EXPLORING THE EFFECTS OF INSULIN AND GLUCOSE ON THE DEVELOPMENT OF IN VITRO PRODUCED DOMESTIC CAT EMBRYOS

BY

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THESIS

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Abstract

It is possible to evaluate the effects of glucose and insulin on feline embryonic development using in vitro fertilization and embryo culture. The objective of this study was to determine if glucose concentrations greater than \( \geq 6 \) mM with or without varying concentrations of insulin would affect cleavage or blastocyst development of feline embryos. We hypothesized that high concentrations of glucose or insulin in culture media would result in a decrease in the proportion of embryos that cleaved and formed blastocysts. Our findings show that 24 mM D-glucose was inhibitory (\( P>0.05 \)) to the early feline embryo in two of three experiments. These effects of glucose were not due to osmotic changes in the media because the same concentration of L-glucose (not taken up by the embryo) did not produce the same effects. The negative effects of glucose were also more pronounced when excess glucose was present as 2-deoxyglucose, which can be metabolized to 2-deoxy glucose 6 phosphate, but not further. From the literature, it was expected the lower doses of insulin to be stimulatory and the higher doses to be inhibitory. In this study, exactly the opposite was found to be observed in feline embryos. There is not much information about insulin signaling in the feline blastocyst, and it is possible that our data was in part due to a response specific to the domestic cat. The concentrations of glucose and insulin in oviductal and uterine fluid are related to plasma concentrations so that metabolic conditions such as insulin resistance, hyperglycemia, and type 2 diabetes that affect circulating concentrations of glucose and insulin may also affect the development of feline embryos in vivo.
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“There are two means of refuge from the misery of life - music and cats.”

— Albert Schweitzer
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CHAPTER 1: General Introduction

**Obesity**

Obesity is described as an disproportionate storage of energy as fat (Chen, Simar et al. 2009). In the United States, 54.9% of the population is considered obese (Reda, Geliebter et al. 2002). This disease has also become a prevalent issue in many of our domestic animal companions, including the cat. It is estimated that 35%-50% of the domestic cat population is considered to be overweight or obese (Brennan, Hoenig et al. 2004). A host of factors may predispose an individual to weight gain and resulting obesity; including genetics, the amount of physical activities, as well as the contents of the diet. There are several devastating diseases associated with obesity, especially in cats, including a 3 to 5 fold increased risk for the development of type 2 diabetes mellitus.(Brennan, Hoenig et al. 2004). Today, 1.2% of cats are considered to be type 2 diabetic (McGarry 2002; Hoenig 2012) and about 5% of the human population also suffers from type 2 diabetes.

**Obesity and the Progression to Type 2 Diabetes**

The cat is an important and unique model for the study of obesity and its progression to type 2 diabetes because the cat shows very similar alterations in glucose and insulin metabolism as people. Obesity has previously been characterized by both a change in insulin action and secretion (Hoenig 2012). Obese cats, like humans also typically experience an increase in the excess fat stored within and around the muscle tissues(Wells 2012). This increase in intra-myocellular and extra-myocellular fat in both humans and cats has been associated with a loss of insulin sensitivity, or the amount of insulin required to lower blood glucose (Hoenig 2006). A rise in body mass index (BMI) of about ~50% in cats, leads to up to a 60% reduction in insulin sensitivity (Hoenig 2002; Hoenig 2006; Kley 2009; Hoenig 2012). Glucose uptake into tissues is also altered by obesity which can cause insulin resistance and hyperglycemia (Leibowitz, Glaser et al. 1995; Hoenig 2002). Insulin resistance (IR) frequently co-occurs with obesity (Curhan, Chertow et al. 1996). IR has previously been defined as the impaired ability of insulin to suppress glucose output from the liver and to promote peripheral glucose disposal (Brennan, Hoenig et al. 2004) (McGarry 2002). In a non-compromised metabolism, the liver’s function is to secrete glucose from the bloodstream and store it as glycogen, or discharge it into the
bloodstream where other tissues can take it up for energy. However, in the pathophysiology of insulin resistance, through a network of signaling pathways; insulin becomes less effective at diverting glucose down its various pathways therefore lowering blood glucose (Chen, Simar et al. 2009). In a state of prolonged IR, the liver and peripheral tissues also become resistant to the action of insulin. Hyperglycemia is also known to develop when the formation of glucose by the body from substrates contributing to blood glucose (BG) levels increases also leading to an increase in endogenous glucose production (EGP) (Dickson, Hewett et al. 2013). In the long run this can precede type 2 diabetes the causes being a resistance to the activity of insulin and an insufficient compensatory insulin response. Long term hyperglycemia can be adequate to cause pathologic effects in several tissues and sometimes occurs with no clinical symptoms for an extended period of time before type 2 diabetes is diagnosed. Throughout this “asymptomatic” phase, an irregularity can become evident in carbohydrate metabolism. This can be tested by measuring plasma glucose after fasting, or, with a challenge with an oral glucose load challenge (Association 2010). When insulin resistance exists, an excess of insulin needs to be produced by the beta cells of the pancreas. Blood glucose concentrations can continue to increase if this compensatory increase in insulin does not occur, (Reaven 1988). This places a long-standing stress on beta cells to secrete extra insulin. This is thought to eventually lead to “exhaustion” of the beta cells and subsequent failure of insulin secretion (Reaven 1988). Insulin secretion can also become abnormal over time (Hoenig 2012). As long as the pancreas can supply enough insulin, even insulin resistant patients can sustain normal blood glucose levels and this has been shown in a number of cases (Hoenig 2002). The existence of insulin resistance can be identified some time prior to the onset of hyperglycemia and the diagnosis of type 2 diabetes (GROOP, SALORANTA et al. 1991) (Solini, Bonora et al. 1997; Paquot, Scheen et al. 2002); (Brennan, Hoenig et al. 2004). However, like cats, not all obese humans will transition from the obese or insulin-resistant state to type 2 diabetes (Brennan, Hoenig et al. 2004; Hoenig 2012). Genetic, environmental, and metabolic factors can considerably influence the response to long-term obesity and insulin resistance (Hoenig 2012). It is probable that the shift from the insulin resistant and glucose intolerant state to the overtly diabetic state also develops gradually over time (Brandao 2004).
The Reproductive Consequences of Maternal Insulin Resistance, Hyperglycemia and Type 2 Diabetes

More women of fertile ages are becoming overweight and obese (Kulie, Slattengren et al. 2011). Overweight and obese women have a greater chance of difficulties during pregnancy. These complications include diagnosis of hypertension and delivery complications such as an increased occurrence of caesarean sections and delayed time of delivery (Linné 2004). These women also have increased risk and occurrence of gestational diabetes, preeclampsia, and fetal overgrowth in comparison to healthy women of a normal weight. The primary pathology of gestational diabetes is a consequence of increased insulin resistance or reduced maternal insulin sensitivity. In the United States alone, 135,000 women per year develop gestational diabetes (Catalano, Kirwan et al. 2003). The combination of obesity and reduced insulin sensitivity during pregnancy increases the probability that these mothers and children will develop problems of hypertension, hyperlipidemia, diabetes, and an accompanying metabolic syndrome including clinical presentation of cardiovascular disease (Catalano, Kirwan et al. 2003). Of special concern are those women with poorly regulated diabetes prior to becoming pregnant. These women often suffer from other reproductive complications, such as miscarriage, embryonic developmental irregularities, and congenital malformations (Wang, Chi et al. 2012). The embryo in a poorly controlled diabetic mother may be exposed to fluctuating levels of insulin (and glucose) during development inside the female reproductive tract. Altering the environment of the early embryo by changing the maternal diet in an animal model, or, the composition of culture media in an in vitro model has been shown to negatively affect early embryo growth, implantation, fetal weight and development, placental morphology, length of gestation (Gluckman, Hanson et al. 2008). After birth, these conditions can even compromise the physical and psychological health of the progeny (Moley 1999; Gardner and Lane 2001; Fraser, Waite et al. 2007; Varghese, Ly et al. 2011).

The Cat as a Model for Metabolic Diseases and their Implications

The overweight or obese cat has been used in other studies as a model to elucidate the causes behind the progression from obesity to type 2 diabetes (Hoenig 2002). Still, there have not been any studies to determine if the domestic cat is also a suitable model for the reproductive
consequences of obesity and type 2 diabetes. Routine production of cat embryos from the oocytes of follicles allows for the domestic cat to be used as a model for metabolic disease and developmental biology (Johnston, O'Brien et al. 1989). Ovarian tissue from domestic cats is often available from veterinary clinics following surgical sterilization, which reduces animal use and cuts down on the costs of the research by eliminating the need to maintain a colony of research cats. In vitro culture allows concentrations of glucose and insulin to be altered independently, avoiding potential interactions between these and other factors, and allows repeated observations of the same embryo, as opposed to single observations following the recovery of embryos grown in vivo.

