High Saturated Fat Diet Induces Gestational Diabetes, Perinatal Skeletal Malformation and Adult-Onset Chronic Diseases

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ABSTRACT

Adult exposure to high fat diet (HFD) has been linked to increased risk of musculoskeletal, cardiovascular, and metabolic diseases; however, the contribution of gestational HFD to elevated oxidative stress (OS), perinatal cardiovascular, skeletal, and metabolic dysfunction as well as long-term effects on adult offspring are incompletely understood. Pathophysiologic mechanisms linking gestational HFD, OS, and insulin resistance to perinatal development and adult-onset chronic diseases are explored in the present study, and maternal antioxidant (quercetin) is offered as a potential preventive dietary supplement to reduce fetal and maternal sequelae of HFD. Female C57BL/6 mice were fed "cafeteria-style" HFD (including 32.1% saturated fat to mimic a typical fast food menu) with or without quercetin for one month prior to conception, and throughout gestation. HFD dams developed gestational diabetes with significantly increased placental OS and vasculopathy. Neonates were smaller at birth than age-matched controls, and surviving offspring developed type 2 diabetes, hypertension and osteoporosis during adulthood, despite having been fed healthy diet throughout their postnatal life. Additional measures of bone using three-dimensionally reconstructed computed tomographic image analysis (microCT) revealed microarchitectural changes of bone at birth, and at 6 and 12 months postnatally. Fetuses from HFD dams displayed diminished bone mineral density (BMD) and disrupted endochondral and intramembranous ossification with significantly shortened distal limb lengths, as compared to offspring of standard rodent chow dams. Skeletal malformation persisted into adulthood despite the fact that both control and HFD offspring were fed conventional rodent chow throughout postnatal life. The offspring gestationally exposed to HFD showed significant decreased femoral BMD at 6 months of age and dysregulation of distal femoral trabecular architecture at 12 months of age, indicating development of osteoporosis. We were able to reduce incidence of placental vasculopathy, fetal maldevelopment and adult-onset type 2 diabetes, hypertension and osteoporosis with concurrent maternal quercetin supplementation during pregnancy. Collectively, these data suggested that maternal HFD increases placental OS

and vascular damage during pregnancy, which are associated with fetal malformation and elevated adult-onset multisystemic chronic diseases. Maternal quercetin supplementation must be further explored as a potential dietary intervention for improved placental integrity, fetal development and lifelong health.

Dedication

To my grandparents, Shengxiang Liang and Haiyun Zhang

In the memory of my grandpa

To my grandma, who has been the greatest love and the most beautiful inspiration in my life
May you always be proud of your child's achievements

I would especially like to express my deep appreciation to my family for their love and continued support.

Thanks to whoever else helped me over the years, but whom I've forgotten to mention. Your encouragement, even your friendly face and smile to me is part of the final result, as well.

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List of Abbreviations

ALP   Alkaline Phosphatase

ANOVA   Analysis of Variance

BHT   Butylated Hydroxytoluene

BMD   Bone Mineral Density

BMP   Bone Morphogenetic Protein

BP   Blood Pressure

BV   Total Bone Volume

CAM   Endothelial Cell Adhesion Molecules

CRP   C-Reactive Protein

CT   Computed Tomography

3-D   Three-Dimensionally

DEXA   Dual Energy X-Ray Absorptiometry

DICOM   Digital Imaging and Communication in Medicine

Dkk1   Dickkopf-1

DOHaD   Developmental Origins of Health and Disease

ELISA   Enzyme-linked Immunosorbent Assay

EndoCAM   Endothelial Cell Adhesion Molecule

EtOH   Ethanol

FFA   Free Fatty Acid

FGF-2   Fibroblast Growth Factor-2

GAPDH   Glyceraldehyde 3-Phosphate Dehydrogenase

G-CSF   Granulocyte-Colony Stimulating Factor

GD	Gestational Day
GDM	Gestational Diabetes Mellitus
GH	Growth Hormone
Glut-2	Glucose transporter 2
GnT-4a	*Mgat4a*-encoded GlcNAcT-IV Glycosyltransferase
ddH$_2$O	Distilled Deionized H$_2$O
HA	Hydroxylapatite
H & E	Hematoxylin-eosin
HFD	High Saturated Fat Diet
HFD/Q	High Saturated Fat Diet with Quercetin Supplementation
IGF	Insulin-like Growth Factor
IL	Interleukin
IP	Intraperitoneal Injection
IUGR	Intrauterine Growth Restriction
Lrp	Lipoprotein-Related Protein
MAP	Mitogen Activated Protein
MCP-1	Monocyte Chemotactic Protein-1
MDA	Malondialdehyde
Micro-CT	Micro-computed Tomographic Image Analysis
MMP	Matrix Metalloproteinase
MNU	Methylnitrosourea
MR	Magnetic Resonance
MSC	Mesenchymal Stem Cells

PBS	Phosphate Buffered Saline
PI	Phosphoinositide
PKB	Protein Kinase B
PKC	Protein Kinase C
NO	Nitric Oxide
NOS	Nitric Oxide Synthases
NTDs	Neural Tube Defects
ONOO$^-$	Peroxynitrite
OS	Oxidative Stress
PDGF	Platelet-Derived Growth Factor
PECAM-1	Platelet-endothelial Cell Adhesion Molecule-1, CD 31
PPAR-gamma	Peroxisome Proliferator-Activated Receptor-Gamma
Q	Quercetin
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
Runx 2	Run-Related Transcription Factor 2
SOD	Superoxide Dismutase
STZ	Streptozotocin
TNF	Tumor-Necrosis Factor
UCP-2	Uncoupling Protein-2
VDR	Vitamin D3 Receptor
VEGF	Vasculoendothelial Growth Factor
Wnt Signaling	Wingless-type Integration Site/beta-catenin Signaling

CHAPTER I

Introduction and Literature Review

Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is a common metabolic abnormality, which is characterized by glucose intolerance first recognized during pregnancy. GDM is associated with increased incidence of fetal malformation, perinatal mortality, and maternal morbidity (Dandrow and O'Sullivan 1966; Stevenson *et al.* 1982). GDM is thought to result from an interaction of genomic and environmental factors, and affects 2–8% of all pregnancies with increased risk of type 2 diabetes in GDM moms in subsequent years, as well as increased risk of diabetes and obesity in offspring (Shao *et al.* 2002). However, it is of considerable concern that recent changes in the gestational environment, including diet, air and water quality may be associated with drastically increased incidence of GDM and may represent a substantive health risk for pregnant women and their offspring (Catalano *et al.* 2003). Relevant dietary factors, including a high fat diet (HFD) intake, have been shown to directly promote glucose intolerance, obesity, and hypertension (Bo *et al.* 2001; Rolo and Palmeira 2006).

