in the cytoplasm of oocytes from some antral but not pre-antral follicles. Cellular retinoic acid binding protein was localized in both the nucleus and cytoplasm of oocytes from primordial follicles but was absent in all other oocytes. No CRABP staining was seen in granulosa cells or thecal cells of any size follicle. Cellular retinoic acid binding protein was also seen in connective tissue in the ovary inclucing the tunica adventitia layer of blood vessels and in the connective tissue surrounding the corpus luteum but no staining was observed in the luteal cells. In addition, CRABP stained intensely in the tunica albuginea but was not seen in the germinal epithelium or cortex of the ovary.

This study suggests retinol and its metabolite, retinoic acid, play a role in normal ovarian function.

INTRODUCTION

Retinol is essential to reproduction in both males and females (reviewed in 1). In the female, vitamin A deficiency can lead to decreased ovarian size, decreased ovarian steroid concentration, and eventually reproductive senescence. In the rat, even mild vitamin A deficiency causes a decrease in activity of ovarian, but not adrenal, steroid enzymes (2-4). This was confirmed by several studies performed in vitro where retinol administration increased progesterone output in luteal cells from swine (5) and cattle (6). In addition, retinoids stimulated progesterone production and enhanced steroid production in response to gonadotropins in rat granulosa cells (7,8). Studies also indicate that retinoids may support early reproductive events. In litter bearing species, administration of retinol or β-carotene prior to ovulation has been reported to increase embryo survival in mice (9), rabbits (10) and swine (11-13). Retinol administration to monotocous animals, when combined with superovulation protocols, increased the number of ovulations in sheep

(14,15) and embryonic quality in both cattle (16) and sheep (14,15). This data implies a fundamental connection between retinol and ovarian function.

Systemic and intercellular transport of retinol is accomplished via a specific transport protein, retinol binding protein (RBP). Cellular retinol binding protein and CRABP are involved in intracellular retinol homeostasis (17). Cellular retinol binding protein has three major functions: retinol accumulation, stimulation of retinol mobilization from retinyl ester stores; and delivery of the retinol to dehydrogenase for conversion to retinal and eventually retinoic acid. The actions of retinoic acid is mediated through two subgroups of nuclear receptors, retinoic acid receptors (RAR) and retinoid X receptors (RXR). Retinoic acid/receptor complexes cause gene repression or activation through association with specific response elements (retinoic acid response element or retinoid X response elements) found in the promoter regions of target genes (17). Cellular transport/metabolism of retinoic acid is accomplished via the small, cytoplasmic proteins cellular retinoic acid binding proteins 1 and 2 (CRABP and CRABP 2). The objective of this study was to immunolocalize RBP, CRBP, and CRABP in the ovine ovary.

MATERIALS AND METHODS

Materials

Bouin fixation fluid was purchased from Baxter Scientific Products (McGaw Park, IL). Paraffin was purchased from Fisher Scientific (Pittsburgh, PA). Histogen^{R} Peroxide anti-Peroxidase Immunostaining Sytem, concentrated biotinylated anti-rabbit immunoglobins, concentrated peroxidase-conjugated streptadvidin, liquid DAB concentrated substrate and their diluents were purchased from BioGenex Laboratories (San Ramon, CA). All other reagents were purchased from Sigma Chemical Corp. (St. Louis, MO).

Methods

Ten sexually mature crossbred ewes that had exhibited at least 2 normal estrous cycles were ovariectomized via mid ventral laparotomy. Ovaries from each animal were immersion fixed in Bouin fixation fluid for 4 - 6 hours and changed into 70% ethanol twice daily until the yellow color had dissipated. Tissues were dehydrated and embedded in paraffin. Retinol binding protein ICC was performed according to Lui and Godkin (18). Briefly, tissue was

sectioned at 4μm and adhered on ProbeOn slides. Anti-RBP serum, diluted (1:200) in phosphate buffered saline containing 1% normal goat serum and 1% bovine serum albumin, was applied to sections and incubated at 37°C for 2 hours. Controls were treated with RBP-adsorbed anti-RBP (1:200 dilution) and incubated under the same conditions. Binding of anti-RBP was detected according to instructions for the Histogen^{R} Peroxide anti-Peroxidase Immunostaining kit. Visualization of the specific interaction between the secondary and primary antiserum was achieved by using 3,3'-diaminobenzidine staining for 3 minutes. The CRBP and CRABP antibodies were kindly provided by John C. Saari (University of Washington, Seattle, WA) and have been characterized in Saari et al (19) and Milam et al (20), respectively. The ICC was performed as above except that biotinylated anti-rabbit immunoglobins and peroxidase-conjugated streptadvidin were used in lieu of the Histogen^{R} Peroxide anti-Peroxidase Immunostaining Sytem. Sections were counterstained with Mayer's Hemotoxylin to visualize nuclei. The ICC procedure was performed such that 1 section from each animal was represented in each analysis. In each replication, all slides were processed in the microprobe slide assembly so that all tissue sections received identical treatment. Similar conditions were applied to each replication and a

representative sample is shown. In order to classify follicles as healthy or atretic, pycnotic nuclei were counted in at least 4 sections of each follicle, averaged and expressed as a percentage (21). Follicles with greater than 10% pycnotic nuclei in the granulosa cell layer did not stain for RBP or CRBP and were considered atretic.

RESULTS

Immunocytochemical analysis for RBP resulted in significant staining of the thecal cell layer of non-atretic antral follicles (figure 3-1). Diffuse staining was also seen in the stromal and granulosa cell layers of these follicles. No staining was noted in follicles that contained greater than 10% atretic granulosa cells (data not shown). Diffuse staining was also demonstrated in the oocyte of antral follicles but not in pre-antral follicles. Significant staining for RBP was displayed in the large cells of the mature corpus luteum. Extremely intense staining was localized in some but not all small luteal cells. The stromal layer of the CL showed diffuse staining similar to that surrounding non-atretic follicles.

A similar immunostaining pattern was observed for CRBP in the ovarian tissue. Intense staining was again localized

to the thecal cell layer of non-atretic follicle with diffuse staining present in stroma surrounding these follicles. This staining was absent in follicles containing greater than 10% atretic granulosa cells (data not shown). However, CRBP staining was absent from granulosa cells and oocytes of all follicle regardless of size or health. Cellular retinol binding protein staining was observed in some but not all of the large luteal cells of mature corpora lutea and absent in small luteal cells (figure 3-1).

The staining pattern of CRABP in ovarian tissue was distinctly different than that of both RBP and CRBP. Intense staining was observed in the nucleus of oocytes from primordial follicles (figure 3-1). Staining was diffuse in the cytoplasm of these oocytes and absent in the single layer of granulosa cells. No staining was observed in oocytes of all other size follicles. Staining was absent from granulosa cells, thecal cells or surrounding stromal cells of any size follicle. No staining was seen in either large or small cells in the corpus luteum but was present in the stromal layer surrounding it. Not all ovarian connective tissue was devoid of CRABP staining. Intense localization was present in the tunica adventitia layer of blood vessels and the tunica albuginea surrounding the entire ovary.

DISCUSSION

Results of this study clearly illustrate the presence of retinoid binding proteins, RBP, CRBP and CRABP, in the ovine ovary. Retinol binding protein and CRBP were immunolocalized in the thecal cells of healthy but not atretic antral follicles. Schweigert and Zucker (22,23) associated bovine follicular fluid retinol concentrations with follicular health. Retinol concentrations were highest in the follicular fluid of healthy follicles, lowest in atretic follicles and highly correlated with estradiol concentration. Similarly, the number of atretic follicles was shown to be highest in vitamin A deficient rats and lowest in the A-hypervitaminotic rats with control animals in between (24). Together, these data indicate that the concentration of retinol within the follicle may be an indicator of follicle health. Our data support this concept and may provide a mechanism for the accumulation and transport of retinol into the follicle. In this model, CRBP accumulates retinol from the blood plasma and RBP transports it across the basement membrane into the follicle (figure 3-2). This model has also been suggested in transportation of retinol through the blood-testes barrier (25).

Retinol administration to animals prior to ovulation and fertilization has been reported to influence the quality of resultant embryos. In swine, a single injection of retinol palmitate given about 5 days prior to estrus increased litter size (12,13). In a subsequent study, it was shown that uterine luminal concentrations of retinol were not affected by this treatment suggesting that the site of retinol action on embryo survival was not the uterus (26). A single injection of retinol given with the first FSH injection five to seven days prior to induced estrus significantly improved the quality of embryos collected from superovulated cattle but had no effect on total number recovered (16). In a series of studies in sheep, administration of Vitamin A in conjunction with pregnant mare serum gonadotropin was reported to influence ovulation rate and decreased the number of tertiary atretic follicles (14,15). Recently, we have shown that administration of Vitamin A in combination with FSH followed by natural mating resulted in embryos with improved capacity to develop to the blastocyst stage, in vitro. These results indicate that retinol administration prior to ovulation improves the in vitro developmental capacity of embryos (27).

In many systems, including the testes, CRABP is involved in retinoic acid homeostatis (28). Holo CRABP is

thought to be a substrate for retinoic acid catabolism and therefore may be present in cells that cannot tolerate high levels of retinoic acid. Retinoic acid is involved in differentiation in many cells types including embryonic stem cells and embryonic carcinoma cells (29). In male rats, retinol deficient diets are often used to synchronize spermatogensis (30). In prolonged vitamin A deficient conditions, spermatogenesis is arrested at preleptotene spermatocytes. In the ovary, oocytes are arrested prior to the first meiotic division until being recruited into the growing phase at which time they undergo either ovulatation or undergo apoptosis. In the present study, CRABP was immunolocalized to oocytes from primordial follicles but not in oocytes from other size follicles. If retinoic acid influences development in the oocyte as it does in the spermatocytes, CRABP may sequester retinoic acid in primordial follicles and prevent its access to nuclear receptors and thus, block its action.

The production of retinoid binding proteins could concentrate retinol for paracrine (RBP) or autocrine (CRBP) functions. Administration of retinol to in vitro cultured luteal cells or luteinizing granulosa cells stimulate progesterone production in swine (31), cattle (32) and rats (33). In the rat, even a mild case of vitamin A deficiency

causes a decrease in activity of ovarian enzymes 3β-hydroxyΔ^5-steroid dehydrogenase (2) and cholesterol side chain cleavage (3) and thus decreases steroid output. Vitamin A may also play a role in the formation and maintenance of the corpus luteum as A-hypervitaminotic rats showed an accelerated occlusion at the point of ovulation, increased vacularization of the CL wall, and more rapid formation of the fibrous cavity surrounding the CL when compared to both control and A-deficient rats (24).

The presence of retinoid-binding proteins in the ovary is not unique to the ewe. These proteins have been recently localized in the rat ovary (34) and bovine corpus luteum (35). In the rat, immunohistochemical staining for CRBP was present in inner follicular granulosa cells and oocytes of all stages of follicular development. In contrast, we observed no immunostaining in these tissues of ovine ovaries. In addition, species differences apparently exist for retinoid binding protein expression in the CL. In the rat, CRBP was located only in the stroma layer found between the CL and the outer cell layer of the ovary (34). In both the bovine (35) and ovine CL, CRBP and RBP were localized in large luteal cells. However in the ewe, RBP was also localized in some small luteal cells. Since species

differences are not uncommon, it is not surprising that the rat and ewe exhibit differential cells localization.

In summary, results from this study demonstrate the presence of retinoid binding proteins RBP, CRBP and CRABP in the ovine ovary. This study, in combination with others, provides evidence to indicate retinoids may function in very early events associated with reproductive success including follicular development and ovum maturation.