*Previous In Vitro Studies of Hyperglycemia and Insulin Resistance*

Most research conducted on insulin resistance and hyperglycemia has taken place focused on murine embryos as a model. (Purcell and Moley 2009) suggest that the major teratogen in diabetic pregnancies is actually elevated concentrations of glucose. A number of reports show that the addition of increased glucose concentrations to the culture medium is detrimental to embryo development (Trocino, Akazawa et al. 1995; Moley, Chi et al. 1998; Gardner and Lane 2001; Gardner, Lane et al. 2003; Gardner and Lane 2004; Fraser, Waite et al. 2007; Lane and Gardner 2007). Diamond et al., (1991) found that two-cell embryos from control mice cultured in high concentrations of glucose are developmentally delayed compared to embryos cultured with lower concentrations of glucose (Diamond, Pettway et al. 1991). The experiments of Diamond et al., (1991) also validated that inadequate metabolic control can alter gestational development even before the period of organogenesis. Lastly, they concluded diabetic women who are not maintaining appropriate metabolic control have blood glucose levels consistent with concentrations sufficient enough to cause dysmorphogenesis of the early embryo. It is worth noting however, that culture of mouse zygotes with insulin in this study failed to alter development as compared with control embryos (Diamond, Pettway et al. 1991). Notably, the precise concentration of exogenous glucose causing morphological and biochemical changes has been defined for a range of developmental stages in murine, bovine, and porcine embryos but it has not yet been defined for cats (Moley, Vaughn et al. 1991; Takahashi and First 1992; Macháty, Day et al. 1998; Chi, Pingsterhaus et al. 2000; Karja, Kikuchi et al. 2006; Cagnone, Dufort et al. 2011). Diamond et al., (1991) cultured 2-cell mice embryos in media containing 110, 220, 440,
and 950 mg d-glucose/dL (6.1 mM; 12.2 mM; 24.4 mM; and 52.7 mM). After culturing embryos for 24, 48, or 72 hours, 220 (12.2 mM) and 440 mg glucose/dL (24.4 mM) reduced embryonic development compared with 110 mg glucose/dL (6.1 mM). Culture media for cattle embryos also containing >5mM glucose arrested development up to the morula stage (Hugentobler, Humpherson et al. 2008; Cagnone, Dufort et al. 2011). Studies in the hamster and the cow have shown that development after compaction is compromised when cleaved embryos are exposed to even very mild hyperglycemia (0.1 mM–5 mM respectively)(Schini and Bavister 1988; Seshagiri and Bavister 1989; Cagnone, Dufort et al. 2011). Somites contain the precursor cells predetermined to become dermis of the skin and essentially all of the skeletal muscles of the body (Ordahl and Le Douarin 1992). Pinter and Reece (1986) cultured rat conceptuses and embryos with only two somites in an excess of D-glucose (19.4 mM, 350 mg/dl), resulting in neural malformations in 20% of rat embryos. During early embryonic development the yolk sac provides the initial nourishment and functions as a primitive circulation system before the internal organs are formed (Brent 1990) (Reece, Pinter et al. 1985). The growth of those embryos cultured in excess of 42 mM D-glucose was also impeded and had multiple anomalies such as abnormal yolk sacs. However, open neural tube was the most frequent deformity and occurred in 50% overall of the rat embryos cultured in hyperglycemic medium (Reece, Pinter et al. 1985). Lastly, increasing the glucose concentration from 1.5 to 6 mM from day 3 to 6 of culture tended to decrease the glycolytic activity of feline blastocysts (Herrick et al., 2007). The ratio of the picomoles of pyruvate oxidized to glucose metabolized through glycolysis provides an overall measure of metabolic activity in feline embryos. Metabolism of pyruvate oxidation through the tricarboxylic acid cycle and glucose via glycolysis was concurrently measured in that study using radiolabeled 2-14C-pyruvate and 5-3H-glucose(Herrick, Bond et al. 2007).Therefore, it was important to determine in this study if further elevations of medium (<6 mM) to high glucose concentrations would lead to a decrease in development to the blastocyst stage.

The mechanism(s) responsible for the inhibition caused by culturing in high glucose are not always clear. Moley et al. (1998) cultured embryos from control or non-diabetic mice in either 3.0 mM or 30 mM D-glucose or 30 mM L-glucose from the two-cell to the blastocyst stage (72 hours). Embryos cultured in vitro in hyperglycemic conditions of 30 mM D-glucose experienced an increase in Bax protein apoptosis (Bax is a member of the Bcl-2 family of
proteins) induced from D-glucose effects (Chi, Pingsterhaus et al. 2000). Additionally, expression of Bax mRNA was 2.5-fold higher in mouse embryos recovered from hyperglycemic diabetic mice 96 hours after mating in comparison to embryos from control mice. Embryos cultured in 30 mM L-glucose, the osmotic control, were not significantly different from embryos cultured in the control medium. This indicates that the effects were due to effects of glucose as a teratogen, rather than due to increased osmolarity of the medium (Moley, Chi et al. 1998). The glucose analog 2-deoxyglucose (DG) (a non-metabolizable analog of glucose) is carried into the mammalian cells of tissues by glucose transporters and can be phosphorylated by hexokinase to 2-deoxyglucose 6-phosphate (DG6P). However, DG6P cannot be used as a substrate for the enzymes in the later steps of the glycolytic pathway, also the pentose phosphate shunt, and it can accumulate in the cytosol (Schini and Bavister 1988). Schini and Bavister (1988) demonstrated that 0.1 mM DG inhibited embryo development (however, in the absence of phosphate). Moley et al. (1998) also showed a significant disparity in the DG uptake when comparing the diabetic and control embryos at 48 and 96 h in vitro. Like studies before this one, it was imperative to culture feline embryos in 22.5 mM L-glucose and 22.5 mM 2-deoxyglucose to determine if inhibitory effects are dependent on glucose uptake or metabolism.

In embryos, similar to other cell types, insulin promotes glucose uptake, stimulates RNA and protein synthesis (Harvey and Kaye 1990; Harvey and Kaye 1991; Baltensperger, Lewis et al. 1992; Dunglison and Kaye 1993), and has mitogenic or growth related and anti-apoptotic (active inhibition of apoptosis) actions in rabbit and murine embryos (Harvey and Kaye 1990; Herrler, Krusche et al. 1998). Recent studies have established that insulin and IGF-1 also regulate glucose transport in mouse blastocysts (Pantaleon and Kaye 1996; Carayannopoulos, Chi et al. 2000; Pinto, Schlein et al. 2002). Glucose transport is known to be specifically regulated by the IGF-1 receptor (IGF-1R) in the mouse blastocyst (Chi, Pingsterhaus et al. 2000). In this study, it was necessary to culture feline embryos with a combination of glucose and insulin to determine if any effects seen were due to increased glucose uptake as stimulated by insulin.

In previous studies, insulin has been added to the culture medium to increase glucose uptake, mimicking in vivo oviductal conditions during a state of increased insulin the blood plasma (Summers and Biggers 2003). It was demonstrated that the addition of insulin to mammalian embryo culture at low doses of insulin, used at a concentration of 1.7 µmol (0.0017
mM) to be stimulatory. Additionally, high doses (similar to insulin resistance/diabetes) (>600 nM; 0.006 mM) are shown to be inhibitory. A previous study chose 200 nM (0.0002 mM) or 950-1500 ng/ml (0.005 - 0.008 mM) range as a two- to threefold elevated physiological range to be indicative of mild insulin resistance (Eng, Sheridan et al. 2007). For this study, both low and high concentrations of insulin were evaluated. It was important to determine if further elevations of medium to high insulin concentrations (1.7 µM – 170 µM; 0.0017 mM- 0.17 mM) would lead to a decrease in the feline blastocyst development.

**Current Study**

*Hypothesis and Specific Aims*

Our overall hypothesis for this series of experiments was that the development of feline embryos, similar to other species, would be inhibited when cultured with supra-physiological concentrations of glucose and/or insulin in vitro. Specifically, we hypothesized that: 1) glucose concentrations greater than ≥ 6 mM would inhibit development of feline embryos; 2) the negative effects of glucose would require uptake and/or metabolism and would not be results of osmotic change; 3) insulin would increase negative effects of glucose by facilitating uptake into the cells; and 4) insulin would have a dose dependent, potentially inhibitory effect on the development of feline embryos.
CHAPTER 2: Literature Review

Overview

Given that the cat is a model for researching other characteristics of obesity and the development of type II diabetes, this carnivore may also be a superb model for the study of the reproductive consequences of these metabolic conditions. There is now literature developed around the use of the laboratory cat as a reproductive model for humans (Comizzoli, Pukazhenthi et al. 2011). This is a unique opportunity to use the feline embryo as a model to investigate the effects of elevated glucose and insulin concentrations as well as the interactions of the two that may occur in obese cats or human women developing diabetes. Domestic cat embryos can progress as far as the hatching blastocyst stage in a broad array of media, however, culture in Feline Optimized Culture Medium (FOCM) has been known to produce a consistently high proportion of cat oocytes developing to the blastocyst stage (Herrick, Bond et al., 2007). Kittens have been produced by embryo transfer following culture in several types of media (Herrick, Bond et al. 2007).

In vivo, the oocyte goes from a somewhat dormant metabolic gamete during ovulation to a quickly metabolizing tissue at the point of implantation in vivo (Leese 2012). Therefore, culture environments that are designed to maintain the oocyte and recently fertilized embryo are typically not ideal for the embryo after the stage of compaction (Roth, Swanson et al. 1994; Gardner and Lane 2002) can actually be inhibitory to the development and differentiation of the blastocyst (Karja, Kikuchi et al. 2006). Composition of the culture medium therefore affects preimplantation and post implantation embryonic morphology, gene expression as well as metabolism at all stages, (Sananmuang, Tharasanit et al. 2011). The environment is oocytes and embryos is also a key regulator of metabolic activity(Gardner and Leese 1990; Lane and Gardner 1998). Culture conditions are known to affect the ability of an embryo to metabolize glucose (Krisher, Lane et al. 1999). Thus, uptake and metabolism of glucose has also been linked to the developmental competence of the in vitro embryo (Krisher, Lane et al., 1999).

