Improved Late-gestation Cardiac Morphology in Fetuses of Diabetic Mothers After Maternal Immune Stimulation: Potential Role of Dysregulated Apoptosis

by

Juan Claudio Gutierrez Toro

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APPROVED BY:

Steven D. Holladay, Chair

M. Renee Prater, Co-Chair

Larry E. Freeman

Bonnie J. Smith

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Juan Claudio Gutierrez Toro

Steven D. Holladay, Committee Chairperson

Department of Biomedical Sciences and Pathobiology,

Virginia-Maryland Regional College of Veterinary Medicine

(ABSTRACT)

The incidence of malformed newborns is higher in human pregnancies complicated by diabetes mellitus, as compared to non-diabetic pregnancies. Neural tube and cardiac defects predominate among the fetal malformations induced by hyperglycemia. Non-specific maternal immune stimulation is protective in mice against birth malformations caused by chemical or physical teratogens, or by maternal diabetes mellitus. Insulin dependent diabetes was induced in ICR females to study the late gestation fetal heart by morphometric analysis. Diabetic females treated with Freund's compete adjuvant (FCA) or interferon-gamma (IFNγ) were also generated to elucidate potential positive effects of maternal immune stimulation during the diabetic pregnancy by morphometric analysis and pathologic scoring. Insulin-dependent CD1 females were generated to analyze late gestation fetal myocardial apoptosis by flow cytometric analysis and by real time-polymerase chain reaction (RT-PCR) analysis of a panel of 5 genes involved in apoptosis/proliferation (Bcl-2, P53, Caspase3, Caspase9 and PkC-e). The morphometric analysis of fetal hearts revealed visibly obvious dilation of ventricular chambers and outflow channel of the left ventricle, and reduction of total myocardial ventricular area in late gestation fetuses, as predominant changes seen in the offspring of diabetic dams. Pathologic scoring revealed that maternal immune stimulation, particularly with FCA, in part alleviated fetal heart changes of cavitary dilation and myocardial reduction. Increased rate of apoptosis/necrosis in the fetal myocardium in late gestation during the diabetic pregnancy was evidenced by flow cytometric analysis. Particularly there was a significant increase in percentage of early apoptotic cells in the fetal myocardium detected by cell markers annexin V and propidium iodide. There was also a significant

increase in percentage of late apoptotic/necrotic fetal myocardial cells in the diabetic group compared to the control group. These results suggest that maternal treatment with FCA may in part protect the heart from high hyperglycemia by reducing the number of myocardial cells undergoing apoptosis and necrosis. The RT-PCR analysis revealed subtle changes in gene expression for all the genes except Bcl-2. A paradoxical and dramatic up-regulation of this anti-apoptotic gene was observed in late gestation fetal myocardium from the insulin-dependent hyperglycemic groups. Possibly, this could be a mechanism to protect the fetal myocardial cell from the chronic exposure to a severe hyperglycemic insult and consequent apoptosis. In conclusion, maternal insulin-dependent diabetes caused morphological changes in the late gestation fetal heart. Such changes were in part related to dysregulation of myocardial apoptosis. Maternal immune stimulation with FCA improved fetal heart morphology, by a mechanism that may in part relate to normalizing fetal myocardial apoptosis.

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BCG	bacillus Calmette Guerin
BMI	body mass index
Casp3	caspase 3
Casp9	caspase 9
CDs	cardiac defects
CNC	cardiac neural crest
CVC	caudal vena cava
ECM	extracellular matrix
FCA	Freund's complete adjuvant
GD	gestation day
GDM	gestational diabetes mellitus
GLUT	glucose transporter
GM-CSF	granulocyte macrophage colony-stimulating factor
HFD	high fat diet
IFNγ	interferon gamma
IGFBP	insulin like growth factor binding protein
IGF-I	insulin like growth factor I
IGF-II	insulin like growth factor II
IL	Interleukin
IVS	interventricular septum
LAu	left auricle
LCVC	left caudal vena cava
LV	left ventricle
MNU	methyl-nitrosourea
NCC	neural crest cells
NTDs	neural tube defects
PC	principal component
PCA	principal component analysis
PkC-e	protein kinase C epsilon

RA	right atrium
RAu	right auricle
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT-PCR	reverse transcriptase PCR
RV	right ventricle
SD	standard diet
STZ	Streptozocin
T1	type 1 diabetes (insulin dependent diabetes)
T2	type 2 diabetes (insulin resistant diabetes)
TGF β	transforming growth factor beta
TNF α	tumor necrosis factor α
VA	valproic acid

CHAPTER 1: HYPOTHESIS AND SPECIFIC OBJECTIVES

1.1: Hypothesis

The incidence of malformed newborns in humans is 6-10% or 3 to 5 times higher in pregnancies complicated by diabetes mellitus, as compared to non diabetic pregnancies (Reece and Eriksson, 1996; Reece et al., 1998). Neural tube and cardiac defects predominate among those malformations related to hyperglycemia (Reece et al., 1998; Punareewattana and Holladay, 2004). Heart defects in particular have been reported as increasing up to five fold among infants of diabetic mothers compared to the general population, representing a significant segment of anomalies that might be reduced with gestational control of maternal blood sugar levels (Becerra et al., 1990; Meyer-Wittkopf et al., 1996). For the most part, available literature suggests the rate of myocardial apoptosis may increase in a hyperglycemic environment (Fiordaliso et al., 2001; Cai et al., 2002; Frustaci et al., 2000). Studies by Frustaci et al. (2000) reveal that apoptosis and necrosis are increased in myocytes, endothelial cells and, fibroblasts of the human adult diabetic heart. Protective effects of maternal immune stimulation and antioxidant therapy are reported in rodent models against congenital malformations by chemical teratogens and maternal diabetes mellitus (Nomura et al., 1990; Holladay et al., 2000; Sharova et al., 2000; Punareewattana and Holladay, 2004; Hrubec et al., 2006).

Rodent models of maternal diabetes are extensively reported in the literature to study the harmful effects of hyperglycemia, and commonly use streptozocin (STZ) as an inducer of non-insulin dependent diabetes (Like and Rossini, 1976; Goldman et al., 1985; Punareewattana and Holladay, 2004). Considering the importance of the study of the effects of gestational hyperglycemia, new alternative models of other varieties of diabetes may be considered.

In this study we hypothesized that diabetes alters the morphology of the late gestation fetal heart and that maternal immune treatment may positively affect the course of maternal hyperglycemia and improve fetal heart development. We also hypothesized that maternal diabetes dysregulates fetal myocardial apoptosis during the course of gestation. We further hypothesized that the use of the combination of high fat diet (HFD)

and low dose of STZ could be an alternative to model insulin-resistant maternal diabetes for the study of fetal development.

1.2: Specific Objectives

These studies were designed to evaluate morphological heart changes in late gestation fetuses due to maternal diabetes with or without maternal immune stimulation. These studies were also designed to study late fetal myocardial apoptosis dysregulation in the diabetic pregnancy with or without maternal immune stimulation. These studies used a classical mouse model of insulin-dependent maternal diabetes and proposed as a new alternative for modeling maternal insulin-resistant diabetes. Study 1 examined fetal cardiac morphology in late gestation in the course of maternal insulin-dependent diabetes. Study 2 examined the effects of maternal immune stimulation in the morphology of the late gestation fetal heart in the course of insulin dependent-diabetes. Study 3 examined and compared the classical model of maternal insulin-dependent diabetes (type 1 diabetes) induced by STZ and a model of maternal insulin-resistant diabetes (type 2 diabetes) induced by the combination of STZ and high fat diet. Study 4 examined fetal myocardial apoptosis in late gestation fetal hearts using insulin-dependent diabetic dams.

1.2.1. Study 1: Effects of insulin-dependent diabetes in late gestation fetal morphology with emphasis in the fetal heart.

A classic mouse model of insulin dependent diabetes induced by STZ was used in this study. Late gestation fetal hearts were analyzed by morphometric analysis using routine histopathologic techniques. Serial sections of the fetal thorax were studied from control and diabetic groups. Fetuses from the diabetic group displaying external malformations (neural tube defects) were analyzed independently.

1.2.2. Study 2: Effects of maternal immune stimulation in late fetal heart development.

A classic mouse model of insulin-dependent diabetes induced by STZ was used in this study. Late gestation fetal hearts were analyzed by morphometric analysis using routine histopathologic techniques. Serial sections of the fetal thorax were studied in the

offspring of control, diabetic and immune-stimulated diabetic females treated with complete Freund's adjuvant (FCA) or interferon γ (INFγ).

1.2.3. Study 3: Non-genetic mouse model of insulin-resistant maternal diabetes.

A classic mouse model of insulin-dependent diabetes induced by STZ was compared with an insulin-resistant model induced by the combination of STZ and HFD using outbred female mice. General maternal and fetal parameters were studied and compared (glucose, insulin, weight). Preliminary results for modeling gestational diabetes mellitus (GDM) were shown.

1.2.4. Study 4: Determination of myocardial apoptosis in fetuses of diabetic mothers

A classic mouse model of insulin dependent diabetes induced by STZ was used in this study. Dysregulation of fetal myocardial apoptosis was determined by flow cytometric analysis and by RT-PCR of a panel of genes involved in apoptosis/proliferation. Effects of maternal immune stimulation with FCA were also determined.

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CHAPTER 2: LITERATURE REVIEW

2.1: Diabetes and Teratogenesis

In pregnancies complicated by diabetes mellitus, the incidence of major birth malformations is 6 – 10%, 3 – 5 fold higher than in the general population (Reece, 1998). Numerous investigators have demonstrated that hyperglycemia and other metabolic fuels produce teratogenic effects during organogenesis (Reece et al., 1996; Reece et al., 1998; Eriksson et al., 1991). The exact mechanisms involved have not been completely elucidated. However, a number of possibilities have been proposed.

Aberrant metabolic fuels present during hyperglycemia and hyperketonemia are toxic factors for the developing embryo. These mechanisms occur via the yolk sac, which appears to be the target site for the injury (Reece et al., 1998). There is a strong relationship between hyperglycemic malformations and yolk sac abnormalities. In rat embryos subjected to a high D-glucose environment, the visceral yolk sac capillaries and vitelline vessels are sparse, patchy and non-uniformly located. In the same embryos, the visceral yolk sac endodermal cells have a reduced number of rough endoplasmic reticulum, ribosomes and mitochondria (Reece et al., 1998). Studies using anti-yolk sac antibodies have shown a secondary embryopathy (Beckam et al., 1990). According to other studies, the injection of the agent trypan blue (a teratogenic agent) results in embryopathy only if the injection affects the yolk sac (Beck et al., 1967; Reece et al., 1994).

Other authors have proposed that the etiology has relationship with nutrient deficient states in membrane lipids such as arachidonic acid and myo-inositol and the generation of excess free oxygen radicals (Reece et al, 1996; Reece et al, 1998). The teratogenic process in maternal diabetes is multifactorial but may follow a common pathway. Reece et al. (1996) have postulated that the teratogenic effect in diabetes is a very specific process of many aberrant events and if these aberrant events are asynchronus, embryopathy may not be manifested. Prevention of birth malformations in diabetic rat offspring has been achieved by glycemic control during the organogenesis process. Similar results may be seen in a hyperglycemic state, where restoration of essential fatty acid/phospholipids deficiency and normalization of excess free radicals

may be achieved by supplementation with polyunsaturated fatty acids, myo-inositol or antioxidants (Reece et al., 1996).

Freinkel et al. (1998) proposed a critical period for hyperglycemia as an inducer of congenital malformations. This period is the organogenesis stage, days 9.5 to 11.5 in rats and days 8 to 9.6 in mice. Myoinositol depletion has been proposed as another mechanism for diabetic embryopathy. Under hyperglycemia, glucose is channeled into nonconventional metabolic pathways such as the polyol pathway which leads to the conversion of glucose into sorbitol (by the action of the enzyme aldose reductase). The increase of sorbitol interferes with some cellular functions (Reece et al., 1998). The increase in sorbitol leads to a proportional inverse reduction in myoinositol, total protein and RNA in rat embryos between days 9.5 and 11.5 in a hyperglycemic medium (Hod et al., 1990). These findings show that myoinositol depletion is a major factor involved in the mechanisms of diabetic embryopathy (Reece et al., 1998; Hod et al., 1990).

Among the malformations induced by hyperglycemia, neural tube defects and cardiac defects are commonly found, and these changes seem to demonstrate a dose-response curve. The pattern for the evidence of birth malformations under a hyperglycemic environment in rats is: 20% malformations rate at glucose levels two fold (300 mg/dl) above normal values (150mg/dl), 50% malformations rate at glucose levels four fold (600 mg/dl) above normal and almost 100% malformations rate at glucose levels greater than six times control (950 mg/dl) (Reece et al, 1998). Hyperketonemia is another suggested teratogenic factor in diabetic animal models but there is not enough evidence in humans (Reece et al., 1998).

Oxidative stress has been proposed to contribute significantly to hyperglycemia-induced teratogenesis. An increased amount of enzyme superoxide dismutase in a hyperglycemic environment protects against malformations in rat (Eriksson et al., 1991) and mouse (Hagay et al., 1995) embryos. Embryos from transgenic mice over-expressing the enzyme superoxide dismutase were protected against teratogenic effects of diabetes. This evidence supports the role of increased reactive oxygen species (ROS) in the diabetic embryopathy (Hagay et al., 1995).

Another molecular mechanism has been proposed for diabetes mellitus-induced embryopathy: altered extracellular matrix (ECM) molecule gene expression and protein

production. These molecules modulate and control cell to cell and cell to matrix interactions, and as such are thought to influence fetal formation. The changes in ECM are critical in morphogenesis because of the architectural importance of these molecules (Cagliero et al., 1993; Smoak, 2004). ECM molecules are proteins such as fibronectin, collagen, glycoproteins and proteoglycans. They can form deposits in different tissues in the course of diabetes mellitus (Chen et al., 2000). As a response to hyperglycemia, fibronectin and collagen accumulate in kidneys, heart, blood vessels and skin (Mauer et al., 1981). The adult heart suffers myocardial hypertrophy, perivascular fibrosis and thickened basement membranes in the diabetic patient (Chen et al., 2000). Transforming growth factor beta (TGF β) is a 25 k-Da protein. TGF β activation promotes cell growth and differentiation. There are three isoforms of this protein that interact with cell surface receptors (TβR-I, II and III). TGF β-1 mediates an increased production and deficient degradation of ECM molecules (Smoak, 2004). Cardiac development and remodeling may be altered by deposition of collagen mediated by TGF β-1 (Booz and Baker, 1995). Mouse embryos examined by immunohistochemistry showed altered protein expression of TGF β-1, 2 and 3 in heart tissue at different developmental time windows: days 9.5, 10.5, 11.5, 12.5 and 13.5 after coitus. In the same experiments, embryos exposed to a hyperglycemic medium (600 mg/dl) for 24 hours showed increased levels of total TGF β-1 and fibronectin in the heart. The authors proposed that TGF β induced the expression of fibronectin mRNA in the embryonic heart (Smoak, 2004).

Heart defects occur in excess in infants of diabetic mothers and represent a segment of anomalies that could be prevented (Becerra et al., 1990; Ferencz et al., 1990). Epidemiological evidence clearly shows that preconceptional maternal diabetes adversely affects the early development of the heart. There are major anomalies of cardiovisceral and atrioventricular concordance, as well as defects of the cardiac outflow tract and atrioventricular valves (Loffredo et al., 2001). Maternal diabetes is an independent factor for cardiovascular malformations and major vulnerability occurs in early pregnancy (Reece et al., 1996). Among the categorization of heart defects, early cardiovascular abnormalities (defects of primary cardiogenesis) are associated with diabetes (Loffredo et al., 2001). The evidence of diabetes-induced major cardiac defects is of urgent clinical significance because preventive care has been demonstrated to protect fetal heart

formation. It is necessary to treat hyperglycemia in women entering their reproductive years as an important target for the prevention of major forms of cardiac and noncardiac anomalies (Loffredo et al., 2001). Studies by Meyer-Wittkpof et al. (1996) reveal that cardiac malformations occurred in 3.1% of a group of 326 human diabetic pregnancies. These authors recommend echocardiography as a screening test in the diabetic pregnancy as a companion to glycemic control. Perinatal mortality related to the diabetic pregnancy has been reduced in recent years due to improvement in diabetic and obstetric care. However, the main factor for perinatal death is congenital malformations, mainly congenital heart disease (Meyer-Wittkpof et al., 1996). Studies by Garcia-Patterson et al. (2004) revealed that among infants of diabetic mothers, the severity of hyperglycemia is a predictor for one or more major congenital malformations (heart, and skeletal malformations) and minor congenital malformations. Nevertheless, in this study another predictor for one or more congenital malformations was an increased pre-pregnancy body mass index (BMI). BMI is a predictor for major congenital malformations (heart and renal/urinary). BMI is also a predictor for minor congenital malformations. The association between pre-pregnant maternal obesity and congenital malformations could be explained by different mechanisms. One of these mechanisms may be the increased supply of nutrients to the fetus from the obese mother. The excess of fuels such as glucose, ketone bodies and amino acids can be embryotoxic (Eriksson, 1995). Another possible mechanism is hyperinsulinemia. An excess of proinsulin in chicken embryos induces teratogenesis by reducing natural apoptosis (Hernandez-Sanchez et al., 2002).

Watkins et al. (2003) reported a significant 2 fold increased risk for heart defects in fetuses from obese and overweight women. One of the explanations for this relationship is metabolic alterations in obese and overweight women such as mild and poorly controlled hyperglycemia and elevated insulin or estrogen levels. Obese women may be unrecognized for diabetes. Obese women also can be deficient in folic acid which is considered to be a necessary nutrient to protect against birth defects (Watkins et al., 2003). These findings consider that obesity and overweight might be other predictors for congenital malformations in addition to hyperglycemia and malnutrition. Increased neural tube defects (NTDs) risk was associated with low values of a diet quality score that reflected lower maternal dietary intake of iron, vitamin B6, calcium, vitamin A, folate

and higher percentage of kilocalorie intakes from fat and sweets. In Mexican-American women there is a strong association between elevated BMI, hyperinsulinemia and NTDs in the offspring (Hendricks et al., 2001). Potential problems in glucose control were associated with NTDs risk even in non-diabetic mothers (Shaw, 2003). A significant 2-fold risk for heart defects in fetuses from obese and overweight women has been reported. Metabolic disorders such as hyperglycemia, hyperinsulinemia or elevated estrogen levels could be possible explanations for this phenomenon. Epidemiologic studies by Shaw et al. (2003) suggest that NTD risk increases as maternal prepregnant BMI increases and, therefore, NTD-affected pregnancy risk is not restricted to obese women. According to these data, the mother must control not only glucose levels, but also her nutrition and weight before and during pregnancy.

2.1.1 Prevention of Birth Defects

It has been proven that non-specific maternal immune stimulation prevents the occurrence of birth defects by teratogens including diabetes mellitus (Torchinsky et al., 1997; Holladay et al., 2000; Sharova et al., 2000; Sharova et al., 2002; Holladay et al., 2002; Punareewattana et al., 2003; Punareewattana and Holladay, 2004). Studies by Prater et al. (2004) support the evidence of immune protection against birth defects from the harmful effects of teratogen methylnitrosourea (MNU), an inducer of oxidative stress. The authors reveal an improvement of distal limb development and in placental integrity when CD1 mice exposed to MNU on gestation day 9 (GD9) are immune stimulated with IFNγ. The authors propose that improved placental function after maternal immune stimulation could be related to the improvement of birth defects associated with MNU. They also propose that improved birth defects in MNU-treated and immune-stimulated mice, may also involve additional mechanisms beyond improved placental structure or function and evaluation of alteration in fetal and maternal gene expression (cytokines and growth factor expression) may help to characterize the underlying mechanisms of maternal immune-protection.

In other studies, Hrubec et al. (2006a) reported the harmful effects of valproic acid (VA) in the offspring of CD1 mice and found these effects were reduced by maternal immune-stimulation with the cytokines granulocyte macrophage colony-stimulating

factor (GM-CSF) or IFNγ. Results in these studies show that at GD17 the incidence of NTDs was 18% in fetuses exposed to VA alone, compared to 3.7% and 2.9% in fetuses exposed to IFNγ + VA, or GM-CSF + VA respectively. Ocular defects were also significantly reduced from 28.0% in VA exposed groups to 9.8% in IFN-γ + VA and 12.5% in GM-CSF + VA groups. In other studies by Hrubec et al. (2006b) maternal immune stimulation with IFNγ or GM-CSF reduced the incidence of diabetes induced craniofacial defects, and GM-CSF prevented hyperglycemia induced shortening of craniofacial length. In these experiments, highly hyperglycemic (>26 mM/L) ICR females produced offspring with craniofacial defects. Among these defects, the most common observation was reduced maxillary and mandibular lengths. The authors concluded that the mechanisms underlying this reduction in diabetes induced craniofacial defects are unclear but may involve maternal or fetal production of cytokines or growth factors that protect the fetus from the dysregulatory effects of the hyperglycemia. Studies by Torchinsky et al. (1997) report that maternal immune stimulation of highly hyperglycemic ICR females (>27.8 mM/L) induced by streptozocin (STZ) protected the litters against fetal malformations. In this report the female mice were immunized with rat splenocytes 3 weeks before breeding. Such maternal immune stimulation reduced the rate of malformations in litters from 63% to 18%. The same study also showed an increase in pregnancy rate in immune stimulated females (69%) compared to non-immune stimulated females (44%), suggesting that STZ may contribute to fetal resorption or early embryonic death.

Studies by Punareewattana and Holladay (2004) made use of three different immune-stimulants in insulin-dependent diabetic pregnant ICR mice. The authors used 20-30 µl FCA by footpad injection, 8000 IU granulocyte-macrophage colony stimulating factor (GM-CSF) by intraperitoneal injection or 1000 IU IFNγ by intraperitoneal injection. In these studies, females received just one of the immune-stimulants. Fetuses were examined at gestation day GD17 for major neural tube defects. The authors detected that maternal immune-stimulation with FCA, GM-CSF or IFNγ produced similar protective results against NTDs (50% of occurrence of NTD on the diabetic group was reduced to 14-23% in the diabetic group with the immune-stimulation treatment).

Maternal antioxidant therapy has also shown reduction of malformations as a result of exposure to teratogens. Studies by Prater at al. (2008) made use of quercetin (2-(3,4-dihydroxyphenyl)-3,5,7;tryhydroxy-4H-1-benzopyran-4-one, Q) a natural polyphenolic flavonoid found in fruits, vegetables and nuts (average human consumption of Q is 1g/day). Among the properties of quercetin is the alteration of signaling pathways such as NFkB, JakSTAT and protein kinase C driving cell proliferation, differentiation and apoptosis (Prater et al., 2008; Dias et al., 2005; Kempuraj et al., 2005). In these studies maternal Q supplementation normalized shortened limbs and distal limb malformations in offspring of C57BL/6 mice exposed to MNU during pregnancy. In the same studies, female supplementation with Q also normalized placental and endothelial and trophoblast damage and normalized placental gene expression of key proteins in placental development and fetal osteogenesis altered by MNU. Studies by Siman et al. (2000) in a morphometric analysis of neural crest-derived organs revealed that fetuses of diabetic rats showed low-set external ears, severely malformed Meckel's cartilage, small thyroid and thymus, absence of parathyroid glands and cardiac anomalies. Cardiac anomalies described by the authors included rightward displacement of the aorta, double outlet right ventricle, persistent truncus arteriosus and ventricular septal defects. However, maternal supplementation with antioxidant vitamin E (α tocopherol) reduced the severity of these malformations. Prater et al. (2005) reported that MNU treated CD1 and C57BL/6N females showed placental improvement and fetal defect (syndactyly, oligodactyly and polydactyly) reduction after supplementation with antioxidant butylated hydroxytoluene (BHT). The authors propose that fetal defects and placental damage are in part related to ROS and that improvement in fetal defects may be related to improved placental integrity and function.

A common theme of the above reports was that mechanisms of maternal immune-stimulation and maternal antioxidant therapy in fetal protection against exposure to teratogens such as diabetes mellitus are not yet completely elucidated and are worth research attention.

2.2 Streptozocin, an Inducer of Diabetes Mellitus in Rodents

Mouse models of maternal diabetes have been used extensively in recent years. The use of streptozocin (STZ) in a high dose to induce insulin-dependent diabetes is considered a classical model of type 1 diabetes (Like and Rossini, 1976; Goldman et al., 1985; Punareewattana and Holladay, 2004). STZ is a monofunctional nitrosourea analogue in which the N-methyl-N-nitrosourea (MNU) moiety is linked to the carbon-2 of a hexose (Lenzen, 2008). STZ is a member of a group of alkylating antineoplastic drugs (Bolzan and Bianchi, 2002).

2.2.1 Streptozocin Mechanisms

Glucose is known to enter the pancreatic beta cell by passive diffusion by a specific membrane protein termed glucose transporters (GLUT). The first four members of the GLUT family are the ones that have been best characterized, and they have distinct affinity for glucose and distinct patterns of expression. Glucose transporter GLUT 2, has a low affinity for glucose and seems to act as a transporter only when plasma glucose levels are relatively high (Greenspan and Gardner, 2004).

Nitrosoureas are usually lipophilic and tissue uptake through the plasma membrane is rapid. However, as a result of the hexose substitution, STZ is less lipophilic and is selectively accumulated in the pancreatic beta cell via GLUT2 (Lenzen, 2008). The toxicity of STZ is dependent upon the DNA alkylating activity of its methylnitrosourea moiety, especially at the O^6 position of guanine. The transfer of the methyl group from STZ to the DNA molecule causes damage which results in DNA fragmentation. Protein glycosylation may be an additional damaging factor. In an attempt to repair DNA, poly (ADP-ribose) polymerase (PARP) is overstimulated. The result is a depletion in NAD^+ and ATP stores. This will cause beta cell necrosis by depletion of cellular energy (Lenzen, 2008).

2.2.2: Streptozocin and High Fat Diet

The use of STZ as a unique inducer of diabetes is a classical rodent model of insulin-dependent (type 1) diabetes (Like and Rossini, 1976; Goldman et al., 1985; Punareewattana and Holladay, 2004). Isolated intraperitoneal (IP) injection of a high dose

of STZ (200 mg/kg) dissolved in cold citrate buffer (0.05M, pH: 4.5) causes severe insulin dependent diabetes in female mice (Punareewattana and Holladay, 2004; Punareewattana et al., 2005; Hrubec et al., 2006b; Gutierrez et al., 2007). The use of a combination of HFD and low dose of STZ was reported for the first time by Luo et al. (1998) to generate a non-genetic mouse model of non-insulin dependent (type 2/insulin resistant) diabetes. The authors indicated that the development of hyperglycemia in mouse models such as C57BL/6J obese (ob/ob) and C57BL/ks (db/db) is genetically determined to a much greater extent than in patients with type 2 diabetes. The experimental approach of this model was an extension of prior studies showing that fructose fed and fat fed rodents become hyperinsulinemic and insulin resistant but are able to maintain normal glucose levels (Zevaroni et al., 1980; Luo et al., 1998). The administration of a relatively low IP dose of STZ (100mg/kg) in high fructose and fat fed mice leads to significant hyperglycemia, whereas the same dose does not decrease the insulin secretory capacity enough to cause hyperglycemia in mice fed a standard rodent diet (Luo et al., 1998). It is important to mention that this model described by Luo et al. (1998) and other authors (Reed et al., 2000; Srinivasan et al., 2005) has made use of male mice and rats. Female rodents (non-pregnant or pregnant) have not been previously used to model insulin resistant diabetes using the combination of HFD and low dose of STZ.

The first two strains of mice used to apply this combination were C57BL/6J (inbred strain) and ICR (outbred strain). Both strains developed insulin resistance and compensatory hyperinsulinemia when fed with diets enriched with fat or fructose. Hyperglycemia developed in fat-fed and fructose fed mice of both strains in response to amounts of STZ that did not change glucose levels in standard diet fed mice (Luo et al., 1998).

2.2.3: Type 2 and GDM Possible Mechanisms

Even though the primary causes for type 2 diabetes and GDM are not known, possible molecular mechanisms have been proposed. Insulin resistance plays an early role in type 2 pathogenesis, and defects in insulin secretion by pancreatic beta cells are instrumental in the progression of hyperglycemia. Skeletal muscle and liver are the two key insulin responsive organs responsible for maintaining normal glucose homeostasis,

and their transition to an insulin resistant state accounts for most of the alterations in glucose metabolism seen in patients with type 2 diabetes (Lowell and Shulman, 2005). Although there may be an initial compensatory increase in beta cell mass, the onset of type 2 diabetes in both human and rodent models is accompanied by a progressive decrease in beta cell mass. As a result the body can no longer adapt to any increases in metabolic load, including insulin resistance associated with obesity (Rhodes et al., 2005). The next step to trigger type 2 diabetes is a loss in b-cell mass by apoptosis. Causes for beta cell apoptosis are multiple and combined in the course from insulin resistance to type 2 diabetes. These combined factors are chronic hyperglycemia, hyperlipidemia and cytokines that interfere with signaling pathways which promote normal cell growth and survival (Linhgor et al., 2002). One of these signaling pathways is led by insulin receptor substrate 2 (IRS2). IRS2 expression in the beta-cell and its tyrosine phosphorylation are required for normal growth and survival. However, chronic hyperglycemia by means of the mammalian target of rapamycin (mTOR) and hyperlipidemia by fatty acyl-CoA-mediated activation of the novel class protein kinase C (PkC) isoforms can lead to increased serine-threonine phosphorylation of IRS2 that then leads to ubiquitination and subsequent degradation. Other cytokines, such as interleukin 1β and TNF-α, may activate c-Jun-N-terminal kinase (jnk)/p38, PkC-delta (and others) which in turn also leads to IRS2 serine-threonine phosphorylation and IRS2 degradation (Rhodes, 2005).

Other possible mechanisms for insulin resistance and type 2 diabetes are associated with dysfunction in mitochondrial function and mitochondrial content in the cell. A chronic increase of fatty acids in the cytosol may trigger their incomplete and decrease beta-oxidation by the mitochondria caused by dysfunction or low mitochondrial number. This decrease in oxidation of fatty acids will raise the intracellular levels of fatty acyl CoA and diacylglycerol. These molecules activate novel PkC which in turn activates a serine kinase cascade leading to serine phosphorylation of IRS1. This blocks IRS1 tyrosine phosphorylation by the insulin receptor (normally done in the insulin pathway) which in turn inhibits the activity of phophatidyl inositol 3 kinase. This inhibition results in suppression of insulin-stimulated glucose transport, the process by which the glucose is removed from the blood (Lowell and Shullman, 2005). Expression of nuclear encoded genes that regulate mitochondrial biogenesis such as peroxisome proliferator-activated

receptor coactivator 1 α (PGC-1α) is decreased in patients with glucose intolerance and type 2 diabetes (Mootha et al., 2003).

