UTILIZATION OF GLUCOSE AND FRUCTOSE BY THE OVINE CONCEPTUS DURING THE PERI-IMPLANTATION PERIOD OF PREGNANCY

A Thesis

by

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ABSTRACT

Early embryonic development in the sheep requires elongation of the conceptus (the embryo and associated extraembryonic membranes) before implantation can take place. This requires energy and biomolecules provided by various metabolic pathways, often initiated by glucose as a preferred metabolic substrate. However, the ovine trophectoderm in the conceptus converts glucose from the maternal vasculature to fructose, resulting in a greater abundance of fructose than glucose, especially in fetal plasma and fetal fluids in later stages of pregnancy. The role of fructose is overlooked due to a preference of glucose over fructose. This study investigated the capability of the ovine conceptus during Days 16 and 17 of gestation to metabolize fructose via the tricarboxylic acid (TCA) cycle and pentose phosphate pathway.

A study was conducted by culturing Day 17 ovine conceptuses with universally labeled glucose and fructose with combinations of unlabeled glucose and fructose. Results suggested that the conceptus metabolizes both glucose and fructose, indicated by the production of $^{14}CO_2$, but glucose is the preferred substrate ($P<0.05$). Immunolocalization of ketohexokinase, which allows fructose to bypass glycolytic regulatory mechanisms, in uncultured conceptuses and endometrium on Days 17, 18, and 30 of pregnancy indicated that ketohexokinase is expressed in the endoderm of the conceptus, as well as the trophectoderm and uterine luminal and glandular epithelium.

A second experiment was conducted to investigate the metabolism of glucose and fructose by Day 16 ovine conceptuses via various metabolic pathways. The conceptuses were cultured similarly to that in the first experiment, using specifically labeled glucose and fructose to estimate metabolism via the pentose phosphate pathway and TCA cycle, and homogenized at the

end of the experiment to evaluate incorporation of glucose and fructose into lactate, pyruvate, lipids, and glycoproteins. The results indicated that the ovine conceptus could metabolize both glucose and fructose by these pathways, with the exception of lipids, with glucose being the preferred substrate. Contribution of fructose to the TCA cycle were greater than the pentose phosphate pathway when glucose was absent ($P<0.05$).

Results of both studies indicated that the ovine conceptus could metabolize fructose to support conceptus development in the sheep.

DEDICATION

In memory of my Papa.

I would have given you a call to let you know I finished, but I think this is just as good.

ACKNOWLEDGEMENTS

It is well known that science is a collaborative effort and is never truly a solo mission. Achieving research goals takes a team, and I have been extremely fortunate and blessed to be a part of an amazing team for the last few years. There are so many people who have played an integral part in not only this project, but in my graduate experience in general.

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Contributors

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CHAPTER I

INTRODUCTION

Conceptus Development in Sheep

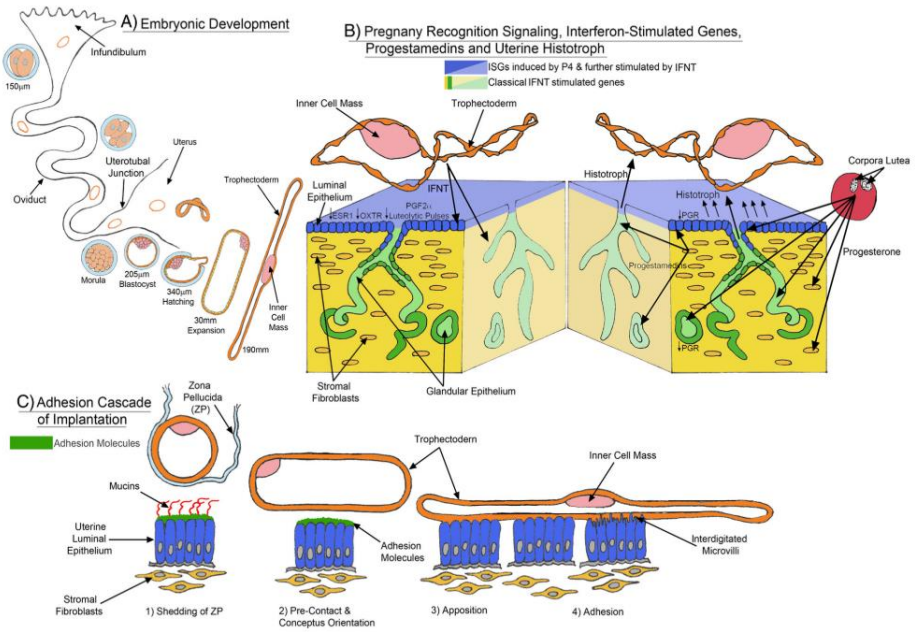
Pregnancy depends on the successful fertilization of the oocyte by the sperm in the oviduct, thus initiating the series of events to develop a blastocyst. Cleavage of the zygote and subsequent increases in numbers of blastomeres result in a morula, which is composed of totipotent cells [1]. The first incidence of cellular differentiation occurs when the inner cells of the morula express connexins to form gap junctions [2], while the outer cells form tight junctions [3]. These innermost cells will eventually form the inner cell mass (ICM), that is fated to become the embryo, and the outer cells will become trophectoderm cells that later become the chorion of the placenta. Formation of the blastocoel due to water influx across the trophectoderm and the presence of tight junctions coupled with sodium transporters increase fluid in the blastocoel that adds pressure to the zona pellucida that, along with proteases from the trophectoderm, eventually rupture the zona pellucida allowing for hatching of the blastocyst [1]. The sheep blastocyst enters the uterus 72 hours post-fertilization and further blastocyst development will occur in the uterus [1, 4, 5].

Development of the blastocyst after hatching from the zona pellucida in sheep requires elongation of the blastocyst (hereafter referred to as the conceptus, a term which encompasses the embryo and associated extraembryonic membranes) [4]. Elongation of the ovine conceptus is needed to increase trophectoderm cell numbers to produce sufficient amounts of interferon tau (IFNT) that prevent regression of the *corpus luteum* (CL) to ensure continued production of

progesterone that is essential for establishment and maintenance of pregnancy, including implantation and placental development [6, 7]. After 10-15 days post-fertilization, the sheep conceptus will develop from a spherical structure to a filamentous structure and continue to elongate through Day 20 of pregnancy to a length of 190 mm (Figure 1) [4]. The conceptus will begin to adhere to the uterine luminal epithelium (LE) at Day 16 [7] and is considered to be fully attached by Day 22 of gestation [8].

This extensive process of cell division and proliferation during elongation requires nutrients to synthesize biomolecules and various growth factors to support cellular proliferation and survival. During this period of development before implantation, the elongating conceptus is supported by histotrophic secretions from the endometrial glands. Histotroph refers to the mixture of water, growth factors, enzymes, and nutrients, such as glucose, fructose, and amino acids secreted by or transported by the uterine luminal and glandular epithelia into the uterine lumen [4, 9]. While the embryonic genome in the sheep is activated before the blastocyst hatches from the zona pellucida, it is able to utilize its own genome to synthesize proteins [10], but it still requires histotroph for nutritional support and survival as the absence of uterine glands and loss of endometrial secretions is fatal to the conceptus [4, 11].

Figure 1. Conceptus development in sheep



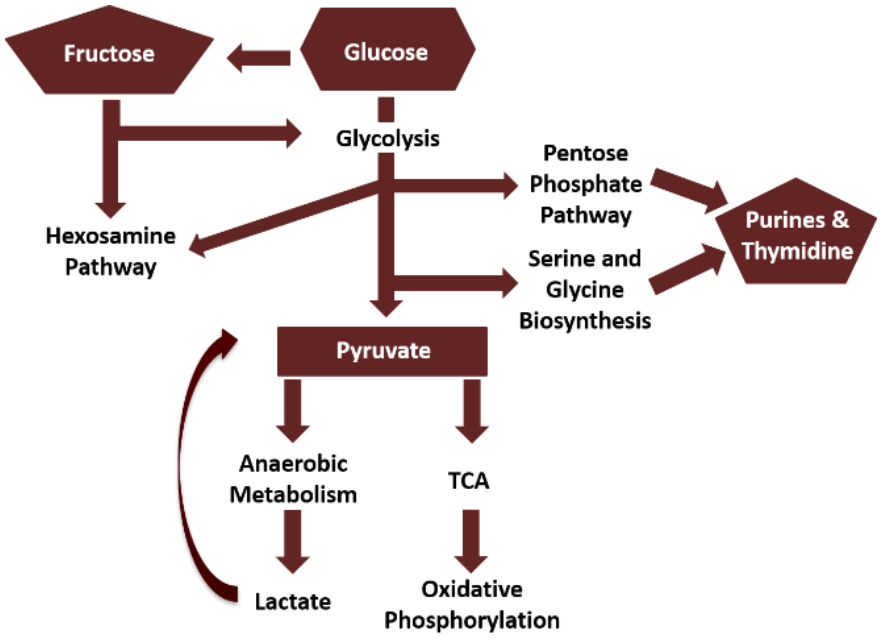
Elongation of the ovine conceptus is required to increase contact of the trophectoderm cells with the maternal uterine luminal epithelia cells. Expansion of the blastocyst after hatching from the zona pellucida results in a spherical structure and then a tubular form of about 30 mm. Conceptuses continue to elongate to 190 mm in length. This long, filamentous structure extends from the uterine horn ipsilateral to the corpus luteum and into the contralateral uterine horn. Reprinted from [4].