Epidemiological studies have shown an increased incidence of GDM (decreased maternal insulin sensitivity/increased insulin resistance) among populations consuming a typical Western diet high in saturated fat (Catalano *et al.* 2003). The percentage of saturated fat is an independent predictor of metabolic abnormalities, as women with an exceptionally high consumption of saturated fat (30% of total kilocalories) develop marked abnormalities in glucose metabolism and insulin resistance (Bo *et al.* 2001). Although the primary cause of GDM is incompletely understood, it is clear that HFD- induced insulin resistance plays an early role in its pathogenesis.

There is evidence that increased fatty acids from HFD appear to cause insulin resistance by directly inhibiting insulin-stimulated glucose transport activity (Dresner *et al.* 1999). This inhibition is likely because intracellular fatty acyl CoAs and diacylglycerol accumulates. That inactivates the tyrosine kinase domain of the insulin receptor and interferes with the insulin signaling pathway, ultimately leading to suppression of insulin signaling and further insulin resistance (Lowell and Shulman 2005). Subjects who develop GDM usually have a complex phenotype with defects in insulin secretion, increased hepatic glucose production, and resistance to the action of insulin, all of which contribute to the development of overt hyperglycemia. Although the precise mechanisms whereby these three factors interact to produce glucose intolerance and diabetes are uncertain, it has been suggested that the final common pathway responsible for the development of GDM and type 2 diabetes is an incomplete compensation of pancreatic beta cells to increase insulin secretion in proportion to elevated circulating glucose levels. This results in manifestation of GDM: hyperglycemia, hyperinsulinemia, and insulin resistance (Saltiel and Olefsky 1996; Kahn 1998; Lebovitz and Banerji 2004).

Animal models provide an important research tool to elucidate pathophysiologic aspects of human diseases. Animal models mimicking diabetes in pregnancy have historically been induced by injecting diabetogenic drugs, particularly streptozotocin (STZ) to pregnant or soon to become pregnant mice. However, diabetogenic drugs such as the alkylating agent STZ induce a severe diabetic syndrome that more closely mimics type 1 rather than type 2 or GDM. This is because STZ works by near-complete ablation of pancreatic beta cells, resulting in hyperglycemia and hyperinsulinemia. In contrast, type 2 and GDM are characterized by intact beta cells but alteration of insulin sensitivity due to dysregulated insulin receptor activity. GDM is also often characterized by modest elevations in blood glucose levels, in contrast to STZ-

induced diabetes, which often results in mean glucose levels exceeding 350 mg/dl (Holemans *et al.* 2004), which further suggests the inadequacy of the STZ model for study of GDM (Holemans *et al.* 2004). Consequently, attempts to improve the STZ model involved lowering the dose of STZ to cause a more modest elevation in circulating glucose, similar to GDM. This modified STZ protocol did produce moderate hyperglycemia, but resulted in variable effects on fetal growth, pancreatic insulin content, and plasma levels of insulin, and therefore is still not considered a suitable model for GDM (Kervran *et al.* 1978; Aerts *et al.* 1988; Oh *et al.* 1988; Oh *et al.* 1991). Different genetic animal models have also been considered for GDM studies. However, use of genetically altered animal models, such as leptin or leptin receptor-altered mice, to study environmental factors of metabolic disorders is also not ideal. On one hand, it is difficult to disentangle the contribution of genes and environment. On the other hand, the genetic modifications seen in these knockout strains are often not represented in human GDM patients (Widdowson 1997; Wilson *et al.* 1999). Therefore, a suitable GDM mouse model has not been available.

Recent studies show that lifestyle and diet are thought to play critical roles in the development of metabolic disorders of pregnancy such as obesity and GDM (Holemans *et al.* 2004). Thus, we hypothesized that maternal high fat diet (HFD) induces GDM and initiated this study with the objective of developing an economical, reproducible, and appropriate mouse model to investigate putative pathophysiologic mechanisms of GDM, and to explore possible preventive or therapeutic regimens to reduce adverse effects of GDM in syngeneic C57BL/6J mice. C57BL/6 is the most widely used inbred strain, is commonly utilized in cardiovascular, developmental, and immunologic research, and is most commonly used to study mechanisms of diabetes, obesity, and osteoporosis. In the present study, clinical signs of GDM were achieved by

feeding female C57BL/6 mice HFD diet before and during pregnancy, which produced insulin resistance and hyperglycemia, similarly to GDM. We then used this mechanism to explore the effects of HFD and GDM on maternal health, and long-term effects on surviving offspring.

Placental function on fetal growth

The placenta is central to fetal development because it plays such a vital role in control of fetal nutrition. The placenta actively regulates the nature and extent of nutrient, gas, and waste transfer between the dam and the fetus (Sibley *et al.* 2005). The placental vasculature and trophoblast also produce hormonal signals that affect maternal and fetal metabolism, and direct placental and fetal signaling pathways of development (Myatt 2006). Appropriate development of the placenta is crucial to normal fetal development, and a properly formed placenta plays several roles on fetal growth, including nutrient, gas, and waste exchange, hormonal control, and immune protection of the fetal allograft (Myatt 2006). In the human placenta, the primary barrier to maternal-fetal exchange is the syncytiotrophoblast, a transporting epithelium covering the placental villi that project into the maternal blood of the intervillous space. Additionally, the placenta contains an extensive network of interdigitating fetal and maternal blood vessels that are closely apposed, to readily facilitate nutrient, gas, and waste exchange between the mother and the fetus during development. Placental nutrient transport has long been known to be dependent on vascular development which determines blood flow to both sides of the placenta and transport of flow-limited substrates. Recent animal experiments have highlighted the involvement of both the vasculature and trophoblast in overall placental function (Myatt 2006).