The transport of glucose across the plasma membrane of mammalian cells represents a central role in cellular homeostasis and metabolism. It is established that the plasma membranes of nearly all mammalian cells have a facilitated diffusion transport system(Pantaleon and Kaye 1998). This allows for the movement of glucose either in or out of cells across the plasma membrane down its chemical gradient (Pantaleon and Kaye 1998). Glucose can also be
transported into cells by several types of glucose transporters. These transporters of glucose are specific for the D-enantiomer of glucose (Purcell and Moley 2009). Two specific insulin responsive glucose transporters (GLUTs) have been detected in pre-implantation embryos in a few species, namely GLUT-4 and GLUT-8. GLUT-8 has been verified in both bovine and rabbit embryos. In bovine embryos it has been recognized at the 2-cell stage continuing through the blastocyst stage (Augustin et al. 2001). However, GLUT-8 was shown only to be expressed at the blastocyst stage in rabbit embryos (Navarrete Santos et al. 2004). GLUT-8 was discovered to play a role in insulin-stimulated glucose uptake in murine blastocysts (Purcell and Moley 2009). This information is not known for the domestic cat embryo. Expression of GLUT-8 emerges simultaneously as the embryo travels from the oviducts to the uterus, the latter of which contains 10-fold higher concentrations of IGF-1 and insulin (Pinto, Carayannopoulos et al. 2002). GLUT-8 has been shown to function just prior to implantation, a time of maximal glucose need (Carayannopoulos, Chi et al. 2000). It seems to translocate particularly in response to increasing IGF-1 levels in vivo (Pinto, Schlein et al. 2002). The appearance of GLUT-8 also coincides with the expression of the IGF-1 receptor in the blastocyst. In an in vitro environment, following treatment with insulin, GLUT-8 has been found to traffic from an intracellular compartment of the cell to the plasma membrane of the trophectoderm. Interestingly, it is thought that at least in the mouse, GLUT-8 may have developed as an anti-apoptotic pathway in the blastocyst (Moley and Mueckler 2000; Pinto, Carayannopoulos et al. 2002). This mechanism could boost glucose consumption at a vital time during development when the embryo metabolically shifts its principal energy substrate consumption from pyruvate to glucose (Pinto, Carayannopoulos et al. 2002).

Given that glucose is the main source of energy during early embryogenesis, an interruption in its transport or utilization could induce the cascade of events that later lead to congenital defects (Freinkel 1988). The effect of hyperglycemia on embryos has been studied during both the pre- and post- implantation periods (Moley, Chi et al. 1998). Excessive glucose levels can result in an increased incidence of apoptosis and cell death (Sananmuang, Tharasanit et al. 2011) and can also impair embryo development by the production of oxidative stress (Gardiner and Reed 1994; Takahashi 2012). It can also cause a decrease in glucose transport which can affect the cellular death program (Moley, Chi et al. 1998; Chi, Pingsterhaus et al. 2000). At a concentration similar to that recorded for plasma in the current study, other
studies have shown that during the first 48 hours after fertilization, the inclusion of glucose in the culture medium can inhibit early cleavage and its exclusion can improved blastocyst yields by up to 31% (Seshagiri and Bavister 1989).

Obesity can produce insulin resistance in the body, so the potential for insulin resistance to cause similar issues in pre-implantation embryos is likely (Jungheim and Moley 2010; Jungheim, Travieso et al. 2012). Data from models of type 2 diabetes have demonstrated that insulin resistance is associated with increased risk of miscarriage (Leddy, Power et al. 2008). Notably, a drug with a clinical application, named Metformin®, an “insulin sensitizer” can reverse this risk (Jungheim and Moley 2010). Earlier studies show that elevated insulin or IGF-I environments cause negative effects on murine pre-implantation embryos both in vitro and in vivo (Schultz, Hogan et al. 1992). A high concentration of IGF-I or insulin can cause the down-regulation the IGF-I receptor in the pre-implantation blastocyst (Chi, Schlein et al. 2000). This is comparable to what occurs in other cell systems, especially in reaction to constant insulin stimulation (Eng, Sheridan et al. 2007). This down-regulation is also accompanied by a decline in insulin-stimulated glucose uptake, which is regulated by the IGF-I receptor (Pantaleon and Kaye 1996) and is consistent with insulin resistance in the blastocyst (Eng, Sheridan et al. 2007).

In mouse blastocysts, the effects of insulin are transmitted by both the insulin and IGF-I receptors, but for different functions (Harvey and Kaye 1990; Augustin, Pocar et al. 2003). The insulin receptor facilitates insulin stimulation of protein synthesis and mitogenesis (Kaye, Bell et al. 1992). Insulin and IGF-I have been shown to cross-react with the corresponding receptor (Harvey and Kaye 1990). But, there is no information available regarding the effects of insulin on feline embryos that are specifically related to the action of insulin and IGF-I and their respective receptors. Unfortunately, not much is known about the insulin receptors in the feline embryo; thus there is little to no information as to the stage in which the early feline embryo becomes responsive to insulin and IGF-1.

*Measurements of oviductal fluid and its relationship to embryonic development*

Glucose, lactate, and pyruvate have been quantified in the oviductal fluid of several mammalian species (Nichol, Hunter et al. 1992; Lighten, Moore et al. 1998; Guérin, El Mouatassim et al. 2001; Aguilar and Reyley 2005). The function of pyruvate, lactate and glucose in the oviduct is to sustain the oocyte, spermatozoa, and early embryo as they journey through
the female reproductive tract (Leese 1988). The fluids of the tract are comprised of a combination of components originating from molecules and plasma produced from the oviduct and uterine epithelium (Harris, Gopichandran et al. 2005). Values for the concentrations of glucose measured in the oviduct of the cow range from 1.87 to 3.17 mM (Hugentobler, Humpherson et al. 2008). These concentrations are also similar to the value of 1.58 mM reported for sheep (Iritani et al., 1969). However, the oviduct glucose concentration recorded in the cow and sheep are about five fold higher than those reported for the pig (Nichol et al., 1992, 1998). The normal concentrations of glucose seen in vivo in oviductal fluid of other animals are typically assumed to be in the 1.0-3.0 mM range as well (Menezo and Guerin, 1997), so we assumed that the normal feline oviduct would contain a similar concentration of glucose.

Oviductal and uterine fluid concentrations of glucose are correlated to blood serum concentrations of glucose in humans, cows, mice, and rabbits (Baker 1963; Robinson, Smith et al. 1990; Brewis, Winston et al. 1992; Hugentobler, Humpherson et al. 2008). The overall channel of glucose from the blood into the lumen of the oviduct is by diffusion through the epithelium of oviduct (Leese 1988; Hugentobler, Humpherson et al. 2008). Concentrations of glucose about three fold lower in the reproductive tract than blood plasma have been reported in both sheep (Iritani et al., 1969) and pigs (Iritani et al., 1974). Oviduct and uterine fluid concentrations of glucose in the cow were about 2.5-fold and 1.5-fold lower, respectively, than those in blood plasma (Hugentobler, Humpherson et al. 2008). Moreover, in the mouse, it was found that glucose was 7–25-fold higher in plasma than in the reproductive tract fluids (Harris, Gopichandran et al. 2005).

Poor embryo quality may originate from the oocyte, but an abnormal tubal and uterine environment may also influence embryo quality (Sutton, Gilchrist et al. 2003). Based on these previous findings, we can assume that glucose concentrations in the reproductive tract fluids of hyperglycemic, hyperinsulinemic, or diabetic cats will be proportional to that of the serum and significantly elevated in a state of metabolic unbalance (>2-3 mM).

**Glucose and insulin in relation to the oocyte and early embryonic development**

Embryo developmental rates for other species are known to be multiphasic with variable cleavage intervals, especially during early periods of development (Swanson, Roth et al. 1994). In cattle, initial cleavage rate to the 4-to 8-cell stage is related to maternal control of protein
synthesis, and slowed growth is associated with the maternal-zygotic transition (Rieger, Loskutoff et al. 1992; Fortune, Cushman et al. 2000). The timing of the maternal-zygotic transition is unknown for the cat, but observed in vivo developmental kinetics indicates that the shift from maternal to embryonic control may well occur at about the 5- to 8- cell stage when the cleavage rate slows substantially (Swanson, Roth et al. 1994). Swanson et al., 1994 found that the developmental rate of cat embryos in vivo appears biphasic, with an initial fast cleavage period followed by a period during which developmental rate within the oviducts was slower. Cat embryos also develop into morulae within the oviducts. When undergoing compaction, cat embryos likely transverse the utero-tubal junctions then enter the uterus as compact morulae or early blastocysts (Roth, Swanson et al. 1994). Therefore, compared to embryos of many domestic species, cat embryos are sustained within the oviducts for a longer period, making the oviductal-to-uterine transition at a relatively advanced stage (Swanson, Roth et al. 1994).