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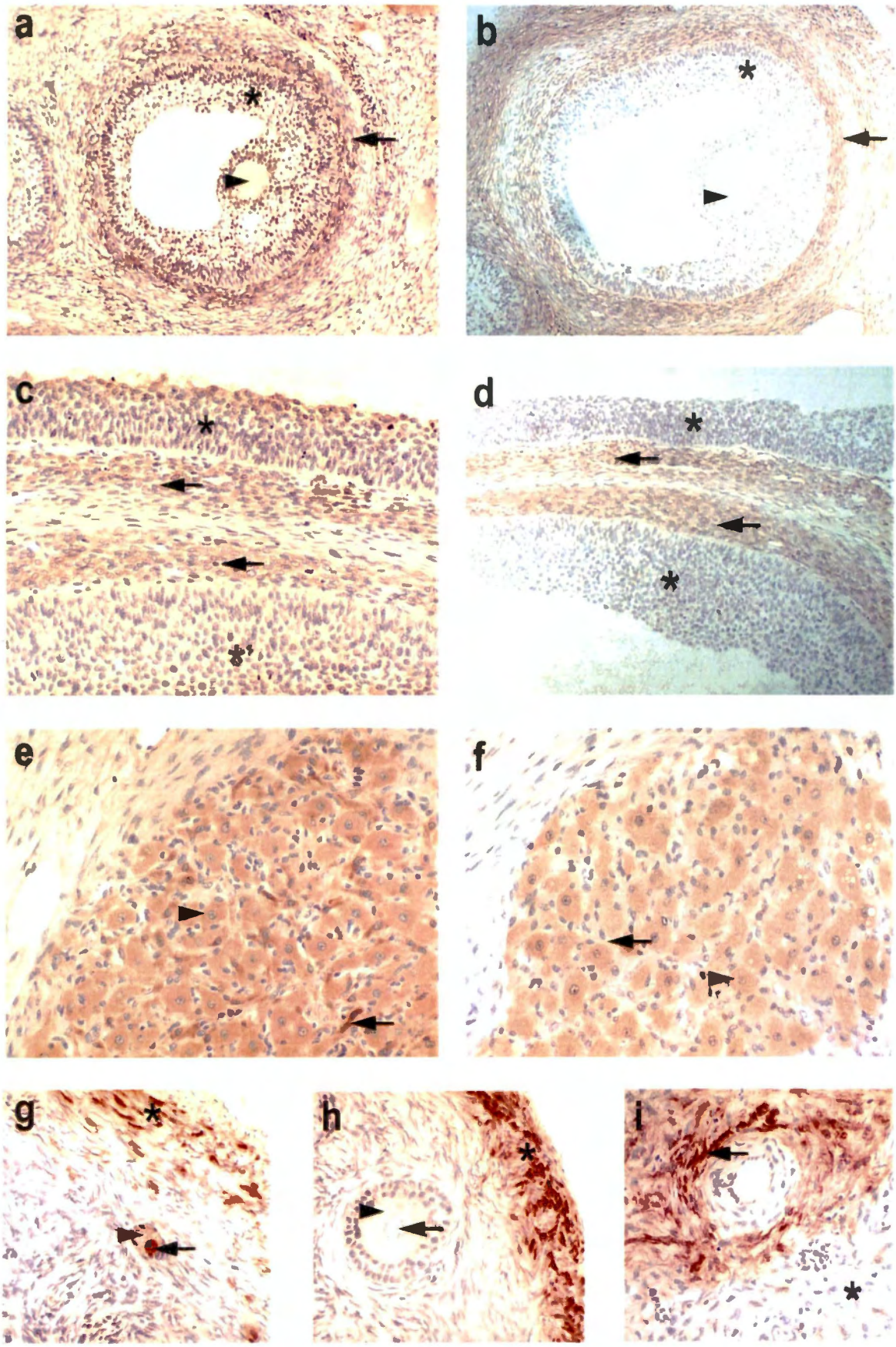
Figure 3-1. Immunocytochemical localization of RBP (a,c,e), CRBP (b,d,f) and CRABP (g,h,i) in ovaries from cycling ewes. a-d) Note strong staining of RBP (a,c) and CRBP (b,d) in the theca cell layer (arrow) of healthy antral follicles. Also, notice diffuse staining for RBP (a,c) but not CRPB-1 (b,d) in granulosa cells (*) and oocytes (arrowhead) of these same follicles. Notice positive staining for RBP (e) and CRBP (f) in the large cells of the corpus luteum (arrowhead). In these same pictures note the strong staining of small luteal cells (arrow) for RBP (e) but not CRBP (f). CRABP staining is apparent in both the nucleus (arrow) and cytoplasm (arrowhead) of the oocyte in a primordial follicle (g) and absent in a growing, pre-antral follicle (h). Also, notice staining in the tunica albuginea (*) in both of these pictures (g,h). (i) Positive staining for CRABP is seen in the tunica adventitia layer of blood vessels and in the stromal cells surrounding the corpus luteum (arrow) but is absent in luteal tissue (*). Counterstain is Mayer's Hemotoxylin. Magnification is × 168 (c,e,f,g,h,i) and × 84 (a,b,d).

Figure 3-2. Model for the delivery of retinol through the basement membrane to granulosa cells and/or follicular fluid. Retinoid proteins represented are: retinol (ROH),

retinol binding protein (RBP) and cellular retinol binding protein (CRBP).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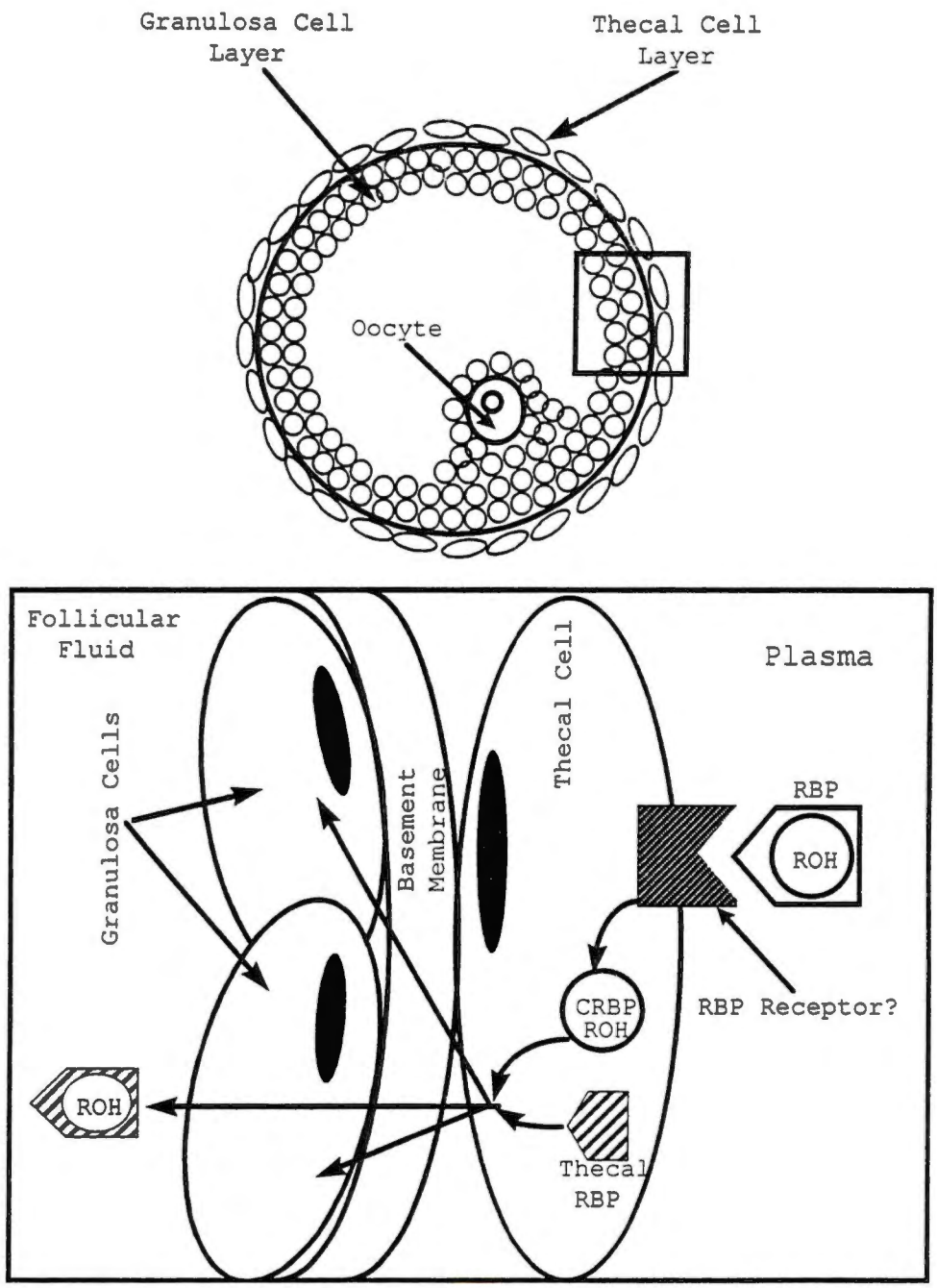

Figure 3-2. Model for the delivery of retinol through the basement membrane to granulosa cells and/or follicular fluid.

Part 4. Localization of retinol-binding protein and cellular retinol-binding protein in the bovine ovary.

ABSTRACT

Ovaries from normal cycling cows were analyzed for the presence of retinol-binding protein (RBP) and cellular retinol-binding protein (CRBP). Thecal and granulosa layers were microdissected apart from 2 to 8 millimeter follicles derived from ovaries of non-pregnant cows. Each layer was homogenized for RNA analysis or cultured for 24 hours for protein analysis. Northern blot analysis demonstrated that thecal cells express RBP mRNA and that granulosa cell expression is vastly lower. Retinol · binding protein synthesis was demonstrated by immunoprecipitation of radiolabeled RBP from the medium of both thecal and granulosa cell cultures. In the second experiment, cows were lutectomized on days 2, 6, 10, or 15 of their respective estrous cycle and luteal tissue dissected and homogenized for RNA analysis, fixed for immunocytochemistry (ICC) or cultured for 24 hours for protein analysis. Slot blot analysis demonstrated no difference in relative RBP mRNA concentrations between the days studied. ICC localized both RBP and CRBP to large luteal cells. Explant culture medium analyzed from each day illustrated the synthesis and secretion of RBP from the bovine CL. This study illustrates the presence of RBP and CRBP in the bovine ovary and

suggests that retinol plays a role in normal ovarian function.

INTRODUCTION

The corpus luteum (CL) is a transient endocrine gland responsible for the early maintenance of pregnancy in all species. The CL is formed by differentiation (luteinization) of the cells of the preovulatory follicle following the primary surge of luteinizing hormone. This process is marked by extensive tissue remodeling, angiogenesis, and cellular growth and differentiation (reviewed in 1). The bovine CL contains an abundance of β-carotene which lends it the characteristic yellow color. Conversion of β-carotene to retinal and ultimately to retinol has been shown in both the intestine and ovary (2).

Several studies, performed in vitro, have indicated a role for retinoids in ovarian steriodogenesis. Retinoids have been shown to stimulate progesterone production in luteal cells from swine (3) and cattle (4). In addition, retinoids stimulated progesterone production and enhanced steroid production in response to gonadotropins in rat granulosa cells (3,5). A classical study performed in rats showed that the number of atretic follicles is highest in the

vitamin A-deficient and lowest in the A-hypervitaminotic animals with control animals in between (6). This study also indicated that vitamin A may play a role in the formation and maintenance of the corpus luteum. A more recent study identified CRABP-2 in the granulosa cells of mature follicles and the early corpus luteum and suggested that vitamin A has a role in CL formation, specifically in granulosa cell remodeling (27).

Systemic and intercellular transport of retinol is accomplished via a specific protein, retinol binding protein (RBP). Cellular retinol binding protein (CRBP) is the cellular binding protein for retinol and is involved in retinol homeostasis through three distinct functions: retinol accumulation from serum RBP, stimulation of retinol mobilization from retinyl ester stores, and delivery of retinol to dehydrogenase for conversion to retinal and eventually retinoic acid (8). The cellular transport/metabolism of retinoic acid is accomplished via the small, cytoplasmic proteins cellular retinoid acid binding proteins 1 and 2 (CRABP and CRABP 2). The actions of retinoic acid is mediated through two subgroups of nuclear receptors, retinoic acid receptors (RAR) and retinoid X receptors (RXR). Retinoic acid/receptor complexes cause gene repression or activation through association with specific

response elements (retinoic acid response element or retinoid X response element) found in the promotor regions of target genes (9). The objective of this study was to identify the proteins that regulate extracellular transport (RBP) and intracellular accumulation (CRBP) of retinol in the bovine CL. Specifically, the goals were to identify cell specific and temporal expression of RBP and CRBP and investigate temporal changes in RBP synthesis and mRNA expression. Investigation of the proteins that mediate retinol action may provide insight into its actions.