Changes in placental growth represent an important link between perturbations in the maternal compartment (such as reduced placental blood flow, altered maternal nutrition and

diabetes) and alterations in fetal growth. Reduced maternal placental blood flow, leading to 'placental insufficiency', probably represents the most common underlying cause of intrauterine growth restriction (IUGR) in Western societies (Jansson and Powell 2007). Conditions such as smoking, obesity, and diabetes mellitus that interfere with placental cell viability, or placental vascular integrity, will directly impact the health and development of the fetus. Recent studies of placental morphology have demonstrated distinct abnormalities of placental villi that could be associated with particular presentations of IUGR (Kingdom *et al.* 2000; Mayhew *et al.* 2004). Environmental exposures or diseases that cause necrosis or apoptosis of growth factor-secreting placental trophoblasts, or cause elevated oxidative stress (OS) and ischemic or inflammatory damage to maternal or fetal labyrinthine placental vasculature, have been shown to result in IUGR and improper fetal organ system development.

A major role of the placenta during organogenesis is to regulate exposure of the early developing embryo to reactive oxygen species (ROS) which are present in the maternal circulation (Jauniaux *et al.* 2006; Tjoa *et al.* 2006). This delicate mechanism of oxygen transfer control protects the embryo from OS, as in the early phases of development embryonic antioxidant capacity is limited. The syncytiotrophoblastic cells that cover the outer surface of the placental villi are in direct contact with the relatively high oxygen concentrations in maternal blood, but have reduced levels of antioxidant enzymes (Jauniaux *et al.* 2006). Hence, they are prone to redox state imbalance and damage if maternal OS is markedly elevated. Because of this delicate balance, states of increased maternal OS are thought to exert significant and direct effects on embryonic and fetal redox status. Increased placental OS has been described in maternal diabetes, in preeclampsia and in other diseases of pregnancy (Ornoy 2007). Several key signaling molecules and growth factors have been shown to contribute to OS-mediated placental

vasculopathy, including vasculoendothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), matrix metalloproteinase-2 (MMP-2), platelet-derived growth factor (PDGF), leptin, interleukin (IL) -1, 3, -11, -12, tumor-necrosis factor (TNF) and granulocyte-colony stimulating factor (G-CSF) (Ribatti *et al.* 2007). These factors have been found to interact synergistically to influence angiogenesis (Cao *et al.* 2001), affect immunomodulation (La Cava *et al.* 2004), alter rate of cell apoptosis (La Cava *et al.* 2004), and play instrumental roles in fetal and placental development. Therefore, it is suggested that infants with OS-mediated abnormal placental phenotypes have a greater risk of neonatal mortality and morbidity and possibly increased future risk of ischemic heart failure and diabetes (Sibley *et al.* 2005). Additional study into the relationship between OS, inflammatory/ischemic placental vascular and trophoblast damage, and their effects on the developing fetus will greatly improve mechanistic understanding of the contribution of environmentally induced OS to fetal development.

High fat diet, oxidative stress and fetal maldevelopment

Oxidative stress refers to a disturbance in the balance between the production of ROS and antioxidant defenses that may lead to tissue injury (Halliwell 1997). Shortly after conception, there is a physiologically necessary burst of ROS that mediates implantation. ROS cause release of MMPs that degrade endometrial connective tissue and permit proper implantation. However, prolonged or excessive OS during pregnancy is considered pathologic, as excessive OS interferes with trophoblast differentiation, alters expression of proinflammatory cytokines and growth factors (Hill *et al.* 1998; Myatt *et al.* 2000; Sikkema *et al.* 2001), and induces lipid peroxidation, protein carbonylation, and DNA damage that alter gene expression related to placental and fetal growth. Specifically, several key components of development are impaired by excessive ROS,

including vascular endothelial health, fetal size and bone density, reduced NAD(P)H reductant capacity, and alteration of mesenchymal stem cell signaling that favors adipocyte rather than osteoblast differentiation. Collectively, these factors increase risk of osteoporosis later in life (Baynes 1991; Cederberg *et al.* 2001; Baer *et al.* 2004). These effects may be attributed either to increased production of ROS, or decreased antioxidant capacity, or both.

It is thought that ROS mediate inflammation and apoptosis through altered cell signaling and activation of NF- kappa B pathway, which causes vascular inflammatory placental damage and enhances tissue-destructive effects by upregulation of multiple immune, protein, inflammatory cytokine, and proapoptotic (Bcl-2 and caspase) genes that are related to fetal development (Barnes and Karin 1997; Haddad 2002; White *et al.* 2002; Coughlan *et al.* 2004; Ribeiro *et al.* 2004; Pustovrh *et al.* 2005). ROS-mediated activation of NF- kappa B pathway, inflammation, and apoptosis inhibits peroxisome proliferator-activated receptor-gamma (PPAR-gamma) that in turn results in upregulation of vascular stress response genes. This pathway is the proposed pathogenesis of human vascular diseases, such as preeclampsia and atherosclerosis (Collins 1993; Rodrigo *et al.* 2005; Gao *et al.* 2006; Takada *et al.* 2006).

