INSULIN-LIKE GROWTH FACTOR-1 STIMULATES GLYCOGEN SYNTHESIS IN THE BOVINE UTERINE EPITHELIUM

BY

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THESIS

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ABSTRACT

Despite interventions and synchronization methods, early embryonic loss in cattle is 30-50%. Embryo development is dependent on proper secretion of glucose into the uterine lumen. Post-conception, the embryo primarily uses pyruvate and lactate. Too much glucose at this stage impairs development. At the morula stage, glucose uptake starts to increase dramatically. However, it is unclear how the bovine uterine epithelium meets the increasing glucose needs of the embryo. Research from our laboratory has found that the epithelial cells in the bovine uterus contain higher levels of glycogen, the storage form of glucose, on day 1 than day 11 of the cycle. During estrus, estradiol (E2) stimulates the production of insulin-like growth factor 1 (IGF-1) in the uterine stroma of other species, which mediates some of the effects of E2 on the uterine epithelium. Therefore, our objective was to evaluate the effect of E2 and IGF-1 on glycogenesis in immortalized bovine uterine epithelial (BUTE) cells. Treatment of BUTE cells with E2 (0.1-10 nM) did not stimulate glycogen synthesis. In contrast, treatment of BUTE cells with IGF-1 (50 or 100 ng/ml) resulted in a >2-fold increase in glycogen levels ($P<0.01$). When treated with both E2 (100 nM) and IGF-1 (50 ng/ml), only the main effect of IGF-1 was significant ($P<0.0001$). We next determined if bovine uterine fibroblast (BFIB) cells produced IGF-1 in response to E2. Treatment of BFIB cells with E2 increased IGF-1 production, and immunohistochemistry revealed that expression of IGF-1 in the stroma was higher on day 1 than on day 11. This agrees with higher levels of glycogen in the epithelium on Day 1. Elucidating the pathway by which IGF-1 increases glycogen synthesis, western blots revealed an increase of approximately 7-fold for phospho-AKT after IGF-1 treatment ($P<0.05$) and an increase of >2-fold for phospho-GSK3β ($P<0.05$). IGF-1 treatment also increased levels of hexokinase and glycogen synthase proteins ($P<0.05$). Metabolomics (GC-MS) showed that IGF-1 increased 3-

phosphoglycerate, lactate, and N-acetyl-glucosamine in BUTE cells, suggesting increased glycolysis and hexosamine biosynthetic pathway activity. IGF-1 also resulted in increased levels of glycosylated proteins in BUTE cells ($P<0.01$). In conclusion, this study demonstrated that glycogen synthesis is the result of IGF-1 produced in the bovine endometrial stroma due to E2. IGF-1 stimulated glycogen synthesis via an AKT/GSK3β pathway and by increasing expression of glycogenic enzymes. Metabolomic (GC-MS) analysis indicated altered flux through glycolysis and the hexamine biosynthetic pathway. By increasing glycogen levels during estrus, the uterine epithelium may be better able to provide glucose to the developing blastocyst. Identifying mechanisms controlling glycogen synthesis and catabolism in the bovine uterus may enable further research toward decreasing early embryonic death in cattle and global revenue loss.

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I would like to dedicate my thesis to my younger self and to my cousins Nevaeh and Eric Jr.

This is proof that Dyslexia is not an indicator of intelligence or something that defines you.

Never let how you learn change, determine, or limit, what you do in life.

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LIST OF ABBREVIATIONS

α: alpha

AI: artificial insemination

AKR1B1: aldo-keto reductase family 1 member B1

ATP: adenosine 5’-triphosphate

β: beta

BCA: bicinchoninic acid assay

BFIB: bovine uterine fibroblast

BSA: bovine serum albumin

BUTE: bovine uterine epithelial

CL: corpus luteum

Coomassie: Coomassie Brilliant Blue

DAB: 3,3’-Diaminobenzidine

DMI: dry matter intake

DMSO: dimethyl sulfoxide

E2: estradiol

EDTA: ethylenediaminetetraacetic acid

EGF: epidermal growth factor

EI: electron impact

ER: estrogen receptor

ERα: estrogen receptor alpha

ERβ: estrogen receptor beta

FBS: fetal bovine serum

FSH: follicle-stimulating hormone

g: gram

G1P: glucose-1-phosphate

G6P: glucose-6-phosphate

G6PC: glucose-6-phosphatase

GC-MS: gas chromatography–mass spectrometry

GE: glandular epithelium

GFP: anti-green fluorescent protein

GLUT: facilitative glucose transporter

GSK3β: glycogen synthase kinase 3 beta

GYS: glycogen synthase

HBP: hexosamine biosynthetic pathway

HK: hexokinase

IFNT: interferon-tau

IGF-1: insulin-like growth factor 1

IGF-1R: insulin-like growth factor 1 receptor

IGFBP: insulin-growth factor binding protein

IHC: immunohistochemistry

IRS: insulin receptor

ITS: insulin transferrin selenous acid

IVF: in vitro fertilization

KEGG: Kyoto Encyclopedia of Genes and Genomes

KOH: potassium hydroxide

LE: luminal epithelium

LH: luteinizing hormone

mg: milligram

MK2206: hydrochloride

ml: milliliter

mM: millimolar

mRNA: messenger RNA

n.d.: no date

Na^+: sodium ion

NADPH: nicotinamide adenine dinucleotide phosphate

ND: non-detectable

ng: nanogram

nM: nanomolar

NVP: NVP-ADW742

P4: progesterone

pAKT: phosphorylated AKT

PAS: periodic acid Schiff

PBS: phosphate buffered saline

PGF: uterine prostaglandin

PGF2α: prostaglandin F2 alpha

PGM: phosphoglucomutase

pGSK3B: phosphorylated glycogen synthase kinase 3 beta

pGYS: phosphorylated glycogen synthase

PPP: pentose phosphate pathway

PPPT: picropodophyllotoxin

PR: progesterone receptor

PR-A: progesterone receptor alpha

PR-B: progesterone receptor beta

PVDF: polyvinylidene difluoride

PYG: glycogen phosphorylase

RIPA: radioimmunoprecipitation assay buffer

RTK: receptor tyrosine kinase

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SGLT: sodium-glucose linked transporters

SORD: sorbitol dehydrogenase

TBS: tris buffer saline

TBS-T: tris buffer saline with tween

TCA: tricarboxylic acid

UDP: uridine diphosphate

UDP-GlcNAc: uridine diphosphate N-acetylglucosamine

μg: microgram

μl: microliter

μM: micromolar

UN: United Nations

US: United States

UTP: uridine 5'-triphosphate

WB: western blot

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CHAPTER 1: INTRODUCTION

Cattle in the Global Economy

Cattle are one of the most valuable livestock species in animal agriculture. Not only do they produce high-quality protein from forage, but they produce numerous animal byproducts and provide work in developing countries. In 2020, there were 12 million head of cattle on feed in the US, producing 27.2 billion pounds of beef, 223.2 billion pounds of milk, valued at $86.6 billion dollars (USDA 2020, USDA, 2021b; USDA 2021a; USDA 2021c). The US produces 18% of the world's beef, using only 6% of the world's cattle (Beef Research, 2021). It is not a question of whether or not cattle are a highly sought-after commodity but the ability of producers to meet global demand. Producers are faced with the challenge of producing more product with less land at an unprecedented rate. Not only are more people around the world eating beef, but the number of people in the world also continues to increase. The UN predicts that the world population will reach 9.7 billion by 2050 and nearly 11 billion by 2100 (Nations, n.d). In order to meet these growing global demands, efficient reproduction in cattle is imperative. In 2020, it was estimated US beef and dairy products contributed to $856.1 billion in global revenue, but embryonic loss was responsible for over $1 billion in US revenue losses (Statistica, 2022; Kincheloe, 2020). Decreasing embryonic mortality is one approach the help meet the increasing global demand.

Conception & Pregnancy Rates

In cattle, conception rates tend to be over 90%; however, rates of successful pregnancies range between 40-60% (Diskin & Morris, 2008). Previous research suggests that many factors affect reproductive performance and embryonic loss in cattle, both biological and environmental.

Biological challenges of a successful pregnancy include ovarian dysfunction, poor embryo attachment, lack of maternal recognition of pregnancy, low body condition, lactation, and infection.

Embryonic mortality can be divided into two categories: early and late loss. Early embryonic loss occurs in cows on or before day 24, while late embryonic loss occurs between days 24 and 42-50 (Santos *et al.*, 2004). Within this period, embryonic death appears to be the highest within the first 3 weeks of gestation, with some reports attributing the greatest losses to the first week, particularly for high-producing dairy cattle. Multiple reports have identified that early embryonic death accounts for most loss that occurs during gestation (Sartori *et al.*, 2002). However, the economic consequences of late embryonic loss are reported to be disproportionately greater because late embryonic loss causes a delay in return to estrus (Silke *et al.*, 2002). Pregnancy losses after day 50 of gestation are considered fetal loss. Better understanding of uterine receptivity and conceptus development in cattle may advance the discovery of strategies to improve reproductive efficiency, minimize embryonic losses, and increase the number of live births.

CHAPTER 2: LITERATURE REVIEW

Bovine Reproduction

Anatomical and Cyclic Characteristics

The major structures of the bovine female reproductive tract include the ovaries, oviducts, uterus, cervix, vagina, and vulva. The reproductive tract lies ventral to the rectum and is separated from it by the recto-genital pouch. The female tract begins with the ovary, a dense ovoid structure that primarily produces female gametes, the ovarian hormones estradiol (E2) and progesterone (P4), and the corpus luteum. The oviduct consists of the infundibulum, ampulla, and isthmus. It is the site where fertilization occurs and transports the newly ovulated oocyte from the ovary to the uterus. The uterus connects the oviducts to the cervix. Cattle have a bicornuate uterus that is characterized by having two uterine horns and a small uterine body (Senger, 2004). The tissues that make up the uterus are the perimetrium, myometrium, and endometrium. The perimetrium, also known as the serosa, is the outer layer of cells that cover the surface of the reproductive tract. Next is the myometrium, also known as the muscularis, a double layer of smooth muscle consisting of an outer longitudinal layer and inner circular layer. The purpose is to provide the uterus with the ability to contract. The contractions are essential for transporting secretory products, gametes, and early embryos. Lastly, the submucosa and mucosa make up the endometrium. The submucosa houses blood vessels, nerves, and lymphatics, while the mucosa is lined with secretory cells. The endometrium is responsible for secreting materials into the lumen of the uterus to enhance embryo development and sperm viability. It contains many different types of cells (epithelial, stromal, immune, endothelium), with the epithelium itself comprised of two distinct cell types: luminal epithelium (LE) and glandular epithelium

(GE) (Kelleher *et al.*, 2019). Caruncles are button-like thickened portions of the endometrium. These regions are highly vascularized and will give rise to the maternal contribution to the placentome if pregnancy occurs. The primary function of the uterus is sperm transport, control of cyclicity, environment for embryo attachment, maternal contribution to the placenta, and expulsion of the fetus and placenta (Senger, 2004). After the uterus is the cervix, which provides lubrication, a flushing system, and a barrier during pregnancy. It is a relatively thick-walled organ that serves as a barrier to sperm transport and isolates the uterus from the external environment during pregnancy. The primary function of the vagina is to serve as a copulatory organ and birth canal. Finally, the vulva is the external part of the female reproductive tract. It consists of two labia that meet in the medial portion of the tract to form sites of union. The labia form a closure that minimizes the entrance of foreign material into the vagina.

Cattle are polyestrous ruminant animals with an average cycle length of 21 days. Estrus occurs 12-18 h before ovulation. There are two phases of the estrous cycle, the follicular and luteal phase. Hendricks *et al.* (1971) found that estrogen concentrations the day before estrus ranged from 15-25 pg/ml but began to decline within 2-5 hours after the onset of estrus. That same study also found that progesterone levels were highest (5-12 ng/ml) on the fourth day prior to estrus, then decreased to nearly non-detectable level the day prior to estrus. E2 and P4 are synthesized from cholesterol in the ovary and can diffuse into a target cell to act on their receptors. The estrogen receptors, ERα and ERβ, and the progesterone receptors, PR-A and PR-B, are ligand-activated transcription factors in the cytoplasm. Hormone binding activates the receptor, promoting receptor dimerization and translocation to the nucleus (Hantak *et al.*, 2014). Activated ER and PR bind to specific genomic sites, termed response elements, to activate or repress the expression of their target genes. Ovarian steroids from pre-ovulatory follicles and

corpus luteum exert classical endocrine control in the endometrium that affects embryo development and pregnancy success (Forde *et al.,* 2009a, Forde *et al.,* 2009b, Mesquita *et al.,* 2015).

During the follicular phase, E2 is the dominant ovarian hormone, and it is primarily produced by antral follicles. The follicular phase is characterized by follicular development, regulation of the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and ovulation. The role of E2 during the cycle is to aid in follicle growth and development, transition into estrus, and stimulate the release of LH and FSH, resulting in ovulation. In the uterus, E2 stimulates the proliferation of endometrial cells. E2 has been reported to induce the expression of various receptors, uterine prostaglandin $F_{2\alpha}$ (PGF2α) production, and many genes involved in uterine extracellular matrix remodeling necessary for embryo growth and a successful pregnancy (Bartol *et al.,* 1981).

During the luteal phase, P4 is the dominant ovarian hormone produced by the corpus luteum (CL). If the female does not become pregnant, the bovine endometrium will produce PGF2α, lysing the CL and resetting the cycle (Senger, 2004). During the luteal phase, the CL forms and produces P4. Adequate P4 secretion is necessary for stimulating endometrial secretions, embryo growth and development, and maintenance of pregnancy by altering endometrial gene expression (Garrett *et al.,* 1988). P4 drives changes in the expression of genes by the endometrial epithelium (LE and GE) that modulate transport of ions, amino acids, glucose, and lipids from serum as well as produce proteins, extracellular vesicles, and other substances (Spencer *et al.,* 2016; Bazer, 1975). Thus, E2 and P4 are important regulators of ovulation, changes in uterine cell morphology, and reproductive capacity.

Events in Early Pregnancy

Embryo development begins in the oviduct, where the egg is fertilized. The newly formed zygote will begin developing, which includes cleavage, compaction, blastulation, expansion, hatching, and elongation (Peippo *et al.*, 2011). Cleavage occurs in the oviduct as the embryo moves toward the uterus. At this stage of development, the embryo preferentially utilize pyruvate and lactate, with glucose becoming the predominant energy source during compaction (Gardner *et al.*, 2011). The bovine embryo then enters the uterus as an early morula approximately 4-5 days post ovulation (Hackett *et al.*, 1993). Around day 6, during the morula stage, the cells of the embryo change shape their shape in a process called compaction, forming a compact ball of cells. Blastulation is believed to occur around day 6-7 (Holm *et al.*, 1998); this leads to the formation of a cavity in the embryo called a blastocoel. Expansion is the increase in diameter of the embryo that results in the thinning of the zona pellcudia beginning around day 7-8 (Peippo *et al.*, 2011). By day 7, the embryo becomes a blastocyst consisting of an inner cell mass that will give rise to the fetus and the trophectoderm, which will form the fetal portion of the placenta (Lonergan *et al.*, 2016). After expansion, the blastocyst hatches from the zona pellucida between days 7.5-11 (Brandão *et al.*, 2004). Once the blastocyst has hatched, it has undergone the process of blastocyst activation, in which the blastocyst acquires implantation competency and attaches to the receptive uterine lumen epithelium (González *et al.*, 2012). Around day 12, the conceptus elongates from a spherical blastocyst to ovoid, tubular, and then filamentous form (Degrelle *et al.*, 2005). The elongated conceptus begins to secrete interferon-tau (IFNT), the maternal recognition of pregnancy in ruminants (Bazer, 2013). It is well established that blastocysts can be produced entirely *in vitro*. However, for elongation to occur, it must be transferred to a receptive uterus to grow and develop (Lonergan *et al.*, 2019). Ramos-Ibeas *et al.* (2022) recently

developed an *in vitro* post-hatching system that allowed sheep embryos to be produced *in vitro* until day 14, around early gastrulation but they did not elongate. Thus, elongation is a complex process requiring interaction with a hormonally primed uterus.

Uterine glands and their secretions have fundamental biological roles in mammalian pregnancy. The bovine endometrium consists of glandular (intercaruncular) and aglandular (caruncular) regions; caruncles are the site of placentation (Wooding, 1992). Within the intercaruncular regions, the endometrium is highly secretory and consists mainly of stroma fibroblast (SF) cells and LE and GE cells (Chaney *et al.,* 2021). The uterine LE cells are separated from the underlying SF by a basement membrane and are continuous with the GE that penetrates down into the stroma forming uterine glands (Chaney *et al.,* 2021; Kelleher *et al.,* 2019). The GE can then secrete proteins and other substances that primarily act on the stroma, immune cells, vascular endothelium, as well as impact embryo and placental development. Uterine glands are involved in the transport of nutrients into the uterine lumen to support conceptus development during the pre-and peri-implantation of pregnancy (Lonergan *et al.,* 2019). Studies on sheep and mice devoid of uterine glands provide direct evidence that they are required for conceptus growth and pregnancy success (Gray *et al.,* 2002; Kelleher *et al.,* 2016).

Embryo survival is highly dependent on the nutrients provided by the maternal uterine environment. Uterine glands directly synthesize and secrete or selectively transport from serum a wide variety of substances, collectively termed the histotroph (Bazer, 1975). Histotroph is complex and comprised of many different substances, including ions, sugars (primarily glucose and fructose), amino acids, extracellular vesicles, lipids, and proteins (Kelleher *et al.,* 2019). Uterine gland secretions have long been implicated in uterine receptivity and blastocyst implantation in various species. Secretions such as PGF2α and glucose play critical roles in the

embryo-maternal interaction. Adequate glucose uptake and metabolism are essential for both the endometrium and embryo. Gao *et al.* (2009a) demonstrated that amount of glucose and arginine increase in the uterine lumen of sheep between days 10 and 16 of pregnancy. Further research found that glucose and arginine stimulate proliferation, migration, and gene transcription in conceptus trophectoderm cells (Kim *et al.,* 2011; Bazer *et al.,* 2012). Fructose may also be a key component of the histotroph. The endometrium in pigs contains the enzymes necessary to convert glucose to fructose (Steinhauser *et al.,* 2016), suggesting that fructose in the uterine lumen could have originated as maternal glucose. Bovine embryos can utilize fructose as an energy source in place of glucose, which is present in the uterine fluid of cattle (Suga and Masaki, 1973; Guyader-Joly *et al.,* 1996). Fructose concentrations are higher in fetuses of species with synepitheliochorial placentas (Goodwin, 1956). In sheep, uterine luminal fluids contain known mitogens, such as IGF-1, LIF, and other factors that support conceptus elongation (Ko *et al.,* 1991). Ultimately, these components of the histotroph function as a source of communication and nourishment during early pregnancy.

The conceptus must produce and secrete IFNT, which acts on the uterus to maintain a functional CL for P4 production during early pregnancy. IFNT stimulates activation of IRF2 within the uterine surface epithelium, silencing E2 and oxytocin receptors (Spencer & Bazer, 2002). Without an embryo, the increased presence of oxytocin receptors triggers an increase in PGF2α production, resulting in luteolysis. The bovine epithelial and stromal cells respond to IFNT by increasing the expression of IFNT stimulated genes (ISGs) (Chaney *et al.,* 2021). ISGs block the secretion of PGF2α from the uterine epithelium, inhibiting the regression of the CL and maintains pregnancy (Spencer & Bazer, 2002). Therefore, IFNT indirectly rescues the CL and supports early embryo development.

