Discovering Metabolic Networks of Bovine Fertilization

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DISCOVERING METABOLIC NETWORKS OF BOVINE FERTILIZATION

by

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Thesis submitted in partial fulfillment of the requirements for the degree of

HONORS IN UNIVERSITY STUDIES WITH DEPARTMENTAL HONORS

in

Bioveterinary Science in the Department of Animal, Dairy & Vet Sciences

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Abstract

At the time of fertilization, a dramatic change occurs in the oocyte that transforms this cell from a metaphase arrested state into a metabolically active and dynamic state. The view of the flow of biological processes within organisms has recently shifted from that of a linear path to a more complex network. Biological processes are no longer thought of in the simple terms of DNA to RNA, RNA to proteins, and proteins to final activity. It is now known that many biological processes involve interconnected networks and feedback loops in which DNA, RNA, proteins, and metabolites perform specific roles. We hypothesized that there are key metabolites and metabolic pathways yet to be determined that are involved in normal mammalian fertilization and embryonic development.

The key objective of this research project was to study the metabolic profiles of unfertilized oocytes and fertilized zygotes to find the differences in the concentration and flux of key metabolites. Examining these differences provides evidence for which metabolic pathways are important during fertilization.

Concentration differences of metabolites were assessed within unfertilized bovine oocytes and \textit{in vitro} fertilized zygotes. Metabolites were identified using gas chromatography-mass spectrometry (GC/MS). Differences in metabolite pools pre- and post-fertilization may be used as intracellular biomarkers of normal embryonic development. It was found that D-glucose, arabinose and fumaric acid were present in lower concentrations in the zygote than the unfertilized oocytes.
Acknowledgments

We wish to thank Dr. Lee Rickords, Benson Morrill, Davin Larsen, Kim Elwood and Marcy Labrum, Utah State University, Logan, UT, for assistance with GC/MS analysis of samples, Dr. Ken White and Ben Sessions for oocyte and zygote collection and URCO (Undergraduate Research & Creative Opportunities) for funding.
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Introduction

Fertilization is the beginning of embryogenesis. An oocyte and sperm fuse to form a zygote, which divides repeatedly in order to produce a new individual/organism (Alberts et al., 2008b). At the time of fertilization, a dramatic change occurs in the oocyte that advances this cell from a metaphase II arrested state into a metabolically active and dynamic state (see Figure 1). It is presumed that several metabolic pathways are initiated upon fertilization. Relatively little information is available about specific metabolites and their networks during mammalian fertilization.

Figure 1. Changes Induced on an Oocyte Upon Fertilization. Upon fertilization, an oocyte moves out of a metabolically inactive state and begins to grow and divide, thus becoming a new organism. (A) an oocyte, (B) an oocyte surrounded by sperm depicting fertilization, (C - F) a depiction of the division that takes place after fertilization, the initial stages of embryogenesis (adapted from Alberts et al., 2008a; Alberts et al., 2008b, copyright Garland Science 2008).

An oocyte is the female germ cell. The oocyte acts as a giant storehouse of materials, thus making it possible to divide into many smaller cells quickly upon activation (Alberts et al., 2008b). Upon oocyte maturation, an oocyte is arrested in meiosis metaphase II (Alberts et al., 2008b, Ferreiraa et al., 2009). The oocyte stores transcripts and proteins in the cell's cytoplasm.
These stored compounds are of great importance for ensuring a normal, healthy progression as embryogenesis begins. Oocytes also store cortical granules, which are derived from the Golgi complex in the cell. They are composed of proteins, structural molecules and enzymes. When the oocyte reaches metaphase II in the meiosis cycle, these granules are dispersed throughout the inner surface of the cell, close to the plasma membrane (Ferreira et al., 2009).

Fertilization is the process in which an oocyte, the female gamete, and a sperm, the male gamete, fuse. After the fusion of the sperm and oocyte, all of the sperm contents are absorbed by the oocyte. This activates the oocyte, causing the cortical granules stored inside to release their contents (Alberts et al., 2008a). Upon fertilization, these granules undergo exocytosis, thus changing the plasma membrane of an oocyte which prevents polyspermy (Ferreira et al., 2009). When the sperm fuses with the plasma membrane of the oocyte, the concentration of inositol 1,4,5, trisphosphate (IP3) increases, which releases calcium that has been stored from the endoplasmic reticulum of the oocyte. The increase of cystolic calcium concentration causes additional calcium channels to open, which allows more calcium to enter the cytosol. The oocyte resumes meiosis and produces a second polar body (Alberts et al., 2008a).

Upon sperm-oocyte fusion, the oocyte is renamed a zygote. A zygote still consists of only a single cell and has not undergone implantation. At fertilization, the sperm contributes a haploid nucleus, called a pronuclei. Fertilization is not complete until the pronuclei of the zygote and sperm fuse. For mammals, the two pronuclei do not fuse until the zygote undergoes its first mitotic division, forming a diploid genome of a new organism (Alberts et al., 2008a). The zygote is then fully active in its developmental program (Alberts et al., 2008b).