Optimal fetal development also relies on an intricately orchestrated availability of nutrients. The developing fetus is highly sensitive to intrauterine conditions during gestation. Maternal health, nutrition, and environmental exposures play an integral role in fetal development, and stresses to the fetus, including malnutrition, exposure to environmental chemicals, and other maternal stressors lead to inadequate fetal development. Busy Western lifestyles have led to an increased reliance on fast food for nutrition, which is high in fat content, especially saturated fat, which increases OS and adversely affects cardiovascular, endocrine, and reproductive health of those who regularly ingest it (Erhardt *et al.* 1997). Increased OS

secondary to HFD and increased plasma free fatty acid (FFA) concentrations are thought to cause mitochondrial dysfunction. It has been suggested that long-term HFD intake increases quantity of electron transfer donors (NADH and $FADH_2$) and further increases the ATP/ADP ratio, which leads to the inhibition of the electron transport chain, which then generates excess ROS by overproduction of superoxide by the mitochondrial electron-transport chain (Rolo and Palmeira 2006). Elevated plasma fatty acids also result in uncoupling protein-2 (UCP-2) dysfunction, which increases production of ROS, and alters gene expression in metabolic and cell signaling pathways important to fetal development (Briaud *et al.* 2002). UCP-2 expression is inversely correlated with the level of ROS generation by respiring mitochondria, and therefore is thought to play a role in defense against adverse effects of excessive ROS (Negre-Salvayre *et al.* 1997). Moreover, HFD is also suggested to activate pro-oxidant enzymes such as NAD(P)H oxidase**,** nitric oxide synthases (NOS), xanthine oxidase and mitochondrial enzymes. This, along with altered glutathione systems, results in increased superoxide and other ROS formation, which inflicts damaging effects on the vasculature (Paravicini and Touyz 2006). OS contributes to vascular damage by promoting cell growth, extracellular matrix protein deposition, activation of matrix metalloproteinases, inflammation, and endothelial dysfunction (Stoker 2005; Tonks 2005; Paravicini and Touyz 2006). The resulting inflammation and vascular damage are related to diminished placental blood flow, placental trophoblast loss, and disruption of endothelium, which together diminish fetal circulation and elevate risk of fetal skeletal malformation (Garcia-Patterson *et al.* 2004).

Chronic consumption of diets high in saturated fats is associated with poorly-controlled hyperglycemia (e.g., >140 mg/dL), hyperinsulinemia, and insulin resistance; these effects, characteristic of type 2 and GDM, are linked to a 3-5-fold increased risk infant mortality.

Moreover, HFD-induced hyperglycemia, hyperinsulinemia and insulin resistance cause increased OS and modification of cell signaling pathways related to fetal development (Styrud *et al.* 1995; Garcia-Patterson *et al.* 2004; Wender-Ozegowska *et al.* 2005; San Martin and Sobrevia 2006). Studies show the relationship between HFD, hyperglycemia, and GDM in the impairment of fetal development, and this is thought to occur because of an upset in the oxidant-antioxidant balance favoring the former. Elevating OS alters expression of proinflammatory cytokines (particularly TNF-alpha, IL-1B, IL-6, IL-8, fibrinogen, e-selectin, C-reactive protein (CRP), and monocyte chemotactic protein-1 (MCP-1)), markers of energy homeostasis (leptin, which influences energy homeostasis; resistin, which impairs glucose tolerance, and adiponectin, which acts as an insulin sensitizing effector) (Bajoria *et al.* 2002; Henson and Castracane 2002; Lappas *et al.* 2002; Catalano *et al.* 2003; Gordeladze and Reseland 2003; Megia *et al.* 2004; Lappas *et al.* 2005; Bastard *et al.* 2006), and growth factors (VEGF, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), FGF-2, insulin-like growth factor-1 (IGF-1) and 2, bone morphogenetic protein-7 (BMP-7)) (Hill *et al.* 1998; Inzerillo and Epstein 2004; Qiu *et al.* 2005).

Increased OS during pregnancy causes placental vascular pathology, decreased fetal-maternal gas and nutrient exchange, increased fetal malformation, and IUGR (Prater *et al.* 2006a). Therefore, adverse fetal outcomes may be due to direct OS effects on the fetus, or indirect effects on the placenta such as vascular damage secondary to increased superoxide generation (Lyall *et al.* 1998). Superoxide dismutase (SOD) is an enzyme that degrades superoxide radicals and outcompetes damaging reactions of superoxide, thus protecting the cell from superoxide toxicity. It is a major source of cytoplasmic and extracellular ROS scavenging (Inoue *et al.* 1996). A study showed that mice genetically altered at the SOD locus (SOD knockout mice) develop massive OS and a wide range of pathologies (Elchuri *et al.* 2005). SOD

aids placental trophoblast differentiation, and diminished placental SOD causes trophoblast syncitialization and increased trophoblast apoptosis, as observed in preeclampsia (Myatt *et al.* 2000). Since trophoblasts direct fetal development through liberation of cytokines and growth factors, trophoblast cell loss is considered to be detrimental to fetal development. Therefore, increased OS, modification of cell signaling pathways, and alterations in inflammatory cytokines and growth factors, collectively result in placental vascular dysfunction and reduction in blood flow and nutrient supply from placenta to levels insufficient to sustain proper fetal skeletal growth and development (Styrud *et al.* 1995; Garcia-Patterson *et al.* 2004; Wender-Ozegowska *et al.* 2005; San Martin and Sobrevia 2006). Based on the previous work, in the present study, we investigated the effect of maternal HFD on placental OS and how this increased OS affects placental vascular integrity and fetal growth and development.