IFNT acts on the uterus in a multitude of ways. IFNT influences uterine gene expression, which prepares the uterus for placental attachment and modifies the uterine immune system to protect the fetus from maternal immune attack (Chen *et al.,* 2006; Spencer *et al.,* 1998; Bazer, 2013). In sheep, intrauterine IFNT infusions elicited gland hypertrophy, increased their secretory function (Spencer & Bazer, 2004) and uterine fluid glucose concentrations (Dorniak *et al.,* 2012). By increasing uterine fluid glucose concentrations, the uterus provides the embryo with an energy source before implantation occurs. Studies have shown that the embryo elicits changes in the bovine endometrium on day 16 and IFNT concentrations increased from day 14 to day 18 (Lonergan & Forde, 2014; Robinson *et al.,* 2006). Increased secretion of IFNT led to maternal recognition of the embryo, which was positively correlated with embryo size. It is important to note that until day 20, there is no anatomical union between the conceptus and the endometrium; thus, signaling molecules, such as IFNT, play a critical role in establishing the first embryo-maternal communication (Sponchiado *et al.,* 2020). Without adequate IFNT concentrations, the embryo will not be able to effectively signal to the endometrium, resulting in early embryonic loss.

The most crucial event leading to a successful pregnancy is ‘implantation,’ the process by which the embryo implants or inserts itself into the endometrium (Peippo *et al.,* 2011). While there are various forms of implantation, in ruminants, implantation is typically described as attachment. Therein, the trophectoderm attaches to the endometrial LE (Lonergan *et al.,* 2016). For the conceptus to be able to attach, the uterus must be in a receptive state. Prior to attachment, the LE undergoes steroid hormone-induced structural and functional changes (Hantak *et al.,* 2014). For instance, the loss in polarity from down-regulation of the cell-to-cell adhesion molecule E-cadherin (Thie *et al.,* 1995) and development of caruncles for embryo attachment. As

the uterus attains receptivity, the trophectoderm layer of the embryo attaches to the uterine LE (Ramathal *et al.,* 2010). The placenta is formed from the trophoblast cells of the developing embryo, and it is the point of contact between the uterus and embryo. Specifically, cattle have a syndesmochorial placenta. It is characterized by numerous button-like structures distributed across the chorion, called fetal cotyledons. The placenta attaches to the endometrium via the fusion of endometrial caruncles and fetal cotyledons to form the placentome. Senger (2004) estimated that 95% of blood flow through the uterus goes through these placentomes and serves as the fetal-maternal interface exchange.

Causes of Pregnancy Failure

While there are many causes of pregnancy failure in cattle, this literature review focuses on identifying those associated with early embryonic loss. Embryo mortality can be attributed to two main influences: low uterine environmental quality or inherent embryo viability (McMillan, 1996; Berg *et al.*, 2010). One contributor to early embryonic loss appears to be associated with the ability of the uterus to provide enough glucose to the developing embryo and/or the embryo's ability to uptake glucose at the blastocyst stage. Other known contributors of embryo mortality are linked to inadequate ovarian support, inadequate uterine support, embryo abnormalities, and embryo-maternal crosstalk.

Inadequate Ovarian Support

In many species, inadequate ovarian support is primarily associated with low levels of P4. P4 is one of the main hormones responsible for establishing pregnancy; P4 stimulates the

production of endometrial secretions that are beneficial for the successful development of the embryo and subsequent survival (Graham & Clarke, 1997; Geisert *et al.*, 1992). In cattle, high P4 concentrations during the luteal phase delayed endometrial gland morphology, which altered the uterine response to oxytocin (Shaham-Albalancy *et al.*, 1997). Increased release of PGF2α would encourage luteolysis and result in early embryonic loss. P4 has also been shown to stimulate IFNT production, necessary for embryo development. During AI and synchronization studies, approximately 30% of the cows lacked a CL at the initiation of the synchronization (Fricke *et al.*, 2003; Vasconcelos *et al.*, 1999). These studies revealed an increased occurrence of asynchronous ovulation in cows lacking CL's and consequently lower pregnancy rates. Therefore, adequate P4 during development of the ovulatory follicle has been linked to failure to prevent luteolysis, poor embryo development, and pregnancy failure (Mann & Lamming, 2001; Mann *et al.*, 1999).

When P4 levels are within optimal range or supplemented to meet the desired threshold, overall fertility and embryonic development improve. Concentrations of P4 during the development of the ovulatory follicle are approximately 3.0 ng/mL greater in cows with a developed CL compared with anovular cows or estrous cyclic cows not in diestrus (Cerri *et al.*, 2009). This study supports the idea that higher P4 concentrations support ovarian function and cyclicity. Shaham-Albalancy *et al.* (1997) reported changes in uterine gland morphology and vascularization in response to P4 supplementation, both of which influence embryo development. Bisinotto *et al.* (2015) and Garrett *et al.* (1988) further demonstrated that P4 supplementation during the growth of the ovulatory follicle improved fertility responses in lactating dairy, enhance conceptus development, and reduce the risk of pregnancy loss. Among

many reasons for early embryonic loss, adequate P4 concentrations are essential in preventing early pregnancy failure.

Inadequate Uterine Support

Conversely, another potential cause of pregnancy failure may be inadequate uterine support. Maternal factors such as the inability of the uterus to support growth and implantation have been observed in natural and assisted pregnancies (Spencer *et al.*, 2017). Uterine support involves the uterus' capacity to serve as a biological sensor to assess embryo quality. Rodriguez-Alonso *et al.* (2020) and Bauersachs *et al.* (2009) showed that endometrial response is based on embryo quality, which may reflect embryo viability. One theory of inadequate uterine support is the ability of the endometrium to uptake glucose. Studies in mice revealed that sufficient glucose uptake and metabolism are essential for the proper differentiation of the uterine endometrium toward a receptive state capable of supporting embryo implantation (Frolova & Moley, 2011). Although the bovine endometrium does not decidualize, it is possible that endometrial glucose uptake could contribute to uterine receptivity.

Alternatively, pregnancy failure may be linked to the uterus' ability to provide adequate glucose concentrations to the developing embryo. It is well known that glucose is required for early embryo development. As a significant source of energy used by the conceptus for growth and development, glucose must be adequately transferred from the maternal circulation (Simpson *et al.*, 2008). Transporters for glucose and amino acids expressed in the endometrium may influence uterine lumen fluid homeostasis and uterine receptivity required for blastocyst implantation (Kelleher *et al.*, 2016).

Abnormalities of the Embryo

Although there are several causes of pregnancy failure in cattle, embryo abnormalities cannot be overlooked. Drost *et al.* (1999) hypothesized that failure of the uterus to establish and maintain pregnancy might be due to poor-quality oocytes or embryos. For example, Rizos *et al.* (2002) demonstrated that blastocyst quality is determined between the zygote and blastocyst stage. This study argues that part of the ability to elongate is intrinsic to the embryo and likely related to the oocyte/embryo quality, suggesting that the oocyte regulates developmental competence. Defects of the embryo are multi-faceted and may serve as a deciding factor of viability.

According to Sartori *et al.* (2002), a significant proportion of embryos degenerated before the blastocyst stage in heat stressed high-producing dairy cows, implying that failure to establish pregnancy may be due to poor quality oocyte or embryo. If this is true, the question then is what portion and why before the blastocyst stage? Stringfellow and Seidel (1998) demonstrated that bovine embryos primarily use pyruvate and lactate to grow and survive on day 5 and glucose beginning on day 6. In Wiebold's (1988) study, it was observed that most embryonic mortality occurred before Day 5. Perhaps the inability of the embryo to uptake glucose contributes to pregnancy failure. Gardner and Leese (1987) examined the relationship between glucose uptake and viability of individual mouse blastocysts. They discovered a positive correlation between glucose uptake by individual mouse blastocysts and subsequent viability. Thus, embryos that lacked the ability to uptake glucose would likely lead to lower pregnancy rates compared to embryos that were able to uptake more glucose. A study in cattle investigated embryo nutrient uptake and post-transfer viability, specifically examining glucose uptake in blastocysts by Day 10 (Renard *et al.* 1980). This study also established a positive relationship between glucose

uptake and subsequent pregnancy. The embryo's ability to uptake glucose is one of many factors that enhances to its development and viability.

Embryo-Maternal Crosstalk

An important aspect of uterine biology is the crosstalk between the developing embryo and endometrium during early pregnancy. Sponchiado *et al.* (2020) illustrated that the bovine embryo can elicit responses to endometrial, oviductal, luteal, and immune cells. Comparison of endometrial responses to bovine conceptuses produced by somatic cell nuclear transfer, IVF, or AI suggest that pregnancy failure in bovine pregnancies may originate from abnormal embryo–maternal crosstalk during the peri-implantation period (day 18–20) (Bauersachs *et al.*, 2009). Moraes *et al.* (2018) illustrated differences in genes regulating the endometrial extracellular matrix of heifers classified as high-fertile and sub-fertile may lead to loss. Thus, the uterus plays a central role in establishing a successful pregnancy via embryo-maternal communication. Embryo-maternal communication is coordinated by the expression of paracrine factors regulated by the steroid hormones E2 and P4 (Hantak *et al.*, 2014). Dysregulation of this crosstalk, such as the inability of stromal factors to regulate epithelial function, often leads to pregnancy loss and infertility (Hantak *et al.*, 2014). These studies support the notion that oocyte quality, uterus receptivity, and crosstalk impact embryo survival. Inadequate communication between the early bovine conceptus and endometrium is believed to contribute to early embryonic mortality.

Role of Glucose in Early Pregnancy

Embryo Development

Before implantation, embryos are reliant on glucose secreted into the uterine lumen for survival. Pyruvate, lactate, and glutamine are preferred to glucose during the early cleavage stages (Rieger *et al.*, 1992). Gardner (1998) performed *in vitro* studies that revealed that glucose uptake increases slowly and becomes the main energy source as the morula develops into a blastocyst. In sheep embryos, glucose uptake was low until the 16-cell stage, when it began to increase rapidly. On day 6, the morula increases glucose uptake dramatically. By day 7, the blastocyst will die if its growing needs for glucose are not met. Blastocysts utilize large amounts of glucose to produce ATP and other substrates necessary for cell proliferation and growth (Gardner *et al.*, 1996).

The sex of an embryo determines the amount of glucose it takes up. There were differences in glucose uptake between male and female mouse embryos (Gardner & Leese, 1987), and bovine embryos (Sturmey *et al.*, 2010). The observed sex-related difference in glucose uptake is consistent with other studies on human embryos (Picton *et al.*, 2010). Female embryos consumed significantly more glucose than their male counterparts did on day 4 of development. These studies demonstrate that glucose is imperative to blastocyst development.

Glucose Concentrations & Availability

The concentration and availability of glucose in the endometrium directly affects fertility. Greater blood glucose concentrations at weeks 3 and 4 postpartum have been associated with shorter days to pregnancy in dairy cows (Cardoso *et al.*, 2013). Therefore, it is crucial to know the optimal range of glucose concentrations for embryo development to occur.

Normal physiological glucose levels in the lumen of the reproductive tract have been measured in various studies. In cattle, Hugentober *et al.* (2008) reported glucose concentrations of 1-3 mM in the oviduct, 3-5 mM in the uterine lumen, and 6-7 mM for plasma. In sheep, glucose concentration inside the uterine lumen ranged between 0.05 and 0.23 mM, (Wales, 1973), while serum concentrations range from 4 to 5 mM (Francis *et al.,* 1999). Collectively, glucose levels within the reproductive tract and plasma demonstrate that glucose is tightly regulated to meet the needs of the developing blastocyst.

Varying concentrations of glucose affect embryos differently. Some studies have shown that glucose concentrations as low as 0.2 mM support maximal development to the blastocyst stage in mouse and livestock (Fraser *et al.*, 2007; Cagnone *et al.,* 2012). In cattle, 5 mM of glucose reduced the percentage of embryos reaching the blastocyst stage, and 4 mM of glucose skewed the sex ratio toward males (Green *et al.,* 2016; Kimura *et al.,* 2005). Others found that increased glucose concentrations in mice (Scott-Drechsel *et al.,* 2012) and human (Ornoy *et al.,* 2015) embryos resulted in developmental delays and embryo death. The same is true when there is not enough glucose. These studies indicate that glucose concentrations in the lumen of the reproductive tract are actively regulated and must be maintained within a narrow range for embryo survival.

Maternal Hyperglycemia

Given glucose's implications on uterine fertility, one solution might be to administer glucose exogenously. However, infusions of exogenous glucose elevated blood glucose concentrations and impaired embryo development in cattle (Leane *et al.,* 2018). In humans, it has been established that women with obesity and type 2 diabetes generally present with

hyperglycemia and have a lower likelihood of conception and a greater chance of pregnancy loss (Poston *et al.*, 2016). So, it is plausible that hyperglycemia during early pregnancy is detrimental to the developing embryo and a potential cause of early pregnancy loss. Leane *et al.* (2018) also found that exogenous glucose infusions did not affect DMI, milk production, CL development, circulating P4 concentrations, uterine lumen glucose concentrations, or the mRNA abundance of glucose transporters in endometrial tissue. Additionally, Moraes *et al.* (2020) illustrated no correlation between plasma glucose concentration and uterine fluid glucose concentrations. These results suggest that maternal hyperglycemia may negatively impact pregnancy rates.

Glucose Metabolism

Formation of Glucose

Glucose is the most abundant monosaccharide in the body; its main function is to serve as fuel to provide energy when oxidized. Circulating glucose is primarily derived from intestinal absorption or gluconeogenesis, the formation of glucose from pyruvate or lactate (Aronoff *et al.*, 2004; Blanco & Blanco, 2017). Another source of circulating glucose stem from the hepatic process of glycogenolysis, the breakdown of glycogen (Aronoff *et al.*, 2004). After a meal, carbohydrates are converted into monosaccharides that are absorbed and transported to the liver (Blanco & Blanco, 2017). The liver is the primary organ for glucose metabolism that takes circulating glucose and transiently stores it by assembling a polymer of glycogen. In ruminants, glucose is primarily derived from propionate via gluconeogenesis in the liver.

Potential Fates of Glucose

Glucose is a critical molecule in several metabolic pathways (Liver Physiology and Energy Metabolism, 2015). After glucose enters the cell, it is phosphorylated by hexokinase to form glucose-6-phosphate (G6P). G6P is an important metabolite capable of entering all pathways that use glucose (Blanco & Blanco, 2017). These pathways are depicted in Supplemental Figure 3.

The metabolic pathway that a single glucose molecule may take depends on the needs of the cell. For example, when there is an excess of glucose, it can be stored as glycogen (glycogenesis) (Engelking, 2015). Glucose can also enter one of the following catabolic pathways: glycolysis, pentose phosphate pathway (PPP), or the hexosamine biosynthetic pathway (HBP). Glycolysis is the catabolism of glucose into pyruvate and lactate, which produces ATP. The PPP leads to glucose oxidation. It serves as a way to generate nicotinamide adenine dinucleotide phosphate (NADPH), pentoses (5-carbon sugars), and ribose 5-phosphate, a precursor for the synthesis of nucleotides (Liver Physiology and Energy Metabolism, 2015; Alfarouk *et al.*, 2020). Lastly, the HBP is responsible for producing UDP-GlcNAc, a key substrate for protein glycosylation (de Queiroz *et al.*, 2019). Together, these pathways demonstrate the potential fates of glucose.

Glucose Transporters: GLUTs

Glucose uptake into cells occurs via glucose transporters, which can be either facilitative glucose transporters (GLUTs, now known as the *SLC2A* gene family) or by sodium glucose linked transporters (SGLTs, now known as the *SLC5A* gene family) (Frolova & Moley, 2011). GLUTs are responsible for glucose transport across cellular membranes via facilitative diffusion.

There are 14 known GLUTs. GLUT1 (*SLC2A*1) is a transporter that is expressed in nearly all cells of the body. It is responsible for basal glucose uptake in most cells (Northrop *et al.*, 2018) and was the first facilitative GLUT discovered with a high affinity for glucose (Mueckler *et al.*, 1985; Mueckler, 1994). GLUT2 is highly expressed in the intestine and pancreas. GLUT2 has a large capacity for glucose transport, making it an efficient mechanism for glucose absorption (Blanco & Blanco, 2017). GLUT3 is a major transporter found in the brain and peripheral nerves. The expression of GLUT3 is prevalent in tissues that have a high demand for glucose, such as the brain, placenta, preimplantation embryos, and some cancers (Pantaleon *et al.* 1997). The affinity of GLUT3 for glucose is higher than GLUTs 1, 2, and 4, making it an effective facilitator of glucose uptake (Frolova & Moley, 2011). GLUT4 has a relatively low affinity for glucose compared to other GLUTs. GLUT4 moves from the intracellular membrane to the cell surface when stimulated by insulin. It is the most studied transporter due to its role in whole-body glucose homeostasis and type II diabetes mellitus (Thorens & Mueckler, 2010).

Of the 14 known GLUTs, only GLUT1, 3, 4, 6, 8, 9, 10, and 12 have been found in the endometrium of various species (Frolova and Moley, 2011). GLUT1 was the first GLUT identified in the endometrium and is the main GLUT responsible for glucose uptake in stromal cells. In fact, endometrial biopsies from women revealed that women with significantly lower GLUT1 protein levels experienced idiopathic infertility than compared to women with non-endometrial infertility (von Wolff *et al.* 2003). Thus, idiopathic infertility could be attributed to decreased glucose uptake by endometrial stromal cells. The GLUT1 protein is found in all three major cell types (LE, GE, and stroma) of the human and rodent endometria. Korgun *et al.* (2005) reported that GLUT1 protein was localized to the LE, the stroma, and the primary decidua in the pregnant rat uterus. During early pregnancy, the endometrium becomes secretory, providing the

developing embryo with nutrients necessary for development. Additionally, E2 treatment has been known to affect glucose utilization in the rat uterus (Welch & Gorski, 1999). They discovered that GLUT1 mRNA and protein increased 3- to 4-fold within 4–8 h after E2 administration. In sheep, abundances of endometrial GLUT1 ranged on different days of the estrous cycle and with pregnancy status (Gao *et al.*, 2009b). In cyclic ewes, GLUT1 expression increased between days 10 and 14 and decreased to day 16, while in pregnant ewes, expression increased between days 10 and 12, remained high through day 16, and decreased at day 20. Throughout various species, it is evident that GLUT1 plays an active role in transporting glucose into the uterus in preparation for pregnancy.

GLUT3 (SLC2A3) is another glucose transporter thought to support storing glycogen in the uterus and embryo. Ganguly *et al.* (2007) showed that mice lacking the SLC2A3 gene had restricted fetal growth and failed pregnancies. Suggesting that endometrial GLUT3 expression facilitates early pregnancy. IHC staining in humans revealed that GLUT3 was more abundant in the uterine stroma than the epithelium and expressed in trophoblasts (Hahn *et al.* 2001, Simpson *et al.* 2008). Brown *et al.* (2011) found that GLUT3 is only expressed in first-trimester trophoblast cells but not in the placenta in both mice and humans. Thus, GLUT3 mediates glucose transport to the developing embryo.

In cattle specifically, GLUTs 5, 7, 8, 9, and 12 are expressed in early pregnancy. Although it is unknown whether the abundance of GLUT5 changes during the estrous cycle, GLUT5 is the only known GLUT to solely transport fructose (Manolescu *et al.*, 2007; Crouse *et al.*, 2017); an important component of the histotroph. Like sheep, the bovine conceptus IFNT may increase the expression of GLUT5 within the endometrium (Bazer *et al.*, 2012; Mathew *et al.*, 2019). Cycling heifers with greater circulating concentrations of P4 have a greater

endometrial expression of GLUT7 (Forde *et al.*, 2014). The upregulation of endometrial transporters, such as GLUT7, may contribute to conceptus growth. The ER-specific GLUT8 localization could provide the glucose necessary for the glycosylation of proteins (Frolova & Moley, 2011).