MATERIALS AND METHODS

Materials

Cesium trifluoroacetate (CsTFA) was obtained from Pharmacia Biotech, Inc (Uppsala, Sweden). Formamide and Random Prime labeling kits were purchased from United States Biochemicals (Cleveland, OH). S & S Nytran membrane was purchased from Schleicher and Schuell, Inc (Keene, NH). Alpha^{P32}-dCTP and ^{3}H-leucine were obtained from ICN Pharmaceuticals, Inc (Costa Mesa, CA). Bouin fixation fluid was purchased from Baxter Scientific Products (McGaw Park, IL). Paraffin was purchased from Fisher Scientific

(Pittsburgh, PA). Histogen® Peroxide Anti-Peroxidase Immunostaining Sytem including concentrated biotinylated anti-rabbit immunoglobins, concentrated peroxidase-conjugated streptadvidin, liquid diaminobenzidine concentrated substrate and their diluents were purchased from BioGenex (San Ramon, CA). Microprobe system including slide holder assembly, 30 well Isolon reagent isolators and ProbeOn™ Microscope Slides were purchased from Fisher Scientific (Pittsburg, PA). All other reagents were purchased from Sigma Chemical Corp. (St. Louis, MO).

Sample collection

Corpora lutea were collected per vagina from normally cycling multiparous Holstein cows administered epidural anesthesia (5 ml 2% Xylocaine) on days 2 (n=7), 6 (n=5), 10 (n=5) or 15 (n=5) of their respective estrous cycles. Corpora lutea were transported to the laboratory on ice and dissected free of connective tissue and prepared for use. All experimental procedures using animals were approved by the Agriculture Animal Care and Use Committee of the University of Tennessee.

Ovaries from non-pregnant, cycling cows (n=10) were collected from the abattoir over a 4 hour period and

transported to the laboratory on ice (mRNA) or at 30°C (immunoprecipitation) in phosphate buffered saline containing 100 units/ml penicillin and 0.1 mg/ml streptomycin. Ovaries were kept separated by cow in statistical analysis of mRNA and proteins samples. Two to 8 mm follicles were dissected from each ovary under sterile conditions. Healthy developing follicles were distinguished from late stage atretic follicles by the presence of a pink vascularized thecal layer and clear follicular fluid free of cell debris. Follicles were punctured with a hypodermic needle and follicular fluid was aspirated. Granulosa and thecal layers were microdissected according to the procedure described by Roberts and Skinner (10). Briefly, granulosa cells were removed by cutting the follicle in half, agitating the follicle in buffer, and gently scraping the follicle wall with a fine plastic loop in a petri dish containing phosphate buffered saline. The thecal cell layer was dissected free of connective tissue and any remaining granulosa cells. Granulosa cells were concentrated by centrifugation. Granulosa cells and thecal layers from each ovary were pooled for analysis.

RNA isolation and analysis

The luteal tissue (day 2, n=3; days 5-15, n=5/day) and thecal cells (n=5 ovaries) were minced and homogenized in 4M guanidine isothiocyanate (4°C) (11). Concentrated granulosa cells (n=5 ovaries) were reconstituted in 4M guanidine isothiocyanate and homogenized as above. Total RNA isolation was performed according to the procedure described by Doré et al (12). Briefly, supernatant was layered on CsTFA (prepared following the manufacturer's instructions to a specific gravity of 1.51) for isopycnic gradient ultracentrifugation. The RNA pellet was resuspended in a solution of 30 mM sodium citrate, 0.1% (w/v) SDS, and 1% (v/v) β-mercaptoethanol. Ethanol precipitation was performed to remove any residual CsTFA. The RNA pellet was dissolved in a solution of 40 mM morpholinopropane sulfate (MOPS), 10 mM sodium acetate, 1 mM EDTA as described by (11) with the modifications 0.1% (w/v) SDS and 1% (v/v) β-mercaptoethanol (12), quantified by absorbance at 260 nm and stored at -100°C.

A DNA probe specific for RBP, designated bcRBP-700 and isolated from a bovine conceptus cDNA library, was used for northern and slot blot analysis (13). The probe DNA was

prepared and random prime labeled with α^{32}P-dCTP to a specific activity of 2.0 x 10^9 cpm/μg DNA as described by Liu et al. (13). Total RNA (10 μg) was electrophoresed in 1.5% (w/v) agarose MOPS/formaldehyde gels (11) and stained with 0.2 μg/ml ethidium bromide to confirm its integrity. Messenger RNA from thecal and granulosa cells was then transferred to a nylon membrane via capillary action. Intact RNA from luteal tissue (10 μg) was loaded onto nylon membranes using a Minifold slot blot apparatus following the procedure recommended by the manufacturer (Schleicher and Schuell, Keene, NH). RNA was cross linked to membranes by UV irradiation (0.12 Joules, UVC 1000; Hoeffer, San Francisco, CA). Membranes were prehybridized, hybridized, and washed as described previously (13). Washed, hybridized membranes were exposed to Kodak X-Omat AR film for 96 hours at -100°C. Slot blot membranes were stripped of the RBP probe by boiling in 0.1% SDS and rehybridized with a rat β-actin (specific activity 1-1.5 x 10^9 CPM/ug DNA) to correct for loading inaccuracies as described by Dore et al (12). Signal intensities from each exposure were analyzed by integration using an LKB Ultrascan Laser Scanning Densitometer (LKB Produkter AB, Bromma, Sweden) and Gelscan XL version 2.0 software package (Pharmacia LKB Biotechnology, Uppsala,

Sweden). Retinol binding protein peak areas from each luteal sample were normalized by the corresponding β-actin areas to allow relative expression levels to be obtained. Corrected values were used to calculate the mean for samples from each day of the estrous cycle.

In vitro culture of ovarian cells and explants

Luteal (day 2, n=2; days 5-15, n=5/day) and thecal tissue (n=5 ovaries) was minced to approximately 1 mm^3 and explants placed in 5 ml of leucine deficient, high glucose Dulbecco's minimum essential medium containing 50 uCi of L-[^{3}H] leucine according to the procedures described previously (13). Tissue samples were cultured for 24 hours at 37°C on a rocking platform in a gaseous atmosphere of 45% O_2, 50% N_2 and 5% CO_2. Granulosa cells (n=5 ovaries) were concentrated by centifugation, resuspended in the same medium as above and cultured for 24 hours in an humidified atmosphere of 5% CO_2 in air. Cultures were terminated by centrifugation at 12,000 x g for 15 minutes and the supernatant stored at -80°C.

Immunoprecipitation

Aliquots of dialyzed medium from tissue cultures were lyophilized and samples resuspended in water such that 200,000 cpm of nondialyzable radioactivity was present in 750 µl aliquots. Aliquots were incubated with 5 µl of anti-RBP or normal rabbit serum overnight at 4°C. The immunprecipitation was performed as described by Lifsy et al (15) and analyzed by one dimensional PAGE (16). Thecal and granulosa cell immunoprecipates from each ovary were run in adjacent lanes (3 ovaries on one gel the remaining 2 on another). Luteal tissue samples were run so that each gel contained 1 animal per treatment (except day 2 which was represented in only two gels; total 5 gels). Fluorography was performed according to the procedure of Chamberlain (17).

Immunocytochemistry

Corpus luteum sections obtained from collection of different days of the estrous cycle (day 2, n=2; days 5-15, n=5/day) were immersion fixed in Bouin's fixation fluid for 4 - 6 hours and changed into 70% ethanol twice daily until yellow color had dissipated. Tissues were dehydrated, embedded in paraffin. Retinol binding protein ICC was

performed according to Lui and Godkin (18). Briefly, tissue was sectioned at 4μm and adhered on ProbeOn slides. Anti-RBP serum , diluted (1:200) in phosphate buffered saline containing 1% normal goat serum and 1% bovine serum albumin, was applied to sections and incubated at 37°C for 2 hours. Controls were treated with RBP-adsorbed anti-RBP (1:200 dilution) and incubated under the same conditions. Binding of anti-RBP was detected according to instructions for the Histogen^{R} Peroxide Anti-Peroxidase Immunostaining kit. Visualization of the specific interaction between the secondary and primary antiserum was achieved by using 3,3'-diaminobenzidine staining for 3 minutes. The CRBP antibody was kindly provided by John C. Saari (University of Washington, Seattle, WA) and has been previously characterized (19). Cellular retinol binding protein ICC was performed as above except that biotinylated anti-rabbit immunoglobins and peroxidase-conjugated streptadvidin were used in lieu of the Histogen^{R} Peroxide anti-Peroxidase Immunostaining System. Sections were counterstained with Mayer's Hemotoxylin to visualize nuclei. The ICC procedure was performed such that 2-3 animals per treatment was represented in each analysis (except day 2 samples in which 1 animal was represented in each of the two analyses). For each replication, all slides were processed in the microprobe

slide assembly so that all tissue sections received identical treatment. Similar conditions were applied to each replication and a representative sample is shown.

Statistical Analyses

Data were analyzed using statistical analysis system (SAS Institute Inc, SAS Circle, Cary, NC). Analysis of variance was performed using the General Linear Models Procedure (PROC GLM) to detect differences in luteal RBP concentration during the estrous cycle (20). Difference among days were tested utilizing protected least significant difference.

RESULTS

Northern Blot Analysis

Northern blot analysis was performed on total RNA prepared from thecal and granulosa cells to identify RBP mRNA expression. Autoradiography revealed that the RBP cDNA probe hybridized to a single transcript of 1.4 kb from thecal cell RNA which was present but barely detectable in

the granulosa cell preparations (figure 4-1). Previous studies have clearly identified bovine and ovine RBP mRNA as a 1.4 kb transcript (12,13).

RBP mRNA Expression

Relative levels of RBP mRNA expression in luteal tissue prepared from different days of the estrous cycle were determined by quantitative slot blot analysis. Mean areas for each day were normalized to β-actin levels and plotted (figure 4-2) No statistically significant differences in relative expression levels were noted throughout the time period studied (days 2-15).

Immunolocalization of RBP and CRBP

Presence of RBP and CRBP in luteal tissue was identified by immunocytochemistry using rabbit anti-RBP and anti-CRBP, respectively. Both RBP and CRBP were localized in the large luteal cells of the bovine corpus luteum (figure 4-3). Retinol binding protein was localized in all large luteal cells; therefore, the number of cells with positive staining increased from day 2 through day 5 and remained relatively constant through day 15. Cellular

retinol binding protein was present only in select large luteal cells.

RBP synthesis in vitro

Granulosa, theca and luteal tissue were incubated for 24 hours in Dulbecco's minimum essential medium with L-[^{3}H] leucine. Immunocomplex precipitation with anti-bpRBP serum, followed by 1D-PAGE and fluorography was used to demonstrate RBP synthesis (figure 4-4). The presence of radiolabeled RBP in conditioned medium from thecal and granulosa cell culture illustrates both tissues synthesize and secrete RBP. Radiolabelled RBP was also present in conditioned media derived from CL explants at all time peroids studied. Lanes designated (a) represent precipitation with rabbit anti-RBP and (b) represent precipitation with normal rabbit serum.

DISCUSSION

Results of this study clearly demonstrate, for the first time, the presence of retinoid binding proteins RBP and CRBP in the bovine corpus luteum. Retinol binding protein and CRBP were immunolocalized to the large or steroid producing cells of the corpus luteum. Production of

these proteins may concentrate retinol for paracrine (RBP) or autocrine (CRBP) functions. Administration of retinol to in vitro cultured luteal cells or luteinizing granulosa cells stimulate progesterone production in swine (4) and cattle (5). In the rat, even mild cases of vitamin A deficiency cause decreased activity of ovarian enzymes 3β-hydroxyΔ^5-steroid dehydrogenase (21) and P450 cholesterol side chain cleavage (22) but seemed to have no effect on normal steroidogensis in the adrenal (23). Vitamin A may also be involved in formation and maintenance of the corpus luteum. A-hypervitaminoic rats showed an accelerated occlusion at the point of ovulation, increased vascularization of the CL wall and a more rapid formation of the connective tissue surrounding the CL in comparison with both control and A-deficient rats. The hyperemia associated with a mature CL occurs earlier and progresses longer in the A-hypervitaminotic animals in comparison with A-deficient animals (24).

Although the overall size of the CL and the number of large luteal cells increased from day 2 to day 15 of the estrous cycle, luteal RBP mRNA expression remained constant throughout the same time period. This discrepancy is not unique to retinol. It has also been reported for oxytocin production in the bovine CL (reviewed in 25). Luteal

oxytocin mRNA peaks 24 hours after ovulation, drops abruptly on day 5-6 and remains low throughout the second half of the cycle. However, production of oxytocin is delayed in reference with its mRNA. Oxytocin is low at ovulation, rises slowly until day 3 and then rapidly increases until day 11 when it peaks and begins a slow decline. In this study, release of newly synthesized RBP remained constant throughout the time period studied. However, immunolocalization of RBP showed a drastic increase in staining on day 5 and remained relatively constant over days 10 and 15. Immunolocalization of CRBP increased as the number of large cells increased through day 10.