Platelet-endothelial cell adhesion molecule-1 (CD31)

CD31 is also called platelet-endothelial cell adhesion molecule-1 (PECAM-1) and endothelial cell adhesion molecule (endoCAM). CD31 is a single-pass transmembrane glycoprotein of ~ 130 kDa with an ectodomain, a transmembrane region, and a cytoplasmic tail (Newman *et al.* 1990; DeLisser *et al.* 1994). It is secreted by trophoblasts and localized on endothelium of large and small vessels in the placenta (Lyall *et al.* 2001). This molecule is also widely expressed by cells associated with the vascular compartment, such as platelets, endothelium, monocytes, neutrophils and lymphocytes, and appears very early during development of the vascular system (DeLisser *et al.* 1994). CD31 is also expressed constitutively on pulmonary arterial, capillary, and venular endothelium (Tasaka *et al.* 2003). Although not all of the physiological roles of CD31 have been determined, studies to date suggest that CD31

functions as a cell adhesion molecule in at least two capacities. Its localization to cell-cell borders of adjacent endothelial cells (Muller *et al.* 1989) suggests that CD31 may play an important role in endothelial cell homotypic interactions in the process of angiogenesis. This is further confirmed by the ability of antibodies against CD31 to inhibit the formation of endothelial cell-cell interactions (Albelda *et al.* 1990). Additionally, experiments both *in vitro* and *in vivo* have implicated CD31 in leukocyte transmigration through the vessel wall during inflammation (Muller *et al.* 1993). In fact, immunoneutralization of CD31 blocks neutrophil and monocyte migration through endothelium (DeLisser *et al.* 1994). CD31 mediates adhesion between several vascular cells, as well as cells of myeloid origin. Recent studies have suggested that CD31 and other endothelial cell adhesion molecules (CAMs) may also play a role in placental spiral artery transformation which is a key step towards establishment of fetal-maternal exchange (Zhou *et al.* 1997). Thus, decreased expression of CD31 is thought to be a marker of endothelial cell dysfunction and vasculopathy, and an indicator of vascular-induced fetal malformation.

CD31 has also been demonstrated to associate with beta-catenin by altering the quantity, activation, phosporylation, and nuclear localization of beta-catenin (Ilan *et al.* 1999; Biswas *et al.* 2003). Studies have suggested an association between diminished expression of beta-catenin and reduced endothelial cell CD31 expression (Biswas *et al.* 2003). Thus, CD31 is thought to serve, at least in part, as a reservoir for beta-catenin (Ilan *et al.* 1999). Beta-catenin is critically involved in canonical Wnt signaling, which mediates several biologic processes including embryonic development, bone formation, glucose and lipid metabolism and insulin secretion. Attenuation of the Wnt/beta-catenin signaling pathway by decreased expression or phosphorylation of beta-catenin has been suggested to link with several features of metabolic

syndrome including hyerlipidemia, hypertension, diabetes and osteoporosis (Manolagas and Almeida 2007). Therefore, decreased CD31 expression may be linked with above diseases through the attenuation of Wnt/beta-catenin signaling. In the present study, the intensity of CD31 immunofluorescense staining of control, HFD, and HFD + antioxidant quercetin was used to explore the effect of HFD and OS on the placental endothelium.

Developmental Originals of Health and Disease (DOHaD)

The developmental origins of health and disease hypothesis describes a concept that was originally put forward by David Barker and colleagues which stated that environmental factors, particularly nutrition, act in early life to program the risks for the early onset of cardiovascular and metabolic disease and reduced life expectancy in adult life (Barker 1995). The developmental environment is thought to determine, in large part, the metabolic parameters of the individual that will persist throughout life and significantly affect life-long risk of adult diseases. The process whereby a stimulus, applied at a critical or sensitive period of development, results in a long-term or permanent effect on the structure or function of the organism is called fetal programming (Ozanne 2001). Numerous epidemiological studies have demonstrated a strong association between IUGR and the later development of metabolic syndrome, consisting of arterial hypertension, coronary heart disease, dyslipidemia, visceral obesity, impaired glucose tolerance, and type 2 diabetes mellitus (Barker *et al.* 1989). Associations between low birth weight in childhood and raised blood pressure (BP) in adult life have been extensively demonstrated around the world (Barker 1998). A study in Sweden reported that the risk of increased diastolic BP was significantly higher in a group of male military recruits who had been small at birth. These authors concluded that being born small for gestational age might be a

valuable predicator of increased BP and increased risk of cardiovascular death in adult life (Gennser *et al.* 1988). Hales, along with Barker, found that subjects who were smaller at birth had a 6-fold increased risk of developing type 2 diabetes than those who were heavier at birth (Hales *et al.* 1991b) and also displayed more than 18-fold increased risk of developing metabolic syndrome. Studies conducted in Denmark with both monozygotic and dizygotic twins in their sixties who were discordant for type 2 diabetes showed that the twin diagnosed with lower birth weight more commonly developed type 2 diabetes than their heavier twin (Poulsen *et al.* 1997). This study indicated that increased risk of diabetes from poor fetal development is independent of genotype as the monozygotic twins are genetically identical. This finding also supports the hypothesis that the fetal environment may be more influential than genotype for the links between fetal maldevelopment and risk of adult-onset diseases.

During the past decade there have been a number of mechanistic frameworks proposed to epidemiologically explain the biological basis of the associations observed between birth weight and health outcomes. In 1992, Hales and Barker coined the term '*the thrifty phenotype hypothesis*' (Hales and Barker 1992). *The 'thrifty phenotype hypothesis'* proposes that alterations in fetal nutrition may result in developmental adaptations that permanently change the physiology and metabolism of the offspring, thereby predisposing individuals to metabolic, endocrine and cardiovascular disorders (Hales and Barker 1992; Barker 1998). According to '*the thrifty phenotype hypothesis*', when the fetal environment is poor, there is an adaptive response, which optimizes the growth of key body organs to the detriment of others and leads to an altered postnatal metabolism, which is designed to enhance postnatal survival under conditions of intermittent or poor nutrition (Hales and Barker 1992; Hales and Barker 2001). These adaptations may confer an initial survival advantage to the fetus, but then may predispose

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the individual to degenerative disease in post-reproductive life, particularly if the postnatal environment does not closely parallel the adverse prenatal environment. Thus, the defective fetal environment would affect long term offspring development and is thought to increase risk of chronic multisystemic disease (Rolo and Palmeira 2006). Extending upon '*the thrifty phenotype hypothesis',* the most recent *'predictive adaptive response hypothesis'* (Gluckman and Hanson 2004b; Gluckman and Hanson 2004a) proposes that the fetus makes adaptations *in utero* (or in the early postnatal developmental period) based on the predicted postnatal environment. When this predictive adaptive response is appropriate, the phenotype is normal; however, when mismatch occurs between the predicted and actual environment, disease manifests. Studies show permanent growth restriction results if maternal protein-restricted diet is continued during lactation, even when the offspring are weaned onto a control diet; however, obesity often results when the maternal diet exceeds that of the prenatal diet (Desai *et al.* 1996). Therefore, the degree of mismatch between the pre and postnatal environments is an important determinant of subsequent diseases.