Metabolism of Glucose and Fructose

Metabolism of sheep conceptuses has been well documented over the years, from as early as the 2-cell stage embryo [12–17]. In vitro experiments indicated that embryos at the 1-2 cell stage rely more on lactate, glucose, and amino acids, specifically glutamine [16]. The early sheep

embryo, at the 8-cell stage, utilizes glucose and lactate at higher rates than during earlier stages [14]. After the morula undergoes compaction, glucose becomes the preferred energy substrate for the blastocyst until implantation occurs [12]. As previously mentioned, following hatching from the zona pellucida, the sheep blastocyst elongates into a filamentous conceptus. During this time, glycolysis increases to support cellular demands for biomolecules, but not for production of adenosine triphosphate (ATP) [13, 15].

Figure 2. General overview of glucose and fructose metabolism



Glucose and fructose can both be used in multiple metabolic pathways. Products and intermediates of glucose and fructose metabolism can be shunted into the pentose phosphate pathway, seine biosynthesis pathway, tricarboxylic acid cycle (TCA) cycle, and hexosamine biosynthesis pathway.

Glucose, through cytoplasmic glycolysis, is a gateway to many different metabolic pathways, from biosynthesis of molecules for cellular processes to production of ATP for energy. Pyruvate produced at the end of glycolysis can progress through the tricarboxylic acid (TCA) cycle if oxygen is available for oxidative phosphorylation. However, if oxygen levels are not sufficient, pyruvate, the glycolytic end product, can be reduced to lactate and regenerate nicotinamide adenine dinucleotide (NAD^+/NADH) (Figure 2) [18, 19]. Due to increases in cellular replication during conceptus development, pathways of interest during this peri-implantation period are the pentose phosphate pathway and the serine biosynthesis pathway.

The pentose phosphate pathway uses glucose-6-phosphate (G6P) synthesized from the first glycolytic step in which hexokinase (HK) phosphorylates glucose, to generate the cofactor nicotinamide adenine dinucleotide phosphate ($NADP^+$/NADPH) and synthesize ribose-5-phosphate (R5P) to produce nucleosides and histidine required by the cells (Figure 2) [20]. The ovine embryo has an active pentose phosphate pathway during the cleavage stages to support DNA replication, but this activity decreases sharply towards implantation [21].

Fructose, another common hexose sugar, can also enter glycolysis directly via phosphorylation by HK to fructose-6-phosphate (F6P) and participate in reactions through the glycolytic route [18]. However, HK has a higher affinity for glucose than fructose, making glucose the preferred substrate for glycolysis [20, 22, 23]. Most of the fructose in fetal fluids is a result of placental conversion of glucose to fructose via the polyol pathway [24–26]. This pathway oxidizes NADPH to $NADP^+$ for use in the pentose phosphate pathway. Fructose is also a starting substrate for the hexosamine biosynthesis pathway. In this pathway, fructose entering the cell can be phosphorylated to F6P via HK, which can then undergo a series of reactions to produce

uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) for enzymatic glycosylation of proteins, a common post-translational modification, and glycosaminoglycans that include hyaluronic acid [4, 27]. Glucose and fructose are both substrates for the production of UDP-GlcNAc used to form *O*-linked glycosylation bonds to serine or threonine [27, 28] and can activate the AKT (proto-oncogenic protein kinase Akt), TSC2 (tuberous sclerosis complex 2), mTOR (mechanistic target of rapamycin; mTORC1) signaling cascade that stimulates proliferation of ovine trophectoderm cells [29].

A glycolytic intermediate, 3-phosphoglycerate (3PG), is a precursor for serine biosynthesis and by extension, glycine [30]. Serine and glycine are crucial to convert uracil to thymidine and donate elements to the ring structure of the purine nitrogen bases for DNA synthesis, respectively (Figure 2) [18]. Highly proliferative cells must generate nucleosides at a fast rate to keep up with mitosis. Amino acids are important for providing the nitrogen bases of nucleosides, with aspartate, glutamate, and glycine contributing a carbon or nitrogen to the purine base [31]. Increased glycolytic activity generates precursors for the serine biosynthesis pathway to support DNA synthesis [32–34]. Serine and glycine are both present in abundant amounts in uterine flushings of pregnant ewes, suggesting production and conversion of these two amino acids during pregnancy by either the conceptus or endometrium, or both [35]. There is evidence that phosphoserine phosphatase (PSPH) in the uterine LE shifts 3PG from glycolysis into serine biosynthesis. However, serine hydroxymethyltransferase (SHMT2), which converts serine to glycine using tetrahydromethylene folate (THF), is found in the trophectoderm. This suggests that serine is produced and released by the uterine LE and then converted to glycine by the trophectoderm [36].

Importance of Fructose During Pregnancy

While glucose has been shown to be an important metabolic substrate, it is not the most abundant hexose sugar in fetal fluids of sheep during pregnancy. The trophectoderm converts glucose provided by the mother to fructose via the polyol pathway [37], which is sequestered in the allantoic fluid as it cannot pass through the placenta to the maternal vasculature [29, 38, 39]. As a result, the amount of fructose increases throughout gestation and is more abundant than glucose with concentrations being 15- to 30-fold greater than for glucose in allantoic fluid in the later stages of pregnancy [38]. Despite being the most abundant hexose sugar in fetal fluids of ungulates, the role of fructose in the trophectoderm has been overlooked due to glucose being the preferred substrate for glycolysis [23, 40, 41]. However, fructose has been shown to be active in the hexosamine biosynthesis pathways of porcine and ovine trophectoderm cells to produce *O*- and *N*-linked glycosaminoglycans, which activate the mechanistic target of rapamycin (mTOR) pathway [29, 42].

It is also important to note that the first trimester of pregnancy is critical for placental development. The sheep placental units formed via the interdigitation of the maternal caruncle and fetal cotyledon form a placentome. These placentomes allow for increased surface area for transfer of nutrients and gases from the maternal vasculature to the fetal-placental vasculature to support growth and development of the conceptus [38, 43]. Until the placental and uterine vasculature is well established, the early conceptus is subjected to a hypoxic environment [44]. Histotroph from the endometrial glands is required during the first trimester to provide nourishment until the placenta can facilitate hemotrophic nourishment [11]. Fructose may play a role in adaptation of the conceptus to the hypoxic conditions of the uterus during the peri-

implantation period of pregnancy, much as it does in naked mole rat metabolism. These rodents live underground in dark, hypoxic environments. When exposed to hypoxic stress, they can shift from a glucose to fructose-1-phosphate (F1P) driven anaerobic glycolysis that is not inhibited by ATP, citrate, and pH. Therefore, this shift in metabolism allows the naked mole rats to and survive longer than their counterparts that live in normoxic environments [26, 45].

Fructose can also be phosphorylated by ketohexokinase (KHK) at the first carbon position, producing F1P, which can enter glycolysis by cleavage via aldolase to yield dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP). KHK is traditionally considered a hepatic enzyme with a higher affinity for fructose than HK, but studies have shown that some extrahepatic tissues also express this enzyme. It has been demonstrated that trophectoderm and placental tissue in pigs express KHK throughout gestation [25, 26]. High levels of ATP produced via the TCA cycle and electron transport chain prevent progression of glycolysis by inhibiting phosphorylation of F6P to fructose-2,6-bisphosphate via phosphofructokinase I (PFK-I). KHK is not affected by high concentrations of ATP, allowing fructose to bypass the first half of glycolysis [46]. It is currently unclear if ovine trophectoderm possesses the same KHK activity as porcine trophectoderm.

Similarities of Metabolism Between Cells of the Conceptus and Cancers

There are similarities in metabolic profiles among cancer cells and the conceptus [47]. Cancer cells have a characteristic metabolic profile, marked with an increase in glycolytic activity and decreased oxidative phosphorylation despite oxygen being available, which is considered to be inefficient as glycolysis yields a net 2 ATP compared to the greater amounts of ATP produced from multiple rounds of oxidative phosphorylation [18, 48, 49]. In some cancers, high fructose

intake can exacerbate uncontrolled cellular proliferation. Colorectal cancers are often associated with high dietary fructose, which increases glycolytic activity by converting fructose to F1P via KHK [50]. One explanation for this is that glycolysis generates ATP faster than oxidative phosphorylation [51]. Another explanation is that rapidly proliferating cells have an increased demand for metabolic precursors to synthesize the biomass needed to sustain survival [52]. Developing conceptuses have a high demand for metabolic precursors to synthesize the biomolecules needed for cellular processes required for proliferation, survival, and differentiation [52].