Sodium-Glucose Linked Transporters: SGLTs

Sodium-glucose linked transporters (SGLTs) are located on the membrane of cells and allow for glucose to accumulate in the cell's cytosol (Blanco & Blanco, 2017). It is a secondary active transporter that uses the Na^+ gradient on the basal side of the membrane of cells (Blanco & Blanco, 2017). SGLTs are also present in the ruminant endometrium. The endometrium of sheep contains SGLT1, in which mRNA abundance increased from days 10-14 during the cycle (Gao *et al.*, 2009b). Cattle with high E2 had increased SGLT1 mRNA located in the GE (Northrop *et al.*, 2018).

Northrop *et al.* (2018) and Gao *et al.* (2009b) suggest that E2, P4, and IFNT most likely play a role in regulating endometrial levels of glucose transporters. An idea supported by the changes in protein levels throughout the cycle and pregnancy. Perhaps the collective effort of GLUTs and SGLTs reinforces the importance of glucose transport during early pregnancy. For example, IFNT increased the expression of glucose transporters (GLUT1 and SGLT1) in uterine epithelial cells (Bazer *et al.*, 2012). It has been shown in pregnant ewes that facilitative transporters (GLUT1, GLUT3, GLUT5) may transport glucose from the plasma across the basal and lateral membranes into endometrial epithelial cells, while sodium-dependent transporters (SGLT1) may facilitate glucose transport from endometrial epithelial cells across the apical membrane into the uterine luminal fluid between days 10 and 16 of gestation (Gao *et al.*, 2009b).

Both transporter types most likely work together to optimize glucose transport into the uterine lumen, which can be utilized for conceptus growth and development (Gao *et al.*, 2009b). Yet, the abundance of GLUTs and SGLTs is unlikely to entirely explain the dynamic changes in glucose metabolism and secretion by the endometrium.

Glycogen Metabolism

Formation of Glycogen

Although glycogenesis primarily takes place in the liver, glucose is taken up and stored by other tissues (Blanco & Blanco, 2017). The process of glycogen synthesis is activated when there is an excess of glucose (Blanco & Blanco, 2017). To be considered a glycogen molecule, at least ten glucose residues must be joined together. Glycogen is found in the cytosol of cells, and each molecule can contain up to 60,000 glucose residues (Engelking, 2015). For a glucose molecule to be stored as glycogen, it is converted to G6P, which is then isomerized by phosphoglucomutase (PGM) to glucose-1-phosphate (G1P). G1P is transferred to uridine 5'-triphosphate (UTP), releasing a phosphate and yielding uridine diphosphate (UDP) glucose. Next, glycogen synthase transfers the UDP-glucose molecule to a preexisting glycogen molecule, forming an α-1,4-glycosidic linkage. The preexisting glycogen molecule will begin to form a branch of 10 or more glucose residues joined by α-1,6-glycosidic linkages. The formation of a glycogen molecule is illustrated in Supplemental Figure 4A.

Glycogenin is a protein previously thought to be required to form a new glycogen molecule (Liver Physiology and Energy Metabolism, 2015). As a glycosyltransferase it would transfer glucose from UDP glucose to itself, yielding a linear chain of glycogen connected by α-

1,4-glycosidic linkages (Adeva-Andany *et al.*, 2016; Skurat *et al.*, 2006). Interestingly, new research has indicated that glycogenin may not be needed for glycogen formation. Testoni *et al.* (2017) discovered that glycogen synthesis still occurred in glycogenin knock-out mouse models. Visuttijai *et al.* (2020) showed that humans with inactivating glycogenin mutations were able to store glycogen in their muscles. These results contradict our previous understanding of glycogen synthesis and indicate that glycogenin is not necessarily needed to form glycogen molecules.

Glycogen Synthesis

Hexokinase is an enzyme that is present in all cells. Glycogen synthesis begins with phosphorylation of glucose by hexokinase, which yields G6P. There are four hexokinases. Hexokinase I-III are inhibited by G6P, a product of their reaction. Hexokinase IV, also known as glucokinase, found exclusively in the liver and pancreatic β cells (Blanco & Blanco, 2017). Unlike hexokinases I-III, glucokinase is not inhibited by G6P. The biological properties of hexokinases I-III allow continued utilization of glucose and a permanent supply of energy to the cells even when blood glucose levels vary (Blanco & Blanco, 2017). Glucokinase is only active when blood levels are significantly high. Hexokinase activity increases the concentration of G6P, the critical step in regulating glycogen synthase activity.

Glycogen synthesis is primarily regulated by modulating the activity of glycogen synthase (GYS) (Blanco & Blanco, 2017). GYS is allosterically activated by G6P and negatively regulated by covalent phosphorylation (Roach *et al.*, 2012). The presence and influx of G6P overrides the inactive form of GYS, allowing GYS to become active. Thus, G6P is a key regulator in GYS activity. Activation of GYS allows glucose residues to be added to the existing glycogen molecule by linking it to a non-reducing residue (Supplemental Figure 4B).

Glycogen synthase kinase 3 beta (GSK3β) is one enzyme that modulates GYS activity. When GSK3β is phosphorylated by a kinase (ex. pAKT), GSK3β becomes inactive. Inhibition of GSK3β helps maintain active GYS (Blanco & Blanco, 2017). Therefore, increased phosphorylation and inhibition of GSK3β allows glycogen synthesis to occur. However, when GSK3β is dephosphorylated, it phosphorylates GYS and inhibits glycogen synthesis. Therefore, when GYS is phosphorylated, it is inactive and therefore reduces glycogen synthesis from occurring. When GYS is dephosphorylated, it is active, and glycogen synthesis more readily occurs. It is important to note that the phosphorylation of an enzyme can increase or decrease its activity but does not change its capacity to function (Supplemental Figure 5).

Glycogenolysis

Glycogenolysis is the breakdown of glycogen, releasing glucose. Mobilization of glucose residues from glycogen stores requires two reactions: shortening the non-reducing ends of the glycosidic chains by glycogen phosphorylase and disassembly of the branch points by glycogen debranching enzyme (Engelking, 2015). Glycogen phosphorylase (PYG) liberates G1P from glycogen. When PYG is phosphorylated, it becomes more active (Liver Physiology and Energy Metabolism, 2015). After G1P has been liberated from glycogen, G1P is isomerized by PGM back into G6P. Glucose-6- phosphatase (G6PC) dephosphorylates G6P yielding glucose (Aronoff *et al.*, 2004). G6PC is primarily found in the liver, kidney, and intestines. G6PC is located on the endoplasmic reticulum membrane (Blanco & Blanco, 2017). The purpose of glycogenolysis differs between muscle and liver tissues. Unlike the liver, muscle does not release free glucose into circulation (Engelking, 2015). In muscle, glycogen breakdown is activated to

meet the energy needs of the muscle, producing pyruvate and lactate. An illustration of glycogenolysis is depicted in Supplemental Figure 4B.

Glycogen in the Uterus

Despite only a few studies illustrating a correlation between fertility rates, embryo survival, and uterine glycogen concentrations, these findings are possibly significant. In rats, uterine glycogen peaks during proestrus to estrus, and is utilized during implantation and pregnancy (Demers et al. 1972; Greenstreet & Fotherby 1973). In mice, glycogen in the uterine epithelium was high during proestrus and decreased during preimplantation (Chen *et al.*, under review). Studies done in mink indicate that endometrial glycogen peaks during estrus and decline during the preimplantation period (Dean *et al.*, 2014). Sandoval *et al.* (2021) demonstrated that cattle near estrus (day 1) had higher glycogen content in the uterine epithelium than cattle in diestrus (day 11). An early study measuring endometrial glycogen concentrations of human patients found that glycogen concentrations were 60% lower during the late secretory phase in infertile patients relative to fertile controls (Maeyama *et al.*, 1977). These results suggest that glycogen reserves may be used to support preimplantation embryo development. Collectively, glycogen reserves in the uterus may serve glucose reservoir and potentially support early embryonic development and thus decrease early embryonic loss.

Since the uterus is a hormonally regulated organ, it is not unreasonable to propose that glycogen storage in the uterus may also be under endocrine control. For example, changes in glycogen levels in the uterine epithelium of cattle between days 1 and 11 may imply that endometrial glycogen metabolism is regulated by ovarian steroids (Sandoval *et al.*, 2021). In

sheep, rats, and mink, E2 treatment resulted in higher quantities of endometrial glycogen (Bitman *et al.*, 1967; Rose *et al.*, 2011). A study in mink indicated P4 treatment results in lower endometrial glycogen stores (Bowman & Rose, 2017). Indicating that glycogen accumulation in the uterus is likely under control of ovarian steroids.

Insulin-like Growth Factor 1

Background Information

Insulin-like growth factor 1 (IGF-1) is a peptide hormone that promotes growth (Baker *et al.*, 1996). Most IGF-1 is produced by the liver and released into circulation. However, this peptide is produced in reproductive organs such as the ovaries, oviducts, uterus, and the hypothalamus (Spicer & Echternkamp, 1995; Watson *et al.*, 1999). The IGF-1 receptor (IGF-1R) is 98% homologous to the insulin receptor (Boron and Boulpaep, 2016). While it remains unclear how the IGF-1R and insulin receptor maintain their specificity, Kim & Accili (2002) did demonstrate that each receptor is functionally distinct and that most effects of each peptide are due to acting through their corresponding receptors.

The IGF-1 receptor is a receptor tyrosine kinase (RTK). RTK receptors have an alpha subunit on the extracellular space of the cell and a beta subunit embedded in the membrane and sticking out into the cytosol of the cell (Boron and Boulpaep, 2016). Binding of IGF-1 to the alpha subunit triggers a conformational change and autophosphorylation transmitted to the beta domain. It then leads to phosphorylation of the tyrosine residues and activation of the beta subunits.

It is well known that glucose stimulates insulin release from the pancreas, and insulin promotes the partitioning of nutrients toward adipose tissue and muscle (Lucy *et al.*, 2012). Insulin signaling is classically mediated via IRS1 and IRS2 autophosphorylation, which activates the PI3K pathway and leads to AKT phosphorylation. AKT substrates trigger the translocation of GLUT4 to the plasma membrane, encouraging glucose uptake (Frolova & Moley, 2011). IGF-1 is also known to regulate glycogen metabolism in various species; in tissues such as the liver, muscle, brain, hepatocytes, and astrocytes (Boron and Boulpaep, 2016; Verspohl *et al.*, 1984; Jaspers *et al.*, 1993; Muhič *et al.*, 2015). After binding of the ligand has occurred and downstream signaling has been initiated, there is an increase in phosphorylated AKT that leads to increased phosphorylated GSK3β, resulting in active GYS (Supplemental Figure 5).

Role in Estrus Cycle

IGF-1 plays a significant role in mammalian reproduction. The oviducts, uterus, and embryo all produce both IGF-1 and a variety of IGF-1 binding proteins (IGFBPs) (Robinson *et al.*, 2000; Meikle *et al.*, 2001), making it likely that local factors govern the availability of IGF-1 within the reproductive tract. Expression of the IGF-1R is detectable in all cell types but is most abundant in the LE and GE (Robinson *et al.*, 2000). High expression of IGF receptors in both the oviduct and uterine endometrial glands (Robinson *et al.*, 2000), suggests that endocrine IGF-1 could have a direct effect on tract secretions, which survival of the early embryo is entirely reliant on (Wathes *et al.*, 1998).

In the uterus, E2 has been shown to stimulate IGF-1 production. IGF-1 appears to be a direct target of ERα, containing several estrogen response elements that show ERα occupancy following E2 stimulation (Hantak *et al.*, 2014). Kapur *et al.* (1992) established that E2 stimulates

the production of IGF-1 in the uterine stroma in mice. Studies performed on cows revealed that endometrial levels of IGF-1 mRNA are higher in the early part of the cycle (Sosa *et al.,* 2010; McCarthy *et al.,* 2012). Therefore, the pre-ovulatory rise of IGF-1 coincides with the rise of E2 at the beginning of estrus.

Previous research indicates that insulin stimulates glycogen synthesis in the uterus. Flannery *et al.* (2018) found that insulin increased glycogen accumulation in the epithelium of humans *in vitro*. Unlike insulin, IGF-1 has been shown to change in a cycle-dependent manner and is stimulated by E2. Sandoval *et al.* (2021) found that the quantity of glycogen stored in the uterine epithelia cattle depends on the stage of the reproductive cycle. Thus, IGF-1 may stimulate glycogen synthesis in the uterus as well. Zhu and Pollard (2007) proposed a mechanism in which IGF-1, produced in the stroma, acts via IGF-1R in the epithelium to stimulate the activation of PI3K/AKT, which in turn phosphorylates and inactivates GSK3β in mice. This mechanism was also investigated in murine hepatocytes, which revealed that IGF-1 stimulates glycogen synthesis (Park *et al.*, 1999). Further supporting the idea that IGF-1 stimulates uterine glycogen synthesis. These studies indicate that IGF-1 may mediate E2-stimulated glycogen synthesis in the endometrium.

Role in Early Pregnancy

IGF-1's influence has been demonstrated through changes in the cycle, glandular development, and appears to influence early pregnancy. The fact that IGF-1R is abundantly located on the uterine epithelium suggests that IGF-1 might be a paracrine mediator of the stromal-epithelial crosstalk involved in epithelial proliferation during the preimplantation phase (Hantak *et al.,* 2014). The role of IGF-1 in early pregnancy is primarily associated with embryo

development. Lucy *et al.* (1995) found that IGF-1 ULF concentrations ranged between 35-40 ng/ml and were not significantly different between pregnant and non-pregnant heifers.

Endocrine IGF-1 could potentially influence embryo viability directly following transfer to the lumen of the reproductive tract or indirectly via actions on the ovary, oviduct, or uterus. Bovine embryos derived *in vitro* demonstrated that IGF-1 can positively effect on preimplantation development and achieve higher pregnancy rates after transfer to recipients (Block *et al.*, 2003; Block *et al.*, 2007). Therefore, the effects of IGF-1 *in vitro* are being mediated, at least in part, by the embryo. This is supported by the presence of IGF-1 receptors on the embryo throughout preimplantation development (Watson *et al.*, 1992). IGF-1 could also potentially influence conceptus development. For example, maternal IGF-1 could alter nutrient availability transferred across the placenta (Bauer *et al.*, 1998). Using a guinea pig model in which IGF-1 was infused during early-mid pregnancy, maternal IGF-1 was increased and appeared to divert nutrients from mother to conceptus (Sferruzzi-Perri *et al.*, 2007). The redirection of nutrients was supported by increased placental transport of both glucose and amino acids. Undoubtedly, IGF-1 is a key peptide during early pregnancy and warrants further research.

CHAPTER 3: EFFECT OF E2 AND IGF-1 ON GLYCOGEN SYNTHESIS IN BOVINE UTERINE EPITHELIAL CELLS

Abstract

Glycogen storage in the uterine epithelium peaks near estrus and is a potential source of glucose for the endometrium and embryos. However, the hormonal regulation of glycogen synthesis in the uterine epithelium is poorly understood. Our objective was to evaluate the effect of estradiol (E2) and insulin-like growth factor 1 (IGF-1) on glycogenesis in immortalized bovine uterine epithelial (BUTE) cells. Treatment of BUTE cells with E2 (0.1-10 nM) did not increase glycogen levels. However, treatment of BUTE cells with IGF-1 (50 or 100 ng/ml) resulted in a >2-fold increase in glycogen. To determine if the uterine stroma produced IGF-1 in response to E2, bovine uterine fibroblasts (BFIBs) were treated with E2, which increased IGF-1 levels. Immunohistochemistry showed higher levels of IGF-1 in the stroma on day 1 than day 11, which coincides with higher glycogen levels in the uterine epithelium. Western blots revealed that IGF-1 treatment increased levels of phospho-AKT, phospho-GSKβ, hexokinase 1, and glycogen synthase in BUTE cells. Metabolomic (GC-MS) analysis showed that IGF-1 increased 3-phosphoglycerate and lactate, potentially indicative of increased flux through glycolysis. We also found higher levels of N-acetyl-glucosamine and protein glycosylation after IGF-1 treatment, indicating increased hexosamine biosynthetic pathway activity. In conclusion, IGF-1 is produced by uterine fibroblasts due to E2, and IGF-1 increases glucose metabolism and glycogenesis in uterine epithelial cells. Glycogen stored in the uterine epithelium due to E2/IGF-1 signaling at estrus could provide glucose to the endometrium or be secreted into the uterine lumen as a component of histotroph.

Introduction

Based on *in vitro* data, preimplantation embryos are dependent on glucose or fructose secreted into the uterine lumen for survival. To date most research has focused on the role of glucose. In sheep embryos, glucose uptake was low until the 16-cell stage, when it began to increase rapidly. By the blastocyst stage, glucose uptake was 22-fold higher than at the zygote stage, and by the hatched blastocyst stage, glucose uptake increased 55-fold over the zygote stage (Gardner *et al.*, 1993). In ruminants, hatched blastocysts then elongate before attachment to the endometrium (Spencer *et al.*, 2004). The glucose uptake of elongating blastocysts has not been measured, but it is likely that glucose uptake continues to increase to support cell proliferation during elongation.

In agreement with increased glucose uptake as the embryo develops, glucose concentrations in the bovine oviduct are reported to be ~1-2 mM, while concentrations in the uterine lumen were ~3-4 mM (Hugentobler *et al.*, 2008). This also suggests that glucose transport from maternal circulation to the uterine lumen is regulated and not due to passive diffusion. In support of this, serum and uterine fluid glucose concentrations are not correlated in heifers (Moraes *et al.*, 2020). Even intravenous infusion of glucose did not change concentrations of glucose in the uterine lumen of lactating dairy cows (Leane *et al.*, 2018). Thus, secretion of glucose by the glandular and luminal epithelial cells appears to be tightly regulated to match the changing needs of the developing embryo.

The bovine endometrium also metabolizes glucose. Chase *et al.* (1992) found that glucose uptake by the endometrium was consistent across the estrous cycle. However, glucose metabolized to lactate was lower during the mid-estrous cycle, while glucose oxidation peaked in the mid-cycle. In bovine endometrial biopsies, lipopolysaccharide challenge increased glucose

consumption, while low glucose concentrations reduced cytokine secretion (Turner *et al.*, 2016). In a bovine endometrium on-a-chip model, high and low glucose concentrations altered the transcriptome of the uterine epithelium and the proteins secreted into the media (De Bem *et al.*, 2021). These results indicate that glucose concentrations also affect endometrial function.

The uterus cannot synthesize glucose *de novo*; therefore, all glucose used by the endometrium or secreted into the uterine lumen must come from maternal circulation (Zimmer and Magnuson, 1990; Yánez *et al.*, 2003). Most research on glucose availability in the endometrium has focused on glucose transporters (GLUTs; gene family *SLC2A*), which transport glucose via facilitated diffusion or sodium-glucose linked transporter 1 (SGLT1; gene symbol *SLC5A1*), which transports glucose into cells via secondary active transport (Forde *et al.*, 2009b; Salker *et al.*, 2017). Several GLUTs and SGLT1 have been shown to be expressed by the uterine epithelium in cows (Forde *et al.*, 2009b; Chankeaw *et al.*, 2021). However, it is unlikely that GLUTs and SGLTs can fully explain the spatiotemporal changes in glucose uptake and secretion by the uterine epithelium (Dean, 2019).

