12-2003

The Effects of Retinol during In Vivo and In Vitro Oocyte Maturation and Embryonic Development.

Tracy Elizabeth Livingston
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Tracy Elizabeth Livingston entitled "The Effects of Retinol during In Vivo and In Vitro 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:

Judy Grizzle, Mary Ann Handel, Patricia Tithof, J. Lannett Edwards

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

To the Graduate Council:

I am submitting herewith a dissertation written by Tracy Elizabeth Livingston entitled "The Effects of Retinol during *In Vivo* and *In Vitro* 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:

Judy Grizzle

Mary Ann Handel

Patricia Tithof

J. Lannett Edwards

Acceptance for the Council:

Anne Mayhew
Vice Provost and
Dean of Graduate Studies

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

THE EFFECTS OF RETINOL DURING *IN VIVO* AND *IN VITRO* OOCYTE MATURATION AND EMBRYONIC DEVELOPMENT

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Tracy Elizabeth Livingston
December 2003

DEDICATION

This dissertation is dedicated to my husband, Bob Fultz, who believed in me, encouraged me, and supported my work and decisions. For my son, Ian Fultz, you alone have brought so much joy to my life. To my parents, George and Nancy Livingston, thank you for believing in me and helping me reach my goals.

ACKNOWLEDGMENTS

First, I would like to sincerely thank my advisor, Dr. Jim Godkin, for his guidance, support, and counsel during these four years. He is not only a remarkable scientist, but also a very pleasant and sincere person. My time as a graduate student has been filled with some personal hardships, which I could not have endured without his encouragement and understanding.

I would like to thank my committee members, Dr. Lannett Edwards, Dr. Judy Grizzle, Dr. Patricia Tithof, and Dr. Mary Ann Handel for their critical evaluation of my work. I would like to thank Dr. Edwards for providing me with valuable laboratory skills, and helping expand my knowledge of reproduction and embryo development. Dr. Grizzle and Dr. Tithof, thank you for your advice, support, and encouragement. I also appreciate the effort of Dr. Handel in the preparation of a manuscript as well as her timely input and guidance.

Mary Roberts deserves special recognition because without her help many of my experiments would not have worked or been conducted properly. She is most affectionately known as "Lab Mom", which is most appropriate. Mary, thank you so much for all of your "chemistry" and "dilution" assistance.

Several graduate students, postdocs, undergraduates, and laboratory technicians have also provided invaluable help and guidance. To mention only a few: Dr. Dawn Eberhardt, Kristin Asbury, Scott MacKenzie , Dr. Magdalena Rambeaud, Dr. Fernando Scenna, Dr. Mitch Hockett, Doug Luther, Janelle Lawrence, Becca Payton, Sara Youngerman, Tommy Jordan, Phil Snow, Jennifer Miller, Carmen Dorado, and TJ Wilson.

Last but not least, I would like to thank my best friend and loving husband, Bob Fultz. Without his support and encouragement my work would not have been completed. My parents, George and Nancy Livingston, as well as my in-laws, Bob and Pam Fultz, deserve recognition for their love and support. To my son Ian: I love you.

ABSTRACT

Previous studies demonstrated that retinol administration to ewes, followed by natural service, resulted in embryos with improved competence to develop *in vitro*. *In vivo* studies with sheep and *in vitro* experiments with bovine embryos, were designed to evaluate the effects of retinol and to understand its mechanism(s) of action.

The primary objective of the first experiment was to analyze ovine oocyte metabolism, and to assess the effects of retinol on this process. Sheep oocytes were matured *in vitro* over a 24 hour period in the presence of different radiolabeled substrates. Results revealed that oxidative metabolism measured by glutamine showed no significant differences over all time periods. Pyruvate oxidation was highest early in maturation and then decreased. Glycolysis was highest at the middle time period. Differences in metabolism between oocytes from retinol-treated ewes and those from control ewes were not detected.

Next, varying concentrations of retinol were added either during *in vitro* maturation (IVM) or *in vitro* culture (IVC) of bovine oocyte and embryos, respectively. Our results demonstrated that 5μM retinol supplementation during IVM tended to improve embryonic development measured by the rate of blastocyst development. This concentration proved even more beneficial if the control blastocyst rate was below 20%. Furthermore, 10μM retinol appeared detrimental during IVC but not during IVM.

In the third experiment, we evaluated the effects of 5μM retinol and 100μM cysteamine on bovine oocyte glutathione content. In addition, we investigated the combinatorial effects of retinol and cysteamine on *in vitro* bovine embryonic

development. We did not observe an increase in glutathione levels in bovine oocytes treated with retinol. However, in the presence of cysteamine bovine oocytes exhibited an increase in glutathione content. Retinol and cysteamine treatment during IVM and IVC increased bovine blastocyst development, which may indicate that retinol is increasing the utilization or uptake of cysteine from the medium.

Next, we evaluated the role of exogenous retinol supplementation to superovulated ewes on the glutathione content of mature oocytes collected from the oviducts. Our results did not reveal differences in glutathione content of oocytes from retinol-treated ewes versus those from control ewes. Antioxidant transcripts encoding for Mn-SOD, Cu-Zn SOD, GS, and GSTp, were detected in ovine oocytes matured *in vivo*. However, retinol did not appear to impact the expression levels of these transcripts.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 6: Summary

CHAPTER 1

Introduction and Literature Review

INTRODUCTION

Retinoids are a group of small organic molecules that play important biological roles in many physiological processes including morphogenesis, cellular growth, and differentiation (Gudas et al., 1994; Sporn and Roberts, 1994). The most common retinoids are: all–*trans* retinol, all-*trans*, 9-*cis*- and 13-*cis*-retinoic acid, and retinal. The two main dietary sources of vitamin A (retinol) include β-carotene found in plants and retinyl esters found in animal products. Intestinal enzymes convert the β-carotene and retinyl esters to retinal and retinol, respectively. Retinol interacts with long-chain fatty acids to form retinyl esters that are packaged into chylomicrons and stored in the liver. Retinol is complexed to retinol binding protein (RBP) in the liver (Blomhoff et al., 1990; Gudas et al., 1994; Wolf, 1996). RBP and intracellular retinoid binding proteins help regulate vitamin A homeostasis by sequestering and stabilizing retinol; thereby, allowing only certain enzymes access to the molecule (Mangelsdorf et al., 1994; Ghyselinck et al., 1999; review Noy, 2000). Several alcohol or retinol dehydrogenases catalyze the conversion of retinol to retinal. Next, retinal can be converted to retinoic acid by retinal dehydrogenases (Ong et al., 1994; Napoli, 1999; review Noy, 2000). Retinoic acid is considered the most biologically active, natural retinoid, and it impacts transcription of a number of genes by binding to and activating two different types of nuclear retinoic acid receptors (Figure 1-1).

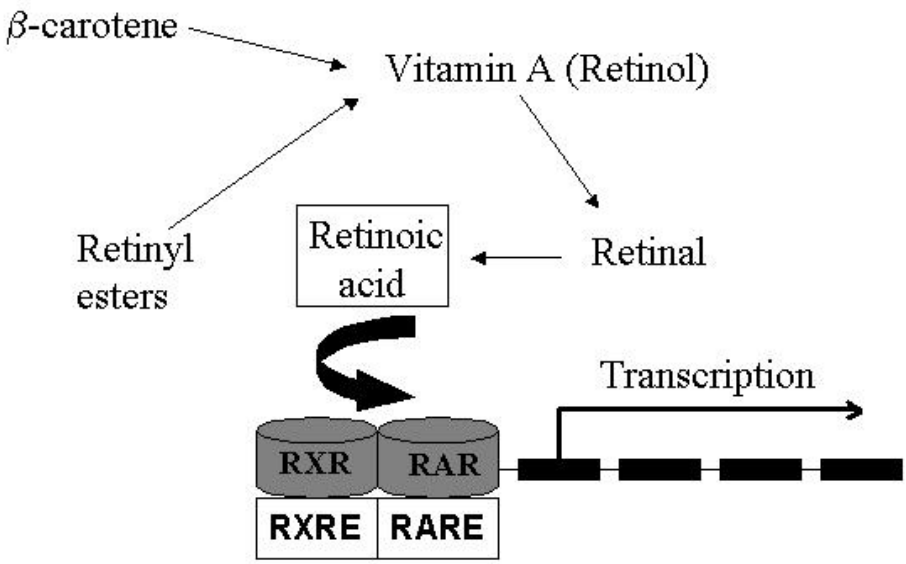

Figure 1-1. Diagram of retinoid metabolism and the mechanism of action of retinoic acid.

Due to an increase in the manipulation of preimplantation embryos both in agricultural and human-related fields, it is of great interest to determine the factors that influence an embryo's viability and developmental competence. Previous studies from our lab and others (Besenfelder et al., 1996; Da Silveira et al., 1998; Eberhardt et al., 1999b; Chew et al., 2001; Livingston et al., 2002) concluded that vitamin A plays a vital role in the reproductive cycles of many different animal species, as well as influencing proper embryo development. Thus, it was the goal of this study to help elucidate the mechanisms of action of retinol on oocyte and early embryonic development. Specifically, the objectives were:

1.) To analyze the metabolism of sheep oocytes throughout their maturation period and to determine if retinol affects this process.
2.) To assess the *in vitro* effects of retinol on bovine embryo development.
3.) To identify mechanisms by which retinol is positively impacting oocyte maturation and early embryonic development.

LITERATURE REVIEW

Vitamin A and Reproduction

The importance of vitamin A in many biological processes has been well characterized since the late 1800's, but observations on the requirement for vitamin A can be traced to early Egyptian times (1500 B.C) in which historians have found references to night blindness being treated with "ox liver" (review Napoli, 1996). In 1907 E.V. McCollum began his research studying the chemical composition of animal feed. He

observed that cows fed a vitamin A deficient diet became blind and gave birth to dead calves (McCollum, 1964). Later, in 1957, Moore reported that vitamin A deficiency led to faulty dark adaptation, decreased growth and weight during development and mucous membrane injuries.

Vitamin A deficiencies have been linked to a variety of reproductive disorders in different species such as decreased size of ovaries and testes in farm animals (Ganguly, 1989). In birds, embryos from hens deprived of retinol die after 48 hours of incubation (Thompson et al., 1969). A recent study in rats demonstrated an association between vitamin A deficient diets during pregnancy and abnormal placental apoptosis (Antipatis et al., 2002). Vitamin A deficiency also led to the discovery that vitamin A plays an important role in the biosynthesis of steroid hormones (Juneja et al., 1966), and cholesterol side-chain cleavage enzyme activity in the ovaries and testes of rats (Jayaram et al., 1973).

Several studies have concluded that vitamin A supplementation of animal diets increases reproductive success. For example, prevention of fetal resorption occurred if retinol was administered to vitamin A-deficient rats prior to Day 10 of gestation (Wellik and DeLuca, 1995). Rabbits treated with β-carotene during superovulation, showed an increase in the number of live births compared to controls (Besenfelder et al., 1993). In sows fed diets supplemented with adequate vitamin A, litter size in gilts was increased by injection of either β-carotene or vitamin A (Coffey and Britt, 1993).

Injections of vitamin A prior to ovulation increased the number of embryos from gilts fed high-energy diets (Whaley et al., 1997). In addition, retinol injections to gilts fed a high-energy diet increased the follicular fluid concentration of progesterone and

insulin-like growth factor (IGF-1) (Whaley et al., 2000). In cattle, retinol injection in combination with FSH-induced superovulation has been reported to improve embryo quality, determined by visual assessment (Shaw et al., 1995), and improve cattle oocyte recovery by ovum pick-up (OPU) (Hidalgo et al., 2002). Increased ovulation rates and increased recovery of embryos were also observed in retinol-treated superovulated mice (Elmarimi et al., 1990).

Retinoic acid (RA), the most bioactive metabolite of retinol is essential for normal embryonic and fetal development (Marshall et al., 1996; Ward and Morriss-Kay, 1997; Maden, 2000). One study reports that certain concentrations of retinoic acid influences the ability of follicle stimulating hormone (FSH) to induce luteinizing hormone receptors in rat granulosa cells (Bagavandoss and Midgley, 1988), and later studies by Minegishi and co-workers observed a RA-induced increase in FSH receptors (Minegishi et al., 2000). Although excessive amounts of retinoic acid provided to embryos *in vitro* is detrimental or even fatal (Ward and Morriss-Kay, 1997; Huang et al., 2001; Huang et al., 2003), recent evidence suggests that 9-*cis*-retinoic acid may improve early embryonic development and quality if provided to meiotically inhibited bovine oocytes (Duque et al., 2002). Furthermore, a cumulative effect of retinol treatment to heifers and *in vitro* retinoic acid treatment of embryos tended to increase blastocyst development (Hidalgo et al., 2002).

Based on previous data collected in our lab, morula stage embryos collected from sheep treated with all-*trans*-retinol prior to ovulation, and then cultured *in vitro* had a higher blastocyst rate (72%) compared to embryos from control sheep (32%). In a similar experiment the oviducts of ewes were flushed, 1-4 cell stage embryos were

collected and cultured *in vitro*. Again, blastocyst development was significantly higher in retinol treated ewes (70%) compared to control (5%). Another intriguing observation was that most embryos from control but not retinol treated ewes, blocked at the eight-cell stage *in vitro* (Eberhardt et al., 1999b). These data suggest that retinol may assist zygotic gene activation in both sheep and cattle. Without proper signals a sheep or cow embryo will block at the 8-16 cell stage *in vitro*, which is when the maternal genome is turned off and the embryonic genome is activated (Telford et al., 1990). Expanding on these results, further experiments were conducted *in vitro* to assess whether similar conclusions could be made about the beneficial effects of retinol.

Oocytes collected from abbatoir cattle ovaries were matured, fertilized, and cultured *in vitro*. Addition of 5μM retinol to the culture medium significantly improved embryo development to the blastocyst stage compared to controls (29% vs. 12%). These particular experiments were performed in a 20% oxygen atmosphere (Livingston et al., 2002). Later studies showed similar results with 5μM retinol addition to the oocyte maturation medium, and subsequent development to the blastocyst stage. This experiment was performed in a low oxygen atmosphere (7%). Interestingly, if the control blastocyst rate was below 20%, possibly an indication of embryonic stress, the effect of retinol treatment was most dramatic (Livingston et al., 2002).

Vitamin A Chemistry

Retinoids are a group of small, lipophilic molecules that play an essential role in many physiological processes such as vision, skin, bone, and nervous system development, epithelial differentiation, reproduction, and immune system function

(review Napoli, 1999). According to Sporn and Roberts (1994), retinoids are "substances that can elicit specific biologic responses by binding to and activating a specific receptor or set of receptors, with the program for the biologic response of the target cell residing in the retinoid receptor rather than in the retinoid ligand itself." Common, naturally occurring, retinoids include vitamin A or retinol (ROH), retinal and retinoic acid (RA). Retinal is one of several unique retinoids required in visual tissues (Saari, 1994). The two most abundant forms of RA include all-*trans*-retinoic acid and 9-*cis*-retinoic acid.

The most common provitamin A molecules, and the two major dietary sources of retinoids are retinyl esters, primarily found in animal fat, and β-carotene found in plants. Retinyl esters are converted to retinol in the intestinal lumen, and β-carotene is cleaved by the intestinal enzyme β-carotene dioxygenase (BCDO) to form two molecules of retinal (Ross et al., 2001). The intestinal enzymes acyl coenzyme A:retinol acyltransferase (ARAT) and lecithin:retinol acyltransferase (LRAT) convert retinol into retinyl esters, which are packaged into chylomicrons. The retinyl esters (the main form is palmitate) can be stored in the liver, the primary storage site of retinoids. LRAT preferentially acts on retinol when complexed to cellular retinoid binding protein (CRBP), while ARAT mainly esterfies uncomplexed retinol (Blomhoff et al., 1990). These enzymes as well as retinoid binding proteins help maintain a delicate balance of free versus stored retinol (Blaner and Olson, 1994; Ong et al., 1994).

Retinoid-Binding Proteins

In parenchymal cells of the liver, retinol is complexed to a retinol binding protein (RBP), a 21,000 Da plasma protein, belonging to a class of proteins called lipocalins. Its

structure has been well characterized as well as its interactions with retinol, specifically all-*trans*-retinol (review Newcomer and Ong, 2000; review Noy, 2000). Before RBP-ROH is transported out of the liver it first interacts with an accessory protein called transthyretin (TTR) (Wolf, 1995). This association is thought to protect the complex from renal filtration (review Noy, 2000). Once the complex reaches its target tissue, TTR dissociates. Exactly how retinol is internalized is still a matter of debate and the mechanism is most likely different for various cell types. In the aqueous environment of the cytosol retinol is bound to specific carrier proteins termed cellular retinol-binding proteins (CRBPs) (Ong et al., 1994).

Studies exist supporting receptor-mediated uptake of retinol from RBP as well as those supporting receptor-independent uptake of retinol from RBP (review Soprano and Blaner, 1994). Using an affinity-tagged recombinant CRBP assay, Sundaram and colleagues provided evidence of a membrane receptor in placental membranes (Sundaram et al., 1998). They demonstrated that the transfer of [^{3}H] retinol from RBP to CRBP was greater in the presence of membranes than in their absence. Furthermore, they suggested that it was a protein receptor because the transfer was greatly inhibited in the presence of protein denaturation agents. Tosetti and co-workers showed evidence of RBP endocytosis but failed to show direct proof of a RBP membrane receptor (Tosetti et al., 1999). In the yolk sac of cultured rat embryos, evidence exists for receptor-mediated RBP uptake, which may explain how embryos acquire and utilize maternal retinol (Ward et al., 1997). On the other hand, in cultured rat Sertoli cells the RBP-retinol complex is recognized at the cell surface, but only retinol is internalized (Shingleton et al., 1989). A

recent study using renal proximal tubal cells discovered that an endocytic receptor called megalin, is responsible for RBP transport in these kidney cells (Marinó et al., 2001).

Noy and colleagues provided evidence that retinol can rapidly dissociate from its binding sites on RBP, and they suggested that free retinol might enter the cell by partitioning in the plasma membrane (Noy and Xu, 1990). Another study using liver cells and a marker for receptor-mediated endocytosis, concluded that RBP is taken up by potocytosis, an endocytic pathway that utilizes caveolae instead of clathrin-coated pits (Malaba et al., 1995).

After retinoids enter the cell, carrier proteins bind and help stabilize these hydrophobic molecules. Retinoids have a detergent-like structure which would be deleterious to cellular and organelle membranes; therefore, since carrier proteins have a high binding affinity, they help regulate the pool of soluble, diffusible retinoid-protein complexes (Ong et al., 1994). In addition, these proteins modulate the metabolism of retinoids, allowing some enzymes access and restricting access of others (Ong et al., 1994; Sundaram et al., 1998).

Cellular retinoid binding protein I and II (CRBP I, II) are 56% identical, they are highly conserved (91%-96% sequence identity) among human, rat, mouse, pig, and chick, and they both interact with all-*trans*-retinol and all-*trans*-retinal (review Noy, 2000). CRBP I is expressed in multiple fetal and adult tissues, including reproductive organs. CRBP II is expressed in the adult small intestine and in some fetal tissues (review Noy, 2000).

The two primary functions of CRBP I are vitamin A storage and retinoic acid synthesis. In vitamin A sufficient animals, CRBP is saturated with retinol and LRAT can

act on the CRBP I-retinol complex to convert retinol to retinyl esters. In contrast, during a deficient state apo-CRBP I will stimulate the liberation of stored retinol from retinyl esters (review Ong et al., 1994; Noy, 2000). CRBP I allows only certain enzymes access to retinol. Since CRBP II is only expressed in the adult small intestine it plays a role in processing vitamin A from food. This protein sequesters retinol, restricting its access to only a few microsomal enzymes. CRBP II also participates in controlling the rate of esterification of retinol in the enterocytes (review Noy, 2000).

Depending on the cell type, one of three isoenzyme families have the potential to oxidize retinol to retinal, the rate-limiting step in retinoic acid synthesis. These include the alcohol dehydrogenases (ADH), retinol dehydrogenases (RoDH), and members of the P450 superfamily (Ross et al., 2001). Actually, P450 enzymes are responsible for metabolizing retinoic acid into more oxidized forms; thus, controlling its intracellular levels and excretion (Fujii et al., 1997). A double null mouse mutant for the genes *Adh1* and *Adh2* was created, and results demonstrated that they have non-overlapping roles. Specifically, ADH1 is responsible for minimizing vitamin A toxicity, and ADH4 is essential during a vitamin A deficient state (Deltour et al., 1999). Recently, ADH3 was determined to be a cytosolic retinol dehydrogenase, which also contributes to RA synthesis (Molotkov et al., 2002).

Retinal can be converted to retinoic acid by the aldehyde dehydrogenases (ALDH). In humans, the retinal dehydrogenase (RaDH) is known as ALDH1 (Ong et al., 1994; review Napoli, 1999; Elizondo et al., 2000; review Noy, 2000). The importance of this enzyme was observed in homozygous knockout mutant mice where the embryos died at midgestation (Neiderreither et al., 1999).

The other important intracellular retinoid proteins are cellular retinoic acid binding protein I and II (CRABP I, II), which primarily bind all-*trans*-retinoic acid. They display a high sequence similarity and are highly conserved across species. Their distribution across tissues is cell type and developmental stage dependent. For example, CRABP I in the adult is expressed almost everywhere, but CRABP II is only expressed in the skin, uterus, ovary, and choroid plexus (review Ong et al., 1994; Bucco et al., 1995; Noy, 2000). In the embryo both are present but in different tissues.

The function of these two binding proteins is still not clearly understood. Like the other retinoid binding proteins they serve to modulate the activities of enzymes that influence the synthesis and availability of retinoic acid. In addition, studies propose that they deliver their ligand to the nucleus where RA can gain access to the nuclear retinoic acid receptors. Noy has proposed two different mechanisms by which this transfer could occur: indirect association between the CRABP and the target receptor or a direct "channeling" process requiring contact between the CRABP and the target receptor (Noy, 2000). In a recent study, Budhu and Noy (Budhu and Noy, 2002) demonstrated that CRABP-II is translocated to the nucleus upon binding to RA, and it modulates retinoic acid receptor mediated biological activities of RA.

The importance of retinoid-binding proteins is observed in knockout (KO) mouse models and in human disease states. For example, low circulating levels of RBP can lead to night blindness even in the presence of sufficient vitamin A (review Ross et al., 2001). CRBP I KO mice are healthy and fertile, but their retinyl ester storage supply decreases by 50%. If these animals are fed a vitamin A-deficient diet they experience vitamin A deficiency syndrome (VAD) and their growth is greatly retarded; therefore, it was

suggested that CRBP I may not be necessary for RA synthesis but it is required for proper retinoid homeostasis (Ghyselinck et al., 1999). In contrast, a null mutation of the CRABP I gene in mice resulted in embryos that were apparently normal, leading to the conclusion that this mutation did not interfere with normal growth, development, and differentiation (de Bruijn et al., 1994). If dietary vitamin A is limited during pregnancy, then offspring of CRBP II knockout mice die within 24 hours after birth (XP et al., 2002).

Retinoic Acid Receptors

Retinoic acid impacts transcription through two nuclear receptor families which are members of the steroid/thyroid hormone receptor superfamily: retinoic acid receptors (RARs) and retinoic acid X receptors (RXRs). RARs consist of three subtypes (RARα, RARβ, RARγ), which are activated by all-trans-RA and 9-*cis*-RA. The RXRs (RXRα, RXRβ, RXRγ) are highly activated by 9-*cis*-RA (Mangelsdorf et al., 1994). These two receptors are surprisingly dissimilar. In fact, RAR is more related to the thyroid hormone receptor than to RXR (Mangelsdorf et al., 1994).

The RARs and RXRs can form homo- or heterodimers depending on the cell type. They bind to retinoic acid response elements (RAREs, RXREs), which flank the promoter regions of a number of RA-inducible genes (Mangelsdorf et al., 1994; Marshall et al., 1996; Rohwedel et al., 1999). RAREs or RXREs consist of a minimal half-site consensus sequence (AGGTCA) configured into a variety of structured motifs (Mangelsdorf et al., 1994). The RAR/RXR response elements are organized into direct repeats spaced 3, 4, or 5 nucleotides apart (Mangelsdorf and Evans, 1995). RXRs can also form heterodimers with vitamin D, thyroid hormone receptors, and peroxisome

proliferator-activated receptor (PPAR), which can act as transcription factors or co-activators of transcription factors. The response elements for these heterodimer partners are organized as a direct repeat as well, but only spaced 1 or 2 nucleotides apart (Mangelsdorf and Evans, 1995).

Once retinoic acid is produced it can directly or indirectly affect the phenotype of a cell. RA binding to RARs or RAR/RXR and subsequent association with RAREs mediates direct action. This process is thought to be very fast (few minutes to few hours) and does not require *de novo* protein synthesis (Gudas et al., 1994). Indirect actions can vary depending on the cell type and its state of differentiation. Examples include activation or repression of transcription factors, translation or post-translational processing, induction of protein synthesis, and even modification (retinoylation) of certain cellular proteins (Gudas et al., 1994). Furthermore, many biological metabolites of retinol exist, like β-glucoronide, which may also mediate or mimic the effects of RA (Blaner and Olson, 1994).

Several studies provide evidence that single mutant knockouts, of either RAR or RXR do not display apparent phenotypic abnormalities, suggesting a functional redundancy of these nuclear receptors (review Maden, 2000; review Ross et al., 2000). For example, if the predominant isoform, RARα1, was knocked out in mice the resulting offspring were normal. However, if the entire RARα gene was disrupted, early embryonic lethality occurred (Lufkin et al., 1993). Interestingly, one study reported that these RARα mutant mice developed lymphoma within two months, and that the tumors displayed a down-regulation of the p53 and bcl-2 genes (Haidar et al., 2000). RARβ mutants were phenotypically normal and fertile; however, if an RARα/RARβ double

mutant was created, the mice developed abnormally (Ghyselinck et al., 1997). Furthermore, double null mutant RXR α-/- and β-/- knockout mice failed to develop properly during embryogenesis (Wendling et al., 1999). One laboratory utilized a retinoid receptor antagonist in the mouse to clarify the roles of retinoic acid receptors during embryogenesis, and they discovered that the antagonist effect was stage and dose dependent (Kochhar et al., 1998).

Extensive work demonstrates that retinoic acid plays an important role in cellular differentiation and embryo body patterning (review Hofmann and Eichele, 1994). Some of the well-known genes that RA regulates or modulates include the homeobox or *Hox* genes, responsible for temporal and spatial body patterning (review Hofmann and Eichele, 1994), transforming growth factor-β, growth hormone, progesterone, enzymes such as alcohol dehydrogenase, transglutaminase, extracellular matrix proteins, and gap junctional proteins (Gudas et al., 1994).

Localization of Retinoids and Their Associated Proteins in Reproductive Tissues

Determining the actual retinoid content of embryos is difficult considering the amount of available tissue present in embryos. Studies have attempted to analyze the retinoid content using HPLC methods (Bucco et al., 1995; Horton and Maden, 1995; Maden, 2000). Results consistently show a graded, relatively non-specific distribution over the entire embryo, but localization of retinoid-associated proteins and their receptors has met with more success. Indeed, since these proteins and receptors mediate the transport, cellular accumulation, metabolism and actions of retinoids, their expression in the reproductive tract has been an area of much investigation.

In presomitic rat embryos, a RBP receptor, CRBP, retinol dehydrogenase, and retinal dehydrogenase were localized in certain tissues (Båvik et al., 1997). Recently, a study characterized the expression of the three subtypes of RXRs, alcohol dehydrogenase (ADH), retinaldehyde dehydrogenase II (RALDH2), and PPARγ in early preattachment bovine embryos (Mohan et al., 2002). This same study demonstrated that RXRα and β isomers, RALDH2, and PPARγ were expressed in all stages of development from the oocyte to the hatched blastocyst. These four transcripts were present in both the inner cell mass and the trophectoderm. These results indicate that the early vertebrate embryo possesses the mechanisms to synthesize and utilize retinoic acid. Furthermore, several studies confirmed the presence of retinoid binding proteins in the ovarian follicle, oviduct, uterus, and placenta; thus, confirming the machinery to mediate the actions of retinol and retinoic acid on the early embryo and developing fetus are present.

Reports from our laboratory illustrated that RBP and CRBP-I was immunolocalized in the thecal cell layers of non-atretic antral follicles in the ovine ovary. Unlike CRBP-I or CRABP, RBP was found in the granulosa cells. RBP was also localized in the cytoplasm of oocytes from antral follicles. CRABP was immunolocalized to oocytes from primordial follicles only (Eberhardt, unpublished). In the bovine ovary RBP was detected in theca and granulosa cells of antral and preantral follicles, CRBP was localized in the granulosa of antral follicles and in large luteal cells, and RBP was also expressed in large and small luteal cells (Brown et al., 2003). Subsequently, northern blot analysis confirmed mRNA expression of these retinoid-binding proteins. Zheng and co-workers provided evidence of CRABP II expression in rat granulosa cells and the uterine epithelium during estrous (Zheng et al., 1999). Studies

in rats detected RBP receptors in embryonic tissues (Morris-Kay & Ward, 1999). CRABP II appeared only in the cytoplasm of luteal cells in the rat ovary corresponding to an increase in progesterone levels (Bucco et al., 1995).

Analysis of bovine follicular fluid revealed the presence of retinol and RBP, and estradiol concentrations were positively correlated suggesting a healthier follicle (Brown et al., 2003). These studies indicate the existence of transport mechanisms that deliver retinol from the plasma into the follicle and ultimately to the oocyte. Once an oocyte is released from the follicle, it travels in the oviduct where fertilization occurs, and then continues to the uterus. Consequently, evidence also exists for the localization of retinoid proteins in the oviduct and uterus during this time period.

Expression of RBP by ovine oviducts was higher on Day 1 of the estrous cycle (Day 0 = estrus) than on Day 5 or Day 10. Furthermore, its expression was upregulated when estrogen was added to the culture medium (Eberhardt et al., 1999a). This suggests that retinol may be playing an important role during early embryo transport. In fact one study provided evidence that the retinol-binding protein 4 (RBP4) gene is one possible marker for increased litter size in pigs (Rothschild et al., 2000).

In cows, RBP was detected in uterine flushings from different days of the estrous cycle (Liu and Godkin, 1992), and RBP was detected in the ovine endometrium (Doré et al., 1994). A study conducted in our laboratory demonstrated RBP synthesis in the bovine occurred in both the uterine luminal and glandular epithelium (Mackenzie et al., 1997). Furthermore, RBP and CRABP were localized in the glandular and surface epithelium of the endometrium during early gestation in pigs (Schweigert et al., 1999).

RBP is present in the placental membranes in both the cow and sheep which suggests a mechanism by which retinol can be internalized and delivered to the placental tissues as well as the fetus. For example, RBP mRNA was observed in expanding bovine conceptuses (Day 13) as well as in several of the extraembryonic membranes (Gao et al., 1991; Liu et al., 1993). The ovine placental membrane epithelium was also found to secrete RBP (Liu et al., 1992). In the rat, during early gestation, the majority of retinol was found in the placenta, but by mid-organogenesis the embryo was found to contain higher levels of retinol (Satre et al., 1992).