Both sheep conceptuses during the peri-implantation period of pregnancy and cancer cells produce high amounts of lactate [14, 15, 53]. Pyruvate dehydrogenase (PDH) incorporates pyruvate into the TCA cycle, whereas lactate dehydrogenase (LDH) reduces pyruvate to lactate. In cancer cells, this could be due to increased pyruvate production from aerobic glycolysis exceeding the metabolic capabilities of PDH. As a result, LDH can use the excess pyruvate to produce lactate and NADH [54]. This increase in pyruvate reduction would regenerate NADH, which may facilitate aerobic glycolysis to meet demands for ATP, as well as regulate gene expression [18, 55].

Furthermore, an increase in production of lactate decreases pH in the uterine lumen and may be necessary for the conceptus to prepare for implantation [56]. Reduction of extracellular pH regulates the expression of vascular endothelial growth factor (*VEGF*) from the endothelial cells and is required to stimulate placental and uterine vascularization [57–59]. Hypoxia-inducible factor-α (HIF-1α), which is upregulated during pregnancy to stimulate pathways to counteract

hypoxic conditions of the uterus [60], has been shown to be affected by lactate by inhibiting prolyl hydroxylase 2 [61], which in normoxic conditions will block HIF-1α activity [62].

Hypothesis

While fructose can be metabolized via the hexosamine biosynthesis pathway during the peri-implantation period of pregnancy, contributions of fructose to other pathways has been overlooked in favor of glucose. It is hypothesized that ovine conceptus trophectoderm can use both glucose and fructose during the peri-implantation period of pregnancy to support conceptus development. Specifically, the conceptus can utilize glucose and fructose to support rapid cellular proliferation via the pentose phosphate pathway. Additionally, it is hypothesized that the products and intermediates of glucose and fructose metabolism can be used for the biosynthesis of substrates for major metabolic pathways including the pentose phosphate pathway, the hexosamine biosynthetic pathway and glycolysis.

CHAPTER II

METABOLISM OF GLUCOSE AND FRUCTOSE BY THE OVINE CONCEPTUS

Methods

Animal Breeding

Experimental procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee. Mature ewes were observed for estrous cycles for 2 mo before synchronization of estrus and breeding. Ewes with normal estrous cycles (n=6) were selected and synchronized by inserting a progesterone Controlled Intravaginal Drug Release (CIDR) device (Zoetis Animal Health, Parsippany, NJ) for 12 days before administering PGF2α analog dinaprost tromethamine (Lutalyse®;Zoetis Animal Health, Parsippany, NJ) at a dose of 20 mg to induce estrus. Ewes were bred to a mature fertile Suffolk rams upon visual detection of estrus (Day 0) and 12h and 24h after detection of estrus.

Tissue Collection

At Day17 of gestation, the ewes were euthanized and hysterectomized. Conceptus tissue was flushed from the uterine horn ipsilateral to the CL following introduction and movement of 15 ml sterile phosphate buffered saline (PBS). Conceptus tissue was minced to ensure homogeneity among cell types and eight aliquots of 27.98 ± 1.61 mg each was placed in 1.5 mL Eppendorf tubes containing 1 ml RPMI 1640 medium with L-glutamic acid (Gibco Life Technologies, ThermoFisher, Waltham, MA) and 5% antibiotic/antimycotic cocktail containing amphotericin B, penicillin, and streptomycin (Gibco, Life Technologies). The remaining conceptus tissue was snap frozen in liquid nitrogen or fixed in 4% paraformaldehyde (PAF) for later analysis for

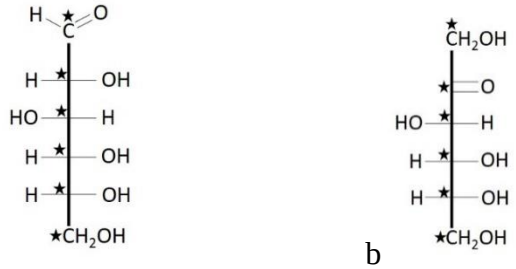
expression of mRNAs and protein, as well as immunolocalization of proteins. A segment of whole uterine tissue from the uterine horn ipsilateral to the CL was fixed in either 4% PAF or optimal cutting temperature (OCT) compound (ThermoFisher Scientific) for future histological analysis. Endometrial tissue was then dissected from the myometrium of the remaining ipsilateral uterine horn and snap frozen in liquid nitrogen for real time-quantitative PCR (RT-qPCR) gene expression analysis.

Isotopic Tracer Experiment

The culture medium was prepared fresh the morning of tissue collection and conceptus tissue was cultured the same day. The culture medium was prepared as noted in Table 1 and included either universally labeled radioactive glucose or fructose (Figure 3) (American Radiological Chemicals, Inc., St. Louis, MO) and unlabeled nutrients in Krebs-Henseleit bicarbonate (KHB) buffer with 1 mM glutamate (Glu). KHB buffer was prepared by sequentially adding 365 ml of 163 mM NaCl, 15 ml of 160 mM KCl, 10 ml of 125 mM $CaCl_2 \bullet 2H_2O$, 5 ml of 120 mM $MgSO_4$, 5 ml of 120 mM KH_2PO_4, 75 ml of 167 mM $NaHCO_3$, and 25 ml of 400 mM Hepes (pH = 7.4). The buffer was then gassed with 95% O_2, 5% CO_2 for 15 min. To make KHB + 1mM Glu, 15 mg of glutamic acid was added to 100 ml of the KHB buffer and mixed well.

Two experimental tubes were set up per treatment condition: one containing conceptus tissue and one was an isotopic control without conceptus tissue. The conceptus tissue was removed from the medium by centrifugation at 500 x g for 20 sec and vacuum aspiration of the RPMI medium. Tissue was then briefly washed in 1 mL KHB without Glu and centrifuged at 500 x g for 20 sec. The supernatant was removed, and the conceptus tissue was added to its respective treatment medium (Table 1).

Figure 3. Universally labeled isotopes for glucose and fructose



a b

Each carbon atom in the radiolabeled glucose (a) and fructose (b) was labelled with a ^{14}C isotope, as noted by the star.

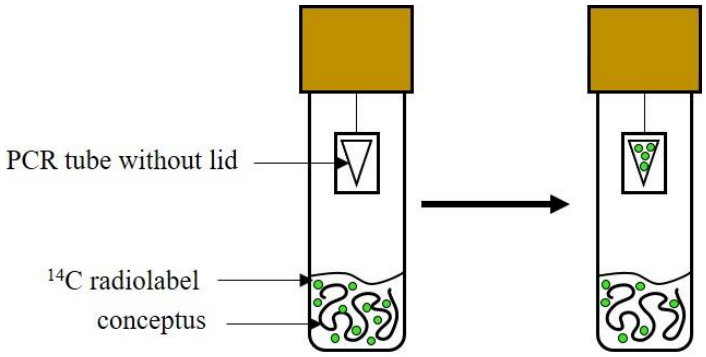
Table 1. Conditions for universal isotope tracer study

Radiolabel (final concentration = 0.1 mCi/ml)	Unlabeled Nutrient (final concentration = 4 mM)	KHB + 1mM Glu
100 µl [U-^{14}C]-Fructose	100 µl Fructose	800 µl
100 µl [U-^{14}C]-Fructose	100 µl Glucose 100 µl Fructose	700 µl
10 µl [U-^{14}C]-Glucose	100 µl Glucose	890 µl
10 µl [U-^{14}C]-Glucose	100 µl Glucose 100 µl Fructose	790 µl

Amounts of each radiolabel, unlabeled nutrient, and KHB + Glu used in 1 ml culture medium.

The cultures were gassed with a 95% O_2/5% CO_2 mixture for 15 sec and the tubes were fitted with a center well stopper cap and a 0.2 ml polymerase chain reaction (PCR) tube without a cap. Tubes were incubated in a shaking water bath for 2h at 37°C. After 2h, 0.2 ml of toluene (PerkinElmer, Waltham, MA) was injected through the cap and into the PCR tube in the center well to capture and solubilize $^{14}CO_2$ (Figure 4). The reactions were terminated by adding 0.2 ml 1.5M $HClO_4$ and allowed to incubate for another 1h in the shaking water bath at 37°C.

Figure 4. Experimental design



The center well held a PCR tube with the cap removed. After 2h, 0.2 ml toluene was injected into each PCR tube in the center well to capture $^{14}CO_2$ gas that was produced during the incubation of conceptus tissue. The PCR tube with toluene and $^{14}CO_2$ was removed and added whole to the PPO/POPOP cocktail.