Gestational diabetes mellitus (GDM) follows the same mechanisms of type 2 diabetes. The difference between type 2 diabetes and GDM is that the mother is not pre-pregnancy diabetic and becomes hyperglycemic in advanced gestation instead (American Association of Diabetes, 2006). Obese and overweight women have greater predisposition to develop GDM. During the course of GDM, the fetus is exposed to hyperglycemia when rapidly growing. Infant macrosomia and organomegaly often result from the exposure to high glucose levels in late gestation. Considering that maternal insulin does not cross the placenta, the fetal pancreas will elaborate extra insulin to decrease blood glucose levels. As such the fetus receives more energy than needed to grow and develop. Infant macrosomia, the classic feature of GDM, is also associated with increased risk of adult type 2 diabetes and obesity in adolescence (Yamashita et al., 2003; Gillman et al., 2003). Mehta and Hussain (2003) have reported 2 cases of infant macrosomia, hypertrophic cardiomyopathy, hepatomegaly and nephromegaly. One of these babies belonged to a mother showing clear signs of GDM. The other mother did not show GDM signs. Both babies were reported with transient hyperinsulinism. Studies by Boloker et al., (2002) indicate that in rats, the altered metabolic milieu of the late diabetic pregnancy causes permanent defects in glucose homeostasis in offspring that lead to the development of diabetes later in life. Gestational diabetes frequently disappears after pregnancy but probably will appear again in future pregnancies. In a few women, the normal metabolic changes during pregnancy uncover type 1 or type 2 diabetes. Many women who have GDM go on to develop type 2 diabetes years later (Catalano et al., 1999).

Insulin sensitivity decreases in obese and lean women during pregnancy. Insulin sensitivity also decreases with advancing gestation (Catalano et al., 1991; Catalano et al., 1999). Women with GDM are less sensitive to insulin than are non-diabetic pregnant women (Ryan et al, 1985). Studies by Catalano et al., (2002) reveal that GDM women have decreased IRS1, which may contribute to reduced insulin suppression of lipolysis with advancing gestation. The authors also propose that decreased peroxisome proliferator-activated receptors (PPARγ), a family of fatty acid sensors (receptors), which

transduce stimuli from fatty acids into changes in gene expression and target genes, may be part of the molecular mechanism to accelerate fat catabolism to meet fetal nutrient demand in late gestation. Maternal circulating insulin like growth factors (IGFs) increase in early pregnancy (Sferruzzi-Perri et al., 2006) and this could be related to GDM mechanisms. Insulin-like growth factors (IGFs) are major endocrine and paracrine regulators of tissue growth and metabolism. IGFs share a high degree of structural homology with insulin (Devedjian et al., 2000; Van Haeften and Twickler, 2004). IGFs and their binding proteins, are crucial for fetal growth and development (Gibson et al., 2001). In mice, ablation of either the IGF I or IGF II genes reduces fetal size to 60% that of normal littermates (Powell et al., 1993). Insulin and IGF receptors belong to the protein tyrosine kinase receptor family. The insulin receptor and IGF-I receptor are homologous and can bind both ligands (insulin and IGF-I). IGF-I receptor has binding sites for IGF-II. IGF-II also has homology with insulin. As the IGF-II receptor has little homology with the other receptors, it can only bind to IGF-II (Van Haeften and Twickler, 2004). IGF-I and IGF-II can bind the insulin receptor at much lower affinities than insulin binding. Very high concentrations of IGF I and II could compete with insulin for binding to insulin receptor (H. Jiang, personal communication). IGFs are transported by a family of high affinity binding proteins (IGFBPs) that protect IGFs from degradation, limit their binding to IGF receptors, and modulate IGFs actions. IGFBP-7, a member of the IGFBP super-family that binds IGF specifically with low affinity, is a high affinity insulin binding protein. IGFBPs with enhanced affinity for insulin may contribute to insulin resistance in pregnancy and type 2 diabetes (Yamanaka et al., 1997).

Studies by Devedjian et al. (2000) indicate that transgenic mice over-expressing IGF II in beta cells show an increase in beta cell mass and in insulin mRNA levels. They also showed enhanced glucose-stimulated insulin secretion. These mice displayed hyperinsulinemia, mild hyperglycemia, and about 30% of these animals developed type 2 diabetes when fed with a high fat diet. As insulin and IGFs have their signaling pathways partially in common, the possibility of reciprocal interference is raised. IGF-I signaling has a major part in common with insulin signaling (Van Haeften and Twickler, 2004). The increase of IGF levels during pregnancy may be an important factor to consider in the phenomenon of insulin resistance and GDM. Considering the lack of maternal non-

genetic animal models of type 2 diabetes and GDM, a combination of HFD and low dose STZ could be an alternative. Such combination has been used previously in males to mimic type 2 diabetes. A similar principle could be applied to females. The HFD exposure and consequent hyperinsulinemia may be combined with a low dose of streptozocin that would mimic and accelerate the loss of insulin sensitivity to recreate hyperglycemia under a non-hypoinsulinemic environment.

2.3: The Development of the Heart

The heart is the first functional organ in the developing embryo. The heart develops from two original endothelial tubes that eventually meet in the medial plane of the embryo to form one single tube. The early development of the cardiovascular system is necessary because the growing embryo needs to obtain nutrients and dispose of waste material (Moore, 1982). The development of the heart begins in the ventral part of the anterior segment of the embryo. This process begins after gastrulation is complete, after which the heart continues to grow from the splanchnopleura (lateral mesoderm and endoderm). A group of endothelial cells within the splanchnopleura form the endothelial tubes (right and left). After an aggregation of cells, one final tube develops from the two original (Moore, 1982; Noden and DeLahunta, 1985).

2.3.1: Cardiogenic plate

The bilateral anterior tips of the unsegmented splanchnic mesoderm become defined as the cardiogenic plates, mainly by the interaction of the gene products of GATA 4/5/6 and NKx2.5 (Laverriere et al., 1994; Jiang et al., 1999; Poelmann and Gittenberger-de Groot, 2005). Vesicles within the cardiogenic plate form the endothelial tubes, with the fusion of the tubes occurring when the lateral body folding and closure of the pharynx initiates. The tubes gradually migrate toward each other and fuse in a cranio-caudal manner (Noden and DeLahunta, 1985).

After the cardiogenic precursors fuse in the midline, they differentiate into cardiomyocytes and endocardial cells. The continued addition of cardiomyocytes to the arterial pole takes place by activity of the adjacent splanchnic mesoderm. Additional endocardial cells are also recruited from the arterial pole endothelium (Poelmann and

Gittenberger-de Groot, 2005). The cardiomyocyte population develops into the functional components of the heart with rhythmic peristaltic contractions early in development and coordinated beats beginning slightly later (Poelmann et al., 2000). The endocardium forms the inner lining of the heart tube (Poelmann and Gittenberger-de Groot, 2005).
The cardiac jelly is a viscous fluid that fills the space between the myocardial and endocardial layers, and is rich in collagen and glycoproteins. The cardiac jelly permits myocardial contractions which help in the formation of the single cardiac tube. The cardiac jelly also contributes to the formation of connective tissue associated with the septa and valves of the heart (Noden and DeLahunta, 1985). Epithelial mesenchymal transformation in the endocardium gives raise to the endocardial cushions (Poelmann and Gittenberger-de Groot, 2005; Hay et al., 2005). The atrioventricular cushions arise in the transitional area between atrium and ventricle (atrioventricular canal) and the outflow tract cushions develop in the conotruncal transition from the heart to the aortic sac (Poelmann et al., 2000).

2.3.2: Extracardiac Constituents of The developing heart

The nervous system is derived from the ectoderm, although, not all ectodermal cells give rise to the nervous system. During neurulation (process of formation of the neural tube from a neural plate), neural crest arise from the dorsal part of the developing neural tube and contribute to the development of the peripheral nervous system (Gammill and Bronner-Faser, 2003). Neural crest cells (NCC) migrate from the fringes of the early neural plate. Epicardium-derived cells migrate from the pro-epicardial organ (a portion of the coelomic wall covering the liver primordium). In the chick embryo the migrating NCC disengage from the caudal rhombencephalic neural plate and travel to the aortic arches. A subpopulation reaches the arterial pole and outflow tract. A secondary subpopulation reaches the venous pole slightly later in development (Poelmann et al., 1998; Poelmann and Gittenberger-de Groot, 1999). A similar pattern is found in mice (Yamauchi et al., 1999). After the emigration of the neural crests, a second cell population emigrates from the ventral part of the cranial neural tube (VENT cells). VENT cells emigrate at the exit site of the cranial nerves of the midbrain (cranial nerves III and IV) and hindbrain (cranial nerves V and XII) (Dickinson et all, 2004). VENT cells have

been detected in larger arteries and veins, indicating a differentiation into vascular smooth muscle (Ali et al., 1999). Some of the VENT cells in the vagus nerve area migrate into the heart (Ali et al., 2003). VENT cells have been detected at different parts of the myocardium and great vessels of the heart. Nevertheless, they have not been detected in the epicardium or endocardium (Dickinson et al., 2004). VENT cells in the myocardium have differentiated into cardiac myocytes (Dickinson et al., 2004). The extirpation of the ventral part of the caudal hindbrain neural tube, which is a source of VENT cells, leads to malformations of the heart and great vessel such as, asymmetrical atria. It also leads to narrowing of the great vessels (Ali et al., 2003). A common defect related to abnormal VENT cells migration is the persistent truncus arteriosus (Dickinson et al., 2004). Epicardial-derived cells take position as interstitial cardiofibroblasts, coronary smooth muscle and adventitial fibroblasts (Poelmann et al., 1998).

2.3.3: Cardiac Tube Morphogenesis

The geometrical changes that occur during cardiogenesis are striking. A single tube can transform into a double pump containing atria, ventricles, valves, and a conduction system (Poelman and Gittenberger-de Groot, 2005). The cardiac tube remains connected to the dorsal body wall by the dorsal mesocardium. The arterial and venous poles provide entrance and exit respectively. After fenestration and large disappearance of the dorsal mesocardium, the cardiac tube loops into a tridimensional curve, bringing the venous and arterial poles close to each other (Poelmann et al., 2000). Four primitive structures will arise from the tridimensional curve, the primordia of the truncus arteriosus, bulboventricle, atrium and sinus venosus. The truncus arteriosus represents the output region of the heart (it will develop into ascending aorta and pulmonary trunk). The bulbus cordis will become part of the right ventricle, the conus arteriosus. The ventricle is an enlargement destined to become the left ventricle. The atrium is an enlargement destined to become the right and left atria. The sinus venosus is a paired region. The left sinus will become the coronary sinus and the right sinus will be part of the right atrium (Noden and DeLahunta, 1985). Septation of the outflow tract requires fusion of the endocardial cushions that contain NCC surrounded by cardiomyocytes. Septation of the atrioventricular canal requires fusion of the atrioventricular cushions including the NCC

and alignment with the muscular primary fold, outflow tract septum and dorsal mesocardium related to the spina vestibuli (which are part of the atrial septation complex).

The cardiomyocytes penetrate the cushions and complete the formation of part of the muscular ventricular septum. The septation of the atria is not completed before birth and involves various septal complexes (Poelmann et al., 2000; Poelmann and Gittenberger-de Groot, 2005). Differentiation of outflow tract semilunar valves, and atrioventricular mitral and tricuspid valves and their tendinous apparatus, involves coordinated interactions of the endocardial cushion cells with migrated NCC, and probably invasion of the epicardial derived cells in conjunction with the surrounding cardiomyocytes (Poelmann and Gittenberger-de Groot, 2005). Coordinated contraction of the atria and ventricles to ensure the cyclic beat of the heart requires the formation of a cardiac conduction system. This system consists of the sinuatrial (SA) node, the atrioventricular (AV) node, the penetrating bundle of His, the bundle branches and the peripheral Purkinje fibers. The conduction system develops from cardiomyocytes that probably need a trigger from the NCC to specialize from contracting myocytes into conducting cells (Poelmann and Gittenberger-de Groot, 1999; Poelmann et al., 2000).

The aortic arches are a result of complex remodeling. A number of arterial segments appear in an orchestrated series of events involving NCC but many disappear in subsequent stages. In early stages of development, only one pair of aortic arteries can be found connecting the heart to the developing microvascular network of the embryo's head. Subsequently, the next pairs of arteries come in to existence but when the fourth pair is developing the first pair starts to disintegrate. During development of the sixth pair, the second starts to disintegrate. The third, fourth and sixth pairs of aortic arches form in a nearly symmetrical fashion (Poelmann and Gittenberger-de Groot, 2005). The third aortic arch (left and right) becomes the internal and external carotid arteries. The left fourth aortic arch becomes the arch of the aorta. The right fourth aortic arch becomes the proximal part of the right subclavian artery (Noden and DeLahunta, 1985). The fifth pair does not develop in mammals and birds (DeRuiter et al., 1993). The left sixth artery persists to give rise to the ductus arteriosus and the pulmonary arteries. The right sixth

artery disappears before birth by apoptosis (Molin et al., 2002). Table 2.1 shows a summary of the embryonic cardiovascular events in the mouse.

2.4: Apoptosis and Heart Development

2.4.1: Two pathways for cell death

Apoptosis is one of the best examples of perfection in living cells. Apoptosis is the programmed death of an animal cell. Apoptotic cell death is found throughout the animal kingdom, and it culminates in the execution, packaging and disposal of the dying cells (Green, 2000). Apoptosis is a cellular disintegration process that differs from necrosis, which is a cytosolytic mechanism in which cells expel their contents into the microenvironment. Apoptosis results in cellular collapse due to DNA fragmentation, cytoskeletal disruption, and metabolic breakdown inside a structurally intact cell membrane (Poelmann and Gittenberger-de Groot, 2005).

The caspases (cysteine proteinases with specificity for aspartic acid residues) are the key in the apoptotic phenomenon. When activated, they cleave a wide variety of proteins. The change in function of key substrates ultimately kills the cell. The caspases remain inactivated in the cell until a protein, usually another caspase, cleaves them. The cleavage can be at 2 points of the caspase structure, between the large and small subunits, or at the level in which the prodomain will be removed. Another way of adaptation is through adapter proteins that bind the caspase and activate it. Apoptosis plays a very important role in cardiac development. In mammals the proteolytic caspases 3, 6 and 7 belong to the effector group that cleaves specific substrates. The activities of these effectors are initiated by activator caspases 8, 9 and 10 (Poelmann and Gittenberger-de Groot, 2005).

The mitochondrial pathway is triggered by pro-apoptotic proteins of the Bcl-2 family: Bid, Bim, Harikari, Noxa and others. These proteins are activated in response to changes in the environment. When activated, they activate other proapoptotic proteins such as Bax and Bak. These proteins reside in the mitochondrial outer membrane or in the cytosol and their oligomerization and insertion in the mitochondrial membrane causes the release of cytochrome c. After the mitochondrial release of cytochrome c, this will bind to protein Apaf-1 to form what is known as the apoptosome complex. The

apoptosome formation supports the catalytic activation of caspase 9 which in turn activates the effector caspase 3 which finally will degrade specific substrate (Green, 2000; Poelmann and Gittenberger-de Groot, 2005). The mitochondrial proteins: SMAC/DIABLO and Omi/HtrA2, are released to the IAP (inhibitor of apoptosis) family member XIAP inhibiting its action. Bcl2 and BclxL, and probably other members of the antiapoptotic family, block cell death by preventing the release of the cytochrome c (Green, 1998).

The external apoptotic pathway requires the action of what are known as membrane death receptors. Among these death receptors we can find: CD95, TNF-R1 and TRAIL. Examples of ligands for these receptors are TNF-α, TGF β and others. Adapter proteins facilitate the death receptor-ligand binding. These are able to bind the activator caspase 8 or 10 forming the death inducing signaling complex (DISC). After caspase 8 is activated, it activates effector caspase 3. Even though the activation of caspase 3 is enough to trigger cell death, sometimes the internal pathway through caspase 8-mediated Bid cleavage is required (Green, 1998; Green, 2000; Poelmann and Gittenberger-de Groot, 2005).

2.4.2: Cardiovascular Mechanisms

Apoptosis plays an important role in heart development. The main locations for apoptosis in chicken, mouse, rat and human are the outflow tract cushions, the atrioventricular cushions, the developing semilunar valves and the walls of the aorta and the pulmonary trunk (Poelmann et al., 2000). Apoptotic patterns are encountered in both the myocardium and the endocardial cushions at various time points in development. During many of the processes such as looping of the primary tube, wedging of the outflow tracts between the atria, ballooning of the chambers, muscularization of the fused endocardial cushions, atrial and ventricular septal formation, induction of the central and peripheral conduction systems, valve differentiation, and formation of the coronary vasculature, apoptosis is part of the differentiation mechanisms (Poelmann and Gittenberger-de Groot, 2005).

After migration of cardiac neural crest (CNC) cells to the heart, these can be traced toward the arterial pole through the pharyngeal arches and toward the venous pole

through the dorsal mesocardium (Poelmann and Gittenberger-de Groot, 1999). Septation of the outflow tract involves a coordinated interaction of the outflow cushions with the population of CNC cells homing into this region. Apoptosis of the CNC cells and cardiomyocytes is very prominent during septation (Poelmann et al., 1998; Poelmann and Gittenberger-de Groot, 2005). Septation of the atria does not involve apoptosis (Poelmann and Gittenberger-de Groot, 2005). Through apoptosis, the CNC cells may release signaling substances. Even though it is believed that apoptotic cells maintain the integrity of the cell membrane, small molecules may be released, resulting in abrupt shrinkage and followed by fragmentation. The apoptotic cell membrane changes dramatically in structure as can be detected when using annexing V for staining. These changes in cell membrane structure may allow the releasing of signaling molecules (Poelmann and Gittenberger-de Groot, 1999). It has been hypothesized by Poelmann and Gittenberger-de Groot (1999) that altruistic apoptosis is a mechanism involved in heart conduction system formation. According to these authors, CNC cells entering through the dorsal mesocardium, induce the final differentiation (separation of the central conduction system and its isolation from the remaining working myocardium) of specialized myocardial conduction cells in the sinoatrial node, atrio-ventricular node, the bundle of His and, the left and right bundle branches. In other words, CNC cells might induce a final differentiation of cardiomyocytes into the specialized conduction system. Apoptotic CNC cells also might be important in the myocardialization of both outflow tract and atrio-ventricular cushions, possibly by activation of growth factors (Poelmann et al., 1998; Poelmann and Gittenberger-de Groot, 1999).

The development of the aortic arches involves complex remodeling by apoptosis as a mechanism in the breakdown of specific segments and involves not only the vascular wall but also the surrounding mesenchyme. Blood flow depends on the diameter of the blood vessels (larger diameter allows more flow). The vessels that are destined to disappear as a result of high incidence of apoptosis always have smaller diameters (Molin et al., 2002). Interestingly, high shear stress protects against apoptosis and turbulent flow promotes apoptosis in these segments (Li et al., 2005). Apoptotic events take place also during the formation of the coronary arteries. A subpopulation of the epicardial cells migrates into the subepicardial space and invades the myocardial wall. This results in

apoptotic events that end in the formation and differentiation of cells that are going to be the coronary arteries. TGF β has been related to apoptosis during cardiovascular development. Apoptosis may change the microenvironment of the transforming area where it occurs. High expression of TGF β has been reported in mesenchymal areas where dominant apoptosis is taking place (Norris et al., 2005; Poelmann and Gittenberger- de Groot, 2005). It is still unclear if apoptosis precedes activation of TGF β or if it is a result of TGF β signaling. Either way, the final result is myocardialization and transformation of the area (Kubalak et al., 2002).

Studies by Lakhani et al. (2006) show that effector caspases 3 and 7 are key mediators in mitochondrial events of apoptosis. The authors generated mice deficient in caspases 3 and 7 which died immediately after birth by defects in cardiac development. Histologic examination of double knockout (for caspase 3 and 7) mouse hearts revealed dilation of the atria and disorganization of and noncompaction of the ventricular musculature. The authors concluded that caspase 3 controls DNA fragmentation and morphologic changes of apoptosis and caspase 7 appears to be more important to the loss of cellular viability although the combined role of both caspases is crucial.

Poelmann and Gittenberger-de Groot (2005) proposed that the exact function of apoptosis is not always clear, although removal of cells seems to be an obvious phenomenon. They also noted the possibility that apoptosis is not a final phase of a developmental process, but functions in itself as a step in further signaling.

2.4.3: Hyperglycemia and Apoptosis

The harmful effects of maternal hyperglycemia on the developing embryo suggest that dysregulation of apoptosis and changes in gene expression may be key factors in congenital malformations. Studies by Fine et al. (1999) reveal that a reduced expression of the regulatory gene for neural tube development, Pax 3, results in increased NTDs during the diabetic pregnancy. These results suggest that congenital malformations may be caused by disruption of regulatory gene expression in the embryo in response to elevated glucose levels. Pax 3 encodes a transcription factor that has recently been shown to inhibit p53 dependent apoptosis. Excess glucose metabolism inhibits expression of Pax 3 which in turn derepresses p53-dependent apoptosis leading to NTDs (Loeken, 2005).

Studies by Fu et al. (2006) show a decreased proliferation of neural stem cells and differentiated cells from mice exposed to high glucose levels (> 16.7 mM/L). The same authors state that apoptosis was increased in neural stem cells but not in differentiated cells. Hyperglycemia causes release of ROS and reactive nitrogen species (RNS). The release of ROS and RNS induces oxidative stress leading to abnormal gene expression, faulty signal transduction and apoptosis of cardiomyocytes.

Hyperglycemia also induces apoptosis via p53 and activation of the cytochrome c-activated caspase 3 pathway (Adeghate, 2004). Apoptosis plays a critical role in cardiac pathogenesis (Cai et al., 2002). According to Cai et al. (2002), apoptosis occurs in the diabetic myocardium of adult FVB mice due to high levels of glucose. The authors propose the activation of mitochondrial release of cytochrome c-caspase 3 under high levels of glucose in cardiac myoblast H9c2 cells may be triggered by ROS derived from high hyperglycemia. Studies by Bojunga et al. (2004) demonstrate that hyperglycemia significantly induces apoptosis in the hearts of diabetic rats by the death receptor and mitochondrial pathways. The authors reveal that hyperglycemia up-regulates CD95 which in turn initiates death receptor-apoptosis. The same authors propose that myocyte loss could be an important mechanism contributing to progressive dilation of the heart and development of diabetic cardiomyopathy in humans.

Studies by Frustaci et al. (2000) reveal that apoptosis and necrosis are increased in the adult diabetic heart. The authors report that apoptosis of myocytes, endothelial cells and, fibroblasts is increased 85-fold, 61-fold, and 26-fold respectively, in diabetic subjects. In the same experiments diabetes increased necrosis by 4-fold in myocytes, 9-fold in endothelial cells, and 6-fold in fibroblasts. The nuclear transcription factor p53 regulates transcription of pro-apoptotic genes and causes expression of inhibitors of survival-related proteins (Bojunga et al., 2004). Studies by Fiordaliso et al. (2001) reveal that cell cultures of myocytes from adult rats subjected to hyperglycemia (25mM/L) show increased myocyte apoptosis mediated by activation of protein p53 and effector responses involving the local renin angiotensin system. Studies by Bojunga et al. (2004) demonstrate an up-regulation of gene p53 in hearts of diabetic rats induced by STZ.

Considering the importance of apoptosis in embryonic and fetal development, these reports suggest in part that dysregulation of apoptosis may be a key factor responsible for maternal diabetes-induced cardiovascular malformations.

2.5: Summary

Maternal diabetes mellitus is associated with increased fetal teratogenesis, including cardiovascular defects. Molecular mechanisms of diabetes-induced cardiovascular malformations are not yet completely elucidated. Rodent models of maternal diabetes have been used to study embryonic and fetal development. Among these models, the use of STZ as an inducer of maternal hyperglycemia in an insulin-dependent environment is a classic one. However, other alternatives have been proposed such as the combination of STZ and high fat diet, to reproduce non-insulin dependent diabetes. Rodent models have demonstrated that non-specific maternal immune stimulation has been associated with protection against congenital malformations by teratogens including maternal diabetes mellitus.

Apoptosis plays an important role in the development of the cardiovascular system and may be affected by maternal hyperglycemia. The study of dysregulation of apoptosis by maternal hyperglycemia may be a key factor for the understanding of the mechanisms underlying maternal diabetes-induced cardiovascular malformations.

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Table 2.1. Summary of the main cardiovascular developmental events in the mouse (summarized from Kaufman, 2003).

Embryonic day	Developmental event
7.5	Cardiogenic plate, two endothelial tubes.
8	Single heart tube, cardiac jelly, sinus venosus, common atrial chamber, ventricular region of the primitive heart, bulbus cordis, paired dorsal aorta.
8.5	Dorsal mesocardium, truncus arteriosus, primitive ventricle.
9	Atrioventricular canal.
9.5	Bulbar cushion tissue, left and right components of common atrium.
10	Aorticopulmonary spiral septum, endocardial cushion tissue, septum primum.
10.5	Left fourth aortic arch, foramen primum, crista terminalis
11	Midline dorsal aorta, visceral pericardium.
11.5	Aortic component, pulmonary component, interventricular septum, right ventricle, left ventricle, right atrium, left atrium, parietal pericardium.
12	Ascending aorta, pulmonary trunk, atrioventricular cushion tissue, foramen secundum.
12.5	Descending aorta, aortic arch, aortic valve, pulmonary, valve, ductus arteriosus, mitral valve, tricuspid valve, septum secundum,
13.5	Muscular component of interventricular septum, foramen ovale.
14.5	Aortic sinus, Aortic valve leaflets, pulmonary valve leaflets, mitral and tricuspid valve leaflets, trabeculae carnae.
15.5	Fibrous pericardium
16.5	Coronary arteries.

CHAPTER 3: AORTIC AND VENTRICULAR DILATION AND MYOCARDIAL REDUCTION IN GESTATION DAY 17 ICR MOUSE FETUSES OF DIABETIC MOTHERS

J. Claudio Gutierrez [1], Terry C. Hrubec [1,2], M. Renee Prater [1,2], Bonnie J. Smith [1], Larry E. Freeman [1] and Steven D. Holladay [1]

[1] Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute & State University, Blacksburg, Virginia

[2] Department of Biomedical Sciences, E. Via Virginia College of Osteopathic Medicine, Blacksburg, Virginia.

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3.1: ABSTRACT

Maternal diabetes mellitus is associated with increased fetal teratogenesis, including cardiovascular defects. Information regarding cardiovascular changes in late gestation fetal mice, related to maternal hyperglycemia, is not present in the literature.
Late-gestation fetal heart and great vessel morphology were analyzed in fetuses from control and diabetic mice. Female ICR mice were injected with streptozocin (200 mg/kg IP) prior to mating to induce diabetes (n=8). Non-hyperglycemic females were used as controls (n=8). At day 17 of gestation, females were euthanized and one fetus was arbitrarily selected per litter to analyze the heart and great vessels. Six additional fetuses from different litters, showing external malformations (spina bifida and/or exencephaly) were also evaluated from the diabetic group. Fetal thoraxes were processed using routine histopathologic techniques, and 7 μm transversal sections were stained with hematoxylin and eosin. Digital images of sections were made and analyzed using NIH Image J software to compare regional cardiac development. Student's *t* tests for means were performed to determine differences between groups ($p < 0.05$).

Maternal hyperglycemia caused a dilation of late-gestation fetal ventricular chambers, a reduction of total ventricular myocardial area and an increase in transversal ascending thoracic aortic area. Three out of six fetuses that displayed external malformations showed an overt cardiac defect, beyond the ventricular and myocardial changes.
Maternal hyperglycemia altered morphology of the late-gestation fetal mouse heart. Postnatal persistence or consequences of late gestation heart chamber dilation and myocardial reduction are not yet known.

Key words: type 1 diabetes, cardiovascular malformations, maternal diabetes mellitus

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3.2: INTRODUCTION

The incidence of malformed newborns is 6-10 % or 3 to 5 times higher in human pregnancies complicated by diabetes mellitus, as compared to non diabetic pregnancies (Reece and Eriksson, 1996; Reece et al, 1998). Neural tube and cardiac defects predominate among those malformations related to hyperglycemia (Reece et al, 1998; Punareewattana and Holladay, 2004). Heart defects in particular have been reported as increasing up to five fold among infants of diabetic mothers compared to the general population, representing a significant segment of anomalies that might be reduced with gestational control of maternal blood sugar levels (Becerra et al, 1990; Meyer-Wittkopf et al, 1996). Identification and treatment of hyperglycemia in women entering their reproductive years has similarly been suggested as a method to prevent major cardiac and non-cardiac anomalies (Loffredo et al, 2001).

Congenital cardiac malformations in human children associated with type 1 diabetic mothers are diverse, and include mitral atresia, double outlet right ventricle, transposition of great vessels, pulmonary valve atresia, aortic and pulmonary stenosis, tetralogy of Fallot, and ventricular and atrioventricular septal defects (Meyer-Wittkopf et al, 1996; Loffredo et al., 2001; Abu-Sulaiman and Subaih, 2004). In humans, possible aberrant migration of neural crest cells may be responsible for such congenital malformations related to maternal hyperglycemia (Meyer-Wittkopf et al., 1996), thus,

hyperglycemia may produce its primary teratogenic effects during organogenesis. It appears that excess fuel molecules in diabetes such as glucose, ketone bodies and amino acids may contribute to embryopathy (Eriksson et al, 1991). Hyperglycemia induces production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which in turn stimulate oxidative stress, leading to abnormal gene expression and increased apoptosis of cardiomyocytes (reviewed by Adeghate, 2004). This may lead to changes in extra-cellular matrix proteins (ECM) such as fibronectin (FN) that requires tightly controlled concentrations for normal development. For instance, over-expression of transforming growth factor β1 (TGF β1) under hyperglycemia mediates increased production of ECM proteins (Smoak, 2004; reviewed by Adeghate, 2004). Excessive ECM proteins in turn accumulate in diabetic tissues including heart, and may affect development (Roy et al, 1990).

The present late gestation fetal hearts from diabetic dams showed dilated ventricles, dilated left ventricular outflow channel (ascending aorta), and reduced transverse sectional myocardial area. These effects on cardiac development have not previously been associated with maternal type 1 diabetes, and raise questions regarding the full spectrum of adverse postnatal cardiac health consequences that may be associated with maternal hyperglycemia.

3.3: MATERIALS AND METHODS

3.3.1: Animal Model

Male and female ICR mice, 6-8 weeks of age, were purchased (Harlan Sprague-Dawley, Indianapolis, IN) and housed individually (males) or at 5 per cage (females) for a 2-week acclimation period. For breeding, males were housed overnight with females, and females checked for vaginal plugs the next morning, which was designated gestation day (GD) 0. Mice were given food (NIH 31 open formula) and distilled water ad libitum, and were maintained under controlled conditions of temperature (72°F), humidity (40-60%) and lighting (14/10 light/dark cycle).

Diabetes was chemically induced in female mice by intraperitoneal (IP) injection with 200 mg/kg streptozocin (STZ) (Sigma, St. Louis, MO) 7 days before breeding. Blood glucose (BG) levels in tail vein blood were determined every 3-5 days using

glucose kits (Sigma), and were used to categorize diabetic mice. STZ-injected mice were hyperglycemic at first bleeding, with blood glucose ranging from 10 - 32 mM/L. BG levels subsequently did not change appreciable over time, with no mouse initially ≥ 26 mM/L falling to under this level or increasing beyond 32 mM/L (data not shown). Mice with initial BG levels ≥ 26 mM/L were previously found to produce fetuses with increased externally visible birth defects (Goldman et al., 1985; Punareewattana and Holladay, 2004) and were used to study heart morphology in the present experiments. These mice were not moribund, and did not show overt clinical signs.

BG levels in the control dams were not monitored in these experiments. Previous experiments performed in our laboratory, under the same conditions, found BG levels in control ICR pregnant dams in the range of 4 mM/L – 7 mM/L (data not shown).

Pregnant mice were euthanized by cervical dislocation at GD 17. Fetuses were collected and preserved in absolute ethanol for later examination. One fetus was selected per litter to analyze the heart and great vessels (n = 8). The selection was arbitrary, with the exception that only fetuses that showed no externally visible defects were selected. Next, six fetuses showing external malformations (spina bifida and/or exencephaly) were selected from 6 diabetic litters, to determine if cardiac defects occurred at an increased rate in fetuses that also expressed an externally visible defect. Fetuses identically collected on GD 17 from non-STZ-injected pregnant females were used as controls (n = 8).