After fertilization, the pre-implantation mammalian embryo, especially during the first 2 weeks, is dependent on the nutrients provided by the fluids if the oviduct and uterine epithelium(Leese, Hugentobler et al. 2007). The early embryo is dependent on these for growth, development, and ultimately, survival (Ellington 1991; Bavister 2002; Leese 2003). Previous in vitro studies in cattle, pigs, and cats show that early development of the embryo requires the ability to metabolize particular energy substrates changes during early development ((Tiffin, Rieger et al. 1991; Thompson, Partridge et al. 1996). Lactate and pyruvate are key sources of energy for embryos up to the blastocyst stage; as demonstrated in cattle and pigs (Takahashi and First 1992; Kim, Funahashi et al. 1993). During early development, consumption of glucose is low but this increases significantly at blastocyst formation (Kim, Funahashi et al. 1993). It has been suggested that glucose may not be a requirement for the pre-implantation of cattle embryos, based on evidence that the concentrations of glucose in the oviduct are much lower than in the cow uterus(Leese 1995). The tendency for a higher uterine glucose concentration is observed not only for cattle embryos, but for those of mammalian species generally (Leese 1988; Leese 2003) and is consistent with increased uptake of glucose by the morula and blastocyst stages. It is also possible that the cow early embryo may be unable to take up glucose due to a lack of or low levels of the glycolytic enzymes hexokinase and pyruvate kinase (Rieger 1992).

Metabolic preferences of embryos appear to change between early and late stages, elevating glucose and oxygen consumption as they begin compaction (Houghton, Thompson et
The increased use of glucose through glycolysis during compaction and blastocyst formation is common in many mammalian species (Gardner 1998; Thompson 2000). From the blastocyst stage and beyond glucose is a main energy source (Tiffin, Rieger et al. 1991; Rieger 1992). Porcine embryos, for example, seem to depend greatly on glucose, metabolizing roughly 20 to 27 times more glucose at the blastocyst stage, and 10 to 18 times more glucose than pyruvate at the morula and eight-cell stages (Swain, Bormann et al. 2002). Domestic cat embryos make use of about twice the amount of glucose as pyruvate at the eight cell stage and approximately five times as much glucose as pyruvate at the blastocyst stage. This data, again, was based on the amount of pyruvate oxidized relative to glucose metabolized through glycolysis (Herrick, Bond et al. 2007).

The pre-implantation embryo also expresses insulin and IGF-1 receptors when the embryo begins to favor glycolytic metabolism to pyruvate oxidation (Purcell and Moley 2009). Evidence suggests that insulin is also an important regulator of growth in vivo and can also stimulate proliferation and growth in a variety of somatic cells in culture (STRAUS 1984). Insulin receptors have been observed to be distributed evenly over the cellular membranes of the blastocyst. The existence of insulin receptors (IR) and IGF-I receptors (IGF-IR) in embryos has been confirmed in oocytes and throughout pre-implantation embryo development in many species including the human (Hogan, Heyner et al. 1991; Schultz, Hogan et al. 1992; Watson 1992; Lighten, Hardy et al. 1997; Yaseen, Wrenzycki et al. 2001). Expression of receptors starts at the 8-cell stage starts in mouse embryos (Schultz, Montgomery et al. 1983; Freinkel 1988; Harvey and Kaye 1990; Hogan, Heyner et al. 1991; Rappolee, Sturm et al. 1991; Schultz, Hogan et al. 1992; Schultz 2002; Markham and Kaye 2003) and rabbit embryos can bind insulin and IGF-I from the morula stage onwards (Navarrete Santos, Tonack et al. 2004; Herrler, Krusche et al. 1998). In mouse blastocysts, the effects of insulin are transmitted by both receptors but for different functions. Insulin and IGF-1 can stimulate glucose uptake in the pre-implantation blastocyst, but operating via the IGF-1R instead of the insulin receptor (Kaye, Bell et al. 1992; Schultz, Hogan et al. 1992; Pantaleon and Kaye 1996; Lighten, Moore et al. 1998; Pantaleon, Jericho et al. 2003). Present on the surface of the trophectoderm of the embryo is the IGF-1 receptor. This receptor can increase glucose uptake in response to insulin or IGF-1 as shown in the mouse embryo (Lighten, Moore et al. 1998; Carayannopoulos, Chi et al. 2000; Riley, Carayannopoulos et al. 2005). Endocrinologists attribute many of the growth-promoting effects
of insulin to its cross-reaction specifically with the type 1 IGF receptor (Pantaleon and Kaye 1996; Carayannopoulos, Chi et al. 2000). The stimulatory effects of exogenous IGF-1 on human blastocyst formation were primarily mediated by the IGF-1R as well (Lighten, Moore et al. 1998). In mouse blastocysts, expression of both insulin and IGF receptors can be indicated by competitive radioligand binding (Mattson et al., 1988). Blastocyst insulin receptors in the mouse embryo mediate insulin's actions at concentrations 100-fold less than that required to activate receptors on classical insulin target tissues (Harvey and Kaye 1990). However, the effects of insulin on embryo development are also usually only seen at very high concentrations in vitro (Fisher 1980).

In a previous mouse study, blastocysts were incubated with 1.7 nM insulin (0.00017 mM) (Pantaleon and Kaye 1996). These mouse blastocysts were cultured in either glucose-free media or media containing 5.6 mM glucose. In this study, blastocysts that had developed with 1.7 nM or the low concentration of insulin (0.0017 mM) contained 9% more cells than those in control medium (Harvey and Kaye 1990; Kaye, Bell et al. 1992; Pantaleon and Kaye 1996). 2 cell embryos cultured to the blastocyst stage and transferred after 48 h culture with insulin, showed a 10% increase in fetal weight and no effect on placental weight (Harvey and Kaye 1990); Gardner and Kaye, 1986).

It is established in other cell types that the IGF-1 receptor plays a critical role in regulating programmed cell death (Elmore 2007). Down-regulation of the insulin receptor by method of antisense oligonucleotides escalates apoptosis in the chicken embryo (de Pablo, Serrano et al. 1990; Chi, Schlein et al. 2000). High concentrations of insulin or IGF-1 can also result in the down-regulation of the IGF-1 receptor in other cell types such as lymphoid cells, FRTL-5 thyroid cells, endothelial cells, and bovine articular chondrocytes as well. Receptor loss induced by a dose dependent effect of high insulin or IGF-1 resulted in a 10–20% reduction at a concentration of 10 nM (0.00001 mM) and 60–85% at 1 µM (0.001 mM) (Chi, Schlein et al. 2000). This can reduce insulin-stimulated glucose uptake and may lead to increased apoptosis in blastocysts as well(Chi, Schlein et al. 2000; Riley and Moley 2006).

Anti-apoptotic signals such as those activated by insulin or IGF-1 receptors have a role in normal embryo metabolism. It is commonly thought that Akt is the chief mediator of the anti-apoptotic signal produced by the PI3-K pathway (Riley and Moley 2006). PI3-K activity is involved in a number of physiologic responses including glucose metabolism, cellular
proliferation, and growth as well (Riley and Moley 2006). Components of the signaling pathway the PI3K/Akt appear in human and mouse embryos from the 1-cell to blastocyst stage (Purcell and Moley 2009). This mechanism has a role in glucose uptake and metabolism (Riley and Moley 2006). Inhibition of this PI3K/Akt pathway can result in a decrease in insulin-stimulated glucose uptake and the activity of the hexokinase enzyme (Riley, Carayannopoulos et al. 2006). This has been observed along with an increase in cell death or apoptosis in the blastocyst as well as trophoblast cell lines (Riley and Moley 2006). Treatment of mouse blastocysts with insulin showed a 2 fold increase in Akt phosphorylation in comparison to the control or not insulin treated blastocysts (Riley et al., 2005). In contrast, rabbit blastocysts, in response to insulin stimulation experience activation of the mitogen-activated protein kinase (MAPK) pathway rather than the PI3-K/Akt pathway (Navarrete Santos et al., 2004). Therefore, it is not completely clear as to which pathway insulin is affecting. Though activation of growth factor receptors like insulin and IGF-I during the pre-implantation stage in mammals are necessary for embryo development and survival, it is likely that too much or too little of either insulin or IGF-1 can cause an inhibitory or stimulatory effect on early embryo development (Riley and Moley 2006). This correlation has not been studied in the feline embryo yet. The mechanism behind the regulation of insulin and glucose homeostasis in feline blastocysts remains to be established. Much of the literature illustrates the intricate patterns of metabolic control that occur within the developing embryo, especially because they are usually species-specific (Herrick, Bond et al. 2007).

Current Study

Restatement of Hypothesis and Specific Aims

Our overall hypothesis for this series of experiments was that the development of feline embryos, similar to other species, would be inhibited when cultured with supra-physiological concentrations of glucose and/or insulin in vitro. Specifically, we hypothesized that: 1) glucose concentrations greater than ≥ 6 mM would inhibit development of feline embryos; 2) the negative effects of glucose would require uptake and/or metabolism and would not be results of osmotic change; 3) insulin would increase negative effects of glucose by facilitating uptake into the cells; and 4) insulin would have a dose dependent, potentially inhibitory effect on the development of feline embryos.
CHAPTER 3: Materials and Methods

Chemicals and Reagents

All chemicals for the media were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Stock solutions were prepared in ultrapure H2O (18.2 MΩhm, <10 ppb organic carbon; EMD Millipore, Billerica, MA) and stored at 4 °C for either 1 week (pyruvate, bicarbonate, cysteine, cysteamine) or 1 month (basal salt solution, D-glucose, 2-deoxyglucose, L-glucose, CaCl2-2H2O, L-lactate (MP Biomedicals. Solon, OH), taurine. A stock solution of alanyl-glutamine was stored at 4 °C and used within 3 months of initial preparation (Herrick 2009). Stock solutions of gentamicin, amino acids (minimal essential medium concentrations (MEM, MP Biomedicals), and ITS (insulin, transferrin, and selenium) (MP Biomedicals), were stored according to supplier’s instructions and used directly for medium preparation. Vitamins (MEM, MP Biomedicals) and fetal calf serum (FCS; Hyclone, Logan, UT) were aliquoted without dilution and stored at -80 °C.