Oocyte Maturation and Embryonic Development

Oocyte and embryonic development *in vivo* and *in vitro* is strongly influenced by the quality of the follicular environment in which the oocyte grows and matures (Boni et al., 2002; Sutton et al., 2003). Interactions of the oocyte and granulosa cells are intricately regulated, and they cannot develop in isolation from one another (Driancourt and Thuel, 1998). The follicular fluid is composed of a variety of proteins, growth factors, cytokines, steroids, energy substrates, and many unknown substances that regulate oocyte development (Sutton et al., 2003). In turn, the oocyte produces many different factors, which affect granulosa cell proliferation, differentiation, and even steroidogenesis, including: factor in the germline alpha (Figα), c-kit, growth differentiation factor –9 (GDF-9), and bone morphogenetic protein (BMP-15) (Eppig, 2001; Matzuk et al., 2002; Wu and Matzuk, 2002; Sutton et al., 2003). Furthermore, communication with somatic cells influences proper development of the egg including transcriptional activity and chromatin remodeling (De La Fuente and Eppig, 2001).

Upon completion of oogenesis an oocyte is arrested in the dictyate stage of prophase I. *In vivo*, the preovulatory surge of gonadotropins, via communication with the granulosa cells, causes resumption of meiosis and release of the oocyte from the follicle. However, physical release of the oocyte followed by *in vitro* culture may also trigger meiosis to resume (Eppig et al., 1996; Driancourt and Thuel, 1998; Duranthon and Renard, 2001). Oocyte maturation involves two distinct yet equally important processes: nuclear and cytoplasmic maturation. Nuclear maturation refers to the progression of meiosis from prophase I to metaphase II (Xu et al., 1986; Dominko and First, 1997). Meiotic resumption in oocytes is regulated by several key factors including, mitogen-activated protein kinase (MAPK) cascade, and the cyclin B-cylin dependent protein kinase-2 (cdc2), sometimes referred to as maturation or mitogenic promoting factor (MPF) (Sirard et al., 1998; Abrieu et al., 2001). The cyclin is the regulatory subunit, and the Cdc2 is the catalytic subunit. Both of these are controlled by somewhat species-specific phosphatases and kinases (Yamashita et al., 2000).

Cytoplasmic maturation involves redistribution of mitochondria, alterations in ATP content (Stojkovic et al., 2001), structural changes such as cortical granule realignment (De Loos et al., 1992; Abbot and Ducibella, 2001), and other organelle rearrangments (De Loos et al., 1992). The timing and assessment of meiotic and cytoplasmic maturation have been characterized in domestic animals, humans, and rodents (Sorensen and Wassarman, 1976; Xu et al., 1986; Dominko and First, 1997; Hunter, 2000; Combelles et al., 2002); however, this developmental process is still not completely understood. Closely associated granulosa cells, called cumulus cells, immediately surround the oocyte and play an important role in maintaining oocyte arrest,

inducing meiotic resumption, and supporting cytoplasmic maturation (Tanghe et al., 2002). These specialized cells provide the growing oocyte with essential nutrients and energy substances necessary for continued development (Sutton et al., 2003). In addition, proper cumulus expansion in most, but not all mammals, is important for proper fertilization (Yanagimachi, 1988).

Both meiotic and cytoplasmic maturation can be altered by substrate concentrations, and studies conducted on oocyte and early embryo metabolism (Leese and Barton, 1984; Rieger and Loskutoff, 1994; Downs and Utecht, 1999; Spindler et al., 2000) have led to the identification of important nutritional requirements as well as improved culture conditions. More recently, certain metabolic events have been linked with better viability after transfer (Krisher and Bavister, 1999; Spindler et al., 2000) and cryopreservation (Gardner et al., 1996). For example, it has been reported that mouse blastocysts with lower glycolytic rates are more viable after transfer (Lane and Gardner, 1996), while others have concluded that higher glycolytic rates in oocytes are more indicative of developmental competence (Krisher and Bavister, 1999; Spindler et al., 2000).

A key aspect of oocyte growth in the tertiary follicle is the accumulation of maternal RNA and proteins necessary for continued development of the embryo. Zygotic gene activation (ZGA) or the maternal to embryonic gene transition occurs at different time intervals, depending on the mammalian species, following fertilization (Telford et al., 1990; Memili and First, 2000; Latham and Schultz, 2001). This critical event is characterized by the initiation of transcription by the embryonic genome. Since the discovery of this genomic reprogramming many studies have described several genes that

are expressed during this time (review Memili and First, 2000; review Latham and Schultz, 2001).

Many studies have been designed to identify factors that influence an oocyte/embryo's developmental competence (review Overström, 2000; review Trounson et al., 2001) such as oocyte diameter (Sorensen and Wassarman, 1976), meiotic maturation (Damiani et al., 1996), ATP content of oocytes (VanBlerkom et al., 1995), follicle size (Pavlok et al., 1992), metabolic activity (Rieger and Guay, 1988; Gardner et al., 1996), and timing of development (McKiernan and Bavister, 1994). Perhaps the most common measure of developmental competence is the ability of an oocyte to progress to the morula and blastocyst stages. Both of these embryonic stages are characterized by morphological and functional changes necessary for continued embryonic development. Briefly, the morula stage embryo is a group of closely adherent cells or blastomeres, connected by tight junctions that allow communication among the cells (Sutherland and Calarco-Gillam, 1983; Crosier et al., 2000). Blastocyst stage embryos are distinguished by formation of a fluid-filled cavity, and differentiation of the inner cell mass (embryo) and the trophectoderm (future extraembryonic membranes) (review Watson, 1992). These developmental stages may be indicative of subsequent development.

Clearly, oocyte and embryonic development is a complex process that is not completely understood; however, with an increase of IVF produced animals, the requirements for successful *in vitro* maturation and culture of oocytes and embryos are becoming more apparent Continued research on the requirements of early embryonic culture conditions will provide the necessary methods needed to improve IVF and other manipulative techniques.

Reactive Oxygen Species Production during Early Embryonic Development

Convincing evidence is provided by many studies that *in vivo* embryo production is superior to *in vitro* embryo culture (review Bavister, 1995; Kubota et al., 1998; Crosier et al., 2000; Khurana and Niemann, 2000; review Duranthon and Renard, 2001; Rizos et al., 2002). One reason for diminished developmental competence *in vitro* may be excessive production of reactive oxygen species (ROS). ROS are generated during normal cellular processes such as the intermediate steps of oxygen reduction; thereby, resulting in production of the superoxide anion, hydrogen peroxide, and hydroxyl radicals (Gutteridge, 1995; review Dröge, 2002).

Endogenous sources of ROS during embryo development include oxidative phosphorylation, NADPH oxidase (produces superoxide anion and hydrogen peroxide), and xanthine oxidase (degrades purine nucleotides). Exogenous sources that may result in production of ROS, particularly during *in vitro* culture, include high oxygen concentration, metallic cations, visible light, and sperm (Salas-Vidal et al., 1998; review Guérin et al., 2001; review Dröge, 2002). The deleterious effects of ROS on preimplantation embryos include protein, lipid, and nucleic acid damage, mitochondrial alterations, cell cycle block, ATP depletion, and excessive apoptosis (Salas-Vidal et al., 1998; Guérin et al., 2001). Increased oxygen radicals in mouse embryos cultured *in vitro* are implicated in developmental blockage that is common during *in vitro* culture (Goto et al., 1993).

In vitro production of embryos has helped investigators define certain requirements for preimplantation embryo survival. Studies have demonstrated that embryos cultured in atmospheric oxygen (20%) undergo cytoplasmic fragmentation and

eventually apoptosis due to a high concentration of ROS (Pabon et al., 1989; Yang et al., 1998; Guérin et al., 2001). Oxidative stress defined as "a disturbance in the prooxidant/antioxidant system" (Sies, 1995), is perhaps the leading cause of early embryonic loss, particularly during *in vitro* culture (Takahashi et al., 2000; Guérin et al., 2001).

In order to regulate the amount of ROS produced, many scientists began culturing embryos in a low oxygen atmosphere (5.0 – 7.5% O_2) and adding antioxidants such as superoxide dismutase SOD (Umaoka et al., 1992), glutathione (review Takahashi et al., 1993; Edwards et al., 2001; Mayor et al., 2001), and vitamins (Guérin et al., 2001). Mouse embryos cultured in high O_2 with antioxidants, such as catalases or other O_2 scavengers, displayed an increased blastocyst development over embryos that did not receive supplemental antioxidants (Orsi and Leese, 2001). Furthermore, an increase in apoptosis in bovine blastocysts was observed in a 20% oxygen atmosphere versus a 5% (Yuan et al., 2003). However, when antioxidants were used during maturation of rat cumulus-oocyte complexes, resumption of meiosis was inhibited (Takami et al., 1999). Increasing concentrations of antioxidants during IVF significantly reduced oocyte penetration rates in bovine oocytes (Blondin et al., 1997). A similar study performed in women undergoing IVF showed that women who became pregnant had higher follicular fluid ROS levels than those who did not; yet, the study also concluded that abnormally high ROS levels may indicate oxidative stress and prohibit conception (Attaran et al., 2000). Consequently, it appears that the presence of ROS during maturation and ovulation may be required, but excessive levels during embryo development may be detrimental.

A recent paper by Harvey and colleagues discusses REDOX (reduction-oxidation) regulation in preimplantation embryos as opposed to the actual amount of ROS present within the embryo environment (Harvey et al., 2002). They defined the intracellular REDOX state as complex interactions of the relative concentrations of various oxidized and reduced molecules. Extreme fluctuations in the REDOX state of a cell can alter the response of a number of genes and/or transcription factors, as well as transport systems like the cystine transport system, which influences intracellular glutathione levels (Dröge, 2002). ROS also serve as critical intracellular signaling molecules.

Reactive oxygen species act as second messengers in signal transduction pathways (Finkel and Sullivan, 2000; Thannickal and Fanburg, 2000), and influence gene expression (Palmer and Paulson, 1997). For instance, a family of proteins called stress-activated protein kinases (SAPK), also known as c-Jun NH_2-terminal kinase (JNK), and p38 are related to the members of the family of mitogen-activated protein kinases (MAPK). Typically, MAPKs respond to growth factors and cytokines resulting in cell proliferation and differentiation. SAPK respond to stressful stimuli, such as an altered redox state, and may participate in apoptosis (Ichijo, 1999). SAPK belong to a family of proline directed serine-threonine kinases that respond to a stimulus by phosphorylation of their threonine and tyrosine residues; thus, altering its conformation resulting in kinase activity that activates transcription factors like nuclear factor κB (NF-κB) and activating protein 1 (AP-1), which play significant roles in cell survival (Hancock, 1999).

Role of Retinoids in Protecting the Early Embryo from Oxidative Stress

Under certain conditions vitamin A and carotenoids participate in a biological antioxidant network. Specifically, carotenoids can quench singlet oxygen molecules as well as interact with other antioxidant compounds (Olson, 1993; Packer, 1995). Retinoids may positively affect preimplantation embryonic development by various mechanisms such as inhibiting apoptosis, inducing and/or cooperating with antioxidant enzymes like superoxide dismutase (SOD), and catalase, and/or by increasing glutathione levels.

Depending on the cell line, retinoids can be anti- or proapoptotic. For example, in some cancerous cell lines retinoic acid has been shown to be proapoptotic (Chiba et al., 1997; Hayden and Satre, 2002; Pratt and Niu, 2003). On the other hand, several studies provide convincing evidence that retinoids suppress apoptosis in a number of tissues and cell lines by different mechanisms. In mesangial cells exposed to hydrogen peroxide (H_2O_2), RA possessed anti-apoptotic effects by inhibiting JNK and activating protein-1 (AP-1) (Moreno-Manzano et al., 1999). AP-1 is composed of the heterodimers, c-Jun and c-Fos, which bind to particular *cis*-elements and initiate transcription of target genes. A follow-up study by the same lab illustrated the role of RARs and RXRs in the suppression of H_2O_2-induced mesangial apoptosis. Again JNK and AP-1 were both involved. In normal human bronchial epithelial cells (HBE), RA treatment inhibited JNK activity and reduced c-*fos* and protein levels by decreasing c-*fos* gene transcription. In addition, agonists of retinoic acid receptors (RARs) and retinoid X receptors (RXRs) suppressed c-*fos* transcription (Lee et al., 1998). Further experiments showed the anti-apoptotic effect of RA was mediated by both receptor-dependent and receptor-

independent mechanisms (Konta et al., 2001). Another study demonstrated in HeLa cells that liganded RARs interfere with c-Jun/c-Jun homodimerization and c-Jun/c-Fos heterodimerization; thereby, preventing AP-1 complexes from binding to DNA and initiating apoptosis (Zhou et al., 1999).

Protein kinase C (PKC) is a well-known enzyme that plays a significant role in signal transduction pathways. It belongs to a family of serine/threonine kinases, and to date consists of eleven different isoforms, categorized as classic, novel, and atypical (Parekh et al., 2000). These isoenzymes regulate a number of different processes such as cellular growth, differentiation, and apoptosis. Retinol treatment of 3T3 cells enhanced the activation of PKCα in response to ROS by binding to its cysteine-rich domain (Imam et al., 2001). Furthermore, investigators proposed a model describing how all-trans RA modulates the activity of PKCα in a line of cancer cells suggesting a pro-apoptotic role for RA (Radominska-Pandya et al., 2000). So, it is possible that RA may impact the actions of other PKC isoforms that are anti-apoptotic.

Another mechanism by which retinoids may exert their antioxidant effects is through modulation of antioxidants like SOD or catalase. Treatment of hippocampal neurons with staurosporine, a chemical that induces apoptosis, reduced Mn-SOD and Cu-Zn SOD levels; however, if the cells were pretreated with retinoic acid, protein levels of both types of SOD remained the same and apoptosis was prevented (Ahlemeyer et al., 2001). Mouse fetuses whose mothers were treated with retinoic acid showed decreased expression of apoptosis-related genes (Sarkar and Sharma, 2002).

Retinoids may also impact oocyte and early embryonic development by modulating glutathione levels. Glutathione (GSH) is the major non-protein sulphydryl

compound found in mammalian cells responsible for strong basal ROS scavenging activity (Dröge, 2002). It is a tripeptide (γ-glutamyl, cysteinyl, glycine) that exists in two forms: an oxidized form (GSSG) and a reduced form (GSH), which is the predominant form found in most tissues. Intracellular glutathione synthesis begins with the transulfuration of homocysteine to cysteine, the major precursor for GSH synthesis (Deplancke and Gaskins, 2002). Next, gamma-glutamyl cysteine synthetase (γ-GCS) combines L-glutamate and cysteine, followed by catalysis of glutamate-cysteine and glycine by glutathione synthetase. γ-GCS is considered the rate-limiting enzyme in glutathione synthesis and its expression is increased under oxidizing conditions (Deplancke and Gaskins, 2002); yet, some believe that glutathione synthetase plays an equally important role (Huang et al., 2000). Other important glutathione-related enzymes include glutathione peroxidase, which converts GSH to GSSG (Arai et al., 1999), glutathione reductase, which can reverse this reaction (Anderson, 1985), and glutathione *S*-transferase-pi (GSTp), which catalyzes the conjugation of GSH with deleterious compounds in order to protect the cell (Bernardini et al., 1999).

Many oxidative stress-related enzymes, including those enzymes that regulate glutathione levels are found in the reproductive tract, its secretions, and even in the oocyte and early embryo (review El Mouatassim et al., 1999; Knappen et al., 1999). For example, different levels of expression of GSH, GSSG, cysteine, and Mn-SOD were found in rabbit and rat embryos in different tissues (Hansen et al., 2001). MII stage *in vivo* produced mouse and human oocytes expressed the following enzymes: Cu-Zn SOD, MnSOD, GPx, and GCS (El Mouatassim et al., 1999). GSTp was found in human

ovarian follicular fluid, and this particular isoform is also important in placental function (review Knappen et al., 1999).

Several reports highlight the importance of having adequate glutathione levels in both oocytes and early embryos. Levels of glutathione vary depending on the embryonic stage, and evidence suggests that gonadotropins may affect glutathione synthesis in the rat ovary by altering levels of key synthetic enzymes (Luderer et al., 2001). For instance, intracytoplasmic levels of glutathione in bovine embryos derived *in vitro* were lowest at the 2-8 cell stage, increased during the 9-16 cell stage, and reached their highest levels at the hatched blastocyst stage (Lim et al., 1996). In hamster oocytes, the GSH content doubled after germinal vesicle breakdown, remained high until after metaphase II, and decreased after fertilization (Zuelke et al., 2003).

Gardiner and colleagues reported the presence of glutathione in mouse oviduct flushings, and if the embryos were deprived of GSH, blastocyst development was retarded compared to embryos that received exogenous GSH (Gardiner et al., 1998). Glutathione addition to caprine oocyte maturation medium increased the GSH content of oocytes, but did not increase the fertilization rate (Mayor et al., 2001). If an inhibitor of glutathione synthesis, buthionine sulfoximine (BSO), was added to the culture medium containing 6-8 cell bovine embryos, blastocyst development was compromised (Takahashi et al., 1993). Further studies confirmed the beneficial effects of higher GSH levels in oocyte maturation medium (bovine, mouse, pig) on subsequent embryo development (Luvoni et al., 1996; Furnus et al., 1998; Knappen et al., 1999; de Matos and Furnus, 2000; Shi et al., 2000; Brad et al., 2003). Glutathione is also necessary for the transformation of the disulfide rich sperm nucleus into the male pronucleus at

fertilization (Calvin et al., 1986; Perreault et al., 1988; Yoshida et al., 1993). Another beneficial effect of glutathione is its potential ability to protect early embryos from heat stress (Ealy et al., 1992; Edwards et al., 2001).

Thiol compounds, particularly cysteine are required for glutathione synthesis (Knappen et al., 1999), and researchers have utilized these precursors to investigate their effect on *in vitro* embryonic development. For example, Takahashi and coworkers concluded that addition of two thiol compounds, ß-mercaptoethanol (ß-ME) and cysteamine, to 6-8 cell bovine embryos improved development to the blastocyst stage (Takahashi et al., 1993). Whenever ß-ME, cysteine, or cystine was added to maturation medium containing bovine oocytes, blastocyst development significantly increased over the control oocytes that did not contain these glutathione precursors (de Matos and Furnus, 2000). Cysteamine addition during oocyte maturation of several species improved significantly embryonic development, as assessed by progression to the blastocyst stage (Gasparrini et al., 2000; de Matos et al., 2002; de Matos et al., 2003). One study cultured bovine embryos in a 5% oxygen atmosphere in the presence of cysteine, and found this increased the number of cells located in the inner cell mass. However, the same study discovered that cysteine addition was detrimental to blastocyst rates and hatching rates (Van Soom et al., 2002).

Evidence exists that retinoids may modulate glutathione levels. Retinoic acid inhibited the depletion of glutathione in cultured neuron cells that were treated with staurosporine; thus, preventing oxidative damage and apoptosis (Ahlemeyer and Krieglstein, 2000). A retinoic acid response element (RARE) was found in the promoter region of a specific isoform of GSTp in a glioblastoma cell line (Lo and Ali-Osman,

1997), and in glutathione peroxidase – Gpx2 (Chu et al., 1999). Additionally, a study provided evidence that retinoic acid modulated glutathione, glutathione peroxidase, and glutathione reductase levels (Texeira et al., 1996).

SUMMARY

With an increase in embryo manipulation and IVF produced animals it is imperative that embryo viability assays be developed (Overström, 1996; review Overström, 2000). Manipulation of mammalian embryos before implantation has risen over the last 10-15 years due to the economic and social pressures placed on the commercial production of animals to produce more milk, meat, and overall genetic gain (Van Soom and de Kruif, 1996). Experimental manipulation of human oocytes and embryos (i.e. intracytoplasmic sperm injection – ICSI and cytoplasmic transfer) has also increased significantly over the last decade. The potential benefits of techniques like *in vitro* fertilization (IVF) and culture include helping infertile couples conceive, rescuing oocytes from women undergoing clinical procedures that place oocytes at risk, and enlarging the endangered wildlife population (Eppig et al., 1996). Even though much research has focused on what influences an oocyte/embryo's developmental competence, it is still not clear what factors are specifically needed for an embryo to become a blastocyst or viable offspring. Furthermore, several laboratories have demonstrated that many stressors impact embryo survival, especially during *in vitro* culture (review Lane, 2001).

Vitamin A (retinol) is essential for reproduction (Ganguly, 1989; Gudas et al., 1994; Maden, 2000), and the natural metabolites of retinol, all-*trans* retinoic acid (RA)

and 9-*cis* RA, are recognized as important signaling molecules in differentiation and development of many cell and organ systems (Blaner and Olson, 1994; Gudas et al., 1994). Recent evidence indicates that retinol and RA influence follicular development and oocytes maturation *in vivo* and *in vitro*. Retinol administration to sheep and cattle, in combination with superovulation and followed by insemination, was shown to improve *in vitro* developmental competence of resultant embryos in the former and embryo quality in the latter (Blaner and Olson, 1994; Gudas et al., 1994; Shaw et al., 1995; Eberhardt et al., 1999b). Retinol administration to swine has been reported to advance resumption of meiosis, alter follicular hormone concentrations (Whaley et al., 2000) and increase litter size in some, but not all, studies (Coffey and Britt, 1993; Pusateri et al., 1999). *In vitro*, addition of retinol to oocyte maturation and embryo culture medium was observed to improve developmental competence of bovine embryos (Livingston et al., 2002). Retinoic acid administration to meiotically blocked bovine oocytes promoted cytoplasmic maturation and enhanced their developmental capacity (Duque et al., 2002).

The objectives of this research were to further characterize the beneficial effects of retinol during oocyte maturation and embryonic development, to evaluate the role of retinol on sheep oocyte metabolism, and to help elucidate its mechanism of action in the oocyte and embryo via oxidative stress signaling pathways. This will help facilitate the identification of factors that not only influence, but also improve an embryo's ability to survive excessive handling, such as *in vitro* culture, cryopreservation, and embryo transfer, which are areas of great importance in both basic and applied reproduction practices.

CHAPTER 2

Metabolism of Sheep Oocytes During *In Vitro* Maturation

ABSTRACT

The purpose of the present study was to evaluate energy metabolism during *in vitro* maturation (IVM) of individual ovine oocytes and to determine effects of *in vivo* retinol administration on this process. Ewes were superovulated with FSH and treated with retinol or vehicle. Oocytes were subjected to metabolic measurements during during early (0-3 hours), mid (9-12 hours), or late (21-24 hours) stages of IVM using hanging-drop procedures. The substrates 5-^{3}H glucose, 1-^{14}C and 6-^{14}C glucose, 2-^{14}C pyruvate, and 3,4 ^{3}H glutamine were used to evaluate metabolism. Results showed that glucose metabolism through the glycolytic pathway changed from early to mid time periods (0.24 to 21.65 pmoles/oocyte/3hours; $p \leq .02$), and early to late time periods (0.24 to 14.24; $p \leq .01$). Glucose oxidation through the Krebs cycle did not change significantly over the twenty-four hour maturation time period (0.29 to – 0.61), and likewise, glucose metabolism via the pentose-phosphate pathway did not change during IVM. Oxidative metabolism measured by glutamine showed no significant differences over all time periods. Pyruvate oxidation was highest early in maturation and then decreased (3.14 to 1.44; $p \leq .14$). Glutamine oxidation appeared to be most active early and then declined. Glycolysis was highest at the middle time period. These results suggest that ovine oocytes use both glycolysis and oxidative pathways throughout IVM.

No significant differences existed between oocytes from retinol-treated ewes and control oocytes.

INTRODUCTION

Preimplantation embryo development is strongly affected by the follicular environment in which the oocyte grows and matures. Upon completion of oogenesis an oocyte is arrested in the dictyate stage of prophase I. *In vivo*, the preovulatory surge of gonadotropins, via communication with the granulosa cells induces resumption of meiosis and release of the oocyte from the follicle. However, physical release of the oocyte followed by *in vitro* maturation (IVM) also triggers meiosis to resume (Eppig et al., 1996; Driancourt and Thuel, 1998; Duranthon and Renard, 2001). Oocyte maturation may be divided into two distinct yet equally important processes: nuclear and cytoplasmic maturation. Nuclear maturation refers to the progression of meiosis from prophase I to metaphase II before fertilization (Xu et al., 1986; Dominko and First, 1997). Cytoplasmic maturation involves redistribution of mitochondria and changes in ATP content (Stojkovic et al., 2001), structural changes such as cortical granule realignment (De Loos et al., 1992; Abbot and Ducibella, 2001), and other organelle rearrangments (De Loos et al., 1992). The timing and assessment of meiotic and cytoplasmic maturation have been characterized in domestic animals, humans, and rodents (Sorensen and Wassarman, 1976; Xu et al., 1986; Dominko and First, 1997; Hunter, 2000; Combelles et al., 2002); however, this developmental process is still not completely understood.

In order to better understand and improve *in vitro* maturation and culture conditions, studies have been performed using different combinations of nutrients, energy sources, hormones, and growth factors. Results from these numerous studies have demonstrated that an oocyte requires certain substrates in order to successfully complete both nuclear and cytoplasmic maturation (Downs and Mastropolo, 1994; Eppig et al., 1994; Downs and Mastropolo, 1997); (Izadyar et al., 1998), and after fertilization complete early embryonic development.

Investigation of oocyte and early embryo metabolism (Leese and Barton, 1984; Rieger and Loskutoff, 1994; Downs and Utecht, 1999; Spindler et al., 2000) has led to the identification of important substrate requirements and better culture conditions. More recently, certain metabolic events have been linked with better viability after transfer (Krisher and Bavister, 1999; Spindler et al., 2000) and cryopreservation (Gardner et al., 1996). For example, it has been reported that mouse blastocysts with lower glycolytic rates are more viable after transfer (Lane and Gardner, 1996), while others have concluded that higher glycolytic rates in oocytes are more indicative of developmental competence (Krisher and Bavister, 1999; Spindler et al., 2000).

Using both fluorescent and radioactive labeling techniques, numerous metabolic studies have been conducted in preimplantation domestic embryos. For instance, non-invasive metabolic measurements of bovine embryos *in vitro* demonstrated a dependence on oxidative phosphorylation until compaction, after which a shift towards glycolysis was observed (Thompson et al., 1996). *In vivo* produced sheep embryos prefer pyruvate as their primary substrate from fertilization until the blastocyst stage (Gardner et al., 1993). Radioactive labeling of glucose in sheep embryos showed no difference in glycolysis

between *in vivo* versus *in vitro* derived embryos, but differences in glucose oxidation were observed (Thompson et al., 1991). A later study also described how culture conditions alter glucose utilization by early sheep embryos (Thompson et al., 1992); thus, many different factors may influence the metabolism of early embryos.

Vitamin A (retinol) is essential for reproduction (Ganguly, 1989; Gudas et al., 1994; Maden, 2000), and the natural metabolites of retinol, all-*trans* retinoic acid (RA) and 9-*cis* RA, are recognized as important signaling molecules in differentiation and development of many cell and organ systems (Blaner and Olson, 1994; Gudas et al., 1994). Recent evidence indicates that retinol and RA influence follicular development and oocytes maturation *in vivo* and *in vitro*. Retinol administration to sheep and cattle, in combination with superovulation and followed by insemination, was shown to improve *in vitro* developmental competence of resultant embryos in the former and embryo quality in the latter (Blaner and Olson, 1994; Gudas et al., 1994; Shaw et al., 1995; Eberhardt et al., 1999b). Retinol administration to swine has been reported to advance resumption of meiosis, alter follicular hormone concentrations (Whaley et al., 2000) and increase litter size in some, but not all, studies (Coffey and Britt, 1993; Pusateri et al., 1999). *In vitro*, addition of retinol to oocyte maturation and embryo culture medium was observed to improve developmental competence of bovine embryos (Livingston et al., 2002). Retinoic acid administration to meiotically blocked bovine oocytes promoted cytoplasmic maturation and enhanced their developmental capacity (Duque et al., 2002).

The actions of retinoids are mediated through two groups of nuclear receptors, retinoic acid receptors (RARs) and retinoic X receptors (RXRs) (review Mangelsdorf et al., 1994). Retinoid-receptor complexes affect gene activation or inactivation through

association with specific response elements (RAREs) found in the promoter regions of target genes. Messenger RNA and gene products for several of the retinoic acid nuclear receptors have been identified in bovine oocytes and early embryos (Mohan et al., 2002) demonstrating that the functional machinery for retinoid signaling is present.

Although it is clear from our previous studies and others that retinoids influence oocyte maturation and embryonic development, the mechanisms involved have yet to be determined. The first goal of this study was to determine the metabolic requirements of sheep oocytes during different time periods of IVM. The second aim was to evaluate the effects of retinol on the oocyte's metabolism and nuclear development *in vitro*. As in previous studies (Rieger and Loskutoff, 1994; Downs and Utecht, 1999; Spindler et al., 2000), we assessed sheep oocyte metabolism using a combination of radiolabeled substrates. The production of $^{3}H_2O$ from 5-^{3}H glucose measures the glucose uptake by the Embden-Meyerhof pathway. This was determined by one study where a tritium radiolabel was used to trace the fate of each glucose hydrogen during glucose metabolism and recycling (Russell and Young, 1990). Earlier experiments demonstrated that release of $^{14}CO_2$ from 1-^{14}C glucose is cycled through glycolysis, Krebs cycle, and the pentose phosphate pathway (PPP) (Katz and Wood, 1963). In addition, the same study showed that metabolism of 6-^{14}C glucose measures the amount of glucose utilized by glycolysis and also by the Krebs cycle. The evolution of $^{3}H_2O$ from the consumption of 3,4 ^{3}H glutamine and $^{14}CO_2$ release from 2-^{14}C pyruvate also measures the activity of the Krebs cycle.

Results from the present study indicate that sheep oocytes utilize different substrates and metabolic pathways over the course of IVM. Furthermore, we did not

observe a difference in metabolism between oocytes from retinol-treated ewes versus oocytes from control ewes.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma Chemical Company, St. Louis, MO unless otherwise noted. Mature cross-bred ewes were provided by the University of Tennessee, Knoxville Experiment Station. Progestin-impregnated vaginal implants (CIDR-G) were purchased from InterAg, Hamilton, New Zealand. Lutalyse® (prostaglandin F2α) was purchased from Pharmacia & Upjohn Co. (Kalamazoo, MI), and follicle stimulating hormone (FSH) was purchased from Sioux Biochemical, Sioux Center, IA. Embryo collection media was composed of modified M199, 4.2 mM $NaHCO_3$, 10 mM HEPES, and 50 μg/mL heparin. Fetal bovine serum (FBS) used in embryo collection medium was purchased from BioWhittaker, Baltimore, MD, and glutamine and penicillin/streptomycin were purchased from Specialty Media, Phillipsburg, NJ. Oocyte maturation medium (OMM) contained M199 and 50 μg/mL of gentamycin, purchased from Specialty Media, 5 μg/mL of FSH purchased from Vetrepharm Canada, Inc. (Ontario, Canada), 0.3 μg/mL of lutenizing hormone (LH) that was generously provided by the USDA, Beltsville, MD., 10% FBS, 0.2 μM sodium pyruvate and 2 mM glutamine. Scintillation fluid (Ultima Gold XR) was purchased from Packard Instrument Co., Meriden, CT.

Radioactive Substrates

D-[5-^{3}H] glucose was purchased from Amersham Pharmacia Biotech, Buckinghamshire, England (1.0 mCi/mL with a specific activity of 77.3 mCi/mg). D-1 – ^{14}C glucose (1.0 mCi/mL; 54.3mCi/mmol) and D-6-^{14}C glucose (1.1 mCi/mL; 56mCi/mL) were purchased from Sigma. Pyruvic acid [2-^{14}C] and Glutamine L-[3,4-^{3}H(N)] were both purchased from New England Nuclear Life Science Products, Boston, MA. Pyruvic acid was purchased in a solid form, and its specific activity was 15.8 mCi/mmol. The concentration of glutamine was 1.0 mCi/mL and its specific activity was 49.9 mCi/mL. The substrates had a final concentration of 0.25 μCi/μl except for 2-^{14}C pyruvate, which was 0.079 μCi/μl. The final concentrations of the labeled plus unlabeled substrates were as follows: glucose (5.5 mM), glutamine (2 mM) and pyruvate (0.2 mM). The $^{3}H_2O$ and $NaH^{14}CO_3$ used to calculate the recovery efficiency were both purchased from Sigma.