Once removed from the water bath, the PCR tubes from the experimental and control cultures were removed and placed in individual vials containing 5.0 mL of a PPO/POPOP liquid scintillation cocktail (made from 500 mL toluene, 500 mL 2-methoxyethanol, 5 g PPO (2,5-diphenyloxazole), and 0.2 mg POPOP (1,4-(di-2-(5-phenyloxazolyl))-benzol)). Vials were incubated overnight at room temperature to prevent chemiluminescence of $^{14}CO_2$ in the liquid scintillation cocktail. Disintegrations per minute (DPM) of $^{14}CO_2$ were measured using a Tri-Carb® 4810 TR PerkinElmer scintillation counter the following day. The remaining culture medium and tissue was neutralized with 0.1 ml 2M K_2CO_3 and stored at -20°C. After culture, the conceptus tissue was homogenized using a glass tissue grinder and centrifuged for 5 min at 600 x g. The resulting supernatants were removed for future analysis.

Specific activity (SA) of the isotopes was determined for each treatment by using the DPM measurements of the $^{14}CO_2$ produced by the conceptus and the isotopic blank, DPM of 50 µl of the isotopic blank media (multiplying this value by 26 to account for a final volume of 1300 µl), and background (20 DPM):

$$\frac{\left(\frac{26 * \text{Isotopic blank media DPM} - 20 \text{ DPM}}{4000 \text{ nmol tracee}}\right) / 0.95}{6} = \text{SA (DPM/nmol C)}$$

The nmol $^{14}CO_2$ produced in a 2h incubation was calculated by dividing the difference in DPM from the experimental PCR tube and blank PCR tube by the SA of one carbon, and then by 0.95 as the liquid scintillation counter has a counting efficiency of 95%:

$$\frac{\left(\frac{\text{DPM}}{\text{SA}}\right)}{0.95} = \text{nmol } ^{14}CO_2/120 \text{ min}$$

That value was divided by the tissue weight to determine $^{14}CO_2$ produced per mg tissue in a 2h incubation.

Analysis of Culture Medium

The supernatants were also analyzed for lactate (MilliporeSigma, Burlington, MA), and pyruvate (MilliporeSigma, Burlington, MA), following the manufacturer's instructions, as well as amino acids following a modified high-performance liquid chromatography (HPLC) protocol [63]. Supernatants of the isotopic blanks were evaluated for glucose (CellBio Labs, Inc., Watertown, MA) or fructose (Bioassay Systems, EnzyChrom, Hawyward, CA) to quantitate the amount of tracee at the start of the cultures (time = 0 min). Concentrations of amino acids were normalized by subtracting detectable amino acids in the water standard from concentrations in the conceptus

samples and isotope blanks. The differences for the sample and blank were then subtracted from each other and finally multiplied by volume of culture medium to determine release of amino acids from conceptus tissue into the culture medium.

Immunohistochemistry of KHK

Immunohistochemistry was used to detect KHK (GTX109591, GeneTex Inc., Irving, CA) in ovine conceptuses and endometria from Days 17, 18, and 30 of pregnancy. The antibody was used at a dilution of 1:100 as described previously [36]. KHK was co-localized with E-cadherin to better visualize the trophectoderm cells. The KHK immunostaining was contrasted with that of a control rabbit IgG used at the same concentration. Images were taken using an Axioplan2 microscope and a Zeiss Imager.M2 (Carl Zeiss, Thornwood, NY) interfaced with an Axioplan HR and an AxioCam HRm digital camera, respectively. This was a collaborative effort with Drs. Greg Johnson and Heewon Seo, Department of Veterinary Integrative Biosciences, Texas A&M University.

Statistical Analysis

Data were analyzed using JMP Statistical Software (SAS Institute, Cary, NC). Data for glucose and fructose in the uterine flushings, production of $^{14}CO_2$, pyruvate, lactate, and all amino acids were evaluated for normality according to the Anderson-Darling Goodness of Fit test and values deemed to be outliers were excluded. Data for analysis of glucose and fructose in Day 17 pregnant uterine flushings was analyzed using a nonparametric Wilcoxon test, while production of $^{14}CO_2$, lactate, pyruvate, and amino acids were analyzed using one-way analysis of variance (ANOVA) and the Student's t-test. Values for are expressed as Least Square (LS) means of logarithmic values ± standard errors of means (SEM).

Results

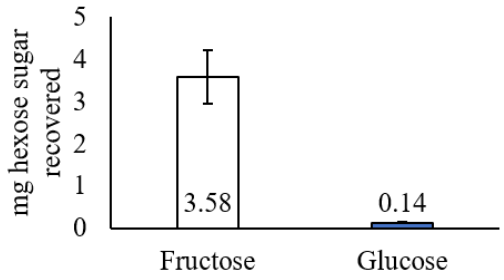
Analysis of Glucose and Fructose in Uterine Flushings

Fructose in Day 17 pregnant uterine flushings was 25.43 ± 4.10-fold more abundant than glucose (Figure 5). Total recoverable fructose in the flushings was 3.58 ± 0.64 mg, compared to 0.14 ± 0.011 mg of total recoverable glucose.

Isotopic Tracer Study

Ovine conceptuses in all treatments metabolized [U-^{14}C]-glucose and [U-^{14}C]-fructose to produce $^{14}CO_2$ (Figure 6). Utilization of [U-^{14}C]-glucose was greater than utilization of [U-^{14}C]-fructose as indicated by increased production of $^{14}CO_2$ from radiolabeled glucose. Conceptus tissue cultured with [U-^{14}C]-fructose in the presence of unlabeled glucose and fructose generated less $^{14}CO_2$ than the other three treatments investigated. Interestingly, conceptus tissue cultured in medium containing only fructose produced detectable $^{14}CO_2$ in the absence of glucose, indicating that it is metabolized although to a lesser extent than glucose.

Figure 5. Total recoverable glucose and fructose in Day 17 pregnant uterine flushings



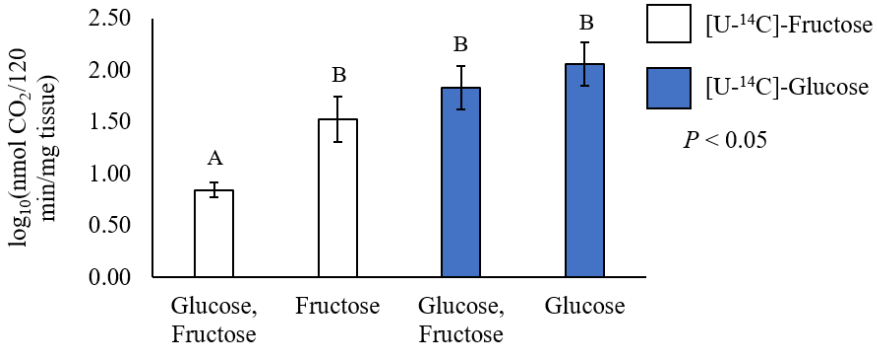
Data are expressed as mean ± SEM. Fructose was 25.43 ± 4.10-fold more abundant than glucose (P<0.05).

Analysis of Culture Medium

Both lactate and pyruvate were detectable in all culture medium after homogenization of conceptus tissue. However, there were no differences in lactate (P>0.05) or pyruvate (P>0.05) among treatments. Across all treatments, lactate was 33- to 44-fold greater in abundance than pyruvate, but composition of the medium did not have a significant effect (P>0.05) (Figure 7).

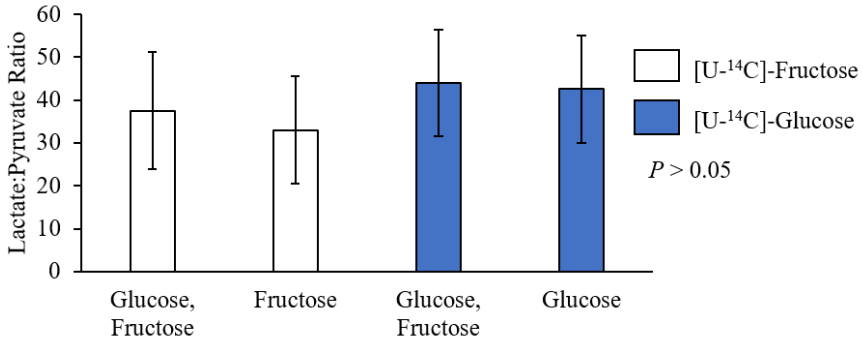
Abundance of amino acids in the culture medium was not affected by treatment (P>0.05) (Table 2). The most abundant amino acids in the culture medium were glutamate, glycine, alanine, and aspartate. In contrast, glutamine and citrulline were lowest in abundance in all medium. Further, citrulline was undetectable in the fructose only treatment. The total amount of amino acids was lowest for conceptus tissue incubated with [U-^{14}C]-glucose, glucose, and fructose, but differences were not significant (P>0.05).

