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Metabolism of Sheep Oocytes During *In vitro* Maturation

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Abstract: The purpose of the present study was to evaluate energy metabolism during *in vitro* maturation (IVM) of individual ovine oocytes and determine effects of *in vivo* retinol administration on this process. Ewes were superovulated with FSH and treated with retinol or vehicle. Oocytes were recovered approximately 12 hours after the last FSH injection, and subjected to metabolic measurements during early (0-3 hours), mid (9-12 hours), or late (21-24 hours) stages of IVM using hanging-drop procedures. Combinations of the following radiolabeled 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 assessed by tracing the fate of the $^{3}H_2O$ from 5-^{3}H glucose, 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; $p \leq .01$). Metabolism of glucose carbon through the Krebs cycle (6-^{14}C glucose) did not significantly change over the twenty-four hour maturation time period (0.29 to - 0.61 pmoles/oocyte/3hours), and likewise, oxidation of 1-^{14}C glucose did not change during IVM. Oxidative metabolism of glutamine appeared to be high early and declined during the later time period, but overall showed no significant differences. Pyruvate oxidation was highest early in maturation and then decreased (3.14 to 1.44; $p \leq .001$). These results suggest that ovine oocytes use both glycolytic and oxidative pathways throughout IVM. No significant differences existed between oocytes from retinol-treated ewes and control oocytes.

Key words: Maturation, Glycolysis, Oxidation, Retinoids

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 and 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 completion of meiosis, specifically progression from prophase I to metaphase 2 before fertilization (Xu *et al*., 1986 and 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 and Abbot and Ducibella, 2001), and other organelle rearrangements (De Loos *et al*., 1992). Although 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 and Combelles *et al*., 2002), 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 can complete both nuclear and cytoplasmic maturation in a variety of energy substrates (Downs and Mastropolo, 1994; Eppig *et al*., 1994; Downs and Mastropolo, 1997 and Izadyar *et al*., 1998)

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 and 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 (Spindler *et al*., 2000 and Krisher and Bavister, 1999).

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 and 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 and 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 (Eberhardt *et al.*, 1999b and Shaw *et al.*, 1995). 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 and 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) (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 primary goal of this study was to determine the metabolic requirements of sheep oocytes during different time periods of IVM; therefore, we used a modified hanging drop procedure (Rieger and Guay, 1988) with varying combinations of ^{14}C and ^{3}H labeled substrates. The second aim was to evaluate the effects of retinol on the oocyte's metabolism and nuclear development *in vitro*.

The present study indicates 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 was purchased from Upjohn/Pharmacia, 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, 0.3 μg/mL of luteinizing hormone (LH) both purchased from 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 approximately 12-15 hours after the last FSH injection, 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 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 each metabolic measurement, oocytes were denuded by gentle pipetting with 10% hyaluronidase, and washed three times in preequilibrated (38.5°C, 5% CO_2 in air) metabolic measurement medium (MMM), which consisted of OMM minus FBS. This procedure 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. The following combinations of the ^{14}C and ^{3}H labeled substrates were used: 6-^{14}Cglucose/3,4 ^{3}H glutamine, 6-^{14}C glucose/5-^{3}H glucose, 1-^{14}C glucose/3,4 ^{3}H glutamine, 2-^{14}C pyruvate/5-^{3}H glucose, and 2-^{14}C pyruvate/3,4 ^{3}H glutamine.

The microcentrifuge lids were gently closed and the tubes placed in the incubator for three hours. The $NaHCO_3$ acted as a trap for $^{14}CO_2$ and $^{3}H_2O$ released by the oocyte. At least two sham and two 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 instrument 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 incubation period, tubes were removed, and oocytes were washed and stained 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 substrate uptake, counts (disintegrations per minute - d.p.m.) from the sham tube were subtracted from the tube containing the oocyte. This difference was divided by the total d.p.m. of labeled substrate added, and multiplied by the total quantity of substrate (labeled plus unlabeled) in 4μl of medium (Tiffin *et al.*, 1991). 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 0.05% Hoechst in HEPES-TALP. The dish was placed under a cover away from light for 5-10 minutes, and then the oocytes were washed to remove excess dye and viewed with a 100X objective under UV light.

Data Analysis: Data were collected in 7 replicates over several days. In each replicate 3-12 oocytes were measured for metabolism. The data were log transformed and analyzed using an incomplete block design blocked on replication with analysis of variance (ANOVA) using mixed model procedure of SAS (2000). Differences were detected using protected least significant differences. Data from retinol treated and control oocytes were combined.