Embryo Collection

Mature cross-bred ewes were synchronized with CIDR-G implants. Lutalyse® was administered 6 days after the CIDR was implanted. FSH was administered four days after prostaglandin injections in decreasing doses, twice daily for three days (5 IU, 4 IU, 3 IU). On the first and last morning of FSH injections each ewe was also administered 500,000 IU of all-trans retinol dissolved in corn oil. Control ewes received only vehicle (corn oil). Implants were left in place until after the ovaries were collected in order to prevent a premature LH surge. All animals were maintained on high-quality hay and fed

ad libitum, with free-choice access to a sheep and goat mineral premix that contained 1 million IU vitamin A per pound.

Ewes were ovariectomized and their cumulus oocyte complexes (COCs) were recovered by slicing only healthy, large follicles (>5mm). The COCs were randomly allocated to different groups, each representing one of the three different time periods (0-3, 9-12, 21-24). The oocytes that were not subjected to a metabolic measurement during the first time period were placed in a four-well plate with 500 μl of preequilibrated (38.5°C, 5% CO_2 in air) OMM. Immediately prior to the metabolism measurement, oocytes were denuded by gentle pipetting with 20% hyaluronidase, and then washed three times in preequilibrated (38.5°C, 5% CO_2 in air) metabolic measurement medium (MMM), which was OMM minus FBS. This was repeated for every time period.

Metabolism Assay

Sheep oocyte metabolism was measured using a modified hanging-drop technique (Rieger and Guay, 1988). Microcentrifuge tubes were filled with 600 μl of 25mM $NaHCO_3$ and preequilibrated for at least one hour at 38.5°C, 5% CO_2 in air. Individual washed oocytes were loaded in 2 μl of MMM, placed on the cap of a microcentrifuge tube, and then 2 μl of the radiolabeled metabolic substrates were added to the cap. Lids were gently closed and the tubes placed in the incubator for three hours. The $NaHCO_3$ acts as a trap for $^{14}CO_2$ and $^{3}H_2O$ released by the oocyte. Sham and total count tubes were also incubated with oocytes from each ewe. The sham tubes contained 2 μl of the MMM and 2 μl of the radiolabel without an oocyte. These accounted for any nonspecific counts including machine background, bacterial contamination, chemiluminescence, or

the spontaneous breakdown of the metabolic substrates. Total count tubes contained 2 μl of the radiolabeled substrates added directly to the $NaHCO_3$.

After the three-hour metabolism period, tubes were removed, and oocytes were washed and stained in order to assess their meiotic stage. The $NaHCO_3$ was immediately mixed with 4 mL of scintillation fluid and counted in a liquid scintillation counter programmed for dual-label counting of ^{14}C and/or ^{3}H. In order to determine mean radioactivity that each oocyte utilized, counts from the sham tube were subtracted from the tube containing the oocyte. This difference was divided by total radioactivity of labeled substrate added, and multiplied by the total quantity of substrate (labeled plus unlabeled) in 4μl of medium. This number was then multiplied by the recovery correction factor (Rieger and Loskutoff, 1994). In order to calculate the recovery efficiency of our system both $^{3}H_2O$ and $NaH^{14}CO_3$ were utilized. Three different concentrations (0.08, 0.25, 0.5 μCi/μl) were used over three days at each of the different time periods. The recovery values for our system were 1.14 and 1.12 for $^{3}H_2O$ and $NaH^{14}CO_3$, respectively.

Staining

In order to determine meiotic stage, oocytes were stained with Hoechst dye. Briefly, each oocyte was placed in approximately 500 μl of 50 μg/ml of *bis*benzamide (Hoechst 33258; Sigma) dissolved in HEPES-TALP. The dish was placed under a cover away from light for 20 minutes, and then the oocytes were washed to remove excess dye and viewed with a 100X objective under UV light.

Data Analysis

Data were checked for normality, log transformed, and analyzed using an incomplete block design blocked on replication with analysis of variance (ANOVA) using mixed model ANOVA methods in SAS (2000). Main effects and all interactions of substrate, time and treatment were tested. Differences were detected using Fisher's protected least significant difference. The means reported are untransformed. A probability of $p<.05$ was considered significant for all statistical tests. Differences due to retinol treatment were not detected; therefore, data from retinol treated and control oocytes were combined.

RESULTS

Oocytes were collected from superovulated, retinol or vehicle-treated ewes, and their metabolism was measured *in vitro* at three separate time points over a twenty-four hour maturation period. Glucose metabolism through the glycolytic pathway (5-^{3}H glucose) dramatically increased from 0.24 to 21.65 pmol/oocyte/3hr ($p<.03$) from the early measurement (0-3 hours) to the middle measurement (9-12 hours), then dropped to 15 pmol/oocyte/3hr at the last time period (21-24 hours) ($p<.001$). During the first three hours of maturation oocytes consumed significantly more 3,4 ^{3}H glutamine compared to 5-^{3}H glucose (15.18 vs 0.24; p<). Glutamine metabolism did not significantly change until the last time period (21-24 hours) when it dropped from 20.22 pmol/oocyte/3hr at 9-12 hours to 7.42 pmol/oocyte/3hr (Figure 2-1)

Figure 2-2 illustrates metabolism of ^{14}C substrates for all oocytes, regardless of treatment. Pyruvate oxidation, measured by 2-^{14}C pyruvate uptake, remained essentially

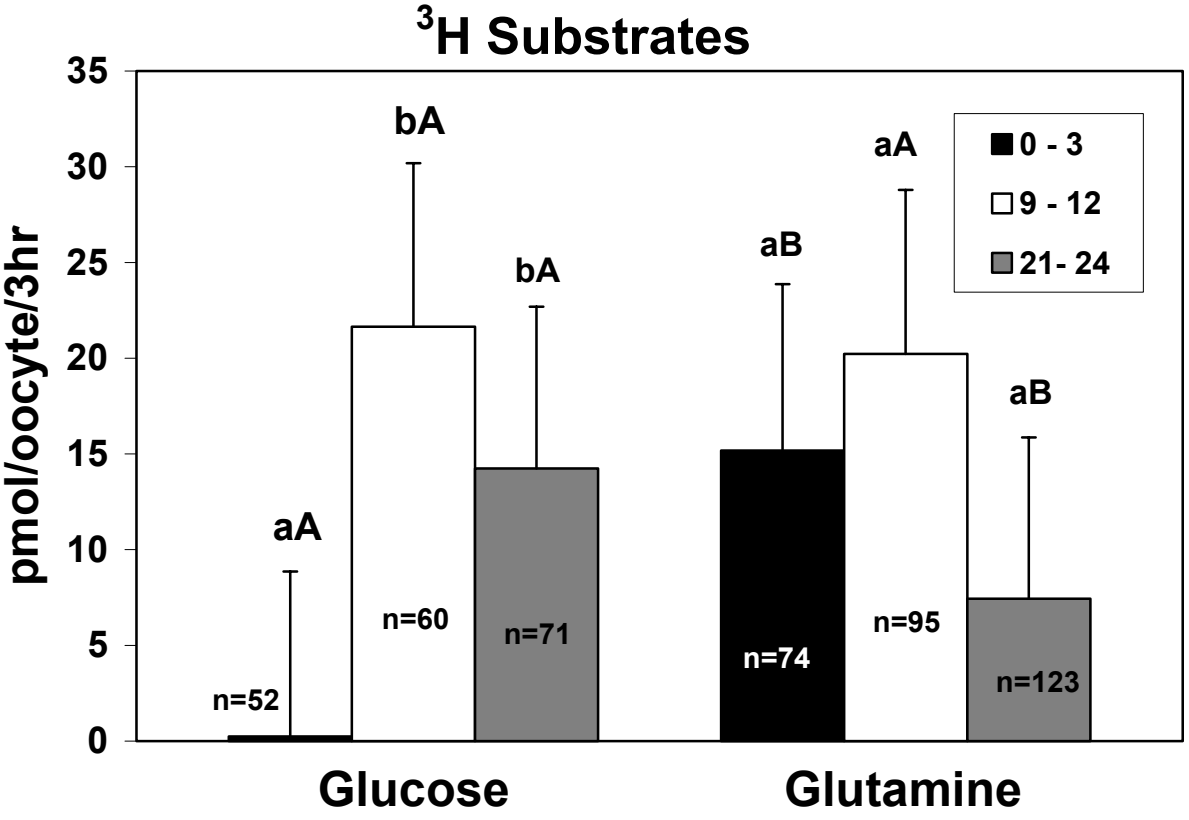

Figure 2-1. The metabolism of 5-^{3}H glucose and 3,4-^{3}Hglutamine by sheep oocytes. Individual sheep oocytes were measured at specific time periods during maturation(0-3, 9-12, and 21-24 hours). Lower case letters indicate differences within the same substrate ($p<.05$). Uppercase letters indicate differences across substrates ($p<.05$). The number of oocytes measured in a given time period is represented as (n).

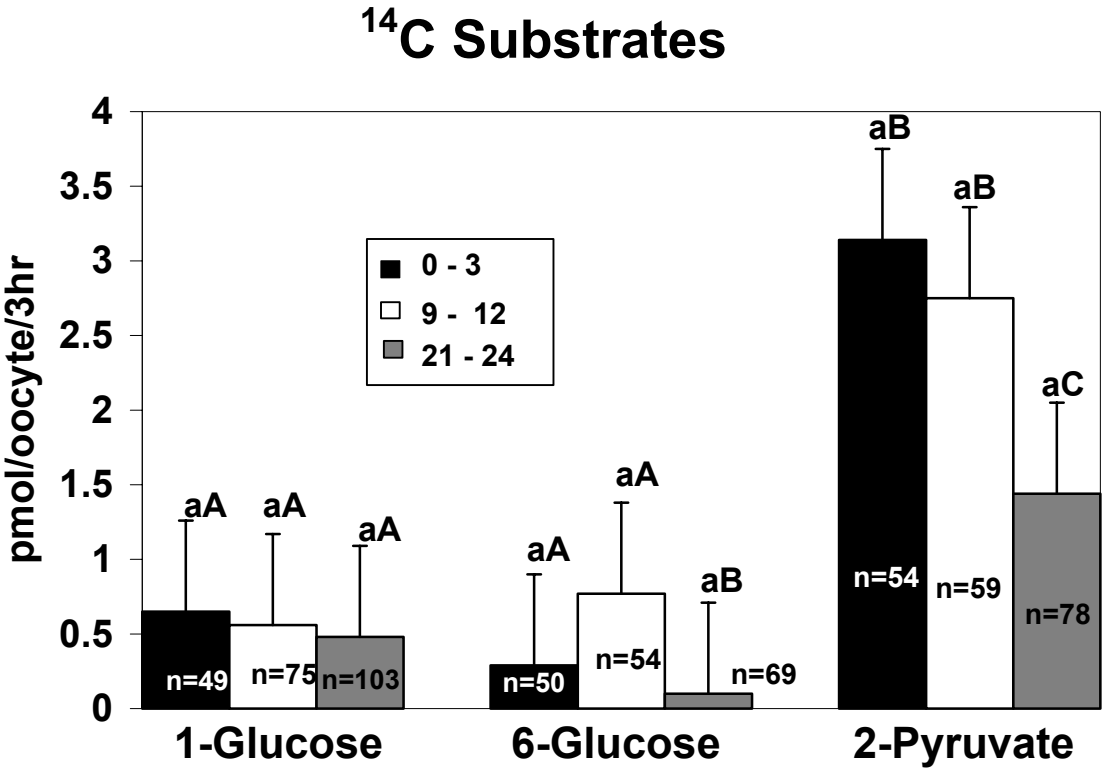

Figure 2-2. The metabolism of 1-^{14}C glucose, 6-^{14}C glucose, and 2-^{14}C pyruvate by sheep oocytes. Individual sheep oocytes were measured at specific time periods during maturation(0-3, 9-12, and 21-24 hours). Lower case letters indicate differences within the same substrate ($p<.05$). Upper case letters indicate differences across substrates ($p<.05$). The number of oocytes measured in a given time period is represented as (n).

the same throughout the entire maturation period until the last time frame when a decrease was observed. From 0-3 hours oocytes consumed 3.14 ± 0.61 pmol/oocyte/3hr of pyruvate, from 9-12 hours they consumed 2.75 ± 0.61 ($p<.01$), and during the last time period pyruvate metabolism dropped to 1.44 ± 0.58 pmol/oocyte/3hr. These values were all significantly higher than the other ^{14}C labeled substrates at all time periods ($p<.001$). Glucose flow through the PPP (indicated by 1-^{14}C glucose) was below 1.0 pmol/oocyte/3hr at all time periods as was glucose flux through the Krebs cycle as indicated by the metabolism of 6-^{14}C glucose.

The effect of retinol on the metabolism of each oocyte was examined by analyzing differences between metabolism of control oocytes and oocytes from retinol-treated ewes, during the same time period within the same substrate. No significant differences existed (data not shown).

Meiotic stage was also assessed for each oocyte (data not shown). Kinetics of meiotic progression from germinal vesicle breakdown (GVBD) to metaphase II (MII) was consistent regardless of the substrate or retinol treatment. After three hours of maturation greater than 90% of the oocytes had progressed to GVBD, after 12 hours they had reached MI, and by 24 hours 70% of the oocytes had progressed to MII.

DISCUSSION

Little information exists concerning sheep oocyte metabolism. Only a few studies have examined the metabolism of *in vivo* matured sheep oocytes (Butler and Williams,

1991; Butler and Williams, 1992; Gardner et al., 1993), and to our knowledge, this is the first in-depth investigation of metabolism of single ovine oocytes over the entire IVM period. We examined the activities of different energy pathways (glycolysis, Krebs, and PPP) of immature and *in vitro* matured sheep oocytes using different combinations of radiolabeled substrates.

Several studies have measured individual oocyte metabolism. Early work in the mouse indicated that a denuded mouse oocyte prefers pyruvate as an energy source (Biggers et al., 1967). Later studies confirmed that pyruvate is essential during mouse oocyte maturation, and it is preferred over glucose (Leese and Barton, 1984; Downs et al., 2002). During *in vitro* maturation of bovine oocytes there is a dependence on pyruvate, but unlike their mouse counterparts, bovine oocytes utilize glucose as well (Rieger and Loskutoff, 1994; Krisher et al., 1999). Studies performed on cat oocytes have demonstrated that these oocytes utilize both glycolysis and oxidation of glucose, glutamine, palmitate, and lactate (Spindler et al., 2000).

In our study, glycolysis values, as indicated by 5-^{3}H glucose utilization during the initial IVM time period (GVBD), were similar to other reported values for denuded oocytes (Rieger and Loskutoff, 1994; Gandolfi et al., 1998; Krisher and Bavister, 1999; Spindler et al., 2000). We observed a dramatic increase in glycolytic metabolism during the middle time period when oocytes reach MI, and then a decrease at the 21-24 hour time period as they reach MII. Glucose uptake, measured by fluorescence techniques in unfertilized sheep ova was consistent with our measurement during the last time period (Butler and Williams, 1992). In contrast, Gardner and colleagues found a much lower glucose uptake in unfertilized sheep ova (Gardner et al., 1993). Only one other study has

evaluated metabolism during the entire maturation period, and that was in bovine oocytes (Rieger and Loskutoff, 1994). Results from this study were different. Specifically, their measurements of bovine oocyte glycolysis were consistently low over the entire maturation period, and higher levels of pyruvate metabolism were observed. However, the concentration of pyruvate used was more than ten times higher than the concentration we used. Since another report showed that decreased levels of pyruvate uptake in bovine oocytes increased glucose utilization via glycolysis (Krisher and Bavister, 1999), this may explain the discrepancy between the two studies. Another possibility includes species differences between the cow and sheep.

Gonadotropin stimulation may also impact the metabolism of 5-^{3}H glucose, as suggested in a study comparing gonadotropin-primed and non-primed mice (Downs and Utecht, 1999). Since the oocytes in our study were recovered from superovulated ewes, this may contribute to differences between our study and others. In addition, higher glycolytic rates may also indicate a hypoxic environment, which limits the use of pyruvate (Leese, 1991). However, this seems unlikely since the oocytes in the present study were matured under atmospheric oxygen conditions.

Oxidation of glucose is measured by the flux of 1-^{14}C glucose and 6-^{14}C glucose through the Krebs cycle. Furthermore, the carbon of the 1-^{14}C glucose is also metabolized via the pentose phosphate pathway (PPP). One study suggests that the PPP is necessary for meiotic induction to occur in the presence of FSH (Downs and Utecht, 1999). In our study utilization of both 1-^{14}C glucose and 6-^{14}C glucose are low throughout the maturation period, which is consistent with results for bovine oocytes (Rieger and Loskutoff, 1994). Proper meiotic progression occurred regardless of the

substrate utilized in our study. Since oxidation of glucose was similar for 1-^{14}C glucose and 6-^{14}C glucose at the middle and early time periods, it can be assumed that both pathways, Krebs and PPP, were being used. During the last time period values for 1-^{14}C glucose were slightly higher than 6-^{14}C glucose, perhaps indicating more flux through the PPP than through the Krebs cycle.

Labeled ^{3}H-glutamine and ^{14}C-pyruvate are also indicators of oxidative metabolism through the Krebs cycle. We observed that glutamine oxidation during the early stages of IVM (GVBD) was higher than both glucose and pyruvate uptake. Bovine oocytes showed lower uptake of glutamine, singly radiolabeled with tritium, than in our study (Rieger and Loskutoff, 1994), but when glutamine was uniformly labeled with ^{14}C (Gandolfi et al., 1998), glutamine oxidation increased, although still lower than our values. When bovine cumulus oocyte complexes were matured in the presence of lutenizing hormone (LH), the denuded oocytes exhibited higher levels of glutamine metabolism (Zuelke and Brackett, 1993). In our study we added additional LH in conjunction with what is present in the fetal bovine serum. Perhaps this extra LH played a role in our higher levels of glutamine oxidation.

Published values for oocyte pyruvate oxidation over the entire maturation period vary greatly, from slightly over 1 pmol/oocyte/3hr (Krisher and Bavister, 1999) to over 30 pmol/oocyte/3hr (Gardner et al., 1993). Values from this study fall within the reported ranges. Some studies have observed that oocytes preferentially utilize pyruvate over glucose during maturation (Rushmer and Brinster, 1973; Leese and Barton, 1984; Rieger and Loskutoff, 1994; Gandolfi et al., 1998; Krisher and Bavister, 1999; Spindler et al., 2000). We too observed that pyruvate utilization during early stages of IVM was

higher than that of 5-^{3}H glucose. Using a microfluorescence technique, Gardner and colleagues examined pyruvate metabolism of mature oocytes collected from the oviducts of ewes. They determined that the pyruvate uptake was 32 pmol/oocyte/3hr (Gardner et al., 1993). In contrast, Butler and Williams, using the same technique, reported much lower values for pyruvate uptake by unfertilized ovine ova (Butler and Williams, 1991). Although our results for an *in vitro* matured oocyte differ from these two papers, the techniques employed may explain some of the dissimilarities (Barnett and Bavister, 1996). In addition, metabolic requirements for *in vivo* derived oocytes have been shown to be different from their *in vitro* matured counterparts (Mermillod et al., 1999; Overström, 2000; Rizos et al., 2002). For example, a report by Khurana and Niemann concluded that glucose metabolism by *in vitro* produced bovine embryos was two-fold higher than their *in vivo* counterparts (Khurana and Niemann, 2000). The buffering system (HEPES versus $NaHCO_3$) employed may alter the metabolic activity of the oocyte, further explaining discrepancies among different studies (Barnett and Bavister, 1996).

This study was the first to evaluate sheep oocyte metabolism over the entire maturation period. Metabolism was chosen as a parameter because it can serve as an indicator of an oocyte's health, and it has been shown that altered oocyte metabolism impacts embryo development (Bavister, 1995; Downs and Utecht, 1999; Krisher and Bavister, 1999; Spindler et al., 2000). We found differences between various substrates during the same time period as well as between different time periods. *In vitro*-matured sheep oocytes preferentially utilized oxidative pathways during early stages of IVM (GVBD) as indicated by elevated levels of glutamine oxidation. In contrast to bovine

oocytes, glucose metabolism during the middle time stage, when oocytes are predominately at MI, was almost entirely derived from glycolysis. However, during this same time period glutamine oxidation also remained high, suggesting the oocytes utilized both pathways. Glutamine oxidation significantly decreased by the 21-24 hour time period, when oocytes reach MII, as did 5-^{3}H glucose metabolism. Unlike their bovine counterparts, sheep oocytes seemed to prefer glucose to pyruvate. The concentration of substrates and metabolic pathways a denuded oocyte uses may vary greatly from the requirements of a cumulus-enclosed oocyte. In addition, since we know IVM conditions are not optimal, these measurements provide only an indication of what substrates and metabolic pathways a sheep oocyte may utilize.

The present study was also performed in order to determine if *in vivo* retinol administration impacted *in vitro* oocyte metabolism. Earlier studies from our laboratory demonstrated that embryos collected from retinol-treated, superovulated ewes had a higher blastocyst developmental rate than did embryos collected from vehicle-treated ewes when cultured *in vitro* (Eberhardt et al., 1999b). In the same study, it was observed from retinol-treated ewes embryos were more competent to progress beyond the 8-16 cell block (Telford et al., 1990) than control embryos. Since maternal factors stored in the egg are recognized to influence the embryo before and after zygotic gene activation (Blondin and Sirard, 1995; Eppig et al., 1996; De Sousa et al., 1998), and the treatment was administered prior to ovulation, it was hypothesized that retinol exerted its affect on the maturing oocyte. However, no significant differences were observed for oocytes from retinol-treated ewes versus oocytes collected from control ewes. Hence, it is concluded that the retinol treatment imposed did not affect oocyte metabolism, overall.

Yet, we are cognizant of the fact that the metabolism assay may not have been sufficiently sensitive to detect differences. The more likely explanation is that positive effects of retinol administration during the final stages of follicular development may be manifested in the embryo, after fertilization. Demonstration of this will require further study and experiments are currently being conducted.

CHAPTER 3

The Effects of Retinol Supplementation on Bovine Embryo Development *In Vitro*

ABSTRACT

Retinoids are recognized as important regulators of vertebrate development, cell differentiation, and tissue function. Previous studies demonstrated that retinol administration to ewes, followed by natural service, resulted in embryos with improved competence to develop *in vitro*. The present study evaluated the *in vitro* effects of retinol addition to media containing maturing oocytes and developing embryos. Bovine oocytes were matured in the presence or absence of varying concentrations of retinol. After a 22-24 hour maturation period the oocytes were fertilized, denuded 18 hours later and cultured in a modified synthetic oviductal fluid (mSOF) in a humidified atmosphere at 38.5°C, 5.5% CO_2, 7% O_2. Cleavage rates as well as the number of eight-cell embryos did not differ between the control and retinol-treated oocytes. The addition of 5μM retinol to the maturation medium (IVM) tended to increase blastocyst formation (blastocyst/putative zygote; 26.1 ± 2.2) compared to the controls (21.9 ± 1.9; p=.07). Cell numbers of day 8 blastocysts did not differ significantly between the two treatments (control, 73.55; 5μM, 79.6). Further analysis revealed when the blastocyst rate fell below 20% in the control group, the 5μM retinol treatment dramatically improved embryonic development, measured by blastocyst/putative zygote rate (14.4 ± 2.1 vs 23.7 ± 2.5; p<.02). The 5μM retinol treatment also enhanced the blastocyst/cleaved rate by nearly

10% (23.7% vs 34.6%; p<.02). The addition of retinol to the embryo culture medium (IVC) did not significantly improve cleavage rates, percent eight-cell, or blastocyst rates, but 5μM retinol significantly increased blastocyst cell number (72.16 ± 5.25 vs 84.78 ± 5.25; p<.03). When 10μM retinol was added to embryos in mSOF, it was detrimental to blastocyst development (25.92% vs 13.92%; p<.002). These studies demonstrate that supplementation of 5μM retinol to the maturation medium may improve embryonic development of bovine oocytes indicated by their increased blastocyst rate, and 5μM retinol addition to the culture medium may improve blastocyst cell number.

INTRODUCTION

Vitamin A (retinol) and its bioactive metabolite, retinoic acid (RA), are essential for reproductive success in both males (Juneja et al., 1966; Jayaram et al., 1973; Livera et al., 2000) and females (Bagavandoss and Midgley, 1988; Keenan, 1997). These lipophilic compounds influence many reproductive steroid hormones such as gonadotropin releasing hormone (Lefèvre et al., 1994), lutenizing hormone and follicle stimulating hormone (Lefèvre et al., 1994; Minegishi et al., 2000). Normal embryonic development also requires a precise, physiological range of retinoids (Bucco et al., 1995; Horton and Maden, 1995; review Maden, 2000; review Ross et al., 2000; review Clagett-Dame and DeLuca, 2002).

β-carotene found in plants and retinyl esters found in animal products provide the primary dietary sources of retinol (Napoli, 1996; Wolf, 1996), and intestinal enzymes convert these precursors to retinal and retinol, respectively. Retinol-binding protein

(RBP) assists in the systemic and intercellular transport of retinol (Blomhoff et al., 1990; Gudas et al., 1994; Wolf, 1996). Cellular retinol binding proteins (CRBPs) and cellular retinoic acid binding proteins (CRABPs) modulate intracellular vitamin A homeostasis by sequestering and stabilizing retinol, only allowing only certain enzymes access to the molecule (Mangelsdorf et al., 1994; Ghyselinck et al., 1999; review Noy, 2000). Several of these cellular retinoid binding proteins have been characterized in reproductive tissues (Gao et al., 1991; Liu et al., 1993; Mackenzie et al., 1997; Zheng et al., 1999; Eberhardt et al., 1999a; Brown et al., 2003). In addition, these proteins along with retinoid-associated enzymes and nuclear retinoic acid receptors (RARs and RXRs) have been localized during preimplantation embryonic development (Horton and Maden, 1995; Rühl et al., 2001; Mohan et al., 2002); thus, suggesting the early embryo can access and utilize retinol and/or its metabolites.

The lack of dietary vitamin A has been associated with reproductive deficiencies such as decreased sizes of ovaries and testes (Ganguly, 1989), decreased steroid biosynthesis (Juneja et al., 1966), increased fetal demise (McCollum, 1964), and most recently abnormal placental apoptosis (Antipatis et al., 2002). The positive effects of vitamin A on reproduction have been reported for several species. For instance, litter-bearing animals receiving either β-carotene or retinol during superovulation displayed an increase in the number of live births (Besenfelder et al., 1993; Coffey and Britt, 1993), and an increase in ovulation rate as well as increased recovery of embryos (Elmarimi et al., 1990). In addition, retinol injections to gilts fed a high-energy diet increased the follicular fluid concentration of progesterone and insulin-like growth factor (IGF-1), both

of which are necessary for normal embryonic development and early pregnancy (Whaley et al., 2000).

Data collected in our lab demonstrated that 1-4 cell and morula stage embryos, collected from the oviduct and uterus, respectively, of all-trans-retinol treated, superovulated ewes exhibited improved capacity to develop to the blastocyst and hatched blastocyst stage during in vitro culture (IVC). In addition, these experiments revealed that most embryos from ewes not treated with retinol (60%) did not progress beyond the eight-cell stage in vitro (Eberhardt et al., 1999b). Despite the numerous *in vivo* studies that have demonstrated the importance of retinoids and its derivatives during embryonic and fetal development (review Hofmann and Eichele, 1994; Neiderreither et al., 1999; Eberhardt et al., 1999b; review Ross et al., 2000; Whaley et al., 2000), few have investigated the effects of *in vitro* retinoid supplementation on preimplantation embryo development (Huang et al., 2001; Duque et al., 2002; Hidalgo et al., 2003; Huang et al., 2003). Furthermore, *in vivo* studies do not allow us to determine if the effect of retinol is on the oocyte, embryo, follicle, reproductive tract and/or its secretions; therefore, *in vitro* experiments were designed to help clarify the effects of retinol.

In the present study, we have investigated the effects of *in vitro* retinol administration to maturing bovine oocytes and early embryos. Results suggest concentration dependent, positive and negative effects on embryonic development.

MATERIALS AND METHODS

Reagents and Media

All chemicals were purchased from Sigma Chemical Company, St. Louis, MO unless otherwise noted. Bovine oocyte collection medium (OCM) was composed of modified M199, 4.2 mM $NaHCO_3$, 12 mM HEPES, and supplemented with 2mM glutamine, 2% fetal bovine serum - FBS (BioWhittaker, Baltimore, MD), and penicillin/streptomycin (Specialty Media, Phillipsburg, NJ). Oocyte maturation medium (OMM) consisted of bicarbonate buffered TCM-199 supplemented with 50 μg/mL of gentamycin, purchased from Specialty Media, 5 μg/mL of FSH purchased from Vetrepharm Inc. (Ontario, Canada), 0.3 μg/mL of lutenizing hormone (LH), generously provided by the USDA, Beltsville, MD, 10% FBS, 0.2 μM sodium pyruvate and 2 mM glutamine. The *in vitro* fertilization (IVF) medium was composed of TALP supplemented with 6 mg/mL of BSA-FAF, 0.2 mM sodium pyruvate, 1.75 U/ml of heparin, and 50 μg/mL of penicillin (10,000 U)/streptomycin (10 mg) (pen/strep). The medium used for *in vitro* culture (IVC) was a modified synthetic oviductal fluid (mSOF) (Tervit et al., 1972) supplemented with 3mg/mL of bovine serum albumin (BSA), 0.6 mM sodium pyruvate, 2% (v/v) BME essential amino acids, 1% (v/v) MEM non-essential amino acids, and 100 μg/mL of penicillin and streptomycin.

All-*trans* retinol was dissolved in 100% ethanol, appropriate dilutions made, and aliquots were stored at -80°C until use. Retinol was prepared fresh each month. The concentration of ethanol during maturation or culture was less than 0.1%.

Collection and In Vitro Maturation (IVM) of Oocytes

For studies on the effects of retinol treatment during oocyte maturation, ovaries from cattle were obtained from an abbatoir and pooled. Cumulus oocyte complexes (COCs) were quickly harvested by slicing healthy follicles (2 –8 mm) with a sterile surgical blade, and collecting them in OCM. Intact COCs with homogeneous ooplasm and two or more layers of cumulus cells were selected, washed, and approximately 50 were transferred to 500 μl of pre-equilibrated OMM, and matured for 22-23 hours in a 38.5°C incubator with an atmosphere of 5.5% CO_2, and humidified air.

For studies on the effect of retinol administration during embryo culture, selected COCs were purchased from BoMed Inc. (Madison, WI). These COCs were shipped overnight in OMM, maintained at 38.5°C by a portable incubator, and arrived mature.

In Vitro Fertilization (IVF)

Fertilization (Day 0) was performed by combining semen from two bulls of proven fertility. Frozen/thawed bull semen were washed in a discontinuous Percoll gradient (45%/90%) by depositing semen on top of the Percoll layers and centrifuged for 15 minutes at 960 *g*. The pellet was removed and resuspended in Sperm-TALP and centrifuged for 8 minutes at 460 *g*. After removal of the supernatant, the sperm sample was reconstituted in 500 μL of IVF-TALP for a final concentration of 1 x 10^6 spermatozoa/mL. To aid in capacitation, 25 μl of PHE (penicillamine, hypotaurine, epinephrine) was added to each well. The plate was incubated for 22 hours at 38.5°C in an atmosphere of 5.5% CO_2 and ambient air with saturated humidity.