Another possible explanation for the constant RBP mRNA expression is the changing functions of the follicular cells (reviewed in 26). Bovine theca cells produce RBP mRNA and are capable of synthesizing and secreting RBP when cultured as explants. Granulosa cells rarely express RBP mRNA, although some RBP is produced after 24 hours in culture. Prior to ovulation, after the LH surge (or after being removed from the follicle and cultured in vitro), granulosa cells begin to luteinize and transform into large luteal cells which produce progesterone. This could account for the lack of RBP mRNA from granulosa cells and the small amount of RBP produced by cultured, and presumably partially

luteinized, granulosa cells. Twenty four to 48 hours after ovulation the corpus luteum is comprised primarily of small luteal cells which are derived from the theca cells (26). It is plausible that the RBP mRNA present in the day 1-2 CL is derived from the theca/small luteal cells and later in the cycle from the transformed granulosa cells (large luteal cells).

The presence of retinoid binding proteins in the bovine CL is not unique. In the ovine ovary, RBP has been immunolocalized in small and large cell while CRBP has been identified in the large cell of a mature CL (27). In this same study these proteins were localized in the thecal but not in the granulosa cells of non atretic antral follicles. In the rat, CRBP staining was only seen in granulosa cells in the interior of large non atretic antral follicles and in the early CL (28).

In summary, results from this study demonstrate the RBP and the mRNA that encodes it are produced by the bovine CL and thecal layer of antral follicles. It further illustrates that CRBP is also present in the CL. This study, in combination with others, provides significant evidence to imply a relationship between retinol and ovarian steroidogensis.

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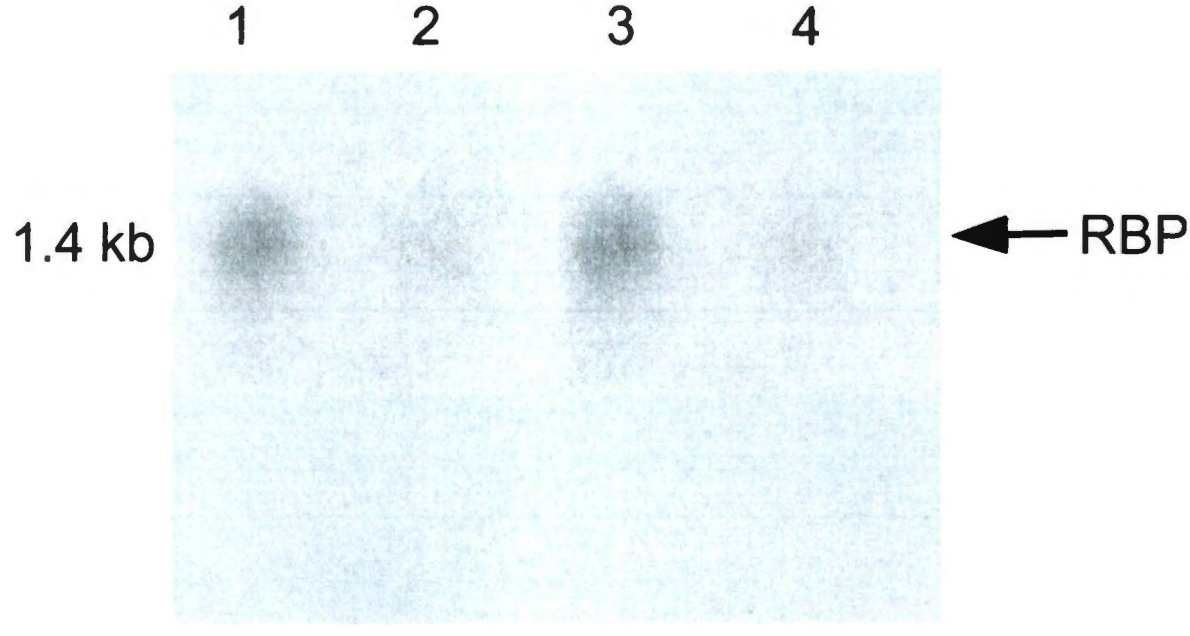
Figure 4-1. Thecal (lanes 1 and 3) and granulosa (lanes 2 and 4) cell expression of RBP mRNA (arrow). Samples contained 10 µg of RNA (see *materials and methods*) and a major transcript of 1.4 kb in length was revealed.

Figure 4-2. Relative concentrations of CL RBP mRNA expression in cyclic Holstein cows. RNA values are means and measured in arbitrary optical units (AU). Error bars represent standard error of the mean. No significant differences in relative concentrations were noted throughout the time period studied.

Figure 4-3. Immunocytochemical localization of RBP (a-d) and CRBP (e-h) in the bovine CL. Day 2 is represented in sections (a and e); day 5 is represented in (b and f); day 10 is represented in (c and g); and day 15 is represented in (d and h). Note RBP was present in all large luteal cells and CRBP was localized only in select large luteal cells. Counterstain is Mayer's Hemotoxylin. Magnification X 168.

Figure 4-4. Production of RBP by granulosa, thecal, and CL explants collected from cycling cows. Metabolically labeled

RBP (arrow) was immunoprecipitated from culture medium, separated by 1D PAGE and identified by fluorography (see *materials and methods*). Lanes 1 and 3 are from granulosa cells; lanes 2 and 4 are from thecal explants; lanes 5, 6, 7 and 8 are from CL explants collected from days 2, 5, 10 and 15, respectively. Lanes designated (a) represent precipitation with rabbit anti-RBP and (b) represent precipitation with normal rabbit serum.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1.4 kb
RBP

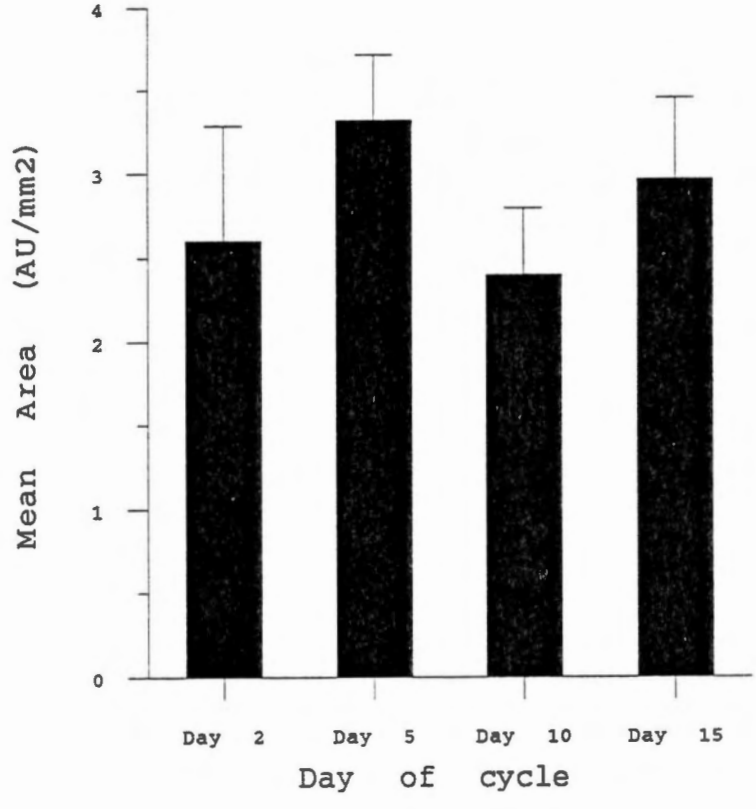
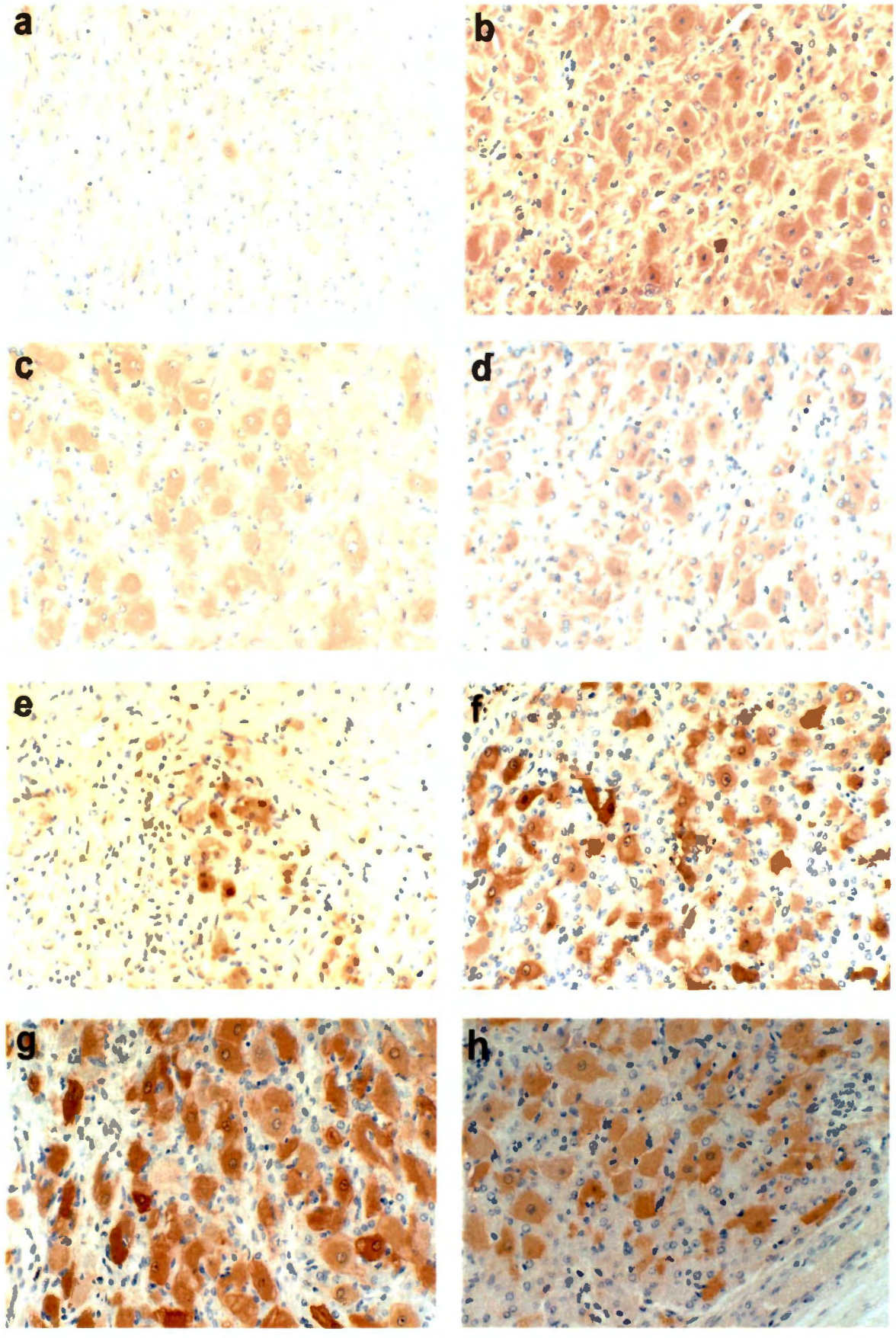