Figure 6. Detectable production of $^{14}CO_2$ by Day 17 ovine conceptuses.



Data are presented as LS($\log_{10}$(means)) ± SEM. Values with different letters indicate significant differences among means. Composition of culture medium had an effect on the amount of $^{14}CO_2$ that was detected (P<0.05)

Figure 7. Ratio of nmol lactate/mg tissue and nmol pyruvate/mg tissue produced by Day 17 conceptuses



Data are presented as LS(means) ± SEM. Lactate production was 33- to 44-fold greater than that for pyruvate. There was no significant effect of treatment (P>0.05).

Table 2. Amounts of amino acids (nmol) in culture medium after a 2h incubation and homogenization of conceptus tissue

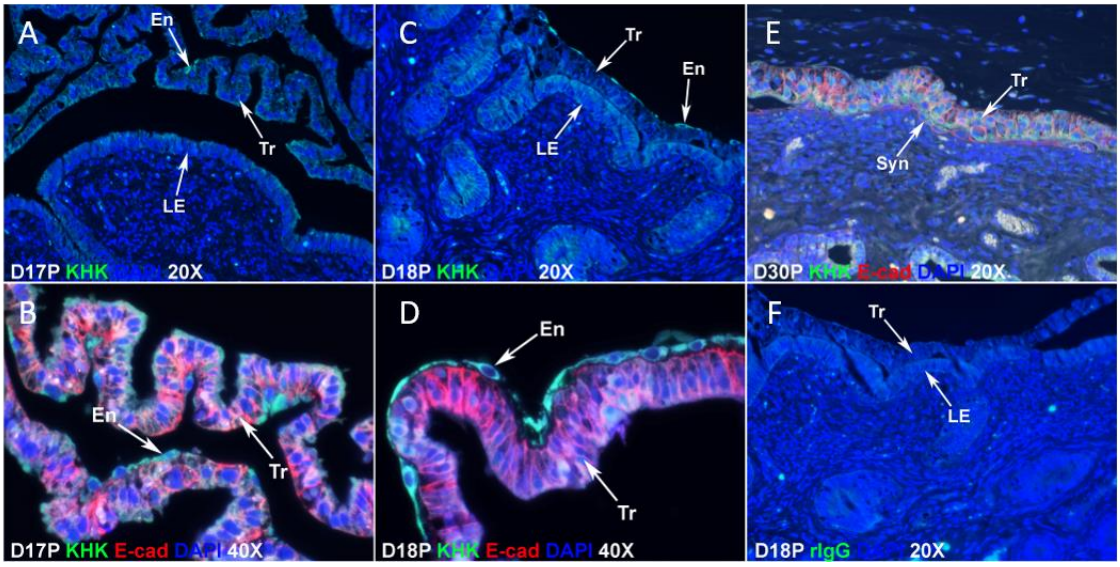
	Compositions of Culture Medium			
Amino Acid	[U-^{14}C]-fructose, glucose, fructose	[U-^{14}C]-fructose, fructose	[U-^{14}C]-glucose, glucose, fructose	[U-^{14}C]-glucose, glucose
Aspartate	1.11 ± 0.29	1.88 ± 0.69	1.36 ± 0.61	1.03 ± 0.45
Glutamate	7.78 ± 2.69	4.06 ± 2.22	9.54 ± 11.08	2.25 ± 1.00
Asparagine	0.04 ± 0.019	0.12 ± 0.060	0.10 ± 0.04	0.06 ± 0.024
Serine	0.43 ± 0.082	1.01 ± 0.39	0.64 ± 0.34	0.47 ± 0.19
Glutamine	0.028 ± 0.025	0.048 ± 0.048	0.0017 ± 0.0017	0.0067 ± 0.0067
Histidine	0.027 ± 0.019	0.057 ± 0.039	0.020 ± 0.013	0.020 ± 0.013
Glycine	3.93 ± 1.20	3.90 ± 1.35	4.28 ± 1.37	3.69 ± 1.31
Threonine	0.855 ± 0.19	1.14 ± 0.44	0.80 ± 0.79	0.69 ± 0.25
Citrulline	0.0067 ± 0.0067	0 ± 0	0.015 ± 0.32	0.0083 ± 0.022
Arginine	0.45 ± 0.083	0.77 ± 0.27	0.32 ± 0.17	0.23 ± 0.086
β-Alanine	0.02 ± 0.013	0.032 ± 0.016	0.023 ± 0.015	0.017 ± 0.011
Taurine	0.12 ± 0.034	0.13 ± 0.061	0.077 ± 0.035	0.043 ± 0.021
Alanine	1.49 ± 0.15	2.41 ± 0.75	1.65 ± 0.58	1.25 ± 0.35
Tyrosine	0.27 ± 0.052	0.34 ± 0.15	0.28 ± 0.12	0.23 ± 0.093
Tryptophan	0.022 ± 0.012	0.056 ± 0.034	0.063 ± 0.022	0.075 ± 0.021
Methionine	0.057 ±0.016	0.10 ± 0.023	0.092 ± 0.032	0.175 ± 0.053
Valine	0.56 ± 0.14	0.85 ± 0.34	0.58 ± 0.28	0.50 ± 0.19
Phenylalanine	0.30 ± 0.08	0.42 ± 0.15	0.28 ± 0.18	0.21 ± 0.089
Isoleucine	0.53 ± 0.10	0.67 ± 0.25	0.50 ± 0.19	0.42 ± 0.12
Leucine	0.78 ± 0.22	1.20 ± 0.53	0.75 ± 0.34	0.58 ± 0.22
Ornithine	0.49 ± 0.080	0.52 ± 0.11	0.49 ± 0.064	0.56 ± 0.050
Lysine	0.63 ± 0.20	1.14 ± 0.42	0.90 ± 0.36	0.74 ± 0.23

Data are presented as means ± SEM. There was no effect of treatment (P>0.05).

Immunohistochemistry of KHK

KHK was localized primarily to the endoderm of the conceptus on Day 17 and Day 18 (Figure 8A and C, respectively) of pregnancy. Although present in the trophectoderm as well, staining intensity was stronger in the endoderm than the trophectoderm of the conceptus on both of those days of pregnancy (Figure 8B and D). At Day 30 of pregnancy, KHK was localized to the trophectoderm (Figure 8E) and expressed weakly by uterine LE and GE (Figure 8A, C, and E).

Figure 8. Immunolocalization of KHK in pregnant sheep conceptuses and endometria



Legend: KHK: ketohexokinase; E-cad: E-cadherin; En: endoderm; LE: luminal epithelium; Tr: trophectoderm; Syn: syncytial plaque; rIgG: rabbit IgG. The enzyme was strongly expressed by the endoderm of the conceptus on Day 17 and Day 18 of pregnancy, and weakly expressed by trophectoderm, uterine LE, and GE. KHK was localized to the trophectoderm and GE on Day 30 of pregnancy.

Discussion

The ovine conceptus undergoes significant cellular proliferation to achieve the surface area necessary for contact between the trophectoderm and uterine LE for implantation [6]. The cells of the conceptus must synthesize the biomolecules required for replication and survival. End products and intermediates from multiple metabolic pathways supply the cells with a pool of substrates to synthesize key biomolecules and stimulate cell signaling pathways [64]. Glucose and fructose are important substrates used in various metabolic pathways to produce biomolecules and ATP for energy. Some fructose is provided by endometrial gland secretions, but the trophectoderm also converts glucose to fructose, resulting in a greater abundance of fructose than glucose in later stages of pregnancy [38]. The greater abundance of fructose than glucose found in the Day 17 uterine flushings suggests that this accumulation of fructose begins during the peri-implantation period of pregnancy. Metabolism of glucose or fructose via either the TCA cycle or the pentose phosphate pathway results in the production of CO_2 as a byproduct [20, 22, 29, 45]. In this study, culture of conceptus tissue with glucose and fructose labeled with a radioactive tracer (^{14}C) provided important new insights into the ability of the conceptus to metabolize glucose and fructose via these pathways [65].

The in vitro results of this study indicate that at Day 17 of gestation, the ovine conceptus can use both glucose and fructose as substrates for both the TCA cycle and the pentose phosphate pathway. Production of detectable $^{14}CO_2$ was greater in the treatments with [U-^{14}C]-glucose than [U-^{14}C]-fructose. HK is the first enzyme of glycolysis as it phosphorylates glucose at carbon number 6 and this glucose is metabolized to pyruvate that can enter the TCA cycle [22]. HK has a higher affinity glucose than fructose [23], which is reflected in the preferential use of glucose

in the experiments described in this chapter. These results demonstrated that when the ovine conceptus is cultured with [U-^{14}C] fructose and glucose, less detectable $^{14}CO_2$, is produced presumably because HK has a higher affinity for glucose and will use it preferentially when it is available. Interestingly, when the ovine conceptus tissue was cultured with [U-^{14}C]-fructose, with and without unlabeled fructose in the absence of glucose, the ovine conceptus utilized fructose to produce $^{14}CO_2$ at a rate comparable to that for conceptuses cultured with [U-^{14}C]-glucose and unlabeled glucose. Previous studies investigating fructose metabolism by the ovine conceptus suggested that fructose cannot be utilized for glycolysis or the TCA cycle [29, 40–42]. However, results of the present study are the first to indicate that fructose can indeed be metabolized via the glycolytic pathway and TCA cycle in the ovine conceptus.