In Vitro Culture (IVC)

Approximately 18 hours after fertilization putative zygotes were denuded of cumulus cells by vortexing in 500 μl of HEPES-TALP for four minutes (Day 1). HEPES was supplemented with 3 mg/mL of BSA, 0.2 mM sodium pyruvate, and 50 μg/mL of pen/strep. Putative zygotes (approximately 25-30) were cultured in 500 μL of mSOF for eight days in a 38.5°C incubator in an atmosphere of 5.5% CO_2, 7% O_2, 88% N_2 with saturated humidity. The mSOF medium was changed every 48 hours. Cleavage was assessed on Day 3 and blastocyst rate was calculated on Day 8.

Blastocyst Cell Number

To determine cell number, nuclei of Day 8 blastocysts were stained with 50 μg/ml of *bis*benzamide (Hoechst 33258; Sigma) dissolved in HEPES-TALP. Briefly, 50 μl of Hoechst was added to 10 mL of HEPES-TALP, and blastocysts were placed in 500 μl under a cover protected from light for 20 minutes, and viewed with a 100X objective under UV light. The nuclei of each blastomere were counted with the assistance of the Metamorph Imaging Software (Universal Imaging Corp., PA).

Treatments

In the first experiment maturation medium was supplemented with all-*trans* retinol (0, 1.0, 5.0, and 10.0 μM) and embryo development to the hatched blastocyst stage (Day 8) was recorded. In the second experiment all-*trans* retinol was added only to

embryo culture medium (0, 1.0, 2.0, 5.0 and 10 μM) on days 1, 3, 5, and 7 and embryo development to the hatched blastocyst stage (Day 8) was recorded.

Data Analysis

Data were checked for normality and analyzed using an incomplete block design, blocked on plate with mixed model ANOVA methods in SAS (2000), as well as randomized block design for the IVC experiments. Differences were detected using Fisher's protected least significant difference. Contrasts were used to test if the average of retinol treatments differed from the control. A probability of $p<.05$ was considered significant for all statistical tests.

RESULTS

The first experiment assessed the effects of retinol addition during IVM and represented twenty-seven replicates. The addition of 5μM retinol during IVM tended to improve ($p=.07$) embryonic development to the blastocyst stage, compared to controls (Table 3-1). The control blastocyst rate was 21.9% compared to 26.1% in 5 μM retinol. Addition of 1μM retinol to the maturation medium did not appear to affect embryonic development compared to controls. 10μM retinol increased blastocyst development, although not significantly. Cleavage rate, 8–16 cell, and cell number of blastocysts did not differ among the four treatments.

Further analysis (13 replicates) revealed (Table 3-2) that if development to the blastocyst stage of controls was below 20%, the 5μM retinol treatment dramatically

Table 3-1. Effect of all-*trans* retinol addition to bovine oocyte maturation medium (mean ± S. E. M.) on embryo development. The number of blastocysts counted is represented by (n).

Retinol concentration (μM)	Putative zygote (n)	Cleavage	8 – 16 cell/putative zygote	Blastocyst/ putative zygote	Blastocyst/ cleaved	Nuclei number (n)
0	1095	66.7 ± 2.7	46.1 ± 2.6	21.9 ± 1.9	32.8 ± 2.2	73.6 (78)
1.0	464	65.5 ± 3.9	47.9 ± 3.6	20.4 ± 2.6	31.7 ± 3.1	91.5 (14)
5.0	1069	68.3 ± 3.2	50.8 ± 3.0	26.1 ± 2.2	37.1 ± 2.5	79.6 (110)
10.0	508	70.1 ± 3.9	46.3 ± 3.7	24.2 ± 2.7	33.8 ± 3.1	71.6 (15)

(n) represents the total number of blastocysts counted.
Values are listed as percentages.

Table 3-2. Effect of all-*trans* retinol addition to bovine oocyte maturation medium on embryo development among replicate groups where less than 20% of control embryos reached the blastocyst stage (mean ± S. E. M.).

Retinol concentration (μM)	Putative zygote (n)	Cleavage	8 – 16 cell/putative zygote	Blastocyst/ putative zygote	Blastocyst/ cleaved
0	516	62.7 ± 3.9	39.4 ± 3.2	14.4 ± 2.1[b]	23.7 ± 2.6[b]
1.0	185	60.0 ± 6.0	37.0 ± 5.1	15.9 ± 3.4[ab]	26.1 ± 4.2[ab]
5.0	530	65.9 ± 4.6	45.9 ± 3.9	23.7 ± 2.5[a]	34.6 ± 3.1[a]
10.0	183	63.3 ± 6.7	35.0 ± 5.7	17.6 ± 3.8[ab]	26.7 ± 4.6[ab]

[ab] Means in the same column with different supercripts are different ($p<.05$).
Values are listed as percentages.

improved ($p<.02$) embryo development (14.4 % vs. 23.7%). When expressed as blastocyst/cleaved the 5μM retinol treatment ($p<.02$) also showed a significant improvement in blastocyst development. Neither 1μM nor 10μM retinol treatment improved embryonic development when compared to those controls that did not achieve a 20% blastocyst rate.

The addition of retinol during IVC only (13 replicates) (Table 3-3), did not improve development to the blastocyst stage compared to controls, at any concentration tested. In fact, a concentration-dependent inhibition of blastocyst development was observed with 5μM and 10μM retinol treatments compared to controls, 1 μM, and 2μM retinol treatments. Conversely, the 5μM retinol treatment increased blastocyst nuclei number ($p<.03$) compared to controls. Cleavage rates as well as development to the 8-cell stage did not differ significantly among embryos treated with and without retinol during culture.

DISCUSSION

In vivo studies performed with laboratory animals (Elmarimi et al., 1990; Besenfelder et al., 1993) and large domestic animals (Coffey and Britt, 1993; Shaw et al., 1995; Eberhardt et al., 1999b; Whaley et al., 2000) have demonstrated that retinol administration prior to ovulation positively impacts preimplantation embryonic development. Previous work in our laboratory provided evidence that retinol administration to ewes, during superovulation, improved embryonic development *in vitro*, and may have been responsible for helping embryos progress beyond the 8-16 cell

Table 3-3. Effects of all-*trans* retinol addition to the culture medium during embryo development only (mean ± S. E. M.).

Retinol concentration (μM)	Putative zygote (n)	Cleavage	8 – 16 cell/putative zygote	Blastocyst/ putative zygote	Blastocyst/ cleaved	Nuclei number (n)
0	567	86.1 ± 2.5	68.2 ± 2.5	26.5 ± 2.4^{ab}	30.7 ± 2.6^{a}	72.2 (47)^{b}
1.0	312	84.7 ± 3.2	67.3 ± 3.3	27.1 ± 3.2^{ab}	32.1 ± 3.5^{a}	76.9 (48)^{ab}
2.0	414	85.3 ± 2.9	69.1 ± 2.9	28.8 ± 2.8^{a}	34.1 ± 3.1^{a}	80.4 (21)^{ab}
5.0	303	80.8 ± 3.2	63.9 ± 3.3	20.2 ± 3.2^{bc}	25.4 ± 3.5^{ab}	84.8 (39)^{a}
10.0	388	81.2 ± 3.0	63.4 ± 3.1	13.5 ± 3.0^{c}	16.2 ± 3.3^{b}	78.3 (21)^{ab}

^{ab} Means in the same column with different supercripts are different ($p<.05$).
(n) represents the total number of blastocysts counted.
Values are listed as percentages.

stage (Eberhardt et al., 1999b), a critical time period when embryos typically block *in vitro* (Telford et al., 1990). Since retinol was administered prior to ovulation, maturing oocytes within ovulatory follicles were considered likely targets of retinoid action. Therefore, over 3000 bovine oocytes were used to evaluate the effects of retinoid supplementation during IVM and IVC on embryonic development to the blastocyst stage.

Maturation of bovine oocytes in the presence of 1μM and 10μM retinol did not increase nor decrease the blastocyst rate; however, 5μM retinol tended to improve blastocyst rate when compared to control oocytes (p=.07) (Table 3-1). A more dramatic effect of the 5μM retinol treatment was observed if control embryos did not achieve a 20% blastocyst rate. In this case, 5μM retinol increased blastocyst development more than 10% (Table 3-2). These results suggest that retinol may not be helping an oocyte already capable of progressing, but rather it is improving the viability of an otherwise incompetent oocyte.

During growth, within the follicle, the oocyte accumulates RNA and proteins in order to complete nuclear and cytoplasmic maturation, progress through fertilization, its early cleavage stages, and the maternal to embryonic gene transition (review Brevini-Gandolfi and Gandolfi, 2001). It is possible that retinol may be impacting nuclear and/or cytoplasmic maturation in a way that is beneficial to the developing cumulus oocyte complex and/or embryo. Vitamin A administration to gilts, fed a high-energy diet, increased the average developmental stage of oocytes, specifically decreasing the number arrested at germinal vesicle stage (Whaley et al., 2000).

Recently, two experiments from the same laboratory demonstrated that pretreatment of bovine oocytes with 9-*cis*-retinoic acid, prior to IVM and resumption of

meiosis, improved blastocyst development (Duque et al., 2002) and pregnancy rates (Hidalgo et al., 2003). Conclusions made from these data suggest that retinoic acid may impact oocyte cytoplasmic maturation, an important event, which must occur during maturation (Eppig et al., 1994; Combelles et al., 2002).

Although vitamin A is essential for normal embryonic development, both excess and deficiency result in abnormal development (Moore, 1957; Wellik and DeLuca, 1995; Huang et al., 2001; Antipatis et al., 2002; Niederreither et al., 2002; Huang et al., 2003). Results from the present study illustrate the requirement for tight regulation of retinol concentrations and indicate that the developing oocyte, within its cumulus investment, and the early embryo respond differently to varying concentrations of retinol.

In this study, 5μM retinol treatment applied during IVM improved embryonic development, and 10μM retinol did not reveal any negative effects. In contrast, 10μM supplementation during IVC was detrimental to embryonic development (Table3-3). Furthermore, 5μM retinol treatment during IVC was detrimental compared to 2μM retinol (Table 3-3). Cumulus cells play a vital role in nuclear and cytoplasmic oocyte maturation, metabolism and conversion of nutrients, and they relay important information (i.e. hormonal and growth factor signaling) between the oocyte and its surrounding follicle (Downs et al., 1997; Tanghe et al., 2002; Sutton et al., 2003). In this study, the cumulus cells may have provided a layer of protection to the maturing oocyte, whereas the denuded embryo was more susceptible to higher concentrations of retinol (10μM and 5μM) in the culture medium.

Even though 5μM retinol administered during IVC decreased blastocyst development compared to controls, it improved blastocyst cell number (Table 3-3). A

recent study demonstrated that 9-*cis*-retinoic acid influences the ratio of the inner cell mass and trophectoderm cells in the blastocyst stage embryo (Hidalgo et al., 2003). Furthermore, several studies have shown that retinoids, retinoic acid in particular, are anti-apoptotic depending on the tissue and concentration (Moreno-Manzano et al., 1999; Zhou et al., 1999; Konta et al., 2001). Retinoic acid can also activate mitosis via the mitogen activated protein kinase pathways (MAPK) (Koshimizu et al., 1995; Morita and Tilly, 1999). Apoptosis during early development is a necessary event in order for the embryo to differentiate properly (Hardy, 1999; Matwee et al., 2000; Van Soom et al., 2002). Although retinol increased the cell number of the blastocyst stage embryo this may not be beneficial, (Van Soom et al., 2002) especially if it is influencing cells normally programmed to undergo apoptosis.

The favorable effects of retinol on *in vitro* bovine blastocyst development may be modulated by growth factors. Since embryo development is characterized by rapid cellular proliferation and cellular differentiation, growth factors are essential for this process (Bavister, 1995; Kane et al., 1997; Teruel et al., 2000). Evidence exists that retinoids affect certain growth factor expression levels. For example, midkine, a member of the heparin-binding growth/differentiation family is induced by retinoic acid, and it has been found to improve bovine oocyte and embryo developmental competence (Ikeda et al., 2000; Ikeda et al., 2000). In addition, retinoic acid was found to regulate epidermal growth factor receptor expression (EGFR), (Roulier et al., 1994) and transforming growth factor (TGF-ß) and insulin-like growth factor binding protein (IGFBP3) expression (Han et al., 1997).

Our results strongly suggest that 5μM retinol addition may modify the environment in which the oocyte is maturing; thus, allowing "incompetent" oocytes to be fertilized and develop to the blastocyst stage. One hypothesis is that this retinol-induced alteration may decrease oxidative stress. According to some, the leading cause of early embryonic loss during IVC may be oxidative stress, generated by reactive oxygen species (ROS) (Takahashi et al., 2000; Guérin et al., 2001). The deleterious effects of ROS on preimplantation embryos include protein, lipid, and nucleic acid damage, mitochondrial alterations, cell cycle block, ATP depletion, and excessive apoptosis (Salas-Vidal et al., 1998; Guérin et al., 2001). Several reports have demonstrated positive effects of antioxidants such as Mn-SOD and/or catalase (Liu and Foote, 1995; Dinara et al., 2001; Orsi and Leese, 2001), vitamins E and C (Olson and Seidel Jr, 2000; Wang et al., 2002), and glutathione (Luvoni et al., 1996; de Matos and Furnus, 2000; de Matos et al., 2002) during IVM and IVC.

Certain studies have highlighted the importance of culturing embryos in a low oxygen atmosphere (5-7%) to increase blastocyst development (Pabon et al., 1989; Voelkel et al., 1992; Liu and Foote, 1995; Takahashi et al., 2002). Since oocyte maturation *in vitro* occurs in a 20% oxygen atmosphere, but embryo culture takes place in a lower oxygen atmosphere (7%), this may partially explain why retinol has a positive effect during maturation but not during embryo culture. Furthermore, it may help elucidate why 5μM and 10μM retinol appear detrimental to the early embryo but not the oocyte. Studies conducted during oocyte and embryonic development have demonstrated negative effects of retinoic acid (Huang et al., 2001; Huang et al., 2003).

The positive effects of retinol on early embryonic development have been observed both *in vivo* when administered to ewes prior to ovulation (Eberhardt et al., 1999b), and in the present study when provided during bovine *in vitro* maturation (Tables 3-1 and 3-2). Retinol may be imparting developmental competence to an otherwise incompetent oocyte through several mechanisms such as modulating growth factors, affecting developmental gene expression, or by an antioxidant mechanism that decreases free radical production in the culture system. In addition, with an increase in blastocyst cell number (Table 3-3) retinol may be acting via retinoic acid to decrease programmed cell death in the embryo. Future studies will be aimed at dissecting the pathways that retinol and its metabolites utilize to improve early embryonic development.

CHAPTER 4

The Effects of Retinol on *In Vitro* Matured Bovine Oocyte Glutathione Content and its Combinatorial Role with Cysteamine During *In Vitro* Bovine Embryonic Development

ABSTRACT

Retinoids serve important roles in many diverse biological functions such as cell growth, morphogenesis, differentiation, and reproduction. Previous studies demonstrated that retinol administration to ewes, followed by natural service, resulted in embryos with improved competence to develop *in vitro*. Glutathione is an important compound found in oocytes and embryos. The present study evaluated the *in vitro* effects of retinol or cysteamine addition to oocyte maturation medium on the glutathione content of mature bovine oocytes. In addition, the combinatorial effects of retinol and cysteamine addition to the maturation medium (IVM) and/or embryo culture (IVC) were assessed. Bovine oocytes were matured in the presence or absence of retinol, denuded, and the glutathione content measured. Results did not show a difference between oocytes matured in the presence or absence of retinol, respectively (4.02 vs 3.98 pmol/oocyte); however, 100μM cysteamine addition during IVM doubled the glutathione content (8.37 pmol/oocyte). In the second experiment, bovine oocytes were matured in the presence or absence of retinol and/or cysteamine. After IVM the oocytes were fertilized, denuded (Day 1), and cultured in a modified synthetic oviductal fluid (mSOF) in a humidified atmosphere at 38.5°C, 5.5% CO_2, 7% O_2. Combinations of retinol and/or cysteamine were added during IVC on

Day 1. Cleavage rates were similar in all treatments except when retinol and cysteamine were both added during IVM and IVC, which displayed a decrease in cleavage. In contrast, this same treatment combination had the highest blastocyst development rate (22.7%), and the highest blastocyst/cleaved rate (38.3%), which was significantly different from the controls. Control blastocyst rates were 14.7% as were treatments that included retinol and cysteamine during IVC (14%), or only retinol during IVM 14.1%).

INTRODUCTION

In vitro culture conditions impose many different stressors on preimplantation embryos such as increased metabolic and osmotic stress, altered pH levels (Lane, 2001), and oxidative stress (review Guérin et al., 2001). Oxidative stress defined as "a disturbance in the prooxidant/antioxidant system" (Sies, 1995), is perhaps the leading cause of early embryonic loss, particularly during *in vitro* culture (review Takahashi et al., 2000; Guérin et al., 2001). Recently, investigators discovered that reducing levels of free radicals during *in vitro* culture improved embryonic development (Iwata et al., 1998; review Guérin et al., 2001; review Lane, 2001; de Matos et al., 2002; review Dröge, 2002).

Reactive oxygen species (ROS) are generated during normal cellular processes resulting in the production of superoxide anion, hydrogen peroxide, and hydroxyl radicals (Gutteridge, 1995; review Dröge, 2002). Endogenous sources of ROS during embryo development include oxidative phosphorylation, NADPH oxidase (produces superoxide anion and hydrogen peroxide), and xanthine oxidase (degrades purine nucleotides). Exogenous sources that may result in production of ROS include high oxygen

concentration, metallic cations, visible light, and sperm (Salas-Vidal et al., 1998; review Guérin et al., 2001; review Dröge, 2002). Deleterious effects of ROS on preimplantation embryos include protein, lipid, and nucleic acid damage, mitochondrial alterations, cell cycle block, ATP depletion, and excessive apoptosis (Salas-Vidal et al., 1998; Guérin et al., 2001). Furthermore, increased oxygen radicals in mouse embryos cultured *in vitro* are implicated in developmental blockage that is common during *in vitro* culture (Goto et al., 1993).

Glutathione (GSH) is the major non-protein sulphydryl compound found in mammalian cells responsible for strong basal ROS scavenging activity (Dröge, 2002), and several reports highlight the importance of maintaining adequate glutathione levels in both oocytes and early embryos. For example, mouse embryos chemically depleted of GSH exhibited retarded development, which was reversed with exogenous GSH (Gardiner et al., 1998). Buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, has been shown to compromise blastocyst development of 6-8 cell bovine embryos (Takahashi et al., 1993). Further studies confirmed the beneficial effects of maintaining GSH levels during IVM (bovine, mouse, pig) on subsequent embryo development (Luvoni et al., 1996; Furnus et al., 1998; Knappen et al., 1999; de Matos and Furnus, 2000; Shi et al., 2000; Brad et al., 2003).

GSH is a tripeptide thiol composed of gluatmic acid, cysteine, and glycine. It is synthesized in the γ-glutamyl pathway, and cysteine availability is a rate-limiting step (Deplancke and Gaskins, 2002). Outside the cell, cysteine is unstable and rapidly oxidized to cystine, a cysteine dimer, in culture medium (Takahashi et al., 1993; Takahashi et al., 2002). Some cell types, including bovine embryos, exhibit low cystine

transport activity, which may negatively impact GSH synthesis (Takahashi et al., 1993). Thiol compounds, such as ß-mercaptoethanol (ß-ME) and cysteamine, reduce cystine to cysteine and promote uptake of both compounds. Addition of ß-ME or cysteamine to culture medium of maturing oocytes, or embryos has resulted in increased glutathione content and improved embryonic development of several species (Gasparrini et al., 2000; de Matos et al., 2002; Takahashi et al., 2002; de Matos et al., 2003).

Retinoids play important roles in bone formation, pattern development, and overall reproductive success (Gudas et al., 1994; Hofmann and Eichele, 1994; Napoli, 1996; Wolf, 1996). Ultimately, the metabolites of vitamin A (retinol), all-*trans*-retinoic acid and 9-*cis*-retinoic acid, together with their receptors (RARs and RXRs), regulate retinoid action at the level of gene expression (Wendling et al., 1999; Konta et al., 2001; review Ross et al., 2001). Vitamin A deficiencies in animals (Moore, 1957; McCollum, 1964; Ross et al., 2000), as well as supplementation with retinol, have clearly defined the importance of this vitamin during early embryonic and fetal development (Wellik and DeLuca, 1995; Besenfelder et al., 1996; Eberhardt et al., 1999b; Whaley et al., 2000). Under certain conditions vitamin A and carotenoids participate in a biological antioxidant network, and have been implicated as important regulators of redox signaling pathways (Imam et al., 2001). Specifically, carotenoids quench singlet oxygen molecules as well as interact with other antioxidant compounds (Olson, 1993).

Studies have demonstrated the beneficial effects of *in vivo* retinoid supplementation to litter-bearing species (Elmarimi et al., 1990; Besenfelder et al., 1996), as well as during *in vitro* preimplantation embryonic development (Hidalgo et al., 2002; Livingston et al., 2002; Hidalgo et al., 2003). In other cell lines retinoids, particularly

retinoic acid, protected cells from oxidative stress-induced apoptosis (Ahlemeyer and Krieglstein, 1998; Ahlemeyer et al., 2001), and inhibited glutathione depletion by preserving the antioxidant, superoxide dismutase (SOD) levels (Ahlemeyer and Krieglstein, 2000). Furthermore, another report provided evidence that retinoids modulate glutathione content in certain cell lines (Texeira et al., 1996). Retinoids suppress apoptosis in a number of cell lines by different mechanisms. For example, a study conducted in mesangial cells exposed to hydrogen peroxide (H_2O_2) demonstrated the anti-apoptotic effects of RA by inhibiting c-Jun NH_2-terminal kinase (JNK) and activating protein (AP-1) (Moreno-Manzano et al., 1999). A retinoic acid response element (RARE) was also found in the promoter region of glutathione transferase pi (GSTp) (Lo and Ali-Osman, 1997), an important enzyme that participates in detoxification of foreign compounds. Additionally, GSTp has also been found to regulate JNK signaling (Adler et al., 1999).

From these studies we hypothesized that retinol may aid in the conversion of cystine to cysteine or promote its uptake; thereby, increasing glutathione levels and improving embryonic development. In the first experiment we measured the glutathione content of *in vitro* matured bovine oocytes after retinol or cysteamine supplementation during IVM. Secondly, we evaluated the combinatorial effects of retinol and cysteamine supplementation during IVM and/or IVC on bovine preimplantation embryonic development.

MATERIALS AND METHODS

Reagents and Media

All chemicals were purchased from Sigma Chemical Company, St. Louis, MO unless otherwise noted. Bovine oocyte collection medium (OCM) was composed of modified M199, 4.2 mM $NaHCO_3$, 12 mM HEPES, and supplemented with 2mM glutamine, 2% fetal bovine serum - FBS (BioWhittaker, Baltimore, MD), and penicillin/streptomycin – pen/strep (Specialty Media, Phillipsburg, NJ). Oocyte maturation medium (OMM) consisted of bicarbonate buffered TCM-199 supplemented with 50 μg/mL of gentamycin, purchased from Specialty Media, 5 μg/mL of FSH purchased from Vetrepharm Inc. (Ontario, Canada), 0.3 μg/mL of lutenizing hormone (LH) (generously provided by the USDA, Beltsville, MD), 10% FBS, 0.2 μM sodium pyruvate and 2 mM glutamine. The *in vitro* fertilization (IVF) medium was composed of TALP supplemented with 6 mg/mL of BSA-FAF, 0.2 mM sodium pyruvate, 1.75 U/ml of heparin, and 50 μg/mL of penicillin (10,000 U)/streptomycin (10mg) (pen/strep). The medium used for *in vitro* culture (IVC) was a modified synthetic oviductal fluid (mSOF) (Tervit et al., 1972) supplemented with 3mg/mL of BSA, 0.6 mM sodium pyruvate, 2% (v/v) BME essential amino acids, 1% (v/v) MEM non-essential amino acids, and 100 μg/mL of penicillin and streptomycin.

All-*trans* retinol was dissolved in dimethylsulfoxide (DMSO), cysteamine was dissolved in water, and both were stored at -20°C until use. Retinol and cysteamine were prepared fresh each week. The concentration of DMSO during maturation or embryo culture did not exceed 0.1%.

Collection and In Vitro Maturation (IVM) of Oocytes

For studies on the effects of retinol and cysteamine treatment during oocyte maturation, ovaries from cattle were obtained from an abbatoir and pooled. Cumulus oocyte complexes (COCs) were quickly harvested by slicing healthy follicles (2–8 mm) with a sterile surgical blade and collecting them in OCM. Intact COCs with homogeneous ooplasm and two or more layers of cumulus cells were selected, washed, and approximately 50 were transferred to 500 µl of pre-equilibrated OMM, and matured for 22-23 hours in a 38.5°C incubator with an atmosphere of 5.5% CO_2, ambient air, and saturated humidity.

In Vitro Fertilization (IVF)

Fertilization (Day 0) was performed by combining semen from two bulls of proven fertility. Frozen/thawed bull semen were washed in a discontinuous Percoll gradient (45%/90%) by depositing semen on top of the Percoll layers and centrifuged for 15 minutes at 960*g*. The pellet was removed and resuspended in Sperm-TALP and centrifuged for 8 minutes at 460*g*. After removal of the supernatant, the sperm sample was reconstituted in 500 µL of IVF-TALP for a final concentration of 1 x 10^6 spermatozoa/mL. To aid in capacitation, 25 µl of PHE (penicillamine, hypotaurine, epinephrine) was added to each well. The plate was incubated for 22 hours at 38.5°C in an atmosphere of 5.5% CO_2 and ambient air with saturated humidity.

In Vitro Culture (IVC)

Approximately 18 hours after fertilization putative zygotes were denuded of cumulus cells by vortexing in 500 µl of HEPES-TALP for approximately four minutes (Day 1). HEPES was supplemented with 3mg/mL of BSA, 0.2mM sodium pyruvate, and 50µg/mL of pen/strep. Putative zygotes (approximately 25-30) were cultured in 500 µL of mSOF for eight days in a 38.5°C incubator in an atmosphere of 5.5% CO_2, 7% O_2, 88% N_2 with saturated humidity. The mSOF medium was changed on Days 1, 3, and 5. Cleavage was assessed on Day 3 and blastocyst rate was calculated on Day 8.

Experiment 1: Assay of Glutathione Content

The glutathione (GSH) content of *in vitro,* matured bovine oocytes was measured to assess the effects of retinol and cysteamine. Briefly, cumulus oocyte complexes were matured in the presence or absence of 5µM all-*trans*-retinol or 100µM cysteamine. Approximately 22-24 hours after IVM, oocytes were stripped of cumulus cells by vortexing in 500 µl of HEPES-TALP supplemented with hyaluronidase (50 µg/ml) for approximately four minutes. The oocytes (30) were washed three times in stock buffer (0.2 M sodium phosphate-0.001 M sodium EDTA, pH 7.2), and transferred to a microcentrifuge tube in 5µl. Phosphoric acid (1.25M, 5µl) was added to achieve a final concentration of 0.625 M H_3PO_4. The oocytes were immediately frozen (-20°C).

The GSH assay described is a modification based on the recycling assay of the enzyme 5,5-dithiobis-(2-nitrobenzoic acid)-glutathione reductase (DTNB-GSSG), described by Tietze (Tietze, 1969) and later modified by Calvin (Calvin et al., 1986) and Anderson (Anderson, 1985). The method explained here is most similar to that of

Anderson (Anderson, 1985). The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): 5,5-dithiobis-(2-nitrobenzoic acid - DTNB), phosphoric acid (H_3PO_4), sodium phosphate hydrated ($NaH_2PO_4H_2O$), sodium phosphate (Na_2HPO_4), ethylenediaminetetraacetic acid (EDTA), reduced glutathione, and β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), and glutathione reductase.

Samples were thawed, centrifuged, and microscopically evaluated to ensure complete lysis of the oocytes. To each sample, 700µl of the NADPH solution (1mL of stock buffer added to 1mg bottle of NADPH), 100µl of 6mM DTNB, 180µl of distilled water, and 10µl of glutathione reductase (266U/ml) were quickly added to a disposable cuvette, placed in a Beckman single-beam DU-640 (Irvine, CA) spectrophotometer, and the increase in absorbance at 412nm was measured every 30 seconds for 2 minutes. Each blank included 5µl of water and 5µl of phosphoric acid, and did not show detectable amounts of GSH-GSSG. Four glutathione (GSH) standards were made fresh (1nmol, 0.5nmol, 0.25nmol, and 0.05nmol), in order to calculate the total GSH content of oocytes based on a standard curve (Tietze, 1969; Anderson, 1985; Calvin et al., 1986).

Experiment 2: Combinatorial Effect of Retinol and Cysteamine

Different combinations of retinol and/or cysteamine were chosen to evaluate and clarify the effect of retinol and cysteamine on *in vitro* blastocyst development. Treatments were added during IVM (Day –1), and after the putative zygotes were denuded (Day 1) during IVC. The mSOF media was changed on Days 1, 3, and 5. Observations from preliminary experiments showed that treatment addition after Day 1

negatively impacted embryonic development; therefore, treatment was only added on Day 1. Ten experimental groups were designed. In the first experimental group, only maturation medium and culture medium were used. In those experimental groups that contained retinol a diluent control was employed, which consisted of DMSO dissolved in either OMM of mSOF.

1.) 0μM retinol and cysteamine and 0μM retinol or cysteamine during IVM and IVC, respectively (-/-).
2.) 0μM cysteamine or retinol and 50μM cysteamine during IVM and IVC, respectively (-/C).
3.) 0μM cysteamine or retinol and 2.5μM retinol during IVM and IVC, respectively (-/R).
4.) 0μM cysteamine or retinol and 2.5μM retinol and 50μM cysteamine during IVM and IVC, respectively (-/R+C).
5.) 100μM cysteamine and 0μM retinol and cysteamine during IVM and IVC, respectively (C/-).
6.) 100μM cysteamine and 50μM cysteamine during IVM and IVC, respectively (C/C).
7.) 2.5μM retinol and 0μM retinol and cysteamine during IVM and IVC, respectively (R/).
8.) 2.5μM retinol and 2.5μM retinol during IVM and IVC, respectively (R/R).
9.) 2.5μM retinol and 100μM cysteamine and 0μM retinol and cysteamine during IVM and IVC, respectively (R+C/-).

10.) 2.5μM retinol and 100μM cysteamine and 2.5μM retinol and 50μM cysteamine during IVM and IVC, respectively (R+C/R+C).

Data Analysis

Data were checked for normality and analyzed using a randomized block design with mixed model ANOVA methods in SAS (2000). Differences were detected using Fisher's protected least significant difference. Contrasts were used to test if the average of the treatments differed from the control. A probability of $p<.05$ was considered significant for all statistical tests.

RESULTS

Intracellular glutathione content of more than 600 bovine oocytes was measured to evaluate the effects of retinol or cysteamine addition during IVM. Results revealed that the GSH content of bovine oocytes matured in the presence of 5μM retinol was similar to control oocytes (4.02 vs 3.98 pmol/oocyte – Figure 4-1). However, glutathione content of oocytes that were matured in the presence of 100μM cysteamine was more than double than that of controls (8.37 pmol/oocyte; $p=0.06$).