Figure 4-2. Relative concentrations of CL RBP mRNA expression in cyclic Holstein cows.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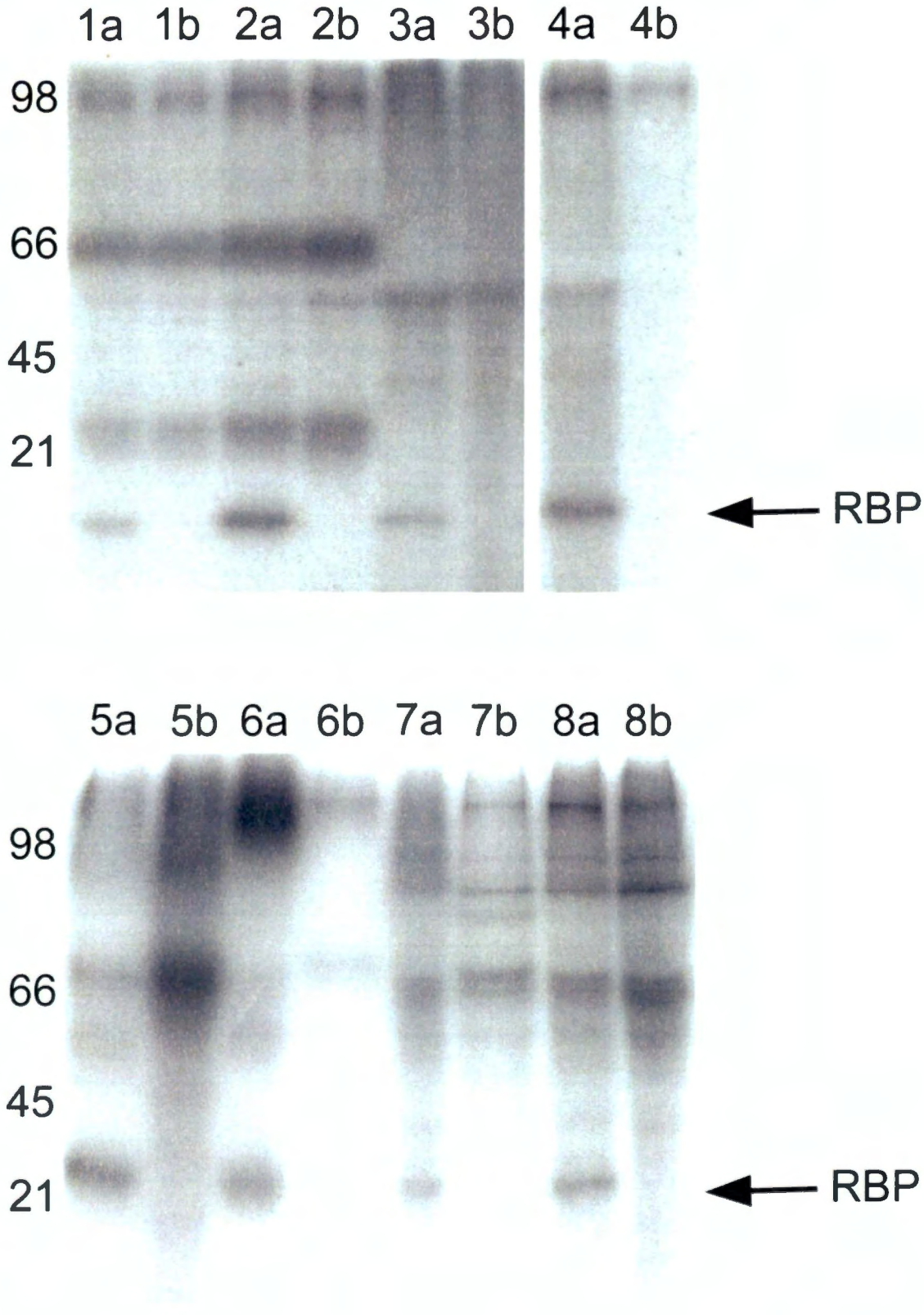
1a 1b 2a 2b 3a 3b 4a 4b
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Part 5: Retinol Administration to Superovulated Ewes Improves in Vitro Embryonic Viability.

ABSTRACT

Retinol (ROH) and its metabolites, all trans retinoic acid (RA) and 9-cis retinoid acid (CIS) are regulators of cellular growth, differentiation and development, and have been implicated in reproductive processes including folliculogenesis and embryonic survival. Three experiments were conducted to identify effects of retinoid treatment of superovulated ewes upon subsequent in vitro embryonic development. Ewes were treated with ROH, RA, CIS or vehicle (CONTROL) on the first and last day of FSH treatment. Embryos were recovered at the morula stage, cultured in vitro for 96 hours and observed for blastocyst formation. Embryos from ROH treated animals had a higher ($p<0.01$) incidence of blastocyst formation than RA, CIS, or CONTROL treated animals (72% vs 27%, 33% and 32%, respectively). In experiment 2, ewes were administered ROH or vehicle and treated as above. ROH treatment resulted in an increased percentage of embryos forming blastocysts (70% vs 22%, $p<0.05$). In experiment 3, ewes were treated with ROH or vehicle, embryos were collected at the 1- to 4-cell stage and cultured for seven days. Retinol treatment resulted in increased blastocyst formation (79% vs 5%, $p<0.05$). The majority of embryos (60% vs 6%; $p<0.01$)) from vehicle treated animals failed to develop

beyond the 8-cell stage in comparison with retinol animals. Retinol treatment of superovulated ewes increased embryonic viability and positively impacted embryonic development.

INTRODUCTION

Retinol and its cellular metabolites, all-trans retinoic acid (RA) and 9-cis retinoic acid (CIS), are collectively known as retinoids. These compounds influence embryonic morphogenesis, cell growth and differentiation in many cell types including embryonic stem cells and embryo carcinoma cells. Differentiation induced by retinoids in vitro has been shown to be accompanied by specific changes in expression of homeobox genes, growth factors and their receptors (reviewed in 1).

Systemic and intercellular transport of retinol is accomplished via a specific protein, retinol binding protein (RBP). Cellular retinol binding protein (CRBP) and cellular retinoic acid binding protein (CRABP) are involved in intracellular retinol homeostasis (reviewed in 2). CRBP has three major functions: retinol accumulation; stimulation of retinol mobilization from retinyl ester stores; and delivery of the retinol to dehydrogenase, for conversion to retinal and eventually retinoic acid which can then undergo

conversion to 9-cis retinoid acid. The cellular transport/metabolism of retinoic acid is accomplished via the small, cytoplasmic proteins cellular retinoic acid binding proteins, CRABP and CRABP-2. The actions of retinoic acid are mediated through two subgroups of nuclear receptors, retinoic acid receptors (RAR) and retinoid X receptors (RXR). Ligand/receptor complexes initiate gene activation or repression through association with specific response elements found in promoter regions of target genes (3).

Retinoids are essential to reproduction in both males and females (4). Deficiencies in vitamin A lead to decreased ovarian size, decreased ovarian steroid concentrations, abortion, and eventually reproductive senescence. Several studies indicate a positive effect of retinol supplementation when diets are adequate in vitamin A. In litter bearing species, administration of retinol or β-carotene has been reported to increase embryo survival in mice (5), rabbits (6) and swine (7-9). In cattle, retinol administration, in combination with superovulation, increased the number of transferable embryos but did not affect ovulation rate in comparison with superovulated control animals (10). Three experiments were conducted to identify the effect of retinoid treatment in combination

with superovulation and natural service upon subsequent ovine embryonic development. The retinoids utilized in this study were chosen because retinol exerts its effect via interaction between its biological active metabolites all-trans retinoic acid and 9-cis retinoic acid and nuclear receptors.

MATERIALS AND METHODS

Materials

Lutalyse ™ was purchased from The Upjohn Company (Kalamazoo, MI). Synchromate B™ was purchased from Rhone Merieux, Inc. (Athens, GA). 9-cis retinoic acid was kindly provided by Hoffmann La Roche (Nutley, NJ). Porcine FSH was purchased from Sioux Biochemical (Sioux City, IA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). Synthetic oviductal fluid (SOF) was purchased from Specialty Media, Inc. (Lavallette, NJ). Falcon organ culture dishes were purchased from Fisher Scientific (Pittsburgh, PA). All-trans retinol and all-trans retinoic acid and all other reagents were purchased from Sigma Chemical Corp. (St. Louis, MO).

Animals

Estrous cycles of sexually mature crossbred ewes were synchronized using progestin implants (Synchromate B™) combined with prostaglandin $F_{2\alpha}$ (Lutalyse™) injections and superovulation induced by multiple FSH injections. Briefly, animals were administered one implant and six days later received two Lutalyse™ injections (15 mg i.m.) twelve hours apart. Superovulation was induced using a total of 24 units of FSH administered twice daily in decreasing doses over 3 days (5,5; 4,4; 3,3 units per injection, respectively) beginning 9 to 11 days after implant administration. Retinoid treatments were administered on the first and last day of FSH injections. Implants were removed at the time of the fifth FSH injection and animals were checked for estrus 24 hours later. Ewes exhibiting behavioral estrus were handbred to intact rams every twelve hours until signs of estrus were no longer detected. All animals were maintained on high quality hay, fed ad libitum, with free choice access to a sheep and goat mineral premix that contained 2.24 million I.U. vitamin A per kilogram.

In the first experiment, performed under decreasing day length (fall), 25 ewes were randomly assigned to receive one the following treatments: 1) all-trans retinol (ROH), 500,000 I.U., n=6; 2) all-trans retinoic acid (RA), 15 mg,

n=6; 3) 9-cis retinoic acid (CIS), 15 mg, n=7; or 4) vehicle (CONTROL), n=6, which was corn oil. Animals were surgically ovahysterectomized at 144 hours post implant removal and uteri were gently flushed twice with culture medium (TCM-199) to collect morula stage embryos. Two ewes from the RA group were dropped from the study, one due to a total lack of response to the FSH treatment, the other due to overstimulation resulting in greater than 50 ovulations of which none of the ova fertilized. This left 4 animals in the RA group, 5 in both the ROH and CONTROL groups, and 7 in the CIS group.

The second experiment was a repetition of the first except that it was performed under increasing day length (winter) and only the ROH and CONTROL treatments were administered (in combination with FSH) to 24 ewes (12/treatment) not used in the previous experiment. Retinoic acid and CIS treatments were not utilized because of failure to improve embryonic viability in experiment 1. One ewe from the CONTROL group was dropped from the study for failure to respond to FSH treatment.

The third study involved two identical studies that were performed sequentially in the fall and winter. Results were not different between seasons and the data were combined. A total of 24 ewes, not used in the previous

experiments, received either ROH (n=12) or CONTROL (n=12) treatment in combination with FSH, followed by natural mating at estrus, as in experiment 2. At 84 hours post implant removal, ewes were salphincectomized and oviducts gently flushed with culture medium in order to recover 1- to 4-cell embryos.

Ovulation and Fertilization Rate

At the time of embryo recovery, ovulation rate was determined by counting corpora lutea (CL) on each ovary. Embryo/oocyte recovery rates were determined by dividing CL number by the embryo/oocyte number. Fertilization rate was determined by dividing the number of cleaved embryos by the total number of embryos/oocytes recovered from each ewe.

Embryo Grading

Embryos (morulae) collected in experiment 1 and 2 were categorized according to morphology, developmental stage and quality based on a procedure developed for bovine embryos (11). Quality grades ranged from 1-4, where 1=excellent, 2=good, 3=poor, 4=degenerate. One individual (DME) who was unaware of treatments at that time, performed all of the grading.

Embryo Culture

In the first two experiments, morula stage embryos were cultured in tissue culture medium 199 (TCM 199) with Earle's salt supplemented with 10% FBS and 1mM glutamine (12). The FBS had been twice stripped of low molecular weight molecules with charcoal and retinol concentrations were below detection levels as determined by fluorescent analysis (13). In the third experiment 1- to 4-cell embryos were cultured in synthetic oviductal fluid (SOF) supplemented with 3 mg/ml BSA and essential and non essential amino acids (14). Both media were prepared weekly, filtered through a 0.2μM filter, and allowed to equilibrate for 2 hours in a humidified atmosphere at 38.5°C containing 5% CO_2 in air.

Morula stage embryos (experiment 1 and 2) were washed a minimum of 3 times in the outer well of organ culture dishes and then transferred with a minimum amount of medium into the inner well which contained 3 ml of TCM 199. Embryos from each ewe were cultured in one dish and there were no significant differences in the average number of embryos/dish between treatments. Embryos were cultured for 96 hours and observed daily for blastocyst formation and complete hatching from the zona pellicuda. No further development was observed after 72 hours in culture and all data presented reflects that time period.

In experiment 3, embryos were treated as above except culture medium was SOF (see above). Embryos were observed every 48 hours until embryos hatched or failed to develop for two consecutive viewings (168 hours maximum).

Statistical Analyses

Data were checked for normality and analyzed using SAS (SAS Institute Inc, SAS Circle, Cary, NC). Analysis of variance was used with mixed models procedure (PROC MIXED) to detect differences in ovulation rate, embryo recovery rate, fertilization rate, embryonic quality, in vitro embryonic development to blastocyst and embryonic hatching due to retinoid treatment (15). Differences due to retinoid treatment were tested utilizing protected least significant difference.

RESULTS

Ovulation rate was not affected by retinoid treatment within any experiment or between experiments and ranged from 8-33 with an average of 19.33 corpora lutea per ewe excluding the animal that did not respond to FSH treatment and the one animal from which >50 unfertilized oocytes were recovered. Embryo/oocyte recovery rates were not different

between treatments ($p<0.50$) or experiments ($p<0.32$) and ranged from 82% to 93%. Fertilization rate was also not influenced by retinoid treatment ($p<0.12$) and ranged from 83% to 93%. No differences were observed in in vivo developmental stage at time of collection or in speed of development (progression to the next developmental stage) in vitro. Results from experiments performed during decreasing day length (fall) were not different from experiments performed during increasing day length (winter).