3.3.2: Morphometric Analysis

Fetal thoraxes were processed using routine histopathologic techniques, and 7 µm transversal sections were prepared and stained with hematoxylin and eosin (Figure 3.1). Digital images of sections were collected using an Olympus microscope and camera, and imported into Photoshop 7.0 to optimize image brightness and contrast. Images were then evaluated using NIH Image J software (free download at http://rsb.info.nih.gov/ij/download.html) designed for morphometric analysis (Figure 3.2). Image J threshold and particle analysis tools were used to measure 3 major features of the heart: left ventricular chamber area, right ventricular chamber area and total myocardial ventricular area. These features were measured in equivalent level transverse

sections. Presence of both, the left and right atrio-ventricular valves was used as the landmark for equivalent transverse level, for morphologic evaluation of these three major areas.

Ten secondary features were next analyzed: regional myocardial ventricular area **(**left free ventricular wall area, right free ventricular wall area and interventricular septum area), transversal thoracic aortic area (both ascending and descending aortic area), aortic valve area, pulmonary trunk diameter, vena cava area (left and right cranial vena cava, and caudal vena cava). For regional myocardial and descending thoracic aortic areas, presence of both atrio-ventricular valves was used as the landmark to compare equivalent transverse levels. For left and right cranial vena cava areas, ascending thoracic aorta area and pulmonary trunk transverse diameter, the pulmonary trunk bifurcation into left and right pulmonary arteries was used as landmark. Sections showing all three aortic valve leaves (valvula semilunaris septalis, valvula semilunaris dextra and valvula semilunaris sinistra) were used to measure aortic valve area. For caudal vena cava area, sections showing caudal limit of left and right ventricular chambers were selected. Kaufman's Atlas of Mouse Development (1995) was used as a guide to precisely compare same level sections among fetuses. Average individual fetal weight was determined by dividing total litter weight by number of fetuses in the litter.

3.3.3: Statistical Analysis

Student's *t* test for comparing means was performed to determine presence or absence of significant difference between groups, for each morphometric variable measured ($p < .05$).

3.4: RESULTS

The present late gestation fetal hearts from diabetic dams showed dilated ventricles, dilated left ventricular outflow channel (ascending aorta), and reduced transverse sectional myocardial area. These effects on cardiac development have not previously been associated with maternal type 1 diabetes, and raise questions regarding the full spectrum of adverse postnatal cardiac health consequences that may be associated with maternal hyperglycemia.

Average fetal weight was moderately but significantly reduced in litters from ≥ 26 mM/L BG mothers compared with control litters. Fetuses from females with lower levels of hyperglycemia (not used for cardiac morphology determinations) did not show depressed body weights (Figure 3.3).

Fetal mice from the hyperglycemic dams, that showed no externally visible defects were first evaluated and compared to controls. Fetuses from the diabetic mothers showed visibly apparent and significant dilation of left and right ventricular chambers, and a corresponding reduction of total ventricular myocardial area (Table 3.1). Left and right mean ventricle chamber areas were both approximately 2.5 times larger in fetal mice from diabetic dams, as compared to corresponding chambers from non-diabetic dams. However, ventricular myocardial area, including the IVS and outer walls, was reduced by about 13% in fetal mice from diabetic dams, as compared to corresponding regions from non-diabetic dams. Representative sections are shown in Figure 3.4.

Similar to ventricular chambers, fetuses of hyperglycemic mothers showed increased transversal ascending thoracic aortic area, representing the outflow channel of the left ventricle. The ascending aorta at pulmonary trunk bifurcation level was 31% greater in mean area in fetal mice from diabetic dams, as compared to corresponding non-diabetic dams (Table 3.1). The pulmonary trunk, representing the outflow channel of the right ventricle, showed a trend toward increased diameter in fetal mice from diabetic dams, with a mean value 11% higher than non-diabetic dams.

The fetal mice showing external defects (exencephaly and/or spina bifida, Figure 3.5) displayed a similar profile of cardiac changes to fetuses without external defects (Table 1). Left and right ventricular chamber area was again increased in the fetal mice from diabetic dams, while total ventricular and septal myocardial area was decreased. Additional cardiac defects were seen in these mice that were not seen in the first group of fetal mice, including dextra-position of the aorta in a fetus with exencephaly, double caudal vena cava in a second fetus with exencephaly, and an interventricular septal defect in a fetus that displayed both exencephaly and spina bifida (Figure 3.6).

3.5: DISCUSSION

Hyperglycemia in adult humans has been related to hypertrophic cardiomyopathy which evolves chronically into a dilated myopathy (Frustaci et al., 2000). These authors further reported cavitary dilation and depressed ventricular performance in adult diabetic humans, effects they related to increased apoptosis of cardiac myocytes, endothelial cells, and fibroblasts. In what may be a related observation, Cai et al. (2002) recently observed enhanced myocardial apoptosis in hearts of hyperglycemic adult mice. Cavitary dilation in fetal human or fetal mouse hearts of hyperglycemic mothers as observed in this study has not been previously reported. Maternal hyperglycemia affects heart development and early postnatal cardiac health in humans. Cardiac-related disease is increased up to five fold among infants of diabetic mothers compared to the general obstetric population (Meyer-Wittkopf et al, 1996). Using duplex Doppler waveform analysis, Macklon et al. (1998) found that fetuses of well controlled type 1 diabetic women continued to show early morphological heart changes of cardiac hypertrophy compared to control fetuses. Jaeggi et al. (2001) similarly reported a significant increase of interventricular septal thickness in mid-trimester fetuses of well-controlled type I diabetic mothers compared to control fetuses. In contrast, Weber et al. (1994) described normal cardiac growth in fetuses of well-controlled diabetic mothers. Wong et al. (2003) observed no difference in fetal ventricular septum thickness or cardiac size between well- and poorly-controlled type I diabetic women. Differences in level of maternal hyperglycemia in these studies may in part explain the divergent findings. Regardless of glucose levels, recent epidemiologic studies by Abu-Sulaiman and Subaih (2004) detected 38% incidence of hypertrophic cardiomyopathy in infants of type 1 diabetic women.

Information is not available in the literature regarding fetal heart development in the STZ induced type 1 diabetic mouse models. This model of type 1 diabetes has been used to study the effects of hyperglycemia during pregnancy for other morphological defects, including diverse craniofacial defects (Hrubec et al., 2006) and neural tube defects (NTDs) (Punareewattana et al., 2003). As these defects may be linked, we used the same model to investigate cardiac defects. Craniofacial defects in mice deficient in one type of endothelin-A receptor have been linked to increased cardiovascular defects. Endothelin genes and their receptors may play a role in neural crest determination

(Clouthier et al, 1998). These findings suggested that STZ-induced hyperglycemia in pregnant mice may also show increased cardiac defects, an idea verified by the present studies.

NTDs in mice have been also linked to increased cardiovascular defects. Henderson et al (2001) indicate increased cardiovascular defects such as ventricular septal defects and double outlet right ventricle among others in Lp/Lp mutant mice used as a model for NTDs. These authors suggest that cardiac looping abnormalities are secondary to failure of neural tube closure and not related to a neural crest migration abnormality. Nevertheless, studies by Cederberg et al (2003) indicate that the development structures derived from neural crest, including cardiac neural crests, are specifically altered during organogenesis in rat embryos of diabetic dams. This may be a consequence of hyperglycemia induced cell death. In particular, studies by Siman et al. (2000) suggest that malformations in organs derived from the neural crest are secondary to reactive oxygen species-related cranial neural crest damage in maternal diabetes, altering normal cell death regulation. Using a rat model, these authors reported rightward displacement of the aorta, double outlet right ventricle, persistent truncus arteriosus and ventricular septal defects in offspring of diabetic rats with BG levels over 20 mM/L. STZ-induced gestational diabetes mellitus was also employed in a rat study by Menezes et al. (2001), in which myocardial hypertrophy was detected in GD 19 and 20 fetuses of the hyperglycemic dams. The latter authors injected dams with STZ on GD 8, with average mid-gestation BG levels in the pregnant rats being 16.65 mMol/L.

In the present study, hyperglycemia was induced in female mice just prior to breeding, similar to Punareewattana and Holladay (2004). These authors used STZ to cause hyperglycemia. Mice with BG>26 mM/L BG represented a high-level of hyperglycemia.

Visibly obvious dilation of ventricular chambers and outflow channel of the left ventricle, and reduction of total myocardial ventricular area in late gestation fetuses from diabetic dams, were the predominant changes seen in the offspring of diabetic dams. Mechanisms underlying this effect are not yet investigated, however enhanced myocardial apoptosis recently reported in hearts of adult hyperglycemic mice (Cai et al., 2002) and adult humans (Frustaci et al., 2000) suggest dysregulated cell death as a possible contributor. In this regard, timed apoptosis is an important mechanism in normal

development of the cardiovascular system, with many sites and timed windows of occurrence (reviewed by Poelmann and Gittenberger-de Groot, 2005). Unlike Menezes et al. (2001) who induced moderate hyperglycemia in rats on GD 8 and observed fetal myocardial hypertrophy, the present mice experienced a high level of hyperglycemia throughout early development and cardiac organogenesis. It is not known if moderate hyperglycemia in mice, during or before the period of cardiac organogenesis, may cause lesser cavitary dilations, or myocardial reduction. Similarly, the postnatal persistence or consequences of late-gestation heart chamber dilation and myocardial reduction are not yet determined and represent an important focus area for future research.

3.6: ACKNOWLEDGMENTS

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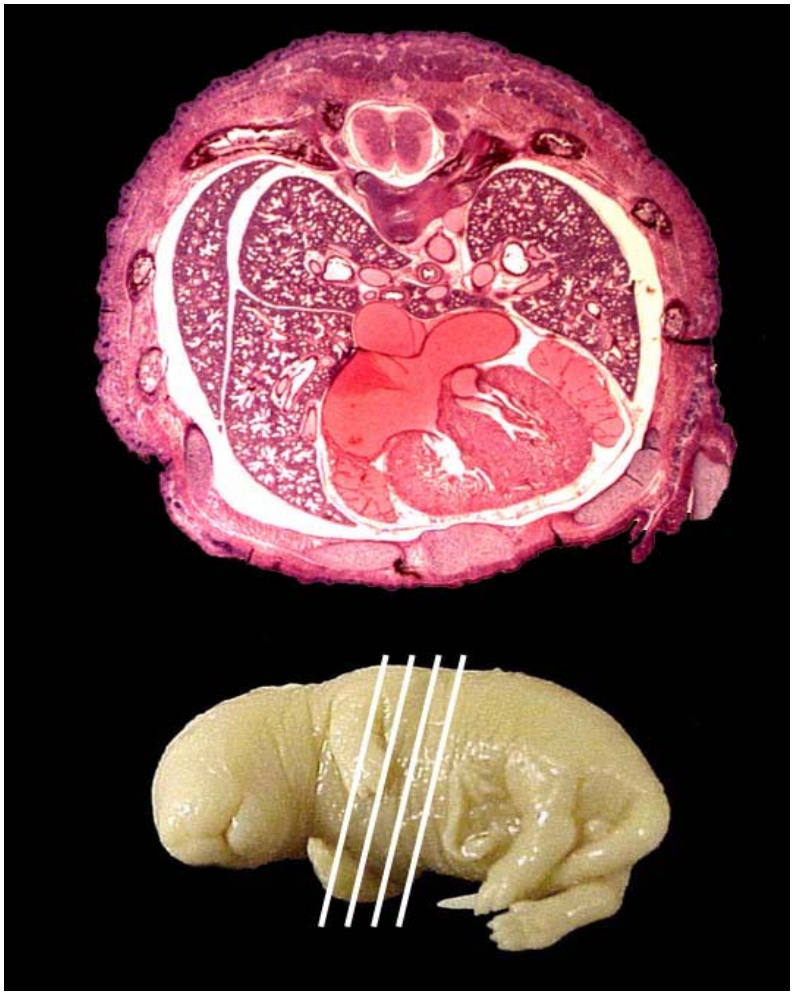

Figure 3.1. Mid-thorax sectioning (7µm) of 17-day-old fetuses for morphometric heart analysis

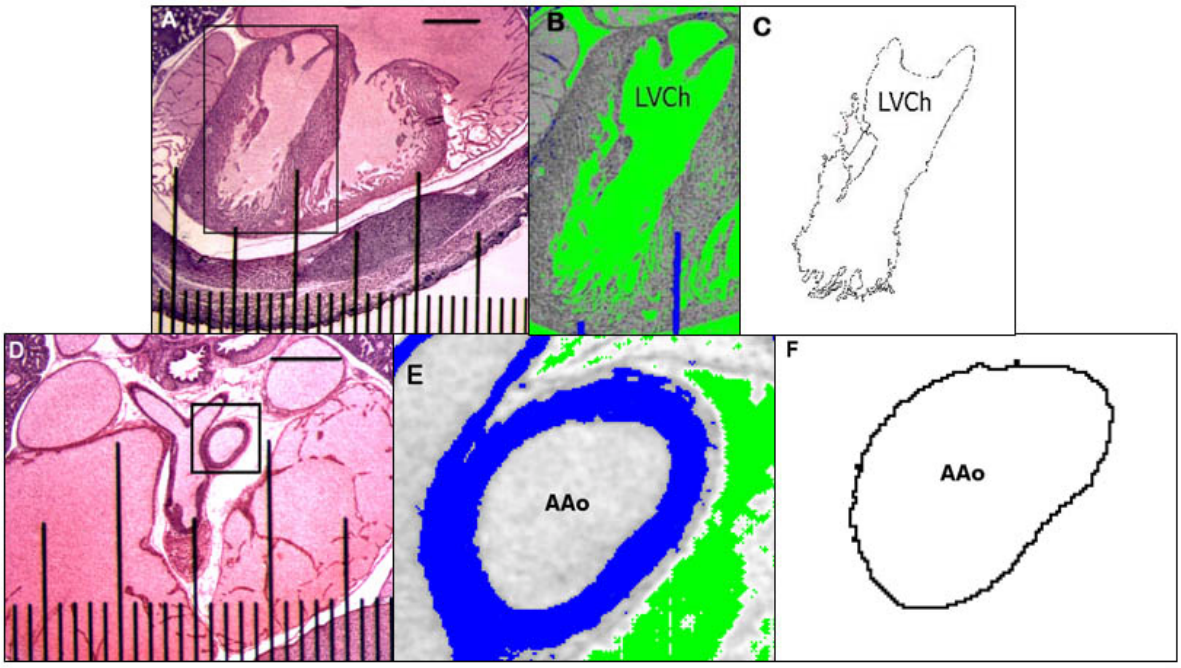

Figure 3.2. Example of Image J tools used to measure different sectional areas. A: original image, long axis view of the heart, B: detail of image A (square region of A), image J threshold analysis of the left ventricle, C: Image J particle analysis of the left ventricular chamber area, D: original image at pulmonary trunk bifurcation level, E: detail of image D (square region of D), image J threshold analysis of the ascending thoracic aorta, F: image J particle analysis of the ascending thoracic aortic area. LVCh: left ventricular chamber, AAo: ascending thoracic aortic area. Scale bars: 500 µm.

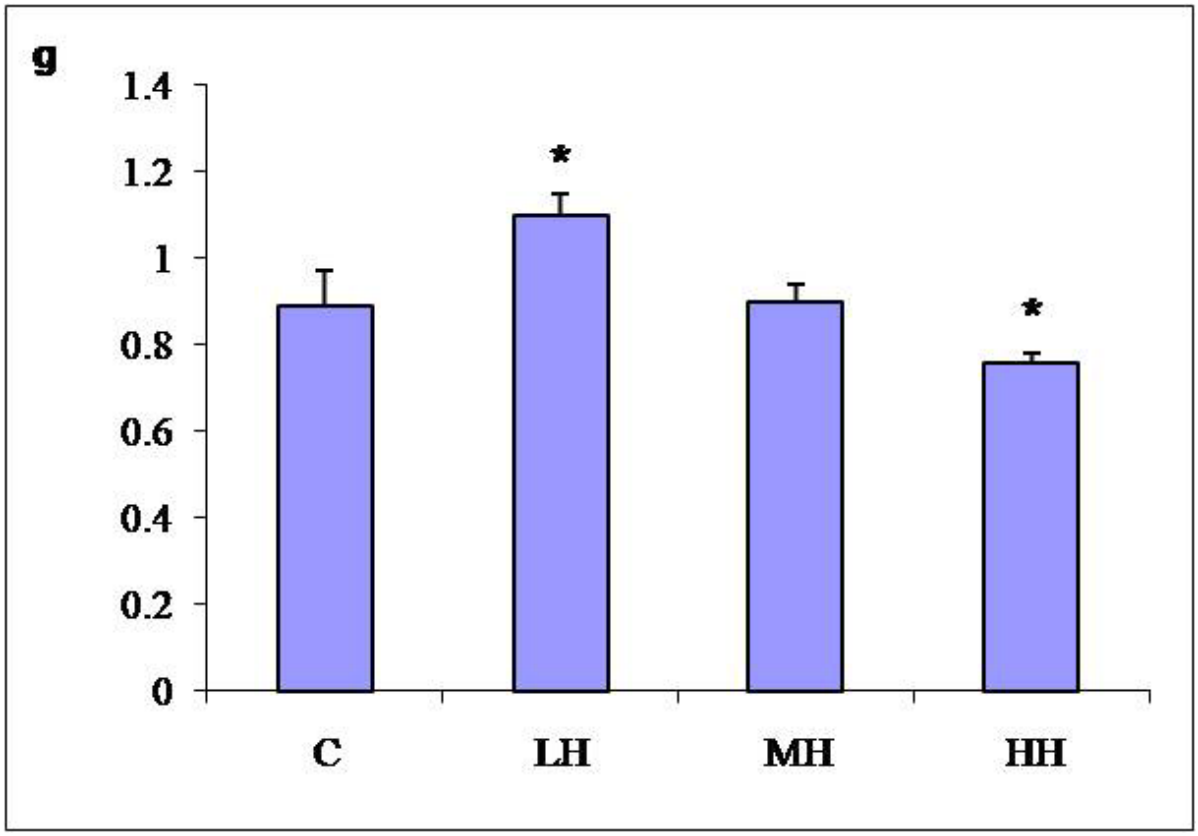

Figure 3.3. Fetal weight in 17 day-old fetuses from control dams (C), low hyperglycemic dams (LH, 10 – 18 mM/L), moderate hyperglycemic dams (MH, 18 – 26 mM/L) and high hyperglycemic dams (HH, > 26 mM/L) (* $p < .05$).

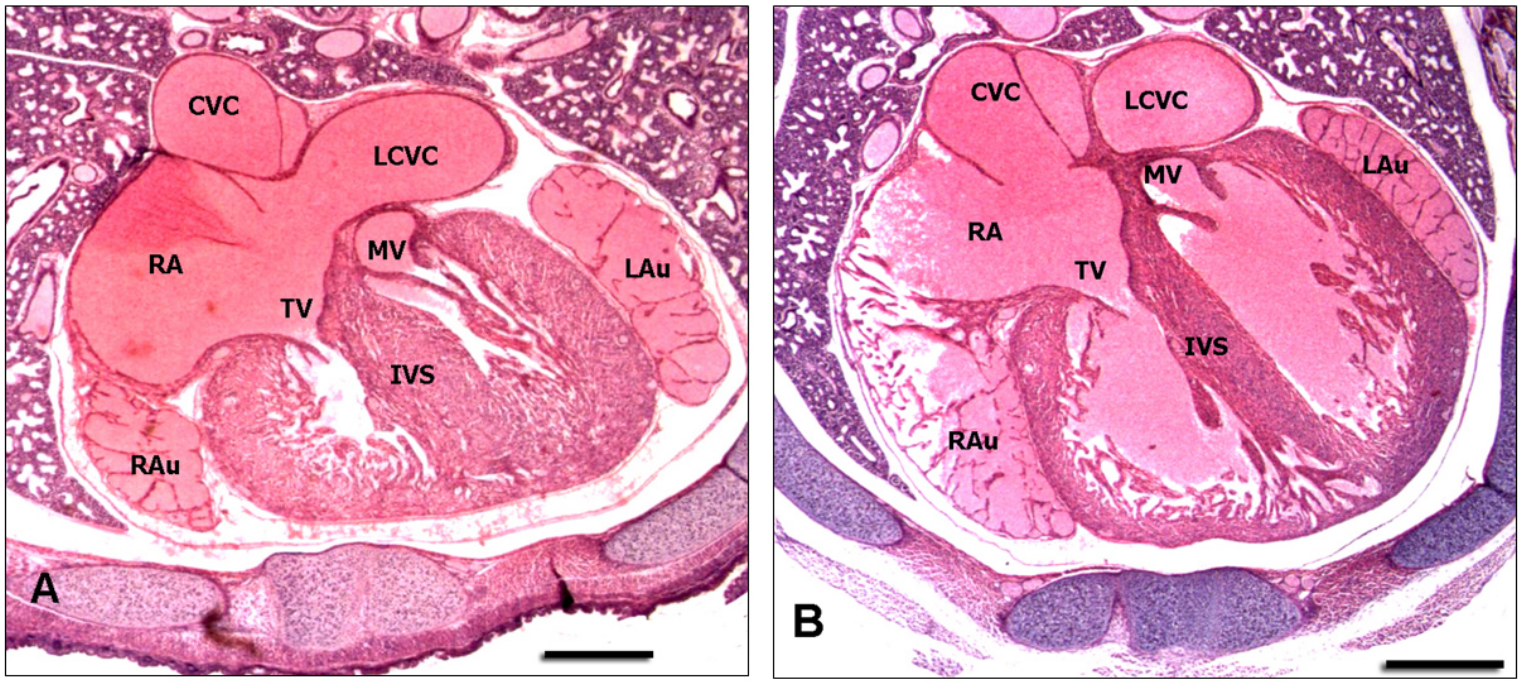

Figure 3.4. Seventeen-day old fetal hearts (long axis) from the control (A) and hyperglycemic (B) groups. CVC: caudal vena cava, LCVC: left cranial vena cava, RA: right atrium, MV: mitral valve, TV: tricuspid valve, IVS: interventricular septum, Lau: left auricle, Rau: right auricle. Scale bars: 500 µm.

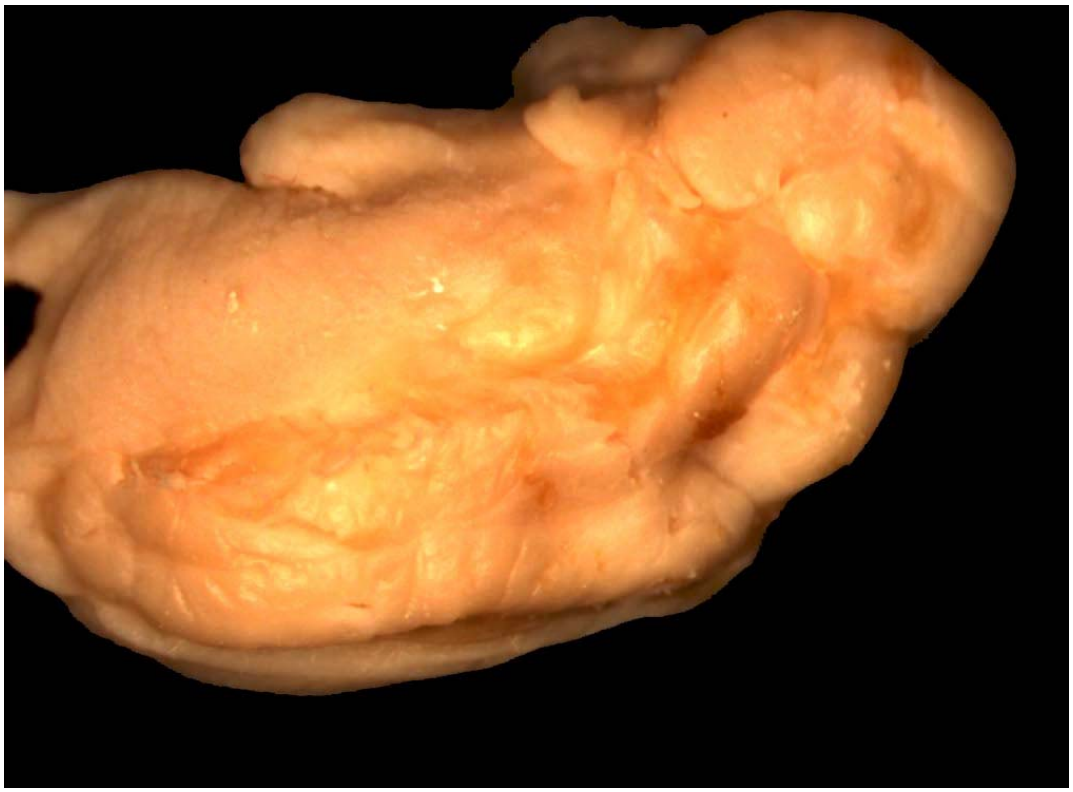

Figure 3.5. Dorsal view of a 17-day-old fetus displaying spina bifida and excencephaly from the diabetic group.

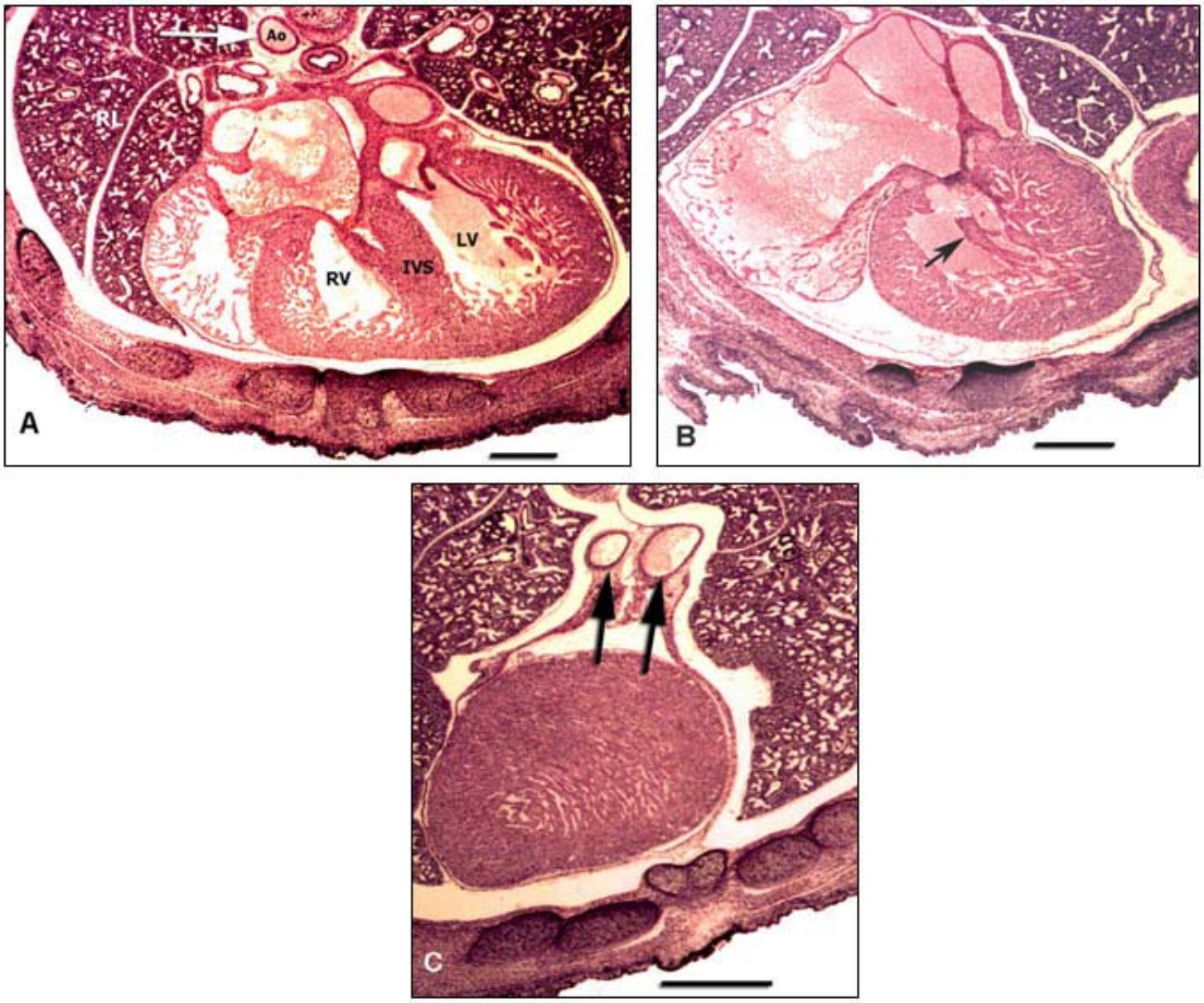

Figure 3.6. A: Dextra-position of the aorta in a fetus with exencephaly (arrow), B: interventricular septal defect in a fetus that displayed both exencephaly and spina bifida (arrow) and C: double caudal vena cava in a fetus with exencephaly (arrows). Ao: descending thoracic aorta, RV: right ventricle, LV: left ventricle, IVS: interventricular septum. Scale bars: 500 µm.

Table 3.1. Cardiovascular endpoints in fetuses of hyperglycemic and control pregnant mice. Fetuses of diabetic mothers showed significant dilation of both left and right ventricular chamber area, reduction in total ventricular myocardial area, and an increase in ascending thoracic aortic area (* $p < .05$, $p < .01$,*** $p < .001$,¶ : µm).**

Variable	Control (um^2)	N	Hyperglycemic group (um^2)	N	Hyperglycemic group with external malformations (um^2)	N
Interventricular septal area	644 ± 149	8	517 ± 102	8	488 ± 59	6
Left free ventricular wall area	798 ± 142	8	743 ± 80	8	714 ± 177	6
Right free ventricular wall area	597 ± 69	8	528 ± 82	8	494 ± 87	6
Left ventricular chamber	229 ± 70	8	565 ± 179***	8	595 ± 267*	6
Right ventricular chamber	193 ± 110	8	476 ± 163**	8	506 ± 178**	6
Total sectional ventricular myocardial area	2031 ± 251	8	1780 ± 195*	8	1690 ± 200*	6
Descending thoracic aorta area	42 ± 5	8	45 ± 12	8	42 ± 7	6
Pulmonary trunk diameter ¶	195 ± 38	8	216 ± 27	5	156 ± 47	5
Ascending thoracic aorta area	35 ± 5	8	46 ± 6**	6	35 ± 4	3
Aortic valve area	59 ± 9	8	65 ± 13	8	55 ± 7	3
Left cranial vena cava area	171 ± 52	8	195 ± 61	8	130 ± 50	6
Right cranial vena cava area	159 ± 31	8	193 ± 38	8	144 ± 48	6
Caudal vena cava	224 ± 62	8	291 ± 105	8	186 ± 95	5

CHAPTER 4: HEART CHANGES IN 17- DAY-OLD FETUSES OF DIABETIC ICR MOTHERS: IMPROVEMENT WITH MATERNAL IMMUNE STIMULATION

J. Claudio Gutierrez [1,3], M. Renee Prater [1,2], Bonnie J. Smith [1], Larry E. Freeman,[1] and Steven D. Holladay [4]

[1] Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute & State University, Blacksburg, Virginia, USA. [2] Department of Biomedical Sciences, E. Via Virginia College of Osteopathic Medicine, Blacksburg, Virginia, USA. [3] Instituto de Farmacología y Morfofisiología, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile. [4] Department of Anatomy and Radiology, University of Georgia, Athens, Georgia, USA.