The intrauterine environment is now thought to be crucial in programming for adult diseases (Barker 1995). The contribution of maternal nutrition to the fetal origins hypothesis has been studied extensively in both the human population and in experimental animals, since it is the critical factor to determine the intrauterine environment. Maternal malnutrition induces abnormal glucose homeostasis (Guo and Jen 1995; Taylor *et al.* 2005), increased BP (Langley-Evans 1996; Khan *et al.* 2005), abnormal serum lipid profiles (Karnik *et al.* 1989; Guo and Jen 1995), increased adiposity (Guo and Jen 1995), pro-atherogenic lesions (Palinski *et al.* 2001), reduced acetylcholine-induced vasodilatation (Taylor *et al.* 2004) and hyperleptinaemia (Taylor *et al.* 2005). Human studies have shown that pregnant women exposed to protein malnutrition, as

in the famine of the Dutch Hunger Winter, have offspring with reduced birth size and an increased risk of glucose intolerance and obesity in adult life (Lumey *et al.* 1993). Conversely, nutritional excesses such as excessive saturated fat intake in pregnant women could compromise the fetus through elevated OS, with long-lasting consequences (Gerber *et al.* 1999). Animal studies indicate endothelial dysfunction in the offspring of dams fed saturated fat (Koukkou *et al.* 1998) and hypertension in male offspring of rats fed saturated fat (coconut oil) (Langley-Evans 1996). Maternal cholesterol over-feeding during pregnancy and lactation in rodents results in phenotypes of offspring that closely resemble human metabolic syndrome (Armitage *et al.* 2005). Reducing the proportion of protein in the diet of pregnant rats results in offspring which have reduced size at birth and also elevated BP (Langley and Jackson 1994) and glucose intolerance (Dahri *et al.* 1991) in adult life. A variety of experimental approaches to induce maternal malnutrition in pregnancy have lead to similar observations in rats (Woodall *et al.* 1996), guinea pigs (Persson and Jansson 1992), and sheep (Bloomfield *et al.* 2003). Thus there can no longer be any doubt that changes in maternal nutrition in animals can both change size at birth and also permanently alter aspects of the physiology of the offspring in a way that is consistent with disease susceptibility observed in human studies.

Novel theories also suggest that an unsatisfactory fetal environment before birth results in insufficient skeletal growth and leads to lifetime deficits in bone volume, density and strength. Maternal chemical exposure or disease during pregnancy alters important fetal skeletal signaling pathways, and may contribute to life-long skeletal fragility of offspring that, when compounded with middle-aged hormonal fluctuations and other nutritional and environmental stressors, becomes clinically significant and leads to elevated risk of fragility fractures in pelvis, limbs, and vertebrae of older adults (Holt 2002). It is reported that about 62% of the variance in bone mass

is determined by the intrauterine environment, while maternal and paternal genetic influences play a less significant role (20% and 18%, respectively) (Davies *et al.* 2005). A recent Finnish cohort study has demonstrated a direct association between poor childhood growth and later risk of hip fracture (Armitage *et al.* 2005). Impaired bone density and architecture result when the fetal environment is less than optimal, and requires the infant to adapt for survival. Perinatal adaptation to fetal growth restriction is often manifested as 'catch-up growth', which favors differentiation of immature cells into fat cells (adipocytes) rather than bone cells (osteoblasts) (Hales and Barker 1992). Additionally, altered cell-signaling pathways early in development by maternal malnutrition favor adipocyte rather than osteoblastic differentiation of mesenchymal stem cells (MSC), leading to decreased bone density and increased fat stores (Lecka-Czernik *et al.* 2002). It is thought that bony defects such as malformation or disorganized bony trabecular architecture that arise during gestation are not typically repaired postnatally, and are thought to increase risk of bone fragility fractures in adulthood (Gluckman *et al.* 2005). Thus, the fetal programming of bone development explored in the present study may provide a tentative link between adverse fetal environment, intrauterine growth restriction (IUGR), skeletal malformation, and increased risk of adult fragility fractures and osteoporosis.

Osteoporosis

Osteoporosis has been defined as a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in risk of fracture. It is a major contributor to morbidity and mortality in the aging US population (Riggs *et al.* 1982) and a leading cause of disability in the US. Nearly 10 million Americans over age 50 currently are diagnosed and an additional 34 million people are

thought to have osteoporosis that remains undiagnosed until they suffer a fragility fracture (approximately 1.5 million cases each year) (www.cdc.gov). Osteoporosis typically becomes symptomatic after middle age in women, when declining perimenopausal estrogen levels result in elevated osteoclastic activity and an imbalance of bone degradation over bone formation. Recent data suggest that there may be an even greater number of young adults with undiagnosed abnormalities in bone density that could predispose them to fractures. These abnormalities in young women are not thought to be caused by hormonal fluctuations, and other factors such as sub-optimal childhood or prenatal nutrition may increase susceptibility to young adult onset osteoporosis. Therefore, symptoms of osteoporosis may result from perimenopausal hormone fluctuations, inadequacy of nutrition and exercise during childhood, and perhaps most importantly, nutrition and environmental factors before birth. A great deal of evidence indicates that osteoporosis is the result of a reciprocal interaction between genetic susceptibility and environmental factors. These evidence suggests that inadequate levels of nutrition or weight-bearing exercise during childhood impair adolescent bone growth (e.g., prior to epiphyseal closure) that are thought to result in insufficient development of total bone mass and strength. Diet composition, in particular fat content, has been shown to act negatively on bone status. Children treated with ketogenic diet (very high fat and low carbohydrate diet) showed a poor bone mineral status (Bertoli *et al.* 2002). In animal models (rat), a western-style diet, i.e. high fat content, results in low bone mass and poor bone quality, demonstrate that diets with high saturated fat content have long-ranging adverse effects on bone mineralization and adult bone health in growing animals (Zernicke *et al.* 1995; Ward *et al.* 2003).