Retinol in combination with superovulation significantly improved embryonic viability as measured by blastocyst formation in vitro. In the first experiment embryos were collected from 21 ewes treated with ROH (n=5), RA (n=4), CIS (n=7), or CONTROL (n=5) resulting in 96, 84, 97 and 93 embryos per treatment group, respectively. Embryos from each treatment were graded immediately after collection and the score was not different between treatments (1.9 ± 0.1, 2.8 ± 0.2, 2.4 ± 0.2 and 2.1 ± 0.1 for ROH, RA, CIS and CONTROL treatment, respectively). Embryos from the ROH treated animals had a greater than two fold increase in vitro blastocyst formation in comparison with RA, CIS or CONTROL animals (72% vs 27%, 33% and 32%; $p<0.05$) (figure 5-1A). In addition, retinol treatment improved ($p<0.05$) embryonic hatching rates in vitro in

comparison with CIS and CONTROL animals but was not different than RA treated animals (73%, 38%, 36% and 55%, respectively) (figure 5-1B).

In the second experiment, treatment of donors with ROH resulted in a dramatic increase ($p<0.05$) in the percentage of embryos that formed blastocysts compared with controls (70% vs 22%; n = 243 and 218, respectively) (figure 5-1C). Retinol treatment resulted in nearly a 3 fold increase in hatching rate in comparison with vehicle treated animals (70% vs 27%, $p<0.05$) (figure 5-1D).

In the third experiment (figure 5-2), the effect of retinol treatment of the dam (24 ewes) on in vitro development of 1- to 4-cell embryos was investigated. Retinol treatment significantly ($p<0.05$) improved the number of embryos that progressed through the 8-cell in vitro block (94% vs 40%). As in the first two experiments, ROH treatment resulted in a dramatic increase ($p<0.05$) in blastocyst formation (79% vs 5%; n = 230 and 202, respectively) and blastocyst hatching (71% vs 0%, respectively).

DISCUSSION

Experiments were performed over a period of two years under conditions of both decreasing (fall) and increasing

(winter) day length and included over 70 ewes producing over 1300 embryos. Results from every experiment demonstrated that retinol treatment, in combination with superovulation, dramatically improved the in vitro developmental competence of resultant embryos. In the first experiment, the incidence of blastocyst formation and hatching of embryos from animals treated with retinol but not retinoic acid, was dramatically higher than embryos from vehicle treated animals. The failure of retinoic acid (either all trans retinoic acid or 9 cis retinoic acid) to increase embryonic development or hatching may be explained by the fact that a specific systemic transport mechanism exists for retinol but not retinoic acid. Systemic and intercellular transport of retinol is accomplished by a specific protein, retinol-binding protein (RBP) (16). RBP is produced predominantly by the liver (17), and it is synthesized in several reproductive tissues including the ovary (18), oviduct (19), endometrium (20,21) and testes (22,23). The differential results of our retinoid treatments may be analogous to studies in which retinol, but not retinoic acid, was effective in restoring reproductive functions in vitamin A deficient rats (24-27).

Previous studies by others have indicated that retinol administration prior to ovulation may positively impact

reproductive performance. In swine, a single injection of retinol palmitate, given about five days prior to estrus, increased litter size (8,9). In cattle, a single injection of retinol palmitate, administered five to seven days prior to induced estrus and in combination with FSH, resulted in an increased number of embryos evaluated to be of high quality when collected on Day 7. Significantly more blastocysts were collected from retinol treated cows but overall ovulation rates and numbers of embryos were not affected (10). We too observed no effect of retinol treatment on either ovulation rates or fertilization rates. In contrast to the study in cattle, we observed no influence of retinol treatment of ewes on the quality, judged by embryo score or quantity of morula collected. However, since we did observe an increase in embryonic viability in vitro it is possible that our embryo scoring technique was not effective in discriminating qualitative differences between embryos. Treatment did not effect the rate (speed) of in vivo development. In vitro development of embryos from control and retinol treated ewes was parallel, time wise, up until the time when controls failed to progress.

In experiments 1 and 2, embryos from vehicle treated ewes (CONTROLS) exhibited a relatively low rate of development from the morula to blastocyst stage. We suggest

this was, in part, the result of culture conditions that included: a) serum that had been twice stripped with charcoal and b) culture of embryos in a relatively large volume (3 ml) of medium. We chose to not expose embryos to retinol-containing medium in vitro because it may mask and/or confound effects of in vivo exposure of oocytes/embryos to exogenous retinol that had been administered to ewes. Hence, the serum component of the medium was twice stripped since a single charcoal treatment did not remove all detectable retinol. Along with removing the remainder of retinol from the serum, it is possible the second charcoal stripping also removed additional beneficial low molecular weight compounds (28). We chose to not culture embryos in microdrops covered with mineral oil because of the potential for the oil to take up the hydrophobic retinol from the embryos. Hence, embryos were cultured in 3 ml of medium in organ culture dishes to minimize evaporation and temperature change during handling. The relatively large volume may have diminished cooperative interactions of embryos which has been reported by some to improve development in vitro (29,30). In addition, other procedures that improve development such as feeder cells (31-33) were not used for similar reasons. We emphasize that all embryos were cultured under identical conditions

and differences in their developmental competence can only be explained by the different treatments administered to the ewes.

Administration of retinol to superovulated ewes improved subsequent embryonic development through the critical transition from maternal to embryonic genome control and development from morula to blastocyst. The switch from maternal to embryonic gene expression is characterized by loss or degradation of maternal mRNA, activation of embryonic transcriptional machinery and alteration in protein synthesis (reviewed in 34). This stage is often associated with developmental arrest or block in embryos cultured in vitro. Sheep and cattle undergo this transition at the eight to sixteen-cell stage (35,36). The majority of 1- to 4-cell embryos from vehicle treated ewes blocked at the eight-cell stage (60%), whereas only 6% of embryos from retinol treated ewes failed to make the transition. In vitro development of morula collected from ewes treated with retinol likewise exhibited greater competence to form blastocysts. The transition from morula to blastocyst is complex and involves numerous structural and functional alterations, including the processes of compaction, blastocoel formation and differentiation of trophectoderm which will contribute to the placenta and

inner cell mass which will give rise to the fetus (reviewed in 37).

The mechanism by which retinol administration contributes to critical transitions in early embryonic development is unknown but the fact that retinol was given prior to ovulation may indicate that it affects the oocyte within the follicle. Maternal factors stored in the egg influence development of the embryo before and after zygotic gene activation (38-40). Sirard and Blondin (40) demonstrated differences in developmental competence of oocytes matured in vivo or in vitro that were expressed several days later in the ability of the resultant embryos to develop beyond the 16 cell stage (41,42) and form blastocysts (40). It was suggested that the intrinsic differences in oocyte competence may result from factors affecting the oocyte during late folliculogenesis. Our results support this hypothesis and suggest that retinol may be one factor that influences oocyte competence.

Superovulation has been shown to alter ovarian function in cattle and sheep resulting in abnormal follicular steriodogenesis (43,44), premature and aberrant oocyte maturation (43,45) and anomalies within the oocyte (44). In the present study, retinol treatments were applied to superovulated animals and it is possible that the exogenous

retinol compensated for or diminished some aberrant ovarian activites resulting from superovulation.

Schweigert and Zucker (46,47) associated bovine follicular fluid retinol concentrations with follicular health. Retinol concentrations were highest in healthy follicles, lowest in atretic follicles and highly correlated with estradiol concentrations. Similarly, we have immunolocalized the binding proteins for retinol (RBP and CRBP) in the thecal cells of healthy but not atretic antral follicles in the ewe and suggested a model in which CRBP accumulates retinol from the blood plasma and RBP transports it across the basement membrane into the follicle fluid where it could influence oocyte maturation/development (18). In addition, retinoids (ROH and RA) have been shown to stimulate steroidogenesis by granulosa cells in vitro and synergistically enhance the ability of FSH to induce LH receptors and stimulate cAMP and progesterone production (48). Together, the above data suggest retinoids play a role in normal follicular development and function.

In summary, results from this study demonstrate that retinol treatment in combination with superovulation improves the developmental competence of resultant embryos. This study, in combination with others, provides evidence to indicate that retinoids may play a role in follicular

development that positively influences embryonic development but does not preclude potential effects in the oviduct and uterus. These results suggest that retinol administration has the potential to positively impact reproductive efficiency and assisted reproduction protocols in domestic animals.

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Figure 5-1. Effect of retinoid treatment of superovulated donors on embryonic development in vitro. Retinol treatment (ROH) increased embryonic development to blastocyst stage in comparison with retinoic acid (RA), 9 cis retinoic acid (CIS), and vehicle (CONTROL) treatments (A). Retinol treatment improved embryonic hatching rates when compared with CIS and CONTROL animals but was not different than RA treatment (B). In the second experiment, retinol treatment improved embryonic development to the blastocyst stage (C) and rate of embryonic hatching (D) in comparison with control treated animals. Animal numbers were ROH=5, RA=4, CIS=7, CONTROL=5, and ROH and CONTROL=12 for experiment 1 and 2, respectively (see Materials and Methods). Error bars represent standard error of the mean. Columns with different letter subscripts are significantly different ($p<0.05$).

Figure 5-2. Effect of retinol treatment of superovulated ewes on subsequent in vitro embryonic development (n=12 ewes/treatment). Columns represent the terminal stage of development. Retinol treatment significantly increased the number of embryos that progressed through the 8-cell in vitro block in comparison with control treatment. Columns within a developmental stage with * are significantly

different ($p<0.05$).

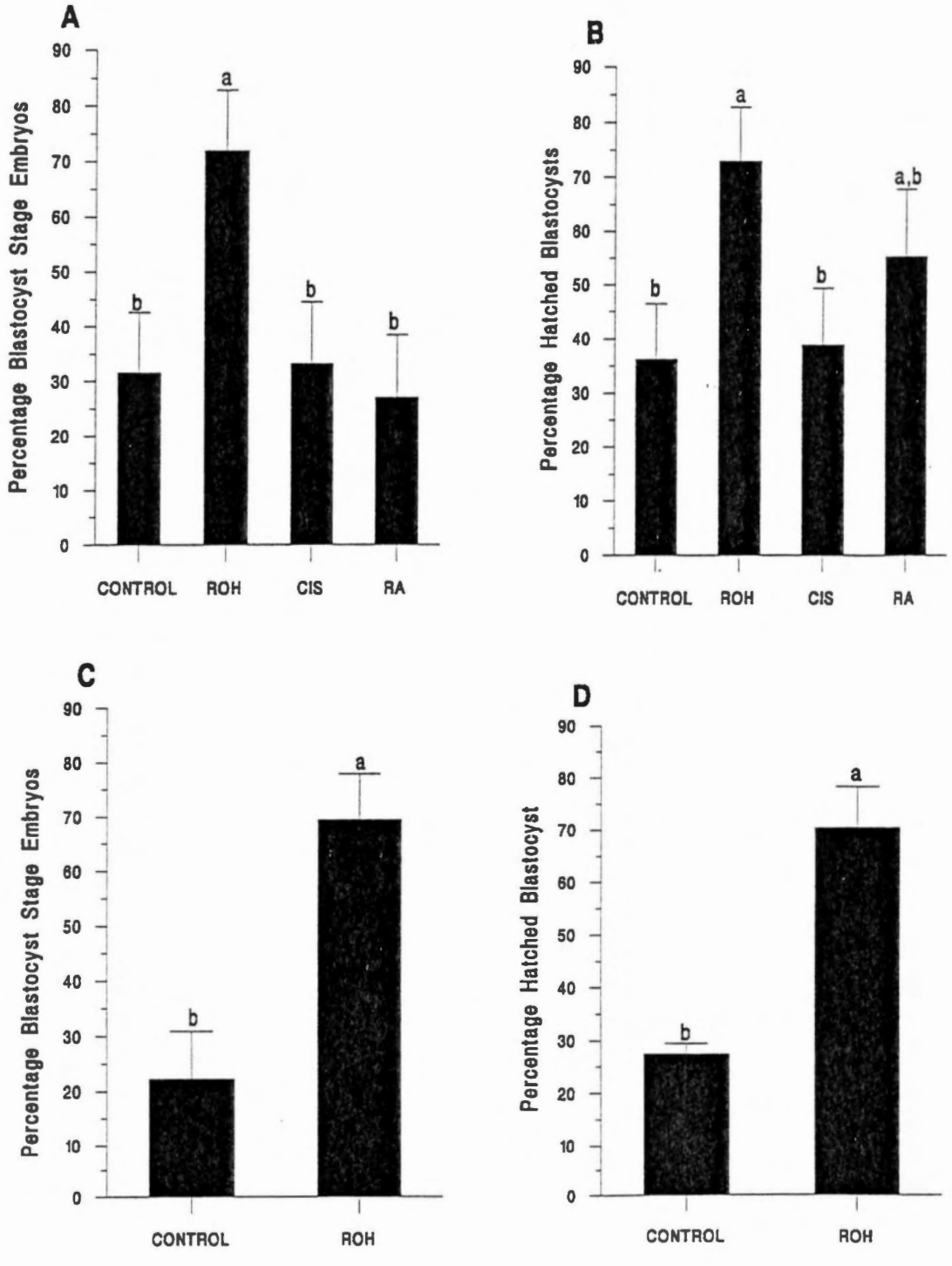

Figure 5-1. Effect of retinoid treatment of superovulated donors on embryonic development in vitro.