Another option is that the uterine epithelium transiently stores glucose as the macromolecule glycogen, which can be catabolized into glucose when needed. We recently showed that the glycogen content of the bovine uterine epithelium changed in a reproductive cycle-dependent manner in Holstein dairy cows (Sandoval *et al.*, 2021). Specifically, the glycogen concentration of the uterine epithelium was higher on day 1 than day 11 of the cycle; leading us to hypothesize that estradiol (E2) stimulates glycogenesis in the uterine epithelium.

In mice, it is well established that E2 stimulates the production of insulin-like growth factor 1 (IGF-1) in the uterine stroma (Kapur *et al.*, 1992). This IGF-1 then mediates some of the proliferative effects of E2 on the uterine epithelium (Murphy and Ghahary, 1990; Zhu and

Pollard, 2007). In cows, endometrial levels of IGF-1 mRNA are higher in the early part of the cycle (Sosa *et al.*, 2010; McCarthy *et al.*, 2012) and *in situ* hybridization localized IGF-1 production to the uterine stroma (Robinson *et al.*, 2000).

IGF-1 is known to regulate glycogen metabolism in other tissues. For example, *in vitro* IGF-1 stimulates glycogen synthesis in cardiomyocytes, HEP-G2 cells, and astrocytes (Verspohl *et al.*, 1984; Jaspers *et al.*, 1993; Muhič *et al.*, 2015). *In vivo*, IGF-1 knockout mice have lower glycogen stores in neurons and chondrocytes than their wildtype counterparts (Wang *et al.*, 1999; Cheng *et al.*, 2000). Thus, IGF-1 may play a role in regulating local glycogen metabolism in specific tissues.

We recently developed bovine uterine epithelial (BUTE) and bovine uterine fibroblast (BFIB) cell lines to investigate the effects of hormones on glycogen metabolism in the uterine epithelium (Berg *et al.*, 2022). Using these cells, our objectives were to 1) test the ability of E2 and IGF-1 to stimulate glycogen synthesis in BUTE cells, 2) confirm that E2 stimulated IGF-1 production in uterine fibroblasts, 3) elucidate the pathway by which IGF-1 stimulates glycogen synthesis in BUTE cells, and 4) identify global changes in metabolite levels of BUTE cells due to IGF-1 treatment.

CHAPTER 3.1: MATERIALS & METHODS

Cell Culture

BUTE and BFIB cells were previously generated from endometrial biopsies collected from a Holstein dairy cow on the University of Illinois Dairy Farm using a pW2 plasmid expressing both the large T and small T antigens (Porrás *et al.*, 1996; Berg *et al.*, 2022). Both cell lines expressed cytokeratin and vimentin as appropriate and expressed glycogen metabolizing enzymes in agreement with *in vivo* findings (Sandoval *et al.*, 2021; Berg *et al.*, 2022).

BUTE and BFIB cells were maintained in αMEM media from the University of Illinois Cell Media Facility. Media was prepared used Caisson Labs αMEM (#MEP10). The composition of αMEM is provided in Supplemental Table 1. Importantly, αMEM contains physiological concentrations of glucose (5.55 mM). Cells were maintained in αMEM supplement with 10% FBS (12103C-500ML, Sigma-Aldrich), 2 mM L-glutamine (GLL01-100ML, Caisson Labs), 10 mg/ml ITS (25-800-CR, Corning) 1.8 ng/ml EGF (20053100UG, Shenandoah Biotechnology), and 18.2 ng/ml E2 (E2758-1G, Sigma). Cells were maintained in T-75 flasks (658170, Greiner Bio-One) in a humidified incubator under 5% CO_2. Cells were passed at confluence using 1x Trypsin/EDTA (TRL02-10ml, Caisson Labs). BUTE and BFIB cells used in the current experiments had been passed less than 30 times.

Hormones and Inhibitors

IGF-1 (2000550UG, Shenandoah Biotechnology) was dissolved in PBS at 100 μg/ml. E2 (E2754, Sigma Aldrich) was dissolved in DMSO at 100 μM and then diluted to 1000x the

desired final concentration in vehicle. NVP-ADW742 (NVP; 18390, Cayman Chemical), MK2206 (11593, Cayman Chemical), picropodophyllotoxin (PPPT, 17329, Cayman Chemical), and Bio-Acetoxmine (16329, Cayman Chemical) were dissolved in DMSO at 10 mM and then diluted to 1000x the final concentration prior to addition to the media. Hormones and inhibitors were added to the media at 1 µl per ml to achieve the desired concentration. In experiments using DMSO as vehicle, the final concentration of DMSO was 0.1%. Control treatments consisted of DMSO and/or PBS as appropriate.

Glycogen Assay

BUTE cells were plated in 10 cm dishes (664160, Greiner Bio-One) and grown to approximately 95% confluency. The media was removed, the monolayer was rinsed twice with 1x PBS, and αMEM without phenol red, FBS, or any other hormones or growth factors were added. After 24 hours of being serum- and steroid-starved, the media was replaced with fresh phenol red-free αMEM containing vehicle and treatment as appropriate. BUTE cells were treated for 48 hours. Then cells were collected with trypsin/EDTA, and then 5 ml PBS was added. Cells were counted and centrifuged. The PBS was aspirated, and the cell pellet was re-suspended in 400 µl of 30% potassium hydroxide (KOH). Samples were stored at -20°C until analysis.

Glycogen was measured based on the protocol developed by Good *et al.* (1933) and previously used by our laboratory (Berg *et al.*, 2022). Samples were heated at 95°C for 30 minutes to inactive glycogen-metabolizing enzymes. Samples were briefly centrifuged, 480 µl of 100% ethanol was added to each tube, and samples were centrifuged again at 15,000 x g for 20 minutes to pellet glycogen. The supernatant was decanted. The pellet was washed with 250 µl of 70% ethanol and centrifuged again to remove intracellular glucose. The supernatant was

decanted, and the tubes were allowed to dry overnight. The next day 50 µl of 1 M HCl was added to each tube, and they were heated at 95°C for 3 hours to hydrolyze glycogen into glucose. Samples were briefly centrifuged, and 50 µl of 1 M NaOH was added to neutralize the acidity. In duplicate, samples (40 µl) were added to a 96-well plate (655180, Greiner Bio-One). To measure the concentration of glucose hydrolyzed from glycogen, 250 µl of Wako Diagnostics Autokit Reagent (99703001, FUJIFILM) was added to each well. The plate was incubated at 37°C for 30 minutes. The absorbance was read at 505 nm (Biotek µQuant Monochromatic Microplate Spectrophotometer, BioTek). To calculate the amount of glycogen isolated from each sample, absorbance was compared to a standard curve of glycogen (6.2 – 200 µg/ml; AAJ1644514, Thermo Scientific). Glycogen (µg) was normalized to the number of cells in each sample and expressed as µg glycogen/10^6 cells.

Like most glycogen assays, this assay breaks glycogen down into glucose and then measures the resulting glucose. To ensure that our assay removed intracellular glucose prior to hydrolyzing the glycogen, we dissolved glycogen (50 µg/ml) or glycogen spiked with glucose (both at 50 µg/ml) in 30% KOH and then measured glycogen. The concentration of glycogen measured in the glycogen and the glycogen + glucose groups was the same (53.2 ± 2.5 and 51.32 ± 2.4 µg/ml, respectively), indicating that free glucose is removed with our assay (Supplemental Figure 1A).

Immunohistochemistry

Procedures were approved by the University of Illinois Urbana-Champaign Institutional Animal Care and Use Committee (no.19036). Endometrial samples from day 1 and 11 of the reproductive cycle were collected and immunohistochemistry was done as described (Sandoval

et al., 2021). Uterine tissue were sectioned (5 µm), deparaffinized, heated in sodium citrate, and incubated in 3% hydrogen peroxide. Tissue sections were incubated in blocking solution (3% BSA, 10% rabbit or goat serum, in tris-buffered saline [TBS]) for 1 hour. Primary antibodies (Table 1) were diluted in blocking solution, added to each slide, and incubated overnight at 4°C. The next day slides were washed three times with TBS with tween (TBS-T), incubated with anti-rabbit or anti-goat secondary antibody (BA-1000 and BA-5000, respectively; Vector Laboratories) diluted 1:200 in block for 30 minutes, and washed three more times in TBS-T. After washing, DAB substrate (SK-4100, Vector Laboratories) was added to each slide. The development time was kept consistent for each antibody. The slides were counterstained with hematoxylin, dehydrated, and mounted. Images were captured with a Zeiss Axioskop upright microscope with an Axiocam 305 color camera.

Negative controls were treated the same as above, except the primary antibody was replaced with an isotype negative control, anti-green fluorescent protein (GFP) antibody. The antibody concentration and development time for the negative controls were the same as the corresponding antibody of interest. The antibodies for IGF-1 and IGF-1 receptor (IGF-1R) were validated by incubating the antibody with its recombinant protein target (1 µg/ml IGF-1, 2000550UG, Shenandoah Biotechnology; 125 µg/ml IGF-1R, 391-GR-050, R&D Systems) overnight at 4°C. The next day the antibody was used as described above. In both cases, immunostaining was eliminated by preincubation with the blocking protein (Supplemental Figure 1B).

Metabolomics

BUTE cells were plated in T-175 flasks (660175, Greiner Bio-One). When they were approximately 95% confluent the cells were steroid, hormone, and serum starved for 24 hours. The next day the media was replaced, and cells were treated with vehicle (PBS) or 50 ng/ml IGF-1. After 48 hours, media was removed from each monolayer, the monolayer was rinsed once with PBS, cells were collected with trypsin, and then 12 ml PBS was added. Cells were counted and centrifuged. The PBS was aspirated, and the cell pellet was re-suspended in 500 µl of a 2:1 chloroform:methanol mixture. Samples were stored at -20°C until analysis.

Gas chromatography–mass spectrometry (GC-MS) was conducted by the University of Illinois Metabolomics Core. Samples were extracted by ultrasound (5 x 1min) with a QSonica Microson X L2000 Ultrasonic Homogenizer (Qsinica, LLC., CT, USA) at 4°C with 1 ml of isopropanol:acetonitrile:water (3:3:2, v/v/v). Samples were centrifuged (5 min at 15,000 x g) and supernatants were evaporated under vacuum. Samples were then derivatized with 100 µl methoxyamine hydrochloride (40 mg/ml in pyridine) for 90 minutes at 500 °C and then with 100 µl MSTFA at 50°C for 120 minutes. Internal standard (20 µl, hentriacontanoic acid, 1 mg/ml) was added to each sample prior to derivatization. Samples were analyzed on a GC-MS system (Agilent Inc, Palo Alto, CA, USA) consisting of an Agilent 7890 gas chromatograph, an Agilent 5975 mass selective detector, and a HP 7683B autosampler. Gas chromatography was performed on a ZB-5MS (60m×0.32mm I.D. and 0.25 µm film thickness) capillary column (Phenomenex, CA, USA). The inlet and MS interface temperatures were 250°C, and the ion source temperature was adjusted to 230°C. An aliquot of 1 µl was injected with the split ratio of 10:1. The helium carrier gas was kept at a constant flow rate of 2.4 ml/min. The temperature program was 5 minutes isothermal heating at 70°C, followed by an oven temperature increase of 5°C/min to

310°C and a final 10 minutes at 310°C. The mass spectrometer was operated in positive electron impact mode (EI) at 69.9 eV ionization energy in m/z 30-800 scan range.

All known artificial peaks were identified and removed. MS peaks were evaluated by AMDIS 2.71 (NIST, Gaithersburg, MD, USA) program and metabolites were identified by custom-built library (520 unique metabolites). To allow comparison between samples, all data were normalized to the internal standard in each chromatogram and then to the number of cells in each sample.

Metabolites identified by GC-MS were mapped to unique identifiers in the Human Metabolome Database (HMDB; https://hmdb.ca/). Metabolite abundance was described using a linear model including the effect of IGF-1 treatment and metabolites that tended to be altered ($P<0.1$) by IGF-1 treatment were identified. Enrichment analysis of the significant metabolites for Kyoto Encyclopedia of Genes and Genomes database (KEGG) human metabolic pathways was performed using MetaboAnalyst Version 5 (https://www.metaboanalyst.ca/) with default specifications (Pang *et al.*, 2021).

Protein Glycosylation. Assay

BUTE cells were plated and treated in the same manner as for western blots. Twenty-five micrograms of protein were separated on 10% SDS-PAGE gels and then stained using the Pierce Glycoprotein Staining kit (24562, Thermo Scientific). After electrophoresis, the gel was fixed in 50% methanol for 30 minutes, washed in 3% acetic acid for 30 minutes and transferred to Oxidizing Solution for 15 minutes. The gel was washed in 3% acetic acid for 15 minutes, placed in periodic acid–Schiff (PAS) glycoprotein stain for 15 minutes, and transferred to Reducing Solution for 5 minutes. It was then incubated in 3% acetic acid for 5 days and washed with

ultrapure water before imaging. The gel was washed, stained with Coomassie Brilliant Blue R-250 (558UA, BRL; 2g/ml Coomassie, 50% methanol, and 10% acetic acid), de-stained (10% methanol and 10% acetic acid), and imaged again. Since PAS stains all glycosylated proteins, the densitometry in the entire length of each lane was measured using ImageJ, and the densitometry for glycoprotein stain was normalized to the densitometry for the Coomassie stain. The positive control (horseradish peroxidase) was supplied by the manufacturer.

Western Blot

Cells were plated in 6-well plates. When they were approximately 95% confluent, the cells were steroid and serum starved in phenol red-free αMEM for 24 hours. The next day the media was replaced and cells were treated as indicated. At the end of treatment, media was removed from each monolayer, the monolayer was rinsed once with PBS, and the cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors (P0044-1ML, SigmaAldrich, and A32953 Thermo Scientific). The protein concentration was determined with a bicinchoninic acid (BCA) assay (23228, Thermo Scientific). Twenty-five micrograms of protein were separated on 10-15% SDS-PAGE gels and then transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for one hour with either 5% powdered milk or 5% bovine serum albumin (BSA) in TBS-T. Membranes were incubated in primary antibodies (Table 1) overnight at 4°C on an orbital shaker. The next day the membranes were rinsed with TBS-T, probed with anti-rabbit secondary antibody (7074S, Cell Signaling Technology) diluted 1:1000 in block for 30 minutes, rinsed with TBS-T, and bands were visualized with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (PI34577, Thermo Scientific).

Densitometry for bands was measured with ImageJ (imagej.nih.gov). Briefly, the densitometry for each band of interest was first normalized to the loading control (β actin) in the same lane. Values were then normalized to the control on the same membrane to account for membrane-to-membrane variation.

Statistical Analysis

Experiments were replicated 3-7 times, and data are expressed as mean ± SEM. Data were analyzed by a student's T-Test, one-way ANOVA followed by a Dunnett's or Tukey's posthoc, or two-way ANOVA using GraphPad Prism Version 9.0.0. $P<0.05$ was considered statistically significant. Analysis was carried out using GraphPad Prism v9.3.

CHAPTER 3.2: RESULTS

IGF-1 Stimulates Glycogen Synthesis

We previously observed that the glycogen content of the bovine uterine epithelium was higher on Day 1 than Day 11 of the cycle (Sandoval *et al.*, 2021), leading us to hypothesize that E2 would stimulate glycogenesis in BUTE cells. In cattle, serum E2 concentrations can reach approximately 0.1 nM at estrus; however, E2 accumulates in the endometrium, reaching concentrations 50-60 fold higher than serum (Henricks *et al.*, 1971; Mann *et al.*, 2007). Therefore, we treated BUTE cells with 0.1-10 nM E2 for 48 hours and measured glycogen. However, E2 had no direct effect on the glycogen concentration of BUTE cells relative to the vehicle control (0 nM; Figure 1A). IGF-1 is produced in the uterine stroma and has stimulated

glycogenesis in other tissues (McCarthy *et al.*, 2012; Muhič *et al.*, 2015). Thus, we tested the ability of physiological concentrations of IGF-1 (10-100 ng/ml) to stimulate glycogen storage in BUTE cells (Willhelm *et al.*, 2021). The lowest concentration of IGF-1 (10 ng/ml) did not affect glycogen concentrations ($P=0.50$). Higher concentrations (50 and 100 ng/ml) increased glycogen levels 2.7- and 2.3-fold, respectively ($P<0.01$; Figure 1B). We also tested for an interaction between E2 and IGF-1. BUTE cells were treated with vehicle (control), 10 nM E2, 50 ng/ml IGF-1, or 10 nM E2 + 50 ng/ml IGF-1. Two-way ANOVA indicated that the main effect of IGF-1 was significant ($P<0.0001$) while the effect of E2 and the interaction did not reach statistical significance (Figure 1C).

Next, we wanted to confirm that the IGF-1R was mediating the effect of IGF-1. To that end, we tested the ability of two purported IGF-1R inhibitors (NVP and PPPT) to inhibit IGF-1-stimulated glycogenesis. First, we determined at what concentration (0, 0.1, 1, and 10 μM) each inhibitor resulted in cell death after 48 hrs. At 0.1 μM PPPT had no visible effects on the integrity of the BUTE monolayer, but at 1 μM PPT the monolayer had completely died. It took 10 μM of NVP to have a similar effect (Supplemental Figure 2A). Next, we tested the ability of both inhibitors to block IGF-1-induced phosphorylation of AKT. NVP (1 μM) completely abrogated IGF-1 induced phospho-AKT, while PPPT (0.1 μM) did not (Supplemental Figure 2B). Therefore, we tested the ability of NVP to block IGF-1-stimulated glycogenesis. As expected, IGF-1 increased glycogen levels in BUTE cells by 2.2-fold ($P<0.05$). NVP (1 μM) by itself did not affect glycogen levels, while concurrent treatment of IGF-1 and NVP resulted in glycogen levels statistically the same as controls (Figure 1D). These results indicate that IGF-1 directly stimulates glycogenesis, not E2, in bovine uterine epithelial cells via IGF-1R.

E2 Stimulates IGF-1 Production in the Uterine Stroma

To determine if E2 stimulates IGF-1 production in uterine fibroblasts, BFIB cells were treated with E2 (0.01-10 nM) for 48 hours. Western blots showed that BFIB cells treated with 5 nM ($P<0.05$) and 10 nM ($P<0.01$) of E2 showed a 2.7- and 3.5-fold increase in IGF-1 levels, respectively ($P<0.05$; Figure 2A). We have previously shown that glycogen content of the uterine epithelium was higher on Day 1 than Day 11 of the cycle in Holstein cows (Sandoval *et al.*, 2021). To determine if that corresponds with changes in IGF-1 signaling, here we localized IGF-1 and the IGF-1R in the bovine endometrium on Day 1 and 11 of the cycle using immunohistochemistry (Figure 2B). The expression of IGF-1 in the endometrium was higher on day 1 than on day 11, with expression highest in the stroma. The expression of IGF-1R was present throughout the endometrium on day 1 and day 11 of the cycle, though immunostaining was more intense in the epithelium. These results support the idea that E2 may stimulate IGF-1 production in the bovine uterine stroma, which could stimulate glycogen synthesis in the adjacent epithelium.

Effects of IGF-1 are Partially Mediated in by AKT/GSK3β Pathway

Classically, IGF-1 activates the AKT/GSK3β pathway. Therefore, we analyzed phospho-AKT and phospho-GSK3β levels over 120 minutes after 50 ng/ml IGF-1 treatment. Western blots revealed an increase of approximately 7-fold for phospho-AKT at 30-120 minutes after IGF-1 treatment ($P<0.05$; Figure 3A). Likewise, IGF-1 treatment increased phospho-GSK3β ($P<0.157$) >2-fold after 30-120 minutes after treatment (Figure 3B).