The base medium for all cultures was Feline Optimized Culture Medium (Herrick, Bond et al. 2007), which contains NaCl (100 mM), KCl (8.0 mM), KH2PO4 (1.0 mM), MgSO4-7H2O (1.0 mM), CaCl2-2H2O (2.0 mM), NaHCO3(25.0 mM), pyruvate (0.1 mM), L-lactate (6.0 mM), alanyl-glutamine (1.0 mM), taurine (0.1 mM), nonessential amino acids (1x MEM), 50 µg/ml gentamicin, and 4.0 mg/ml BSA (True Cohn Fraction V, MP Biomedicals). This base medium was further supplemented for each stage of embryo production (oocyte maturation, fertilization, and embryo culture). Media was prepared for each replicate, filtered (0.22 µm MillexGV; EMD Millipore, Billerica, MA), and equilibrated in the appropriate gas atmosphere at 38.5 °C for at least 3–4 hours and not longer than 24 hours before use. Cumulus-oocyte complexes (5 per drop) or embryos (8 to 12 per drop) were cultured in 50µl drops of medium covered with 4 or 10 ml Embryo-Tested, Filtered mineral oil (Sigma, M-5310) in 35 or 60 mm plastic dishes (Falcon 1008 or 1007; Becton Dickinson Labware, Franklin Lakes, NJ), maintained at 38.7 °C with 6% CO2 in air (maturation and fertilization) or 6% CO2, 5% O2, and 89% N2 (embryo culture). Most embryo media utilize a bicarbonate/CO2 buffer system to maintain a physiological pH of between 7.2 and 7.4 in the medium. The inclusion of sodium bicarbonate in the medium requires the use of a CO2 incubator to maintain a 5–7% CO2 atmosphere. An advantage of the bicarbonate/CO2 system is that it is the physiological buffering system in fluid surrounding mammalian cells. The use of an oil overlay to reduce gas exchange when culture dishes are taken
out of the incubator, e.g., for embryo scoring (Gardner, Lane et al. 2003; Gardner and Lane 2004).

Collection of Oocytes and In Vitro Maturation

IACUC approval was not required for this study because the tissue was collected opportunistically. For this study, feline ovaries were obtained from a local trap/spay/neuter program during the breeding (January–August) and non-breeding season (September to December). Only ovaries from cats at least 6 months of age were selected for use in the study. Ovaries were collected from spay/neuter clinics following routine, elective ovariohysterectomies of females >6 months old, placed in 0.9% NaCl with 50 µg/ml gentamicin, and maintained at 4 ºC for transport to the laboratory (~2 hrs). Cumulus oocyte complexes (COCs) were recovered by repeatedly slicing the ovaries with a scalpel blade in HEPES-buffered FOCM (FOCMH) supplemented with 40 U/ml heparin for aspiration or cutting medium.

Only COCs containing an oocyte with a dark, uniform ooplasm surrounded by multiple layers of cumulus cells were used for these experiments. For in vitro maturation, COCs were cultured for 24 h in maturation media [glucose (6.0 mM), 0.5x MEM essential amino acids, 1x MEM vitamins, 10 µg/mL insulin, 5.5 µg/mL transferrin and 5.0 ng/mL selenium(ITS; 100x-Sigma Chemicals (Herrick, Bond et al. 2007), cysteine (0.6 mM), cysteamine (0.1 mM), EGF (25 ng/ml), and eCG (1.0 IU/ml)]. Following this incubation, matured cumulus-oocyte complexes were washed twice in FOCMH, three times in the fertilization medium (IVF), and placed into 45 µl drops of IVF and maintained at 38.5 ºC in 6% CO2 in air until insemination.

In Vitro Fertilization

Straws of frozen domestic cat sperm were thawed in air for 10 seconds, then placed into a 38 ºC water bath for 30 seconds. Thawed samples were slowly diluted with 150 µl warm FOCMH and then centrifuged for 10 minutes at 300 x g. The supernatant was removed and the resulting pellet was diluted with 25-50 µl FOCMH. Sperm concentration (hemocytometer) and motility (percentage of motile spermatozoa, 0-100%) were determined and the sperm concentration was adjusted to 5 x 10^6 motile sperm/ml with FOCM IVF. Aliquots (5 µl) of this solution were added to 45 µl drops of FOCM IVF containing COCs.
Embryo Culture

At 18–22 h after insemination, presumptive zygotes were placed into a 1.5-ml microcentrifuge tube containing 100 µl FOCMH and 25 µl hyaluronidase (~750-1500 U/mg) and vortexed for 2–3 min to remove loosely bound spermatozoa and remaining cumulus cells. Denuded zygotes were washed twice in FOCMH and randomly allocated to treatments for embryo culture IVC1 with D-glucose (1.5, 6.0, 12.0, 18.0, or 24.0 mM), insulin (recombinant human, 1.7, 17, and 170 µM insulin), L-glucose (22.5 mM), or deoxyglucose (22.5 mM).

Embryos were cultured (a total of at least 50 embryo from at least 3 replicates) for 48 h in media containing the appropriate experimental treatments. On Day 3 (day 0 = day of IVF), the proportion of embryos that have cleaved (≥2 cells) was determined and embryos with more than 4 cells were transferred to FOCM containing FCS (5% v/v) instead of BSA with the same concentration (s) of glucose and/or insulin used for the first 3 days of culture until Day 7.

Evaluating Embryo Development

On day 7 post-insemination, the proportion of embryos reaching the blastocyst stage was evaluated. Blastocyst cell number was determined on day 7 as an additional measure of embryo viability. For blastocyst evaluation, embryos were placed on a siliconized (Sigmacote, Sigma Chemicals) glass slide, counterstained with Trypan Blue (0.1% in 2.3% Na-citrate) for 1 min, stained with Hoechst 33342 (0.01 mg/ml in 1.73% Na-citrate and 25% ethanol), and covered with a coverslip in Permount (Fisher Scientific) (Wall, Pursel et al. 1985) et al., 1985). Cells numbers were determined using a fluorescent microscope. Only embryos containing ≥50 cells with a visible blastocoel cavity were considered blastocysts.

Experimental Design

Experiment 1: The Effects of Elevated Glucose Concentrations (Hyperglycemia)

The purpose of this experiment was to determine if higher (>6 mM) glucose concentrations would affect development of domestic cat embryos. Following in vitro maturation and fertilization, embryos were cultured in FOCM1 containing 1.5 (control), 6.0, 12.0, 18.0, or 24.0 mM D-glucose. On day 3, cleaved (≥2 cells) embryos were moved to FOCM2 with the
same concentration of glucose used for the first 3 days of culture. Each treatment was replicated 10-12 times for a total of 100-150 embryos per treatment.

**Experiment 2: Interactions between Insulin and Glucose**

The purpose of this experiment was to determine if feline embryo development was negatively affected by interactions of increased glucose and insulin in the media. Embryos were cultured in 1.5 mM D-glucose (control); 24 mM D-glucose; 1.5 mM D-glucose + 1.7 µM Insulin; 24 mM D-glucose + 1.7 µM Insulin. On day 3, cleaved (≥2 cells) embryos were moved to FOCM2 with the same combination of glucose and insulin used for the first 3 days of culture. Each treatment was replicated 10 times for a total of 90-100 embryos per treatment.

**Experiment 3: Glucose Analogs**

The purpose of this experiment was to determine if the inhibitory effects of 24 mM glucose observed in experiments 1 and 2 were due to uptake or metabolism of glucose by the cell, or increased medium osmolarity. Based on the results of experiment 2, embryos were cultured with 1.5 mM D-glucose, 24 mM D-glucose, 22.5 mM L-glucose (not transported into the cell), or 22.5 mM 2-deoxyglucose (transported into the cell and metabolized by hexokinase but not further.). Media containing L-glucose or 2-deoxyglucose were supplemented with 1.5 mM D-glucose, so the total concentration of glucose in all treatments was 24.0 mM. The same concentrations of glucose were used in both FOCM1 and FOCM2. Each treatment was replicated at least 10 times for a total of at least 90-100 embryos per treatment.

**Experiment 4: Elevated Insulin (Insulin Resistance)**

The purpose of this experiment was to determine if feline embryos were affected by variations of concentrations of insulin the culture media. In other species, 1.7 µM insulin has been shown to stimulate embryonic development (Matsui, Takahashi et al. 1995). Embryos were cultured in FOCM1 with 1.5 mM D-glucose supplemented with 0, 1.7, 17, and 170 µM insulin. The insulin used was recombinant human insulin that arrives in a solution of 10 mg/ml (or 10 g/L). This is equivalent to 1.7 mM. For this experiment, 2 µM insulin is diluted into 2 ml of culture media (1:1000 dilution) for a final concentration of 1.7 µM (shown to be stimulatory in other species). On day 3, cleaved (≥2 cells) embryos were moved to FOCM2 with the same
concentration of insulin used for the first 3 days of culture. Each treatment was replicated 10 times for a total of 90-100 embryos per treatment.