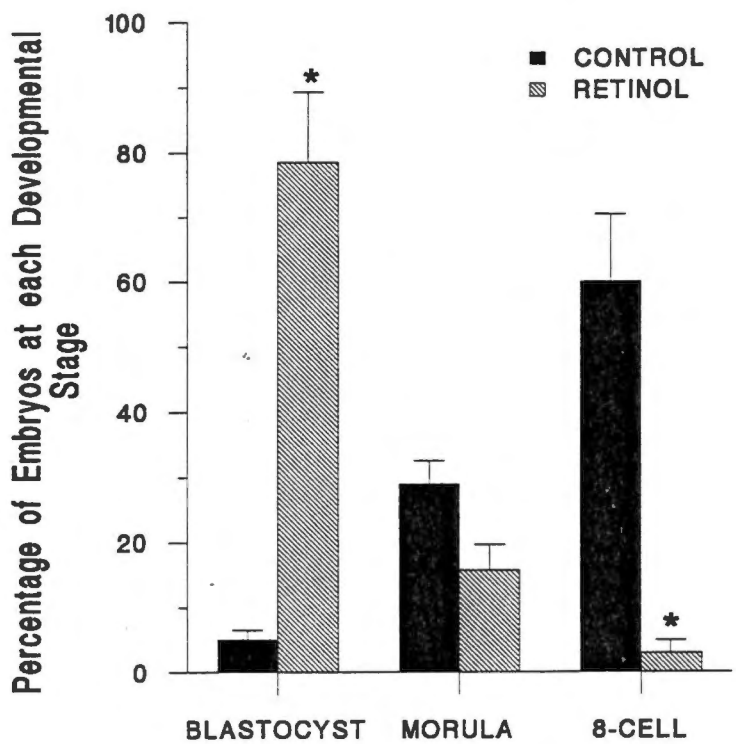

Figure 5-2. Effect of retinol treatment of superovulated ewes on subsequent in vitro embryonic development.

Part 6: Effects of retinoid supplementation on in vitro maturation, fertilization and subsequent development of bovine embryos.

ABSTRACT

The effects of all-trans retinol (100 μM and 10 μM ROH) and all-trans retinoic acid (10 μM and 1 μM RA) on the maturation, fertilization and subsequent development of cattle oocytes/embryos were evaluated in vitro. Cumulus-oocyte complexes (COC) were collected from cattle ovaries, matured for 22 hours, fertilized and cultured to blastocyst in the presence and absence of retinoids. Maturation and fertilization of oocytes in the presence of the high concentrations of retinoids (100 μM ROH or 10 μM RA) or culture of embryos in the presence of 100 μM ROH resulted in no blastocyst formation. The addition of 10 μM ROH to the maturation and fertilization media diminished ($p<0.01$) blastocyst formation in comparison with maturation and fertilization medium alone or in combination with 1 μM RA (6.4% ± 1.8% vs 22.4% ± 2.6% and 25.7% ± 2.7%, respectively). Embryos cultured in the presence of 10 μM ROH or 1 μM RA, regardless of maturation and fertilization treatment, exhibited increased ($p<0.01$) blastocyst formation in comparison with culture medium alone or in combination with 10 μM RA (18.6% ± 2.5% and 33.9% ± 3.0% vs 13.5% ± 2.2%

and 12.5% ± 2.6%). These results suggest a role for retinoids in preimplantation embryo development.

INTRODUCTION

Retinol (ROH) and its metabolite, all trans retinoic acid (RA), are regulators of cellular growth, differentiation and development and are essential to reproduction in both males and females (1). These retinoids have been implicated in reproductive processes including folliculogenesis and embryonic survival. Follicular fluid retinol concentrations and presence of retinol binding proteins in follicular cells have been associated with follicular health (2-4). Supplementation of retinol or β-carotene to animals fed a diet adequate in vitamin A, has been reported to increase embryonic survival in rats (5), rabbits (6) and swine (7-9). In cattle and sheep, administration of retinol, in combination with superovulation, increased embryonic quality as judged by embryonic score or subsequent in vitro development (10,11). Retinoids have also been associated with embryonic morphogenesis, cell growth and differentiation in many cell types including embryonic stem cells and embryo carcinoma cells (12). Differentiation induced by retinoids in vitro

has been shown to be accompanied by specific changes in expression of homeobox genes, growth factors and their receptors (12). This data suggests a connection between retinol and early reproductive events such as folliculogenesis/oocyte maturation and/or embryonic development/survival.

Systemic and intercellular transport of retinol is accomplished via a specific protein, retinol binding protein (RBP). Cellular retinol binding protein (CRBP) is responsible for intracellular retinol homeostasis and has three major functions: retinol accumulation from serum RBP, stimulation of retinol mobilization form retinyl ester stores, and delivery of the retinol to dehydrogenase for conversion to retinal and eventually retinoic acid (13). The cellular transport/metabolism of retinoic acid is accomplished via small, cytoplasmic proteins cellular retinoic acid binding proteins 1 and 2 (CRABP and CRABP-2) (14). The actions of retinoic acid are mediated through two subgroups of nuclear receptors, retinoic acid receptors (RAR) and retinoid X receptors (RXR). Homo- or hetero-dimer retinoic acid/receptor complexes cause gene repression or activation through association with specific response elements (retinoic acid response element or retinoid X response element) found in the promoter regions of target

genes (15). The objective of this study was to evaluate the effects of all-trans retinol and all-trans retinoic acid on the maturation, fertilization and subsequent development of cattle oocytes/embryos in vitro.

MATERIALS AND METHODS

Materials

Fetal bovine serum, synthetic oviduct fluid (SOF) and modified Tyrodes media (IVF-TALP, SP-TALP, and TALP-HEPES) were purchased from Specialty Media, Inc (Lavallette, NJ). Tissue culture medium 199 with Earle's salts (TCM-199), hormones (LH, FSH and estradiol 17β), antibiotics (penicillin/streptomycin and gentamicin), and other media supplements (bovine serum albumin BSA, pyruvate, penicillamine, hypotaurine, epinephrine, essential amino acids and non-essential amino acids) were purchased from Sigma Chemical Company (St. Louis, MO). All-trans retinol and all-trans retinoic acid were also purchased from Sigma Chemical Company (St. Louis, MO). Nalgene 50 mm bottletop filters (0.2μM) were purchased form Nalgene Nunc International (Rochester, NY). Nunc 4 well multidishes were purchased from Nunclon (Fisher Scientific, Pittsburgh, PA).

Falcon 150 X 25 mm and 60 X 15 mm Petri Dishes were purchased from Becton Dickinson and Co. (Bedford, MA).

Culture Media

Oocyte maturation medium (OMM) was tissue culture medium 199 (TCM-199) supplemented with 0.2 mM pyruvate, 5.0 μg/ml LH, 0.5μg/ml FSH, 1.0 μg/ml estradiol 17β and 10% fetal bovine serum (16). The fetal bovine serum had been twice stripped of low molecular weight molecules with charcoal and retinol concentrations were below detection levels as determined by fluorescent analysis (17). Modifications of TALP used for sperm cultures (SP-TALP), working with oocytes/embryos outside the incubator (TALP-HEPES) and for in vitro fertilization (IVF-TALP) are described elsewhere (18). Embryo culture medium was synthetic oviduct fluid (SOF) described by Takahashi and First (19) and modified by Carolan et al (20) containing 3 mg/ml bovine serum albumin fraction V, essential amino acids and nonessential amino acids. All media was filter sterilized through a 0.2 μM filter prior to use.

Treatments

Four retinoid treatments were utilized in this experiment: 1) 100 μM retinol (100ROH); 2) 10μM retinol

(10ROH); 3) 10μM retinoic acid (10RA); and 4) 1μM retinoic acid (1RA). Oocytes/embryos were matured and fertilized and cultured in the treatments in Table 6-1:

Oocyte Collection and in vitro Maturation

Ovaries were collected from slaughtered heifers and cows over a 4 hour period and transported to the laboratory at 30°C in phosphate buffered saline containing 100 units/ml penicillin and 0.1 mg/ml streptomycin. Immature oocytes (n~300/day) were aspirated from 2 to 6 mm follicles, placed in a 25 ml glass graduated cylinder and allowed to sediment for 20 minutes in a tissue culture incubator (95% air, 5% CO_2) and the supernatant removed. The COC suspension was divided into 150 X 25 mm petri dishes and searched for COC under a dissecting scope. Only COC with multilayered compact cumulus cells and evenly granulated cytoplasm (21) were selected for in vitro fertilization. COC were then washed a minimum of 4 times in TALP-HEPES and once in OMM. Fifty COC were cultured in each well of a 4 well dish containing 0.5 ml of OMM with or without retinoid treatment for 22 hours at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

In vitro Fertilization

Sperm were prepared according to the method described by Parrish et al. (22). Briefly, semen straws from the same five bulls were thawed in a 35°C water bath and combined. One half ml of the semen solution was layered under 1 ml of SP-TALP in 15 ml conical tubes, tilted at a 45° angle and placed into a 38.5°C incubator. After 1 hour the top 0.85 ml of medium was removed, pooled and centrifuged at 200 X g for 10 minutes. The sperm pellet was brought to a volume of 1 ml with IVF-TALP, counted on a hemocytometer and diluted with IVF-TALP to 25 X 10^6 sperm/ml. Twenty five ul of the sperm preparation was added to each well containing IVF-TALP resulting in approximately 1 X 10^6 sperm/ml. Matured COC were washed 3 times in TALP-HEPES and once with fertilization medium (IVF-TALP). Thirty COC were cultured in each well of a 4 well dish containing 0.6 ml of IVF-TALP with and without retinoid treatment and 1 X 10^6 sperm/ml. Gametes were co-incubated for 8-10 hours in 5% CO_2 in humidified air at 38.5°C.

Embryo Culture

Eight to 10 hours after insemination, presumptive zygotes were denuded of cumulus cells by vortexing for 4

minutes in 200 µl TALP-HEPES and subsequently washed twice with TALP-HEPES and twice with SOF. Embryos/oocytes were then transferred into 4 well dishes at a density of 1 embryo per 10 µl SOF with and without retinoid treatment in groups of 40-60 embryos and cultured at 38.5°C in humidified air containing 5% CO_2. Culture medium was changed every two days. Cleavage and blastocyst rates were assessed on day 2 and days 7 and 8 (insemination = day 0) and expressed as a percentage. Blastocyst cell numbers were determined in representative samples (10-15 per treatment) of day 8 blastocysts after fixation and staining with Hoechst dye #33342 as described in Pursel et al (23).

Statistical Analysis

Data were analyzed using SAS (SAS Institute Inc, SAS Circle, Cary, NC). Analysis of variance was used with mixed models procedure to detect differences in blastocyst cell number due to retinoid treatment. Differences due to retinoid treatment were tested utilizing protected least significant difference (24). Cleavage rate and blastocyst yield were compared by Chi Square (X^2) analysis.

RESULTS

The results presented in table 6-2A are cleavage rate and rate of blastocyst formation based on retinoid treatment during maturation and fertilization of oocytes, regardless of embryo culture treatment. Oocytes matured and fertilized in the presence of high concentrations of retinoids (100ROH and 10RA) underwent no embryonic cleavage and therefore no blastocyst formation. The addition of 10 μM retinol to maturation and fertilization media significantly decreased ($p<0.01$) embryonic cleavage rate and dramatically reduced ($p<0.01$) blastocyst formation in comparison with maturation and fertilization media with or without 1 μM retinoic acid (10ROH = 58.9% ± 0.1% and 6.4% ± 1.8% vs 1RA = 62.7% ± 2.4% and 25.8% ± 2.7% or OMM = 61.0% ± 1.5% and 22.4% ± 2.6%).