To determine if the AKT/GSK3β pathway was mediating the effects of IGF-1, BUTE cells were treated with vehicle (control), IGF-1 (50 ng/ml), MK2206 (AKT inhibitor, 10 μM),

and IGF-1 + MK2206. Again, IGF-1 increased glycogen levels as expected. MK2206 did not affect glycogen levels. Co-treatment with IGF-1 and MK2206 resulted in an intermediate glycogen level statistically similar to both control and IGF-1 treatments (Figure 3C). Addition of 10 μM MK2206 did not appear to affect cell survival. GSK3β inhibits glycogen synthase activity. To confirm that GSK3β played a role in glycogen synthesis, we tested the effect of GSK3 inhibitor (Bio-Acetoxime, 1 μM). Bio-Acetoxime treatment resulted in a 4-fold increase in glycogen levels in treated cells compared to control ($P<0.05$; Figure 3D). These results indicate that IGF-1 activates the AKT/GSK3β pathway in BUTE cells and that this pathway mediates part of the effect of IGF-1 on glycogen synthesis.

IGF-1 Increases Expression of Glycogen Synthesizing Enzymes

Next, we evaluated the ability of IGF-1 to change the levels of the two major glycogen synthesizing enzymes—hexokinase 1 and glycogen synthase. After treating BUTE cells with IGF-1 (50 ng/ml) for 12 and 24 hours, there was an increase in hexokinase 1 levels ($P<0.05$; Figure 4A). Similarly, after 24 hours of IGF-1 treatment, there was an increase in glycogen synthase expression ($P<0.001$; Figure 4B). These results indicate that IGF-1 can also increase glycogen synthesis by increasing the expression of glycogen synthesizing enzymes.

IGF-1 Changes Overall Glucose Metabolism in BUTE Cells

The above results demonstrate that IGF-1 affects glucose metabolism of BUTE cells. To begin to characterize these changes, we performed metabolomics (GC-MS profiling) of BUTE cells treated with vehicle (PBS) or 50 ng/ml IGF-1 for 48 hrs. A total of 145 metabolites were identified in at least one sample, but only 66 metabolites were detected in all 12 samples (6

control and 6 IGF-1 treated). The complete list of metabolites is provided in Supplemental Table 2. Of the 145 metabolites identified by GC-MS, 123 metabolites were identified in the HMDB. Changes in eleven metabolites were associated ($P<0.1$) with IGF-1 treatment. Glucose was detectable in all 12 samples, but there was no significant difference in levels. Glucose-6-phosphate was detectable in only 2 out of the 6 control samples. It was readily detected in all 6 IGF-1 treated samples, suggesting an increase in glucose-6-phosphate levels due to IGF-1 treatment. Related to glucose metabolism, there was a significant increase in 3-phosphoglycerate levels (Figure 5A) and a trend for increased levels of lactic acid ($P=0.066$; Figure 5B), both of these can play a role in glycolysis. Interestingly, there also tended to be higher levels of three glucogenic amino acids (threonine, tryptophan, and tyrosine) in BUTE cells treated with IGF-1 ($P<0.1$; Figure 5C-E).

IGF-1 treatment also resulted in increased levels of N-acetyl-glucosamine (Figure 6A), an important intermediate in the hexosamine biosynthetic pathway. PAS staining of an SDS-PAGE gel showed that IGF-1 increased global protein glycosylation levels 82% over controls in BUTE cells ($P<0.01$; Figure 6B). These results indicate that IGF-1 increases glucose uptake and utilization via multiple pathways.

CHAPTER 3.3: DISCUSSION

In the bovine uterus, glycogen levels change in a reproductive cycle-dependent manner (Sandoval *et al.*, 2021). In mink, endometrial glycogen is mobilized during the preimplantation period (Dean *et al.*, 2014). This could suggest that glycogen is used to support preimplantation

embryo development. In support of this contention, in both species, endometrial glycogen stores are primarily localized to the uterine epithelium. In cows, the uterine epithelium also expresses glucose-6-phosphatase (Sandoval *et al.*, 2021), which is required to dephosphorylate and secrete the glucose moiety liberated from glycogen. Additionally global knockout of glucose-6-phosphatase in mice results in a smaller litter size (Jun *et al.*, 2012). However, the factors that stimulate the synthesis and subsequent breakdown of glycogen in the uterine epithelium have not been fully clarified.

Maternal hormones likely play a role in endometrial glucose metabolism. Here we show that E2/IGF-1 signaling stimulates glucose storage in the uterine epithelium. The factor(s) regulating the subsequent breakdown of glycogen remain to be elucidated but we have shown that epithelial glycogen content was lower during the luteal phase in cows (Sandoval *et al.*, 2021). Exogenous P4 increased the concentration of glucose and metabolites related to glycogen metabolism in uterine fluid (Simintiras *et al.*, 2019a, b). Thus, it is likely that P4 stimulates glycogenolysis in the uterine epithelium and/or stimulates direct transport of glucose from maternal circulation to uterine fluid.

There also appears to be complex interactions between the embryo and glucose secreted into the uterine lumen. Glucose concentrations in uterine fluid are correlated with the presence of a conceptus (Snider *et al.*, 2022). In a study categorizing heifers as high fertile or sub-fertile, Moraes *et al.* (2020) found conceptus length was associated with luminal glucose concentrations in high fertile but not sub-fertile heifers. These associations suggest that either bovine embryos secrete factors that stimulate glucose secretion by the uterine epithelium or that higher glucose concentrations increase embryo survival and growth. Both could be true, creating a positive feedback loop between conceptus growth and glucose secretion. Finally, uterine fluid is

composed of metabolites and enzymes that can act on those metabolites. Therefore, uterine fluid is semi-autonomous; its components can continue to change after it is secreted (Simintiras *et al.*, 2022).

A recent metabolomics study of media conditioned with bovine conceptuses found that bovine embryos released glucose into the media (Simintiras *et al.*, 2021). Early studies found that murine embryos can store glycogen (Edirisinghe *et al.*, 1984). Hence, it is unclear if glucose secreted by the bovine embryo was produced via gluconeogenesis or was from the breakdown of glycogen synthesized *in utero* before the embryos were collected.

In the present investigation, we found that E2 increased IGF-1 levels in uterine fibroblasts (BFIB cells), and immunohistochemistry suggested higher levels of IGF-1 production in the uterine stroma on day 1 than day 11 of the cycle. The high expression of IGF-1 on Day 1 correlates with high glycogen levels in the epithelium on day 1 that we observed previously (Sandoval *et al.*, 2021). While there was also some IGF-1 immunostaining in the epithelium, this is likely due to secreted IGF-1 because *in situ* hybridization has localized IGF-1 mRNA exclusively to the stroma (Robinson *et al.*, 2000). Higher endometrial IGF-1 protein levels on day 1 agree with northern blot and qPCR studies showing higher IGF-1 mRNA during the first five days of the cycle (Meikle *et al.*, 2001; McCarthy *et al.*, 2012). IGF-1R protein was highly expressed in the epithelium with lower expression in the stroma, agreeing with an earlier study (McCarthy *et al.*, 2012).

We found that IGF-1 increased glycogen stores in BUTE cells, and IGF-1 increased levels of pAKT and pGSK3β. The effect of IGF-1 was partially reversed by the AKT inhibitor MK2206. GSK3β is a negative regulator of glycogen synthase activity. AKT typically inhibits GSK3β activity through phosphorylation. Here the GSK3β inhibitor Bio-Acetoxime increased

glycogen concentrations. This suggests that part of the effect of IGF-1 is mediated by the AKT/GSK3β/glycogen synthase pathway. IGF-1 treatment also increased levels of hexokinase and glycogen synthase, which would also contribute to increased glycogen levels. Collectively these data offer a scenario *in vivo* where E2 stimulates stromal production of IGF-1, which increases glycogen synthesis in the uterine epithelium at estrus (Sandoval *et al.*, 2021).

Insulin is a well-known stimulator of glycogenesis in tissues such as the liver and muscle. Insulin has been shown to increase glycogen concentrations in uterine epithelial cells from mink and humans (Dean and Rose, 2018; Flannery *et al.*, 2018). We have previously shown that insulin stimulates glycogen stores in BUTE cells (Berg *et al.*, 2022). The effect of insulin in our previous study and the effect of IGF-1 seen here were of similar magnitude. This agrees with many other cell types that both insulin and IGF-1 can stimulate glycogen synthesis to similar degrees (Verspohl *et al.*, 1984; Jaspers *et al.*, 1993; Muhič *et al.*, 2015). The relative importance of each hormone *in vivo* is unclear. But given the reproductive cycle-dependent changes in glycogen levels of the uterine epithelium (Sandoval *et al.*, 2021), we hypothesize that E2-stimulated IGF-1 is an important regulator of glycogen metabolism in the uterine epithelium. In support of this, a recent study in mice found that uterine deletion of insulin receptor (IR) using P4 receptor Cre had minimal effects on the uterus, while IGF-1R knockout dramatically reduced fertility (Sekulovski *et al.*, 2021). However, the role of insulin in regulating uterine epithelial glycogen cannot be ruled out.

Preimplantation embryos need carbohydrates to survive, but which monosaccharide is used *in vivo* is unclear. Numerous studies have shown that preimplantation embryos can survive in culture using glucose. However, in cattle, fructose concentrations are much higher than glucose concentrations in fetal plasma and amniotic fluid, while fructose is undetectable in

maternal plasma (Bertolini *et al.*, 2004). Experiments comparing the development of bovine embryos cultured in glucose or fructose have found that a higher percentage of embryos reach the blastocyst stage with fructose (Kimura *et al.*, 2005; Barceló-Fimbres and Seidel, 2007). Interestingly, fructose does not skew the ratio of surviving embryos to males like glucose does (Kimura *et al.*, 2005).

Since fructose is not present in maternal plasma, the carbohydrate secreted into the uterine lumen must come from maternal glucose. Glucose liberated from glycogen, or glucose coming directly from maternal circulation, could be converted to fructose via the polyol pathway. In pigs, the uterine epithelium and placenta express aldo-keto reductase family 1 member B1 (*AKR1B1*) and sorbitol dehydrogenase (*SORD*) (Steinhauser *et al.*, 2016). However, expression and regulation of the polyol pathway enzymes in the bovine uterus remain to be characterized.

Our metabolomic analysis identified changes associated with the hexosamine biosynthetic pathway due to IGF-1 treatment. We confirmed an increase in protein glycosylation in BUTE cells after IGF-1 treatment. Protein glycosylation is considered a metabolic sensor that can have far-reaching effects on cell function (Ong *et al.*, 2018). In human and mouse models, protein glycosylation is critical for embryo attachment and implantation due to the regulation of integrin and leukemia inhibitor factor receptor (LIFR) pathways (Yu *et al.*, 2020). Thus, IGF-1-stimulated glycosylation may have essential roles in preparing the uterine epithelium for pregnancy.

The increased hexokinase expression combined with increases in glucose-6-phosphate, 3-phosphoglycerate, and lactate levels after IGF-1 treatment potentially indicate that IGF-1 increases glycolysis. Interestingly, we also found a trend for increased levels of three glucogenic

amino acids. We speculate that the increased levels of these amino acids may be due to a sparing effect due to switching from using amino acids to glucose for energy. In any case, the effects of IGF-1 on glycolysis in the uterine epithelium remain to be confirmed.

In conclusion, E2 stimulated IGF-1 production by uterine fibroblasts (Supplemental Figure 6). IGF-1 is expressed in the endometrial stroma near estrus and the IGF-1R is highly expressed in the uterine epithelium. IGF-1 stimulates glycogenesis in uterine epithelial cells (Supplemental Figure 7). IGF-1 increases glycogen synthesis by activating the AKT/GSK3β pathway and increasing the expression of the key glycogenic enzymes—hexokinase and glycogen synthase. In addition, IGF-1 alters overall glucose metabolism in the uterine epithelium. The purpose of glycogen stored in the uterine epithelium remains to be determined but could be used by the endometrium or secreted into the uterine lumen.

FIGURES

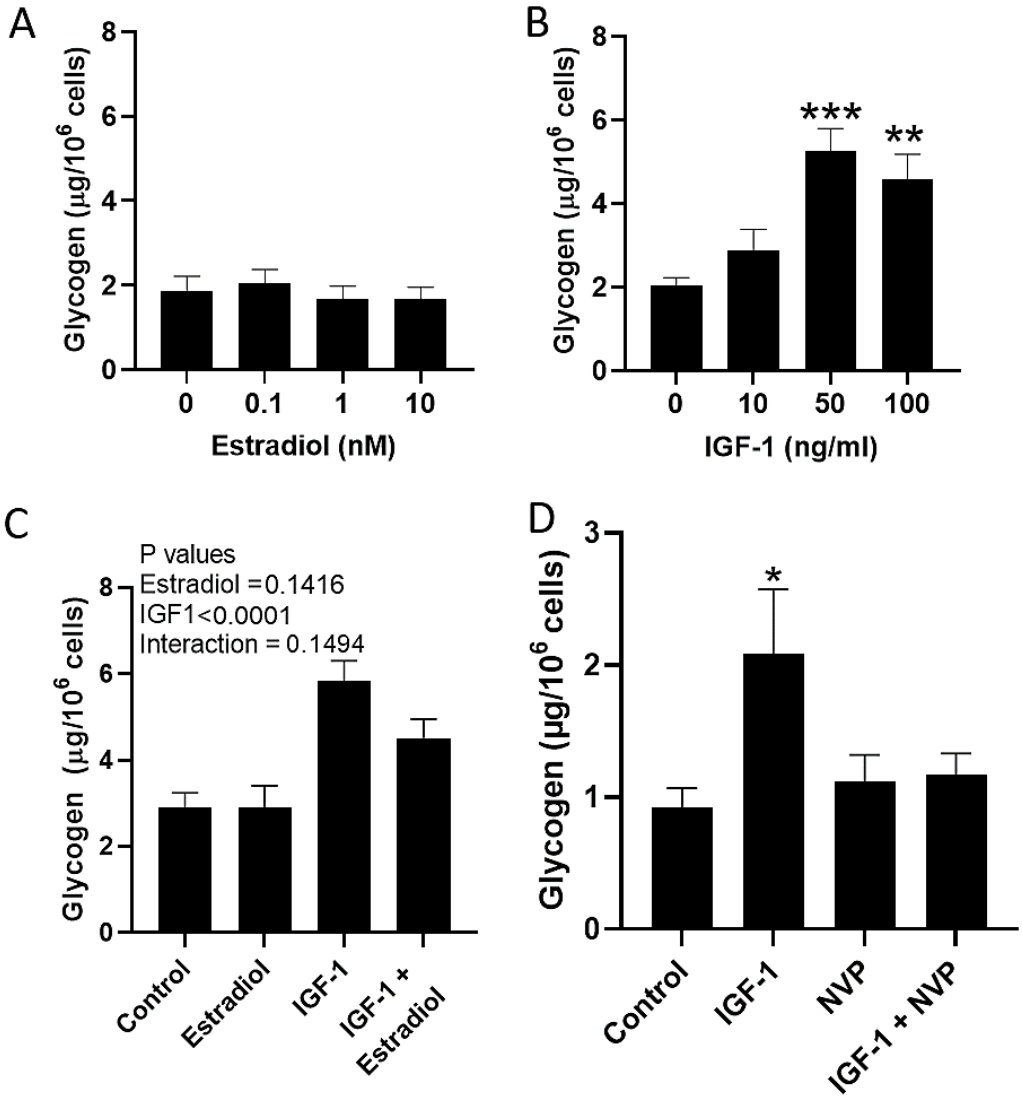

Figure 1. IGF-1, not E2, stimulates glycogen synthesis in BUTE cells. (A-B) Glycogen levels in BUTE cells treated with E2 (A) or IGF-1 (B) at indicated concentrations for 48 hours. (C) Glycogen levels in BUTE cells treated with vehicle (control), E2 (10 nM), or IGF-1 (50 ng/ml) for 48 hours. (D) Glycogen levels in BUTE cells treated with IGF-1 (50 ng/ml) or the IGF-1R inhibitor NVP-ADW742 (NVP; 1 µM) for 48 hours. The concentrations of vehicle (DMSO and/or PBS) was the same in all treatments. *P<0.05; **P<0.01; ***P<0.001 relative to Control. n=5-7.

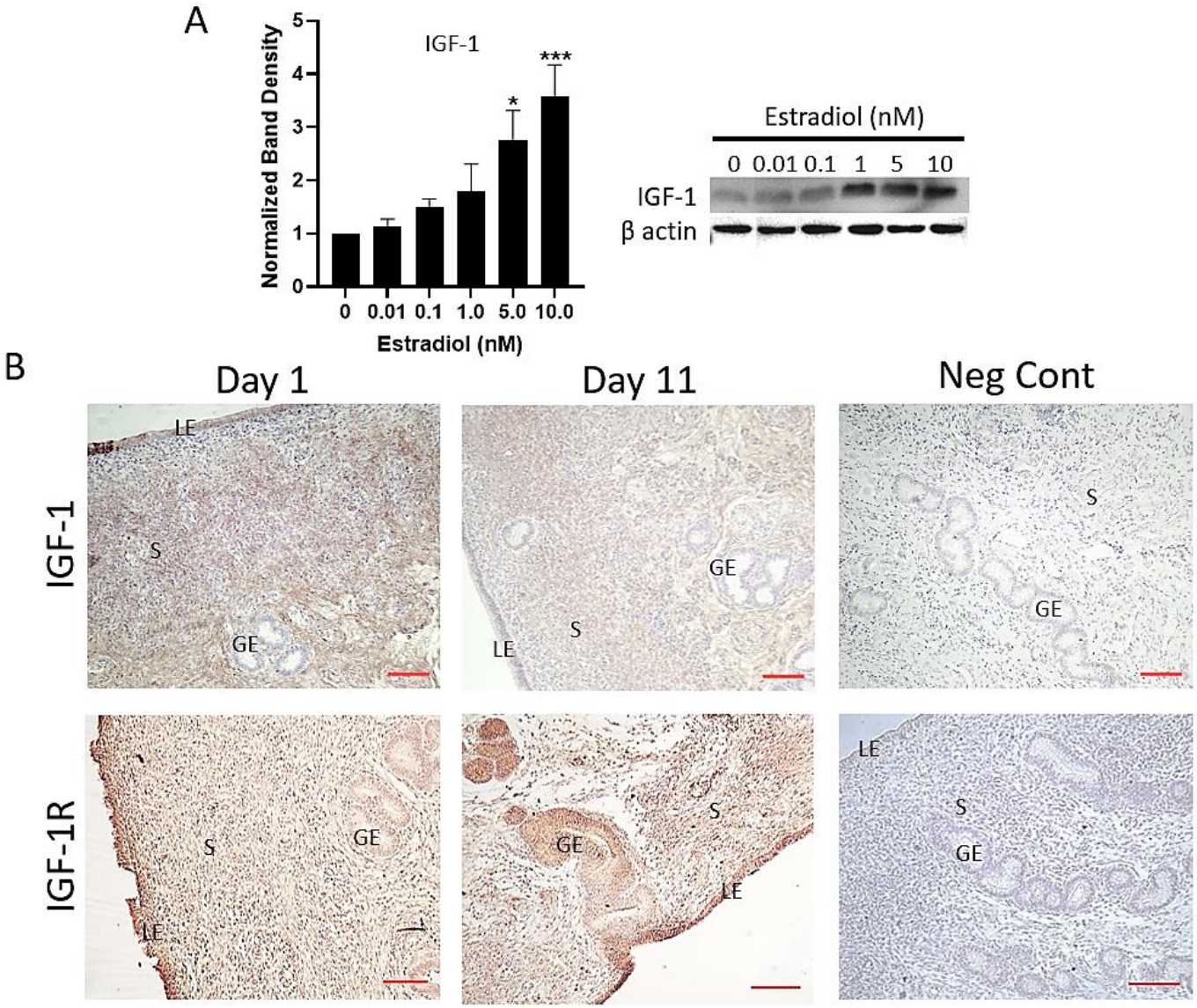

Figure 2. E2 stimulates IGF-1 production in uterine fibroblasts. (A) Normalized band density for IGF-1 in BFIB cells after being treated with E2 at indicated concentrations for 48 hours (left), with representative images (right). The final concentration of DMSO was 0.1% in all treatments. n=5. *P<0.05; ***P<0.001 relative to 0 nM. (B) Immunohistochemistry for IGF-1 and IGF-1R in bovine endometrial samples collected on Day 1 or Day 11 of the reproductive cycle. Images representative of 4 animals. GE, glandular epithelium; LE, luminal epithelial; S, stroma. Neg Cont, Negative Control. Scale bar = 100 µm.