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4.1: ABSTRACT

Maternal diabetes mellitus is associated with increased fetal teratogenesis, including cardiovascular defects. Non specific maternal immune stimulation with Freund's complete adjuvant (FCA) or interferon gamma (IFNγ) has been associated with protection against birth malformations.

Using a diabetic mouse model, late-gestation fetal heart and great vessel morphology were analyzed. Four groups of mice were used: Non-diabetic females as a control group, hyperglycemic females induced by streptozotocin as a diabetic group and diabetic females injected either with FCA or IFNγ. At day 17 of gestation, females were euthanized and one fetus was arbitrarily selected per litter for fixation and sectioning. Treatment-induced changes in cardiac development were assessed from digital images of serial sections taken at standardized levels in the thorax. One way parametric and non-parametric ANOVA and ordinal logistic regression were performed to compare the difference among groups ($p < 0.05$).

Maternal hyperglycemia altered morphology of the late-gestation fetal mouse heart by causing ventricular chamber dilation, sectional myocardial reduction, and an increase in transversal aortic area. FCA protected the fetal heart from cavitary dilation in diabetic mothers. FCA and IFNγ protected the fetal heart against reduction of myocardial area, and ascending thoracic aorta dilation. Consequences of late gestation heart chamber dilation and myocardial reduction are not yet known. Maternal immune stimulation partially protected against these developmental defects by mechanisms that remain unclear.

Key words: type 1 diabetes, cardiovascular malformations, diabetes and pregnancy, FCA, IFNγ

4.2: INTRODUCTION

Non specific maternal immune stimulation is protective in mice against birth malformations by chemical teratogens (Nomura et al., 1990; Holladay et al., 2000; Sharova et al., 2000; Sharova et al., 2002; Prater et al., 2004; Hrubec et al., 2006a). Studies by Hrubec et al. (2006b) indicate a reduction of NTDs (neural tube defects) and ocular defects induced by valproic acid in CD1 mice when dams were treated either with interferon γ (IFNγ) or granulocyte-macrophage colony stimulating factor (GM-CSF). Prater et al., (2004) similarly found a reduction of distal limb defects in offspring of IFNγ treated ICR mice exposed to methylnitrosourea (MNU).

Pre-pregnancy diabetes mellitus can cause increased fetal teratogenesis. If the mother suffers pre-pregnancy diabetes (type 1 or type 2), the embryo is affected by hyperglycemia related events from the first stages of development. There is a 3 to 5 times higher incidence of malformed newborns in women with type 1 diabetes as compared to non diabetic women (Reece and Eriksson, 1996; Reece et al., 1998).

NTDs and cardiac defects (CDs) are more frequent in the diabetic pregnancy than in non diabetic pregnancy (Reece et al., 1998; Punareewattana and Holladay, 2004). Heart disease is increased up to five fold among infants of diabetic mothers compared to the general obstetric population (Meyer-Wittkopf et al., 1996). Fetal heart cavitary dilation and myocardial reduction were recently described in late-gestation fetuses of diabetic

pregnant mice (with blood glucose (BG) > 26 mM/L) (Gutierrez et al., 2007). Reports regarding human fetal and infant heart changes under maternal hyperglycemia are divergent but toward the description of fetal myocardial hypertrophy (Macklon et al., 1998; Jaeggi et al. 2001; Abu-Sulaiman and Subaih 2004).

Punareewattana and Holladay (2004) made use of Freund's complete adjuvant (FCA), GM-CSF or IFNγ to induce a non specific immune-response in insulin-deficient diabetic ICR dams. In these studies fetuses were examined at gestation day (GD) 17 for major NTDs. Highly significant reduction of NTDs occurred, from 50% in non-immune stimulated diabetic dams to 14-23 % in immune stimulated dams. Torchinsky et al. (1997) made use of male rat splenocytes to immune stimulate ICR insulin-deficient diabetic female mice 3 weeks before breeding. The authors found a reduction of malformed litters and an increase in pregnancy rate in immune stimulated females compared to non-immunized females.

Hrubec et al. (2006a) reported a reduction of craniofacial embryopathy in offspring of diabetic dams immune-stimulated with IFNγ or GM-CSF.

The present study examining hyperglycemia-induced fetal heart cavitary dilation and myocardial reduction also demonstrates that these changes are in part alleviated by maternal immune stimulation with FCA or IFNγ. The mechanism for the protective effect of maternal immune stimulation is not known. The collective available literature suggests that hyperglycemia may enhance myocardial apoptosis, and immune stimulation may in part normalize this process (Frustaci et al., 2000; Cai et al., 2002). Maternal immune stimulation has prevented alteration in expression of genes regulating apoptosis in other teratogenic models (Sharova et al., 2002). A similar mechanism may be involved in preventing hyperglycemia induced myocardial malformations.

4.3: MATERIALS AND METHODS

4.3.1: Animal Model

Six to 8 week old outbred ICR mice (Harlan Sprague-Dawley, Indianapolis, IN) were housed individually (males) or at 5 per cage (females) for a 2-week acclimation period. Mice were given food (NIH 31 open formula) and tap water *ad libitum*, and were maintained at 22°C, 40-60% relative humidity with a 12/12 light/dark cycle.

Diabetes was chemically induced in female mice by intraperitoneal (IP) injection with 200 mg/kg streptozotocin (STZ) (Sigma, St. Louis, MO) 7 days before breeding. Blood glucose (BG) levels in tail vein blood were determined every 3-5 days using glucose kits (Sigma), and were used to categorize diabetic mice. Mice with BG levels ≥ 26 mM/L were previously found to produce fetuses with increased externally visible birth defects (Punareewattana and Holladay, 2004) and were selected for this experiment.

Four groups of animals were used in these experiments (n = 8 in each group): non-STZ-injected, non-diabetic females as a control group (C group), hyperglycemic females without additional treatment as a diabetic group (D group), and diabetic females injected either with FCA (FCA group) (20-30 μl, by footpad injection) or IFNγ (IFNγ group) (1000 units by IP injection). Immune stimulants were administered twice, first at 1 week and again at 1 day before STZ administration (Nomura et al. 1990). Pregnant mice were euthanized by cervical dislocation at GD 17. Fetuses were collected and preserved in absolute ethanol for later examination.

4.3.2: Morphometric Analysis

One fetus was selected per litter to analyze the heart and great vessels morphology (n = 8 per treatment). The selection was arbitrary, with the exception that only fetuses showing no externally visible defects were selected. Complete fetal thoraxes were processed using routine histopathologic techniques, and serial 7 μm transversal sections were prepared and stained with hematoxylin and eosin. Digital images of sections were collected using an Olympus Zoom Stereo Microscope SZX7 and camera (Olympus America Inc., Melville, NY), and imported into Photoshop 7.0 to optimize image brightness and contrast. Images were then evaluated using NIH Image J software (http://rsb.info.nih.gov/ij/download.html) designed for morphometric analysis. Three major features of the heart were measured: sectional myocardial area, left ventricular chamber area and right ventricular chamber area in equivalent level transverse sections. Presence of both the left and right atrio-ventricular valves was used as the landmark for equivalent transverse level.

Seven secondary features were also analyzed: transversal ascending thoracic aortic area, transversal thoracic descending aortic area, aortic valve area, pulmonary trunk diameter,

left cranial vena cava, right cranial vena cava and caudal vena cava. For descending thoracic aortic area, presence of both atrio-ventricular valves was again used as the landmark to compare equivalent transverse levels. For left and right cranial vena cava areas, ascending thoracic aorta area and pulmonary trunk diameter, the pulmonary trunk bifurcation into left and right pulmonary arteries was used as the landmark. Sections showing all three aortic valve leaves (valvula semilunaris septalis, valvula semilunaris dextra and valvula semilunaris sinistra) were used to measure aortic valve area. For caudal vena cava area, sections showing the caudal limit of left and right ventricular chambers were selected. Kaufman's Atlas of Mouse Development (2003) was used as a guide to precisely compare same-level sections among fetuses.

4.3.3: Pathologic Scoring

In addition to morphometric analysis of regional heart areas, a visual categorization of fetal hearts was made. Briefly, visual categorization was performed using a 0 - 3 scale based on a series of predetermined morphologic parameters. The evaluation was performed blindly by a veterinary pathologist (Co-author M. Prater) and results were verified separately by a veterinary anatomist (Co-author B. Smith).

For this analysis, same level long axis sections of the fetal heart were observed (n = 8 per treatment group). Fetal hearts were assigned a number based on ventricular chamber dilation and myocardial reduction: 0 implied no apparent ventricular chamber dilation + myocardial reduction, 1 was assigned to hearts with mild ventricular chamber dilation + myocardial reduction, 2 was assigned to hearts with moderate ventricular chamber dilation + myocardial reduction, and 3 was assigned to hearts with marked ventricular chamber dilation + myocardial reduction.

All procedures involving mice were reviewed by and conducted in compliance with the guidelines of the Virginia Tech Animal Care and Use Committee at the VA-MD Regional College of Veterinary Medicine.

4.3.4: Statistical Analysis

Statistical software SAS 9.1 was used to run either one way ANOVA or non-parametric ANOVA (Kruskal-Wallis statistical test) to detect differences among groups.

When a significant difference was observed ($p < .05$), a Scheffes statistical test was used to analyze differences among groups. Statistical software MINITAB 15 was used to run descriptive statistics and ordinal logistic regression test for the visual pathologic categorization. Ordinal endpoints for all groups were compared. Ordinal endpoints were compared in the following pairs of groups: C/D, C/FCA, C/IFNγ, D/FCA, D/IFNγ, FCA/IFNγ.

4.4: RESULTS

The morphometric analysis made with Image J, using threshold and particle analysis tools (Gutierrez et al., 2007) revealed that: maternal hyperglycemia caused increased fetal ventricular chamber and ascending thoracic aortic area; effects that were sometimes alleviated by maternal immune stimulation. Representative sections of 17-day-old fetal hearts from the different groups are shown in Figure 4.1.

Protection was offered by FCA and IFNγ against myocardial reduction and dilation of the left out-flow channel (Figure 4.2 and Figure 4.3). The diabetic group without additional treatment showed a significant increase in ascending thoracic aortic transversal area compared to the other groups. Left and right mean ventricle chamber areas were both approximately 2.5 times larger in fetal mice from diabetic dams without additional treatment (D group), as compared to corresponding chambers from non-diabetic dams (C group). Particularly, there was no statistical difference in left and right ventricular chamber area between the C group and the FCA group (Figure 4.4 and Figure 4.5). Nevertheless, there was also no statistical difference among the FCA group, the diabetic group and the IFNγ group. These results are not conclusive regarding possible protective value of the FCA treatment against ventricular chamber dilation. However, the pathologic scoring (Table 4.1) of fetal hearts supported the morphometric data and ANOVA results. With this analysis, FCA was determined to be protective by visual staging (Figure 4.6). Very close concordance of visual scoring results was found between the two evaluators (veterinary pathologist and veterinary anatomist). Ordinal logistic regression, using ordinal endpoints from the different groups (factors) showed the FCA group to be different from the D group ($p < .05$, for the covariate and after Log-likelihood test). This

indicates that the lower lesion score in the FCA group was related to the immune treatment. There was no difference between D and IFNγ groups ($p > .05$).

The following endpoints: aortic valve area, descending thoracic aortic area, pulmonary trunk diameter, left cranial vena cava area, right cranial vena cava area, and caudal vena cava area, were not affected by the different treatments (Table 4.2). Non-cardiac maternal and fetal parameters are shown in Table 4.3.

4.5: DISCUSSION

The first description of immune stimulation as protective against teratogen-induced congenital malformations was by Nomura et al. (1990). The authors found that immune stimulation as long as 3 weeks before breeding reduced birth defects. In those experiments, ICR and CL/Fr mice offspring showed a reduction of radiation and chemical induced birth defects including cleft palate and digit anomalies. The pregnant females were pre-treated with Pyran copolymer or Bacillus Calmette-Guérin to induce a non-specific maternal immune response. These authors suggested that activated maternal macrophages may cross the placenta, recognize and destroy embryonic pre-teratogenic cells. Later, however, Holladay et al. (2000) were unable to detect maternal leukocytes in fetal blood of teratogen-challenged C57BL/6N mice, using a fluorescent cell tracking probe.

Mechanisms by which maternal immune stimulation reduces embryonic/fetal maldevelopment in mice remain unclear. Sharova et al. (2003) found that the teratogen, urethane, shifted placental cytokine gene expression profile toward Th1 cytokines in pregnant ICR mice, including IFNγ and interleukin 2 (IL-2). However maternal immune stimulation in the urethane-exposed females induced expression of placental genes for Th2 cytokines: IL-4, IL-10 and IL-13; and decreased cleft palate caused by the urethane. These authors also reported damage to placental structure by urethane, particularly at the level of the syn-trophoblast, that was decreased by maternal FCA or IFNγ treatment.

Laudermilch et al. (2005) found that maternal immune stimulation improved placental damage caused by the alkylating agent and teratogen, methylnitrosourea (MNU). In particular, maternal immune stimulation using IFNγ restored placental weight and diminished the number of placental necrotic cells in C57BL/6N females exposed to

MNU. These authors also observed a reduction of fetal distal limb defects in MNU exposed mice after maternal IFNγ treatment, and suggested placental improvement may explain the reduced limb defects. Together, these urethane and MNU data suggested that placental improvement due to the maternal immune stimulation may play a protective role in fetal development in teratogen-challenged mice. In contrast, Punareewattana et al. (2005) were unable to detect improved placental function in diabetic ICR mice subjected to various immune stimulations, and suggested that non-placental mechanisms may explain reduced birth defects in the hyperglycemic dams. Sharova et al. (2000) evaluated the expression of a panel of genes that regulate cell cycle and apoptosis in fetal heads from urethane-treated dams. Urethane exposure decreased *Bcl-2α/P53*, *Bcl-2β/P53* and *PKCα/P53* gene expression ratios in gestation day 14 fetal heads, thereby shifting expression toward enhanced apoptosis and increased incidence of cleft palate. Maternal IFNγ treatment normalized the altered gene expression ratios and reduced both severity and incidence of cleft palate lesions. Based on these outcomes, the authors suggested that maternal immune stimulation reduced urethane-induced teratogenesis by overcoming dysregulated apoptosis caused by urethane.

Neural tube defects in the diabetic pregnancy may be associated with reduced expression of the gene Pax 3 leading to neuroepithelial apoptosis (Fine et al., 1999). The incidence of apoptosis is increased in neural stem cells treated with high glucose levels (Fu et al., 2006). Hyperglycemia has been reported as an inducer of myocardial apoptosis in adult mice (Cai et al. 2002). Hyperglycemia also affects gene expression in the embryonic mouse heart leading to changes in extra-cellular matrix proteins (ECM) such as fibronectin. Over-expression of transforming growth factor β1 (TGF β1) under hyperglycemia has been reported. TGF β1 mediates an increased production of ECM proteins (Smoak, 2004). Prater et al. (2006) later reported approximately equal protection against MNU-induced distal limb defects in mice by maternal IFNγ treatment or maternal antioxidant therapy. These authors noted that both MNU and urethane cause oxidative stress, and that increased reactive oxygen species (ROS) up-regulate *P53* (pro-apoptotic) (Bianchi et al., 2003) and down-regulate *Bcl-2* (anti-apoptotic) (Cicchillitti et al., 2003) gene expression in proliferating fetal tissues.

Similar to MNU- and urethane-treated mice, the present fetal mice showed hyperglycemia-induced cardiac changes that were improved by maternal immune stimulation. Mechanisms that may explain this improved developmental outcome remain unknown. However, hyperglycemia is an inducer of reactive oxygen species and reactive nitrogen species, both of which are proposed mediators of abnormal gene expression and apoptosis in cardiomyocytes (reviewed by Adeghate, 2004; Malhotra et al., 2005). Cai (2002) similarly found that high levels of hyperglycemia directly induce apoptotic cell death in myocardial cells. This cell death was related to activation of the cytochrome c-activated caspase 3 pathway. Activation of *PKC-ε* in diabetic hearts has also been related to inactivation of mitochondrial protein, bad, and inhibition of cytochrome c release (Malhotra et al., 2005). Bad protein forms heterodimers with proteins *Bcl-2 and Bcl-x*, inhibiting their antiapoptotic actions, which again links diabetes-related cardiac pathology to dysregulated apoptosis.

In adult humans, chronic hyperglycemia has been associated with hypertrophic cardiomyopathy, which evolves into a dilated myopathy in the adult heart (Frustaci et al., 2000). The latter authors indicate the affected adult diabetic heart shows progressive changes including cardiac hypertrophy and fibrosis, cavitary dilation, and depressed ventricular performance due to increase in apoptosis of myocytes, endothelial cells, and fibroblasts. The present observation of significant and visually evident cavitary dilation in fetal hearts of highly hyperglycemic dams may suggest dysregulated myocardial apoptosis as an operating mechanism worthy of further investigation, similar to the chronic lesion expressed in adult hearts. The improvement of fetal heart cavitary dilation and myocardial reduction by maternal immune stimulants FCA or IFNγ, interventions which have been linked to improved regulation of apoptosis with other teratogens, add support to this hypothesis.

Additional studies will be required to determine the postnatal persistence of the dilative heart chamber lesion observed in fetuses of severely diabetic dams, as well as the possibility that lower levels of hyperglycemia may contribute to similar, but reduced, myocardial damage, with uncertain consequences to postnatal health.

4.6: ACKNOWLEDGMENTS

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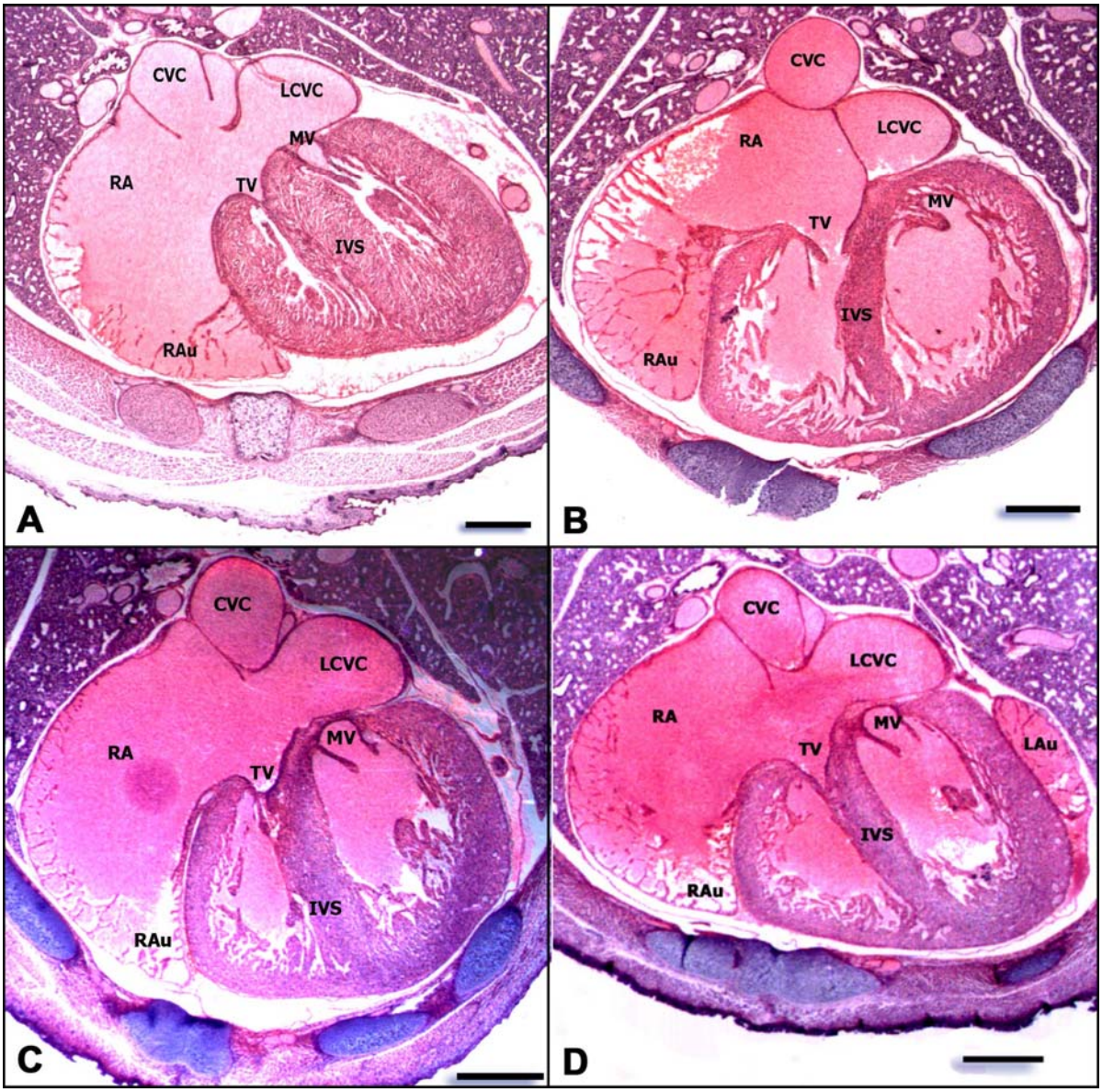

Figure. 4.1. Seventeen day old fetal heart (long axis) from the control group (A), diabetic group without additional treatment (B), FCA group (diabetic group treated with FCA) (C) and IFNγ group (diabetic group treated with IFNγ) (D). CVC: caudal vena cava, LCVC: left cranial vena cava, RA: right atrium, MV: mitral valve, TV: tricuspid valve, IVS: interventricular septum, LAu: left auricle, RAu: right auricle. Scale bars: 500 µm.

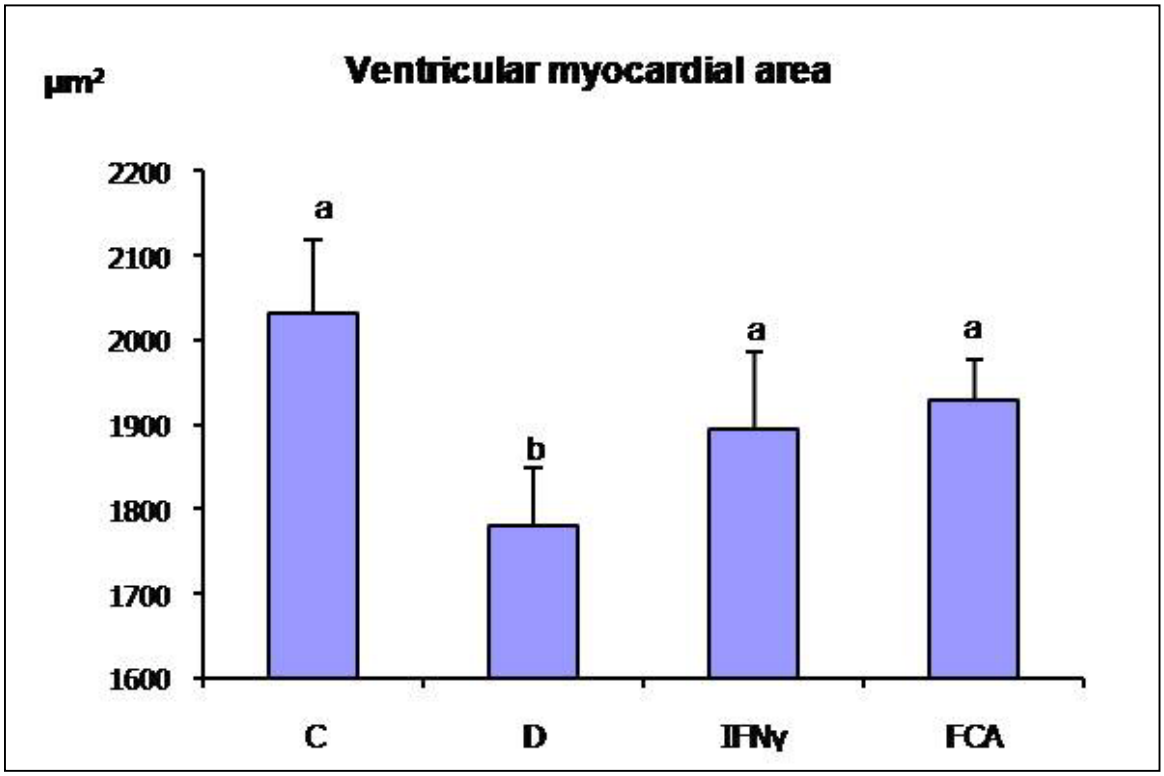

Figure 4.2. Cardiovascular endpoints for sectional ventricular myocardial area (n = 8 in each group). C: control group, D: diabetic group without additional treatment (a contrast test was performed for this group versus each of the other groups after ANOVA), IFNγ: diabetic group treated with IFNγ and FCA: diabetic group treated with FCA. Bars sharing the same letter are not significantly different. Bars represent mean ± SD.

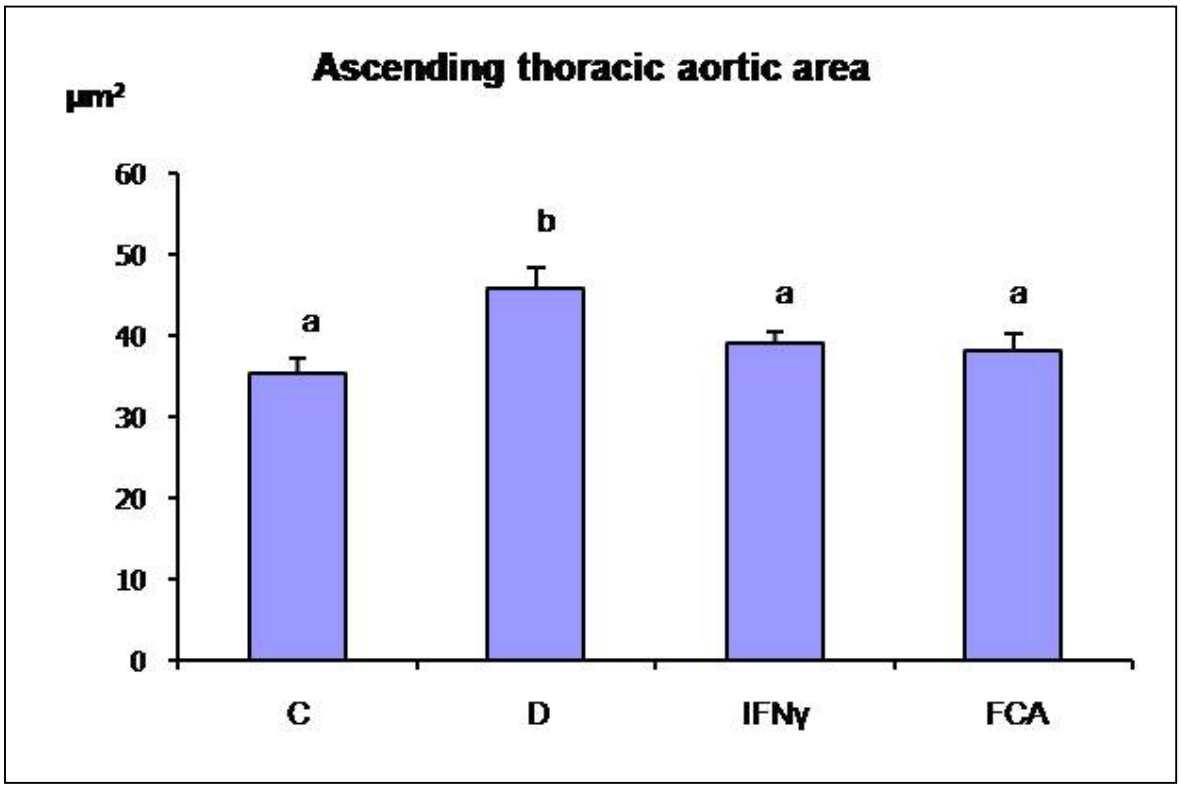

Figure 4.3. Cardiovascular endpoints for ascending thoracic aorta area (n = 8 in each group). C: control group, D: diabetic group without additional treatment, IFNγ: diabetic group treated with IFNγ and FCA: diabetic group treated with FCA. Bars sharing the same letter are not significantly different. Bars represent mean ± SD.

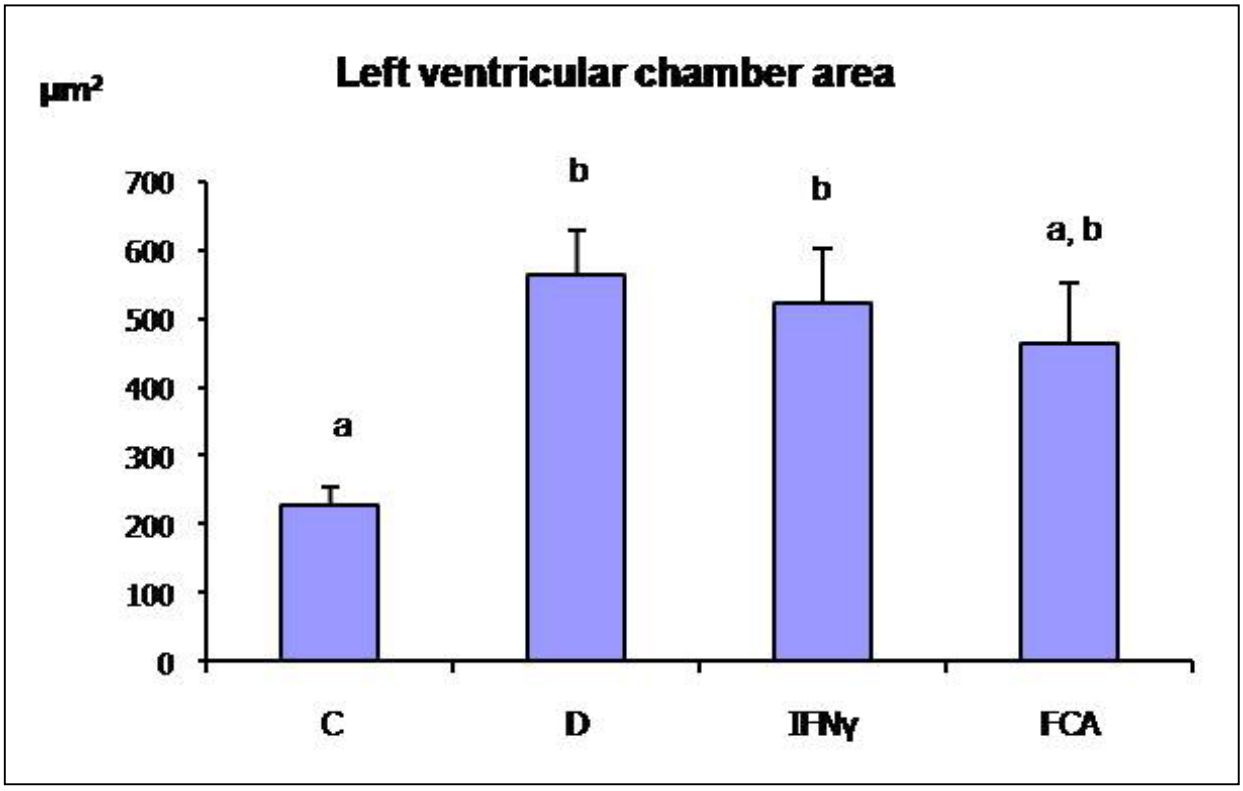

Figure 4.4. Cardiovascular endpoints for left ventricular chamber area (n = 8 in each group). C: control group, D: diabetic group without additional treatment, IFNγ: diabetic group treated with IFNγ and FCA: diabetic group treated with FCA. Bars sharing the same letter are not significantly different. Bars represent mean ± SD.