The results presented in table 6-2B are percent blastocyst formation in embryo culture medium (SOF) with or without retinoid treatment, irrespective of retinoid treatment during maturation and fertilization. Addition of the low concentration of retinoids (10ROH and 1RA) resulted in an increase ($p<0.01$) in blastocyst formation when compared with SOF alone (18.6% ± 2.5% and 33.9% ± 3.0% vs 13.5% ± 2.2%, respectively). Blastocyst formation of

embryos cultured in the presence of high retinol concentrations was either not different (10RA; $p>0.50$) or significantly reduced (100ROH; $p<0.01$) in comparison with SOF alone (12.5% ± 2.6% and 0.0% ± 0.0% vs 13.5% ± 2.2%, respectively).

Table 6-3 represents percent blastocyst formation and blastocyst cell number of oocytes matured and fertilized/embryos cultured in each of the retinoid treatment combinations. In treatment combinations with blastocyst formation, no difference ($p>0.20$) was observed in blastocyst cell number. Once again oocytes matured and fertilized in the presence of high concentrations of retinoids or cultured in the presence of high retinol concentrations (100ROH/100ROH, 100ROH/SOF, OMM/100ROH, 10RA/10RA, and 10RA/SOF) underwent no embryonic cleavage and therefore no blastocyst formation. Blastocyst formation of embryos cultured in the presence of the high concentration of retinoic acid or resulting from oocytes matured and fertilized in the presence of the low concentration of retinoic acid was not different ($p<0.50$) than control embryos (OMM/10RA = 15.2% ± 2.2% and 1RA/SOF = 17.7% ± 3.3% vs OMM/SOF = 12.5% ± 1.8%). When oocytes were matured and fertilized in the presence of the low concentration of retinol, regardless of embryonic culture media, the

subsequent embryos exhibited a lower rate of blastocyst formation in comparison with embryos cultured in SOF from oocytes matured and fertilized in the absence of retinoid treatment (10ROH/10ROH = 4.7% ± 2.0% and 10ROH/SOF = 7.9% ± 2.5% vs OMM/SOF = 12.5% ± 1.8%). Embryos cultured in 10 μM retinol and 1 μM retinoic acid, unless oocytes were matured and fertilized in retinol, had a greater than 2 fold increase in blastocyst formation when compared with control embryos (OMM/1RA = 33.0% ± 4.2%, OMM/10ROH = 29.6% ± 3.9% and 1RA/1RA = 34.7% ± 4.3% vs OMM/SOF = 12.5% ± 1.8%).

DISCUSSION

Results from this study clearly illustrate that addition of the retinoids, all-trans retinol and all-trans retinoic acid, to embryonic culture medium results in a dramatic increase in embryonic viability as measured by blastocyst formation. The addition of high concentrations of these retinoids (10 μM RA and 100 μM ROH) resulted in either no improvement in blastocyst formation over culture medium alone or in no blastocyst formation. The inclusion of vitamins in embryonic culture media is not a new idea. Addition of water soluble vitamins have been reported to

facilitate blastocyst expansion and/or hatching in rabbit (25-27) and hamster embryos (28,29). An equally impressive number of studies have reported no effect of these vitamins in cattle (19,30), mice (31,32), and hamster (33). In the case of sheep, addition of minimum essential medium vitamins to serum-free culture medium resulted in no improvement in blastocyst formation and no effect on embryonic morphology but did induce a significant increase in blastocyst metabolism as measured by increased glucose uptake and increased lactate production per cell (34). In all of these studies, the vitamins used were water soluble vitamins which do not contain vitamin A (retinol).

Retinol and retinoic acid influence embryonic morphogenesis, cell growth and differentiation in many cell types including embryonic stem cells and embryo carcinoma cells. Differentiation induced by retinoids in vitro has been shown to be accompanied by specific changes in expression of homeobox genes, growth factors and their receptors (12). Preimplantation embryos express a myriad of growth factors and receptors (reviewed in 35) that have been implicated in the autocrine/paracrine effects seen during culture of embryos in groups and in microdrops (34,36). The increase in embryonic development caused by addition of retinol/retinoic acid to embryonic culture medium may be due

to an up regulation of growth factor synthesis or receptor expression.

In vivo, administration of retinol prior to ovulation has been linked to improved viability in subsequent embryo production. In swine, a single injection of retinol palmitate, given about five days prior to estrus, increased litter size (8,9). In cattle, a single injection of retinol palmitate, administered five to seven days prior to induced estrus and in combination with FSH, resulted in an increased number of embryos evaluated to be of high quality when collected on day 7. Significantly more blastocysts were collected from retinol treated cows but overall ovulation rates and numbers of embryos were not affected (10). In sheep, two retinol injections given on the first and last day of FSH treatment, resulted in an increase in embryonic viability as measured by in vitro blastocyst formation. No effect was seen on either ovulation or fertilization rates (11). These results indicate that retinol administration prior to ovulation, improves developmental capacity of the resultant embryos.

A series of studies support the idea that retinol has a role in folliculogenesis and/or oocyte competence. Schweigert and Zucker (2,3) associated bovine follicular fluid retinol concentration with follicular health. Retinol

concentrations were highest in healthy follicles, lowest in atretic follicles and highly correlated with estradiol concentrations. In addition, we have recently immunolocalized the binding proteins for retinol (RBP and CRBP) in the thecal cells of healthy but not atretic follicles in the ewe and suggested a model in which CRBP accumulates retinol from the blood plasma and RBP transports it across the basement membrane into the follicular fluid (4). Retinol influences follicular steriodogenesis. Administration of retinoids to in vitro cultured granulosa cells stimulated steriodogenesis and synergistically enhanced the ability of FSH to induce LH receptors and stimulate cAMP and progesterone production (37). In the rat, even a mild case of vitamin A deficiency causes a decrease in activity of the ovarian enzymes 3β-hydroxyΔ^5-steroid dehydrogenase and cholesterol side chain cleavage and therefore decreases steroid output (38,39).

The addition of retinoids to oocyte maturation medium was not beneficial to subsequent embryo cleavage and development and at the higher doses appeared to be cytotoxic. A lower dose (10 μM) of ROH during maturation decreased both embryonic cleavage and blastocyst formation. Retinoic acid has at least 10 times the biological activity of ROH, but is ineffective in replacing retinol in systems

that require facilitated transport, a process served by RBP (12). It is possible that oocyte maturation was more sensitive to ROH than RA because the former was delivered to the oocyte from cumulus cells by RBP while the latter was not.

The relatively low rate of development to blastocyst observed in control treatments (no retinoids) may have resulted from culture conditions that included: a) serum that had been twice stripped with charcoal and b) culture under relatively high (around 20%) oxygen conditions. The serum component of the oocyte maturation medium was twice stripped because a single charcoal treatment was insufficient to remove all detectable retinol. Along with removing the remainder of retinol from the serum, it is possible the second charcoal stripping also removed additional beneficial low molecular weight compounds (40). Removal of all detectable retinol was necessary to determine retinol's effect, if any, on oocyte maturation. We chose to use an atmosphere of 5% CO_2 in air in order to take advantage of retinol's antioxidant qualities (41). Spermatozoa produce both superoxide anion and hydrogen peroxide free radicals during normal motility (42). The production of these free radicals has been suggested to play a role in mediating sperm-zona interactions at fertilization

(42,43). However, oxygen free radicals have been implicated in the loss of motility of sperm cells (44-46) and in the impaired development of preimplantation embryos in vitro (47-49). Addition of free radical scavengers such as superoxide dismutase, catalyse, and glutathione have resulted in an increase in both blastocyst formation and cell number (48,50,51). These studies illustrate oxygen free radical formation reduces embryonic development. In addition, other procedures that improve development such as co-culture with somatic cells (52-54), the use of conditioned medium (55-57), the addition of growth factors to medium (35) and supplementation of medium with serum (58,59) were not utilized. We emphasize that all embryos were cultured under identical conditions and differences in their developmental competence can only be explained by the addition of retinoids.

In summary, results from this study demonstrate that addition of lower concentrations of the retinoids, all-trans retinol and all-trans retinoic acid to embryonic culture medium improves in vitro embryonic development to the blastocyst stage. This study in combination with others provides evidence that retinol positively effect embryonic development/survival and suggests that retinol has the potential to positively impact reproductive efficiency and

assisted reproduction protocols in domestic animals. Further study is needed to determine if these retinoids have any effect on oocyte competence.

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Table 6-1. Maturation/fertilization and embryonic culture treatments. Four retinoid treatments were utilized in this experiment: 1) 100 μM retinol (100ROH); 2) 10μM retinol (10ROH); 3) 10μM Retinoic acid (10RA); and 4) 1μM retinoic acid (1RA). Culture medium used: oocyte maturation medium (OMM which consists of TCM-199 with supplements) and embryo culture medium (SOF which consists of synthetic oviductal fluid with supplements). See *materials and methods* for recipes.

Table 6-2. Effect of retinoid supplementation during oocyte maturation/embryonic culture. (A) Effect of retinoid supplementation during oocyte maturation on subsequent embryonic cleavage and development. OMM = oocyte maturation medium; 10ROH and 100ROH = 10 μM retinol and 100 μM retinol; 1RA and 10RA = 1μM retinoic acid and 10 μM retinoic acid. (B) Effect of retinoid supplementation during embryo culture irrespective of oocyte maturation treatment. SOF = synthetic oviductal fluid; 10ROH and 100ROH = 10 μM retinol and 100 μM retinol; 1RA and 10RA = 1μM retinoic acid and 10 μM retinoic acid. ± represents standard error of the mean. Columns with different letter subscripts are significantly different ($p<0.01$).

Table 6-1. Maturation/fertilization and embryonic culture treatments.

	Oocyte Maturation and Fertilization Treatment	Embryo Culture Treatment
1.	100 µM ROH	100 µM ROH
	100 µM ROH	SOF
	OMM	100 µM ROH
	OMM	SOF
2.	10 µM ROH	10µM ROH
	10 µM ROH	SOF
	OMM	10 µM ROH
	OMM	SOF
3.	10 µM RA	10 µM RA
	10 µM RA	SOF
	OMM	10 µM RA
	OMM	SOF
4.	1 µM RA	1 µM RA
	1 µM RA	SOF
	OMM	1 µM RA
	OMM	SOF

Table 6-2. Effect of retinoid supplementation during oocyte maturation/embryo culture.

	Oocytes per Treatment	Cleavage Rate (%)	Development to Blastocyst(%)
A. Maturation & Fertilization Treatment			
OMM	409	61.01 ± 1.50[a]	22.40± 2.56[a]
100ROH	100	0.00 ± 0.00[c]	0.00 ± 0.00[c]
10ROH	196	58.88 ± 0.06[b]	6.36 ± 1.85[b]
10RA	120	0.00 ± 0.00[c]	0.00 ± 0.00[c]
1RA	153	62.68 ± 2.38[a]	25.68 ± 2.72[a]
B. Embryo Culture Treatment			
SOF	445	-----	13.52 ± 2.18[c]
100ROH	100	-----	0.00 ± 0.00[d]
10ROH	197	-----	18.60 ± 2.50[b]
10RA	120	-----	12.50 ± 2.58[c]
1RA	164	-----	33.87 ± 3.00[a]

Table 6-3. Effect of retinoid treatment combinations on subsequent embryonic development.

Treatment Combination	Oocytes per Treatment	Development to Blastocyst(%)	Blastocyst Cell Number
OMM/SOF	523	12.50 ± 1.85[b]	96.75 ± 2.33
100ROH/100ROH	50	0.00 ± 0.00[d]	-----
100ROH/SOF	50	0.00 ± 0.00[d]	-----
OMM/100ROH	50	0.00 ± 0.00[d]	-----
10ROH/10ROH	208	4.67 ± 2.04[c]	90.80 ± 6.35
10ROH/SOF	208	7.96 ± 2.54[c]	106.67 ± 3.67
OMM/10ROH	208	29.63 ± 3.93[a]	89.55 ± 4.73
10RA/10RA	60	0.00 ± 0.00[d]	-----
10RA/SOF	60	0.00 ± 0.00[d]	-----
OMM/10RA	60	15.15 ± 6.24[b]	89.78 ± 4.25
1RA/1RA	205	34.71 ± 4.33[a]	104.40 ± 3.67
1RA/SOF	205	17.65 ± 3.27[b]	106.40 ± 3.67
OMM/1RA	208	33.07 ± 4.18[a]	102.67 ± 3.67

VITA

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