Fructose can also enter glycolysis after phosphorylation of fructose at the first carbon position by KHK and enter glycolysis by bypassing the regulatory mechanisms of PFK. Immunolocalization of KHK in the endoderm and trophectoderm of the sheep conceptus suggest that the conceptus can metabolize fructose in this manner and enter glycolysis to produce pyruvate for entry into the TCA cycle. The extent of fructose utilization by the ovine conceptus may be further appreciated if one incubates conceptus or placental tissues with physiological amounts of glucose and fructose as fructose is 11- to 33-fold more abundant than glucose in fetal fluids during gestation [38], and on Day 17 of gestation in the present study, 25-fold more abundant than glucose in uterine flushings.

It has been previously shown that the conceptus can utilize glucose in an in vitro culture system and produce lactate under hypoxic conditions [53]. The results obtained from the present research indicate that the ovine conceptus is capable of utilizing both glucose and fructose to

produce pyruvate, the end product of glycolysis, and further reduce it to lactate. The conceptus also favors reduction of pyruvate to lactate which regenerates NAD^+ for use by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in glycolysis, as indicated by the large lactate to pyruvate ratio. The results also suggest that the initial substrate does not affect the ability of the conceptus to produce pyruvate. When fructose is the only available substrate, pyruvate production and its reduction to lactate is comparable to that for conceptus tissue incubated with glucose. Although pyruvate can also be produced from glucogenic amino acids such as alanine [66], the culture medium in this study did not contain supplemental amino acids to allow assessment of the full gluconeogenic capabilities of the ovine conceptus.

Increased proliferation of the trophectoderm during the peri-implantation period of pregnancy requires an increase in synthesis of nucleotides to support DNA replication. This provides an explanation for the abundances of aspartate and glycine for generating the purine bases, both of which, along with glutamate, donate a carbon or nitrogen to the structure [18, 31]. The high abundance of glutamate in the culture medium is likely due to the addition of 1mM glutamate to the KHB which results in an over-estimation of the amount of glutamate produced by the conceptus. While glutamate is highly abundant in Day 16 pregnant ovine uterine flushings, it should be noted that there is also likely a contribution of glutamate from histotroph from the uterine endometrial glands [35]. The abundance of glycine suggests the conversion of serine to glycine, which is supported by the low amounts of serine found in the culture medium. In the later stages of gestation in sheep, glycine the most abundant amino acid in fetal plasma and increases with gestational day [67].

The experiments described in this chapter have clearly demonstrated that both glucose and fructose can be metabolized by the ovine conceptus via the TCA cycle and the pentose phosphate pathway. However, the radiolabeled carbon tracer used for these experiments was universally labelled. That is, all carbons on glucose or fructose were radiolabeled with ^{14}C. This poses an obstacle in determining which metabolic pathway is contributing the detectable $^{14}CO_2$. To overcome this experimental limitation, a further experiment was performed (see Chapter 3) utilizing specifically labelled radioactive carbons on glucose and fructose at the carbon 1 or carbon 6 position to allow determination of relative contributions of each hexose sugar to the pentose phosphate pathway and the TCA cycle [21, 68].

CHAPTER III

METABOLIC CONTRIBUTIONS OF GLUCOSE AND FRUCTOSE IN THE OVINE CONCEPTUS

Methods

Animal Breeding

Experimental procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee. Sexually mature Suffolk ewes were observed for estrous cycles for 2 mo before synchronization of estrus and breeding, as outlined in Chapter 2. Thirty-two ewes were bred to mature, intact Suffolk rams, in six groups of four each (n=6, Table 3) when detected in estrus (Day 0) and 12h and 24 h later.

Tissue Collection

On Day 16 of gestation, ewes were euthanized for tissue collection. Conceptuses from pregnant ewes (Table 3) were collected by clamping the cervix and flushing the uterine horns with 15 mL sterile PBS. The conceptuses were collected whole in sterile RPMI 1640 medium with L-glutamine (ThermoFisher Scientific, Waltham, MA) and 5% antibiotic/antimycotic cocktail containing amphotericin B, penicillin, and streptomycin (ThermoFisher Scientific, Waltham, MA) cocktail. Whole uterine tissue sections from the uterine horn ipsilateral to the CL were fixed in 4% PAF and endometrial tissue dissected from the myometrium was snap frozen in liquid nitrogen. All conceptus tissue from pregnant ewes was pooled within groups to achieve sufficient amounts of conceptus tissue for the isotopic tracer experiments.

Table 3. Breeding groups and confirmed pregnancies for Day 16 of gestation.

Group number	Ewes bred	Confirmed pregnancies at necropsy
1	4	3
2	4	3
3	3	2
4	4	4
5	4	4
6	4	2

Multiple ewes were bred for each group to ensure there was sufficient conceptus tissue for all tracer treatment conditions.

Isotopic Tracer Study

The conceptuses from individual ewes in each group were pooled in a grid dish and washed in KHB buffer. The tissue was then finely cut and mixed well to ensure homogeneity across cell types and individual conceptuses. Each treatment condition (Table 4) had 19.93 ± 0.33 mg of tissue cultured in KHB + Glu buffer, radiolabeled tracer (Figure 9) (American Radiological Chemicals, Inc., St. Louis, MO), and unlabeled nutrients, as outlined in in Table 4. Culture procedures and subsequent homogenization of tissue were carried out as outlined in Chapter 2.

The production of $^{14}CO_2$ was calculated as outlined in Chapter 2. Activity of the pentose phosphate pathway was estimated by subtracting nmol $^{14}CO_2$ produced in the 2 h incubation per mg of tissue of [1-$^{14}CO_2$] – [6-$^{14}CO_2$], otherwise known as the Larrabee approach [68]. The carbon at the first position (Figure 9a) is released as $^{14}CO_2$ in both the pentose phosphate pathway and TCA cycle. However, the carbon at the sixth position (Figure 9b) can only be

released as $^{14}CO_2$ in the TCA cycle. Thus, the difference of [1-$^{14}CO_2$] – [6-$^{14}CO_2$] is an estimate of each hexose sugar's contribution to the pentose phosphate pathway.

Table 4. Conditions for specifically labeled isotope tracer study

Radiolabel (final concentration = 0.1 mCi/ml)	Unlabeled Nutrient (final concentration = 4 mM)	KHB + 1mM Glu
100 µl [1-^{14}C]-Fructose	100 µl Fructose	800 µl
100 µl [6-^{14}C]-Fructose	100 µl Fructose	800 µl
100 µl [1-^{14}C]-Fructose	100 µl Glucose 100 µl Fructose	700 µl
100 µl [6-^{14}C]-Fructose	100 µl Glucose 100 µl Fructose	700 µl
100 µl [1-^{14}C]-Glucose	100 µl Glucose	800 µl
100 µl [6-^{14}C]-Glucose	100 µl Glucose	800 µl
100 µl [1-^{14}C]-Glucose	100 µl Glucose 100 µl Fructose	700 µl
100 µl [6-^{14}C]-Glucose	100 µl Glucose 100 µl Fructose	700 µl

Amounts of each radiolabel, unlabeled nutrient, and KHB + Glu used in 1 ml culture medium.

Figure 9. Specifically radiolabeled glucose and fructose.

a. [1-^{14}C]-Glucose [1-^{14}C]-Fructose b. [6-^{14}C]-Glucose [6-^{14}C]-Fructose

a) ^{14}C label at the first carbon position (noted by a star) can be released as $^{14}CO_2$ in the pentose phosphate pathway activity and TCA cycle. b) ^{14}C label at the sixth carbon position (noted by a star) can only be released as $^{14}CO_2$ from the TCA cycle.

Analysis of Culture Medium

To track metabolic contributions of glucose and fructose by the conceptus, the supernatants from conceptus homogenates of the glucose only and fructose only treatments were analyzed at the end of the 2 h incubation for glucose (CellBio Labs, Inc., Watertown, MA), fructose (Bioassay Systems, EnzyChrom, Hawyward, CA), lactate (MilliporeSigma, Burlington, MA), and pyruvate (MilliporeSigma, Burlington, MA), following the manufacturer's instructions, as well as ^{14}C-lipids and ^{14}C-glycoproteins.