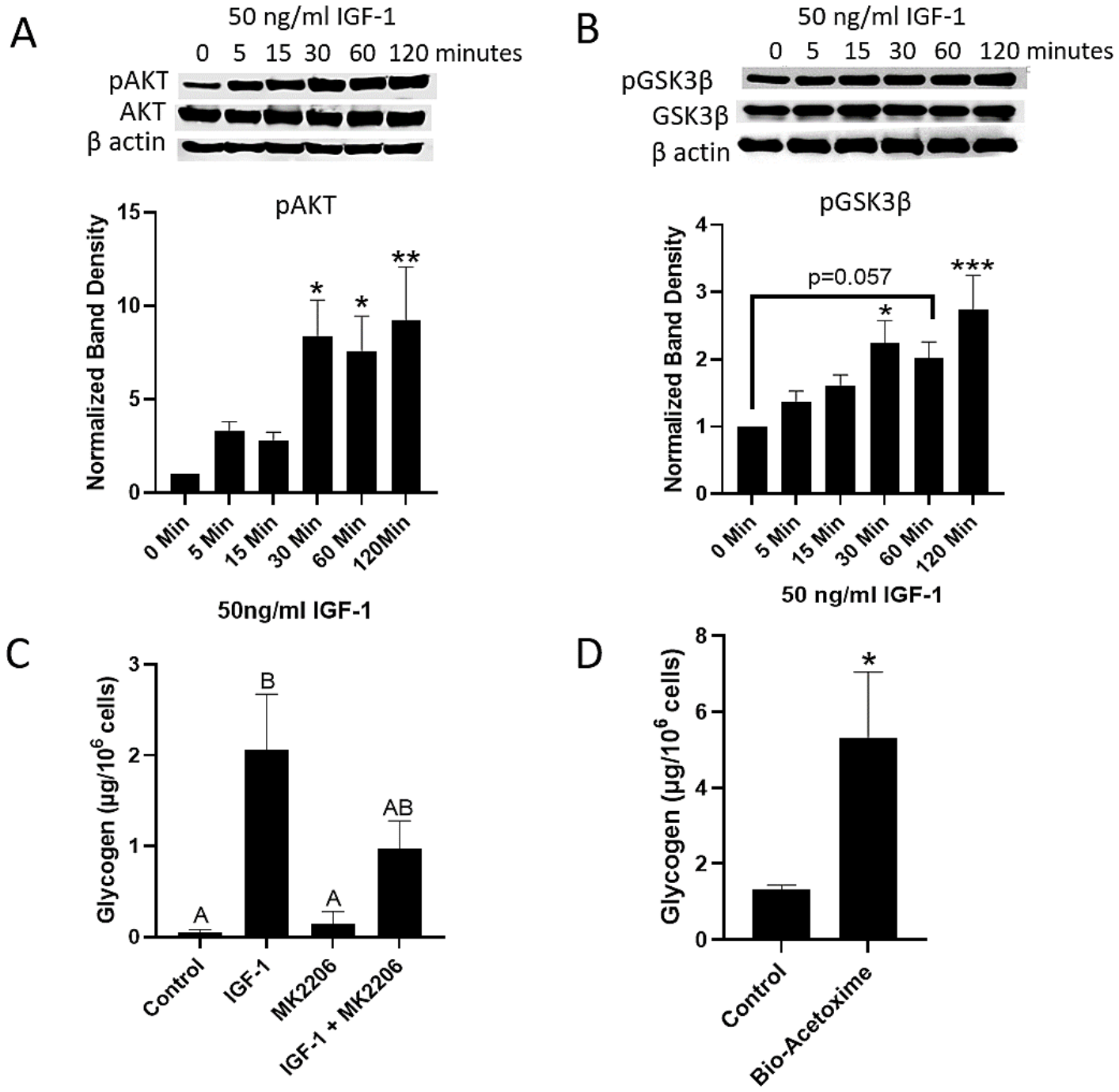

Figure 3. Effects of IGF-1 are partly mediated by the AKT/GSK3β pathway in BUTE cells. (A-B) Levels of phospho-AKT (A) and phospho-GSK3β (B) in BUTE cells treated with IGF-1 (50 ng/ml) for 0, 5, 15, 30, 60, and 120 minutes as determined by western blot. (C-D) Glycogen levels in BUTE cells treated with IGF-1 (50 ng/ml), MK2206 (AKT inhibitor), or Bio-Acetoxime (GSK inhibitor) as indicated. The concentrations of vehicle (DMSO and/or PBS) was the same in all treatments. A, B, D)* $P < 0.05$, ** $P < 0.01$, ***$P<0.001$ relative to control. C)[A-B]Bars without a common letter significantly differ $P<0.05$. n=3-6.

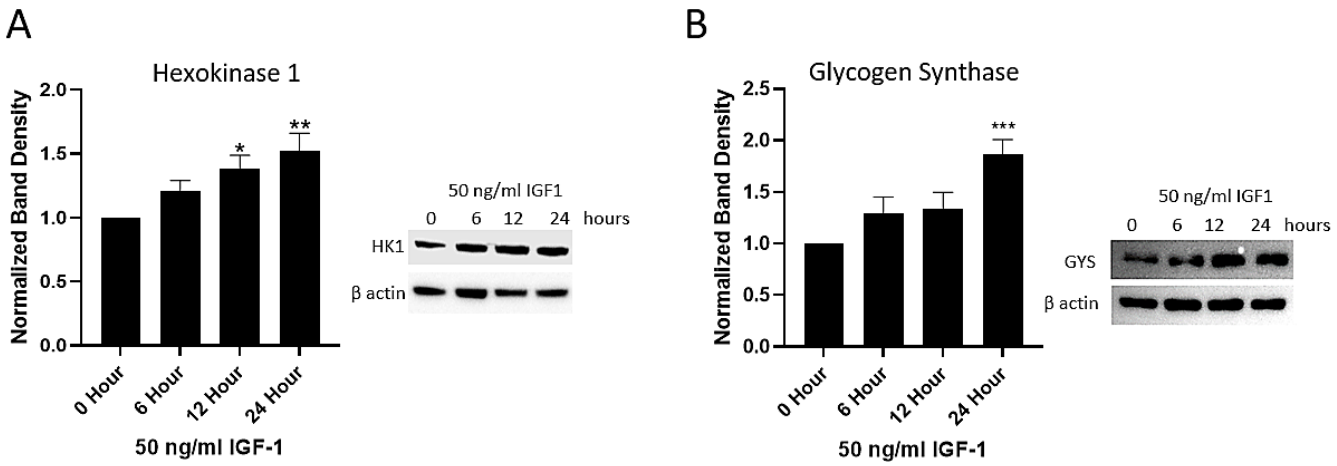

Figure 4. IGF-1 increases expression of glycogen synthesizing enzymes. (A-B) BUTE cells were treated with IGF-1 (50 ng/ml) for 0, 6, 12, and 24 hours and analyzed by western blot for levels of hexokinase 1 (HK1; A) or glycogen synthase (GYS; B). *P < 0.05, **P < 0.01, ***P<0.001 relative to 0 Hour. n= 4-6.

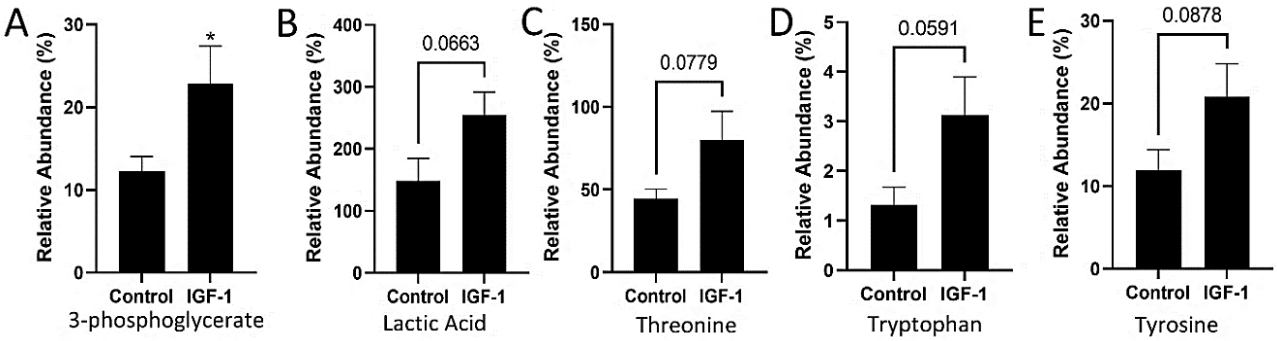

Figure 5. IGF-1 may influence glycolysis in BUTE cells. (A-B) Relative levels of 3-phosphoglycerate (A) and lactic acid (B) in BUTE cells after 48 hours of 50 ng/ml IGF-1 treatment. (C-E) Relative levels of three glycogenic amino acids (threonine, tryptophan, and tyrosine) in BUTE cells after treatment with IGF-1. *P < 0.05 relative to Control. n=6.

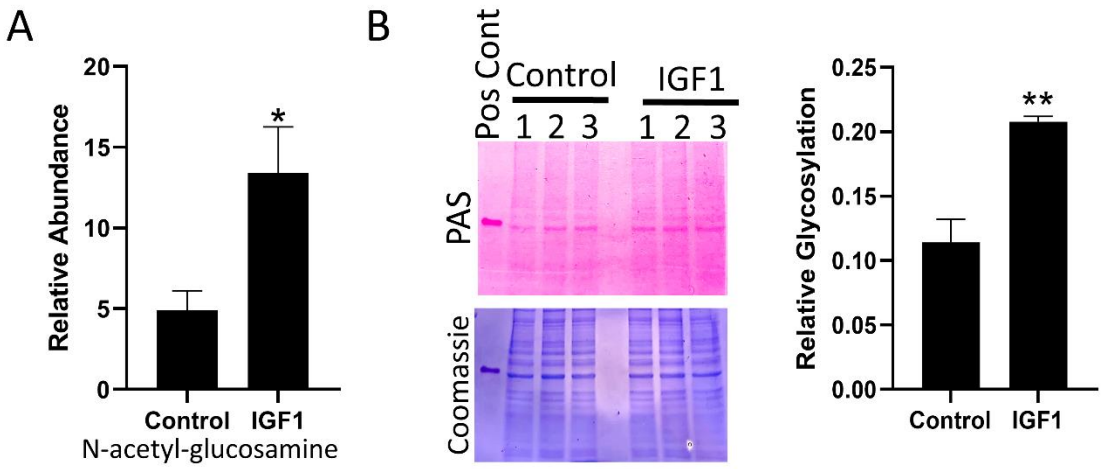

Figure 6. IGF-1 treatment increases protein glycosylation in BUTE cells. (A) Relative levels of N-acetyl-glucosamine in BUTE cells after 48-hour treatment with PBS or 50 ng/ml IGF-1 measured by GC-MS. n=6. (B) SDS-PAGE gel stained with PAS to detect glycosylated proteins and Coomassie Brilliant Blue for total protein content. Densitometry for BUTE cells treated with vehicle or 50 ng/ml IGF-1. n=3. Pos Cont, Positive Control; *P<0.05. **<0.01.

TABLE

Table 1. Primary antibodies and summary of conditions used for western blot and immunohistochemistry.

Antigen	Catalog No. and Company	Technique	Antibody Dilution and Block
IGF-1	A0830 AbClonal	WB	1:250 BSA
		IHC	1:50 Goat Serum Block
β actin	A2066 Sigma Aldrich	WB	1:1000 milk
pAKT	4060 Cell Signaling	WB	1:1000 milk
AKT	4691 Cell Signaling	WB	1:1000 milk
pGSK3β	9323 Cell Signaling	WB	1:1000 BSA
GSK3β	12456 Cell Signaling	WB	1:1000 BSA
pGYS	3891 Cell Signaling	WB	1:1000 milk
GYS	3886S Cell Signaling	WB	1:1000 BSA
IGF-1R	A0243 AbClonal	IHC	1:100 Goat Serum Block
GFP	29356 Cell Signaling	IHC	variable
Hexokinase 1	2024 Cell Signaling	WB	1:1000 BSA

Block consisted of 5% powdered milk or 5% BSA in TBST.

CHAPTER 4: CONCLUSIONS, IMPLICATIONS, & FUTURE RESEARCH

Conclusions

In conclusion, E2 likely stimulates IGF-1 production in the stroma. IGF-1 then stimulates glycogenesis in the adjacent uterine epithelial cells. Overall, it was discovered that E2 and IG-1 promote glycogen synthesis in the bovine endometrium.

Implications

Defects in uterine glycogen storage may lead to reduced fertility in cattle. By increasing glycogen levels during estrus, the uterine epithelium may be better able to provide glucose to the developing blastocyst. Identifying mechanisms regulating glycogen synthesis and catabolism in the bovine uterus may enable further research toward decreasing early embryonic death and global economic loss.

Future Research

With the creation of the BUTE and BFIB cell lines, bovine endometrial research is now easily accessible and feasible. Our study replicated endometrial responses occurring throughout the estrous cycle. Going forward there several studies that could be performed to continue to investigate uterine glycogen metabolism. To investigate increasing glycogen levels during estrus, E2 could be injected before ovulation to enhance glycogen stores or supplement with estradiol-17β before the last gonadotropin-releasing hormone injection of the Ovsynch protocol in lactating dairy cows. One could also explore endometrial glycogen levels in BUTE cells after

treating them with IGF-1, IFNT, and prostaglandin E2 (PGE2). Potentially this would allow researchers to understand glycogen metabolism during elongation. Perhaps further research into the abundance of GLUT5 in the endometrium throughout the estrous cycle and confirm that IFNT increases its expression. In mice, it may be possible to study the temporal upregulation of IGF-1 and the effects on early embryonic glycogen availability. The collection of uterine tissue at different points during pregnancy would also allow for any even greater understanding of how glycogen metabolism changes. Analysis of uterine, placental, embryo, and fetal tissue could provide a more comprehensive picture of the role glycogen plays during pregnancy.

Continued research into glycogen metabolism in the uterus will advance our knowledge of uterine receptivity and conceptus development in cattle. Ultimately, it may contribute to the discovery of strategies to improve reproductive efficiency, minimize embryonic losses, and increase the number of live births.

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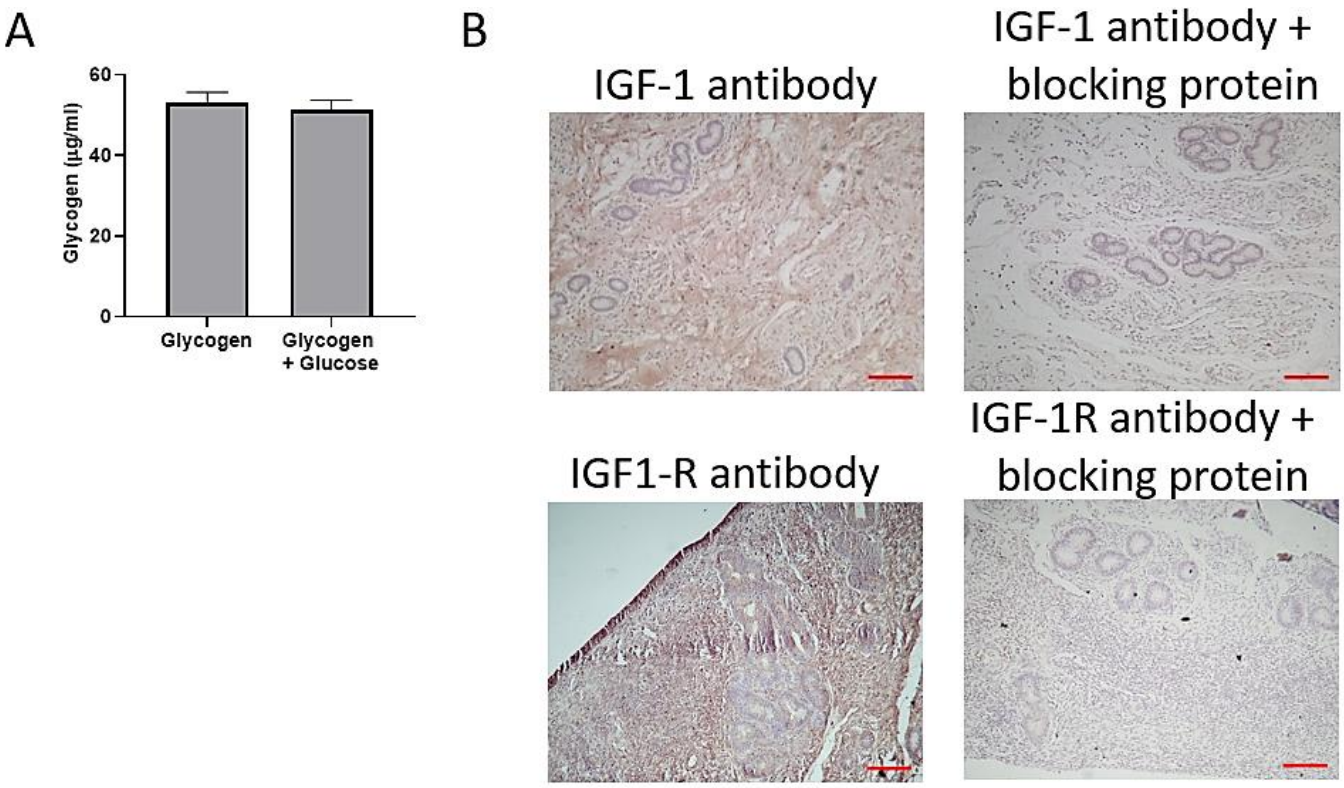
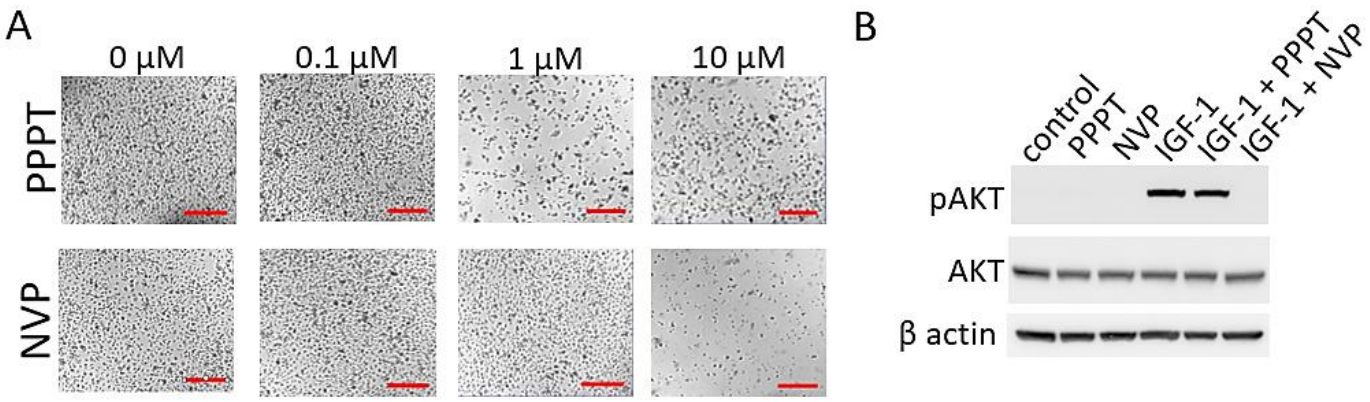
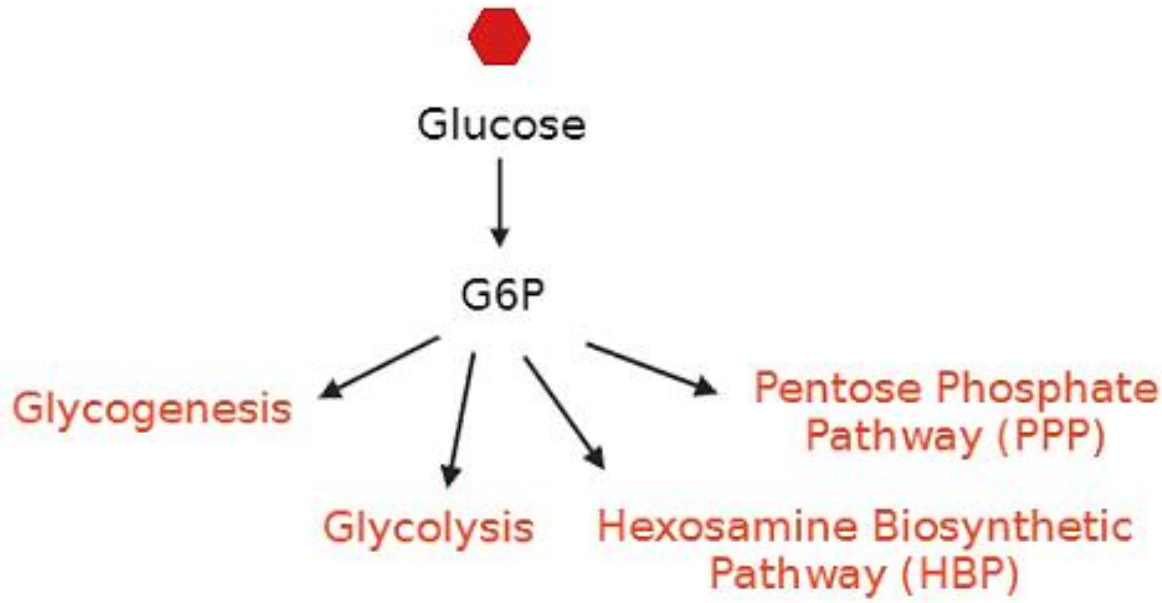
APPENDIX A: SUPPLEMENTAL FIGURES