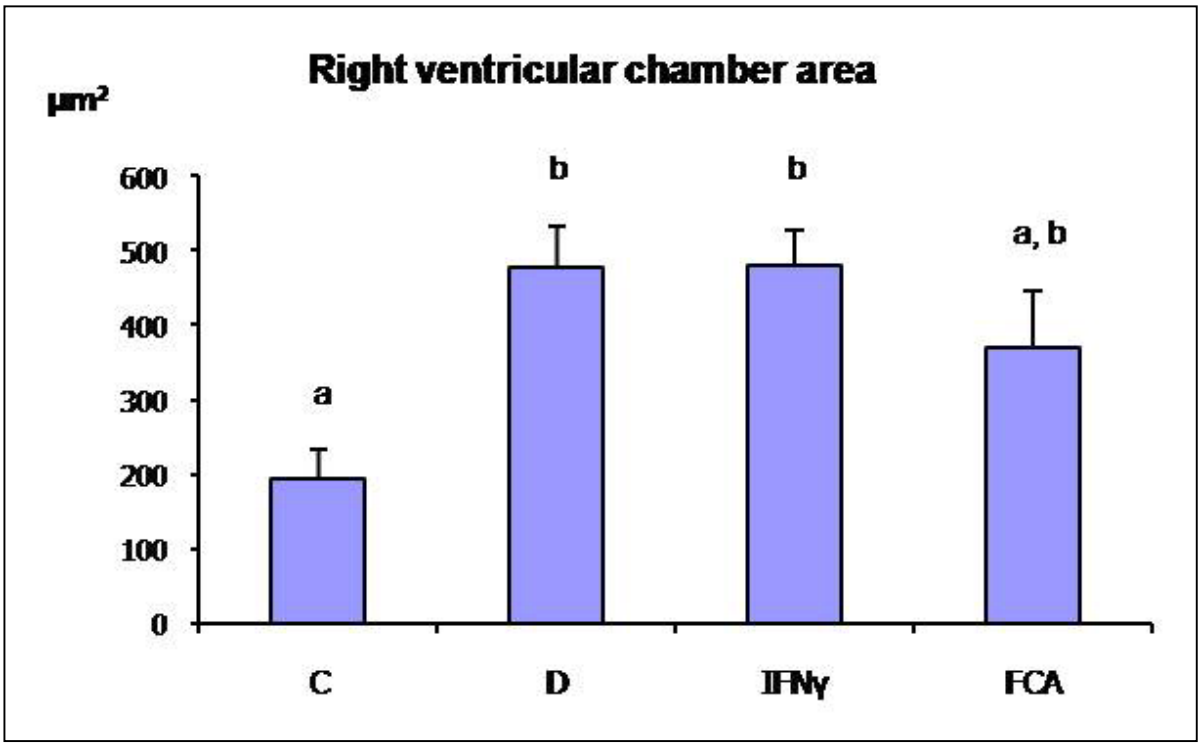

Figure 4.5. Cardiovascular endpoints for right ventricular chamber area (n = 8 in each group). C: control group, D: diabetic group without additional treatment, IFNγ: diabetic group treated with IFNγ and FCA: diabetic group treated with FCA. Bars sharing the same letter are not significantly different. Bars represent mean ± SD.

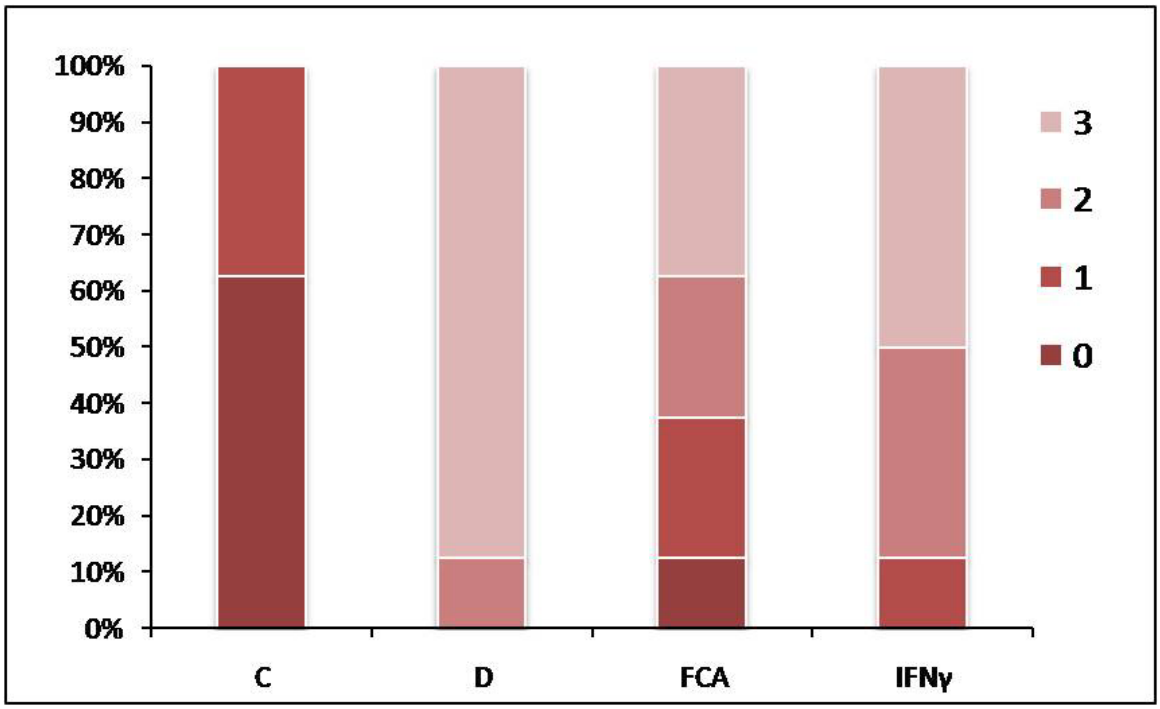

Figure 4.6. Seventeen-day-old fetal heart pathologic categorization (n = 8 in each group): 0: no apparent ventricular chamber dilation + myocardial reduction, 1: mild ventricular chamber dilation + myocardial reduction, 2: moderate ventricular chamber dilation + myocardial reduction and 3: marked ventricular chamber dilation + myocardial reduction. C: control group, D: diabetic group without additional treatment, FCA: diabetic group treated with FCA and IFNγ: diabetic group treated with IFNγ. D group vs FCA group: G = 5.282, DF = 1, $p < .05$ which means the difference is associated with the treatment. D group vs IFNγ group: G = 2.945, DF = 1, $p > .05$.

Table 4.1. Summary of pathologic scoring of fetal hearts (n = 8 in each group): 0: no apparent ventricular chamber dilation + myocardial reduction, 1: mild ventricular chamber dilation + myocardial reduction, 2: moderate ventricular chamber dilation + myocardial reduction and 3: marked ventricular chamber dilation + myocardial reduction. C: control group, D: diabetic group without additional treatment, FCA: diabetic group treated with FCA and IFNγ: diabetic group treated with IFNγ.

Scoring	C	D	FCA	IFNγ
0	5	0	1	0
1	3	0	2	1
2	0	1	2	3
3	0	7	3	4

Table 4.2. Cardiovascular endpoints for aortic valve area (*AoV*), descending thoracic aortic area (*DAo*), pulmonary trunk diameter (*PT*), caudal vena cava area (*CVC*), left cranial vena cava area (*LCVC*) and right cranial vena cava area (*RCVC*). C: control group, D: diabetic group without additional treatment, IFNγ: diabetic group treated with IFNγ and FCA: diabetic group treated with FCA. No differences among groups were found in these variables (*P* > .05). Data represent mean ± SD.

AoV	N	mean (μm^2)	DAo	N	mean (μm^2)
C	8	58.6 ± 9.3	C	8	42.3 ± 5.4
D	8	65.4 ± 13.5	D	8	45.3 ± 12.6
IFNγ	7	65.3 ± 6.5	IFNγ	8	39.6 ± 9.2
FCA	8	62 ± 10.5	FCA	8	39.3 ± 5.7
PT	N	mean (μm)	CVC	N	mean (μm^2)
C	8	194.8 ± 38.3	C	8	223.6 ± 62.6
D	5	216.4 ± 27.7	D	8	291.4 ± 105.6
IFNγ	8	170.5 ± 24	IFNγ	8	239.9 ± 75.1
FCA	8	174.9 ± 31.2	FCA	8	221.9 ± 60.7
LCVC	N	mean (μm^2)	RCVC	N	mean (μm^2)
C	8	170.8 ± 52.6	C	8	159 ± 31.9
D	8	195.3 ± 61.8	D	8	193.1 ± 38.4
IFNγ	8	161.6 ± 31.1	IFNγ	8	181.1 ± 27.5
FCA	8	167.3 ± 31.1	FCA	8	169.5 ± 32

Table 4.3. Maternal and fetal parameters at GD 17 in STZ-induced diabetic ICR mice, with or without maternal immune stimulation. D: diabetic group without additional treatment, IFNγ: diabetic group treated with IFNγ and FCA: diabetic group treated with FCA. * significantly different from control ($p < .05$); ** significantly different from diabetic group ($p < .05$).

	C	D	IFNγ	FCA
N° of females	8	8	8	8
Pregnancy rate (%)	100	32*	42*,**	42*,**
N° of total fetuses/dam	11.2 ± 0.6	12.0 ± 0.3	11.8 ± 0.5	10.6 ± 0.6
N° of live fetuses/dam	10.8 ± 0.7	9.6 ± 0.4	9.0 ± 1.1	8.3 ± 0.8
Fetal weight (g)	0.89 ± 0.1	0.76 ± 0.02*	0.69 ± 0.05*	0.79 ± 0.06*

CHAPTER 5: PRODUCTION OF A TYPE 2 MATERNAL DIABETES RODENT MODEL USING THE COMBINATION OF HIGH FAT DIET AND MODERATE DOSE OF STREPTOZOCIN

J. Claudio Gutierrez [1, 3], M. Renee Prater [1, 2] and Steven D. Holladay [4]

[1] Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute & State University, Blacksburg, VA 24061.

[2] Edward Via Virginia College of Osteopathic Medicine, Department of Biomedical Sciences 2265 Kraft Drive, Blacksburg, VA 24060.

[3] Universidad Austral de Chile, Instituto de Farmacología y Morfofisiología, Valdivia, Chile.

[4] Department of Anatomy and Radiology, University of Georgia, Athens, Georgia, USA.

5.1: ABSTRACT

Pregnancy may be complicated by maternal diabetes mellitus under three different scenarios: type 1 diabetes, an insulin dependent phenomenon, type 2 diabetes, an insulin resistant phenomenon and gestational diabetes mellitus (GDM), also an insulin resistant phenomenon and probably an early stage of type 2 diabetes. The following experiments were performed in an attempt to produce mouse models that most closely mimic these scenarios: Experiment 1: CD1 females were put on a high fat diet (HFD) during breeding and pregnancy. Maternal insulin levels were measured at gestational day (GD) 17. Maternal weight was monitored throughout gestation. Females fed a standard diet (SD) were used as a control group. There was a significant increase of insulin levels in the HFD fed females. Experiment 2: CD1 females received 200 mg/kg streptozocin (STZ) to model insulin dependent (type 1) diabetes (T1 group). Another group of females (T2 group) was put on a HFD 4 weeks before receiving 100 mg/kg STZ. After 4 additional weeks of HFD, hyperglycemic females were separated and bred. Normoglycemic females on a SD served as control groups. About 25% of the females on a HFD became

hyperglycemic after 4 weeks of the injection of the moderate dose of STZ. Experiment 3: CD1 females were fed a HFD for 4 weeks before receiving an intravenous (GDM1 group) or intraperitoneal (GDM2 group) injection of 100 mg/kg STZ after 4 weeks of being on a HFD. Females from GDM1 group were bred at the same day of the STZ injection. Females from GDM2 group were bred 4 weeks after the STZ injection. Both groups continue on a HFD during pregnancy. Normoglycemic females on a SD served as control groups. Fifty percent of the females from GDM1 group reached hyperglycemic levels of greater than 250 mg/dl during pregnancy. The use of the combination of HFD and moderate dose of STZ in CD1 female mice therefore produced hyperglycemic females that might serve to model T2 diabetes, however numbers of these mice were somewhat low.

Key words: type 1 diabetes, type 2 diabetes, diabetes and pregnancy, mouse models of diabetes

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5.2: INTRODUCTION

Pregnancy may be complicated by maternal diabetes mellitus under three different scenarios. In type 1 diabetes (insulin dependent diabetes), the mother produces vastly inadequate insulin. Without supplementary insulin treatment, she will continue to be hyperglycemic during pregnancy. Thus, the mother suffers pre-pregnancy diabetes and the embryo might be affected by hyperglycemia related events from the first stages of development. Type 2 diabetes (insulin resistant diabetes) is the most common form of diabetes (90 – 95% of all diagnosed cases), in which the individual either does not produce enough insulin or target cells are not responsive to insulin (defective insulin receptors). Thus in pregnancy, the mother suffers pre-pregnancy diabetes and the embryo might be affected by hyperglycemia from the first stages of development (American Diabetes Association, 2006). In gestational diabetes mellitus (GDM) (insulin resistant, probably a variety of type 2 diabetes), the mother develops diabetes in late gestation (third trimester). The mother does not have pre-gestational diabetes but instead develops hyperglycemia during advanced gestation. In this case, the fetus is affected by

hyperglycemia when rapidly growing (American Diabetes Association, 2006). About 135,000 cases of GDM are documented in the United States each year, and GDM is thought to occur in 1-14% of all US pregnancies (Hillier et al., 2008). Under any of the scenarios above, the embryo/fetus may suffer harmful effects of hyperglycemia.

Type 2 diabetes is the combination of resistance to insulin action and an inadequate compensatory insulin secretory response (American Diabetes Association, 2006). GDM refers to any degree of glucose intolerance with onset of first recognition during pregnancy. GDM is a special phenomenon and probably an early stage of type 2 diabetes, because women with GDM demonstrate increased risk of developing type 2 diabetes mellitus after pregnancy. For reasons that remain poorly understood, diabetes is not preexisting but develops during advanced pregnancy. Obese and overweight women have greater predisposition to develop GDM (American Diabetes Association, 2006; Hillier et al., 2008). During the course of GDM, the fetus is exposed to hyperglycemia when rapidly growing. It has been also suggested that hyperinsulinemia is a primary defect in type 2 diabetes and that insulin resistance develops secondarily to chronic hyperinsulinemia (Devedijian et al., 2000).

Fetal effects of GDM include infant macrosomia and organomegaly and often result from the exposure to high glucose levels in late gestation. Resultant infant macrosomia, the classic feature of GDM, is associated with increased risk of adult type 2 diabetes and obesity in adolescence (Yamashita et al., 2003; Gillman et al., 2003). Insulin sensitivity decreases in obese and lean women during pregnancy. Insulin sensitivity also decreases with advancing gestation (Catalano et al., 1991; Catalano et al., 1999). Women with GDM are less sensitive to insulin than non-diabetic pregnant women (Ryan et al., 1985). According to these data, the mother would be advised to control not only glucose levels, but also her nutrition and weight before and during pregnancy.

Rodent models of maternal diabetes using high levels of STZ as an inducer of pancreatic beta-cell death are extensively reported in the literature (Like and Rossini, 1976; Goldman et al., 1985; Punareewattana and Holladay, 2004). This model mimics insulin dependent diabetes. Studies by Luo et al. (1998) reveal an interesting non-genetic male mouse model of type 2 diabetes using ICR and C57BL/6J mice. According to this approach, a combination of high fat diet (HFD) and moderate dose STZ was an

alternative to model non-insulin dependent diabetes. The concept behind this idea: HFD induces hyperinsulinemia which saturates the insulin-glucose control system. Next, the moderate dose of STZ reduces insulin levels close to normality and hyperglycemia begins. This model was proposed to more closely mimic non-insulin dependent diabetes, however was not studied in female mice or under conditions of pregnancy.

The present experiments use the combination of HFD and moderate dose of STZ in CD1 females and compared the results with the classical use of high dose of STZ to induce insulin dependent diabetes. Results suggest the combination of HFD and moderate dose of STZ may be used to model type 2 diabetes and GDM in pregnant female mice. This model could have considerable value to better understand the effects of late gestation non-type 1 hyperglycemia in fetal development, as a result of maternal decreased insulin sensitivity in the course of gestation.

5.3: MATERIALS AND METHODS

5.3.1: Experiment 1: HFD-induced Hyperinsulinemia in CD1 Females

Six- to seven-week old CD1 females (Charles River) were used in these experiments. Mice were housed 5 per cage for a 1-week acclimation period. Mice were fed either with a high fat diet (HFD, 20% protein and 60% of total fat, 37.1 % of saturated fat, Research Diets Lab, New Brunswick, NJ, Table 5.1) or with a standard rodent diet (SD, Harlan Teklad Global Diet 2018: 18% protein and 5 % fat, Madison, WI). Tap water was given ad libitum.

Mice were maintained under controlled conditions of temperature (22 °C), humidity (40-60 %) and lighting (12/12 hour light/dark cycle). For breeding, males were housed overnight with females, and females checked for vaginal plugs the next morning, which was designated day 0 of gestation (GD 0). Plug positive females were separated from males and divided into the following treatment groups in a randomized complete block design.

Control group (C): females were fed during breeding and pregnancy with a SD (n = 5).

HFD group (HFD): females were fed with HFD during breeding and pregnancy (n = 5).

At GD 17, females were euthanized by CO_2 inhalation. Parameters evaluated were: Maternal body weight and plasma insulin. Blood samples were taken post-mortem

directly from the heart and great vessels using capillary heparinized tubes. Plasma samples were stored at –20 °C for insulin analysis. Maternal insulin levels at GD 17 were measured by radio immune-assay (RIA) according to the manual using 100 µl of plasma per sample. Maternal weight was determined at GD 0, 5, 10, 15 and 17.

Statistical Analysis

Student's *t* test for means was performed to determine presence or absence of significant difference between the control and treatment group ($p < .05$).

5.3.2: Experiment 2: The Use of Streptozocin and HFD to Model Insulin Dependent and Insulin Resistant Diabetes Using CD1 Females

Six- to seven-week old CD1 females (Charles River) were used in these experiments. Mice were fed either a HFD or a SD. Tap water was given ad libitum. Husbandry and breeding conditions were the same as for experiment 1.

Experimental groups:

Main control group (C group): SD fed CD1 females (n = 6) were injected intraperitoneally (IP) a citrate buffer (0.05 M, pH: 4.5) and served as a main control group: C group (n = 6).

Insulin dependent group (T1 group): Diabetes was induced in SD fed CD1 females (n = 10) using the classical dose of 200 mg/kg STZ. Females were injected by intraperitoneal (IP) injection with STZ (Sigma, St. Louis, MO) dissolved in a citrate buffer (0.05 M, pH: 4.5) 7 days before breeding, as previously reported (Punareewattana and Holladay, 2004). Blood glucose (BG) levels in tail vein blood were determined every 3-5 days (as determined by Accuchek compact blood glucose monitoring following tail venipuncture).
Type 2 diabetes group (T2 group): CD1 females (n = 24) were fed a HFD for 4 weeks. At the end of the fourth week these mice were injected a moderate dose of STZ (100 mg/kg IP) disolved in a citrate buffer (0.05 M, pH: 4.5) (Luo et al., 1998; Mu et al., 2006). Four weeks after the STZ injection, hyperglycemic females were separated and breeding initiated. Femlaes were continued on the HFD during pregnancy.

Secondary control group (CSTZ group): SD fed CD1 females (n = 6) were injected with a 100 mg/kg IP STZ dissolved in citrate buffer (0.05 M, pH: 4.5), one week before breeding.

At GD 17, females were euthanized by CO_2 inhalation and fetuses collected. Parameters evaluated were: Maternal and fetal body weight, blood glucose (as determined by Accu-Check compact glucometer, Roche laboratories, distributed by www.americandiabeteswholesale.com) following tail venipuncture, and plasma insulin (as determined by ELISA mouse ultrasensitive kit, ALPCO diagnostics, Windham, NH). ELISA replaced RIA in experiment 2 because of the small amount of plasma required and its convenience in serial sampling (5 µl for each duplicated sample).

Blood samples were taken from the tail vein using capillary tubes at breeding day 1 (BD1) and gestation days (GD) 10 and 17. Blood samples were taken at noon ±1 hr of each sampling day. These procedures were performed to verify onset of gestational hyperglycemia and hyperinsulinemia. Maternal and fetal weight were determined at GD 17. Average individual fetal weight was determined by dividing total litter weight by number of fetuses in the litter at GD 17. Fetuses were examined using a SZ Olympus microscope.

Statistical Analysis

Statistical software SAS 9.1 was used to run one-way ANOVA to detect differences among groups. When a significant difference was observed ($p < .05$), a Scheffes statistical test was used to further analyze differences among groups.

5.3.3: Experiment 3: Induction of Hyperglycemia in a Gestational Diabetes Profile

Six- to seven-week old CD1 females (Charles River) were used in these experiments. Mice were fed either with a HFD or with a SD. Husbandry and breeding conditions were the same as for experiment 1.

Experimental groups:

Main control group (C): Female CD1 females (n = 4) on a standard diet (SD) were injected IV with 100 µl of a citrate buffer (0.05 M, pH: 4.5) and served as a main control group.

Secondary control group (CSTZ): SD fed CD1 females (n = 4) were injected with 100 mg/kg IV STZ dissolved in citrate buffer (0.05 M, pH: 4.5) one week before breeding.

Gestational diabetes group 1 group (GDM1): female CD1 mice (n = 12) were placed on the HFD for 4 weeks before breeding. At breeding day 1 (BD 1), these mice were IV injected with 100 mg/kg STZ dissolved in in citrate buffer (0.05 M, pH: 4.5) and then continued on the HFD during throughout pregnancy (Luo et al., 1998; Mu et al., 2006).

Gestational diabetes group 2 (GDM2): female CD1 (n = 12) were placed on the HFD for 4 weeks and then IP injected with 100 mg/kg STZ dissolved in citrate buffer (0.05 M, pH: 4.5) (Luo et al., 1998; Mu et al., 2006). After additional 4 weeks on HFD, BG levels were determined. Approximately 25% of these mice had BG exceeding 250 mg/dl, and were eliminated from the study because they were hyperglycemic before pregnancy. Remaining non-hyperglycemic mice (n = 8) were bred and continued on the HFD during pregnancy. The same controls were used for GDM1 and GDM2 groups.

At GD 17, females were euthanized by CO_2 inhalation and fetuses collected. Parameters evaluated were: Maternal and fetal body weight, blood glucose (as determined by Accuchek compact blood glucose monitoring following tail venipuncture), and blood insulin (as determined by ELISA mouse ultrasensitive kit, ALPCO diagnostics). Maternal blood samples were obtained from the tail vein using capillary tubes 1 day before HFD exposure, at the end of the 4^{th} week of HFD exposure, at BD1 and at GD 9, 13 and 17 to measure plasma glucose (Accu-Check compact) and plasma insulin levels (ELISA). Blood samples were taken at noon ±1 hr on the corresponding sampling day. Females of each group were weighed at the same times during the experiment. Average individual fetal weight was determined by dividing total litter weight by number of fetuses in the litter at GD 17. Fetuses were examined using a SZ Olympus microscope.

Statistical Analysis

Statistical software SAS 9.1 was used to run one-way ANOVA to detect differences among groups. When a significant difference was observed ($p < .05$), a Scheffes statistical test was used to further analyze differences among groups.

5.4: RESULTS

In experiment 1, the statistical analysis demonstrated no difference in maternal weight increase from GD 0 to GD 17 between groups (Figure 5.1). Insulin levels in the same experiment were significantly higher in the HFD fed group as determined by RIA (Figure 5.2) (levels of insulin were transformed from μIU/L to ng/ml using the conversion factor: 0.0417).

In experiment 2, 90% of the females from T1 group became highly hyperglycemic (> 400 mg/dl) after 1 week of the high dose STZ injection. However, only 25% of the females from the type 2 diabetic model (T2 group) became hyperglycemic after 4 weeks of the STZ injection (moderate dose), and were used for breeding. When comparing glucose levels at BD 1, GD10 and GD17, differences among groups were found *(*$p < .001$). At BD 1, as expected, control groups (C and CSTZ) showed significantly lower levels of glucose compared to the diabetic groups. Among the diabetic females, T1 glucose levels were significantly higher than the T2 at BD1. At GD 10 and GD 17, glucose levels from T1 group continued to be significantly higher among the diabetic groups (glucose levels shown in Figure 5.3).

Maternal weight at GD 17 was significantly decreased in females from T1 group (Table 5.2) and fetal weight at GD 17 was also significantly decreased in T1 group ($p < .05$, Figure. 5.4). Insulin levels showed no statistical difference among the T2 group and the control groups when compared at BD1, GD10 and GD17 (Table 5.2).

In experiment 3, 50% of the females from GDM1 group reached blood glucose levels ≥ 250 mg/dl during pregnancy. The tendency of those females was to recover normal glucose levels by GD 17 (Figure 5.5). In GDM2 group, hyperglycemia during pregnancy was modest and females did not reached glucose levels as in GDM1 group (Figure 5.5). When comparing glucose levels among groups, no significant difference was found. However, when the subgroup of females from GDM1 group that reached glucose levels ≥ 250 mg/dl (50% of the group) was considered independently, differences were found: a significant difference in glucose levels was found between this subgroup and the C group at GD9 ($p < .05$). Also, at GD13, the subgroup was different from C and CSTZ groups ($p < .05$). One female from the CSTZ group reached 252 mg/dl at GD 13 but was normalized at GD 17.

There was no significant difference in fetal weight among the different groups ($p > .05$, Figure 5.6). There was also no significant difference among groups in maternal weight ($p > .05$) (Table 5.3).

Plasma insulin levels were significantly higher in GDM 2 group at BD1 and at GD 9 when compared to the rest of the groups ($p < .05$). No difference was found among the rest of the groups ($p > .05$) (Table 5.3).

5.5: DISCUSSION

Pre-gestational diabetes is a term used to describe diabetes diagnosed before pregnancy. Gestational diabetes however, is a term used to describe any degree of glucose intolerance recognized during the course of pregnancy (US Preventive Task Force, 2008).

Unlike women with type 1 diabetes, women suffering non-insulin dependent diabetes have adequate or higher insulin levels than normal. However, the effect of their insulin is partially blocked by a variety of other hormones made in the placenta. Human placental lactogen is suggested as one of the hormones primarily responsible for decreased insulin sensitivity with advancing gestation (Yamashita et al., 2000).

Maternal type 2 and GDM are insulin resistant phenomena; a mouse model should maintain that premise if at all possible. Hyperglycemia should not be a result of hypoinsulinemia as in the traditional use of STZ to induce insulin-dependent diabetes, but rather, hyperglycemia must be resultant in a normal insulin environment. Type 2 diabetes ranges from insulin resistance to predominantly an insulin secretory defect plus insulin resistance (American Association of Diabetes, 2006). GDM is pathophysiologically similar to type 2 diabetes and probably an early stage of the same affliction. Human patients with recent GDM have shown no difference in insulin levels when compared with control mothers (Eroglu and Zeyneloglu, 2006). Another study revealed that insulin receptor tyrosine phosphorylation was significantly decreased in subjects with GDM compared with pregnant and non-pregnant control subjects (Shao et al., 2000).

Streptozocin is an inducer of insulin-dependent diabetes in rats and mice when injected IP in a high dose (200 mg/kg in mice). This model has been widely used to study neural tube, cardiac and other alterations in the offspring of diabetic females

(Punareewattana et al., 2003; Punareewattana and Holladay, 2004; Hrubec et al., 2006, Gutierrez et al., 2007). In ICR mice exposed only to a HFD, hyperinsulinemia occurs but hyperglycemia is modest and well controlled by the organism. Studies by Park et al. (2006) indicate that HFD alone induced modest hyperglycemia in ICR mice (11.5 ± 0.2 mM/L ~ 200 mg/dl plasma glucose).

In the model of non-insulin dependent diabetes, first proposed by Luo et al. (1998), ICR males on a HFD developed high and maintained hyperglycemia (> 400 mg/dl) if injected with STZ (100 mg/kg IV). In the same mice, high hyperglycemia was reached but insulin levels were normal. Male mice on a standard diet (SD) injected with STZ (100 mg/kg) did not develop hyperglycemia. A similar model has been described with good results in rats (Reed et al., 2000; Srinivasan et al., 2005).

The present results from experiment 1 confirmed that the use of HFD for a short period of time (breeding and pregnancy) causes a significant increase in insulin levels (Luo et al.1998; Mu et al., 2004). The use of a combination of HFD and moderate dose of STZ has not been previously used in non-pregnant or pregnant females. Experiment 2 results reveal that 25% of these females become hyperglycemic under this combination after 4 weeks of receiving an IP injection of moderate dose of STZ. This differed from the above-described male mice, which almost all became hyperglycemic. Males are considered to be less sensitive to insulin than females, which may explain the higher number that become hyperglycemic under the combination of HFD/moderate STZ (Luo et al., 1998; Mu et al., 2004; G. Reaven, personal communication). Further manipulation of the present female model, including time on the HFD or dose of STZ used, may increase the percentage of hyperglycemic mice that result.

When comparing the T2 groups with the classical induction of hyperglycemia seen in the T1 group, clear differences in glucose levels were found. T1 females displayed levels of hyperglycemia that were significantly higher than those displayed by T2 females during the course of gestation. This clearly has to do with the high dose of STZ received by T1 group and its increased damage to pancreatic beta cells. STZ is a glucose methyl-nitrosourea which enters the pancreatic beta cells through glucose transporter GLUT2. Once inside the beta cell, STZ is split into its glucose and

methylnitrosourea moiety. This ends with fragmentation of DNA and beta cell destruction causing a state of insulin-dependent diabetes (Lenzen, 2008).

When comparing maternal weight at GD17 among groups from experiment 2, a decrease in weight occurred in females from T1 group. This is likely explained by the severity of hyperglycemia experienced by these females compared to the T2 and control groups. This severe hyperglycemia induced weight loss and continued polydipsia and polyuria during the course of pregnancy was not observed in the T2 groups.

When comparing fetal weight at GD17 among groups from experiment 2, 17-day-old fetuses from T1 females were significantly smaller. This is again described in the literature (Punareewattana and Holladay, 2004; Gutierrez et al., 2007) and is a result of the severe hyperglycemia displayed by the diabetic dams. Insulin levels among T2 groups and control groups were not significantly different. This assures that hyperglycemia in the T2 groups was not a result of hypoinsulinemia as in the classical use of high dose STZ.

GDM rodent models using STZ during pregnancy have been described in the literature (Menezes et al., 2001; Caluwaerts et al., 2003). These models represent a great effort to reproduce the GDM phenomenon in order to acquire better understanding of the mechanisms of the disease. Nevertheless, the disadvantage of using only STZ is that it produces a model closer to type 1 diabetes rather than GDM. The heterozygous leptin receptor-deficient mice, C57BL/KsJ-Lepr$^{db/+}$, develop spontaneous hyperglycemia during pregnancy (Yamashita et al., 2003). This mouse is a good alternative to model GDM. However, genetic mouse models are determined to a much greater extent to become hyperglycemic than patients with GDM (Luo et al., 1998). In experiment 3, mice from GDM1 group developed hyperglycemia during pregnancy. Those mice were injected with STZ intravenously. Hyperglycemia in GDM2 group was modest. However, hyperinsulinemia was higher in GDM2 group. This might be a consequence of a longer exposure to the HFD. According to these results the IV injection was more effective for generating hyperglycemic females, as compared to IP injection.