Lipids were extracted by adding 100 µl of the supernatant to 500 µl of an isopropyl alcohol, heptane, and 1N H_2SO_4 mixture at a ratio of 40:10:1, respectively. The mixture was vortexed and allowed to sit for 10 min. Next, following addition of 300 µl of heptane and 300 µl of ultrapure water, the samples were vortexed once more, and rested for 10 min for layers to separate. The upper heptane layer was collected and evaporated to less than 100 µl and added to 4.5 ml of Hionic Fluor scintillation cocktail (PerkinElmer, Waltham, MA) to measure DPM via

liquid scintillation for 5 min to determine incorporation of ^{14}C-glucose or ^{14}C-fructose for lipid synthesis.

The remaining conceptus tissue pellet was analyzed for incorporation of ^{14}C-glucose or ^{14}C-fructose into glycoproteins. The pellet was resuspended in 5 ml (2%, weight/volume) trichloroacetic acid and centrifuged at 600 x g for 5 min, after which the supernatant was discarded, and the pellet saved. This was repeated twice. After the third wash, the pellet was dissolved in 0.5 ml of 1M NaOH by gently mixing. The solutions were allowed to dissolve further overnight at room temperature. The following day, 200 µl of the solution was mixed with 4.5 mL Hionic Fluor liquid scintillation cocktail in a scintillation vial and incubated for 2h at room temperature. DPMs were measured in a liquid scintillation counter.

Production of ^{14}C-glycoproteins was calculated similarly to that for production of $^{14}CO_2$. The DPM difference between the conceptus and isotope blank were multiplied by a factor of 2.5, as 500 µl of 1 M NaOH was used to solubilize the protein pellet, and 200 µl of this solution was used for liquid scintillation counting.

Consumption of glucose and fructose by the conceptus tissue was determined by evaluating the production of pyruvate, lactate, ^{14}C-lipids, ^{14}C-glycoproteins, and $^{14}CO_2$ from glucose or fructose via either the pentose phosphate pathway or the TCA cycle.

Statistical Analysis

Production of $^{14}CO_2$ via the pentose phosphate pathway was analyzed using JMP Statistical Software (SAS Institute, Cary, NC). The data were analyzed for normality according to the Anderson-Darling Goodness of Fit test and values determined to be outliers were excluded from

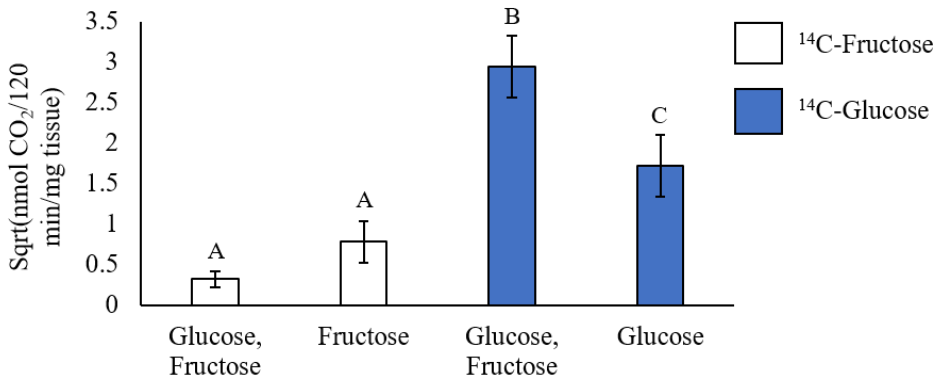
the analysis. Resulting data were transformed as needed to ensure normality and treatment effects were analyzed using one-way ANOVA and the Student's t-test. Data are expressed as LS means ± SEM.

Results

Isotopic Tracer Study

The tracer used had a significant effect on the amount of $^{14}CO_2$ detected, as shown in Figure 10 ($P<0.05$). Detectable $^{14}CO_2$ produced via the pentose phosphate pathway for Day 16 ovine conceptus tissue in in vitro cultures was greatest when ^{14}C-glucose was the tracer with unlabeled glucose and fructose in the medium. The pentose phosphate pathway in ovine conceptuses cultured with ^{14}C-fructose was also active, but less than for conceptuses cultured in the presence of ^{14}C-glucose. There were no differences in pentose phosphate pathway activity among the two and ^{14}C-fructose conditions, that is, those with only unlabeled fructose or with unlabeled fructose and glucose (Figure 10).

Figure 10. Pentose phosphate pathway activity of Day 16 ovine conceptuses.

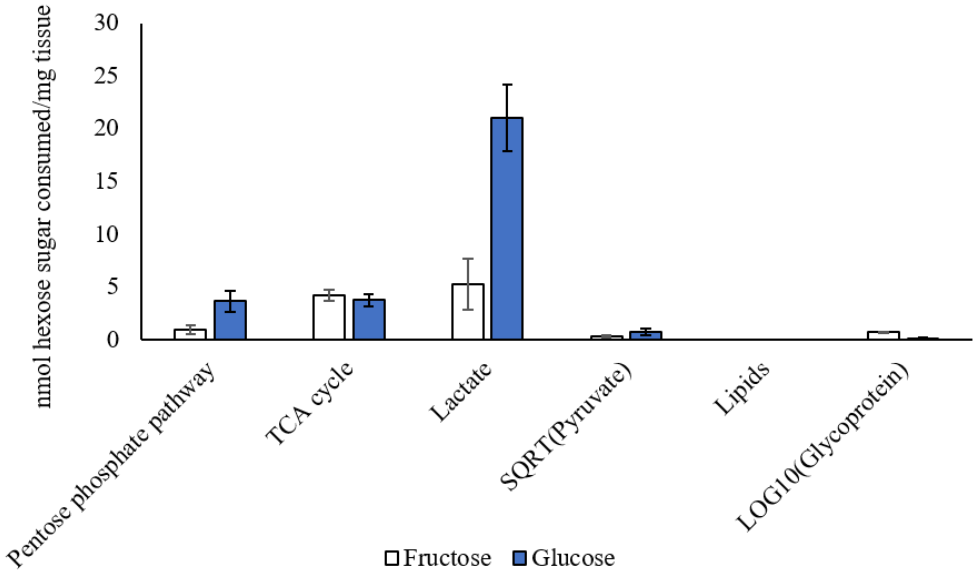


Values are expressed as LS(means) ± SEM. Different letters indicate significant differences among means. There was a significant effect of composition of the medium on the estimated activity of the pentose phosphate pathway ($P<0.05$). The presence of glucose in the medium with ^{14}C-fructose decreased the amount of detectable $^{14}CO_2$ produced. The ovine conceptus was able to utilize fructose via the pentose phosphate pathway; however, its rate of metabolism in this metabolic pathway was less than that for glucose.

Analysis of Culture Medium

The ovine conceptus metabolized glucose and fructose differently when the other hexose sugar was not present. Overall, ovine conceptus tissue utilized more glucose than fructose ($P<0.05$), except in the case of TCA cycle activity, for which there were no significant differences between glucose and fructose metabolism ($P>0.05$) (Figure 11).

Figure 11. Metabolism of glucose and fructose via various metabolic pathways by Day 16 ovine conceptuses.



Metabolism of glucose was greater than fructose via the pentose phosphate pathway ($P<0.05$), and for lactate production ($P<0.05$), pyruvate production ($P<0.05$), and incorporation into glycoproteins ($P<0.05$). There were no differences between metabolism of glucose and fructose via the TCA cycle ($P>0.05$) and there was no detectable lipid synthesis from glucose and fructose.

When only glucose was available in the culture medium (Table 5), its contributions to the pentose phosphate pathway and TCA cycle were similar ($P>0.05$). There was, on average, a 69.52 ± 32.98-fold greater abundance of lactate than pyruvate in the culture medium. The contribution of glucose to glycosylation of proteins accounted for 8.04 ± 0.22 nmol glucose/mg tissue, while de novo synthesis of lipids from glucose was not detectable.

Table 5. Metabolism of glucose by Day 16 ovine conceptuses after a 2h incubation.

	nmol glucose metabolized/mg tissue
Pentose phosphate pathway	3.67 ± 2.46
TCA cycle	3.74 ± 0.57
Lactate	17.99 ± 0.88
Pyruvate	0.67 ± 0.22
Lipids	0
Glycoproteins	8.04 ± 0.22
Other pathways or not utilized	64.46 ± 7.11

Values are expressed as means ± SEM. Glucose metabolism by the pentose phosphate pathway and TCA cycle were similar. Lactate production accounted for a significant amount of glucose metabolism via the metabolic pathways evaluated. There was no detectable contribution of glucose to the synthesis of lipids but, glucose was utilized in the glycosylation of proteins.

When only fructose was present in the culture medium (Table 6), there was less metabolic activity overall compared to that for glucose. TCA cycle activity was 2.90 ± 0.71-fold greater than for pentose phosphate activity when fructose was the substrate ($P<0.05$). Lactate production from metabolism of fructose was, on average, 52.77 ± 15.40-fold greater than that for pyruvate. The conceptuses did not use fructose for lipid synthesis, but did utilize it for the glycosylation of proteins.