**Statistical Analysis**

Embryos from each replicate (day of ovary collection) were divided among as many treatments as possible. Therefore, each treatment contained an equivalent number of embryos (≥20 per treatment per replicate). All treatments were assigned out once before any were replicated for each experiment. All comparisons were made by analysis of variance in the PROC MIXED procedure of the SAS System. Cleavage, blastocyst, and cell number development were evaluated for all experiments. An ANOVA F-Test was used to determine if treatment effects were statistical significant (P<0.05). Pairwise comparisons between individual treatments were made by Fisher’s protected (only used if the F-test is significant) least square difference test. Each treatment (e.g., glucose, insulin) and interactions of those treatments (e.g., Glucose*insulin, etc.) were considered fixed factors. Replicate (all endpoints) and the interaction between replicate and the treatments (e.g., replicate*) were considered random factors. For statistical analysis, individual oocytes were assigned a 1 or 0 depending on the stage of development. “1” was assigned to oocytes that developed to at least the 2-cell stage (cleavage) or the blastocyst stage. 0’s were assigned to oocytes that did not cleave or embryos that did not develop to the blastocyst stage. Blastocyst cell number per embryo was subjectively counted under fluorescent microscope. Blastocyst development on Day 7 was recorded per recovered oocyte and per cleaved embryo for each treatment in all replicates. Proportions are represented as mean ± standard error of the mean. The program used in SAS; probit link function, is similar to a transformation and error designated as binomial. We did not need to test for a normal distribution because the data set was designed to have a binomial distribution.
CHAPTER 4: Results

Experiment 1: The Effects of Elevated Glucose Concentrations (Hyperglycemia)

The proportion of oocytes that cleaved was 85.45% ± 8.1%; this was not affected (P>0.05) by treatment. Blastocyst development per cleaved embryo ranged from 38.02% ± 11.4% (6 mM D-glucose) to 50.91% ± 8.5% (1.5 mM D-glucose) with no significant differences (P>0.05) observed between treatments (1.5, 6, 12, 18, 24 mM D-Glucose). There were also no differences (P>0.05) observed for cell numbers between treatments (75.31± 3.68, 12 mM D-glucose to 89.84% ± 5.92, 18 mM D-glucose). Although there were no significant effects of increasing glucose concentrations on embryonic development or cell number (P>0.05), increasing glucose concentrations caused a downward trend in the proportions of feline embryos developing to the blastocyst stage.

<table>
<thead>
<tr>
<th>Glucose (mM)</th>
<th>Cleavage (cl)</th>
<th>Blastocyst/Oocyte (bl/oo)</th>
<th>Blastocyst/Cleaved (bl/cl)</th>
<th>Blastocyst cell number (#)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>85.45±8.1%</td>
<td>45.49±8.5%</td>
<td>50.91±8.5%</td>
<td>77.99±3.32</td>
</tr>
<tr>
<td>6</td>
<td>81.64±8.7%</td>
<td>32.80±11.8%</td>
<td>38.02±11.4%</td>
<td>77.34±3.29</td>
</tr>
<tr>
<td>12</td>
<td>84.08±5.3%</td>
<td>36.47±12.7%</td>
<td>41.06±12.6%</td>
<td>75.30±3.67</td>
</tr>
<tr>
<td>18</td>
<td>83.39±6.8%</td>
<td>35.04±10.8%</td>
<td>39.35±10.9%</td>
<td>89.84±5.91</td>
</tr>
<tr>
<td>24</td>
<td>90.43±3.8%</td>
<td>37.57±10.2%</td>
<td>40.24±9.9%</td>
<td>82.98±4.32</td>
</tr>
</tbody>
</table>

No significant differences (P>0.05) observed between treatments (1.5, 6, 12, 18, 24 mM D-Glucose).
Experiment 2: Culturing in the Presence of Insulin and Glucose

In contrast to the first experiment, embryo development was significantly affected by the concentration of glucose. Increasing glucose concentrations from 1.5 to 24 mM D-glucose significantly decreased the proportion of oocytes and cleaved embryos that developed to the blastocysts stage (P<0.05). However, cell numbers were not significantly affected by glucose (P>0.05). The addition of insulin had no significant effect on embryonic development or cell number (P>0.05).

Table 2: Culturing the presence of both insulin and glucose.

<table>
<thead>
<tr>
<th>Treatment (mM; µM)</th>
<th>Cleavage (cl)</th>
<th>Blastocyst/Oocyte (bl/oo)</th>
<th>Blastocyst/Cleaved (bl/cl)</th>
<th>Blastocyst Cell number (#)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>89.72±3.2%   A</td>
<td>29.74±9.2% A</td>
<td>32.94±9.6% A</td>
<td>91.73±5.60 A</td>
</tr>
<tr>
<td>1.5 + 1.7 I</td>
<td>91.09±3.2%   A</td>
<td>40.25±5.5% A</td>
<td>44.75±6.7% A</td>
<td>71.90±2.84 A</td>
</tr>
<tr>
<td>24</td>
<td>84.68±6.5%   A</td>
<td>14.69±3.5% B</td>
<td>17.76±4.3% B</td>
<td>81.75±4.59 A</td>
</tr>
<tr>
<td>24 + 1.7 I</td>
<td>89.52±3.5%   A</td>
<td>13.58±4.1% B</td>
<td>15.59±5.2% B</td>
<td>73.46±5.50 A</td>
</tr>
</tbody>
</table>

Treatments with different letters are significantly (P<0.05).

Experiment 3: Glucose Analogs

Compared to control (1.5G; 43.80±3.7% blastocyst/cleaved), both 24 mM 2DXG and 24G resulted in poor development (3.45±1.5% and 26.59±5.4%, respectively; P<0.05). Development of embryos cultured in 24LG (43.07% ± 8.4%) was not significantly different (P>0.05) from 1.5G (43.80% ± 3.7%). Culture made with 22.5 2 mM DXG (2.24%±1.0% bl/oo; 3.45%±1.5% bl/cl) was the most detrimental to feline embryo development.

Table 3: Glucose analogs (determining effects due to uptake; test of osmolarity).

<table>
<thead>
<tr>
<th>Treatment (mM)</th>
<th>Cleavage (cl)</th>
<th>Blastocyst /Oocyte</th>
<th>Blastocyst /Cleaved</th>
<th>Blastocyst Cell number (#)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>77.21±2.7%   A</td>
<td>33.60±3.0% A</td>
<td>43.80±3.7% A</td>
<td>74.16±1.41 A</td>
</tr>
<tr>
<td>24</td>
<td>66.82±5.9%   A</td>
<td>18.39±4.4% B</td>
<td>26.59±5.4% B</td>
<td>61.83±2.97 B</td>
</tr>
<tr>
<td>24 deoxyG</td>
<td>64.68±3.4%   A</td>
<td>2.24±1.0% C</td>
<td>3.45±1.5% C</td>
<td>77.70±2.25 A,B</td>
</tr>
<tr>
<td>24LG</td>
<td>72.12±4.4%   A</td>
<td>29.96±5.2% A</td>
<td>43.07±8.4% A,B</td>
<td>58.50±3.61 A</td>
</tr>
</tbody>
</table>

Treatments denoted with different letters are significantly (P<0.05) different.
Experiment 4: Modeling Insulin Resistance

Low dose insulin (1.7 µM or 0.0017 mM) resulted in significantly worse development (17.75% ± 1.8 % blastocyst/oocyte) than embryos cultured with 170 µm (0.17 mM) insulin(P<0.05)). However, a high dose of insulin (170 µM 31.27±6.0%) did not differ from midrange dose (17 µM; 0.017 mM, 22.46±4.2%) or control (75.62±2.6%, (P>0.05)). Development of embryos to blastocyst stage in treatments containing insulin 1.7 µM, 17 µM; (0.0017 mM, 0.017 mM) (P>0.05) were significantly worse than control (P<0.05).

Table 4: Modeling insulin resistance.

<table>
<thead>
<tr>
<th>Treatment (mM;µM)</th>
<th>Cleavage (cl)</th>
<th>Blastocyst/Oocyte (bl/oo)</th>
<th>Blastocyst/Cleaved (bl/cl)</th>
<th>Blastocyst Cell number (#)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>75.62±2.6%(^{A})</td>
<td>36.91±2.4%(^{A})</td>
<td>48.54±2.3%(^{A})</td>
<td>80.47±1.64(^{A})</td>
</tr>
<tr>
<td>1.5 + 1.7I</td>
<td>67.22±6.8%(^{A})</td>
<td>17.74±1.8%(^{B})</td>
<td>27.11±3.0%(^{B})</td>
<td>73.19±2.44(^{A})</td>
</tr>
<tr>
<td>1.5 + 17 I</td>
<td>67.43±5.3%(^{A})</td>
<td>22.46±4.2%(^{B, C})</td>
<td>32.39±4.4%(^{B, C})</td>
<td>83.28±3.29(^{A})</td>
</tr>
<tr>
<td>1.5 + 170I</td>
<td>73.35±7.5%(^{A})</td>
<td>31.27±6.0%(^{A, C})</td>
<td>42.52±7.3% (^{A, C})</td>
<td>79.85±2.67(^{A})</td>
</tr>
</tbody>
</table>

Treatments denoted with different letters are significantly different (P<0.05).
CHAPTER 5: Discussion

Insulin resistance and gestational diabetes are a common condition in pregnant women (Setji, Brown et al. 2005). In diabetic pregnancies, adverse outcomes are 3 to 9 times more likely than in non-diabetic or healthy pregnancies (Dunne, Brydon et al. 2003). Hyperglycemia in pre-implantation mouse embryos causes down-regulation of glucose transporters (GLUTs), resulting in abnormal metabolism, lower intracellular glucose, and an increase in apoptosis at the blastocyst stage (Purcell and Moley 2009). Interestingly though, in a mouse model of the obese hyperinsulinemic intrauterine environment, fetuses became hyperinsulinemic but did not have significant changes in plasma glucose levels (NANDI, KITAMURA et al. 2004). However, many studies have shown that hyperglycemia in the embryo’s environment can induce apoptosis of progenitor cells, affecting differentiation of the remaining cells. This can eventually result in fetal abnormalities or miscarriage (Purcell and Moley 2011). Gestational diabetes can also lead to a drop in maternal insulin sensitivity as may also be accountable for an increased risk of fetal overgrowth and adiposity (Catalano, Kirwan et al. 2003).