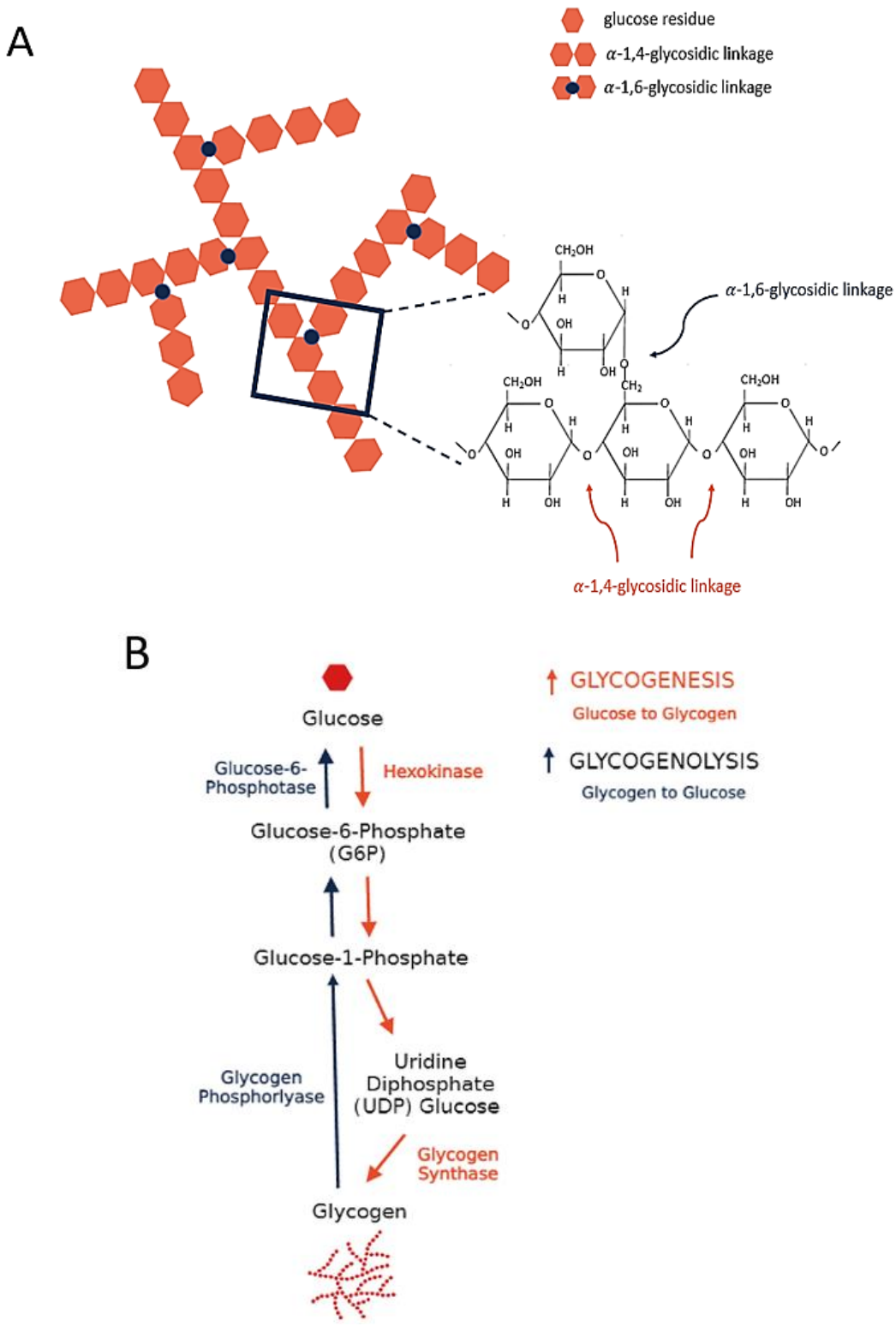
Supplemental Figure 1. Glycogen assay and IHC validation. (A) Glycogen (50 µg/ml) or glycogen + glucose (both at 50 µg/ml) was dissolved in 30% KOH and measured via our glycogen assay. There was no difference in the concentration of glycogen detected. (B) Immunohistochemistry for IGF-1 and IGF-1R using antibodies or antibodies preincubated with the recombinant target protein. Scale bar = 100 µm.



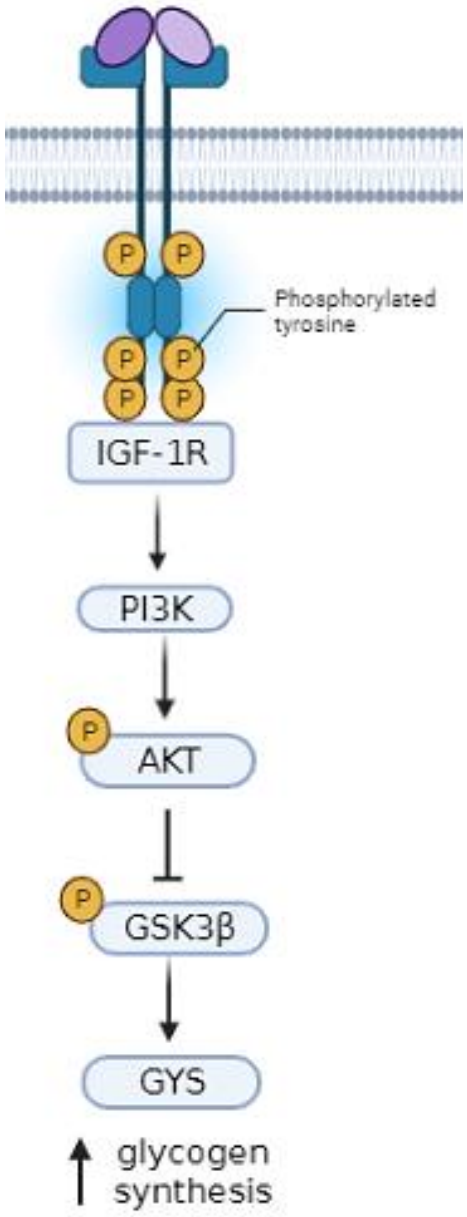
Supplemental Figure 2. Inhibitor validation. (A) Monolayers of BUTE cells after being treated with 0-10 µM picropodophyllotoxin (PPPT) or NVP-ADW742 (NVP) for 48 hours. Scale bar = 500 µM. (B) Western blot for phospho-AKT, AKT, and β actin in BUTE cells treated with 50 ng/ml IGF-1, 0.1 µM picropodophyllotoxin, and 1 µM NVP-ADW742 as indicated for 30 minutes.



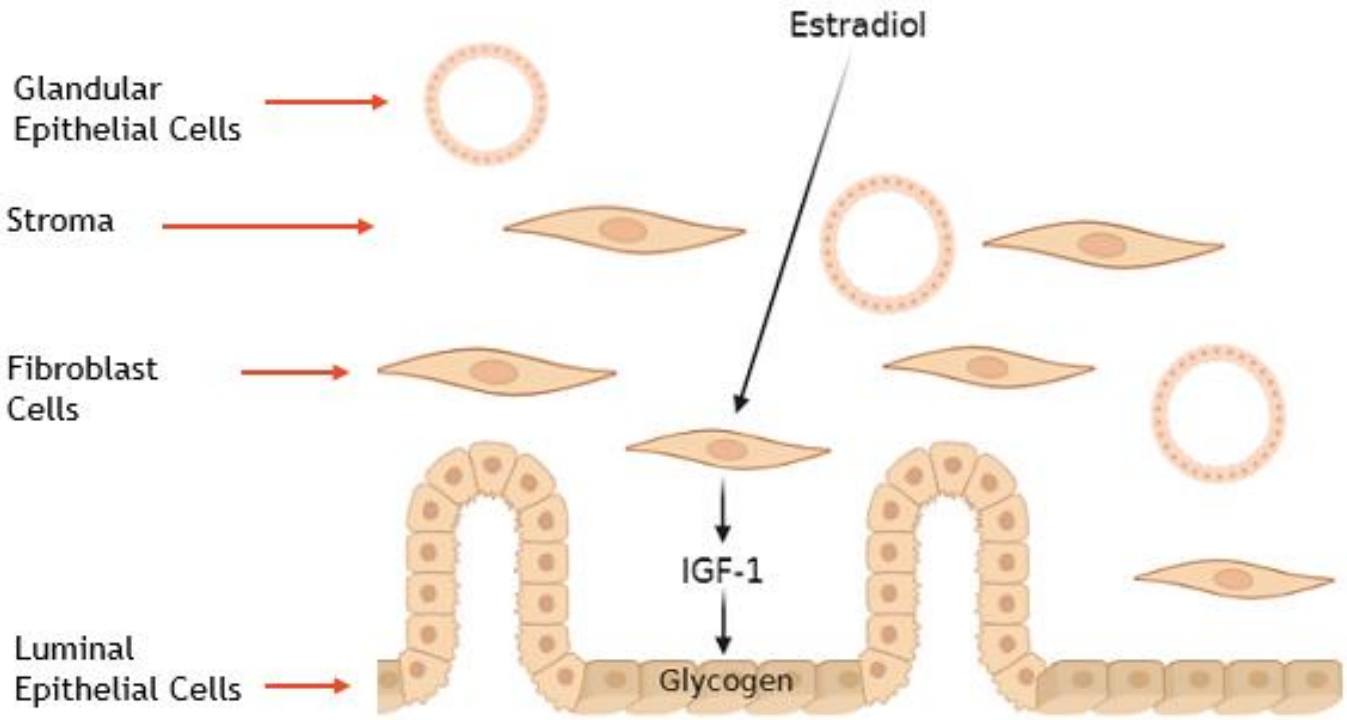
Supplemental Figure 3. Potential fates of glucose. Illustration of glucose converted to G6P and the metabolic pathways that use glucose: Glycogenesis, Glycolysis, HBP, and PPP.



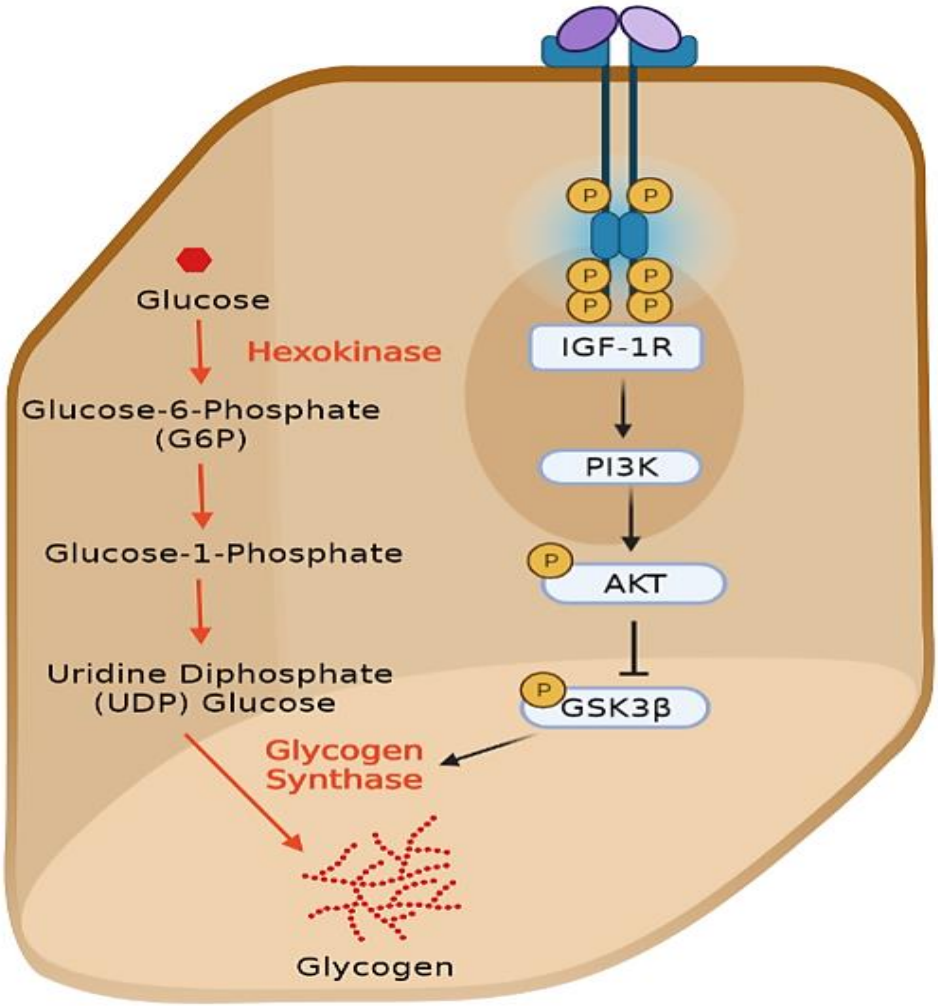
Supplemental Figure 4. Glycogen formation and metabolism. (A) Formation of a glycogen molecule connected by different glycosidic linkages. (B) A single glucose molecule synthesized into glycogen and glycogen catabolized into glucose.



Supplemental Figure 5. Activation of glycogen synthesis. IGF-1 binds to the IGF-1R, initiating downstream signaling and phosphorylation of AKT. pAKT then phosphorylates GSK3β. pGSK3β is inhibited from phosphorylating GYS, allowing GYS to remain active. Active GYS allows glycogen synthesis to occur. ⟶ activates/increases activity, ⟞ inhibits/decreases activity.



Supplemental Figure 6. Proposed stromal-epithelial cell signaling in the endometrium that resulted in increased glycogen stores in the epithelium. E2 stimulates IGF-1 production in the uterine stroma. IGF-1 then stimulates glycogen synthesis in uterine epithelial cells.



Supplemental Figure 7. Mechanisms of glycogen synthesis. Detailed illustration of mechanisms in which IGF-1 stimulates glycogen synthesis within the uterine epithelial cells.

APPENDIX B: SUPPLEMENTAL TABLES

Supplemental Table 1. Composition of αMEM media.