More than 1200 oocytes were used in five independent replicates to evaluate the effects of retinol and/or cysteamine on bovine embryonic development. All treatment combinations had similar cleavage rates except for R/- and R+C/R+C, which exhibited significantly lower cleavage (Table 4-1). Development to the 8-16 cell stage did not

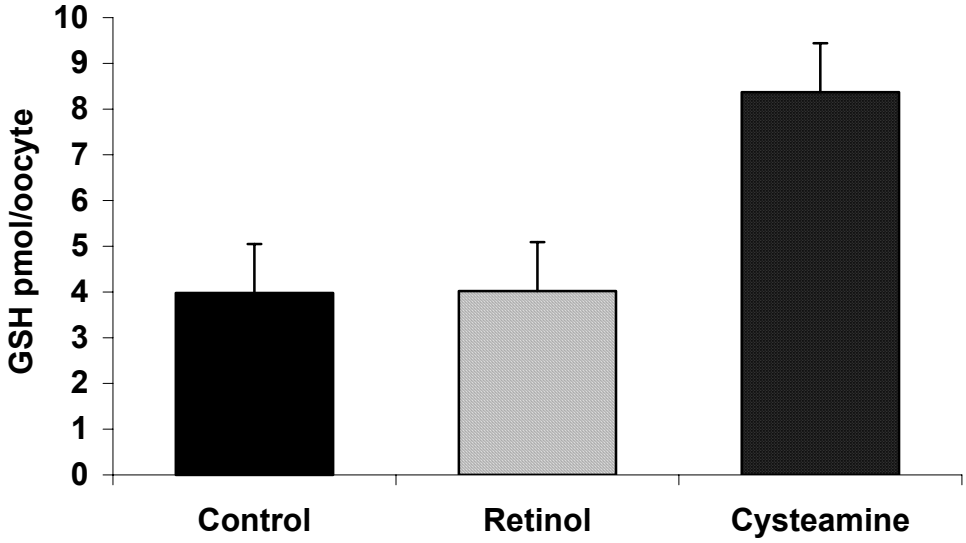

Figure 4-1. Glutathione levels of *in vitro* bovine oocytes matured in the presence of retinol, cysteamine, or maturation medium only.

Table 4-1. Effect of all-*trans* retinol (R) and cysteamine (C) during bovine IVM and IVC (mean ± S. E. M.)

Treatment IVM / IVC	Putative zygote (n)	Cleavage	8 – 16 cell/putative zygote	Blastocyst/ putative zygote	Blastocyst/ cleaved
- / -	118	72.6 ± 5.3^{a}	45.2 ± 5.9^{abc}	14.7 ± 4.1^{ab}	21.2 ± 4.9^{b}
- / C	117	74.3 ± 5.3^{ab}	50.7 ± 5.9^{abc}	17.8 ± 4.1^{ab}	24.1 ± 4.9^{b}
- / R	128	73.6 ± 5.3^{ab}	57.5 ± 5.9^{a}	20.8 ± 4.1^{ab}	27.0 ± 4.9^{ab}
- / R+C	125	72.9 ± 5.3^{abc}	57.1 ± 5.9^{a}	14.0 ± 4.1^{ab}	17.6 ± 4.9^{b}
C / -	121	76.4 ± 5.3^{a}	56.2 ± 5.9^{ab}	19.9 ± 4.1^{ab}	25.7 ± 4.9^{ab}
C / C	117	76.5 ± 5.3^{a}	55.3 ± 5.9^{ab}	19.5 ± 4.1^{ab}	25.9 ± 4.9^{ab}
R / -	118	60.0 ± 5.3^{bc}	39.3 ± 5.9^{bc}	14.1 ± 4.1^{ab}	23.4 ± 4.9^{b}
R / R	118	68.9 ± 5.3^{abc}	48.3 ± 5.9^{abc}	10.9 ± 4.1^{b}	15.2 ± 4.9^{b}
R+C / -	125	69.8 ± 5.9^{abc}	37.4 ± 6.7^{bc}	19.2 ± 4.6^{ab}	26.7 ± 5.5^{ab}
R+C / R+C	152	58.2 ± 5.3^{c}	37.4 ± 5.9^{c}	22.7 + 4.1^{a}	38.3 ± 4.9^{a}

^{abc} Means in the same column with different supercripts are different ($p<.05$).
Values are listed as percentages.

exhibit significant differences when compared to controls, but was numerically higher when retinol alone (-/R) was added during IVC or retinol with cysteamine (-/R+C). Similar development to the 8-16 cell stage was observed if cysteamine was present during IVC (-/C) or IVMC (C/C). Retinol and cysteamine addition during IVM (R+C/ -) or during IVMC (R+C/R+C), reduced 8-16 cell development; however, the treatment combination of R+C/R+C exhibited the highest blastocyst development (22.7%).

Retinol and cysteamine addition (R+C/-) during IVM had a blastocyst rate of 19.2%, which was comparable to cysteamine treatment alone during maturation (C/-), retinol treatment alone during IVC (-/R), and cysteamine treatment during maturation and culture (C/C). Control blastocyst rate (-/-) and retinol treatment alone during IVM (R/-) had similar values (14.7 and 14.0, respectively). Retinol addition during IVMC (R/R) had the lowest blastocyst rate (10.9%). This was significantly lower than the R+C/R+C treatment combination ($p<.05$).

Retinol and cysteamine together during IVM and IVC (R+C/R+C) showed a higher blastocyst/cleaved rate than controls (-/-), cysteamine alone during IVC (-/C), retinol alone during IVM (R/-), retinol treatment during IVMC (R/R), and retinol and cysteamine during IVC (-/R+C).

DISCUSSION

Recent studies by our laboratory (Eberhardt et al., 1999b; Livingston et al., 2002) and others (Whaley et al., 2000; Duque et al., 2002; Hidalgo et al., 2002) have demonstrated that retinoid treatment of oocytes and embryos positively impacts *in vitro* development. In other cell systems, retinoic acid treatment decreased apoptosis through

participation in cellular defense mechanisms against oxidative stress. For example, retinoic acid, protected certain cell lines from oxidative stress-induced apoptosis (Ahlemeyer and Krieglstein, 1998; Ahlemeyer et al., 2001), and inhibited glutathione depletion by preserving the antioxidant, SOD, levels (Ahlemeyer and Krieglstein, 2000). Texiera and colleagues provided evidence that retinoids modulate glutathione content in chondrocytes (Texeira et al., 1996). Furthermore, a study conducted in mesangial cells exposed to hydrogen peroxide (H_2O_2), demonstrated anti-apoptotic effects of RA by inhibiting c-Jun NH_2-terminal kinase (JNK) and activating protein (AP-1) (Moreno-Manzano et al., 1999). Based on this information, it was hypothesized that retinol may affect glutathione concentrations in maturing oocytes.

In the first experiment, retinol supplementation (5μM) during IVM of bovine oocytes did not increase glutathione levels (Figure 4-1). In contrast, cysteamine (100μM) treatment doubled the glutathione content. Our control values are similar to other reports (de Matos et al., 1995; de Matos and Furnus, 2000; Hashimoto et al., 2000). The failure of retinol to influence glutathione concentrations in maturing oocytes may have resulted from an inability of the oocyte to take up and utilize cysteine. Outside the cell, cysteine is readily oxidized and converted to cystine, forming a dimer, which is difficult for the oocyte or embryo to utilize (de Matos et al., 1995; Takahashi et al., 2002). Additions of thiol compounds such as beta-mercaptoethanol (β-ME) and cysteamine have been employed to increase cysteine availability, increase glutathione synthesis, and improve embryo development (de Matos et al., 1995; de Matos et al., 2002; Takahashi et al., 2002; de Matos et al., 2003). It was hypothesized that dual treatment with cysteamine and

retinol may promote cysteine uptake, and improve utilization and redox regulation in embryos; resulting in improved blastocyst development.

Retinol addition alone during IVM (R/-) significantly reduced cleavage ($p<.05$), but displayed similar blastocyst rates as controls (Table4-1). This result is contradictory to an earlier study (Livingston et al., 2002), which showed that retinol addition (5μM) during IVM tended to improve blastocyst development. The disagreement between the two studies may reflect concentration dependent effects of retinoids (Bagavandoss and Midgley, 1988). High performance liquid chromatography (HPLC) analysis revealed an 82% recovery of retinol (data not shown) after 48 hours in culture, suggesting that it is relatively stable; yet, breakdown products may exist and account for some of the observed discrepancies.

Retinol supplementation during IVM and IVC (R/R) did not adversely affect cleavage, but did diminish blastocyst development (Table 4-1). The concentration dependent effects of retinol may partially explain this result, because in our previous study higher concentrations of retinol (10μM) during IVC were detrimental to embryonic development (Livingston et al., 2002). In certain cell lines retinoids influence the levels of manganese superoxide dismutase (Mn-SOD) (Ahlemeyer et al., 2001); thus, in the present study retinol may have increased SOD levels. Dinara and colleagues reported that higher levels of SOD were harmful to oocytes because they increased H_2O_2 production, which can ultimately result in an increase in OH• radical formation, leading to significant cellular damage (Dinara et al., 2001).

Results from Experiment 2 also indicate that cysteamine supplementation during IVM or IVC only, or during both (C/C) did not significantly improve embryonic

development, although a numerical increase was observed (Table 4-1). The concentration of cysteamine in our study was similar to de Matos and colleagues (de Matos et al., 2002); however, other studies have demonstrated that lower as well as higher concentrations of thiol compounds improve embryonic development (Takahashi et al., 1993; de Matos and Furnus, 2000), respectively.

Retinol and cysteamine addition to IVM alone (R+C/-) or only during IVC (-/R+C) did not affect cleavage rates compared to controls (-/-); however, if both were added during IVM and IVC (R+C/R+C), cleavage rates dropped by 10%, which was significantly different from controls. Interestingly, this treatment combination (R+C/R+C) demonstrated the highest blastocyst/putative zygote rate (22.7%), and when measured as blastocyst/cleaved rate it was significantly higher than the controls (38% vs 21%; $p<.02$). These results suggest that a combination of retinol and cysteamine during IVMC may be detrimental for cleavage, but of those embryos that cleave, more are likely to continue development if supplemented with retinol and cysteamine during the first three days of culture. This suggests that retinol and cysteamine are impacting oocyte developmental competence, perhaps by decreasing intracellular free radical content. Superoxide dismutase (SOD) treatment during IVM of bovine embryos did not increase cleavage nor blastocyst/putative zygote rates, but it did improve blastocyst/cleaved rates (Luvoni et al., 1996); therefore, it was hypothesized that SOD improved the quality of the maturing oocytes. Van Soom and colleagues observed detrimental effects after adding cysteine to culture medium (mSOF) (Van Soom et al., 2002). They reasoned that mSOF contains pyruvate and citrate, two factors that serve as a protectant against ROS; therefore, the protective effects of cysteine were diminished. We did not employ a

treatment combination of cysteamine during IVM and retinol during IVC, which may have clarified the effects of these compounds.

Retinol can bind to the cysteine-rich (Zn-finger) regulatory domains of protein kinase C (PKC) and cRaf, facilitating the redox activation of these molecules (Hoyos et al., 2000). Therefore, if retinol increases the sensitivity of redox activation, then perhaps in our study, addition of retinol and cysteamine decreased free radical production too much, and altered the redox state of the embryo. The intracellular REDOX (reduction-oxidation) state describes complex interactions of the relative concentrations of various oxidized and reduced molecules (Harvey et al., 2002). Extreme fluctuations in the REDOX state of a cell can alter the response of a number of genes and/or transcription factors, as well as transport systems such as the cystine transport system which influences intracellular glutathione levels (Dröge, 2002). ROS also serve as critical intracellular signaling molecules in signal transduction pathways (Finkel and Sullivan, 2000; Thannickal and Fanburg, 2000), and influence gene expression (Palmer and Paulson, 1997); therefore, slight disruptions may be detrimental to preimplantation embryonic development. This may partially explain negative effects on cleavage by the R+C/R+C treatment when applied on Day 1. ROS may be critical players in signaling pathways, which are involved in the embryonic gene transition (Latham and Schultz, 2001), and R+C/R+C may alter levels of ROS to the extent that proper cleavage cannot occur.

On the other hand, the negative combinatorial effects of retinol and cysteamine on embryo cleavage may impact mechanisms in addition to protection from oxidative stress. Retinoids have been shown to modulate growth factor expression as well as other genes important to preimplantation development. Several reports have described the role of

peptide growth factors during embryonic development (review Teruel et al., 2000); however, investigators are still unsure of their specific function(s), especially during *in vitro* culture (Kane et al., 1997; Teruel et al., 2000). Midkine, a member of the heparin-binding growth/differentiation family is induced by retinoic acid, and it has been found to improve bovine oocyte and embryo developmental competence (Ikeda et al., 2000; Ikeda et al., 2000). In addition, retinoic acid was found to regulate epidermal growth factor receptor expression (EGFR), (Roulier et al., 1994) and transforming growth factor (TGF-ß), insulin-like growth factor binding protein (IGFBP3) expression (Han et al., 1997), and follicle stimulating hormone receptor (FSHR) expression in granulosa cells (Minegishi et al., 2000).

Retinoids are important regulators of vertebrate cellular development, and research has shown its beneficial effects on early embryonic development *in vivo* (Elmarimi et al., 1990; Shaw et al., 1995; Besenfelder et al., 1996; Eberhardt et al., 1999b), and during *in vitro* embryo culture (Duque et al., 2002; Livingston et al., 2002). In addition, the advantageous effects of maintaining adequate glutathione levels during IVM and IVC have been discovered. In the present study retinol treatment during IVM did not appear to increase or decrease glutathione levels of *in vitro* matured bovine oocytes, despite the supporting evidence that retinoids modulated glutathione levels in other cell lines (Texeira et al., 1996; Ahlemeyer and Krieglstein, 2000). However, after analyzing our results it would be interesting to examine the glutathione content of the resulting blastocysts from retinol and cysteamine treatments.

Different combinations of retinol and cysteamine were employed in an attempt to improve early embryonic development of *in vitro* produced bovine embryos. The

combination of retinol and cysteamine during IVM and IVC was beneficial as assessed by the blastocyst rate; however, the interpretation of this result must be guarded because this same treatment appeared harmful to early cleavage stage embryos. Nevertheless, addition of antioxidants to embryo culture medium seems to warrant further consideration and evaluation.

CHAPTER 5

Glutathione Levels of *In Vivo* Sheep Oocytes and the Expression of Manganese Superoxide Dismutase, Cu-Zn Superoxide Dismutase, Glutathione Synthetase, and Glutathione Transferase-pi

ABSTRACT

Retinoids play important roles in many diverse biological functions such as cell growth, morphogenesis, differentiation, and reproduction. Previous studies demonstrated that retinol administration to ewes, followed by natural service, resulted in embryos with improved competence to develop in vitro. Glutathione is an important non-protein, sulphydryl compound found in oocytes and embryos, which acts to decrease oxidative stress. The purpose of the present study was to evaluate the effects of retinol administration to ewes on the glutathione content and expression of glutathione-related and antioxidant enzymes in mature ovine oocytes. Briefly, ewes were administered retinol or vehicle during superovulation, and after 60 hours the oviducts removed and mature oocytes collected. Glutathione content did not differ significantly between oocytes collected from retinol-treated ewes (6.78 ± 3.81 pmol/oocyte) and oocytes from control ewes (6.38 ± 1.58 pmol/oocyte). Transcripts encoding for manganese superoxide dismutase (Mn-SOD), copper zinc superoxide dismutase (Cu-Zn SOD), glutathione synthetase (GS), and glutathione transferase pi (GSTp) were detected in single ovine

oocytes; however, semi-quantitative RT-PCR analysis did not reveal any significant differences between oocytes from retinol-treated ewes and those from control ewes.

INTRODUCTION

Production of reactive oxygen species (ROS) is the natural result of normal, physiological cellular processes (review Dröge, 2002). However, if left unchecked, ROS may adversely affect preimplantation embryonic development, especially during *in vitro* culture. Deleterious effects of ROS include protein, lipid, and nucleic acid damage, mitochondrial alterations, cell cycle block, ATP depletion, and excessive apoptosis (Salas-Vidal et al., 1998; Guérin et al., 2001). During *in vivo* embryonic development, enzymatic as well as non-enzymatic antioxidants are present in follicular fluid (Schweigert and Zucker, 1988; Carolan et al., 1996; Bisseling et al., 1997; Cassano et al., 1999), and in reproductive tract secretions to help control the propagation of free radical reactions (Gardiner et al., 1998; El Mouatassim et al., 2000); thus, keeping a tight regulation on the redox state of the embryo.

Retinoids play important roles in vision, metabolism, reproduction, bone formation, and pattern development during embryogenesis (Gudas et al., 1994; Napoli, 1996; Wolf, 1996). Ultimately, the metabolites of vitamin A (retinol), all-*trans*-retinoic acid and 9-*cis*-retinoic acid, together with their receptors (RARs and RXRs), regulate retinoid action at the level of gene expression (Wendling et al., 1999; Konta et al., 2001; review Ross et al., 2001). Vitamin A deficiencies in animals (Moore, 1957; McCollum, 1964; Ross et al., 2000; Takahashi et al., 2000; Guérin et al., 2001), as well as

supplementation with retinol, have clearly defined the importance of this vitamin during early embryonic and fetal development (Wellik and DeLuca, 1995; Besenfelder et al., 1996; Eberhardt et al., 1999b; Whaley et al., 2000).

Glutathione (GSH), the major non-protein sulphydryl compound found in mammalian cells, cycles from a reduced form (GSH) to an oxidized form (GSSG) (Deplancke and Gaskins, 2002). One of the primary responsibilities of glutathione is strong basal reactive oxygen species (ROS) scavenging activity (Dröge, 2002), and several reports highlight the necessity of maintaining adequate glutathione levels in both oocytes and early embryos (Luvoni et al., 1996; Furnus et al., 1998; Knappen et al., 1999; de Matos and Furnus, 2000; Shi et al., 2000; Brad et al., 2003).

Intracellular glutathione synthesis begins with the transulfuration of homocysteine to cysteine, the major precursor for GSH synthesis (Deplancke and Gaskins, 2002). Next, gamma-glutamyl cysteine synthetase (γ-GCS) combines L-glutamate and cysteine, followed by catalysis of glutamate-cysteine and glycine by glutathione synthetase (GS). Other important glutathione-related enzymes include glutathione peroxidase, which converts GSH to the oxidized form (GSSG) (Arai et al., 1999), glutathione reductase, which can reverse this reaction (Anderson, 1985), and glutathione *S*-transferase-pi (GSTp), which catalyzes the conjugation of GSH with deleterious compounds in order to protect the cell (Bernardini et al., 1999).

Many oxidative stress-related enzymes, including those that regulate glutathione levels, are found in the reproductive tract, its secretions. In addition, their transcripts and/or proteins have been localized in the oocyte and early embryo (review El Mouatassim et al., 1999; Knappen et al., 1999; El Mouatassim et al., 2000). For

example, different levels of expression of GSH, GSSG, cysteine, and Mn-SOD were found in different tissues in rabbit and rat embryos (Hansen et al., 2001). MII stage *in vivo* produced mouse and human oocytes expressed the following mRNA transcripts: Cu-Zn SOD, MnSOD, GPx, and GCS (El Mouatassim et al., 1999). GSTp has been localized in human ovarian follicular fluid, and this particular isoform is also important in placental function (review Knappen et al., 1999).

Litter-bearing animals receiving either β-carotene or retinol during superovulation displayed an increase in the number of live births (Besenfelder et al., 1993; Coffey and Britt, 1993), and an increase in ovulation rate as well as increased recovery of embryos (Elmarimi et al., 1990). Data collected in our lab demonstrated that 1-4 cell and morula stage embryos, collected from the oviduct and uterus, respectively, of all-*trans*-retinol treated, superovulated ewes exhibited improved capacity to develop to the blastocyst and hatched blastocyst stage during *in vitro* culture (IVC). In addition, these experiments revealed that most embryos from ewes not treated with retinol (60%) did not progress beyond the eight-cell stage *in vitro* (Eberhardt et al., 1999b).

Under certain conditions vitamin A, and carotenoids participate in a biological antioxidant network, and have been implicated as important regulators of redox signaling pathways (Imam et al., 2001). Specifically, carotenoids quench singlet oxygen molecules as well as interact with other antioxidant compounds (Olson, 1993). Furthermore, retinoid addition to cultured neurons prevented glutathione depletion after staurosporine treatment (Ahlemeyer and Krieglstein, 2000), and retinoids were also found to decrease ROS content and increase SOD levels (Ahlemeyer et al., 2001). In chondrocyte culture,

retinoids increased the activity of glutathione reductase, catalase, and SOD (Texeira et al., 1996).

Based on prior data, which demonstrated *in vivo* retinol administration improved ovine embryonic development (Eberhardt et al., 1999b), and *in vitro* results, which showed that retinol supplementation improved bovine embryonic development (Livingston et al., 2002), we speculated that retinol was impacting the follicular environment of the developing oocyte. We hypothesized that retinol may increase the glutathione content, and upregulate levels of particular antioxidant enzymes in the *in vivo* matured ovine oocyte; thus, improving future embryonic development. *In vivo* matured sheep oocytes were collected from oviducts, and the glutathione content measured. In addition, semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on *in vivo* matured, single ovine oocytes to evaluate the effects of retinol on the expression of manganese superoxide dismutase (Mn-SOD), copper-zinc SOD (Cu/Zn SOD), glutathione synthetase (GS), and glutathione transferase pi (GSTp) transcripts.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma Chemical Company, St. Louis, MO unless otherwise noted. Mature cross-bred ewes were provided by the University of Tennessee, Knoxville Experiment Station. All animals were maintained on high-quality hay fed ad libitum, with free-choice access to a sheep and goat mineral premix that

contained 1 million IU of vitamin A per pound. Progestin-impregnated vaginal implants (CIDR-G) were purchased from InterAg, Hamilton, New Zealand. Follicle stimulating hormone (FSH) was purchased from Sioux Biochemical, Sioux Center, IA. Dulbecco's phosphate buffered saline without Ca^{++} or Mg^{++} (DPBS) was purchased from Gibco BRL (Grand Island, NY). Fetal bovine serum (FBS) used in oocyte collection medium was purchased from BioWhittaker, Baltimore, MD. Reverse-transcriptase polymerase chain reaction (RT-PCR) kits were purchased from Qiagen, Valencia, CA. Recombinant Rnasin® ribonuclease inhibitor (RNase) and electrophoresis standards were purchased from Promega, Madison, WI. Oligonucleotide primer pairs were purchased from Oligos Etc., Wilsonville, OR.

Ovine Oocyte Collection

Mature cross-bred ewes were synchronized with CIDR-G implants for fourteen days. FSH was administered in decreasing doses, twice daily for three days (5 IU, 4 IU, 3 IU). On the first and last morning of FSH injections each ewe was also administered 500,000 IU of all-trans retinol dissolved in corn oil. Control ewes received only vehicle (corn oil). The implant was changed after 9 days, the second implant was removed on the last FSH injection, and surgery was performed approximately 60 hours after implant removal. After the number of corpora lutea were recorded, each oviduct was removed and gently flushed with DPBS containing 1% FBS to collect the oocytes. Oocytes were washed three times in DPBS, 1% FBS. Any remaining cumulus cells were manually denuded with a narrow-bore glass pipette.

Experiment 1: GSH Assay

The GSH assay described is a modification based on the recycling assay of the enzyme 5,5-dithiobis-(2-nitrobenzoic acid)-glutathione reductase (DTNB-GSSG), described by Tietze (Tietze, 1969) and Calvin (Calvin et al., 1986). The method explained here is most similar to that of Anderson (Anderson, 1985). The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): 5,5-dithiobis-(2-nitrobenzoic acid - DTNB), phosphoric acid (H_3PO_4), sodium phosphate hydrated ($NaH_2PO_4H_2O$), sodium phosphate (Na_2HPO_4), ethylenediaminetetraacetic acid (EDTA), reduced glutathione, and β-Nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), and glutathione reductase.

After collection, the oocytes were washed three times in stock buffer (0.2 M sodium phosphate-0.001 M sodium EDTA, pH 7.2), and transferred to a microcentrifuge tube in 5µl. Phosphoric acid (1.25M, 5µl) was added to achieve a final concentration of 0.625 M H_3PO_4. The oocytes were immediately frozen (-20°C).

Samples were thawed, centrifuged, and microscopically evaluated to ensure complete lysis of the oocytes. To each sample, 700µl of the NADPH solution (1mL of stock buffer added to 1mg bottle of NADPH), 100µl of 6mM DTNB, 180µl of distilled water, and 10µl of glutathione reductase (266U/ml) were quickly added to a cuvette, placed in a Beckman single-beam DU-640 (Irvine, CA) spectrophotometer, and the increase in absorbance (412nm) was measured every 30 seconds for 2 minutes. Each blank included 5µl of water and 5µl of phosphoric acid, and did not show detectable amounts of GSH-GSSG. Four glutathione (GSH) standards were made fresh on the day of the assay (1nmol, 0.5nmol, 0.25nmol, and 0.05nmol), in order to calculate the total

GSH content of oocytes based on a standard curve (Tietze, 1969; Anderson, 1985; Calvin et al., 1986).

Experiment 2: Oocyte mRNA Isolation and RT-PCR Analysis

Based on a modification by Fiorenza and Mangia (Fiorenza and Mangia, 1998), mRNA was obtained from single ovine oocytes. Briefly, individual oocytes were placed on the cap of a diethylpyrocarbonate (DEPC) treated microcentrifuge tube (200µl) containing 10 µl of RNase-free water, and the remaining buffer (DPBS with 1% FBS) was aspirated from the cap. The tube was centrifuged for 30 seconds at 8950 g, and promptly frozen at -80°C until analysis. Before analysis, 10U of RNase inhibitor (RNaseI) was added to each tube and frozen/thawed two additional times to ensure complete lysis of each oocyte.

Detection of Transcripts Encoding Cu/Zn and Mn Superoxide Dismutases, Histone (H2a), Glutathione Synthetase, and Glutathione Transferase-Pi

Following the manufacturer's (Qiagen) suggestions, each tube received 40µl of a mixture containing RT-PCR buffer (1x), dNTP mix (400µM of each dNTP), 0.6µM of each primer, enzyme mix (Omniscript™ and Sensiscript™ reverse transcriptase, HotStartTaq™ DNA polymerase), and RNaseI (10U). All tubes, except for the primer pair, Mn-SOD, also received an additional chemical called Q-solution, which is a proprietary additive used to improve RT-PCR conditions.

Primer pairs for Mn-SOD, Cu/Zn-SOD, and Histone H2a were similar to those used in other papers with minor modifications (Hallewell et al., 1991; Meyrick and

Magnuson, 1994; Lequarré et al., 2001; Robert et al., 2002), respectively. Glutathione synthetase (GS) was based on a mouse GS sequence, glutathione transferase pi (GSTp) was based on a bovine sequence (Table 5-1), and both primer pairs were designed according to criteria in the software program, Gene Runner, version 3.05 (Hastings Research, Inc., Moraga, CA). Identification of the amplification products was confirmed by sequencing (Genetic Analyzer ABI 3100, University of Tennessee Molecular Biology Resource Facility, Knoxville, TN).

RNA was reverse-transcribed for 30 minutes at 50°C, reverse transcriptase was inactivated and DNA polymerase activated at 95°C for 15 minutes, followed by 30-40 cycles of DNA amplification (depending on the primer pair) run according to manufacturers suggestions: DNA was denatured at 94°C for 1 minute, reannealing was achieved at 47°-50.5°C, depending on the primer pair, and extension at 72°C for 1 minute, and a final extension was performed for 7 minutes at 72°C. The amplification products were separated on either a 2% agarose gel (Mn-SOD, Histone, GS, GSTp) or a 4% low-melting agarose gel (Cu/Zn SOD, Histone), and visualized with ethidium bromide. Under these conditions each transcript was detected from a single oocyte. The software used to analyze each band was Quantity One ™ (Bio-Rad Laboratories, Hercules, CA). The abundance of each transcript was determined by dividing the intensity of the band of interest by the intensity of the standard (histone) (Knijn et al., 2002).

Table 5-1. Antioxidant-related transcripts detected in singe sheep oocytes.

Gene	Primer sequence	Reference	Product size (bp)	Number of Cycles	Annealing Temperature (°C)
Histone H2a		Mouse (Robert et al., 2002)	182	Depended on primer pair	47.0 - 50.5°C
Sense	5′ GTCGTGGCAAGCAAGGAG 3′				
Antisense	5′ GATCTCGGCCGTTAGGTACTC 3′				
Mn-SOD		Bovine (Meyrick & Magnuson, 1994)	409	30	47.0°C
Sense	5′ CACCACAGCAAGCACCA 3′				
Antisense	5′ TCCCACACGTCAATCCC 3′				
Cu/Zn-SOD		Bovine (Hallewell et al., 1991)	226	30	47.0°C
Sense	5′ GAAGAGAGGCATGTTGGAG 3′				
Antisense	5′ CCAATTACACCACGAGCC 3′				
GS		GenBank Accession number NM_008180	598	40	50.0°C
Sense	5′ GGTGCTACTGATTGCTCAAG 3′				
Antisense	5′ GCTCAGGTTCAATCTTCTCC 3′				
GSTp		GenBank Accession number NM_177516	292	35	50.5C
Sense	5′ CCTCCCTACACCATCGTC 3′				
Antisense	5′ TCCTCTACACCGTCATTCAC 3′				

Data Analysis

Data from Experiment 1 and 2 both were analyzed using chi-square analysis to detect differences between oocytes from retinol treated ewes and control ewes.

RESULTS

The glutathione content of *in vivo* matured sheep oocytes did not significantly differ between oocytes collected from ewes treated with retinol (6.78 ± 3.81 pmol/oocyte) versus ewes not exposed to retinol (6.38 ± 1.58) (Figure 5-1). Together, over 260 oocytes were collected and assayed for glutathione.

The transcripts of Histone H2a, Mn-SOD, Cu-Zn SOD, GS, and GSTp were detected in *in vivo* mature ovine oocytes. As depicted in Table 5-1 all PCR products showed the expected size, and their identity was confirmed by sequencing. The sequence of each product was confirmed by a BLAST search in GenBank. The GS transcript expressed 89% homology with previously published sequences of GS, GSTp (98%), Mn-SOD (98%), Cu-Zn SOD (98%), and Histone H2a (87%).