Once the oocyte has been fertilized, the cell divides, producing many cells from the original. These new cells specialize and differentiate to perform specific biological functions.
They interact with their neighboring cells and migrate to specific regions necessary for normal development. This all begins to happen at fertilization, and is common to almost all animals (Alberts et al., 2008a).

Metabolic pathways act as the sources of building blocks -- sugars, amino acids, and lipids-- that form cellular structures and macromolecules for regulatory function for the cells in all organisms. They allow the assimilation of simple and modified monosaccharides, amino acids, nucleotides and modified lipids to form complex molecules such as polysaccharides, lipids, and proteins.

The view of the flow of biological processes within organisms has recently shifted from that of a linear path to a more complex network. Biological processes are no longer thought of in the simple terms of DNA to RNA, RNA to proteins, and proteins to final activity. It is now known that many biological processes involve interconnected networks and feedback loops in which DNA, RNA, proteins, and metabolites perform specific roles (Barabási, 2004). While genomics, the study of an organism's complete set of genes, and proteomics, the study of an organism's complete set of proteins, have proven to be helpful in understanding the functions of many genes and proteins in various biological systems, it has also been shown that differences in gene transcription and protein levels do not necessarily lead to changes in phenotype (Sumner et al., 2003; Gygi et al., 1999). The field of metabolomics offers end-point analysis of metabolites at a specific time within the organism and therefore provides a unique approach for comprehending how genes and environment affect the cellular phenotype.

Although we have reached a point in the study of mammalian reproduction where we are able to understand and manipulate many reproductive processes, the low efficiencies of success with manipulating these processes, such as somatic cell nuclear transfer (mammalian cloning)
(Beyhan et al., 2007), provide compelling evidence that our understanding of these processes is still lacking. Therefore, we hypothesized that small molecule metabolites and their pathway networks play a critical role in normal mammalian fertilization. To test this hypothesis, we investigated the differences in metabolites present in a mature oocyte and those present in a zygote. The data obtained from these experiments will provide important information regarding metabolic pathways that are vital to mammalian fertilization and early development. This would aid in improving media for other embryonic or stem cell studies, which could be used for various applications in both research and human health.

The bovine model was used to represent mammalian systems in this research project. One of the revelations of the past few decades has been that much of the basic machinery of development is essentially the same across species. Homologous proteins have been found to function interchangeably between very different species (Alberts et al. 2008b). The bovine model has been utilized for over twenty years and has achieved high in vitro developmental rates and good success to term (Reed et al., 1996; Aston et al., 2006a; Aston et al., 2006b). Therefore, the bovine model is a good choice to study metabolites and cofactors associated with mammalian fertilization and early embryonic development.

While there is sufficient knowledge available on the involvement of small molecule substrates, relatively little is known about the exact role of these molecules in mammalian embryonic development (Sinclair et al., 2003). We hypothesized that small molecule metabolites and their pathway networks were involved in normal fertilization and preimplantation embryo development. We tested our hypothesis using the bovine in vitro fertilization and culture model to determine the molecular events associated with mammalian development.

Recently, the development of high throughput technologies that allow large scale
profiling of physiology have facilitated rapid advances in understanding functions of cellular systems on a much broader scale. Gas chromatography has the potential to have high resolution, high efficiency, reproducibility, and small sample size (Issaq et al., 2009). Gas chromatography coupled with mass spectrometry becomes a valuable and reliable analytical technique for separation, detection, and identification. GC/MS, however, is limited to small compounds that are thermally stable, volatile, or can be made volatile through derivatization (Issaq et al., 2009). A sample destined for GC/MS is first derivatized, and then injected into a GC/MS machine (see Figure 2). The volatilized sample is carried by a carrier gas, normally helium, through a heated column where the compounds are separated by mass to charge ratio. A detector at the end of the column records the mass and time that each compound took to travel through the column. The readout of this process is a chromatogram (McLafferty, 1980).

Figure 2. A Diagram of a Gas Chromatography Mass Spectrometry Machine. A sample is injected into the machine, carried by a gas, normally helium, through a heated column. A detector at the end records the time and mass of each sample that exits the machine (adapted from weather.nmsu.edu)

Different types of cells from humans and other animals have been studied using this technique in vivo; examples include vascular smooth muscle cells, adipocytes, T-lymphocytes,
hippocampal neurons, hepatocytes, endothelial cells, mammary epithelial cells, the cerebral
cortex, spermatocytes, pituitary tumors, chronic lymphocytic leukemia cells and others (Issaq et
al., 2009). Analysis by GC/MS is sensitive, reproducible and quantitative, and label
incorporation and die-away approaches are simple to use and interpret.

Materials and Methods

Fertilized zygotes and unfertilized oocytes were provided by the laboratory of Dr. Kenneth L. White (Utah State University). There were 300 oocytes and 250 zygotes in each representative sample. Samples were frozen at -80°C in PB1 media until metabolite extraction was performed.

Ice cold 100% methanol was added to thawed cell samples to create a 50% methanol solution, after which cellular metabolites were extracted using an equivalent volume of chloroform.