Results

Oocytes were collected from superovulated, retinol or vehicle-treated ewes, and substrate metabolism was measured *in vitro* at three separate time points over a twenty-four hour maturation period. Fig. 1 represents utilization of the ^{3}H substrates for all oocytes, regardless of treatment. 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 substrate metabolism via the Krebs cycle was significantly higher compared to the glycolytic pathway, when comparing 3,4 ^{3}H glutamine to 5-^{3}H glucose, respectively (15.18 vs 0.24; $p<0.001$). Glutamine metabolism did not significantly change until the last

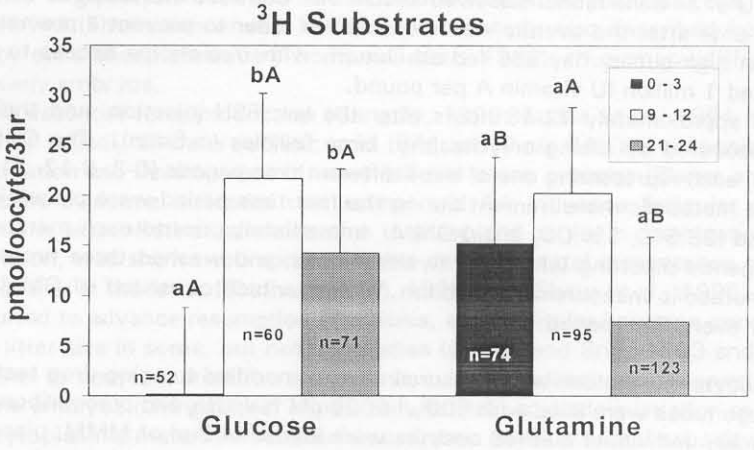

Fig. 1: The metabolism of 5-^{3}H glucose and 3,4-^{3}Hglutamine by sheep oocytes. Individual sheep oocytes were measured at specific time periods during maturation (0-3, 9-12, and 21-24 hours). Lower case letters indicate differences within the same substrate ($p<.05$). Uppercase letters indicate differences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

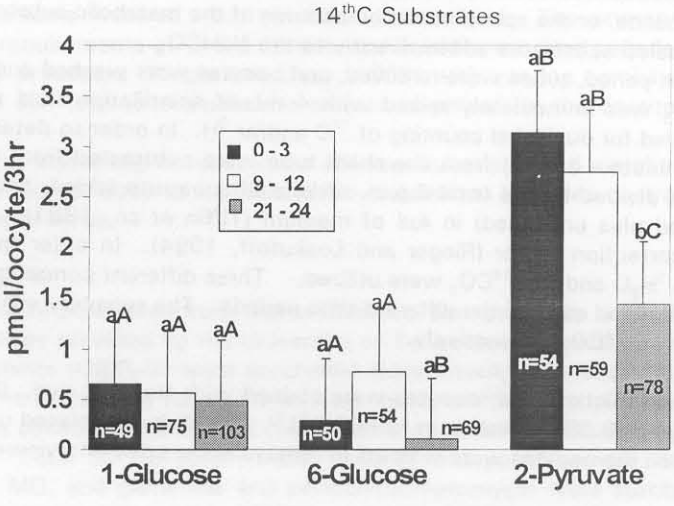

Fig. 2: The metabolism of 1-^{14}C glucose, 6-^{14}C glucose, and 2-^{14}C pyruvate by sheep oocytes. Individual sheep oocytes were measured at specific time periods during maturation(0-3, 9-12, and 21-24 hours). Lower case letters indicate differences within the same substrate ($p<.05$). Upper case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

time period (21-24 hours) when it dropped from 20.22 pmol/oocyte/3hr at 9-12 hours to 7.42 pmol/oocyte/3hr ($p<.001$).

Fig. 2 illustrates metabolism of ^{14}C substrates for all oocytes, regardless of treatment. Pyruvate oxidation remained essentially 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, and during the last time period pyruvate metabolism dropped to 1.44 ± 0.58 pmol/oocyte/3hr which was significantly lower than the earlier time periods ($p<0.01$). Compared to the other ^{14}C labeled substrates pyruvate oxidation was higher at all time periods ($p<.001$). Utilization of 1-^{14}C glucose was below 1.0 pmol/oocyte/3hr at all time periods as was glucose flux through the Krebs cycle, 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 the substrate metabolism of control oocytes and oocytes from retinol-treated ewes, during the same time period within the same substrate. No significant differences were detected (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 2 (M2) 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 M 2.