Importantly, other very recent studies are now examining the contribution of the fetal environment to increased risk of osteoporosis. Novel theories suggest that an unsatisfactory fetal

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environment results in a permanent alteration of fetal growth that results in low birth weight babies, insufficient skeletal growth that is not repaired postnatally, and lifetime deficits in bone volume, density and strength. Exposure of the developing fetus to environmental toxins such as second hand cigarette smoke or maternal malnutrition is a significant problem that is now believed to contribute significantly to prenatal dysregulation of osteogenesis, growth restriction, and low BMD. Maternal chemical exposure or disease during pregnancy alters important fetal skeletal signaling pathways, and may contribute to life-long skeletal fragility of offspring that, when compounded with middle-aged hormonal fluctuations and other nutritional and environmental stressors, becomes clinically significant and leads to elevated risk of fragility fractures in pelvis, limbs, and vertebrae of older adults. Previous work has implicated leptin, a hormone released from fat cells following maternal HFD, as a central mediator of growth factor signaling in bone development (Alexe *et al.* 2006; Vickers *et al.* 2008). Diets rich in saturated fats promote adipocytic release of the hormone leptin. Perinatal leptin release appears to be influenced by maternal fat stores and diet. Leptin has been proposed to mediate anti-osteogenic effects on periosteal, trabecular, and epiphyseal bone formation and remodeling (Ducy *et al.* 2000; Alexe *et al.* 2006). Therefore, gestational HFD together with leptin may interfere with fetal skeletal development by altering osteogenic cell signaling pathways. In this respect, maternal dietary nutrients or food components linked to bone health might play a fundamental role in the prevention of this disease. These include calcium, vitamin D, protein, sodium, magnesium and the most recently studied antioxidant quercetin, which hails from the flavonoid family (Kanter *et al.* 2007). In the current study, the effect of maternal supplementation with quercetin on fetal bone development and adult osteoporosis was examined.

Quercetin

Flavonoids are a group of naturally occurring compounds that are widely distributed as secondary metabolites in the plant kingdom. They have been recognized for their interesting clinical properties, including anti-inflammatory, anti-allergic, anti-viral, anti-bacterial, and anti-tumoral activities (Middleton 1998). Flavonoids, and more recently their metabolites have been reported to function at the phosphoinositide 3-kinase (PI 3-kinase), protein kinase B (PKB), tyrosine kinases, protein kinase C (PKC), and mitogen activated protein kinase (MAP kinase) signaling cascades. Inhibitory or stimulatory effects at these pathways are likely to modulate cellular functions profoundly, via alterations of the phosphorylation states of target molecules, and via the modulation of gene expression (Kim *et al.* 2006). Within the flavonoid family, quercetin is a potent scavenger of ROS, including superoxide, and reactive nitrogen species (RNS) like NO and ONOO⁻. It is ubiquitously present in foods, such as vegetables, fruit, tea and wine as well as food supplements (Pawlikowska-Pawlega *et al.* 2003). Quercetin is thought to promote optimal health, partly via its antioxidant effects in protecting cellular components against ROS (Hertog and Hollman 1996). Quercetin was reported to scavenge superoxide in ischemia-reperfusion injury (Huk *et al.* 1998), to protect against OS induced by ultraviolet light (Erden Inal and Kahraman 2000), to reduce spontaneous hypertension (Garcia-Saura *et al.* 2005), to decrease incidence of secondary biliary cirrhosis (Peres *et al.* 2000), to mitigate adverse effects of bacterial lipopolysaccharide (Wadsworth *et al.* 2001), and to inhibit angiogenesis (Igura *et al.* 2001), carcinogenesis (Yang *et al.* 2000), and portal hypertensive gastropathy (Moreira *et al.* 2004). A recent report indicates that quercetin is also able to partially prevent serum nitric oxide increases in STZ-treated rats (Coskun *et al.* 2005). Quercetin is thought to reduce oxidant injury and cell death by several mechanisms. These mechanisms include

scavenging of oxygen radicals (Andrade *et al.* 2007), protecting against lipid peroxidation (Coskun *et al.* 2005), and chelating metal ions (Afanas'ev *et al.* 1989). The antioxidative effects of quercetin are attributed to the presence of two antioxidant pharmacophores within the molecule that have the optimal configuration for free radical scavenging. Moreover, quercetin is suggested to substantially empower the endogenous antioxidant shield due to its contribution to the total plasma antioxidant capacity (Paravicini and Touyz 2006). It is thought that quercetin readily passes through the placenta, and as such, the developing fetus may benefit from both indirect (placental effects of quercetin exposure) and direct (transplacental quercetin delivery) antioxidant effects of maternal quercetin ingestion (Skibola and Smith 2000). Studies showed that serum levels of 0.65µmol/L quercetin can provide highly efficient protection against OS (Manach *et al.* 1997).

It has recently been demonstrated that quercetin effects a reduction in osteoclastic bone resorption *in vitro* via the direct targeting of the mature osteoclasts by a mechanism involving, at least in part, the estrogen receptor (Wattel *et al.* 2003). Notoya et al. (Notoya *et al.* 2004) reported that quercetin inhibits the proliferation, differentiation, and mineralization of rat calvarial osteoclast-like cells *in vitro*, whereas it inhibits osteoclastic activity. Prouillet et al. (Prouillet *et al.* 2004) reported that quercetin induced an increase in alkaline phosphatase (ALP) activity in human osteoblasts via the activation of the estrogen receptor. These results, together with those previously reported by Wattel and colleagues (Wattel *et al.* 2003) suggest that quercetin can both decrease osteoclastic activity and stimulate osteoblastic activity. Thus, quercetin may represent a new pharmacological tool for the treatment or prevention of osteoporosis.