Although numerous studies have been done to evaluate the effects of glucose and/or insulin on the development of rodent embryos, few studies have addressed these issues in other model species (Bavister 1995). Given the utility of the domestic cat as a model for numerous aspects of obesity and diabetes, we wanted to determine if the feline embryo is sensitive to glucose and/or insulin, an initial step in the development of another model for studies of the reproductive consequences of obesity, insulin resistance, hyperglycemia, and diabetes. Our findings from Exp 2 and 3 have shown that 24 mM D-glucose is inhibitory to the feline early embryo however this finding was not significant in Exp 1. It has been shown experimentally that “the nutrients an embryo selects to consume, and their rates of consumption, vary extensively and are affected by the makeup of the nutrients, as well as other components of the culture medium” (Leese, 2003). The finding that 24 mM D-glucose is inhibitory to feline embryos is important as it is lower than the inhibitory dose of D-glucose for mice (early embryos 27 mM) (Pantaleon and Kaye 1996; Pantaleon and Kaye 1998), but is higher than the dose for cows (5.56 mM) or pigs (<10 mM). Results from a previous pig study demonstrated that the optimal concentration of glucose likely varies according to the stage of development (Karja, Kikuchi et al. 2006). Karja et al., (2006) suggested that the “concentration of glucose in the medium utilized
by days 1–2 pig embryos is limited to 3.5 mM and exposure to higher glucose concentrations did not improve embryo development” (Karja, Kikuchi et al. 2006). It is thought that pig embryos have a limited ability to use glucose, meaning that early embryos only use a fraction of the glucose that they take up (Karja, Kikuchi et al. 2006; Karja, Kikuchi et al. 2011). This could potentially explain the lack of response of early pig embryos to high glucose concentrations in that study (Karja, Kikuchi et al. 2006). However, later in development in most species and especially in the cat, glucose (through glycolysis) becomes increasingly utilized to support compaction and blastocyst formation (Herrick, Bond et al. 2007). Again, this is common among species. (Herrick, Bond et al. 2007), using the quantity of pyruvate oxidized relative to the amount of glucose metabolized (through glycolysis), found that feline embryos metabolize about twice as much glucose as pyruvate on Day 3 of development. By Day 6, in vitro, cat embryos metabolize nearly five times the amount of glucose in comparison to metabolism of pyruvate. Due to this increased reliance of glucose during the culture period, it is possible that feline embryos are able to utilize the glucose necessary for growth, but are able to mediate the negative effects to an extent. The cells of the early embryo can develop mechanisms to promote survival to alleviate the effects of a hyperglycemic condition and subsequent induction of cell death. Another explanation is the less explored role of GLUT-1 in feline embryos. This may also imply there is a variable sensitivity of each of the GLUT members in feline embryos. Sananmuang et al., (2011) suggested that feline blastocysts can balance glucose metabolism even in the suboptimal glucose condition through GLUT-1 expression (Sananmuang, Tharasanit et al. 2011).

The mechanism behind poor embryo development under high glucose conditions is still unclear, although it has been proposed that increased glucose concentrations may down regulate the glucose uptake into cells and generate oxidative stress which can result in insufficient metabolic conditions (Sananmuang, Tharasanit et al. 2011). Glucose, at increased concentrations, has been shown to cause a decrease of glucose transporter and metabolism in mouse blastocysts, and increase cell death and oxidative stress (Moley and Mueckler 2000). Elevated glucose concentration could also promote an imbalance of inorganic phosphate which in a previous study, was suggested to affect the development of feline embryos cultured in vitro (Sananmuang, Tharasanit et al. 2011). Previous studies show that an increasing glucose concentration lowered glycolytic activity during blastocyst formation in cat embryos (Herrick, Bond et al. 2007). Moreover, it also tended to reduce hatching blastocyst rate and increased the fragmented nuclei
of blastocysts. Increasing the glucose concentration (>6 mM) in this study negatively affected feline embryo development by reducing the blastocyst rate and cell number of blastocysts in our studies similar to previous studies by Sananmuang et al., (2011) and (Herrick, Bond et al. 2007). These results correspond to previous reports in other species demonstrating the detrimental effects of a high glucose condition on embryo development (Reece, Pinter et al. 1985; Pinter, Reece et al. 1986; Moley, Chi et al. 1998; Moley, Chi et al. 1998; Keim, Chi et al. 2001; Moley 2001; Jimenez, Madrid-Bury et al. 2003; King and Loeken 2004; Fraser, Waite et al. 2007; Cagnone, Dufort et al. 2011). One report states that increased concentrations of glucose would be assumed to be promptly phosphorylated by hexokinase to form glucose-6-phosphate. This could lead to increased concentrations of glucose-6-phosphate, and likely inhibiting hexokinase which would decrease glycolysis (Krisher, Brad et al. 2007).

Cells regulate glucose uptake by glucose transporter proteins (GLUTs) on the cell plasma membrane and also by modifying glycolytic enzyme activity (Carayannopoulos, Chi et al. 2000). Moley et al., (1998), using a mouse diabetes model found that during the blastocyst stage hyperglycemia is related to a decrease in glucose transport. This also was linked to decreased GLUT1 protein expression (Moley et al. 1998). As a consequence of the resulting down-regulation of glucose transporters, blastocysts can also experience a decrease in glucose uptake. This decrease can also cause a concurrent increase in apoptosis in the embryo (Chi et al. 2000). A decrease in glucose transport has been previously demonstrated in other reports using hyperglycemic culture conditions; the consequences being that a greater degree of apoptotic mechanisms were activated in the early mouse embryo (Doblado and Moley 2007). This can have long-lasting detrimental effects on embryo development such as fetal malformations or increased occurrence of pregnancy reabsorption (Chi, Schlein et al. 2000). The current study however, did not examine glucose transport of the feline embryo in a hyperglycemic culture condition.

The different responses of feline embryos to glucose in Experiment 1 compared to Experiments 2 and 3 may have also been due to an effect of seasonality on oocyte quality. Oocytes that came from a cat in a reproductively inactive part of the season may be more stressed by change in media substrate concentrations because the time of year has an impact on the quality of domestic cat oocytes. Spindler, Pukazhenthhi et al., (2000) established that feline oocytes remain resistant to in vitro maturation and fertilization during the natural cycle of
seasonal reproductive quiescence in the domestic cat. Even free from maternal influences, oocytes collected during suboptimal point in the breeding season continue to have diminished developmental competence in vitro (Spindler and Wildt 1999). Reduced developmental competence of the cat oocyte during the non-breeding season could have very well exaggerated any deleterious effects that elevated glucose may have had on embryos in this experiment. It is proposed that the poor development of human and mouse embryos in a high glucose condition is the consequence of altered expression of glucose transport genes (Moley, Chi et al. 1998). Alterations of glucose uptake via the glucose transport gene have not been previously examined in cat embryos either.

One more premise for the etiology of type 2 diabetes-related deformities is hyperglycemia induced apoptosis (Moley et al. 1998; Pampfer, Cordi et al. 2001). This can result in resorption of the fetus or defects due to the death of crucial progenitor cells. An in vitro hyperglycemic condition increases the incidence of nuclear fragmentation and apoptosis in murine blastocysts. An increase in the occurrence of apoptotic cells was also observed in pig embryos cultured in high glucose (Karja, Kikuchi et al. 2006). Mice embryos from hyperglycemic culture (30 mM D-glucose for 72 hours) showed about a 6-fold to 10-fold increase in the percentage of apoptotic or TUNEL-positive nuclei when compared to controls. Embryos from obesity induced diabetic mice also experience a 9.2-fold increase in the proportion of TUNEL positive (or apoptotic) nuclei (Moley, Chi et al. 1998; Moley, Chi et al. 1998). In our study, we did not measure apoptotic index nor did we see a significant difference in cell numbers. The only effects seen were observed in the decrease in the proportion of embryos reaching the blastocyst stage.

Although a significant effect of 24 mM D-glucose on feline embryos was observed, it is still unclear how glucose was exerting these inhibitory effects. Previous studies have relied on glucose analogs, 2-deoxy glucose and L-glucose, to measure glucose accumulation inside the embryo and as an osmotic control. Upon entry into cells, the analog 2-deoxyglucose may be phosphorylated, preventing subsequent efflux (Pantaleon and Kaye 1998). Moley, Chi et al. (1998) determined that the damage induced by hyperglycemia in the early mouse embryo is due to impairment in glucose transport because equal concentrations of L-glucose had no effect on the embryos. Therefore, the effect of high glucose concentrations on mouse embryos was not likely due to osmotic effect. Feline embryos were cultured with L-glucose, which is not taken up
by the embryo, and 2-deoxyglucose, which is taken up by the embryo, but cannot be metabolized through or past the first step of glycolysis. We tested these glucose analogs on our feline embryos to also determine if effects from high glucose were due to problems with uptake and metabolism of glucose, or if could be at all related to an increase in medium osmolarity. In this study, like studies before ours; L-glucose had no effect on feline embryos. Culture with 24 mM 2-deoxyglucose was detrimental to feline embryo development. A likely explanation for this is that 2-deoxyglucose can be phosphorylated to 6-phosphodeoxyglucose. However, the compound 6-phosphodeoxyglucose cannot be metabolized through the first step of glycolysis, so ATP cannot be produced. This leads to a net loss of ATP from this pathway (Schini and Bavister 1988).