Examining the relative abundance of each transcript did not reveal differences between oocytes from retinol-treated ewes versus controls. The transcripts encoding for Mn-SOD (Figure 5-2) were detected in all oocytes tested (24/24), for Cu-Zn SOD 94% (17/18) of the oocytes expressed the transcript (Figure 5-3), GS transcripts (Figure 5-4) were found in 96% (27/28) of the oocytes analyzed, and 100% of the oocytes (16/16) expressed GSTp (Figure 5-5). Histone (Figures 5-2 – 5-5) was used as an internal control because it had previously been detected at all stages of bovine embryo

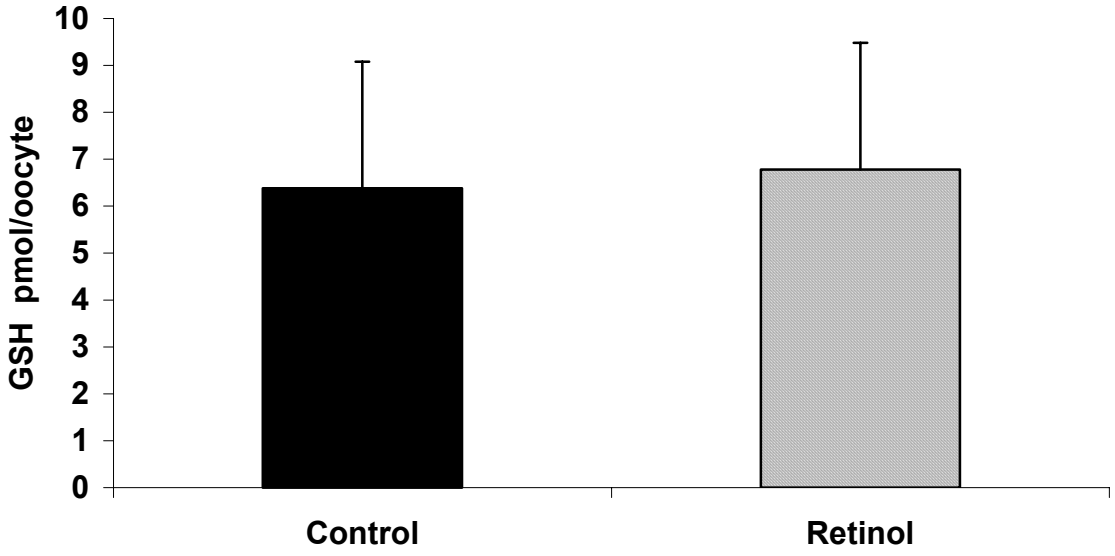

Figure 5-1. Glutathione content of *in vivo* matured sheep oocytes.

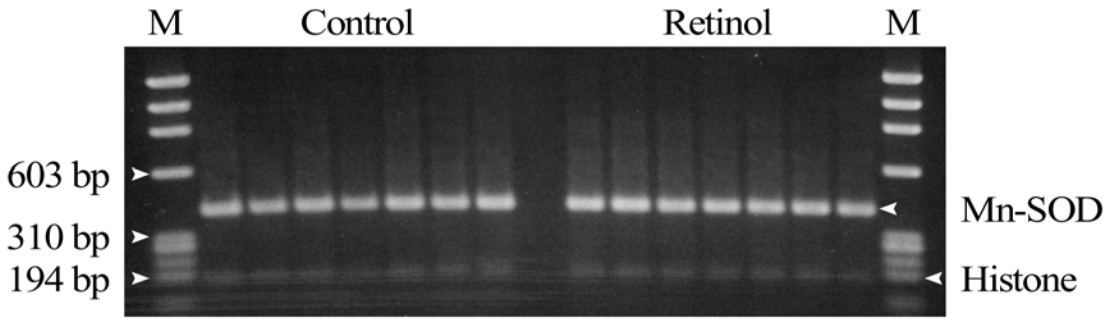

Figure 5-2. Expression of the Mn-SOD and Histone H2A transcripts in single, mature sheep oocytes. Lanes 1 & 17 (M): Marker. Lanes 2-8: Oocytes from control ewes. Lane 9: Blank. Lanes 10-16: Oocytes from retinol-treated ewes.

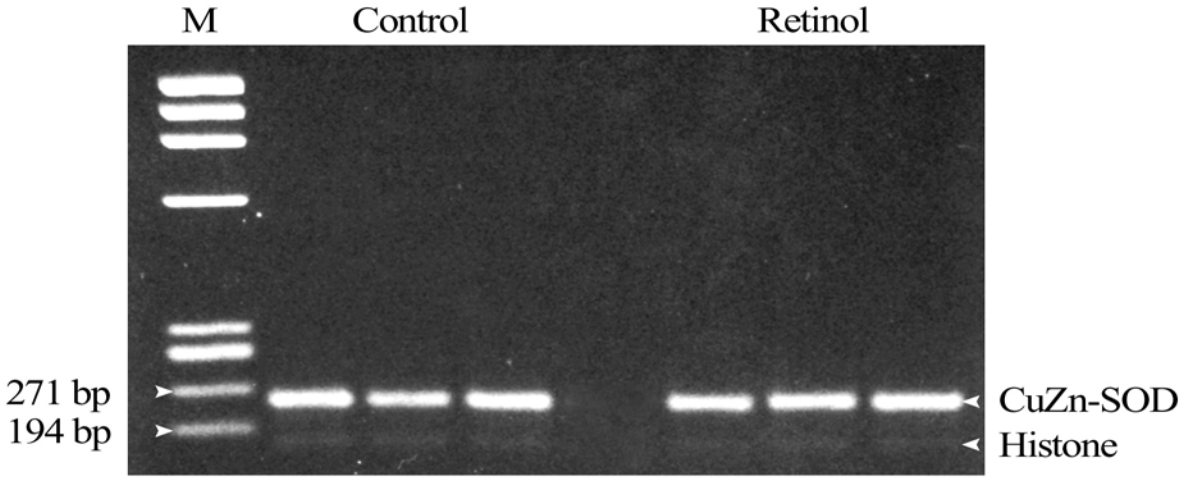

Figure 5-3. Expression of the Cu-Zn SOD and Histone H2A transcripts in single, mature sheep oocytes. Lanes 1 (M): Marker. Lanes 2-4: Oocytes from control ewes. Lane 5: Blank. Lanes 6-8: Oocytes from retinol-treated ewes.

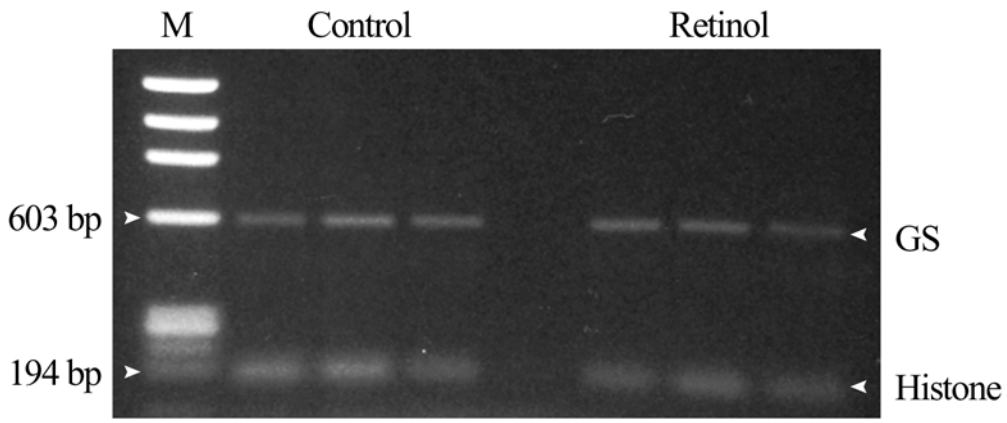

Figure 5-4. Expression of the GS and Histone H2A transcripts in single, mature sheep oocytes. Lanes 1 (M): Marker. Lanes 2-4: Oocytes from control ewes. Lane 5: Blank. Lanes 6-8: Oocytes from retinol-treated ewes.

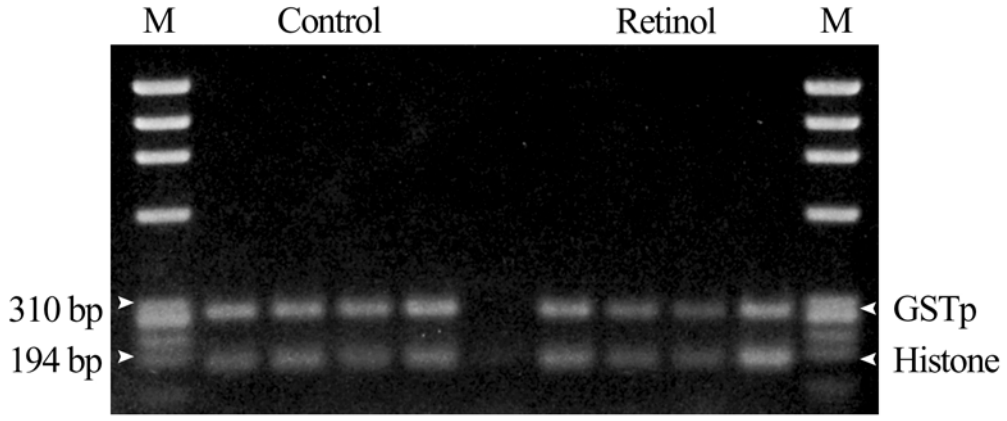

Figure 5-5. Expression of the GSTp and Histone H2A transcripts in single, mature sheep oocytes. Lanes 1 (M): Marker. Lanes 2-4: Oocytes from control ewes. Lane 5: Blank. Lanes 6-8: Oocytes from retinol-treated ewes.

development to the blastocyst stage in the same quantity (Robert et al., 2002). Our results showed that this transcript did not appear to differ between treatments; therefore, it was an appropriate internal control to use in this study.

DISCUSSION

Retinol administration to ewes during gonadotropin-induced follicular maturation resulted in an increased development to the blastocyst stage *in vitro* (Eberhardt et al., 1999b). In addition, retinol supplementation to bovine oocytes during *in vitro* maturation tended to improve embryonic development (Livingston et al., 2002); therefore, maturing oocytes within the follicular environment were considered likely targets of retinoid action. Based on previous reports in other cell lines that retinoids modulated glutathione levels (Ahlemeyer and Krieglstein, 2000; Ahlemeyer et al., 2001), and other antioxidants (Texeira et al., 1996), we hypothesized that retinol may increase the glutathione levels in oocytes, and/or increase levels of antioxidant enzymes that ultimately enhance embryonic development.

The glutathione content of *in vivo* matured ovine oocytes (Figure 5-1) from retinol-treated ewes (6.78 ± 3.81 pmol/oocyte) did not differ significantly from control oocytes (6.38 ± 1.58 pmol/oocyte). To our knowledge, this is the first time glutathione content has been measured in superovulated, *in vivo* derived ovine oocytes. Our values are similar to those reported after *in vitro* maturation of ovine oocytes (de Matos et al., 2002). In contrast, Brad and colleagues observed that *in vivo* matured porcine oocytes had an increased concentration of glutathione compared to their *in vitro* matured counterparts

(Brad et al., 2003). This apparent contradiction in GSH content of *in vivo* versus *in vitro* matured oocytes between sheep and swine could result from species differences, methodology employed, or treatment effects. Superovulation may also have affected the GSH content because Foote and Ellington detected differences between normal ovulated bovine oocytes versus superovulated ones (Foote and Ellington, 1988).

In experiment 2, mature ovine oocytes were collected from the oviducts of superovulated ewes to evaluate the effects of retinol on Mn-SOD, Cu-Zn SOD, GS, and GSTp expression. RT-PCR analysis on single oocytes detected transcripts for each gene (Figures 5-2 – 5-5). In fact, using this procedure each transcript was detected in over 95% of the oocytes analyzed, demonstrating the efficacy of this technique.

Mn-SOD is present in the mitochondria and Cu-Zn SOD is located in the cytoplasm (McCord and Fridovich, 1969; Weisiger and Fridovich, 1973). Both are responsible for converting the superoxide anion (O^{2-}) to H_2O_2, which is then removed by catalase and/or glutathione peroxidase (Guérin et al., 2001). Studies have demonstrated benefits of Mn-SOD addition to simple culture medium (Luvoni et al., 1996; Orsi and Leese, 2001). In the current study, we detected Mn-SOD transcripts in 24/24 oocytes, and Cu-Zn SOD was found in 17/18 oocytes analyzed. Transcripts for Mn-SOD and Cu-Zn SOD have been detected in *in vitro* matured (IVM) bovine oocytes (Lequarré et al., 2001), and in human and mouse *in vivo* matured oocytes (El Mouatassim et al., 1999). The presence of these transcripts in bovine, ovine, mouse, and human suggests these defense mechanisms are conserved, and important for early embryonic development. However, Mn-SOD was detected in only 60% of *in vitro* matured bovine oocytes

(Lequarré et al., 2001), which may indicate a significant difference between *in vivo* and *in vitro* derived oocytes.

Few studies have studied the transcriptional expression or regulation of glutathione synthetase (GS), particularly during embryonic development. This enzyme catalyzes the formation of γ-glutamylcysteine and L-glycine, the final step in glutathione synthesis. In this study, the transcript for GS was detected in 27/28 oocytes analyzed. Other studies have confirmed the presence of the rate-limiting enzyme, γ-glutamylcysteine synthetase, in mature human, mouse, and bovine oocytes (Harvey et al., 1995; El Mouatassim et al., 1999).

Glutathione transferase pi (GSTp) is one of nine gene families that exist in the GST superfamily (Ketterer, 2001). The GSTs are well known xenobiotic metabolic enzymes, playing a role in the detoxification process by catalyzing the conjugation of glutathione to toxic or foreign compounds (Knappen et al., 1999). GSTp has also been shown to protect cells from oxidative stress-induced cell death (Yin et al., 2001). High levels of GSTp1 expression have been found in fetal tissues (Knappen et al., 1999), and correlated with physiological cellular proliferation (Ketterer, 2001). GSTp and GST-alpha were both detected in the follicular fluid of women undergoing infertility treatments (Bisseling et al., 1997). Furthermore, GSTp was found in the greatest amounts in early human embryonic and fetal tissues (8 and 13 weeks gestational age) (Raijmakers et al., 2001). Ours is the first study to demonstrate that at least one gene family of GSTs, GSTp, is expressed in mature ovine oocytes; thus, suggesting the oocyte has the capacity for detoxifying toxic compounds, and managing oxidative stress. An allelic variant of GSTp was found to contain a retinoic acid response element (RARE),

suggesting retinoic acid may play a role in the molecular regulation of GSTp (Lo and Ali-Osman, 1997).

Retinol administration during superovulation to ewes did not appear to affect the mRNA expression of transcripts for the antioxidant enzymes studied. However, it is possible that the protein levels of one or more of these enzymes are modified in a way that improves future embryonic development. In fact, in one report RA supplementation during cell culture did not alter SOD transcript levels, but it upregulated SOD protein levels (Ahlemeyer et al., 2001).

Alternatively, retinol may not be acting directly on the oocyte, but rather indirectly affecting other reproductive tract cells, or the granulosa and/or theca cells in a way that ultimately benefits the oocyte. In addition, retinoids may directly or indirectly modulate antioxidant-inducible genes (review Primiano et al., 1997). Antioxidant-inducible genes include GSTs, cytochrome P450s, aldehyde dehydrogenases, glutathione-related enzymes, and many others (review Primiano et al., 1997). Although the positive effects previously detected by retinol supplementation during *in vitro* culture (Livingston et al., 2002) may be explained by an upregulation of antioxidant-induced enzymes, the question remains: does the same hold true for an *in vivo* derived oocyte or embryo that is already surrounded by protective mechanisms present in the follicular and oviductal fluid? If not, then the mechanism of action *in vivo* may be different than during *in vitro* culture.

Retinoids are known to impact a variety of genes and/or transcription factors such as midkine, a member of the heparin-binding growth/differentiation family, which is induced by retinoic acid, and has been found to improve bovine oocyte and embryo

developmental competence (Ikeda et al., 2000; Ikeda et al., 2000). In addition, retinoic acid was found to regulate epidermal growth factor receptor expression (EGFR), (Roulier et al., 1994) and transforming growth factor (TGF-ß), insulin-like growth factor binding protein (IGFBP3) expression (Han et al., 1997), and follicle stimulating hormone receptor (FSHR) expression in granulosa cells (Minegishi et al., 2000). Retinoic acid also modulated the expression of cell cycle proteins such as MAPK (Alsayed et al., 2001). Perhaps, retinol is impacting the oocyte in a way to synchronize its cell cycle, in order to endure sub-optimal *in vitro* culture conditions.

For the first time, glutathione content was measured in *in vivo* derived, mature ovine oocytes, and the values correspond to other published values (de Matos et al., 2002). In addition, transcripts encoding for Mn-SOD, Cu-Zn SOD, GS, and GSTp were detected in over 95% oocytes collected; however, differences between oocytes collected from retinol-treated ewes and control oocytes were not detected based on a semi-quantitative RT-PCR analysis. The presence of these transcripts suggests that an endogenous pool of antioxidant enzymes exist in the oocyte, stored as mRNA, presumably to assist future development.

CHAPTER 6

Summary

The effects of retinoids on embryogenesis and fetal development have been studied extensively over the past 20-30 years (review Hofmann and Eichele, 1994; Båvik et al., 1996; Ghyselinck et al., 1997; Neiderreither et al., 1999; review Ross et al., 2000). Retinoids impact embryogenesis, providing critical positional information by influencing *Hox* genes, which control anterior/posterior body patterning (review Hofmann and Eichele, 1994; review Marshall et al., 1996). In addition, retinoic acid regulates WNT genes (Katoh, 2002), which serve important roles during neural differentiation, and have been characterized in the ovary (Ricken et al., 2002). Oct-4, a member of the POU transcription factor family is correlated with an undifferentiated phenotype in embryos (Ovitt and Scholer, 1998; Kirchhof et al., 2000). Evidence exists that this protein may be modulated by retinoic acid (Ovitt and Scholer, 1998).

Studies have confirmed the necessity of maintaining a homeostatic balance of retinoid metabolizing enzymes, binding proteins and retinoid receptors in order to avoid a vitamin A deficient or excessive state (Mangelsdorf et al., 1994; Ghyselinck et al., 1999; review Noy, 2000). Our laboratory and others have presented evidence that retinol supplementation during gonadotropin-induced follicular maturation improves embryonic development and subsequent pregnancies (Elmarimi et al., 1990; Besenfelder et al., 1996; Whaley et al., 1997; Eberhardt et al., 1999b; Whaley et al., 2000; Hidalgo et al., 2002;

Hidalgo et al., 2003). Addition of retinoids during *in vitro* culture also proved beneficial for embryonic development to the blastocyst stage (Duque et al., 2002; Livingston et al., 2002). Based on this data, we hypothesized that retinol may be exerting its effect on the maturing oocyte; therefore, we designed *in vivo* studies with sheep and *in vitro* experiments with bovine embryos to evaluate the effects of retinol, in an effort to understand its mechanism(s) of action.

The first study measured sheep oocyte metabolism, and evaluated the role of retinol on this process. Using radiolabeled substrates to measure the evolution of $^{14}CO_2$ and $^{3}H_2O$, this was the first time sheep oocyte metabolism had been measured over the entire twenty-four hour maturation period. Results showed that oxidative metabolism measured by glutamine showed no significant differences over all time periods. Pyruvate oxidation was highest early in maturation and then decreased. Glutamine oxidation appeared to be most active early and then declined. Glycolysis was highest at the middle time period. These results suggest that ovine oocytes use both glycolysis and oxidative pathways throughout IVM; thus, providing insight into the nutritional and energy requirements of the maturing ovine oocyte. Perhaps due to a lack of sensitivity of our assay, we did not detect significant differences in metabolism between oocytes from retinol-treated ewes and those from control ewes.

Our next study was performed in order to determine the *in vitro* effects of retinol on bovine preimplantation development. Varying concentrations of retinol were added either during IVM or IVC. Our results demonstrated that 5μM retinol supplementation during IVM tended to improve embryonic development measured by the rate of blastocyst development. Interestingly, this concentration proved even more beneficial if

the control blastocyst rate was below 20%. Furthermore, 10μM retinol appeared detrimental during IVC but not during IVM. We postulated this was due to a protective effect of cumulus cells.

Based on previous *in vivo* and *in vitro* retinoid experiments, and data from studies demonstrating the importance of glutathione (de Matos and Furnus, 2000; de Matos et al., 2002), we hypothesized that retinol addition to the medium may increase oocyte glutathione levels. In addition, we investigated the combinatorial effects of retinol and cysteamine on *in vitro* bovine embryonic development. We did not observe an increase in glutathione levels in bovine oocytes treated with retinol, perhaps due to a decreased uptake of cysteine from the culture medium. However, in the presence of cysteamine bovine oocytes exhibited an increase in glutathione content. Retinol and cysteamine treatment during IVM and IVC increased bovine blastocyst development, which may indicate that retinol increases the utilization or uptake of cysteine from the medium.

Next, we evaluated the role of exogenous retinol supplementation to superovulated ewes on the glutathione content of mature oocytes collected from the oviducts. Our results did not reveal differences in glutathione content of oocytes from retinol-treated versus those from vehicle-treated ewes. Antioxidant transcripts encoding for Mn-SOD, Cu-Zn SOD, GS, and GSTp, were detected in *in vivo* matured ovine oocytes. However, retinol did not appear to impact the expression levels of these transcripts.

Overall, retinol addition during IVM appeared beneficial for embryonic development, but its effects during IVC were concentration dependent. Although glutathione levels were not altered significantly during IVM, the effects of retinol may

become more apparent later in development, perhaps during the blastocyst stage when glutathione levels increase. Adding both retinol and cysteamine during IVM and IVC proved beneficial. The *in vivo* metabolism study did not detect any significant differences between oocytes from retinol-treated versus control ewes, nor did our *in vivo* data confirm that retinol was altering glutathione levels in mature ovine oocytes.

In vivo conditions differ from culture conditions, but an embryo must still respond appropriately to the varying environment of the oviduct and uterus. Retinol may modulate growth factors, hormones, or other substances that are necessary for embryonic development. Additionally, the positive effect of retinol administration may have a more indirect effect on the oocyte or follicle, impacting the follicular environment and/or the oviductal secretions. A study conducted in our laboratory, demonstrating that more pregnancies and subsequent births resulted from frozen embryos collected from retinol-treated ewes (unpublished) suggests that retinol is positively influencing early preimplantation development; however, its mechanism of action is still unclear.

Organisms living in an aerobic environment are exposed to reactive oxygen species (ROS) that are produced by normal, cellular processes such as aerobic metabolism (Martindale and Holbrook, 2002). *In vitro* culture exposes cells to further ROS by exogenous stimuli such as light and experimental manipulation (Guérin et al., 2001). Depending on their concentration, ROS serve important regulatory functions (Thannickal and Fanburg, 2000; Dröge, 2002; Martindale and Holbrook, 2002), by altering the intracellular redox state or creating oxidative modifications in proteins (Thannickal and Fanburg, 2000). The redox state of a cell is important in terms of how it respond to signals, i.e., apoptosis, proliferation, or differentiation. Some studies have

suggested that cells contain a redox sensor, an unidentified protein or complex that senses the redox state of the cell and responds appropriately (Gutteridge, 1995; Primiano et al., 1997) (Figure 6-1).

Retinoids can act in a variety of ways that ultimately impacts cellular physiology such as altering levels of kinases, contractile elements, and gene expression (Gudas et al., 1994). Retinol may be affecting embryonic development in one of three ways: modulating apoptosis, acting as an antioxidant, and/or regulating growth factor expression.

Several reports have provided clear evidence that retinoids, particularly retinoic acid (RA), suppressed apoptosis in a number of cell lines (Moreno-Manzano et al., 1999; Ahlemeyer and Krieglstein, 2000; Ahlemeyer et al., 2001; Antipatis et al., 2002). Furthermore, many studies, especially those *in vitro* demonstrated that excessive apoptosis during embryonic development is detrimental (Hardy, 1999; Morita and Tilly, 1999; Matwee et al., 2000; Van Soom et al., 2002). The biochemical pathway that mediates the retinoid suppression of apoptosis most likely involves the stress-activated kinase, known as c-jun terminal kinase (JNK). For example, RA was shown to inhibit JNK activation in mesangial cells exposed to H_2O_2 (Figure 6-1). Specifically, the study found RA decreased c-Jun/c-Fos heterodimerization (Moreno-Manzano et al., 1999), which are necessary components of activating protein 1 (AP-1) (Figure 6-2). AP-1 is regarded as a redox-sensitive transcription factor that regulates the expression of several genes, including those involved in apoptosis (Palmer and Paulson, 1997). Moreover, RA was found to increase the levels of mitogen-activated protein kinase phosphatase 1

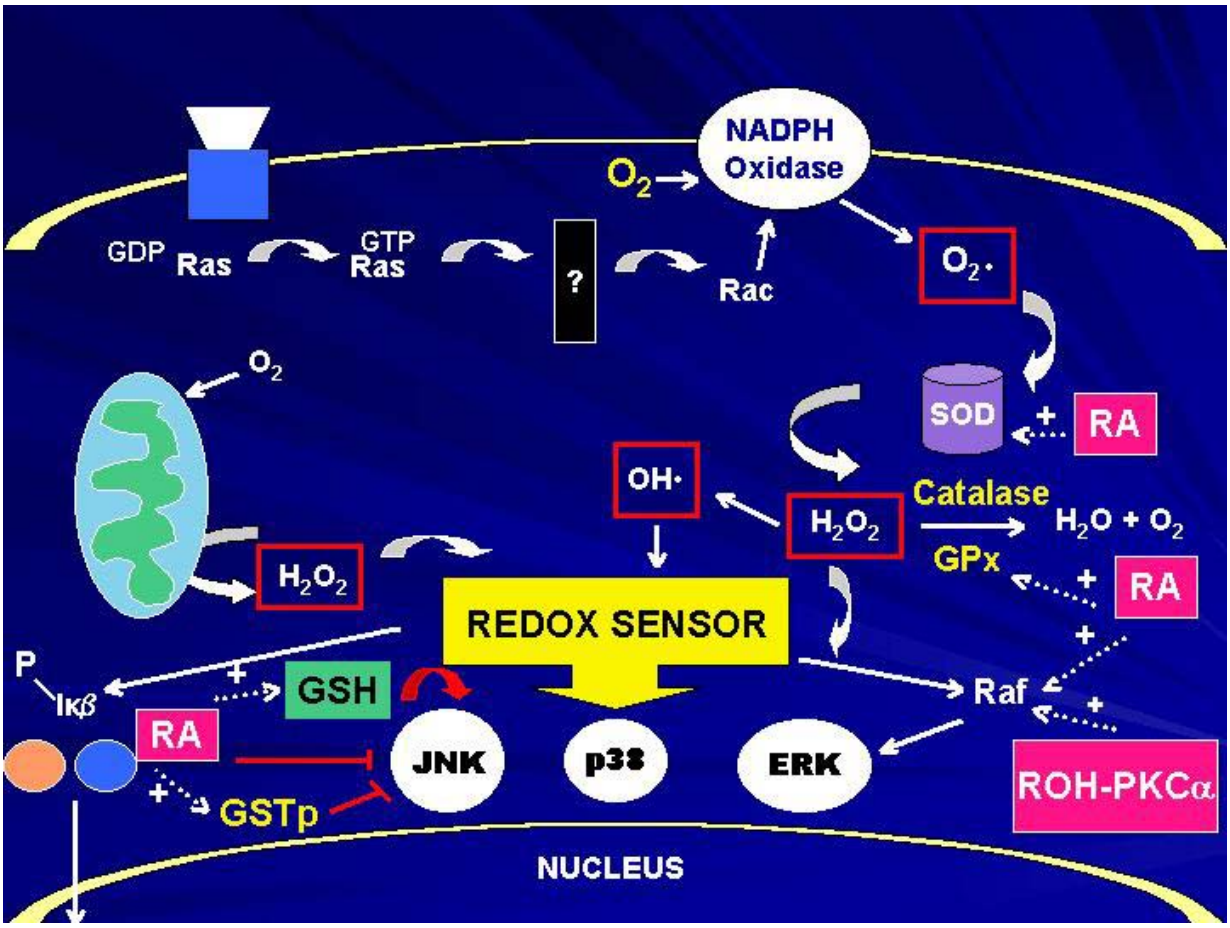

Figure 6-1. The redox pathway and potential mechanisms of action of retinoids in the cytoplasm during embryonic development.

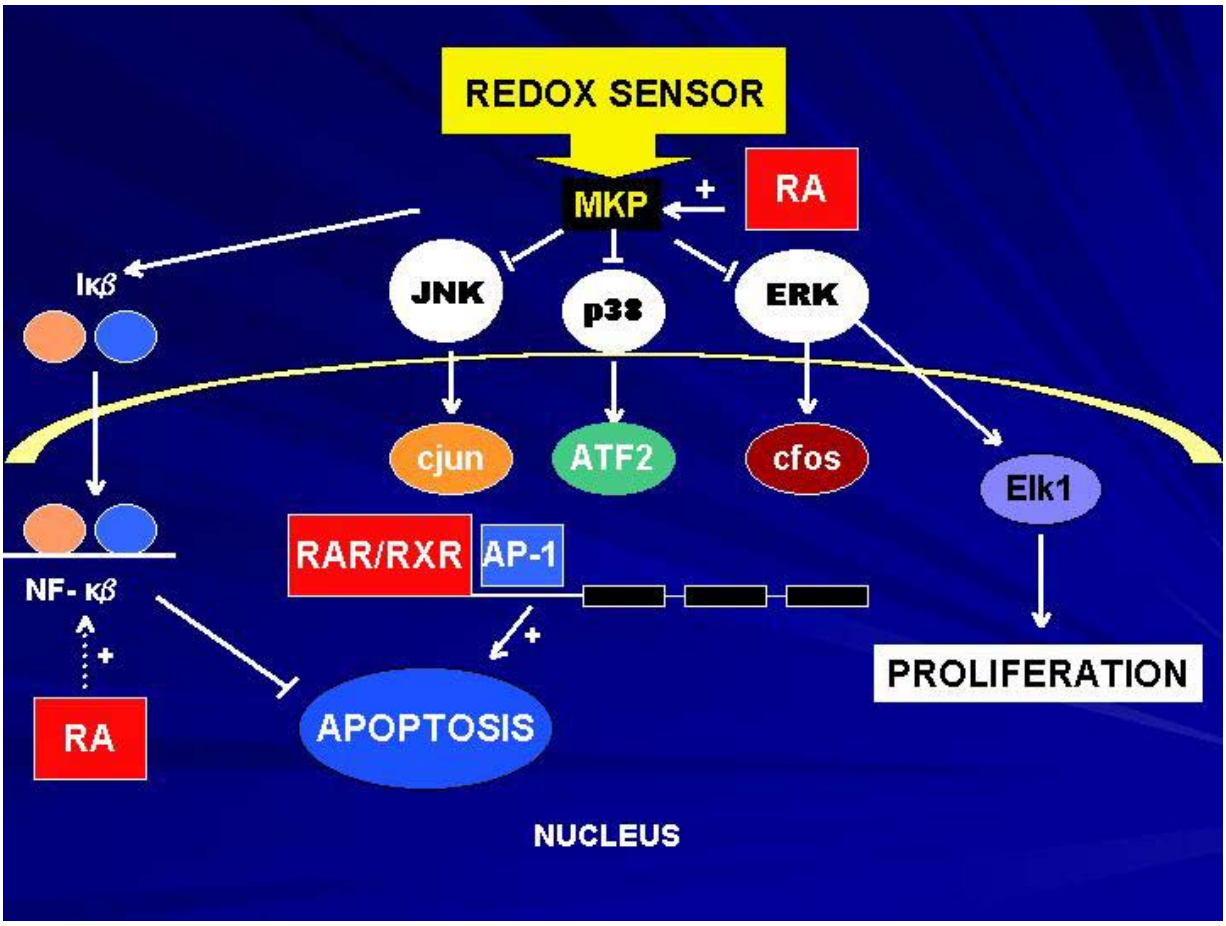

Figure 6-2. The redox pathway and potential mechanisms of action of retinoids in the nucleus during embryonic development.

(MKP-1), which inactivates MAPKs such as JNK and p38 MAPK (Xu et al., 2002) (Figure 6-2).

Interestingly, AP-1 can also increase the levels of xenobiotic metabolizing enzymes, such as glutathione transferase pi (GSTp), which ultimately influence the levels of intracellular glutathione (Figure 6-1). Under normal cellular conditions GSTp inhibits JNK activity (Adler et al., 1999), but in an oxidative stress-induced environment GSTp allows JNK to phosphorylate c-Jun, and AP-1 to be expressed. GSTp was found to contain a RARE (Lo and Ali-Osman, 1997), which was induced by RA treatment, suggesting another mechanism through which retinol may be acting.