In the GDM1 group, a very well defined wave of hyperglycemia was generated during pregnancy. These results are interesting considering the lack of animal models to reproduce GDM. According to the classical findings of human GDM, infant macrosomia

is common (Yamashita et al., 2001; Mehta and Hussain, 2003). When comparing late fetal weight at GD17, no differences were found among groups of experiment 3. This may be explained by the fact that the human pancreas produces adequate levels of insulin before birth with the result of macrosomic babies. This might not be the case until after birth in the mouse (Prasadan et al., 2002).

In conclusion, the combination of HFD/moderate STZ is an alternative to generate type 2 diabetic female mice and GDM in pregnant mice. This combination produced a relatively low number of hyperglycemic CD1 females after 4 weeks of the moderate IP injection of STZ, which was improved by the IV injection. Further modifications of the HFD duration and/or STZ dosing may improve the percentage of T2 diabetic female mice generated.

5.6: ACKNOWLEDGMENTS

The authors acknowledge veterinary technician Mr. Dixon Smiley for his support and knowledge involving mice procedures and DVM student Mrs. Javiera Bahamonde for her help with animal husbandry in these experiments. Supported by NIH # R21-PAR-03-121, NIH # K01RR017018 and Harvey Peters Foundation.

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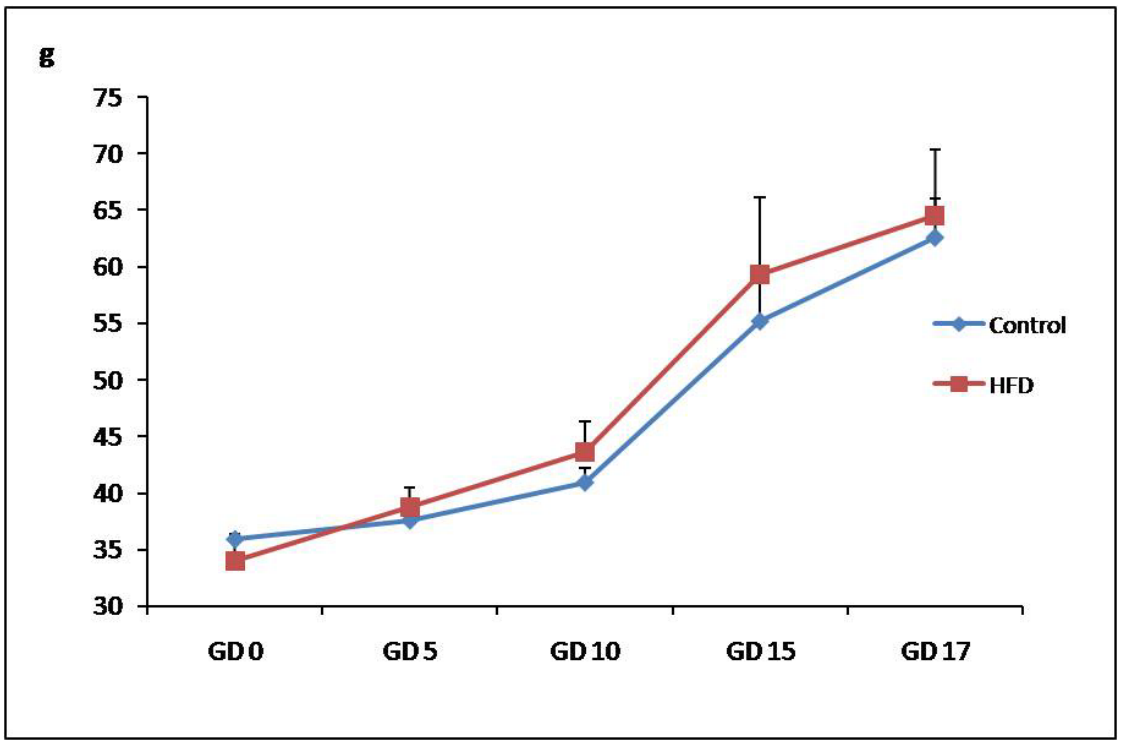

Figure 5.1. Maternal weight in experiment 1. No significant difference was found between groups (n = 5 in each group, $p > .05$).

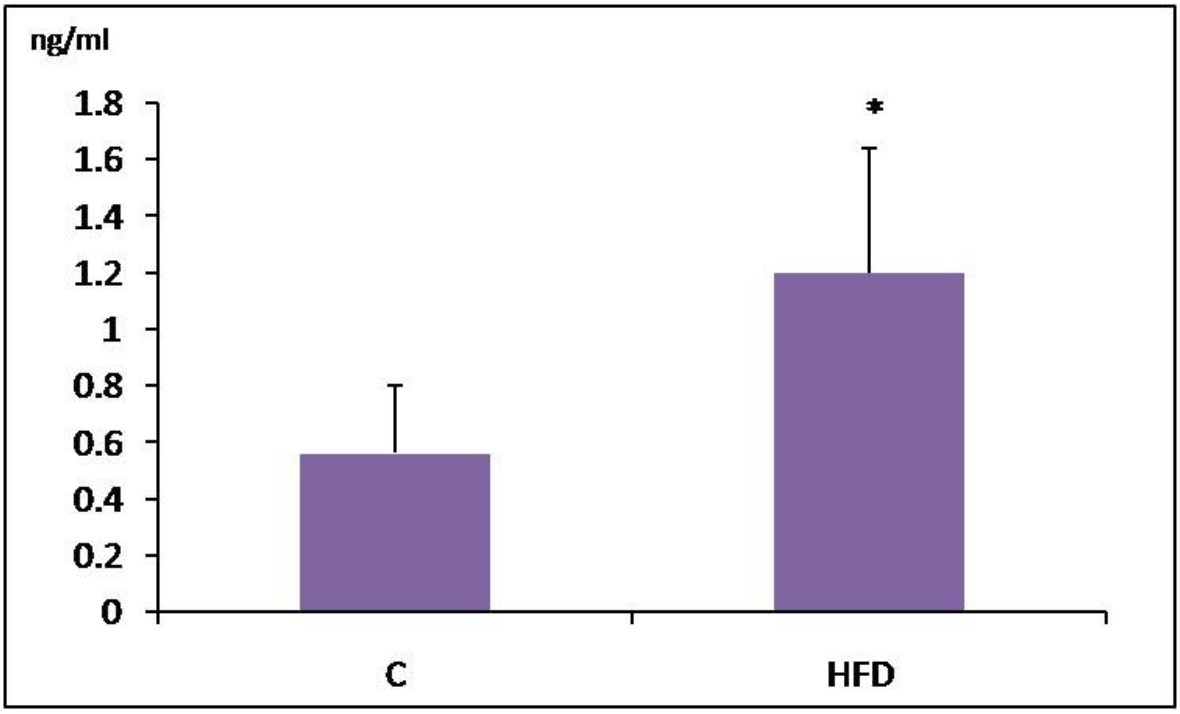

Figure 5.2. Maternal insulin levels in experiment 1. Insulin levels were significantly increased in the HFD group (n = 5 in each group, $p < .05$). Levels of insulin were transformed from μIU/L to ng/ml using the conversion factor: 0.0417.

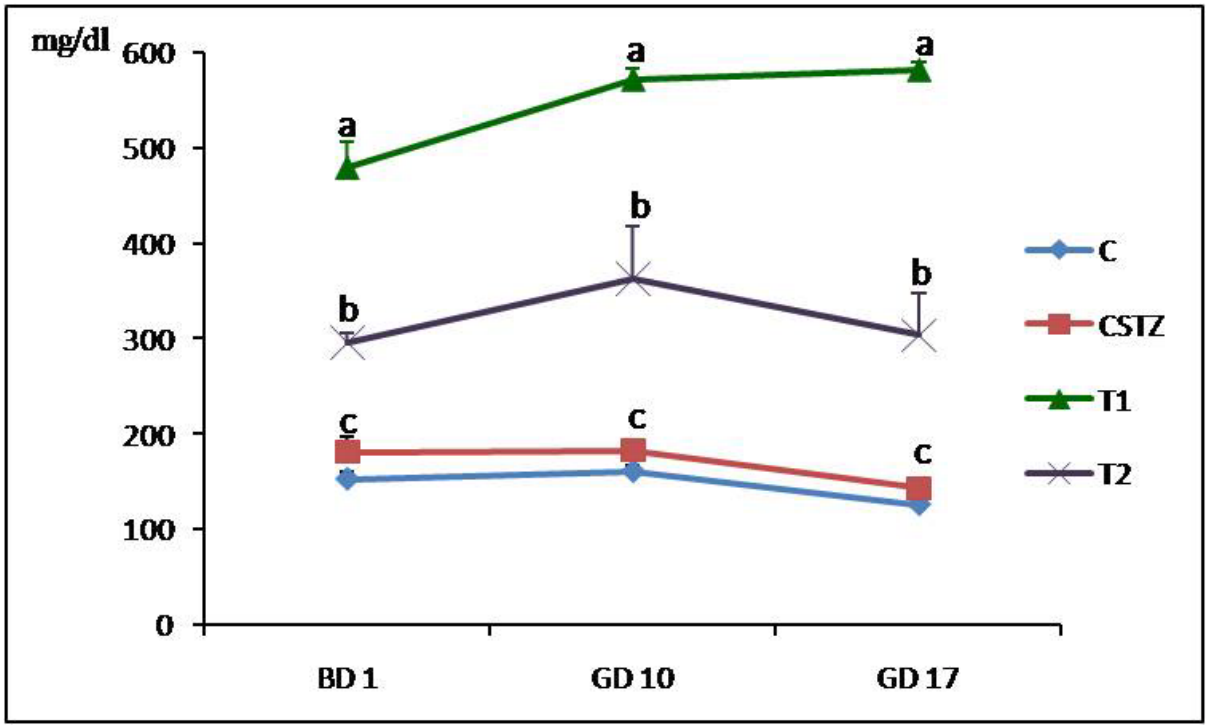

Figure 5.3. Comparison of maternal glucose levels for experiment 2. C: main control group, CSTZ: secondary control group, T1: type 1 diabetic group, T2: type 2 diabetic group, BD: breeding day, GD: gestation day. Groups sharing the same letter are not significantly different.

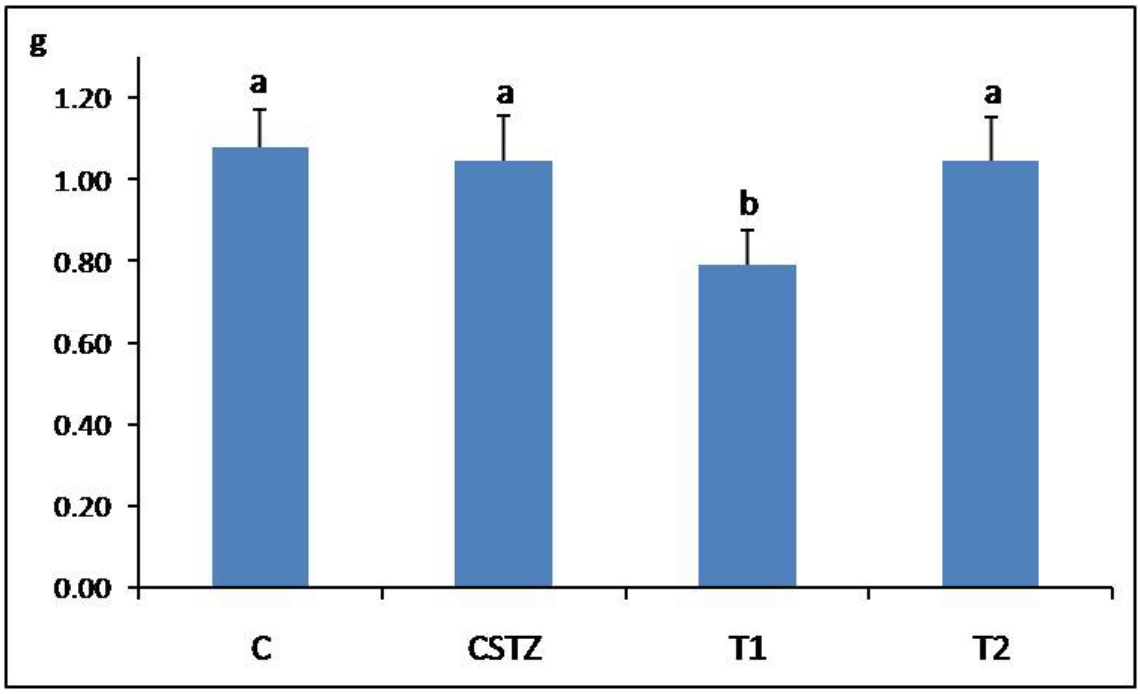

Figure 5.4. Fetal weight at GD 17 for experiment 2. C: control buffer group, CSTZ: control streptozocin group, T1: type 1 diabetic group, T2: type 2 diabetic group. Groups sharing the same letter are not significantly different.

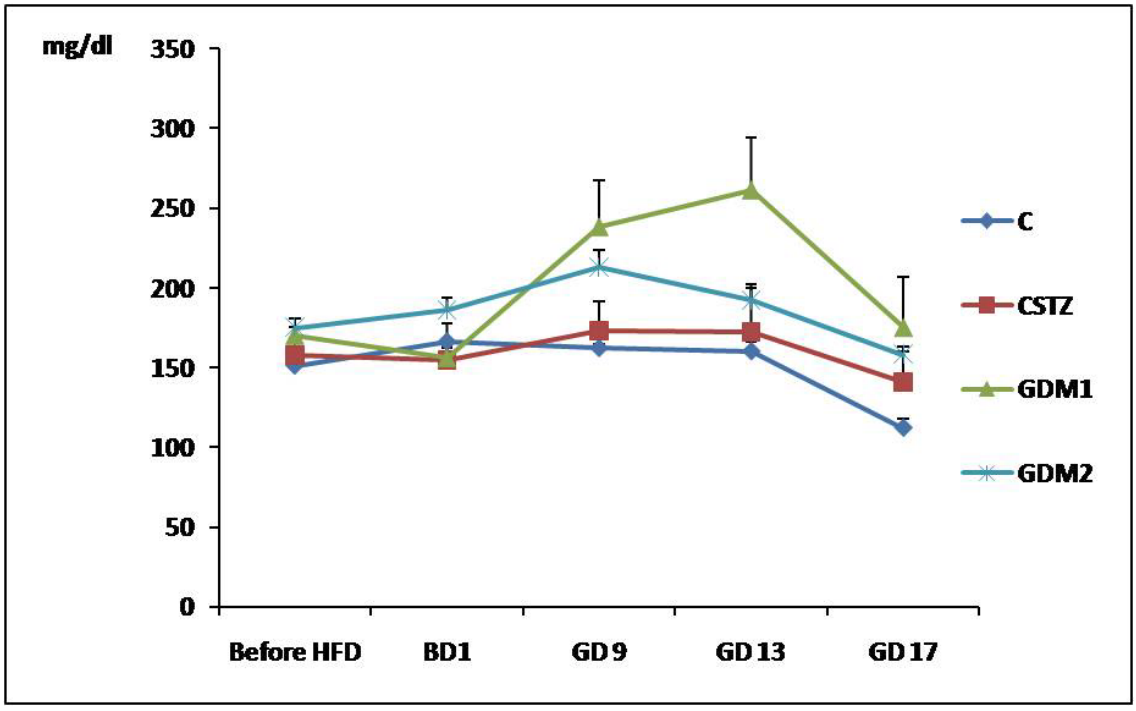

Figure 5.5. Comparison of maternal glucose levels for experiment 3. C: main control group, CSTZ: secondary control group, GDM1: gestational diabetes group 1, GDM2: gestational diabetes group 2, BD breeding day, GD: gestation day.

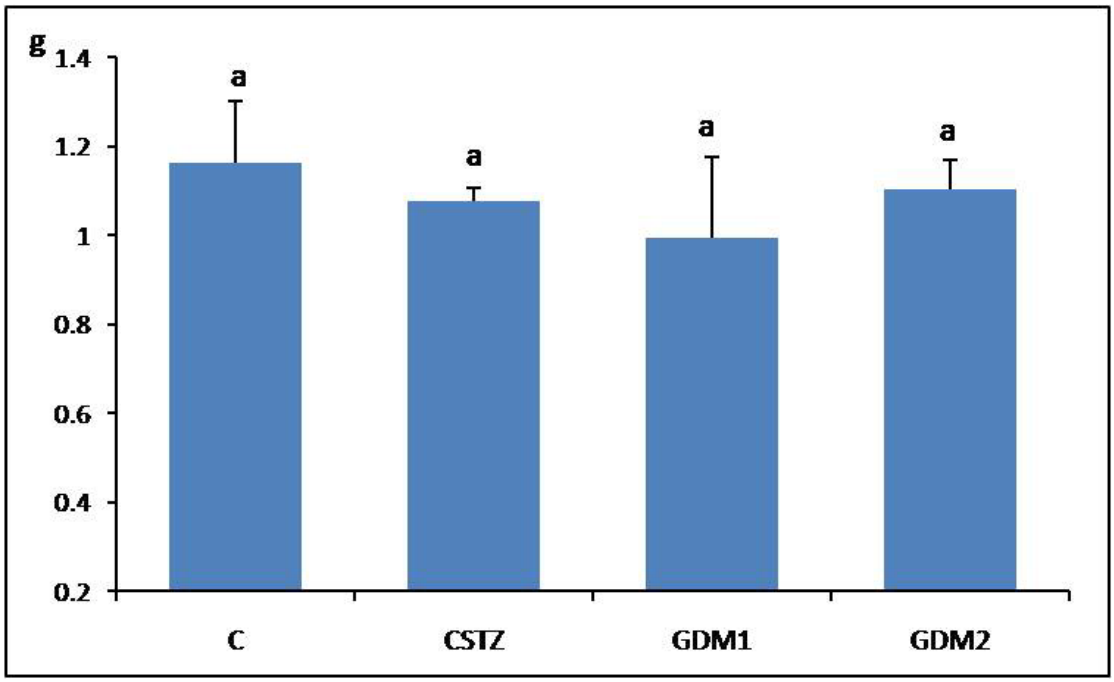

Figure 5.6. Fetal weight at GD 17 for experiment 3. C: control buffer group, CSTZ: control streptozocin group, GDM1: gestational diabetes group 1 and GDM2: gestational diabetes group 2.

Table 5.1. High fat diet composition.

High Fat Diet	gm%	Kcal%
Protein	26.2	20
Carbohydrate	26.3	20
Fat	34.9	60
Fat composition:		%
Saturated		37.1
Monounsaturated		46
Polyunsaturated		16.9

Table 5.2. Maternal plasma insulin levels (ng/ml) and maternal weight (g) for experiment 2. T1: type 1 diabetic group, T2: type 2 diabetic group, C: main control group, CSTZ: secondary control group, BD: breeding day, GD: gestation day and MW: maternal weight. Different letters mean differences among groups.

Parameter/group	N (at GD17)	BD 1	GD 10	GD 17
Insulin/T2	6	1.36 ± 0.4	1.53 ± 0.4	1.2 ± 0.4
Insulin/C	6	0.86 ± 0.03	1.13 ± 0.3	1.12 ± 0.5
Insulin/CSTZ	6	0.77 ± 0.3	1.38 ± 0.5	1.39 ± 0.5
MW/T2	6			53.9 ± 5 [a]
MW/T1	7			40.1 ± 6.1 [b]
MW/C	6			58.5 ± 3.9 [a]
MW/CSTZ	6			59.1 ± 4.8 [a]

Table 5.3. Maternal plasma insulin levels (ng/ml) and maternal weight (g) for experiment 3. HFD: high fat diet, C: main control group, CSTZ: secondary control group, GDM1: gestational diabetes group 1, GDM2: gestational diabetes group 2, HFD: high fat diet, BD: breeding day, GD: gestation day and MW: maternal weight. Different letters mean differences among groups.

Parameter/group	N	Before HFD	BD1	GD 9	GD 13	GD 17
Insulin/ C	4		0.72 ± 0.3 [a]	0.68 ± 0.5 [a]		0.8 ± 0.2 [a]
Insulin/ CSTZ	4		0.7 ± 0.2 [a]	0.75 ± 0.5 [a]		0.9 ± 0.4 [a]
Insulin/ GDM1	6		1.03 ± 0.5 [a]	0.85 ± 0.3 [a]		0.8 ± 0.2 [a]
Insulin/ GDM2	8		1.65 ± 0.5 [b]	1.6 ± 0.6 [b]		1.12 ± 0.4 [a]
MW/C	4	23 ± 1.4	28 ± 3.5	33 ± 2.7	41 ± 5.7	55 ± 8.9
MW/CSTZ	4	23 ± 2.1	28 ± 4.3	33 ± 3.3	43 ± 4	57 ± 7.5
MW/GDM1	12	23 ± 1.1	31 ± 4	34 ± 3.6	40 ± 4.5	50 ± 6.3
MW/GDM2	12	24 ± 1.6	35 ± 3.6	37 ± 3.6	44 ± 4.2	56 ± 7

CHAPTER 6: INCREASED MYOCARDYAL APOPTOSIS IN 17-DAY-OLD FETUSES OF DIABETIC CD1 MICE: PARADOXICAL UPREGULATION OF ANTI-APOPTOTIC GENE EXPRESSION

J. Claudio Gutierrez [1,3], M. Renee Prater [1,2], Bonnie J. Smith [1], Larry E. Freeman,[1] and Steven D. Holladay [4]

[1] Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute & State University, Blacksburg, Virginia, USA. [2] Department of Biomedical Sciences, E. Via Virginia College of Osteopathic Medicine, Blacksburg, Virginia, USA. [3] Instituto de Farmacología y Morfofisiología, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile. [4] Department of Anatomy and Radiology, University of Georgia, Athens, Georgia, USA.

6.1: ABSTRACT

Maternal diabetes mellitus is associated with increased fetal teratogenesis, including cardiovascular defects. Previous work in our laboratory revealed that insulin-dependent maternal diabetes caused dilation of late-gestation fetal ventricular chambers and reduction of total ventricular myocardium. Maternal treatment with complete Freund's adjuvant (FCA) reduced the severity of these fetal heart lesions. The following experiments were performed to analyze maternal diabetes-induced changes in apoptotic cell death in the late gestation fetal heart. Experiment 1: Female CD1 mice were injected intraperitoneally (IP) with 200 mg/kg of streptozocin (STZ) to induce insulin-dependent (type 1) diabetes. Half of these females were immune stimulated with FCA prior to the STZ injection. Females were bred one week after the STZ injection. At gestation day (GD) 17, females were euthanized and fetal hearts were collected. Fetal myocardial cells were suspended and examined for level of apoptosis by flow cytometry. Early apoptotic cells and late apoptotic/necrotic cells were both significantly increased ($p < .05$) in fetal hearts from diabetic mothers compared to controls. Experiment 2: Female CD1 mice

were injected with STZ and FCA as in Experiment 1, to induce diabetes. One week after the STZ injection, females were bred. Fetal hearts were collected on GD 17 and a panel of apoptosis regulatory genes (Bcl-2, p53, Casp3, Casp9, PkC-e) was examined using RT-PCR. Expression of pro-apoptotic genes Casp3 and Casp9 was decreased by diabetes, while the anti-apoptotic gene Bcl-2 was increased. FCA did not change the pattern of fetal heart gene expression. Thus, while flow cytometry of fetal myocardial cells indicated enhanced cell death as a contributor to the observed heart defects, the selected panel of genes paradoxically indicated reduced myocardial apoptosis.

Key words: Bcl-2, apoptosis, fetal heart, mouse, hyperglycemia, diabetes

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6.2: INTRODUCTION

Cardiovascular complication is one of the most common causes of morbidity and mortality in diabetic patients (reviewed by Adeghate, 2004). Maternal hyperglycemia is an inducer of teratogenesis with a high incidence of cardiovascular malformations. Heart defects occur in excess in infants of diabetic mothers and represent a segment of anomalies that could be prevented (Becerra et al, 1990; Ferencz et al, 1990). Epidemiological evidence clearly shows that pre-conceptional maternal diabetes, adversely affects the early development of the heart. This includes major anomalies of cardiovisceral and atrioventricular concordance, as well as defects of the cardiac outflow tract and atrioventricular valves (Loffredo et al, 2001). Maternal diabetes is an independent factor for cardiovascular malformations and major vulnerability occurs in early pregnancy (Reece et al, 1996).

Studies in our laboratory have revealed that cavitary dilation and myocardial reduction are observed in late gestation fetal hearts from highly hyperglycemic females using a mouse model (Gutierrez et al., 2007). According to the literature, the rate of myocardial apoptosis may increase in a hyperglycemic environment (Fiordaliso et al., 2001; Cai et al., 2002; Frustaci et al., 2000). Studies by Frustaci et al. (2000) reveal that apoptosis and necrosis is increased in myocytes, endothelial cells and fibroblasts of the human adult diabetic heart. However, in other studies, positive effects on the myocardial

cell have been reported as a result of a chronic exposure to a hyperglycemic insult (Shaffer et al., 2000; Ricci et al., 2008).

Diverse forms of maternal immune stimulation in rodents offer protection against birth malformations caused by chemical teratogens and maternal diabetes (Nomura et al., 1990; Holladay et al., 2000; Sharova et al., 2000; Sharova et al., 2002; Prater et al., 2004; Punareewattana and Holladay, 2004; Hrubec et al., 2006). This protection has been associated with improved regulation of apoptosis in fetal target tissues of the teratogens (Sharova et al., 2000). In our laboratory, maternal treatment with FCA also improved diabetes-related fetal heart structural changes of cavitary dilation and myocardial reduction (Gutierrez et al., 2009 in press). We therefore hypothesized that dysregulation of apoptosis may be in part responsible for myocardial changes in late gestation fetal mouse hearts during the course of the diabetic pregnancy. We also hypothesized that alleviation of such changes by maternal treatment with FCA may be in part related to normalization of fetal myocardial apoptosis. A classic mouse model of insulin dependent diabetes was used to analyze myocardial apoptosis by flow cytometry, and by RT-PCR analysis of a panel of 5 genes involved directly in apoptosis.

6.3: MATERIALS AND METHODS

6.3.1: Experiment 1: Flow Cytometric Determination of Myocardial Apoptosis in 17-day-old Fetal Hearts

Six- to seven-week old CD1 female mice (Charles River) were used in these experiments. Mice were housed 5 per cage for a 1-week acclimation period. Mice were fed a standard rodent diet (SD, Harlan Teklad Global Diet 2018: 18% protein and 5 % fat, Madison, WI). Tap water was given ad libitum. Mice were maintained under controlled conditions of temperature (22 °C), humidity (40-60 %) and lighting (12/12 hour light/dark cycle). For breeding, males were housed overnight with females, and females checked for vaginal plugs the next morning, which was designated day 0 of gestation (GD 0). Mice were divided into the following treatment groups in a randomized complete block design:

Control group (C group): Female mice injected intraperitoneally (IP) with a citrate buffer (0.05 M, pH: 4.5) served as the control group (n = 6).

Diabetic group (STZ group): Diabetes was induced in female mice using the classical IP injection of STZ. Females were injected with 200 mg/kg STZ (Sigma, St. Louis, MO) dissolved in a citrate buffer (0.05 M, pH: 4.5) 7 days before breeding (Punareewattana and Holladay, 2004). Blood glucose (BG) levels in tail vein blood were determined every 3-5 days (as determined by Accuchek compact blood glucose monitoring following tail venipuncture) (n = 6).

Immune stimulation using Freund's complete adjuvant (STZ+FCA group): Diabetic females were injected twice (30 µl dose IP) with FCA, first at 1 week and again at 1 day before STZ administration (n =3).

At GD 17, females from all groups were euthanized and fetal hearts (5 per litter) were collected arbitrarily by micro-dissection (Figure 6.1) using an Olympus Zoom Stereo Microscope SZX7 (Olympus America Inc., Melville, NY). Hearts were collected in DMEM media with 10% fetal bovine serum at 37 °C, after removing left and right auricles to remove blood in the ventricles. Next, hearts were removed from the media and placed in 1ml solution of enzyme dispase II (Roche Applied Sciences, Indianapolis, IN) for 30 minutes incubation at 37 °C. Propidium iodide (35 µl) and annexin V (5 µl) were used as cell markers for detection of necrosis/late apoptosis and early apoptosis respectively. Samples were analyzed by flow cytometry (FACS Aria, BD Biosciences, Franklin Lakes, NJ). Annexin V is a 35-36 kDa Ca2+ dependent phospholipid-binding protein that has a high affinity for phospholipid-like phosphatidylserine (PS) exposed on cell surfaces during apoptosis, and binds to cells with exposed PS. Propidium Iodide (PI) binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA. PI is membrane impermeant and generally excluded from viable cells.

Statistical analysis

JMP software from the SAS family was used to run ANOVA. When a significant difference was observed ($p < .05$), a Tukey's statistical test was used to further analyze differences among groups.

6.3.2: Experiment 2: Expression of a Panel of Apoptosis/Proliferation Related Genes in Late Fetal Hearts of Insulin-dependent Diabetic Mothers.

Six- to seven-week old CD1 female mice were used in these experiments. Husbandry and breeding conditions were the same as for experiment 1. Experimental groups:

Control group (C group): Female mice were injected IP with a citrate buffer (0.05 M, pH: 4.5) and served as the control group (n = 6).

Diabetic group (STZ group): Diabetes was induced in female mice as in Experiment 1 (n = 6).

Immune stimulation using Freund's complete adjuvant (STZ+FCA group): Immune stimulation with FCA was also the same as in Experiment 1 (n = 6).

Blood glucose levels were determined in all the experiments by Accuchek compact blood glucose glucometer (Roche laboratories, distributed by www.americandiabeteswholesale.com) following tail venipuncture.

Extraction of RNA

Seventeen day-old fetal hearts were collected by micro-dissection (Figure 6.1) under sterile conditions. During heart micro-dissection, left and right auricles and the origin of the great vessels were removed. The ventricles were placed in sterile saline for 1 to 2 minutes and then placed in RNA later and stored at – 20 °C for later analysis. The RNeasy fibrous tissue mini kit was used to extract RNA (Qiagen, Valencia, CA). Hearts were removed from RNAlater and weighed to appropriately determine an amount of 20-30 mg of tissue (6-7 hearts per litter). Tissue was placed in a collection tube and 400 uL of β-mercaptoethanol + RLT buffer were added. Hearts were immediately homogenized in the collection tube using a tissue disruptor (Qiagen, Valencia, CA) for 1 minute. RNase free water and 10 ul of proteinase K were added to the mix for a better disruption of the heart tissue. Mini Spin Columns were used according to the protocol in the next steps. Incubation of the sample with DNase I for 15 minutes at room temperature was also a part of the RNA extraction protocol. Samples of RNA (30 ul of RNA) were stored at – 80 °C for posterior analysis. RNA quality and quantity were read at a Biophotometer (Eppendorf, Westbury, NY)

The script cDNA synthesis kit from Bio-rad (Hercules, CA) was used in these experiments from 1 μg of RNA in each sample.

RT-PCR and primer design

Primers were designed using software Beacon designer and sequences submitted to Invitrogen (Carlsbad, CA) for elaboration (primer sequences are shown in Table 6.1). Hearts were analyzed by real time polymerase chain reaction (RT-PCR) for quantification of a preliminary panel of apoptosis regulatory genes (Bcl-2, P53, Casp3, Casp9 and PkC-e) using the SYBRgreen supermix from Bio-rad (Hercules, CA).