An excessive concentration of glucose is also related to an increase in oxidative stress in mouse blastocysts. This is consistent with porcine in vitro research as well. The concept that oxidative stress is linked with diabetes has been proposed by numerous others authors (Takahashi 2012). Porcine embryos cultured with elevated glucose concentrations experience a rise in reactive oxygen species (ROS) generation, especially at the one-cell stage (Cagnone, Dufort et al. 2011). Providing oocytes and cumulus cells with mechanisms for coping with the oxidative stress of in vitro culture is therefore a critical component of a maturation medium. The dysmorphogenesis found in rodent embryos exposed to high glucose concentrations in vitro has been diminished by addition of inositol, arachidonic acid, or other antioxidants to the culture medium (Eriksson and Borg 1991; Guérin, El Mouatassim et al. 2001; Lojkic, Getz et al. 2012). Cysteamine reduces cysteine to cysteine, so maintaining greater concentrations in the medium can support glutathione (GSH) synthesis (De Matos et al., 2002). The generally reported positive effects of cysteine and cysteamine is probably attributed to enhanced GSH synthesis, decreased oxidative stress, and improved cytoplasmic maturation (Guérin, El Mouatassim et al. 2001). Thus, it is advisable to include both cysteine and cysteamine in maturation medium to support sufficient GSH synthesis (Krisher, Brad et al. 2007). Many authors also reported increased blastocyst development following cysteamine supplementation to the IVM medium. For example, this has been observed in cattle, buffalos, and mice (de Matos et al. 2003; Gasparrini, Sayoud et al. 2003; Urdaneta, Jiménez et al. 2003(Gasparrini, Sayoud et al. 2003). Most commonly, IVM media have been supplemented with a dose of 100 µM of cysteamine in cattle, buffalo, goats, horses, pigs, mice, dogs, and cats (Gardiner and Reed 1995; Trocino, Akazawa et
al. 1995; Van Soom, Yuan et al. 2002). Cysteine also has a positive effect on oocyte maturation in vitro in the pig (Yoshida, Cran et al. 1993). Cysteine and cysteamine are reported to promote cell viability by a direct antioxidant action (Takahashi et al., 1993). It is possible that supplementing our medium with cysteine & cysteamine had a protective antioxidant effect as many authors report increased blastocyst development following cysteamine supplementation to IVM. The current study supplemented IVM with 0.6 mM cysteine and 0.1 mM cysteamine though we cannot determine whether or not this addition had protective effects. Data supports the idea that diabetic pregnancy involves excess radicals at a late stage contributing to the teratogenic process. However, this diabetes-induced dysmorphogenesis can be considerably reduced by opposing the excess radicals with antioxidants as well (Eriksson and Borg 1991; Eriksson, Cederberg et al. 2003). It is possible that adding more antioxidants to the media could lead to feline embryos being more resistant to the effects of glucose. The offspring of diabetic rodents show less dysmorphogenesis if the mother is supplied with inositol, arachidonic acid, or antioxidants during pregnancy (Eriksson, Cederberg et al. 2003) but this was not tested in the course of this study.

We investigated the effects of elevated glucose and insulin concentrations on the development of the feline embryo in vitro. Effects of insulin were evaluated in the presence of glucose to determine if there were effects due to insulin stimulated glucose uptake. Insulin and IGF-1 have been shown to promote uptake of glucose in the blastocyst, but this uptake, at least in the mouse, has been shown to operate via the IGF-1 receptor rather than the insulin receptor in a previous study (Chi, Schlein et al. 2000). The action of insulin in bovine embryos is also shown to be mediated by the IGF-I receptor, as it was blocked by an anti-IGF-I receptor antibody (alphaIR-3) (Matsui et al., 1997). Positive effects of insulin on development of bovine embryos, however, were observed only when it was accompanied by glucose in the culture media (Matsui et al., 1995; (Augustin, Pocar et al. 2001; Augustin, Pocar et al. 2003). However, previous studies found that supra-physiological levels of insulin and IGF-I causes a reduction in protein expression of the IGF-I receptor in the mouse blastocyst. This leads to an overall decrease insulin and IGF-I signaling in the embryo (Chi, Schlein et al. 2000). In response to a high concentration of insulin or IGF-1, as seen in other in vitro and in vivo model systems of hyperinsulinemia, down-regulation of the IGF-I receptor is observed (Harvey and Kaye 1991). These findings are consistent with insulin resistance in the blastocyst. Feline embryos were cultured in 0, 1.7, 17,
and 170 insulin µM to represent alterations in insulin sensitivity. The rate of development for blastocyst per cleaved embryo observed was poor for those feline embryos cultured in the low and midrange dose of insulin. From the literature, it was expected the lower doses to be stimulatory and the higher doses (similar to insulin resistance or type 2 diabetes) to be inhibitory (Harvey and Kaye 1990; Moley, Vaughn et al. 1991; Kaye and Gardner 1999; Catalano, Kirwan et al. 2003; NANDI, KITAMURA et al. 2004). For this experiment, exactly the opposite was observed in feline embryos. However, most other studies used extremely elevated in vitro levels of insulin (as high as 600 nm), to show a decrease cell number and adversely affect development. It is likely that with the lower concentrations we used, the abundance of insulin was enough to down-regulate the insulin receptors (and Akt/PI3K signaling), but not enough to trigger the IGF-1/growth promoting pathways in the feline embryo. Another previous study demonstrated that two cell mouse embryos incubated for 72 hours in intermediate doses of insulin (350 nm) also displayed TUNEL-positive nuclei. Apoptotic rates in were about 50% of those seen for high and low insulin in the mouse. Exposure to high concentrations of insulin or IGF-1 causes apoptosis of blastocyst nuclei in mouse embryos(Chi, Schlein et al. 2000). In a state of hyperinsulinemia, the IGF-1 and IGF-1R signaling mechanisms necessary to traffic GLUT-8 to the plasma membrane are malfunctioning (Carayannopoulos, Chi et al. 2000). Subsequently, the critical increase in glucose uptake by GLUT-8 does not occur and a lower threshold of glucose is reached. This can trigger programmed cell death or apoptosis. If there is a significant degree of programmed cell death within the inner cell mass (ICM), a pregnancy could result in a fetal resorption (Chi, Schlein et al. 2000). We did not measure insulin related apoptotic index for feline embryos in this study though.

Insulin enhances embryonic development and these effects are mediated via receptors which are sensitive to extremely low insulin concentrations suggesting a mechanism by which the embryo may be continually responsive to basal maternal insulin levels (Harvey and Kaye 1990). A disruption of this normal, but steady baseline exposure to insulin may lead to such deleterious effects that we observed in our experiments. It is not discernible as to why the low and medium (1.7 and 17 µM; 0.0017 mM- 0.017 mM) insulin concentrations so negatively affected feline embryos in comparison to control. Additional research must be carried out in order to investigate insulin and its receptor actions in feline embryos. Lastly, high concentrations of insulin are likely to engage the IGF-1 receptors, promoting growth; an effect we likely saw in
our embryos cultured in 170 μM (0.17 mM). Dose response and anti-insulin receptor antiserum studies revealed that this occurs via the IGF-1R and not the insulin receptor in the mouse. IGF-1 can stimulate glucose transport half maximally at a very low concentration (1.0 pm; 1 x 10^-9 mM) in vitro, a value in the scope for standard physiologically relevant stimulation of the IGF-1 receptor. Yet, to see the same effect with insulin, a 1,000-fold level higher is required (Chi, Schlein et al. 2000). It seems plausible that overabundance of insulin ends up triggering the IGF-1 receptor, resulting in stimulatory or growth promoting effects (Harvey and Kaye 1990; Schultz, Hogan et al. 1992; Pantaleon and Kaye 1996; Herrler, Krusche et al. 1998; Chi, Schlein et al. 2000; Navarrete Santos, Tonack et al. 2004; Navarrete Santos, Ramin et al. 2008).

The pathology of insulin resistance, hyperglycemia, and gestational and/or type 2 diabetes has led to a significant increase in neonatal mortality rates. These conditions generate a host of reproductive anomalies that can result in congenital abnormalities of the fetus. This also increases the risk for spontaneous abortion (Fuhrmann, Reiher et al. 1983). The negative effects of maternal diabetes can take place as early as the oocyte stage, but can continue to occur throughout implantation and organ development in the embryo. Progress can be expected in the future with the continued use of improved in vitro animal models. This will improve our understanding behind the biochemical range of responses to alterations in glucose and insulin metabolism. Several theories are being explored that cover a majority of the major cellular steps for glucose- stimulated insulin secretion and insulin stimulated glucose transport. Topics being studied include exploring deviations from normal glucose transport, storage, and metabolism.

We found that the cat is sensitive to glucose and insulin, suggesting it may be a good model for further explorations of the effects of obesity on fertility in humans. No standalone postulation is currently able to explain all of the aberrations the embryo can experience due to an alteration in glucose and/or insulin metabolism. It would be important to elucidate mechanisms that determine insulin resistance & glucose signaling in the feline embryo, the knowledge from this study should contribute to the understanding of effects of insulin and high glucose on feline embryos. More information will lead to innovative new strategies for therapy of maternal obesity and related metabolic derangements in utero.
Bibliography