The intracellular defense program must be intricately regulated to maintain homeostasis; therefore, multiple and complementary protection mechanisms are present in the oocyte and embryo and its environment (Guérin et al., 2001), including antioxidants. ROS exist in the mitochondria, cytoplasm, and cell membrane. They can be highly reactive such as OH•, and some ROS have different ½ lives; therefore, cells need diverse antioxidants to manage different kinds and levels of ROS (Gutteridge, 1995). For example, Vitamin E and its metabolites serve as radical chain breakers, radical scavengers include Ubiquinol 10, and β-carotene and retinoids provide defense against singlet oxygen quenchers.

The difficulty in assessing whether retinol is acting as an antioxidant either *in vitro* or *in vivo* is that vitamins, depending on their concentration and the state of the cell, can act as prooxidants or antioxidants (Herbert, 1996; Primiano et al., 1997). In fact, the chemistry of retinol, with its *pi* electrons allows it to exhibit both pro- and antioxidant

properties (Hoyos et al., 2000). One report even states that precautions must be taken when adding single antioxidants to the medium as they could have prooxidant effects (Guérin et al., 2001).

Recently, it was observed that retinol binds to the cysteine residues in the catalytic sites of the serine/threonine kinases, protein kinase C (PKC) and c-Raf (Hoyos et al., 2000; Imam et al., 2001); however, only under oxidizing conditions did retinol activate and increase the activity of these proteins. Raf ultimately leads to extracellular regulated kinase (ERK) activation and cellular proliferation (Finkel and Sullivan, 2000; Scita et al., 2000); thus, a possible mechanism by which retinol may be influencing embryonic development is through cellular proliferation (Figure 6-1).

Intracellular levels of glutathione play important roles in determining the redox state of the cell. Retinoids have been shown to inhibit the depletion of glutathione (Ahlemeyer and Krieglstein, 2000) and modulate glutathione peroxidase levels (Texeira et al., 1996) (Figure 6-1). One study provided evidence that glutathione depletion caused a hyperinduction of the JNK pathway, but if the cells were pretreated with N-acetylcysteine or glutathione this pathway was inhibited (Wilhelm et al., 1997). Thus, in our system, retinol may be converted to retinoic acid or another derivative that ultimately binds to and activates RAR/RXR. This receptor complex may then bind to antioxidant response elements (ARE) of certain antioxidant inducible genes (Primiano et al., 1997), which influence JNK and p38 MAPK pathways.

Retinoids have been shown to modulate growth factor expression as well as other genes important to preimplantation development. Several reports have described the role of peptide growth factors during embryonic development (review Teruel et al., 2000);

however, investigators are still unsure of their specific function(s), especially during *in vitro* culture (Kane et al., 1997; Teruel et al., 2000). Midkine, a member of the heparin-binding growth/differentiation family is induced by retinoic acid, and has been found to improve bovine oocyte and embryo developmental competence (Ikeda et al., 2000; Ikeda et al., 2000). In addition, retinoic acid was found to regulate epidermal growth factor receptor expression (EGFR), (Roulier et al., 1994), transforming growth factor (TGF-ß), insulin-like growth factor binding protein (IGFBP3) expression (Han et al., 1997), and follicle stimulating hormone receptor (FSHR) expression in granulosa cells (Minegishi et al., 2000).

Future work to define and clarify retinol's mechanism of action may include working in a completely defined system to evaluate retinol's affects, because serum and bovine serum albumin (BSA) may contain protective compounds (Ahlemeyer and Krieglstein, 2000). Comparing gene expression of the antioxidant transcripts between *in vivo* and *in vitro* derived oocytes may also provide necessary clues about how retinol is influencing early embryonic development. In fact, LeQuarre and colleagues have discovered that *in vitro* matured bovine oocytes are heterogeneous in their expression of certain genes, and this may correlate with developmental capacity (Lequarré et al., 1997). DNA microarray may also be very useful in discerning the mechanistic actions of retinol. Although *in vitro* culture induces oxidative stress, future experiments may include imposing oxidative stress with H_2O_2 or other compounds, and evaluating the effects of retinol. Furthermore, using specific mice strains that are null mutants for one or more retinoid related enzyme(s) or receptor(s) may also help dissect the mechanistic effects of retinol.

REFERENCES

Abbot AL, Ducibella T. 2001. Calcium and the control of mammalian cortical granule exocytosis. Front Biosci 6: d792-d806.

Abrieu A, Dorée M, Fisher D. 2001. The interplay between cyclin-B-Cdc2 kinase (MPF) and MAP kinase during maturation of oocytes. J Cell Sci 114(2): 257-267.

Adler V, Yin Z, Fuchs SY, Benezra M, Rosario L, Tew K, Pincus MR, Sardana M, Henderson CJ, Wolf CR, Davis RJ, Ronai Z. 1999. Regulation of JNK signaling by GSTp. The EMBO Journal 18(5): 1321-1334.

Ahlemeyer B, Bauerback E, Plath M, Steuber M, Heers C, Tegtmeier F, Krieglstein J. 2001. Retinoic acid reduces apoptosis and oxidative stress by preservation of SOD protein level. Free Radic Biol Med 30(10): 1067-1077.

Ahlemeyer B, Krieglstein J. 1998. Retinoic acid reduces staurosporine-induced apoptotic damge in chick embryonic neurons by suppressing reactive oxygen species. Neurosci Lett 246: 93-96.

Ahlemeyer B, Krieglstein J. 2000. Inhibition of glutathione depletion by retinoic acid and tocopherol protects cultured neurons from staurosporine-induced oxidative stress and apoptosis. Neurochem Int 36: 1-5.

Alsayed Y, Uddin S, Mahmud N, Lekmine F, Kalvakolanu D, Minucci S, Bokoch G, Platanias L. 2001. Activation of Rac1 and the p38 mitogen-activated protein kinase pathway in response to all-trans-retinoic acid. J Biol Chem 276(6): 4012-4019.

Anderson ME. 1985. Determination of glutathione and glutathione disulfide in biological samples. A. Meister. Methods Enzymol: Academic Press, Inc. 113**:**pp 548-585.

Antipatis C, Ashworth CJ, Riley SC, Hannah L, Hoggard N, Lea RG. 2002. Vitamin A deficiency during rat pregnancy alters placental TNF-alpha signalling and apoptosis. Am J Reprod Immunol 47: 151-158.

Arai M, Imai H, Koumoura T, Yoshida M, Emoto K, Umeda M, Chiba N, Nakagawa Y. 1999. Mitochondrial phospholipid hydroperoxide glutathione peroxidase plays a major role in preventing oxidative injury to cells. J Biol Chem 274(8): 4924-4933.

Attaran M, Pasqualotto E, Falcone T, Goldberg JM, Miller KF, Agarwal A, Sharma Rk. 2000. The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. Int J Fertil 45(5): 314-320.

Bagavandoss P, Midgley AR. 1988. Biphasic action of retinoids on gonadotropin receptor induction in rat granulosa cells in vitro. Life Sci 43: 1607-1614.

Barnett D, Bavister BD. 1996. What is the relationship between the metabolism of preimplantation embyros and their developmental competence? Mol Reprod Dev 43: 105-133.

Båvik C, Ward SJ, Chambon P. 1996. Developmental abnormalities in cultured mouse embryos deprived of retinoic acid by inhibition of yolk-sac retinol binding protein synthesis. Proc Natl Acad Sci U S A 93: 3110-3114.

Båvik C, Ward SJ, Ong DE. 1997. Identification of a mechanism to localize generation of retinoic acid in rat embryos. Mech Dev 69: 155-167.

Bavister BD. 1995. Culture of preimplantation embryos: facts and artifacts. Hum Reprod 1(2): 91-148.

Bernardini S, Melino G, Cortese C, Ballerini S, Annicchiarico-Petruzzelli M, Bernassola F, Corazzari M, Federici G. 1999. Modulation of glutathione transferase P1-1 activity by retinoic acid in neuroblastoma cells. J Cell Biochem 75: 375-381.

Besenfelder U, Solti L, Seregi J, Brem G. 1993. Influence of ß-carotene on fertility in rabbits when using embryo transfer programs. Theriogenology 39: 1093-1109.

Besenfelder U, Solti L, Seregi J, Müller M, Brem G. 1996. Different roles for ß-carotene and vitamin A in the reproduction on rabbits. Theriogenology 45: 1583-1591.

Biggers JD, Whittingham DG, Donahue RP. 1967. The pattern of energy metabolism in the mouse oocyte and zygote. Proc Natl Acad Sci U S A 58: 560-567.

Bisseling JGA, Knapen MFCM, Goverde HJM, Mulder TPJ, Peters WHM, Willemsen WNP, Thomas CMG, Steegers EAP. 1997. Glutathione S-transferases in human ovarian follicular fluid. Fertil Steril 68(5): 907-911.

Blaner WS, Olson JA. 1994. Retinol and retinoic acid metabolism. M. B. Sporn, A. B. Roberts and D. J. Goodman. The Retinoids: Biology, Chemistry, and Medicine. New York: Raven Press, Ltd. 2**:**pp 229-256.

Blomhoff R, Green MH, Berg T, Norum KR. 1990. Transport and storage of vitamin A. Science 250: 399-404.

Blondin P, Coenen K, Sirard MA. 1997. The impact of reactive oxygen species on bovine sperm fertilizing ability and oocyte maturation. J Androl 18(4): 454-460.

Blondin P, Sirard MA. 1995. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. Mol Reprod Dev 41: 54-62.

Boni R, Cuomoo A, Tosti E. 2002. Developmental potential in bovine oocytes is related to cumulus-oocyte complex grade, calcium current activity, and calcium stores. Biol Reprod 66: 836-842.

Brad AM, Bormann CL, Swain JE, Durkin RE, Johnson AE, Clifford AL, Krisher RL. 2003. Glutathione and adenosine triphosphate content of in vivo and in vitro matured porcine oocytes. Mol Reprod Dev 64: 492-498.

Brevini-Gandolfi TAL, Gandolfi F. 2001. The maternal legacy to the embryo: cytoplasmic components and their effects on early development. Theriogenology 55: 1255-1276.

Brown JA, Eberhardt DM, Schrick FN, Roberts MP, Godkin JD. 2003. Expression of retinol-binding protein and cellular retinol-binding protein in the bovine ovary. Mol Reprod Dev 64: 261-269.

Bucco R, Melner MH, Gordon DS, Leers-Sucheta S, Ong DE. 1995. Inducible expression of cellular retinoic acid-binding protein II in rat ovary: gonadotropin regulation during luteal development. Endocrinology 136(6): 2730-2740.

Budhu A, Noy N. 2002. Direct channeling of retinoic acid between cellular retinoic acid-binding protein II and retinoic acid acid receptor sensitizes mammary carcinoma cells to retinoic-acid induced growth. Mol Cell Biol 22(8): 2632-2641.

Butler JE, Williams JE. 1991. Noninvasive measurement of pyruvate uptake by ovine preimplantation embryos and unfertilized ova. Theriogenology 36(6): 1043-1048.

Butler JE, Williams JE. 1992. Noninvasive measurement of glucose uptake by preimplantation sheep embryos and unfertilized ova. Sm Rum Res 7: 347-352.

Calvin HI, Grosshans K, Blake EJ. 1986. Estimation and manipulation of glutathione levels in prepubertal mouse ovaries and ova: relevance to sperm nucleus transformation in the fertilized egg. Gamete Res 14: 265-275.

Carolan C, Lonergan P, Monget P, Monniaux D, Mermillod P. 1996. Effect of follicle size and quality on the ability of follicular fluid to support cytoplasmic maturation of bovine oocytes. Mol Reprod Dev 43: 477-483.

Cassano E, Tosto L, Balestrieri M, Zicarelli L, Abrescia P. 1999. Antioxidant defense in the follicular fluid of water buffalo. Cell Physiol Biochem 9: 106-116.

Chew BP, Weng BBC, Kim HW, Wong TS, Park JS, Lepine AJ. 2001. Uptake of ß-carotene by ovarian and uterine tissues and effects on steroidogenesis during the estrous cycle in cats. American Journal of Veterinary Research 62(7): 1063-1067.

Chiba H, Clifford J, Metzger D, Chambon P. 1997. Specific and redundant functions of retinoid X receptor/retinoic acid receptor heterodimers in differentiation, proliferation, and apoptosis of F9 embryonal carcinoma cells. J Cell Biol 139(3): 735-747.

Chu FF, Esworthy RS, Lee L, Wilczynski S. 1999. Retinoic acid induces *Gpx2* gene expression in MCF-7 human breast cancer cells. J Nutr 129: 1846-1854.

Clagett-Dame M, DeLuca HF. 2002. The role of vitamin A in mammalian reproduction and embryonic development. Annu Rev Nutr 22: 347-381.

Coffey MT, Britt JH. 1993. Enhancement of sow reproductive performance by ß-carotene or Vitamin A. J Anim Sci 71: 1198-1202.

Combelles CMH, Cekleniak NA, Albertini DF. 2002. Assessment of nuclear and cytoplasmic maturation in in-vitro matured human oocytes. Hum Reprod 17(4): 1006-1016.

Crosier AE, Farin PW, Dykstra MJ, Alexander JE, Farin CE. 2000. Ultrastructural morphometry of bovine compact morulae produced in vivo or in vitro. Biol Reprod 62: 1459-1465.

Da Silveira PRS, Fernandes LCO, de Moraes Filho JC, Junior WB. 1998. Reproductive performance of sows injected with vitamin A. R Brasialian Zootec 27(4): 743-748.

Damiani P, Fissore RA, Cibelli JB, Long CR, Balise JJ, Robl JM, Duby RT. 1996. Evaluation of developmental competence, nuclear and ooplasmic maturation of calf oocytes. Mol Reprod Dev 45: 521-534.

de Bruijn DRH, Oerlemans F, Hendricks W, Baats E, Ploemacher R, Wieringa B, van Kessel AG. 1994. Normal development, growth and reproduction in cellular retinoic acid binding protein-I (CRABPI) null mutant mice. Differentiation 58: 141-148.

De La Fuente R, Eppig JJ. 2001. Transcriptional activity of the mouse oocyte genome: companion granulosa cells modulate transcription and chromatin remodeling. Dev Biol 229: 224-236.

De Loos F, Van Maurik P, Van Beneden T, Kruip TAM. 1992. Structural aspects of bovine oocyte maturation in vitro. Mol Reprod Dev 31: 208-214.

de Matos DG, Cortvrindt R, Smitz J, Van Soom A, Nogueira D, Pasqualini SR. 2002. Cysteamine supplementation during in vitro maturation and embryo culture: a useful tool for increasing the efficiency of bovine in vitro embryo production. Mol Reprod Dev 62: 203-209.

de Matos DG, Furnus CC. 2000. The importance of having high glutathioine (GSH) level after bovine in vitro maturation on embryo development: effect of ß-mercaptoethanol, cysteine and cystine. Theriogenology 53: 761-771.

de Matos DG, Furnus CC, Moses DF, Baldassarre H. 1995. Effect of cysteamine on glutathione level and developmental capacity of bovine oocytes matured in vitro. Mol Reprod Dev 42: 432-436.

de Matos DG, Gasparrini B, Pasqualini SR, Thompson JG. 2002. Effect of glutathione synthesis stimulation during in vitro maturation of ovine oocytes on embryo development and intracellular peroxide content. Theriogenology 57: 1443-1451.

de Matos DG, Nogueira D, Cortvrindt R, Herrera C, Adriaenssens T, Pasqualini RS, Smitz J. 2003. Capacity of adult and prepubertal mouse oocytes to undergo embryo development in the presence of cysteamine. Mol Reprod Dev 64: 214-218.

De Sousa PA, Watson AJ, Schultz GA, Bilodeau-Goeseels S. 1998. Oogenetic and zygotic gene expression directing early bovine embryogenesis: A review. Mol Reprod Dev 51: 112-121.

Deltour L, Foglio MH, Duester G. 1999. Impaired retinol utilization in Adh4 alcohol dehydrogenase mutant mice. Dev Genet 25: 1-10.

Deplancke B, Gaskins HR. 2002. Redox control of the transsulfuration and glutathione biosynthesis pathways. Curr Opin Clin Nut Met Ca 5: 85-92.

Dinara S, Sengoku K, Tamate K, Horikawa M, Ishikawa M. 2001. Effects of supplementation with free radical scavengers on the survival and fertilization rates of mouse cryopreserved oocytes. Hum Reprod 16(9): 1976-1981.

Dominko T, First NL. 1997. Timing of meiotic progression in bovine oocytes and its effect on early embryo development. Mol Reprod Dev 47: 456-467.

Doré JJE, Roberts MP, Godkin JD. 1994. Early gestational expression of retinol-binding protein mRNA by the ovine conceptus and endometrium. Mol Reprod Dev 38: 24-29.

Downs SM, Houghton FD, Humpherson PG, Leese HJ. 1997. Substrate utilization and maturation of cumulus cell-enclosed mouse oocytes: evidence that pyruvate oxidation does not mediate meiotic induction. J Reprod Fertil 110: 1-10.

Downs SM, Humpherson PG, Leese HJ. 2002. Pyruvate utilization by mouse oocytes is influenced by meiotic status and the cumulus oophorus. Mol Reprod Dev 62: 113-123.

Downs SM, Mastropolo AM. 1994. The participation of energy substrates in the control of meiotic maturation in murine oocytes. Dev Biol 162: 154-168.

Downs SM, Mastropolo AM. 1997. Culture conditions affect meiotic regulation in cumulus cell-enclosed mouse oocytes. Mol Reprod Dev 46: 551-566.

Downs SM, Utecht AM. 1999. Metabolism of radiolabled glucose by mouse oocytes and oocyte-cumulus cell complexes. Biol Reprod 60: 1446-1452.

Driancourt MA, Thuel B. 1998. Control of oocyte growth and maturation by follicular cells and molecules present in follicular fluid. A review. Reprod Nutr Dev 38: 345-362.

Dröge W. 2002. Free radicals in the physiological control of cell function. Physiol Rev 82: 47-95.

Duque P, Díez C, Royo L, Lorenzo PL, Carneiro G, Hidalgo CO, Facal N, Gómez E. 2002. Enhancement of developmental capacity of meiotically inhibited bovine oocytes by retinoic acid. Hum Reprod 17(10): 2706-2714.

Duranthon V, Renard J. 2001. The developmental competence of mammalian oocytes: a convenient but biologically fuzzy concept. Theriogenology 55: 1277-1289.

Ealy AD, Drost M, Barros CM, Hansen PJ. 1992. Thermoprotection of preimplantation bovine embryos from heat shock by glutathione and taurine. Cell Biol Int Rep 16(2): 125-131.

Eberhardt DM, Jacobs WG, Godkin JE. 1999a. Steroid regulation of retinol-binding protein in the ovine oviduct. Biol Reprod 60: 714-720.

Eberhardt DM, Will WA, Godkin JD. 1999b. Retinol administration to superovulated ewes improves in vitro embryonic viablility. Biol Reprod 60: 1483-1487.

Edwards JL, King WA, Kawarsky SJ, Ealy AD. 2001. Responsiveness of early embryos to environmental insults: potential protective roles of HSP70 and glutathione. Theriogenology 55: 209-223.

El Mouatassim S, Guérin P, Ménézo Y. 1999. Expression of genes encoding antioxidant enzymes in human and mouse oocytes during the final stages of maturation. Mol Hum Reprod 5(8): 720-725.

El Mouatassim S, Guérin P, Ménézo Y. 2000. Mammalian oviduct and protection against free oxygen radicals: expression of genes encoding antioxidant enzymes in human and mouse. Eur J Obstet Gynecol Reprod Biol 89: 1-6.

Elizondo G, Corchero J, Sterneck E, Gonzalez FJ. 2000. Feedback inhibition of the retinaldehyde dehydrogenase gene *ALDH1* through retinoic acid receptor alpha and CCAAT/Enhancer-binding protein ß. J Biol Chem 275(50): 39747-39753.

Elmarimi AA, Holdas S, Vén E, Imrik P. 1990. Effect of vitamin A supplementation on mice embryo production and viability. Reproduction of Domestic Animals 25: 247-248.

Eppig JJ. 2001. Oocyte control of ovarian follicular development and function in mammals. Reproduction 122: 829-838.

Eppig JJ, O'Brien MO, Wigglesworth K. 1996. Mammalian oocyte growth and development. Mol Reprod Dev 44: 260-273.

Eppig JJ, Schultz RM, O'Brien M, Chesnel F. 1994. Relationship between the developmental programs controlling nuclear and cytoplasmic maturation of mouse oocytes. Dev Biol 164: 1-9.

Finkel T, Sullivan DM. 2000. Reactive oxygen species in proliferative signaling. J. S. Gutkind. Signaling networks and cell cycle control: The molecular basis of cancer and other diseases. Totowa: Humana Press.

Fiorenza MT, Mangia F. 1998. Quantitative RT-PCR amplification of RNA in single mouse oocytes and preimplantation embryos. Biotechniques 24(4): 618-623.

Foote RH, Ellington JE. 1988. Is a superovulated oocyte normal? Theriogenology 29(1): 111-123.

Fujii H, Sata T, Kaneko S, Gotoh O, Fujii-Kuriyama Y, Osawa K, Kata S, Hamda H. 1997. Metabolic inactivation of retinoic acid by a novel P450 differentially expressed in developing mouse embryos. EMBO J 16(14): 4163-4173.

Furnus CC, de Matos DG, Moses DF. 1998. Cumulus expansion during in vitro maturation of bovine oocytes: relationship with intracellular glutathione level and its role on subsequent embryo development. Mol Reprod Dev 51: 76-83.

Gandolfi F, Milanesi E, Pocar P, Luciano AM, Brevini TAL, Acocella F, Lauria A, Armstrong DT. 1998. Comparative analysis of calf and cow oocytes during in vitro maturation. Mol Reprod Dev 49: 168-175.

Ganguly J. 1989. Biochemistry of Vitamin A. Boca Raton: CRC Press.

Gao K, Liu KH, Godkin JD. 1991. Immunohistochemical localization of bovine placental retinol-binding protein. Int J Dev Biol 35: 485-489.

Gardiner CS, Salmen JJ, Brandt CJ, Stover SK. 1998. Glutathione is present in reproductive tract secretions and improves development of mouse embryos after chemically induced glutathione depletion. Biol Reprod 59: 431-436.

Gardner DK, Lane M, Batt P. 1993. Uptake and metabolism of pyruvate and glucose by individual sheep preattachment embryos developed in vivo. Mol Reprod Dev 36: 313-319.

Gardner DK, Pawelczynski M, Trouson AO. 1996. Nutrient uptake and utilization can be used to select viable day 7 bovine blastocysts after cryopreservation. Mol Reprod Dev 44: 472-475.

Gasparrini B, Neglia G, Di Palo R, Campanile G, Zicarelli L. 2000. Effect of cysteamine during in vitro maturation on buffalo embryo development. Theriogenology 54: 1537-1542.

Ghyselinck NB, Båvik C, Sapin V, Mark M, Bonnier D, Hindelang C, Dierich A, Nilsson CB, Håkansson H, Sauvant P, Azaïs-Braesco V, Frasson M, Picaud S, Chambon P. 1999. Cellular retinol-binding protein I is essential for vitamin A homeostasis. EMBO Journal 18(18): 4903-4914.

Ghyselinck NB, Dupé V, Dierich A, Messaddeq N, Garnier JM, Rochette-Eagly C, Chambon P, Mark M. 1997. Role of retinoic acid receptor beta (RARß) during mouse development. Int J Dev Biol 41: 425-447.

Goto Y, Noda Y, Mori T, Nakano M. 1993. Increased generation of reactive oxygen species in embryos cultured in vitro. Free Radic Biol Med 15: 69-75.

Gudas LJ, Sporn MB, Roberts AB. 1994. Cellular biology and biochemistry of the retinoids. M. B. Sporn, A. B. Roberts and D. J. Goodman. The Retinoids: Biology, Chemistry, and Medicine. New York: Raven Press, Ltd. 2**:**pp 443-520.

Guérin P, El Mouatassim S, Ménézo Y. 2001. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum Reprod Update 7(2): 175-189.

Gutteridge JMC. 1995. Signal, messenger and trigger molecules from free radical reactions and their control by antioxidants. L. Packer and K. Wirtz. Signalling mechanisms - from transcription factors to oxidative stress. Heidelberg: Springer-Verlag. 92**:**pp 157-164.

Haidar MA, Manshouri T, Keating MJ, Kantarjian HM, Freireich EJ, Mehta K, Albitar M. 2000. Downregulation of the p53 tumor suppressor gene and upregulation of the bcl-2 gene in retinoic acid receptor alpha deficient transgenic mice. Int J Oncol 16: 561-565.

Hallewell RA, Implay KC, Lee P, Fong NM, Gallegos C, Getzoff ED, Tainer JA, Cabelli DE, Tekamp-Olson P, Mullenbach GT, Cousen LS. 1991. Thermostabilization of recombinant human and bovine Cu/Zn superoxide dismutatases by replacement of free cysteines. Biochemical and Biophysical Research Communications 181: 474-480.

Han G, Dohi D, Lee H, Rajah R, Walsh G, Hong W, Cohen P, Kurie J. 1997. All-trans-retinoic acid increases transforming growth factor-b2 and insulin-like growth factor binding protein-3 expression through a retinoic acid receptor dependent signaling pathway. J Biol Chem 272(21): 13711-13716.

Hancock JF. 1999. Kinase cascades and apoptosis. D. Watters and M. Lavin. Signalling pathways in apoptosis. Herston: Harwood Academic Publishers. 5**:**pp 123-146.

Hansen JM, Choe HS, Carney EW, Harris C. 2001. Differential antioxidant enzyme activities and glutathione content between rat and rabbit conceptuses. Free Radic Biol Med 30(10): 1078-1088.

Hardy K. 1999. Apoptosis in the human embryo. Rev Reprod 4: 125-134.

Harvey MB, Arcellan-Panlilio MY, Zhang X, Schultz GA, Watson AJ. 1995. Expression of genes encoding antioxidant enzymes in preimplantation mouse and cow embryos and primary oviduct cultures employed for embryo coculture. Biol Reprod 53: 532-540.

Harvey MB, Kind KL, Thompson JG. 2002. REDOX regulation of early embryo development. Reproduction 123: 479-486.

Hashimoto S, Minami N, Yamada M, Imai H. 2000. Excessive concentration of glucose during in vitro maturation impairs the developmental competence of bovine oocytes after in vitro fertilization: relevance to intracellular reactive oxygen species and glutathione contents. Mol Reprod Dev 56: 520-526.

Hayden LJ, Satre MA. 2002. Alterations in cellular retinol metabolism contribute to differential retinoid responsiveness in normal human mammary epithelial cells versus breast cancer cells. Breast Cancer Res Treat 75: 95-105.

Herbert V. 1996. Prooxidant effects of antioxidant vitamins. J Nutr 126: 1197-1200.

Hidalgo CO, Diéz c, Duque P, Facal N, Gómez E. 2003. Pregnancies and improved early embryonic development with bovine oocytes matured in vitro with 9-cis-retinoic acid. Reproduction 125: 409-416.

Hidalgo CO, Diez C, Duque P, Facal N, Prendes JM, Fernandez I, Gomez E. 2002. Improved cumulus-oocyte complex yields from heifers treated with retinol. Theriogenology 57: 672.

Hofmann C, Eichele G. 1994. Retinoids in development. M. B. Sporn, A. B. Roberts and D. J. Goodman. The Retinoids: Biology, Chemistry, and Medicine. New York: Raven Press, Ltd. 2**:**pp 387-442.

Horton C, Maden M. 1995. Endogenous distribution of retinoids during normal development and teratogenesis in the mouse embryo. Dev Dyn 202: 312-323.

Hoyos B, Imam A, Chua R, Swenson C, Tong GX, Levi E, Noy N, Hämmerling U. 2000. The cysteine-rich regions of the regulatory domains of raf and protein kinase C as retinoid receptors. J Exp Med 192(6): 835-845.

Huang FJ, Shen CC, Chang SY, Wu TCJ, Hsuuw YD. 2003. Retinoic acid decreases the viability of mouse blastocysts in vitro. Hum Reprod 18(1): 130-136.

Huang FJ, Wu TCJ, Tsai MY. 2001. Effect of retinoic acid on implantation and post-implantation development of mouse embryos *in vitro*. Hum Reprod 16(10): 2171-2176.

Huang ZZ, Yang HW, Chen C, Zeng Z, Lu SC. 2000. Inducers of gamma-glutamylcysteine synthetase and their effects on glutathione synthetase expression. Biochemica et Biophysica Acta 1493: 48-55.

Hunter MG. 2000. Oocyte maturation and ovum quality in pigs. Rev Reprod 5: 122-130.

Ichijo H. 1999. From receptors to stress-activated MAP kinases. Oncogene 18(45): 6087-6093.

Ikeda S, Ichihara-Tanaka K, Azuma T, Muramatsu T, Yamada M. 2000. Effect of midkine during in vitro maturation of bovine oocytes on subsequent developmental competence. Biol Reprod 63: 1067-1074.

Ikeda S, Nishikimi A, Ichihara-Tanaka K, Muramatsu T, Yamada M. 2000. cDNA cloning of bovine midkine and production of the recombinant protein, which affects in vitro maturation of bovine oocytes. Mol Reprod Dev 57: 99-107.

Imam A, Hoyos B, Swenson C, Levi E, Chua R, Viriya E, Hammerling U. 2001. Retinoids as ligands and coactivators of protein kinase C alpha. FASEB J 15: 28-30.

Iwata H, Akamatsu S, Minami N, Yamada M. 1998. Effects of antioxidants on the development of bovine IVM/IVF embryos in various concentrations of glucose. Theriogenology 50: 365-375.

Izadyar F, Hage WJ, Colenbrander B, Bevers MM. 1998. The promotory effect of growth hormone on the developmental competence of in vitro matured bovine oocytes is due to improved cytoplasmic maturation. Mol Reprod Dev 49: 444-453.

Jayaram M, Murthy SK, Ganguly J. 1973. Effect of vitamin A deprivation on the cholesterol side-chain cleavage enzyme activity of testes and ovaries of rats. Biochem J 136: 221-223.

Juneja HS, Murthy SK, Ganguly J. 1966. The effect of vitamin A deficiency on the biosynthesis of steroid hormones in rats. Biochem J 99: 138-145.

Kane MT, Morgan PM, Coonan C. 1997. Peptide growth factors and preimplantation development. Hum Reprod Update 3(2): 137-157.

Katoh M. 2002. Regulation of WNT signaling molecules by retinoic acid during neuronal differentiation in NT2 cells: Threshold model of WNT action (reveiw). Int J Mol Med 10(6): 683-687.

Katz J, Wood HG. 1963. The use of $C^{14}O_2$ yields from glucose-1- and -6-C^{14} for the evaluation of the pathways of glucose metabolism. J Biol Chem 238(2): 517-523.

Keenan DL. 1997. Dietary carrot results in diminished ovarian progesterone secretion, whereas a metabolite, retinoic acid, stimulates progesterone secretion in the in vitro perfused rabbit ovary. Fertil Steril 68(2): 358-363.

Ketterer B. 2001. A bird's eye view of the glutathione transferase field. Chem Biol Interact 138: 27-42.

Khurana NK, Niemann H. 2000. Energy metabolism in preimplantation bovine embryos in vitro or in vivo. Biol Reprod 62: 847-856.

Kirchhof N, Carnwath JW, Lemme E, Anastassiadis K, Scholer H, Niemann H. 2000. Expression pattern of Oct-4 in preimplanation embryos of different species. Biol Reprod 63(6): 1698-1705.