Statistical analysis

Statistical software JMP from the SAS family was used to run one-way ANOVA to detect differences among groups. When a significant difference was observed ($p < .05$), a Tukey's statistical test was used to further analyze differences among groups. JMP software was also used to run principal component analysis (PCA) to analyze cluster changes in gene expression. The PCA test is designed for condensation of raw data, generated from numerous genes, to a coordinately meaningful and more readily interpretable set of parameters. PCA of the correlation matrix of gene expression was used for visualization of gene expression change under the different treatments. Next, ANOVA of PCA scores was used to determine which of the principal components were affected by treatment (Sharova et al., 2000).

The raw data used to run ANOVA and PCA was the output obtained from the delta CT (ΔCT) equation of gene expression. However, all the charts showing expression of individual genes or ratio of gene expression have been created using the delta-delta CT (ΔΔCT) output to have a clearer idea of up or down gene regulation compared to the main control group.

All procedures involving mice were reviewed by and conducted in compliance with the guidelines of the Virginia Tech Animal Care and Use Committee at the VA-MD Regional College of Veterinary Medicine.

6.4: RESULTS

Experiment 1 diabetic groups showed high levels of hyperglycemia during the whole pregnancy (> 450 mg/dl, data not shown). There was a significant increase in percentage of early apoptotic cells in the fetal myocardium from STZ and STZ + FCA groups compared to the C group (Figure 6.2), detected by cell markers annexin V and propidium iodide. There was also a significant increase in the percentage of late apoptosis/necrosis GD 17 fetal myocardial cells in the STZ group compared to the C group. However, fetal heart samples from diabetic females treated with FCA did not show a difference with the C group in percentage of late apoptotic/necrotic myocardial cells suggesting some protection was rendered by FCA (Figure 6.3). Representative charts from the flow cytometric analysis are shown in Figure 6.4.

Experiment 2 examined gene expression in fetal myocardial tissue. Results by ANOVA show a subtle difference in gene expression for all genes except Bcl2. The level of expression in late gestation fetal myocardial tissue for Bcl2 was significantly up-regulated in both hyperglycemic groups (STZ and STZ + FCA, Figure 6.5). The high expression of Bcl2 in the STZ and STZ + FCA groups was corroborated by ANOVA of gene expression ratio (data not shown). The expression of pro-apoptotic gene P53 did not show significant differences between the diabetic groups and the control group (Figure 6.5). The expression of pro-apoptotic gene effector Casp3 was significantly reduced in the STZ + FCA group when compared with the control group but did not differ from the STZ group (Figure 6.5). The expression of pro-apoptotic gene activator Casp9 was significantly reduced in the STZ group when compared to the control group but did not differ from the STZ + FCA group (Figure 6.5). The expression of cardio-protective anti-apoptotic gene PkC-e demonstrated no difference among groups (Figure 6.5). Maternal glucose levels at GD17 are shown in Figure 6.8.

Principal Component Analysis (PCA) for Experiment 2.

The results of PCA correlate well with the analysis of individual gene expression and gene ratio. Three principal components (PC1-3) explained 89% of the variation in gene expression due to the different treatments (Table 6.2). The first principal component (PC1) represents the average level of expression for all genes but is biased slightly

towards P53, Casp3 and Casp9 and their ratio to Bcl-2. PC2 evidences mostly Bcl-2 with a high representation and PkC-e with a lower representation and their ratio to Casp3 and 9. PC3 represents mostly gene P53. ANOVA indicated that treatment affected PC1 and PC2. PC1 and PC2 explained 70% of the variation in gene expression due to the different treatments (Table 6.2). A scatter plot of PC1 and PC2 was created to visualize the effects of the different treatments (Figure 6.7). The plot shows a clear effect of maternal hyperglycemia in relation to PC1 and PC2 axis. The diabetic groups tended to be shifted to the right along the PC1 axis. This is a reflection of the dramatic up-regulation of Bcl-2 in the diabetic groups. The diabetic groups also tended to be more shifted along the PC2 axis when compared to the control group. Particularly, the STZ + FCA group was considerably shifted to the bottom of the PC2 axis. Again this is a reflection of the up-regulation of gene Bcl2 in the diabetic groups and a tendency for a higher gene ratio of PkC-e in the STZ + FCA group (Figure 6.6).

6.5: DISCUSSION

In a previous report, we have studied the late gestation fetal heart by morphometric analysis and determined that cavitary dilation and myocardial reduction is a feature in fetuses from diabetic mothers (Gutierrez et al., 2007). We hypothesized that such changes may be in part related to increased fetal myocardial apoptosis in the course of the diabetic pregnancy. Results from the flow cytometric analysis revealed that late gestation fetal hearts from highly hyperglycemic mothers show increased number of early and late apoptotic and necrotic myocardial cells when compared with fetal hearts from control mothers. These results supported our hypothesis regarding the origins of fetal heart cavitary dilation and myocardial reduction in the diabetic pregnancy. The literature describes increased cardiac apoptosis as a result of a hyperglycemic insult (Fiordaliso et al., 2001; Frustaci et al., 2000; Cai et al., 2002;). Studies by Cai et al. (2002) describe increased myocardial apoptosis in adult diabetic hearts from FVB mice (>12 mM/L). The authors describe structural abnormalities and TUNEL-assay-detected apoptotic cells in the diabetic hearts compared to controls. The same authors also describe increased apoptosis (in vitro) using cardiac myoblast H9c2 cells exposed to high levels of glucose (22-33 mM/L). The authors propose that hyperglycemia-induced apoptosis is in part

mediated by the activation of the cytochrome c release-caspase 3 pathway triggered by increased reactive oxygen species (ROS). In other studies, Frustaci et al. (2000) detected increased apoptosis and necrosis in myocytes, endothelial cells and fibroblasts of adult human hearts of diabetic and diabetic/hypertensive patients. These authors proposed that local increases in angiotensin II with diabetes, and diabetes and hypertension, may enhance oxidative stress, activating cardiac cell apoptosis and necrosis.

The present flow cytometric results suggest that maternal treatment with FCA may improve the harmful effects of hyperglycemia on the fetal myocardium by reducing the percentage of late apoptotic/necrotic cells. These results correlate well with previous work at our laboratory regarding maternal immune stimulation with FCA and improvement of fetal heart cavitary dilation and myocardial reduction in the diabetic pregnancy (Gutierrez et al., in press, 2009). Reduction rate of late apoptosis/necrosis by FCA treatment may explain in part the structural improvement of the fetal heart from such changes.

Gene analysis experiments revealed changes in gene expression in the fetal heart induced by maternal hyperglycemia. There was an unexpected and dramatic up-regulation of the anti-apoptotic gene Bcl-2 in both hyperglycemic groups (STZ and STZ + FCA). The highest expression of Bcl-2 was detected in the STZ + FCA group and can be seen in the PC2 axis of the PCA. This finding appeared paradoxical considering results by flow cytometric analysis revealing increased fetal myocardial apoptosis and necrosis under the same levels of hyperglycemia. However, the literature supports upregulation of Bcl-2 under high hyperglycemia conditions. Schaffer et al. (2000) used neonatal Wistar rat cardiomyocytes exposed for 3 days to a highly hyperglycemic media (25 mM/L), and showed an up-regulation of anti-apoptotic factor Bcl-2. In those studies, two pro-apoptotic factors, Bax and Bad, remained unaltered under the same hyperglycemic conditions. However Bad was shifted in favor of its inactive form under hyperglycemia. Ricci et al. (2008) similarly found that myocytes from newborn and adult rats over-expressed Bcl-2 and Akt factor under chronic exposure to a highly hyperglycemic media. Bojunga et al. (2004) also showed increased levels of anti-apoptotic factor Bcl-2 in the heart of adult diabetic rats (26 mM/L) compared to controls.

In the same study pro-apoptotic genes Bax and Bak showed increased levels under the same hyperglycemic levels.

The present hyperglycemic groups also showed lower expression of pro-apoptotic factors Casp3 (in the STZ + FCA group) and 9 (in the STZ group) when compared to control. Studies by Bojunga et al. (2004) describe increased activity of Casp9 but not of Casp3 in the diabetic heart of adult rats. The gene analysis also revealed a tendency toward a higher gene ratio of PkC-e/Caspase3 in the STZ + FCA group when compared with the control group. This ratio of PkC-e versus pro-apoptotic factors is shown in the PC2 axis of the PCA and may be a positive effect of maternal immune-stimulation with FCA. Malhotra et al. (2004) proposed that activation of PkC-e protects cell cultures of adult rat ventricular myocytes from hyperglycemia-induced death signal. In other studies, Malhotra et al. (2005) demonstrate in vivo that PkC-e has a cardio-protective effect in the myocardium of diabetic rats. In those studies, diabetic rats showing increased PKC-e activity have reduced levels of markers of oxidative stress and protection from hyperglycemia-induced apoptosis.

In conclusion, high levels of maternal hyperglycemia increased fetal myocardial apoptosis, evidenced by flow cytometry of myocardial cells. An unexpected up-regulation of anti-apoptotic gene Bcl-2 in late gestation was simultaneously present, as was down-regulation of Casp3 and Casp9. These gene expression results suggest the fetal myocardial cells were actively upregulating the mitochondrial anti-apoptotic pathway. Bcl2 among other functions has a positive impact in mitochondrial cell death protection, preventing the releasing of cytochrome-c and interfering with protein Apaf1, blocking the formation of the apoptosome and in turn mitochondrial mediated apoptotic pathway activation (reviewed by Reed, 1998). Thus, the present increased Bcl-2 and subsequent decreased Casp3 and Casp9, may represent an internal attempt by the cell to overcome external apoptotic pathways (e.g., FAS- or TNFα-mediated). Additional experiments will be required to analyze a broader panel of gene expression in fetal myocardial cells in the diabetic pregnancy, to understand mechanisms leading to increased death of these cells.

6.6: ACKNOWLEDGMENTS

The authors acknowledge Dr. Wen Li for her support with the RT-PCR protocols and, Melissa Makris and Dr. Murali Mallela for their support with the flow cytometric analysis. Supported by NIH # R21-PAR-03-121, NIH # K01RR017018 and Harvey Peters Foundation.

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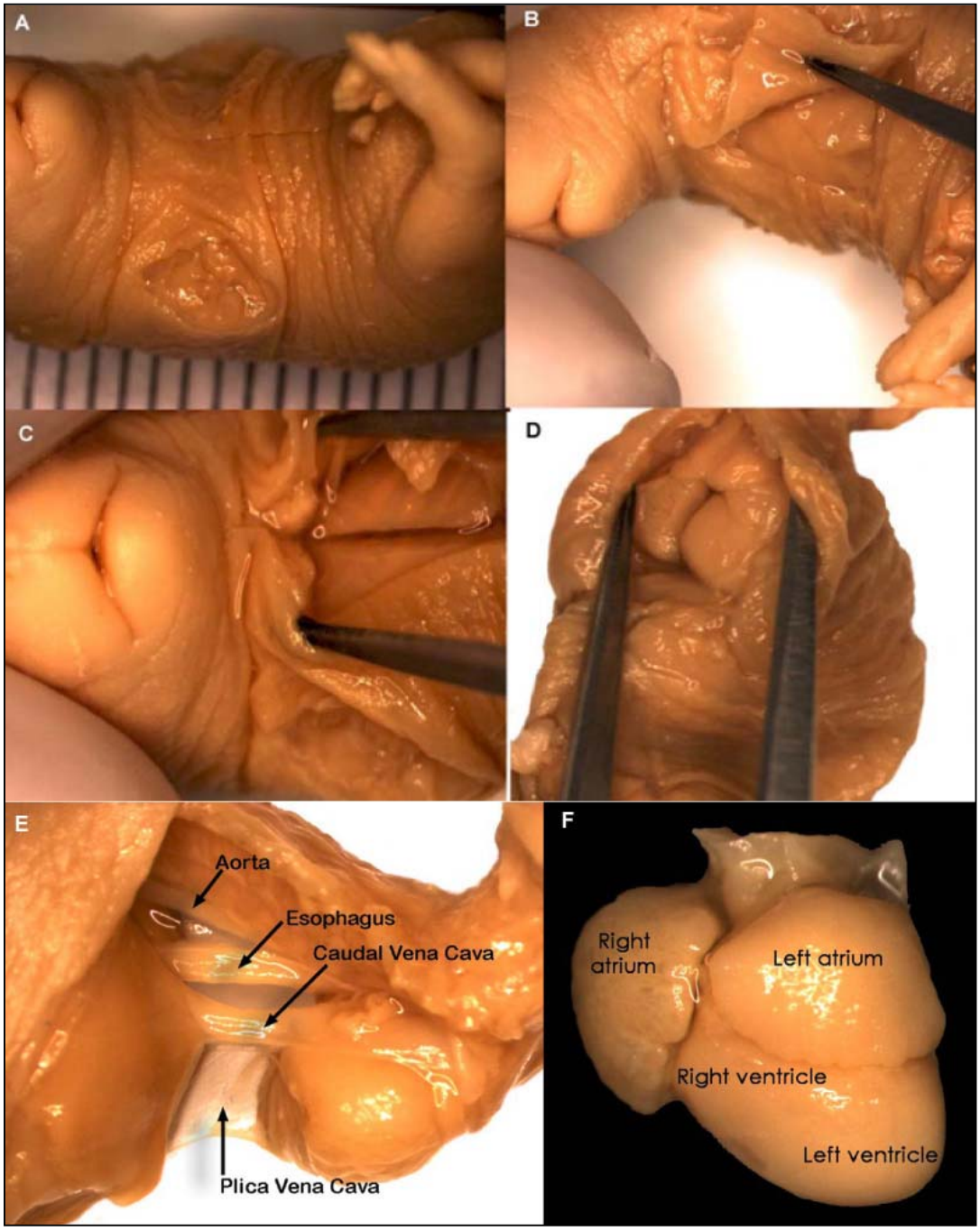

Figure 6.1. Demonstration of micro-dissection of a 17-day-old control fetus (performed on a fixed fetus to clarify the micro-dissection technique). A: first medial incision (each line represents one millimeter), B-C: accessing the rib cage, D: accessing the fetal heart for extraction, E: right thorax view and F: heart isolated.

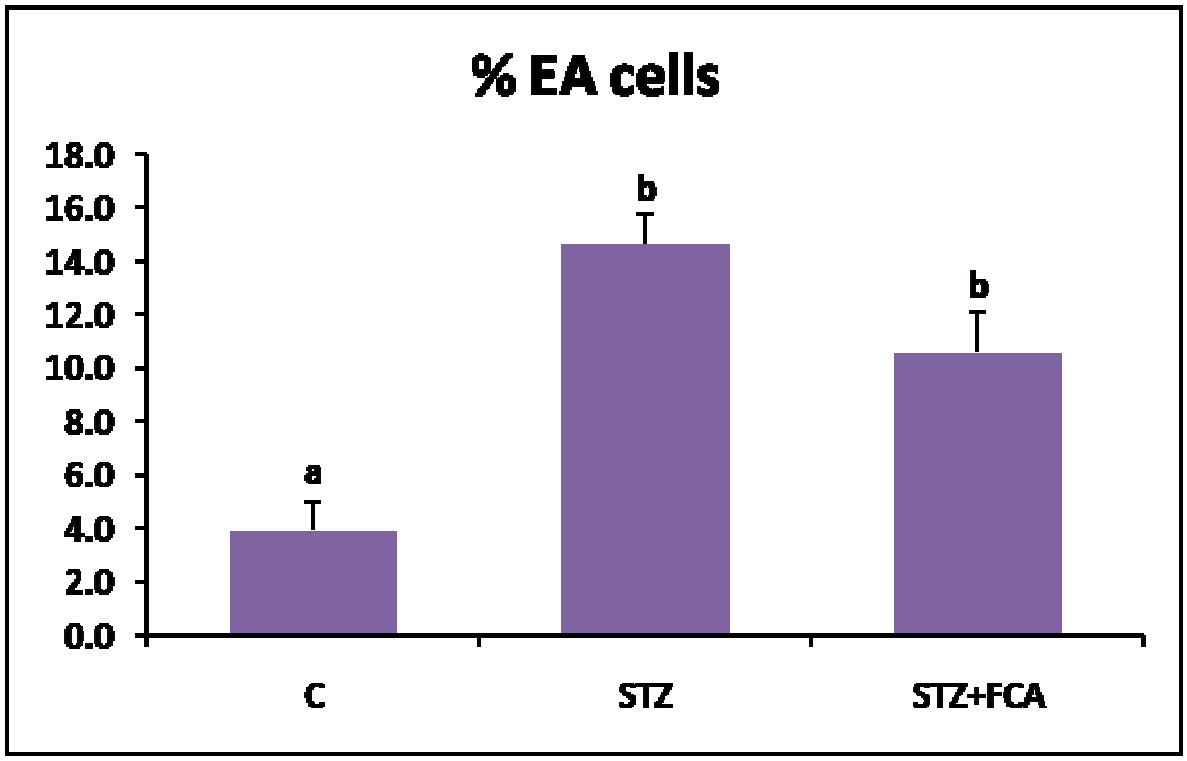

Figure 6.2. Percentage of early apoptotic myocardial cells. There was a significant increase (p < .01) in late apoptotic-necrotic cells in the STZ group (n = 6) and STZ+FCA group (n = 3) compared to the C group (n = 6).

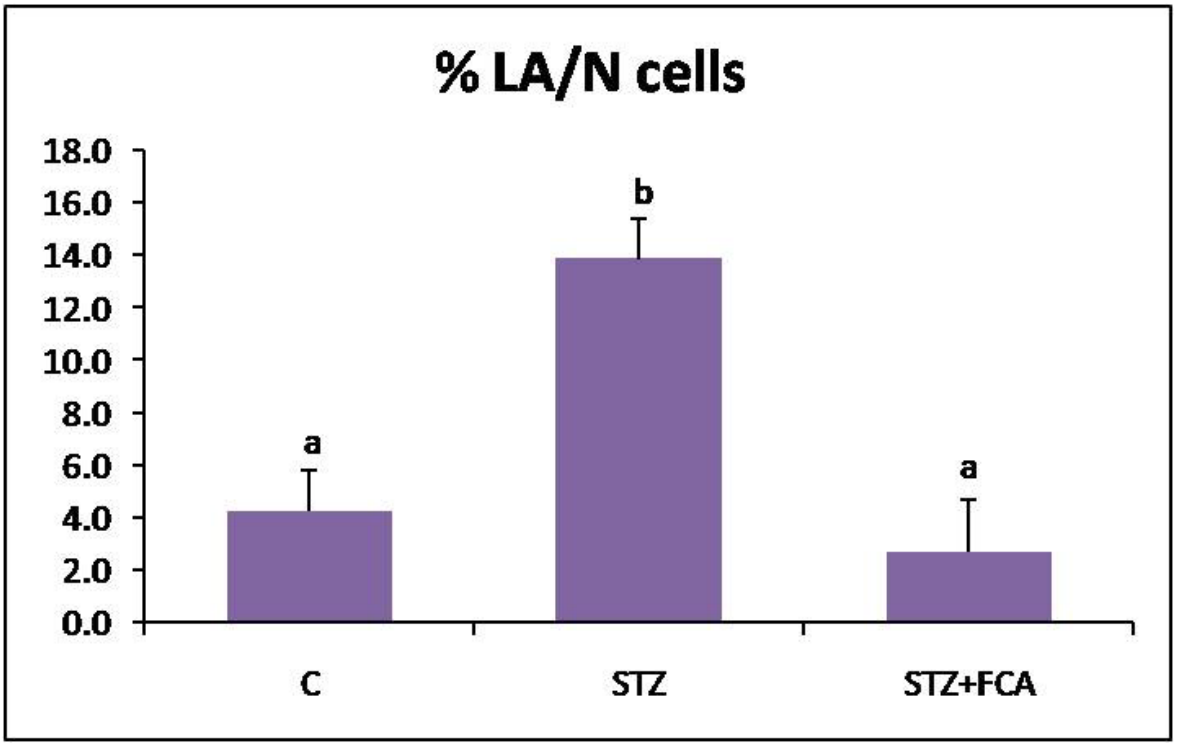

Figure 6.3. Percentage of late apoptotic-necrotic myocardial cells. There was a significant increase ($p < .01$) in late apoptotic-necrotic cells in the STZ group (n = 6) compared to the C group (n = 6) and STZ+FCA group (n = 3).

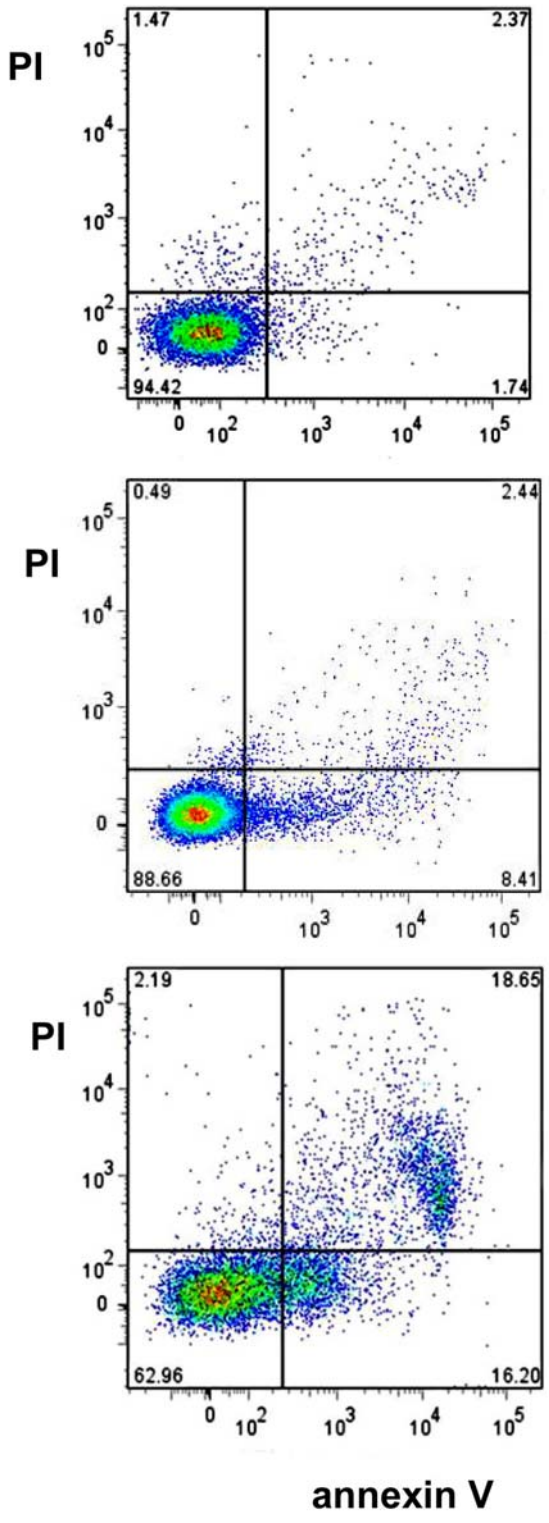

Figure 6.4. Fetal myocardial apoptosis determined by flow cytometry. Top: sample from the C group, middle: sample from STZ+FCA group and bottom: sample from the STZ group. PI: propidium iodide.

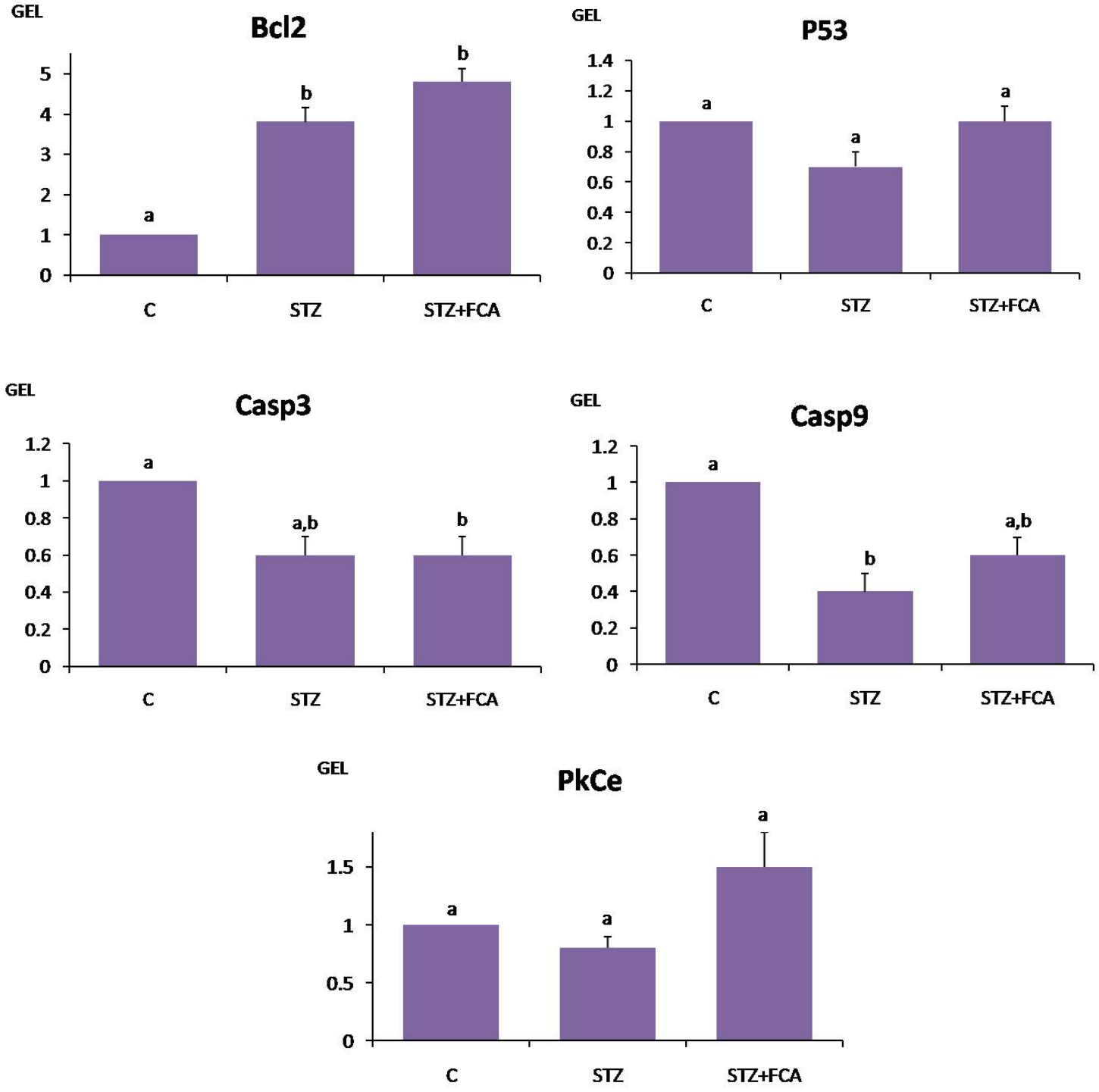

Figure 6.5. ANOVA results for gene expression in experiment 2. The graphs were created using the ΔΔCT output. GEL: gene expression level, C: control group, CSTZ: secondary control group, STZ: diabetic group, STZ+FCA: diabetic group treated with FCA. Different letters mean difference among groups.

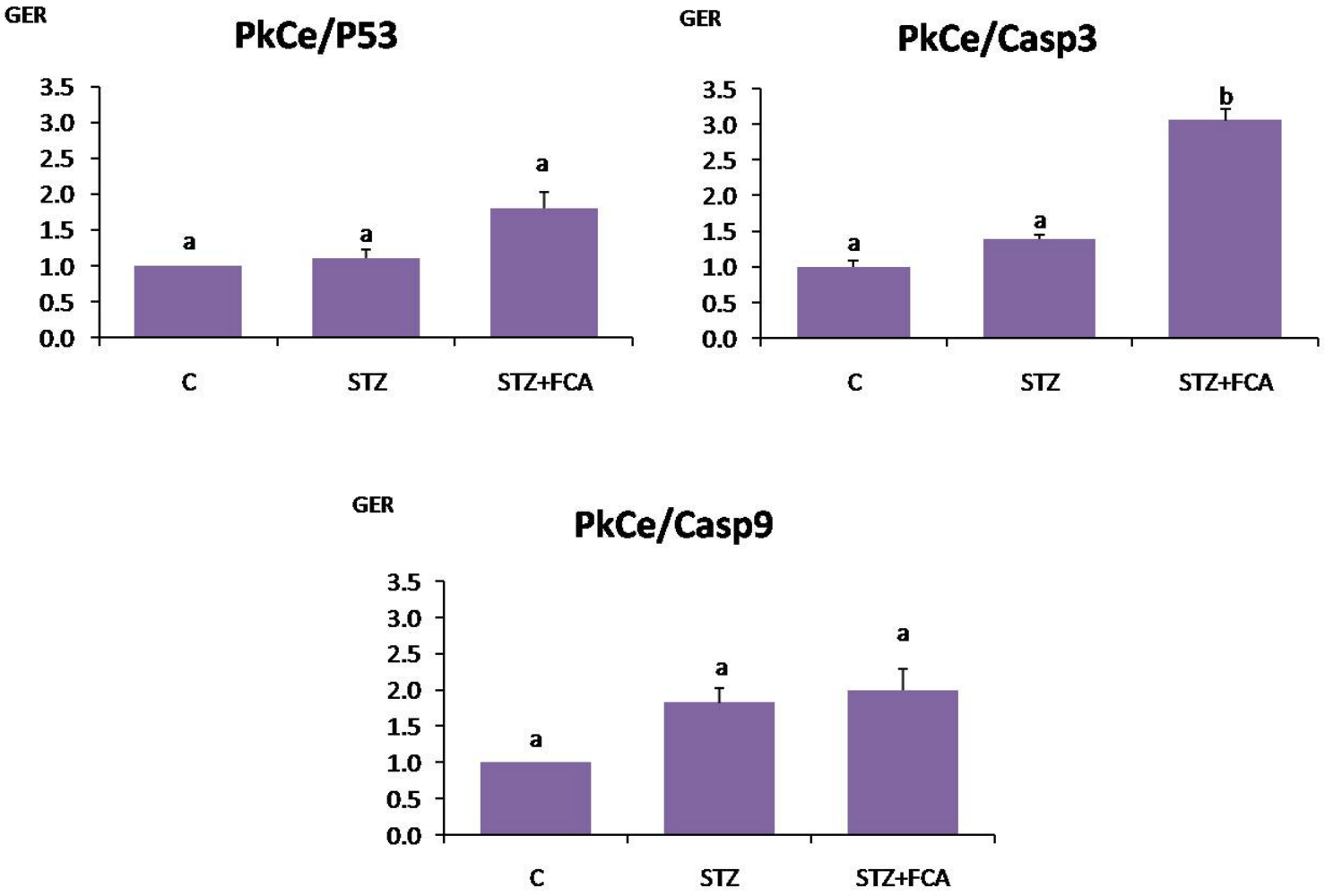

Figure 6.6. ANOVA results for gene expression ratio of PkC-e in experiment 2. The graphs were created using the ΔΔCT output. GEL: gene expression level, C: control group, STZ: type 1 diabetic group, STZ+FCA: type 1 diabetic group treated with FCA. Different letters mean difference among groups.