Discussion

Little information exists concerning sheep oocyte metabolism. Only a few studies have examined energy substrate utilization 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 by single ovine oocytes over the entire IVM period. As in previous studies (Rieger and Loskutoff, 1994; Downs and Utecht, 1999; Spindler *et al.*, 2000), we used a combination of radiolabeled substrates to measure the different energy pathways the denuded oocyte utilizes during maturation. The production of $^{3}H_2O$ from 5-^{3}H glucose measures glucose utilization by the Embden-Meyerhof (EM) pathway. This was determined in a study using a tritium radiolabel to trace the fate of each glucose hydrogen during glucose metabolism and recycling (Russell and Young, 1990). Tiffin and colleagues used 5-^{3}Hglucose as a measure of total glucose metabolism, and calculated the pentose phosphate pathway contribution as a ratio of 1-^{14}C glucose/5-^{3}Hglucose (Tiffin *et al.*, 1991). 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). Some have suggested that the generation of $^{14}CO_2$ from 1-^{14}C glucose reflects total glucose oxidation (Downs and Utecht, 1999). Katz and Wood also investigated the flow of glucose carbon labeled in the sixth position (6-^{14}C glucose), and they showed that it cycles through glycolysis/Krebs pathways (Katz and Wood, 1963). 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 oxidation also measures the activity of the Krebs cycle.
Several studies have measured individual oocyte metabolism. Early work in the mouse indicated that a denuded mouse oocyte prefers pyruvate as an energy source, indicated by its ability to progress to metaphase 2 (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 and 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 and 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 reached MI, and then a decrease at the 21-24 hour time period as they progressed to MII. Little to no $^{14}CO_2$ generated from 6-^{14}C glucose was produced compared to 5-^{3}H glucose at all stages except for the first time period demonstrating that glucose is metabolized via the EM pathway until phosphoenolpyruvate, but that the glucose carbon is not passed onto the Krebs cycle (Rieger *et al.*, 1992). Only one other study evaluated metabolism using radiolabeled substrates during the entire maturation period, which was in bovine oocytes (Rieger and Loskutoff, 1994). Results from that study were different from ours. 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.
The glucose carbon from 1-^{14}C glucose goes first through the EM pathway then the Krebs, and it can also be 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); however, in our study proper meiotic progression occurred regardless of the energy substrate being measured. 6-^{14}C glucose is used to measure the

activity of the EM and Krebs cycle, and our results showed that both 1-^{14}C glucose and 6-^{14}C glucose were low throughout the maturation period, which is consistent with results for bovine oocytes (Rieger and Loskutoff, 1994). Since oxidation of glucose was similar for 1-^{14}C glucose and 6-^{14}C glucose at the early and middle time periods, it can be assumed that glucose was being cycled by both pathways, Krebs and PPP. 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.

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 and 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 explain 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 and 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 is the first time sheep oocyte metabolism has been measured 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 and 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 and pyruvate metabolism. In contrast to bovine oocytes, glucose metabolism during the middle time stage, when oocytes were predominately at the MI stage, 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 preferentially utilized glucose versus pyruvate except during the initial time period. Further study must be conducted to completely understand the role of the pentose phosphate pathway in sheep oocytes. 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.*, 1999). 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 and 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. It is possible that retinol may be impacting follicular recruitment, or another likely explanation is that the beneficial 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.

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References

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

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

Bavister, B. D., 1995. Culture of preimplantation embryos: Facts and artifacts. Hum. Reprod, 1: 91-148.

Biggers, J. D., D. G. Whittingham and R. P. Donahue, 1967. The pattern of energy metabolism in the mouse oocyte and zygote. Proc. Natl. Acad Sci. USA, 58: 560-567.

Blaner, W. S. and J. A. Olson, 1994. Retinol and retinoic acid metabolism. In: M. B. Sporn, A. B. Roberts and D. J. Goodman. (Eds), The Retinoids: Biology, Chemistry, and Medicine, New York, Second Edition, Raven Press, Ltd., pp: 229-256.

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

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

Butler, J. E. and J. E. Williams, 1991. Noninvasive measurement of pyruvate uptake by ovine preimplantation embryos and unfertilized ova. Theriogenology, 36: 1043-1048.