Table 6. Metabolism of fructose by Day 16 ovine conceptuses after a 2h incubation.

	nmol fructose metabolized/mg tissue
Pentose phosphate pathway	0.95 ± 0.38
TCA cycle	4.13 ± 0.57
Lactate	5.29 ± 0.99
Pyruvate	0.13 ± 0.03
Lipids	0
Glycoproteins	5.15 ± 0.62
Other pathways or not utilized	77.66 ± 17.11

Values are expressed as means ± SEM. Metabolism of fructose via the TCA cycle was greater than that for the pentose phosphate pathway ($P<0.05$). Fructose metabolism contributed to the production of pyruvate that was reduced to lactate. Fructose was not used by the ovine conceptus to synthesize lipids; however, fructose was utilized for glycosylation of proteins.

Discussion

Metabolism is not static, nor is it a "one pathway at a time" process. There are multiple metabolic reactions occurring at once to ensure that cells maintain homeostasis and synthesize the molecules needed to survive and function. While some metabolic reactions are shared, not all cell types and tissues have exactly the same requirements for survival and function. It is important, therefore, to understand how different nutrients contribute to various metabolic pathways in cells of interest.

A key metabolic pathway required for proper conceptus development is the pentose phosphate pathway, as it provides NADPH, a reducing agent and cofactor, and R5P for the synthesis of

nucleosides [20]. This pathway is active in highly proliferating tissues, such as the elongating conceptus, to support cellular functions and survival [21, 49, 52, 69]. NADPH is also required for conversion of glucose to fructose by aldose reductase in the polyol pathway [20], which is active in the sheep placenta to convert maternal glucose into fructose [24, 37, 38].

The HK enzyme is responsible for phosphorylation of glucose to G6P, which is the first substrate in the pentose phosphate pathway. Fructose can also be phosphorylated to F6P by HK, however, HK has a higher affinity for glucose [23]. In order for fructose to enter the pentose phosphate pathway, F6P would need to be converted to G6P via phosphohexose isomerase (PGI). In the present study, the production of detectable $^{14}CO_2$ via the pentose phosphate pathway was greater for conceptuses in medium containing ^{14}C-glucose as compared to ^{14}C-fructose. Adding unlabeled glucose to medium with ^{14}C-fructose decreased the ability of the conceptuses to metabolize fructose via the pentose phosphate pathway, as glucose was the preferred substrate.

Interestingly, there was an increase in production of $^{14}CO_2$ by conceptuses in medium containing ^{14}C-glucose, unlabeled glucose, and unlabeled fructose compared to the other treatments. With fructose in the medium to compete with glucose for the active site on HK, it was hypothesized that production of $^{14}CO_2$ would be less than that for medium containing only glucose. One explanation could be that in the conceptus, fructose or F6P allosterically activates the first enzyme of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PDH), and thus increases the activity of this metabolic pathway. This a potential topic for future research.

Of particular interest in this study was the question of whether ^{14}C-fructose and unlabeled fructose could be metabolized via the TCA cycle and pentose phosphate pathways. Production of $^{14}CO_2$ from metabolism of radiolabeled fructose indicated that, in the absence of glucose, the

Day 16 conceptuses metabolized fructose via the pentose phosphate pathway. This is a novel finding as the role of fructose is currently overlooked and poorly understood since glucose is the preferred metabolic substrate for oxidative glycolysis and aerobic glycolysis.

Individual contributions of glucose and fructose were determined by analyzing Day 16 conceptus conditioned culture medium after tissue homogenization when there was not a competing hexose sugar. As expected, the metabolic activities of glucose contributing to the pentose phosphate pathway, TCA cycle, lipid synthesis, glycosylation of proteins, and pyruvate to lactate production were greater than for fructose.

Much as for Day 17 conceptus conditioned culture medium, there was a greater lactate to pyruvate ratio in medium in which Day 16 conceptuses were cultured with glucose only and fructose only, indicating that the reduction of pyruvate generated from glucose or fructose in ovine conceptuses to lactate is favored. Conceptuses cultured with either glucose or fructose had active TCA cycles and pentose phosphate pathways. Interestingly, there was a shift in metabolic pathway preference between glucose and fructose. Contributions of glucose to the TCA cycle and pentose phosphate pathway in the conceptuses were similar. In contrast, when fructose was the only available substrate for these pathways, TCA cycle activity was about 3-fold greater than pentose phosphate activity. This suggests that fructose metabolism via the pentose phosphate pathway is possible, but not the preferred metabolic pathway. This shift in metabolism by conceptuses further supports the hypothesis that the ovine conceptus can use fructose as a substrate for both the TCA cycle and pentose phosphate pathway.

Lipids have multiple roles in cell survival, including oxidation for ATP production, signaling pathways, and cell membrane maintenance [70]. Synthesis of lipids from glucose and fructose

was not detectable in the present study. Histotroph from the uterine endometrial glands is the main source of lipids for the conceptus during the peri-implantation period and de novo synthesis in the conceptus is low [6, 70–72]. The culture medium in this study did not have an exogenous source of substrate for synthesis of lipids by the conceptuses and the time in culture was only 2h.

Glycosylation of proteins in this study was greater if glucose was the available substrate in the medium. However, there was detectable utilization of fructose for glycosylation of proteins if it was the only available substrate. Entry of fructose into the hexosamine biosynthesis pathway requires phosphorylation via HK to produce F6P, which can then directly react with glutamine via glutamine:F6P aminotransferase (GFAT) to proceed through the hexosamine biosynthesis pathway to produce UDP-GlcNAc for glycosylation of proteins as well as glycosaminoglycans, such as hyaluronic acid [29, 42].

While glucose the preferred metabolic substrate for the ovine conceptuses during the peri-implantation period of pregnancy, results of this study indicate that fructose likely plays an important role as well. This is likely true in vivo as fructose is 11- to 33-fold more abundant than glucose in fetal blood and fetal fluids [38]. Fructose is a product of the polyol pathway, which is active in the trophectoderm, and is able to contribute to critical metabolic pathways required for development, growth, and survival of the conceptus. Based on available evidence, the conceptus does not likely survive on fructose alone, as metabolism of fructose is less than that for glucose. However, if glucose stores are low or depleted, the great abundance of fructose in the fetal fluids, particularly allantoic fluid, with increasing gestational age could support metabolic requirements of conceptuses in sheep and other species of ungulates and cetaceans.

CHAPTER IV

CONCLUSIONS

Ovine conceptus development during the peri-implantation period of pregnancy is extremely dynamic in both structural and metabolic changes. Elongation of the conceptus during this time requires sufficient nutrients provided by multiple metabolic pathways to support the increased cellular proliferation occurring, as well as survival, differentiation, and development. Glucose and fructose are both important substrates for multiple metabolic pathways. During gestation, glucose is converted to fructose by the ovine conceptus, resulting in a great abundance of fructose in fetal fluids [38] that can play a role to support conceptus development.

Analysis of the culture medium provided insights into the metabolic profiles of the conceptus. Lactate was more abundant in culture medium than pyruvate in all medium regardless of composition. Pyruvate produced from glycolysis may be either oxidized via glycolysis or reduced to lactate. While CO_2 production in the isotopic tracer studies indicated that the conceptus could produce pyruvate from metabolism of glucose or fructose, it is mostly reduced to lactate. Pyruvate produced from glucose was favored for entry into the TCA cycle. In contrast, pyruvate production from fructose favored reduction to lactate.

Of interest, protein glycosylation is important in conceptus development to stimulate cell signaling pathways. The conceptus could metabolize fructose in the absence of glucose and use it to glycosylate proteins. The ovine conceptus did not use either glucose or fructose in the culture medium to synthesize lipids, as lipids and precursors available for the conceptus to produce lipids originate from maternal endometrial gland secretions.

Conceptus tissue, at the end of the experiments, was subjected to acid-base neutralization. Thus, conceptus tissue was not available to determine relative expression of mRNAs and proteins. Culturing conceptus tissue or ovine trophectoderm cell lines in similar and physiological concentrations of glucose and fructose could provide insights into changes in gene expression and protein synthesis of key metabolic enzymes when fructose is the only available substrate. Experiments were also performed under normoxic conditions, however, it has been shown that the uterus is hypoxic before placentation and development of the vasculature is established [44]. A better understanding of utilization of glucose and fructose by the conceptus could be gained by performing the conceptus culture experiments under hypoxic conditions.

The role of fructose in conceptus metabolism is overlooked as the conceptus utilizes glucose at a higher rate [12]. The experiments in this study investigated the ability of ovine conceptus tissue to metabolize fructose. The results from the present research indicate that glucose was the preferred metabolic substrate for the conceptus. However, the conceptus can indeed metabolize fructose via the TCA cycle and pentose phosphate pathway, as well as glycosylate proteins.

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