Component	Concentration (mg/L)
2'-Deoxyadenosine	10
2'-Deoxycytidine	11
2'-Deoxyguanosine	10
Adenosine	10
Biotin	0.1
Calcium Chloride	200
Choline Chloride	1
Cytidine	10
D-Calcium Pantothenate	1
D-Glucose	1,000
Folic Acid	1
Glycine	50
Guanosine	10
L-Alanine	25
L-Arginine	127
L-Ascorbic acid	50
L-Asparagine	50
L-Aspartic acid	30
L-Cysteine	100
L-Cystine	31
L-Glutamine	292
L-Histidine	42
L-Isoleucine	52
L-Leucine	52
L-Lysine	73
L-Methionine	15
L-Phenylalanine	32
L-Proline	40
L-Serine	25
L-Threonine	48
L-Tryptophan	10
L-Tyrosine	52
L-Valine	46
Lipoic Acid	0.2
Magnesium Sulfate	98
Myo-Inositol	2
Niacinamide	1
Phenol Red	10
Potassium Chloride	400
Pyridoxal	1
Pyruvic acid	110
Riboflavin	0.1
Sodium Chloride	6,800
Sodium Phosphate	140
Thiamine	1
Thymidine	10
Uridine	10
Vitamin B12	1.4
Sodium Bicarbonate	2,200

Supplemental Table 2. All metabolites identified by GC-MS.

relative concentration/cell count	BUTE DMSO 7/24 F1A	BUTE IGF-1 7/24 F1A	BUTE DMSO 7/25 F1B	BUTE IGF-1 7/25 F1B	BUTE DMSO 7/27 F1A	BUTE IGF-1 7/27 F1A	BUTE DMSO 7/27 F1B	BUTE IGF-1 7/27 F1B	BUTE DMSO 7/28 F2A	BUTE IGF-1 7/28 F2A	BUTE DMSO 7/28 F2B	BUTE IGF-1 7/28 F2B
1,6-Anhydroglucose	4.32	5.91	2.93	5.65	4.73	5.89	3.74	0.70	3.28	2.10	2.10	2.59
10-Heptadecenoic acid	3.59	ND	ND	ND	ND	ND	3.44	0.34	2.83	6.70	1.26	1.83
10-Nonadecenoic acid	1.58	ND	ND	ND	ND	ND	2.87	ND	ND	1.19	1.71	1.97
11,14,17-Eicosatrienoic acid	35.38	ND	ND	ND	ND	ND	30.31	ND	11.81	17.42	ND	ND
11,14-Eicosadienoic acid	21.70	ND	ND	ND	ND	4.61	10.89	ND	5.39	16.15	6.91	13.05
11-Eicosenoic acid	48.35	10.03	ND	9.23	ND	27.28	30.29	7.00	16.47	32.31	21.23	27.51
13-Docosenoic acid	6.25	ND	ND	ND	ND	3.72	7.25	1.02	4.75	4.35	6.32	6.14
15-Tetracosenoic acid	4.59	ND	ND	ND	ND	ND	4.41	ND	1.56	ND	4.89	4.14
1-Monohexadecanoylglycerol	24.92	11.08	3.00	9.71	2.93	7.11	7.18	2.32	3.90	11.82	7.53	6.66
1-Monooctadecanoylglycerol	18.09	22.84	3.85	10.30	1.43	9.07	10.19	2.57	1.63	12.06	7.23	6.37
1-O-Hexadecenylglycerol	19.30	ND	ND	ND	ND	ND	5.40	ND	0.81	5.56	2.08	3.81
1-Oleylglycerol	18.96	11.49	ND	ND	ND	4.73	6.48	0.97	2.31	10.12	4.37	2.48
1-O-Octadecylglycerol	91.52	93.05	52.98	103.15	79.54	69.71	54.95	33.11	56.13	100.42	71.63	62.78
2-Amino-4,6-dihydroxypyrimidine	ND	1.08	ND	ND	1.09	ND	ND	ND	ND	ND	ND	ND
2-Aminoadipic acid	1.38	1.92	1.01	2.15	ND	0.28	0.45	ND	ND	0.62	0.73	0.36
2-Hexadecanoylglycerol	ND	ND	ND	ND	ND	5.46	ND	2.02	ND	10.57	ND	ND
2-Methyl-1,2-butanediol	1.78	2.68	ND	2.78	ND	1.18	1.05	0.86	ND	1.47	ND	1.05
2-Methylsuccinic acid	5.75	ND	ND	ND	ND	ND	5.31	1.16	2.87	0.32	ND	3.85
2-Pyrrolidinone, 1-(9-octadecenyl)	13.19	4.88	ND	ND	ND	5.90	10.45	0.73	ND	14.58	3.06	5.79
3-Hydroxyybutanoic acid	1.52	2.55	ND	0.58	3.80	0.92	0.27	0.27	ND	ND	ND	0.71
3-Phosphoglycerate	20.38	40.43	10.47	23.82	13.83	25.98	9.62	8.56	10.08	25.10	9.35	13.02
4,7,10,13,16,19-Docosahexaenoic acid	37.87	ND	ND	ND	ND	ND	44.39	ND	ND	ND	34.66	ND
5,8,11,14,17-Eicosapentaenoic acid	ND	ND	11.24	ND	ND	ND	15.06	5.41	ND	11.45	6.95	17.15
5,8,11,14-Eicosatetraenoic acid	113.14	40.25	10.56	28.79	6.66	37.82	76.70	16.80	33.55	63.87	46.44	56.73
7,10,13,16-Docosatetraenoic acid	10.69	ND	ND	ND	ND	ND	ND	ND	ND	2.42	0.77	1.04
9,12-Octadecadienoic acid	36.17	6.27	10.30	10.52	32.23	12.32	10.63	4.39	9.54	17.13	14.45	26.67
Adenine	3.21	2.64	ND	2.53	ND	2.19	1.70	0.63	ND	2.50	1.23	1.40
Adenosine	34.24	31.68	ND	22.71	ND	10.20	4.86	2.99	6.58	11.49	11.49	8.97
Adenosine-3-monophosphate	5.73	41.21	ND	10.16	ND	3.03	ND	ND	ND	0.67	ND	1.37
Adenosine-5-monophosphate	71.64	463.28	67.33	411.20	87.49	112.23	7.23	36.25	40.91	66.01	53.15	41.58
Adipic acid	8.51	ND	ND	ND	ND	ND	5.47	ND	ND	6.72	4.19	10.13
Alanine	128.57	221.91	233.84	378.97	292.38	118.49	34.17	22.61	26.97	151.71	102.91	86.88
Aminomalonic acid	1.86	ND	ND	9.02	ND	1.60	1.06	0.81	0.24	ND	1.09	1.97
Arginine	ND	ND	ND	ND	ND	0.99	ND	0.29	ND	0.38	ND	ND
Aspartic acid	621.04	1125.47	416.08	1023.96	188.06	440.83	313.57	166.42	202.90	599.98	392.03	350.85
Azelaic acid	5.30	1.00	ND	ND	0.62	2.15	4.07	1.12	2.03	3.99	3.50	3.73
b-Alanine	4.70	5.30	ND	ND	ND	3.84	2.84	1.85	1.32	2.79	1.19	2.91
Benzene-1,2,4-triol	2.19	ND	ND	ND	ND	0.75	1.19	ND	0.95	1.83	1.18	1.14
Benzoic acid	10.11	6.62	4.79	4.75	15.31	2.97	5.56	1.80	2.62	4.45	3.48	5.79
Benzoic acid, 2-methyl	6.80	6.87	1.22	2.31	23.67	3.60	1.65	0.68	0.79	2.06	0.80	2.09
Butanoic acid, 3-methyl-3-hydroxy	20.82	20.73	6.30	ND	38.78	10.78	3.09	3.28	2.71	5.43	1.36	9.93
C16:1 #1	55.97	ND	ND	ND	ND	17.15	32.98	5.63	21.19	28.01	17.71	28.53
C16:1 #2	79.23	25.34	4.07	11.49	3.89	24.77	55.49	10.45	24.31	47.04	32.02	40.39
C16:1 #3	5.25	ND	ND	ND	ND	1.03	4.58	0.53	1.58	2.31	2.16	2.89
C18:1 (11) [Vaccenic acid]	327.80	109.74	40.10	72.18	35.30	114.68	231.05	67.13	128.55	227.57	159.47	184.71
C18:1	10.85	1.90	ND	ND	ND	ND	14.95	3.48	6.73	5.82	5.97	6.56
C18:1 (9) [Oleic acid]	1088.39	382.68	188.63	342.92	143.07	405.46	719.25	218.94	450.06	763.08	545.41	645.75
C18:2	11.87	ND	ND	ND	ND	ND	9.43	2.77	3.83	6.86	9.60	5.85
Cholecalciferol	215.99	268.49	66.05	293.75	71.50	107.99	114.40	46.44	84.84	210.68	116.36	87.10
Cholesta-3,5-diene	ND	ND	ND	ND	ND	13.52	8.34	1.89	7.12	ND	9.41	ND
Cholesterol	3143.79	4576.73	2354.96	4290.32	3010.59	3531.88	1924.07	1255.65	1352.47	3690.54	1649.92	1843.68
Citraconic acid	10.74	1.64	0.96	2.26	4.05	5.38	9.09	3.68	4.10	9.92	3.90	5.01
Citric acid	39.98	205.38	63.97	202.83	148.56	36.13	15.02	10.30	11.15	44.31	18.13	16.53
Cytindine-5'-monophosphate	0.63	4.95	1.10	5.39	1.77	1.50	ND	0.49	0.53	0.72	0.75	0.48
Decanoic acid	1.48	ND	ND	ND	ND	0.86	0.69	ND	ND	ND	0.25	ND
Diethyleneglycol	7.85	8.44	7.49	9.15	7.38	5.03	4.36	3.07	5.71	8.14	5.29	4.69
Docosanoic acid	32.22	12.10	4.08	10.41	4.63	9.48	16.29	5.08	11.51	18.83	14.42	14.39
Dodecanoic acid	11.46	5.46	2.25	6.10	5.48	5.92	7.20	1.86	2.95	5.97	4.21	5.89
Eicosanoic acid	53.00	24.13	8.45	20.30	13.01	22.90	39.06	9.87	20.13	32.50	24.36	30.62
Eicosanol	0.77	ND	ND	ND	ND	0.30	0.49	0.28	0.25	1.03	ND	0.27
Erythronic acid	1.42	2.18	0.81	3.41	ND	ND	ND	ND	ND	ND	ND	ND
Ethanolamine	321.77	268.08	314.36	246.46	282.20	177.10	147.09	140.84	182.51	320.96	181.71	247.75
Ethanolamine-glycerophosphate	21.19	33.93	8.00	24.99	7.97	14.65	8.27	5.00	7.06	19.73	10.95	9.52
Fructose	2.28	1.45	0.71	0.94	8.40	1.29	ND	0.45	0.42	1.10	0.23	1.21
Fumaric acid	3.65	8.07	3.36	12.04	7.43	3.34	2.37	2.19	3.08	5.00	1.93	2.78
Glucose	55.97	19.29	10.22	9.96	34.17	12.92	8.41	4.13	24.91	28.61	15.41	8.82
Glucose, 2-amino-2-deoxy	3.41	5.27	ND	4.43	ND	5.07	ND	ND	0.62	ND	2.16	ND
Glucose-6-p	ND	3.65	ND	2.76	1.79	3.03	0.88	0.51	ND	0.87	ND	0.82
Glutamic acid	378.22	477.65	183.46	570.49	8.35	217.91	182.12	84.45	77.17	387.39	224.68	169.93
Glutamine	381.57	851.73	338.93	962.65	362.13	333.94	214.70	134.99	175.23	371.71	253.81	252.32
Glutaric acid	3.20	ND	ND	ND	ND	1.08	4.03	0.58	1.70	2.68	2.73	3.32
Glutaric acid, 2-hydroxy	1.80	ND	ND	4.44	ND	0.57	0.33	ND	0.07	ND	ND	ND
Glycerol	5.81	ND	23.94	34.14	28.03	9.06	11.97	10.50	8.77	18.11	17.80	10.69
Glycerol-2-P	ND	ND	ND	ND	ND	ND	0.23	0.51	ND	0.57	0.41	ND
Glycerol-3-p	23.25	54.28	21.47	56.81	54.63	41.84	18.67	18.85	9.04	34.88	17.64	21.72
Glycerone phosphate	ND	ND	ND	ND	ND	0.27	0.15	0.06	ND	0.28	ND	0.13
Glycine	220.00	325.92	125.43	387.70	120.92	174.12	124.30	72.31	71.68	224.21	137.37	130.17
Glycolic acid	19.89	21.07	15.40	19.62	21.37	16.78	10.47	7.61	11.65	14.91	7.99	15.99
Glyoxylic acid	30.79	75.05	60.76	100.23	108.61	38.87	29.11	15.65	36.32	31.67	21.72	26.55

Supplemental Table 2 (cont.).

relative concentration/cell count	BUTE DMSO 7/24 F1A	BUTE IGF-1 7/24 F1A	BUTE DMSO 7/25 F1B	BUTE IGF-1 7/25 F1B	BUTE DMSO 7/27 F1A	BUTE IGF-1 7/27 F1A	BUTE DMSO 7/27 F1B	BUTE IGF-1 7/27 F1B	BUTE DMSO 7/28 F2A	BUTE IGF-1 7/28 F2A	BUTE DMSO 7/28 F2B	BUTE IGF-1ssdf 7/28 F2B
Heptadecanoic acid	4543.92	59.29	41.76	67.52	54.78	321.01	4247.34	244.36	632.26	1506.98	1470.41	2045.82
Heptanoic acid	0.94	ND	0.64	ND	ND	ND	0.09	0.04	ND	0.81	0.74	ND
Hexacosanoic acid	1.04	ND	ND	ND	ND	ND	2.88	ND	ND	0.66	ND	ND
Hexadecanol	5.33	6.73	1.41	4.64	2.78	5.44	3.55	2.14	2.81	6.62	3.39	4.32
Hexoanic acid	2.55	4.07	6.05	5.19	4.81	1.45	1.51	2.88	4.38	1.99	4.91	2.60
Inositol, #1	15.06	ND	ND	ND	ND	ND	10.96	ND	ND	ND	ND	5.65
Inositol, #2	0.92	1.73	ND	1.75	ND	0.59	0.29	0.19	ND	0.62	0.47	ND
Inositol, myo	143.39	226.40	88.95	306.61	93.79	135.91	92.28	56.58	55.97	154.13	119.96	108.95
Inositol-p	181.22	520.04	188.04	486.02	239.36	177.55	42.21	64.30	79.79	187.96	85.94	74.50
Isoleucine	5.10	8.07	ND	5.97	ND	4.14	13.45	1.53	1.74	6.97	2.76	2.18
Itaconic acid	ND	3.56	6.28	5.76	9.09	ND	ND	ND	ND	ND	ND	ND
Lactic acid	276.15	279.71	121.84	413.29	244.34	269.76	108.32	171.78	75.25	216.41	63.97	178.96
Lactose	1165.02	1809.26	682.32	1158.11	1014.94	616.16	530.51	267.60	330.34	882.85	585.66	616.81
Lanosterol	416.32	611.06	279.02	644.08	250.04	238.80	262.12	88.37	208.22	429.24	273.14	208.51
Leucine	8.40	12.23	ND	14.38	ND	10.14	8.58	4.14	5.09	9.75	5.53	5.68
Lysine	78.74	87.98	26.46	74.02	18.86	53.85	39.04	23.84	19.04	71.07	37.05	30.80
Malic acid	33.92	82.22	25.31	125.64	53.29	31.85	18.37	14.64	10.91	45.67	19.14	17.62
Maltitol	11.18	6.92	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Mannose	0.54	ND	ND	ND	ND	0.91	0.23	ND	ND	0.52	0.27	ND
Mimosine	66.32	161.31	21.91	106.27	30.69	61.87	24.28	17.19	15.80	48.26	21.49	23.51
Monomethylphosphate	150.75	134.79	42.09	51.96	22.39	76.32	35.44	20.52	40.60	101.68	58.07	51.37
N-acetyl-D-glucosamine	9.88	10.55	1.82	11.29	5.02	24.12	4.11	7.98	2.35	19.88	6.24	6.60
N-Acetylglutamic acid	122.60	189.64	51.02	272.47	36.62	90.96	65.71	34.45	32.32	116.94	76.50	67.51
Nicotinamide	9.96	ND	ND	11.57	ND	10.74	7.22	3.88	3.87	8.58	6.61	5.16
Nonadecanoic acid	11.45	ND	ND	ND	ND	ND	8.38	ND	3.04	4.18	4.37	5.51
Nonanoic acid	ND	ND	ND	ND	ND	4.04	ND	4.15	26.60	ND	4.05	9.88
Octadecanol	18.53	25.75	6.66	29.31	34.43	23.93	15.00	7.56	10.43	22.72	11.55	19.66
Oleamide	8.52	10.79	ND	ND	ND	1.55	1.06	ND	ND	8.64	1.84	2.99
O-phosphocolamine	86.53	177.58	56.05	163.01	56.43	67.57	22.94	24.67	29.98	71.64	35.70	34.37
O-phospho-L-serine	ND	0.88	ND	0.87	ND	0.05	ND	ND	ND	ND	ND	ND
Ornithine	2.83	ND	ND	ND	ND	2.33	1.53	1.44	1.05	3.10	1.66	1.80
Oxalic acid	10.79	21.62	ND	24.85	19.35	8.37	4.40	3.55	5.83	7.48	6.43	5.76
Palmidrol	7.55	8.18	ND	ND	ND	ND	4.14	4.13	ND	6.30	1.64	5.80
Palmitic acid	3227.90	1279.16	1248.38	1570.94	1930.72	1733.18	2939.60	682.83	1382.48	1870.05	1630.90	2094.47
Pentadecanoic acid	7.62	3.45	2.57	3.71	3.18	5.10	8.02	1.64	5.59	4.71	4.47	4.87
Phenylalanine	12.47	17.68	5.15	10.16	ND	6.88	6.66	3.60	5.43	17.74	6.11	4.37
Phosphoenolpyruvate	2.26	6.48	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Phosphoglycolic acid	ND	ND	ND	ND	ND	1.53	0.96	0.64	1.42	2.02	0.57	1.18
Proline	6.82	9.51	ND	15.77	ND	6.39	6.12	2.24	2.72	8.29	7.15	4.30
Propan-1,2-diol	8.28	3.22	2.95	1.81	46.97	5.08	1.57	2.80	4.56	4.63	3.06	4.74
Putrescine	27.42	46.66	10.72	55.48	10.78	17.33	12.10	6.80	5.85	30.15	13.19	15.22
Pyrophosphate (4:1)	307.60	106.80	19.47	60.94	21.64	186.19	156.89	92.55	213.92	221.26	204.75	182.41
Pyrrole-2-carboxylic acid	1.56	ND	1.85	2.33	6.87	1.97	0.91	0.98	1.52	1.54	0.74	1.35
Pyruvic acid	3.86	3.40	2.07	2.77	1.82	1.83	1.28	0.68	0.99	1.79	1.12	1.38
Ribose-5-p	4.52	4.80	ND	3.07	1.30	2.25	3.46	0.56	0.93	1.98	ND	1.89
Serine	21.51	30.05	8.12	34.32	7.55	16.52	17.53	7.53	7.15	18.17	13.60	8.59
Sorbitol	ND	1.68	0.60	1.90	0.80	ND	ND	ND	ND	ND	0.91	0.83
Spermidine	34.00	70.15	12.10	72.23	2.32	8.15	3.27	5.33	7.32	27.67	9.83	10.15
Stearic acid	3045.85	1114.84	1079.61	1358.04	1651.90	1493.52	2712.91	620.18	1286.44	1716.62	1542.67	1957.65
Suberic acid	3.31	ND	ND	ND	ND	ND	3.06	0.36	2.13	2.26	1.24	2.90
Succinic acid	54.92	43.74	27.40	65.92	24.12	33.23	42.01	18.55	23.75	53.86	39.87	37.39
Sucrose	29.80	3.46	9.05	4.15	33.20	14.16	ND	ND	ND	ND	ND	ND
Tetracosanoic acid	21.08	11.66	3.63	9.93	4.80	8.72	11.23	4.24	8.26	15.25	10.84	10.36
Tetradecanoic acid	1300.40	61.07	33.51	56.95	48.53	119.77	1311.03	74.59	174.94	393.82	391.76	572.40
Threonic acid	ND	ND	ND	ND	11.05	24.41	18.79	14.85	1.87	13.76	5.00	8.15
Threonine	67.92	98.09	35.26	150.51	40.45	66.25	46.56	31.86	28.91	85.91	48.92	48.10
Thymine	0.13	ND	ND	ND	ND	ND	0.42	ND	0.16	ND	ND	ND
Thymol	17.10	18.92	4.47	23.69	8.96	17.88	11.10	6.23	7.90	11.51	2.78	8.66
Tryptophan	2.59	6.00	2.00	4.21	0.89	2.56	0.66	1.20	0.26	3.66	1.49	1.11
Tyrosine	22.33	32.08	7.98	27.82	7.92	19.52	14.67	8.06	6.03	26.66	12.53	10.60
Uracil	1.51	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.74	ND
Urea	32.75	14.84	8.78	ND	4.90	6.31	4.92	5.83	9.63	0.42	ND	1.88
Uric acid	ND	2.11	ND	0.57	ND	0.18	ND	ND	ND	ND	ND	ND
Uridine	ND	ND	ND	ND	ND	4.56	ND	ND	ND	ND	1.53	ND
Uridine-5'-monophosphate	10.23	38.42	16.11	20.98	ND	6.68	ND	2.30	0.93	7.69	ND	ND
Valine	13.72	17.23	2.51	20.40	0.67	12.86	10.02	5.53	7.43	16.87	10.21	2.29