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

Combelles, C. M. H., N. A. Cekleniak and D. F. Albertini, 2002. Assessment of nuclear and cytoplasmic maturation in *in vitro* matured human oocytes. Hum. Reprod, 17: 1006-1016.

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

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

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

Downs, S. M. and P. G. Humpherson, H. J. Leese, 2002. Pyruvate utilization by mouse oocytes is influenced by meiotic status and the cumulus oophorus. Mol. Reprod Dev., 62: 113-123.

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

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

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

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

Duque, P., C. Dí ez, L. Royo, P. L. Lorenzo, G. Carneiro, C. O. Hidalgo, N. Facal and E. Gómez, 2002. Enhancement of developmental capacity of meiotically inhibited bovine oocytes by retinoic acid. Hum. Reprod, 17: 2706-2714.

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

Eberhardt, D. M., W. A. Will and J. D. Godkin, 1999. Retinol administration to superovulated ewes improves *in vitro* embryonic viablility. Biol. Reprod, 60: 1483-1487.

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

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

Gandolfi, F., E. Milanesi, P. Pocar, A. M. Luciano, T. A. L. Brevini, F. Acocella, A. Lauria and D. T. Armstrong, 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.

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

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

Gudas, L. J., M. B. Sporn, A. B. Roberts, 1994. Cellular biology and biochemistry of the retinoids. In: M. B. Sporn, A. B. Roberts and D. J. Goodman (Eds). The Retinoids: Biology, Chemistry, and Medicine. New York, Second Edition, Raven Press, Ltd., pp: 443-520.

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

Izadyar, F., W. J. Hage, B. Colenbrander, M. M. Bevers, 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.

Katz, J., H. G. Wood, 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: 517-523.

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

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

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

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

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

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

Livingston, T. E., D. M. Eberhardt, J. D. Godkin, 2002. The effects of retinol on bovine embryo development *In vitro*. Biol. Reprod, 66(Supp 1): 18.

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

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

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

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

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

Pusateri, A. E., M. A. Diekman, W. L. Singleton, 1999. Failure of vitamin A to increase litter size in sows receiving injections at various stages of gestation. J. Anim. Sci., 77: 1532-1535.

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

Rieger, D., N. M. Loskutoff, K. J. Betteridge, 1992. Developmentally related changes in the uptake and metabolism of glucose, glutamine and pyruvate by cattle embryos produced *in vitro*. Reprod Fert. Dev., 4: 547-557.

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

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

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

Shaw, D. W., P. W. Farin, S. P. Washburn, J. H. Britt, 1995. Effect of retinol palmitate on ovulation rate and embryo quality in superovulated cattle. Theriogenology, 44: 51-58.

Sorensen, R. A., P. M. Wassarman, 1976. Relationship between growth and meiotic maturation of the mouse oocyte. Dev. Biol., 50: 531-537.

Spindler, R. E., B. S. Pukazhenthi, D. E. Wildt, 2000. Oocyte metabolism predicts the development of cat embryos to blastocyst in vitro. Mol. Reprod Dev., 56: 163-171.

Stojkovic, M., S. A. Machado, P. Stojkovic, V. Zakhartchenko, P. Hutzler, P. B. Gonçalves, E. Wolf, 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.

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

Thompson, J. G., R. J. Partridge, F. D. Houghton, C. I. Cox, H. J. Leese, 1996. Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. J. Reprod Fertil., 106: 299-306.

Thompson, J. G., A. C. Simpson, P. A. Pugh, H. R. Tervit, 1992. Requirement for glucose during in vitro culture of sheep preimplantation embryos. Mol. Reprod Dev., 31: 253-257.

Thompson, J.G., Simpson, A.C., Pugh, P.A., Wright, R.W., Tervit, H.R., 1991. Glucose utilization by sheep embryos derived in vivo and in vitro. Reproduction Fertility and Development (3) 571-576.

Tiffin, G. J., D. Rieger, K. J. Betteridge, B. R. Yadav, W. A. King, 1991. Glucose and glutamine metabolism in pre-attachment cattle embryos in relation to sex and stage of development. J. Reprod Fertil., 93: 125-132.

Whaley, S. L., V. S. Hedgpeth, C. E. Farin, N. S. Martus, F. C. L. Jayes, J. H. Britt, 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.

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

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