Knappen MFCM, Zusterzeel PLM, Peters WHM, Steegers EAP. 1999. Glutathione and glutathione-related enzymes in reproduction: a review. Eur J Obstet Gynecol Reprod Biol 82: 171-184.

Knijn HM, Wrenzycki C, Hendricksen PJM, Vos PLAM, Herrmann D, van der Weijden GC, Niemann H, Dieleman SJ. 2002. Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocysts derived in vitro or in vivo. Reproduction 124: 365-375.

Kochhar DM, Jiang H, Penner JD, Johnson AT, Chandraratna RAS. 1998. The use of a retinoid receptor antagonist in a new model to study vitamin A-dependent developmental events. Int J Dev Biol 42: 601-608.

Konta T, Xu Q, Furusu A, Nakayama K, Kitamura M. 2001. Selective roles of retinoic acid receptor and retinoid X receptor in the suppression of apoptosis by all-*trans*-retinoic acid. J Biol Chem 276(16): 12697-12701.

Koshimizu U, Watanabe M, Nakatsuji N. 1995. Retinoic acid is a potent growth activator of mouse primordial germ cells *in vitro*. Dev Biol 168: 683-685.

Krisher RL, Bavister BD. 1999. Enhanced glycolysis after maturation of bovine oocytes in vitro is associated with increased developmental competence. Mol Reprod Dev 53: 19-26.

Krisher RL, Lane M, Bavister BD. 1999. Developmental competence and metabolism of bovine embryos cultured in semi-defined and defined media. Biol Reprod 60: 1345-1352.

Kubota C, Yang X, Dinnyés A, Todoroki J, Yamakuchi H, Mizoshita K, Inohae S, Tabara N. 1998. In vitro and in vivo survival of frozen-thawed bovine oocytes after IVF, nuclear transfer, and parthenogenetic activation. Mol Reprod Dev 51: 281-286.

Lane M. 2001. Mechanisms for managing celluar and homeostatic stress in vitro. Theriogenology 55: 225-236.

Lane M, Gardner DK. 1996. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. Hum Reprod 11(9): 1975-1978.

Latham KE, Schultz RM. 2001. Embryonic gene activation. Front Biosci 6: d748-d759.

Lee HY, Walsh GL, Dawson MI, Kong WK, Kurie JM. 1998. All-*trans*-retinoic acid inhibits Jun N-terminal kinase-dependent signaling pathways. J Biol Chem 273(12): 7066-7071.

Leese HJ. 1991. Metabolism of the preimplantation mammalian embryo. S. R. Milligan. Oxf Rev Reprod Biol: Oxford University Press. 13**:**pp 35-72.

Leese HJ, Barton AM. 1984. Pyruvate and glucose uptake by mouse ova and preimplantation embryos. J Reprod Fertil 72: 9-13.

Lefèvre A, Rogier E, Astraudo C, Duquenne C, Finaz C. 1994. Regulation by retinoids of lutenizing hormone/chorionic gonadotropin receptor, cholesterol side-chain cleavage cytochrome P-450, 3ß-hydroxysteroid dehydrogenase/ $^{5-4}$-isomerase and 17alpha-hydroxylase/C_{17-20} lyase cytochrome P-450 messenger ribonucleic acid levels in the K9 mouse leydig cell line. Mol Cell Endocrinol 106: 31-39.

Lequarré AS, Feugang JM, Malhomme O, Donnay I, Massip A, Dessy F, Van Langendonckt A. 2001. Expression of Cu/Zu and Mn superoxide dismutases during bovine embryo development: influence of in vitro culture. Mol Reprod Dev 58: 45-53.

Lequarré AS, Grisart B, Moreau B, Schuurbiers N, Massip A, Dessy F. 1997. Glucose metabolism during bovine preimplantation development: analysis of gene expression in single oocytes and embryos. Mol Reprod Dev 48: 216-226.

Lim JM, Liou SS, Hansel W. 1996. Intracytoplasmic glutathione concentration and the role of ß-mercaptoethanol in preimplantation development of bovine embryos. Theriogenology 46: 429-439.

Liu KH, Doré JJE, Roberts MP, Krishnan R, Hopkins FM, Godkin JD. 1993. Expression and cellular localization of retinol-binding protein messenger ribonucleic acid in bovine blastocysts and extraembryonic membranes. Biol Reprod 49: 393-400.

Liu KH, Gao K, Baumbach GA, Godkin JD. 1992. Purification and immunolocalization of ovine placental retinol-binding protein. Biol Reprod 46: 23-29.

Liu KH, Godkin JD. 1992. Characterization and immunolocalization of bovine uterine retinol-binding protein. Biol Reprod 47: 1099-1104.

Liu Z, Foote RH. 1995. Development of bovine embryos in KSOM with added superoxide dismutase and taurine and with five and twenty percent O_2. Biol Reprod 53: 786-790.

Livera G, Rouiller-Fabre V, Durand P, Habert R. 2000. Multiple effects of retinoids on the development of sertoli, germ, and leydig cells of fetal and neonatal rat testis in culture. Biol Reprod 62: 1303-1314.

Livingston TE, Eberhardt DM, Godkin JD. 2002. The effects of retinol on bovine embryo development in vitro. Biol Reprod 66(Supp 1): 18.

Lo HW, Ali-Osman F. 1997. Genomic cloning of *hGSTP1*C*, an allelic human Pi class glutathione *s*-transferase gene variant and functional characterization of its retinoic acid response elements. J Biol Chem 272(52): 32743-32749.

Luderer U, Kavanaugh TJ, White CC, Faustman EM. 2001. Gonadotropin regulation of glutathione synthesis in the rat ovary. Reprod Toxicol 15: 495-504.

Lufkin T, Lohnes D, Mark M, Dierich A, Gorry P, Gaub M, LeMeur M, Chambon P. 1993. High postnatal lethality and testis degeneration in retinoic acid receptor alpha mutant mice. Proc Natl Acad Sci U S A 90: 7225-7229.

Luvoni GC, Keskintepe L, Brackett BG. 1996. Improvement in bovine embryo production in vitro by glutathione-containing culture media. Mol Reprod Dev 43: 437-443.

Mackenzie SH, Roberts MP, Liu KH, Doré JJE, Godkin JD. 1997. Bovine endometrial retinol-binding protein secretion, messenger ribonucleic acid expression, and cellular localization during the estrous cycle and early pregnancy. Biol Reprod 57: 1445-1450.

Maden M. 2000. The role of retinoic acid in embryonic and post-embryonic development. Proc Nutr Soc 59: 65-73.

Malaba L, Smeland S, Senoo H, Norum KR, Berg T, Blomhoff R, Kindberg GM. 1995. Retinol-binding protein and asialo-orosomucoid are taken up by different pathways in liver cells. J Biol Chem 270(26): 15686-15692.

Mangelsdorf DJ, Evans RM. 1995. The RXR heterodimers and orphan receptors. Cell 83: 841-850.

Mangelsdorf DJ, Umesono K, Evans RM. 1994. The retinoid receptors. M. B. Sporn, A. B. Roberts and D. J. Goodman. The Retinoids: Biology, Chemistry, and Medicine. New York: Raven Press, Ltd. 2**:**pp 319-350.

Marinó M, Andrews D, Brown D, McCluskey RT. 2001. Transcytosis of retinol-binding protein across renal proximal tubule cells after megalin (gp 330)-mediated endocytosis. J Am Soc Nephrol 12: 637-648.

Marshall H, Morrison A, Studer M, Popperl H, Krumlauf R. 1996. Retinoids and Hox genes. FASEB 10: 969-978.

Martindale JL, Holbrook NJ. 2002. Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol 192: 1-15.

Matwee C, Betts DH, King WA. 2000. Apoptosis in the early bovine embryo. Zygote 8: 57-68.

Matzuk MM, Burns KH, Viveiros MM, Eppig JJ. 2002. Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science 296: 2178-2180.

Mayor P, López-Béjar M, Rodríguez-González E, Paramio MT. 2001. Effects of the addition of glutathione during maturation on *in vitro* fertilisation of prepubertal goat oocytes. Zygote 9: 323-330.

McCollum E. 1964. From Kansas Farm Boy to Scientist. Lawrence: University of Kansas.

McCord JM, Fridovich I. 1969. Superoxide dismutase. An enzymatic function for erythrocuprein. J Biol Chem 244: 6049-6055.

McKiernan SH, Bavister BD. 1994. Timing of development is a critical parameter for predicting successful embryogenesis. Hum Reprod 9(11): 2123-2129.

Memili E, First NL. 2000. Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species. Zygote 8: 87-96.

Mermillod P, Oussaid B, Cognié Y. 1999. Aspects of follicular and oocyte maturation that affect the developmental potential of embryos. J Reprod Fertil 54: 449-460.

Meyrick B, Magnuson MA. 1994. Identification and functional characterization of the bovine manganous superoxide dismutase promoter. Am J Respir Cell Mol Biol 10: 13-21.

Minegishi T, Hirakawa T, Kishi H, Abe K, Tano M, Abe Y, Miyamoto K. 2000. The mechanisms of retinoic acid-induced regulation on the follicle stimulating hormone receptor in rat granulosa cells. Biochemica et Biophysica Acta 1495(3): 203-211.

Mohan M, Malayer JR, Geisert RD, Morgan GL. 2002. Expression patterns of retinoid X receptors, retinaldehyde dehydrogenase, and peroxisome proliferator activated receptor gamma in bovine preattachment embryos. Biol Reprod 66: 692-700.

Molotkov A, Deltour L, Foglio MH, Cuenca AE, Duester G. 2002. Distinct retinoid metabolic functions for alcohol dehydrogenase genes *Adh1* and *Adh4* in protection against vitamin A toxicity or deficiency revealed in double null mutant mice. J Biol Chem 277(16): 13804-13811.

Moore T. 1957. Vitamin A. Amsterdam: Elsevier Publishing Company.

Moreno-Manzano V, Ishikawa Y, Lucio-Cazana J, Kitamura M. 1999. Suppression of apoptosis by all-*trans*-retinoic acid. J Biol Chem 274(29): 20251-20258.

Morita Y, Tilly JL. 1999. Oocyte apoptosis: like sand through an hourglass. Dev Biol 213: 1-17.

Morita Y, Tilly JL. 1999. Segregation of retinoic acid effects on fetal ovarian germ cell mitosis *versus* apoptosis by requirement for new macromolecular synthesis. Endocrinology 140(6): 2696-2703.

Napoli JL. 1996. Retinoic acid biosynthesis and metabolism. FASEB 10: 993-1001.

Napoli JL. 1999. Interactions of retinoid binding proteins and enzymes in retinoid metabolism. Biochemica et Biophysica Acta 1440: 139-162.

Neiderreither K, Subbarayan V, Dollé P, Chambon P. 1999. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. Nat Genet 21: 444-448.

Newcomer ME, Ong DE. 2000. Plasma retinol binding protein: structure and function of the prototypic lipocalin. Biochemica et Biophysica Acta 1482: 57-64.

Niederreither K, Abu-Abed S, Schuhbaur B, Petkovich M, Chambon P, Dollé P. 2002. Genetic evidence that oxidative derivatives of retinoic acid are not involved in retinoid signaling during mouse development. Nat Genet 31: 84-88.

Noy N. 2000. Retinoid-binding proteins: mediators of retinoid action. Biochem J 348: 481-495.

Noy N, Xu ZJ. 1990. Interactions of retinol with binding proteins: implications for the mechanism of uptake by cells. Biochemistry (Mosc) 29(16): 3878-3883.

Olson JA. 1993. Vitamin A and carotenoids as antioxidants in a physiological context. J Nutr Sci Vitaminol (Tokyo) 39: S57-S65.

Olson SE, Seidel Jr GE. 2000. Culture of in vitro-produced bovine embryos with Vitamin E improves development in vitro and after transfer to recipients. Biol Reprod 62: 248-252.

Ong DE, Newcomer ME, Chytil F. 1994. Cellular retinoid-binding proteins. M. B. Sporn, A. B. Roberts and D. J. Goodman. The Retinoids: Biology, Chemistry, and Medicine. New York: Raven Press, Ltd. 2**:**pp 283-318.

Orsi NM, Leese HJ. 2001. Protection against reactive oxygen species during mouse preimplantation embryo development: role of EDTA, oxygen tension, catalase, superoxide dismutase and pyruvate. Mol Reprod Dev 59: 44-53.

Overström EW. 1996. In vitro assessment of embryo viability. Theriogenology 45: 3-16.

Overström EW. 2000. Evaluation of embryo quality in vitro: an update. Proceedings of the American Embryo Transfer Association annual convention.

Ovitt C, Scholer H. 1998. The molecular biology of Oct-4 in the early mouse embryo. Mol Hum Reprod 4(11): 1021-1031.

Pabon JE, Findley WE, Gibbons WE. 1989. The toxic effect of short exposures to the atmospheric oxygen concentration on early mouse embryonic development. Fertil Steril 51(5): 896-900.

Packer L. 1995. Antioxidants: molecular interactions between vitamins E and C, Biothiols and Carotenes. L. Packer and K. Wirtz. Signalling mechanisms - from transcription factors to oxidative stress. Heidelberg: Springer-Verlag. 92**:**pp 187-216.

Palmer HJ, Paulson KE. 1997. Reactive oxygen species and antioxidants in signal transduction and gene expression. Nutr Rev 55(10): 353-361.

Parekh DB, Ziegler W, Parker PJ. 2000. Multiple pathways control protein kinase C phosphorylation. EMBO J 19(4): 496-503.

Pavlok A, Lucas-Hahn A, Niemann H. 1992. Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. Mol Reprod Dev 31: 63-67.

Perreault SD, Barbee RR, Slott VL. 1988. Importance of glutathione in the acquisition and maintenance of sperm nuclear decondensing activity in maturing hamster oocytes. Dev Biol 125: 181-186.

Pratt CMA, Niu MY. 2003. Bcl-2 controls caspase activation following a p53-dependent cyclin D1-induced death signal. J Biol Chem 278(16): 14219-14229.

Primiano T, Sutter TR, Kensler TW. 1997. Antioxidant-inducible genes. Adv Pharmacol 38: 293-328.

Pusateri AE, Diekman MA, Singleton WL. 1999. Failure of vitamin A to increase litter size in sows receiving injections at various stages of gestation. J Anim Sci 77(6): 1532-1535.

Radominska-Pandya A, Chen G, Czernik PJ, Little JM, Samokyszyn VM, Carter CA, Nowak G. 2000. Direct interaction of all-*trans*-retinoic acid with protein kinase C (PKC). J Biol Chem 275(29): 22324-22330.

Raijmakers MTM, Steegers EAP, Peters WHM. 2001. Glutathione S-transferase and thiol concentrations in embryonic and early fetal tissues. Hum Reprod 16(11): 2445-2450.

Ricken A, Lochhead P, Kontogiannea M, Farookhi R. 2002. Wnt signaling in the ovary: identification and compartmentalized expression of wnt-2, wnt-2b, and frizzled 4 mRNAs. Endocrinology 143(7): 2741-2749.

Rieger D, Guay P. 1988. Measurement of the metabolism of energy substrates in individual bovine blastocysts. J Reprod Fertil 83: 585-591.

Rieger D, Loskutoff NM. 1994. Changes in the metabolism of glucose, pyruvate, glutamine and glycine during maturation of cattle oocytes *in vitro*. J Reprod Fertil 100: 257-262.

Rizos D, Fair T, Papadopoulos S, Boland MP, Lonergan P. 2002. Developmental, qualitative, and ultrastructural differences between ovine and bovine embryos produced in vivo or in vitro. Mol Reprod Dev 62: 320-327.

Robert C, McGraw S, Massicotte L, Pravetoni M, Gandolfi F, Sirard MA. 2002. Quantification of housekeeping transcript levels during the development of bovine preimplantation embryos. Biol Reprod 67: 1465-1472.

Rohwedel J, Guan K, Wobus AM. 1999. Induction of cellular differentiation by retinoic acid in vitro. Cells Tissues Organs 165: 190-202.

Ross AC, Zolfaghari R, Weisz J. 2001. Vitamin A: recent advances in the biotransformation, transport, and metabolism of retinoids. Curr Opin Gastr 17: 184-192.

Ross SA, McCaffery PJ, Drager UC, DeLuca LM. 2000. Retinoids in embryonal development. Physiol Rev 80(3): 1021-1054.

Rothschild MF, Messer L, Day A, Wales RG, Short T, Southwood O, Plastow G. 2000. Investigation of the retinol-binding protein 4 (*RBP4*) gene as a candidate gene for increased litter size in pigs. Mamm Genome 11: 75-77.

Roulier S, Rochette-Egly C, Rebut-Bonneton D, Porquet D, Evain-Brion D. 1994. Nuclear retinoic acid receptor characterization in cultured human trophoblast cells: effect of retinoic acid on epidermal growth factor receptor expression. Mol Cell Endocrinol 105: 165-173.

Rühl R, Plum C, Elmazar MMA, Nau H. 2001. Embryonic subcellular distribution of 13-*cis*- and all-*trans*-retinoic acid indicates differential cytosolic/nuclear localization. Toxicol Sci 63: 82-89.

Rushmer RA, Brinster RL. 1973. Carbon dioxide production from pyruvate and glucose by bovine oocytes. Exp Cell Res 82: 252-254.

Russell RW, Young JW. 1990. A review of metabolism of labeled glucose for use in measuring glucose recycling. J Dairy Sci 73: 1005-1016.

Saari JC. 1994. Retinoids in photsensitive systems. M. B. Sporn, A. B. Roberts and D. J. Goodman. The Retinoids: Biology, Chemistry, and Medicine. New York: Raven Press, Ltd. 1**:**pp 351-379.

Salas-Vidal E, Lomelí H, Castro-Obregón S, Cuervo R, Escalante-Alcalde D, Covarrubias L. 1998. Reactive oxygen species participate in the control of mouse embryonic cell death. Exp Cell Res 238: 136-147.

Sarkar SA, Sharma RP. 2002. Expression of c-myc and other apoptosis-related genes in Swiss Webster mouse fetuses after maternal exposure to all *trans*-retinoic acid. Reprod Toxicol 16: 245-252.

Satre MA, Ugen KE, Kochhar DM. 1992. Developmental changes in endogenous retinoids during pregnancy and embryogenesis in the mouse. Biol Reprod 46: 802-810.

Schweigert F, Bonitz K, Siegling C, Buchholz I. 1999. Distribution of vitamin A, retinol-binding protein, cellular retinoic acid-binding protein I, and retinoid X receptor ß in the porcine uterus during early gestation. Biol Reprod 61: 901-911.

Schweigert F, Zucker H. 1988. Concentrations of vitamin A, ß-carotene and vitamin E in individual bovine follicles of different quality. J Reprod Fertil 82: 575-579.

Scita G, Tenca P, Frittoli E, Tocchetti A, Innocenti M, Giardina G, Di Fiore PP. 2000. Signaling from Ras to Rac and beyond: not just a matter of GEFs. EMBO J 19(11): 2393-2398.

Shaw DW, Farin PW, Washburn SP, Britt JH. 1995. Effect of retinol palmitate on ovulation rate and embryo quality in superovulated cattle. Theriogenology 44: 51-58.

Shi ZZ, Osei-Frimpong J, Kala G, Kala SV, Barrios RJ, Habib GM, Lukin DJ, Danney CM, Matzuk MM, Lieberman MW. 2000. Glutathione synthesis is essential for mouse development but not for cell growth in culture. Proc Natl Acad Sci U S A 97(10): 5101-5106.

Shingleton JL, Skinner MK, Ong DE. 1989. Characteristics of retinol accumulation from serum retinol-binding protein by cultured sertoli cells. Biochemistry (Mosc) 28: 9641-9647.

Sies H. 1995. Strategies of antioxidant defense: relations to oxidative stress. L. Packer and K. Wirtz. Signalling mechanisms - from transcription factors to oxidative stress. Heidelberg: Springer-Verlag. 92**:**pp 165-186.

Sirard MA, Richard F, Mayes MA. 1998. Controlling meiotic resumption in bovine oocytes: a review. Theriogenology 49: 483-497.

Soprano DR, Blaner WS. 1994. Plasma retinol-binding protein. M. B. Sporn, A. B. Roberts and D. J. Goodman. The Retinoids: Biology, Chemistry, and Medicine. New York: Raven Press, Ltd. 2**:**pp 257-282.

Sorensen RA, Wassarman PM. 1976. Relationship between growth and meiotic maturation of the mouse oocyte. Dev Biol 50: 531-537.

Spindler RE, Pukazhenthi BS, Wildt DE. 2000. Oocyte metabolism predicts the development of cat embryos to blastocyst in vitro. Mol Reprod Dev 56: 163-171.

Sporn MB, Roberts AB. 1994. Introduction. M. B. Sporn, A. B. Roberts and D. J. Goodman. The Retinoids: Biology, Chemistry, and Medicine. New York: Raven Press, LTD. 2**:**pp 1-3.

Stojkovic M, A MS, Stojkovic P, Zakhartchenko V, Hutzler P, Gonçalves PB, Wolf E. 2001. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. Biol Reprod 64: 904-909.

Sundaram M, Sivaprasadarao A, DeSousa MM, Findlay JBC. 1998. The transfer of retinol from serum retinol-binding protein to cellular retinol-binding protein is mediated by a membrane receptor. J Biol Chem 273(6): 3336-3342.

Sutherland AE, Calarco-Gillam PG. 1983. Analysis of compaction in the preimplantation mouse embryo. Dev Biol 100: 328-338.

Sutton ML, Gilchrist RB, Thompson JG. 2003. Effects of in vivo and in vitro environments on the metabolism of the cumulus-oocyte complex and it influence on oocyte developmental capacity. Hum Reprod Update 9(1): 35-48.

Takahashi M, Keicho K, Takahashi H, Ogawa H, Schultz RM, Okano A. 2000. Effect of oxidative stress on development and DNA damage in in vitro cultured bovine embryos by comet assay. Theriogenology 54: 137-145.

Takahashi M, Nagai T, Hamano S, Kuwayama M, Okamura N, Okano A. 1993. Effect of thiol compounds on in vitro development and intracellular glutathione content of bovine embryos. Biol Reprod 49: 228-232.

Takahashi M, Nagai T, Okamura N, Takahashi H, Okano A. 2002. Promoting effect of ß-mercaptoethanol on in vitro development under oxidative stress and cystine uptake of bovine embryos. Biol Reprod 66: 562-567.

Takami M, Preston SL, Toyloy VA, Behrman HR. 1999. Antioxidants reversibly inhibit the spontaneous resumption of meiosis. American Journal of Physiology 276: E684-E688.

Tanghe S, Van Soom A, Nauwynck H, Coryn M, de Kruif A. 2002. Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. Mol Reprod Dev 61: 414-424.

Telford NA, Watson AJ, Schultz GA. 1990. Transition from maternal to embryonic control in early mammalian development: a comparison of several species. Mol Reprod Dev 26: 90-100.

Teruel M, Smith R, Catalano R. 2000. Growth factors and early embryo development. Biocell 24(2): 107-122.

Tervit H, Whittingham DG, Rowson L. 1972. Successful culture in vitro of sheep and cattle ova. J Reprod Fertil 30: 493-497.

Texeira CC, Shapiro IM, Hatori M, Rajpurohit R, Koch C. 1996. Retinoic acid modulation of glutathione and cysteine metabolism in chondrocytes. Biochem J 314: 21-26.

Thannickal VJ, Fanburg BL. 2000. Reactive oxygen species in cell signaling. American Journal of Physiology Lung Cell Molecular Physiology 279: L1005-L1028.

Thompson JG, Partridge RJ, Houghton FD, Cox CI, Leese HJ. 1996. Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. J Reprod Fertil 106: 299-306.

Thompson JG, Simpson AC, Pugh PA, Tervit HR. 1992. Requirement for glucose during in vitro culture of sheep preimplantation embryos. Mol Reprod Dev 31: 253-257.

Thompson JG, Simpson AC, Pugh PA, Wright RW, Tervit HR. 1991. Glucose utilization by sheep embryos derived in vivo and in vitro. Reprod Fertil Dev 3: 571-576.

Thompson JN, Howell J, Pitt GAJ, McLaughlin CI. 1969. The biological activity of retinoic acid in the domestic fowl and the effects of vitamin A deficiency on the chick embryo. British Journal of Nutrition 23: 471-492.

Tietze F. 1969. Enzymic method for quantitaive determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. Anal Biochem 27: 502-522.

Tosetti F, Campelli F, Levi G. 1999. Studies on the cellular uptake of retinol binding protein and retinol. Exp Cell Res 250: 423-433.

Trounson AO, Anderiesz C, Jones G. 2001. Maturation of human oocytes in vitro and their developmental competence. Reproduction 121: 51-75.

Umaoka Y, Noda Y, Narimoto K, Mori T. 1992. Effects of oxygen toxicity on early development of mouse embryos. Mol Reprod Dev 31: 28-33.

Van Soom A, de Kruif A. 1996. Oocyte maturation, sperm capacitation and pre-implantation development in the bovine: implications for in vitro production of embryos. Reproduction of Domestic Animals 31: 687-701.

Van Soom A, Yuan YQ, Peelman LJ, de Matos DG, DeWulf J, Laevens H, de Kruif A. 2002. Prevalence of apoptosis and inner cell allocation in bovine embryos cultured under different oxygen tensions with or without cysteine addition. Theriogenology 57: 1453-1465.

VanBlerkom J, Davis PW, Lee J. 1995. ATP content of human oocytes and developmental potential and outcome after in vitro fertilization and embryo-transfer. Hum Reprod 10(2): 415-424.

Voelkel SA, Hu YX, Moore K, Bondioli KR. 1992. Freeze survival of bovine embryos produced by in vitro maturation, fertilization, and culture of oocytes. Theriogenology 37(1): 317.

Wang X, Falcone T, Attaran M, Goldberg JM, Agarwal A, Sharma Rk. 2002. Vitamin C and Vitamin E supplementation reduce oxidative stress-induced embryo toxicity and improve blastocyst development rate. Fertil Steril 78(6): 1272-1277.

Ward SJ, Chambon P, Ong DE, Båvik C. 1997. A retinol-binding protein receptor-mediated mechanism for uptake of vitamin A to postimplantation rat embryos. Biol Reprod 57: 751-755.

Ward SJ, Morriss-Kay GM. 1997. The functional basis of tissue-specific retinoic acid signalling in embryos. Semin Cell Dev Biol 8(4): 429-435.

Watson AJ. 1992. The cell biology of blastocyst development. Mol Reprod Dev 33: 492-504.

Weisiger RA, Fridovich I. 1973. Mitochondrial superoxide dismutase. Site of synthesis and intramitochondrial localization. J Biol Chem 248: 4791-4793.

Wellik DM, DeLuca HF. 1995. Retinol in addition to retinoic acid is required for successful gestation in vitamin A-deficient rats. Biol Reprod 53: 1392-1397.

Wendling O, Chambon P, Mark M. 1999. Retinoid X receptors are essential for early mouse development and placentogenesis. Proc Natl Acad Sci U S A 96: 547-551.

Whaley SL, Hedgpeth VS, Britt JH. 1997. Evidence that injection of vitamin A before mating may improve embryo survival in gilts fed normal or high-energy diets. J Anim Sci 75: 1071-1077.

Whaley SL, Hedgpeth VS, Farin CE, Martus NS, Jayes FCL, Britt JH. 2000. Influence of vitamin A injection before mating on oocyte development, follicular hormones, and ovulation in gilts fed high-energy diets. J Anim Sci 78: 1598-1607.

Wilhelm D, Bender K, Knebel A, Angel P. 1997. The level of intracellular glutathione is a key regulator for the induction of stress-activated signal transduction pathways including jun n-terminal protein kinases and p38 kinase by alkylating agents. Mol Cell Biol 17(8): 4792-4800.

Wolf G. 1995. Retinol transport and metabolism in transthyretin-knockout mice. Nutr Rev 53(4): 98-99.

Wolf G. 1996. A history of vitamin A and retinoids. The FASEB Journal 10: 1102-1107.

Wu X, Matzuk MM. 2002. GDF-9 and BMP-15: Oocyte organizers. Rev Endo Met Dis 3: 27-32.

XP E, Zhang L, Lu JY, Tso P, Blaner WS, Levin MS, Li E. 2002. Increased neonatal mortality in mice lacking cellular retinol-binding protein II. J Biol Chem 277(39): 36617-36623.

Xu KP, Greve T, Smith S, Hyttel P. 1986. Chronological changes of bovine follicular oocyte maturation in vitro. Acta Vet Scand 27: 505-519.

Xu Q, Konta T, Furusu A, Nakayama K, Lucio-Cazana J, Fine LG, Kitamura M. 2002. Transcriptional induction of mitogen-activated protein kinase phosphatase 1 by retinoids. J Biol Chem 277(44): 41693-41700.

Yamashita M, Mita K, Yoshida A, Kondo T. 2000. Molecular mechanisms of the initiation of oocyte maturation: general and species-specific aspects. L. Meijer, A. Jézéquel and B. Ducommun. Prog Cell Cycle Res. New York: Plenum Publishers. 4**:**pp 115-129.

Yanagimachi R. 1988. Mammalian fertilization. E. Knobil and J. D. Neill. The physiology of reproduction. New York: Raven Press**:**pp 135-185.

Yang HW, Hwang KJ, Kwon HC, Kim HS, Choi KW, Oh KS. 1998. Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. Hum Reprod 13(4): 998-1002.

Yin Z, Ivanov VN, Habelhah H, Tew K, Ronai Z. 2001. Glutathione S-transferase p elicits protection against H_2O_2 -induced cell death via coordinated regulation of stress kinases. Cancer Res 60: 4053-4057.

Yoshida M, Ishigaki K, Nagai T, Chikyu M, Pursel VG. 1993. Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. Biol Reprod 49: 89-94.

Yuan YQ, Van Soom A, Coopman FOJ, Mintiens K, Boerjan ML, Van Zeveren A, de Kruif A, Peelman LJ. 2003. Influence of oxygen tension on apoptosis and hatching in bovine embryos cultured in vitro. Theriogenology 59: 1585-1596.

Zheng WL, Bucco RA, Sierra-Rievera E, Osteen KG, Melner MH, Ong DE. 1999. Synthesis of retinoic acid by rat ovarian cells that express cellular retinoic acid-binding protein II. Biol Reprod 60: 110-114.

Zhou XF, Shen XQ, Shemshedini L. 1999. Ligand-activated retinoic acid receptor inhibits AP-1 transactivation by disrupting c-Jun/c-Fos dimerization. Mol Endocrinol 13(2): 276-285.

Zuelke KA, Brackett BG. 1993. Increased glutamine metabolism in bovine cumulus cell-enclosed and denuded oocytes after in vitro maturation with luteinizing hormone. Biol Reprod 48: 815-820.

Zuelke KA, Jeffay SC, Zucker RM, Perreault SD. 2003. Glutathione (GSH) concentrations vary with the cell cycle in maturing hamster oocytes, zygotes, and pre-implantation stage embryos. Mol Reprod Dev 64: 106-112.

VITA

Tracy Livingston was born in Ashland, Kentucky on May 27, 1970, and was raised in Ironton, Ohio. She graduated from Green High School in 1988. Tracy earned her Bachelor of Science and Master of Science degrees from the University of Kentucky. After completing four years of adjunct teaching, she changed careers and became a criminalist with the Charleston Police Department in Charleston, SC. In 1999, Tracy married Bob Fultz and in December 2002 they had a son, named Ian. In 2003 she earned her Doctor of Philosophy degree in Animal Science from the University of Tennessee.