Aqueous and organic layers were then separated and dried. Dry extracts were then derivatized using methoxyamine hydrochloride in pyridine, and incubated at 42°C for 90 minutes. Samples were silylated in N-Methyl-N-TMS-Trifluoroacetamide (MSTFA) at 42°C for 60 minutes. Because the low cell number of these oocyte samples did not give the sensitivity we expected, samples were concentrated by re-drying in a speedvac overnight and dissolved in 40 ul MSTFA and 60 ul pyridine.

After derivatization, samples were analyzed by GC/MS (GCT Premier, Waters) at the Center for Integrated BioSystems (CIB, Utah State University). 1 µl aliquots were injected into a DB5-MS capillary column (30 m x 250 µm i.d. x 0.25 µm film thickness). The initial GC oven
temperature was 70°C and 5 min after injection the temperature was increased 5°C/min to 310°C and held for 5 minutes at 310°C. Helium was used as a carrier gas and pressure was programmed such that the helium flow was kept constant at a flow rate of 1 ml/min. Detection was achieved using MS detection in electron impact mode and full scan monitoring mode (m/z 50-800). The temperature of the ion source was set at 250°C and to 200°C for the mass analyzer.

Results

A chromatogram was obtained by running samples through GC/MS (see Figure 3). This chromatogram was analyzed for differences in the concentrations of compounds between the oocytes and zygotes. The peaks were integrated to determine relative amounts (see Table 1 and Figure 4).

Figure 3. The Resulting Chromatograms of the Metabolites of the Collected Oocytes and Zygotes. Chromatograms of Oocytes (top) and Zygotes (bottom). TIC – Total Ion current.
Figure 4. The Relative Integration Values of the Compounds of Interest Identified in the Generated Chromatogram.
Comparisons of peak areas of bovine oocytes and zygotes of the compounds of interest.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Oocyte Integration</th>
<th>Zygote Integration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>8068</td>
<td>2450</td>
</tr>
<tr>
<td>D-glucose</td>
<td>639</td>
<td>161</td>
</tr>
<tr>
<td>Fumaric Acid</td>
<td>1055</td>
<td>315</td>
</tr>
</tbody>
</table>

Table 1. The Relative Integration Values of the Compounds of Interest Identified in the Generated Chromatogram. A list of compounds found in the chromatogram with their corresponding peak integration values. A peak integration value is a measurement of peak area, which is considered proportional to the amount of compound present in each sample.

Conclusion

In an oocyte, important compounds are pooled and stored for the future developing embryo. Upon fertilization, the fusion of a sperm and oocyte causes the oocyte to becomes active and move out of Metaphase II. The zygote begins to use its stored compounds as embryogenesis begins. This is evidenced by a reduction in D-glucose, Arabinose, and Fumaric Acid (see Figure 5). Glucose is a major source of energy for a growing mammalian embryo.
D-glucose undergoes glycolysis and is used in the TCA cycle, which generates ATP, an energy source for the cell. Fumaric acid can be changed into fumarate, which is part of the TCA cycle and can also be used for energy (Kenealy et al. 1986). Arabinose can be isomerized by the Arabinose isomerase enzyme into D-ribose which is utilized in the pentose phosphate pathway to generate glucose and energy or used to synthesize nucleic acids, both of which would be needed in a rapidly growing and dividing cell population (Tozzi et al. 2006). When an oocyte is fertilized it is more active, and uses its stores of important compounds to grow and develop.

Biological samples are complex, and contain hundreds of metabolites which cannot be adequately separated using a single GC/MS experiment to detect all the metabolites (Issaq et al., 2009). Although only one set of samples were run, and this study is not conclusive, we were able to identify three compounds that differed in concentration between the oocyte and zygote. In this study of the difference in metabolite concentrations of an oocyte pre- and post-fertilization, we saw evidence of significant metabolic activity of three specific compounds, arabinose, D-glucose and fumaric acid in the fertilized zygotes.
Sources Cited:


Biography:

Erin Young came to Utah State on a Presidential Scholarship for the opportunity to live away from home and develop her talents. She was introduced to undergraduate research her sophomore year. Erin found that she enjoyed the laboratory setting and her aspirations became more research oriented. She has spent time as an undergraduate researcher in both Dr. Quinton Winger’s and Dr. Lee Rickord’s laboratories. Erin has presented her research at the Utah Conference of Undergraduate Research and has been given the Undergraduate Research Scholar Transcript Designation. In addition to her experience at Utah State University, she was able to participate in the Integrated Cancer Biology Program's 2009 Summer Program, where she moved to Stanford University Campus to study acute myelogenous leukemia. Erin graduated with a Major in Bioveterinary Science with an emphasis in Biotechnology and minors in Biology, Chemistry and Mathematics. Erin grew up in Bountiful, Utah and graduated from Bountiful High School in 2004. Outside of class, Erin has a love for travel, theater and Aggie ice cream.

Image 1. A Photograph of Erin Young, the Author.

Photograph of Erin Young taken on Stanford University Campus July 2, 2009.