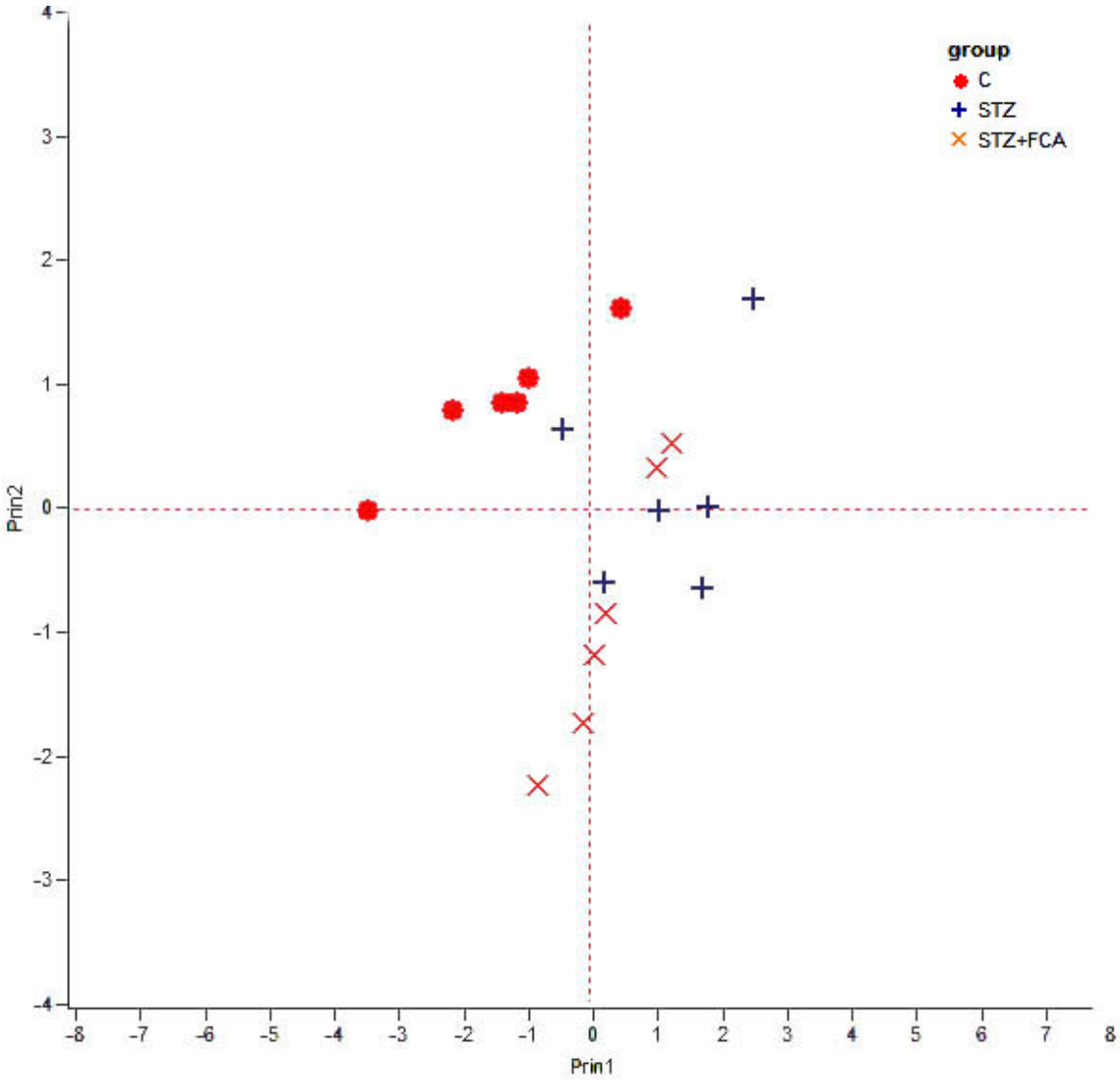

Figure 6.7. Principal component analysis (PCA) of the effect of maternal diabetes and treatment with FCA on fetal myocardial gene expression in experiment 2. PC1 and PC2 representing the first and second principal components are shown. C: control group, STZ: diabetic group, STZ+FCA: diabetic group treated with FCA.

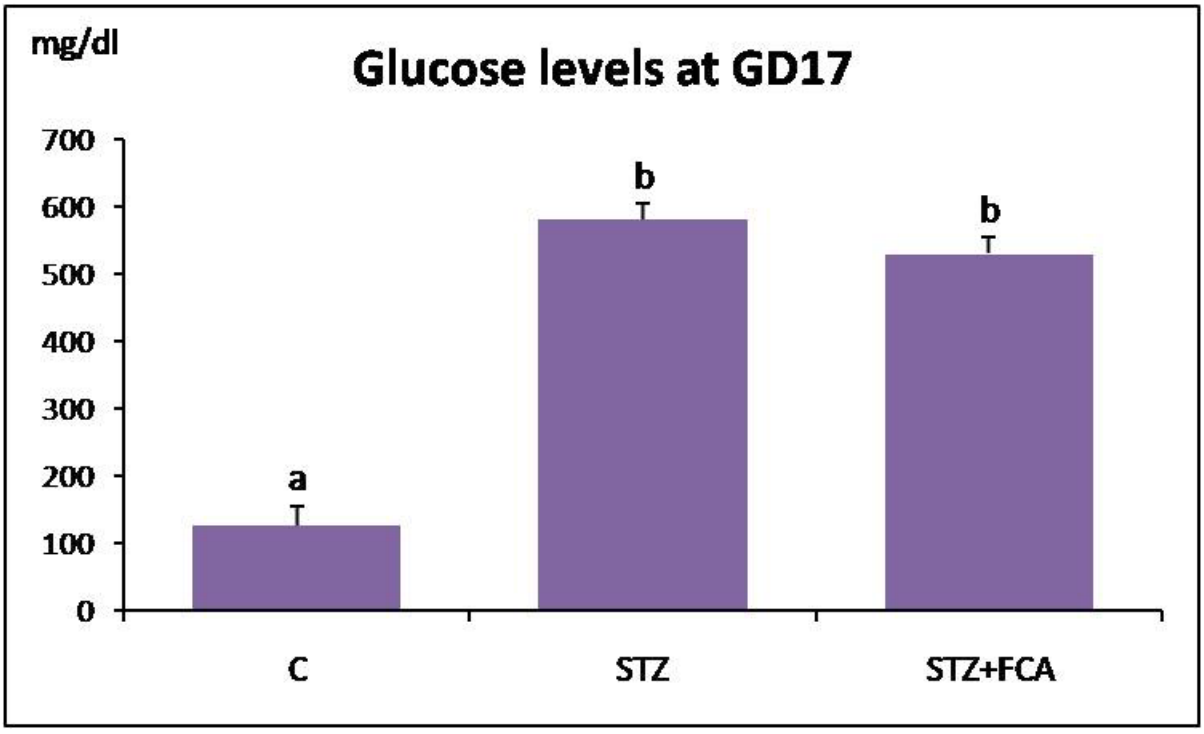

Figure 6.8. Maternal glucose levels for experiment 2 at GD17. C: control group, STZ: diabetic group, STZ+FCA: diabetic group treated with FCA. Different letters mean difference among groups.

Table 6.1. Target genes and primer sequences used in experiments 2.

Gene	Gene function	Primer 5'-3' (forward and reverse)
Bcl2	Anti-apoptotic gene, prevents releasing of cytochrome c	CCT TGG CGT GTC TCT CTG TCC TGT GAT TCT CCC TTC TTC
P53	Pro-apoptotic gene	ATG AAC CGC CGA CCT ATC C CAC AAA CAC GAA CCT CAA AGC
Caspase3	Pro-apoptotic gene, effector caspase	GAC TTC CTG TAT GCT TAC TCT AC ATT CCG TTG CCA CCT TCC
Caspase9	Pro-apoptotic gene, activator caspase	GGT GGA GGT GAA GAA CGA C TCA ATG GAC ACG GAG CAT C
PkCe	Anti-apoptotic cardio-protector gene	ACC TCC CCT TGT GAC CAG TGC TCC TCT CCT CGG TTG
B-actin	Housekeeping gene	CGT GCG TGA CAT CAA AGA G TGC CAC AGG ATT CCA TAC C

Table 6.2: Treatment alters gene expression in experiment 2, PCA analysis.

Genes	PC1	PC2	PC3	PC4	PC5
Bcl2	-0.2030	0.8176	0.0817	0.0198	0.5321
P53	0.41631	0.2446	0.7022	0.3979	-0.3396
Casp3	0.54470	-0.0356	0.2431	-0.7606	0.2535
Casp9	0.57505	-0.1894	-0.3003	0.5039	0.5378
PkCe	0.39758	0.4842	-0.5922	-0.0932	-0.4978
Eigenvalue	2.196	1.327	0.921	0.498	0.055
Cumulative % of variation explained	43.939	70.486	88.916	98.885	100
ANOVA probability that treatment had no effect (*p* value)	$P < 0.01$*	$p < 0.05$*	$p < 0.95$	$p < 0.059$	p: 0.72

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

The incidence of malformed newborns is higher in human pregnancies complicated by diabetes mellitus, as compared to non-diabetic pregnancies. Neural tube and cardiac defects predominate among the fetal malformations related to hyperglycemia. Heart defects in particular have been reported as increasing up to five-fold among infants of diabetic mothers compared to the general population, representing a significant segment of anomalies that might be reduced with gestational control of maternal blood sugar levels. Non-specific maternal immune stimulation is protective in mice against birth malformations caused by chemical teratogens or by maternal diabetes mellitus.

This study was designed to evaluate the hypotheses that 1) maternal hyperglycemia alters the morphology of the late gestation fetal heart using a mouse model of insulin-dependent diabetes; 2) maternal immune treatment may positively affect the course of maternal hyperglycemia and improve fetal heart development; 3) maternal diabetes dysregulates fetal myocardial apoptosis during the course of gestation; and 4) the use of the combination of high fat diet and low dose of STZ could be an alternative to model insulin resistant maternal diabetes for the study of fetal development as has been used previously in adult males. The experiments made use of a classical model of insulin dependent-diabetes induced by STZ (ICR/CD1 mice). Highly hyperglycemic females were used in the experiments to study the harmful effects of hyperglycemia in 17-day-old fetal hearts.

The morphometric analysis revealed visibly obvious dilation of ventricular chambers and outflow channel of the left ventricle, and reduction of total myocardial ventricular area in late gestation fetuses from diabetic dams, as the predominant changes seen in the offspring of diabetic dams. Fetal mice showing external defects (exencephaly and/or spina bifida) displayed a similar profile of cardiac changes to fetuses without external defects. Left and right ventricular chamber area was again increased in the fetal mice from diabetic dams, while total ventricular and septal myocardial area was decreased. Additional cardiac defects were seen in these mice that were not seen in the first group of fetal mice, including dextra-position of the aorta in a fetus with

exencephaly, double caudal vena cava in a second fetus with exencephaly, and an interventricular septal defect in a fetus that displayed both exencephaly and spina bifida.

In a second experiment the same model of diabetes was used to test 2 different immune stimulants in insulin dependent diabetic ICR females: FCA and IFNγ. The morphometric analysis revealed that protection was offered by FCA and IFNγ against myocardial reduction and dilation of the left out-flow channel. The diabetic group without the immune stimulation treatment showed a significant increase in ascending thoracic aortic transversal area compared to the other groups. Left and right mean ventricle chamber areas were both approximately 2.5 times larger in fetal mice from diabetic dams without additional treatment, as compared to corresponding chambers from non-diabetic dams. Particularly, there was no statistical difference in left and right ventricular chamber area between the control and the diabetic group treated with FCA. Nevertheless, there was also no statistical difference among the FCA group, the diabetic group and the IFNγ group. Pathologic scoring revealed that FCA was more beneficial than IFNγ in improving fetal heart cavitary dilation and myocardial reduction.

We developed another experiment to produce insulin resistant (type 2 diabetic) CD1 females using the combination of high fat diet (HFD) and low dose of STZ. The results of our experiment showed that the combination of HFD/moderate STZ is an alternative to generate type 2 diabetic females using CD1 mice. Nevertheless, this combination produced a low number of hyperglycemic CD1 females after 4 weeks of the moderate injection of STZ. Hyperglycemic females generated by this combination did not reach the extremely high glucose levels as diabetic females under a standard diet induced by the classic use of a high dose of STZ. We also obtained preliminary results for a new non-genetic mouse model for gestational diabetes mellitus (GDM).

In our last experiments we analyzed late gestation fetal myocardial apoptosis by flow cytometry and by RT-PCR analysis of a panel of 5 genes involved in apoptosis/proliferation (Bcl-2, P53, Casp3, Casp9 and PkC-e). We also tested maternal immune stimulant FCA, which had been reported to improve teratogen-induced dysregulation of fetal apoptosis. Results revealed that high levels of maternal hyperglycemia induced dysregulation of fetal myocardial apoptosis. An increased rate of late apoptosis/necrosis in the fetal myocardium in late gestation was also evidenced by

flow cytometric analysis. Particularly there was a significant increase in percentage of early apoptotic cells in the fetal myocardium detected by cell markers annexin V and propidium iodide. There was also a significant increase in percentage of late apoptosis/necrosis fetal myocardial cells in the diabetic group compared to the control group. FCA appears to have a positive effect on hyperglycemia-challenged fetal myocardial cells. Fetal samples from diabetic females treated with FCA did not show a difference with the control group in percentage of late apoptotic/necrotic myocardial cells. We observed paradoxical up-regulation of anti-apoptotic gene Bcl-2 in late gestation fetal myocardium from the hyperglycemic groups.

The first two hypotheses were proven considering that evident morphological changes of cavitary dilation and myocardial reduction of the fetal heart were found. We determined that maternal immune stimulation with FCA and IFNγ protected the fetal heart from such structural changes. Evidence supporting the third hypothesis was also produced. Particularly, the flow cytometric analysis demonstrated increased fetal myocardial apoptosis in fetuses from highly hyperglycemic females. This explains in part the structural changes of cavitary dilation and myocardial reduction observed in late gestation. However, the gene analysis showed subtle differences in gene expression for all the genes but Bcl-2. Gene Bcl-2 interestingly, showed a dramatic up-regulation in the fetal heart of the highly hyperglycemic groups, along with decreased Casp3 and Casp9. Possibly, these results may indicate the myocardial cells are attempting to activate internal anti-apoptotic pathways in the presence of external pro-apoptotic challenge.

The fourth hypothesis was proven, however results were at the same time not strongly encouraging. We generated insulin resistant diabetic females (type 2 diabetic females). However, the number of females that became hyperglycemic by the combination of HFD and low dose of streptozocin were very low and would limit utility of the type 2 model. We also generated insulin resistant females in a GDM profile.

The studies must be extended to better understand the effects of maternal hyperglycemia in the fetal heart, including increased myocardial apoptosis in the presence of upregulated mitochondrial anti-apoptotic pathways.

APPENDIX A

Future Directions: Preliminary Study Results

Preliminary studies suggested that insulin-resistant diabetes may alter fetal heart gene expression for apoptosis, similar to insulin-dependent diabetes mellitus.

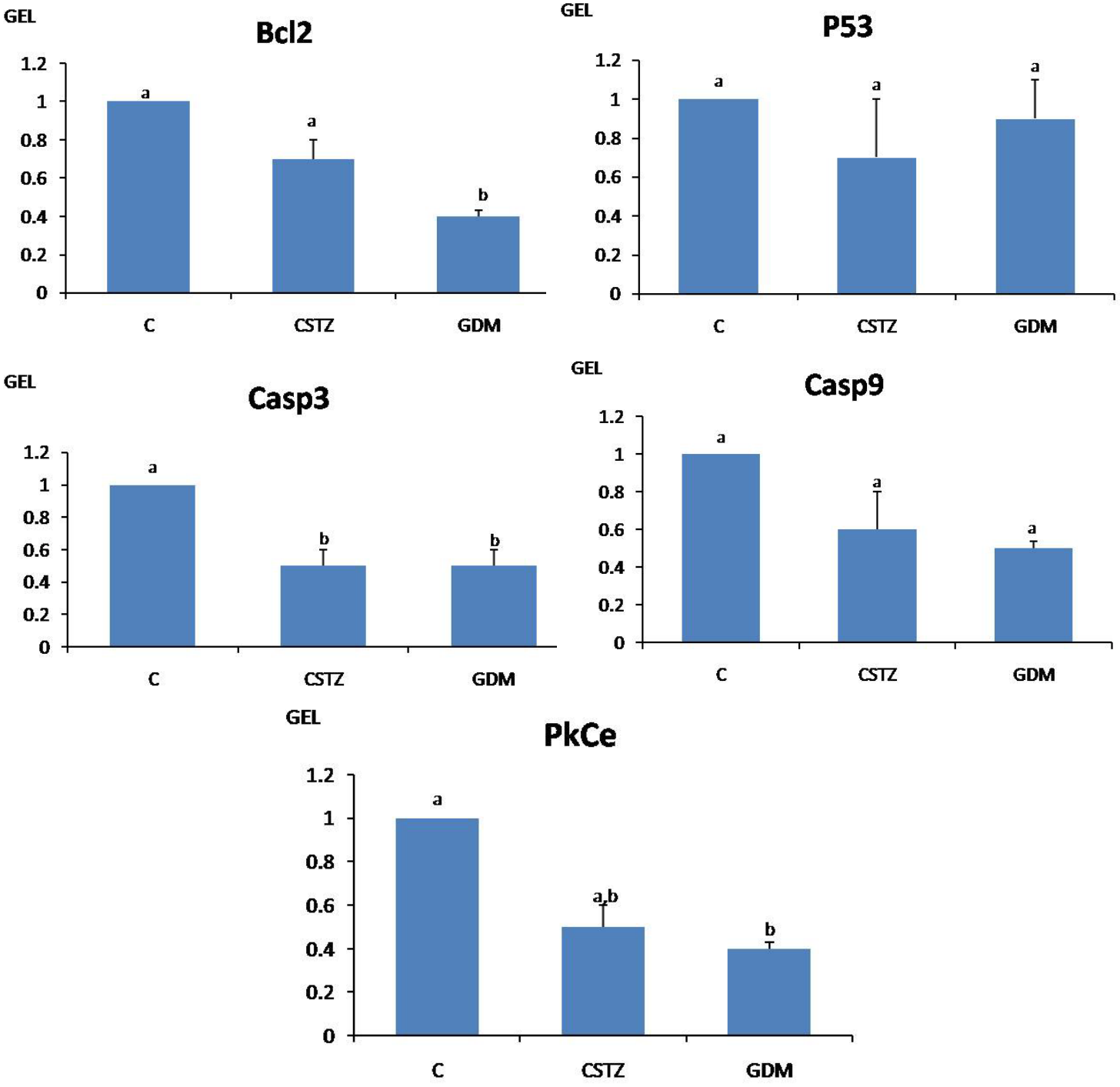

Figure 1. ANOVA results for gene expression in the GDM groups. The graphs were created using the ΔΔCT output. GEL: gene expression level, C: main control group, CSTZ: secondary control group, GDM: gestational diabetes mellitus group. Different letters mean difference among groups.

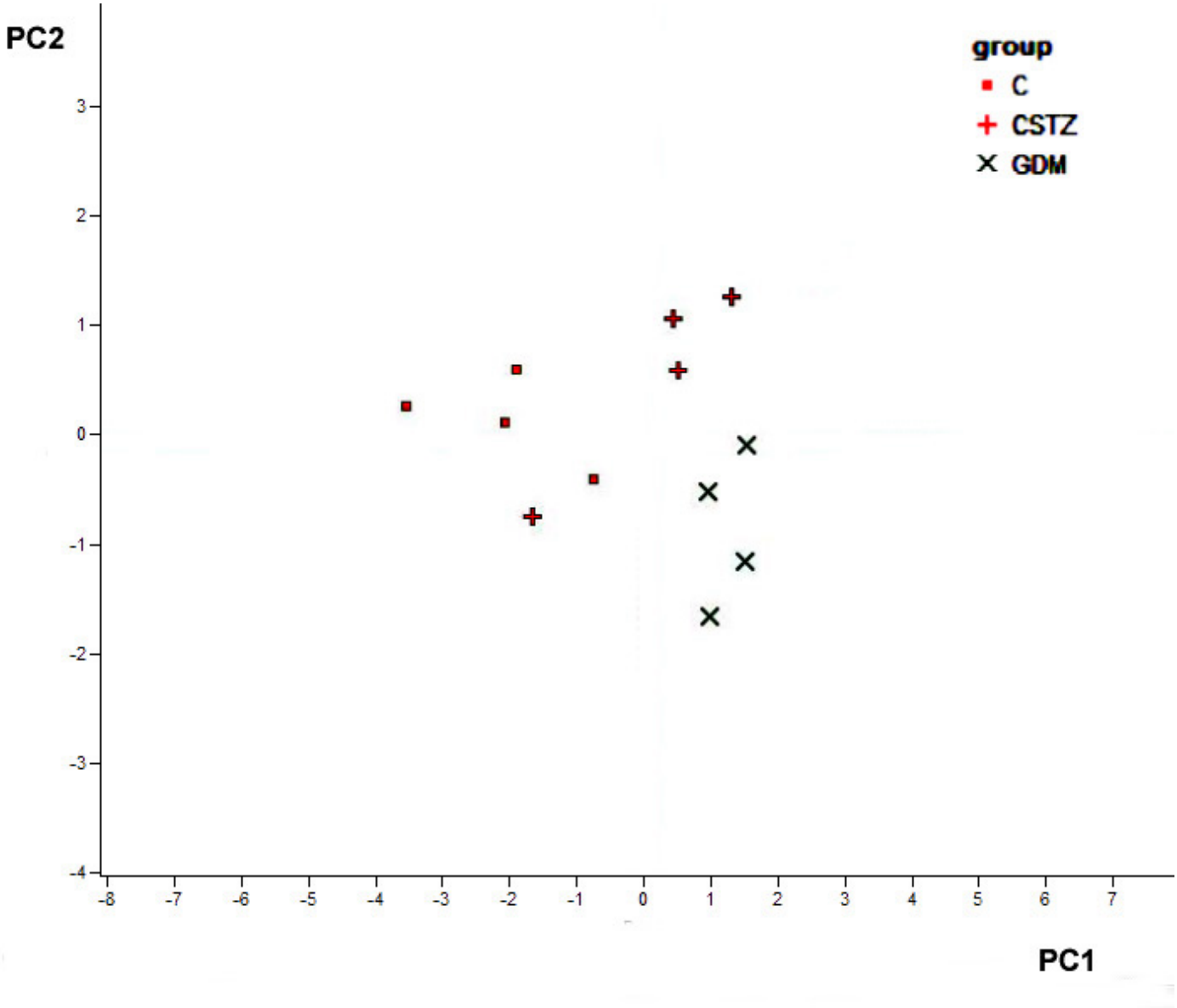

Figure 2. Principal component analysis (PCA) of the effect of maternal GDM on fetal myocardial gene expression. PC1 and PC2 representing the first and second principal components are shown. , C: main control group, CSTZ: secondary control group, GDM: gestational diabetes mellitus group.

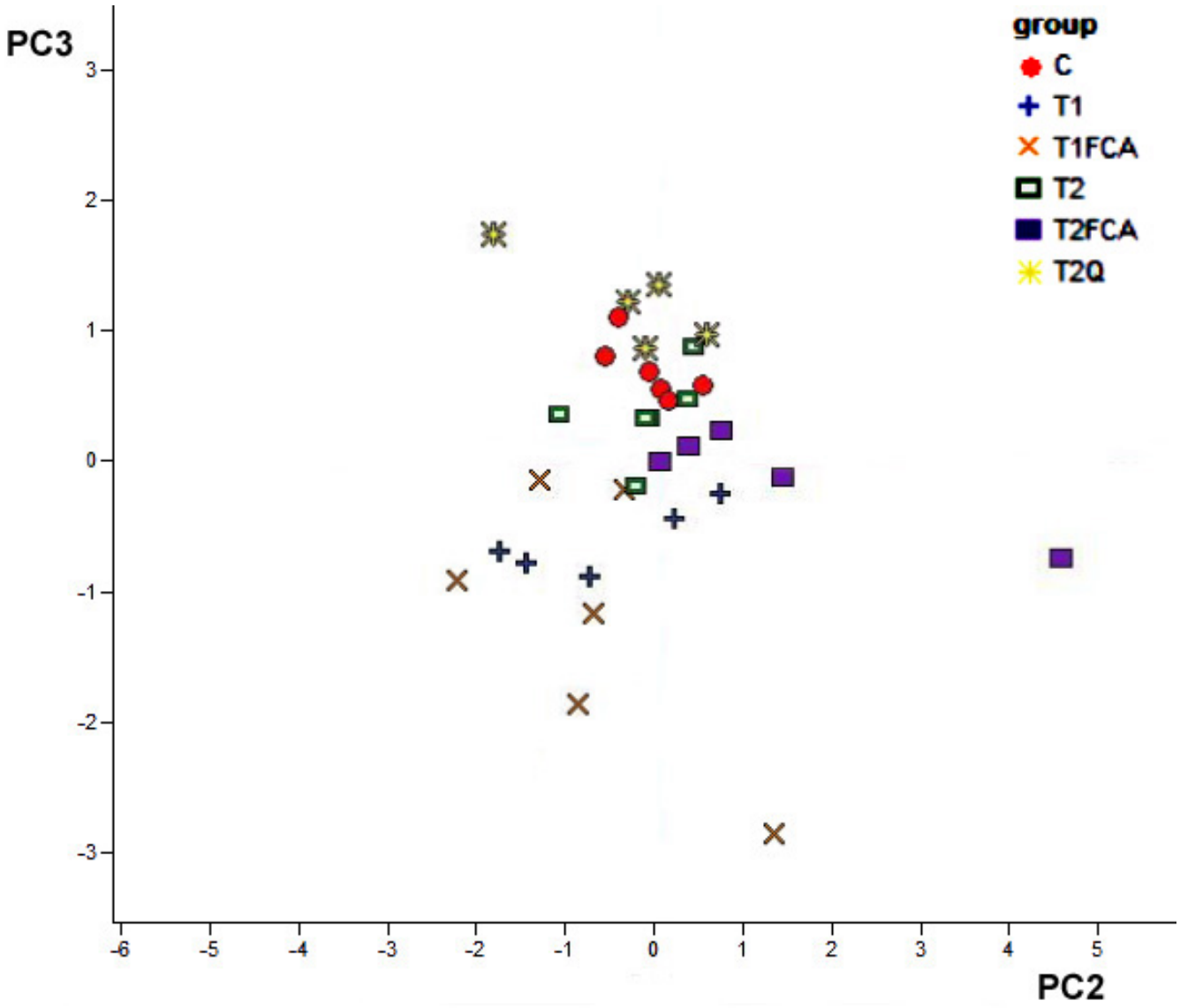

Figure 3. Principal component analysis (PCA) of the effect of insulin-dependent and insulin resistant maternal diabetes and treatments with FCA and quercetin on fetal myocardial gene expression. PC2 and PC3 representing the second and third principal components are shown. C: main control group, CSTZ: secondary control group, T1: type 1 diabetic group, T1FCA: type 1 diabetic group treated with FCA, T2: type 2 diabetic group, T2FCA: type 2 diabetic group treated with FCA, T2Q: type 2 diabetic group supplemented with quercetin.

Table 1: Treatment alters gene expression for the GDM groups. PCA analysis.

Genes	PC1	PC2	PC3	PC4	PC5
Bcl2	0.4524	-0.4233	0.4212	0.5482	-0.3714
P53	0.3107	0.8077	0.5002	0.0016	0.0276
Casp3	0.4771	0.1510	-0.5670	0.4680	0.4570
Casp9	0.4964	0.0891	-0.4168	-0.4381	-0.6163
PkCe	0.4738	-0.3709	0.2774	-0.5369	0.5220
Eigenvalue	3.4992	0.8541	0.4185	0.1517	0.0763
Cumulative % of variation explained	69.9840	87.0674	95.4382	98.4730	100
ANOVA probability that treatment had no effect (*p* value)	*$p < .05$	*$p < .05$	*p*: 0.7304	*p*: 7494	*p*: 1216

Table 2: Treatment alters gene expression for T1 and T2 groups. PCA analysis.

Genes	PC1	PC2	PC3	PC4	PC5
Bcl2	0.1981	0.3876	0.7977	0.0764	0.4101
P53	0.3871	0.5613	-0.2970	0.6179	-0.2548
Casp3	0.5589	0.3101	-0.2223	-0.7361	0.0064
Casp9	0.5129	-0.484	-0.2333	0.2609	0.6157
PkCe	0.4851	-0.450	0.4140	0.0479	-0.6225
Eigenvalue	1.8915	1.4511	1.0552	0.4010	0.2013
Cumulative % of variation explained	37.82996	66.8511	87.9543	95.9747	100
ANOVA probability that treatment had no effect (*p* value)	*p*: 0.144	$p < 0.05$*	$p < 0.0001$*	$p < 0.05$*	*p*: 0.573

APPENDIX B

Future Directions: Preliminary Study Results

Preliminary studies suggested that supplementation of insulin-resistant diabetic CD1 females with quercetin, had a positive impact on lowering maternal glucose levels during pregnancy.

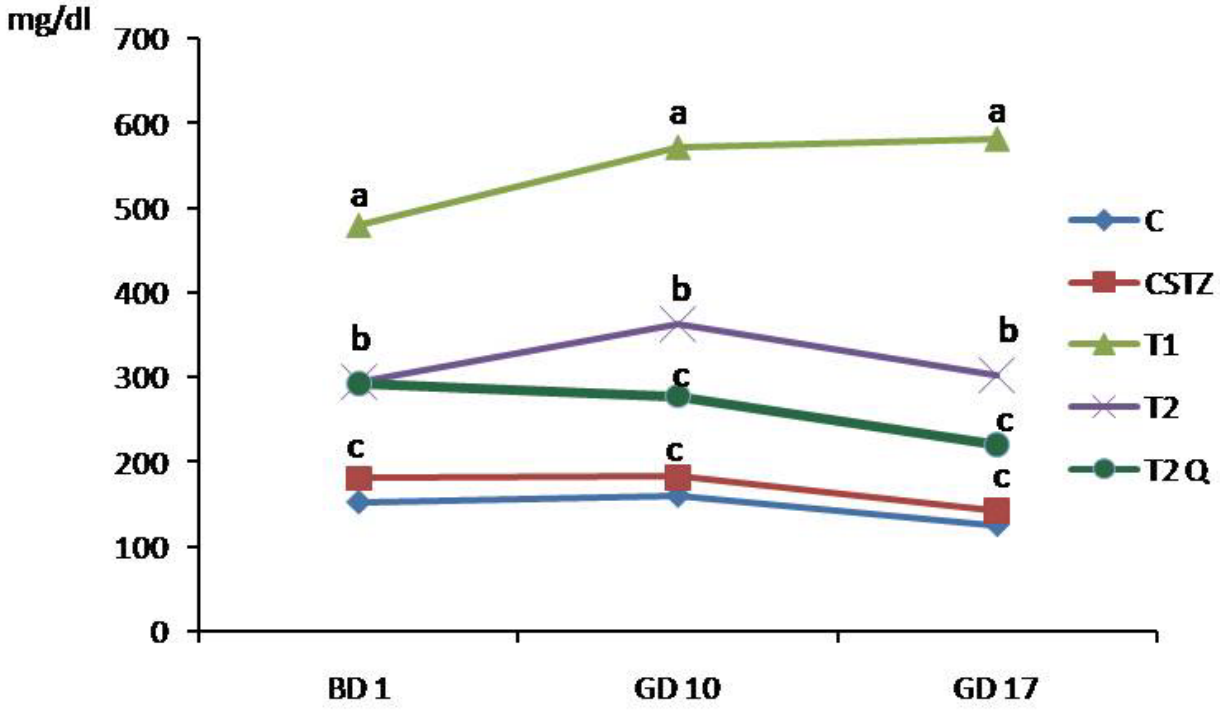

Figure 1. Maternal glucose levels during pregnancy. C: main control group, CSTZ: secondary control group, T1: type 1 diabetic group, T2: type 2 diabetic group, T2Q: type 2 diabetic group supplemented with quercetin, BD: breeding day, GD: gestation day. Groups sharing the same letter are not significantly different.