Micro CT

Traditional techniques to quantify murine fetal skeletal development, such as gross measurements and clear-staining have been widely used in the past, where ethanol-fixed fetuses are taken through a series of chemical immersions to clear soft tissue. Then they are double-stained for ossified and cartilaginous tissues (Trueman *et al.* 1999). The significant long staining time, loss of tissue integrity in the processed fetus and inconsistent results due to excessive or insufficient tissue clearing and staining have limited the quality and extent of data we can collect on environmental effects influencing fetal development. Additionally, these crude methods of evaluating fetal formation exclude visualization of internal morphologic features, and as such offer an incomplete and often inconsistent evaluation of fetal development. Imaging modalities that have been customized for small animal research include tomographic imaging (CT), high field magnetic resonance imaging (MR) and ultrasonography. MR has been validated for analysis of endochondral ossification and bone anatomy in fetal mice (Orita *et al.* 1996; Ichikawa *et al.* 2004). However, the scanning procedure takes long time to perform. Ultrasonography has been validated for cardiac phenotyping in fetal mice (Leatherbury *et al.* 2003). However, this imaging technique is of limited value for skeletal phenotyping because bone surfaces block transmission of sound waves. Therefore, novel noninvasive microcomputed tomographic imaging (micro-CT) methodologies were developed to more specifically and efficiently evaluate fetal skeletal development. Micro-CT is considered a superior method of murine skeletal imaging (Oest *et al.* 2008). Micro-CT is similar to medical CT in that x-rays are collimated to a narrow slice band and projected through the subject over 360 degrees. The transmitted x-ray energy is recorded by detectors, which convert the energy to an electrical signal, which is then converted to numeric data by a computer, which is converted to visible

images based on densities relative to water (Ritman 2004). Micro-CT differs from medical CT in that the x-ray beam energy range is narrow, the field of view is smaller, detectors are much more sensitive, and slice thickness capabilities are much smaller. Micro-CT scanning also offers fine voxel resolutions up to 10µm, so that finer trabecular and cortical detail can now for the first time be observed (Ford-Hutchinson *et al.* 2003). Imaging murine fetal skeletons using micro-CT enables the researcher to nondestructively quantify fetal skeletal development parameters including limb length, total bone volume and average bone mineral density, and permits the investigator to completely evaluate the skeletal system for malformation (Ford-Hutchinson *et al.* 2003). Meanwhile, the nondestructive nature of this technique preserves the fetus for further experiments of soft tissue or histological processing (Oest *et al.* 2008). Micro-CT measurement of fetal limb lengths correlates well with traditional gross measurements, but decreases variability in measurement, decreases data acquisition time by eliminating the time needed for tissue processing, and can preserve the intact fixed fetus for further analysis. Moreover, micro-CT is a power tool for assessing trabecular microarchitectural morphology (such as trabecular number, thickness, spacing, connectivity density and degree of anisotropy) in mature animals (Oest *et al.* 2008). Qualitative and quantitative analyses of skeletal structure can be performed using post-processing software. Three- dimensional reconstructions can be generated and rotated in space so that all aspects of the skeleton can be readily observed and measured (Ford-Hutchinson *et al.* 2003). Therefore, micro-CT provides an accurate, nondestructive method for determining the developmental state of the murine skeleton using not only limb lengths and identification of malformation, but also total skeletal bone volume, average skeletal mineral density, and bone microarchitecture as well. As such, micro-CT was utilized in the present study

to quantify diet-induced changes in fetal skeleton, and adult offspring of control, HFD, or HFD + quercetin fed dams.

Hypothesis

In this study, we hypothesized that maternal HFD in an inbred syngeneic rodent strain of C57 BL/6 mice for one month prior to and through pregnancy will result in the development of GDM, and will serve as a novel model for improving mechanistic understanding of oxidative and metabolic effects of HFD. This will offer avenues to study novel treatments and preventions of GDM on placental and fetal development. More specifically, we hypothesize that concurrent antioxidant supplementation during gestation will at least partially reduce maternal hyperglycemia and hyperinsulinemia induced by HFD.

We also hypothesized that gestational maternal HFD will increase placental OS, inflammation, and induce placental vasculopathy, which may be associated with indirect adverse effects on fetal formation as well as elevate risk of adult-onset type 2 diabetes, hypertension, and osteoporosis; we further hypothesize that maternal antioxidant supplementation with the flavonoid quercetin will partially protect against HFD induced placental OS, inflammation and vasculopathy and protect fetal development as well as long-term cardiovascular, endocrine, and skeletal health in developing and adult offspring.

References

Aerts, L., F. Sodoyez-Goffaux, J. C. Sodoyez, W. J. Malaisse and F. A. Van Assche (1988) The diabetic intrauterine milieu has a long-lasting effect on insulin secretion by B cells and on insulin uptake by target tissues. *Am J Obstet Gynecol*. **159**, 1287-92.

Afanas'ev, I. B., A. I. Dorozhko, A. V. Brodskii, V. A. Kostyuk and A. I. Potapovitch (1989) Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem Pharmacol*. **38**, 1763-9.

Albelda, S. M., P. D. Oliver, L. H. Romer and C. A. Buck (1990) EndoCAM: a novel endothelial cell-cell adhesion molecule. *J Cell Biol*. **110**, 1227-37.

Alexe, D. M., G. Syridou and E. T. Petridou (2006) Determinants of early life leptin levels and later life degenerative outcomes. *Clin Med Res*. **4**, 326-35.

Andrade, A. C., O. Nilsson, K. M. Barnes and J. Baron (2007) Wnt gene expression in the post-natal growth plate: regulation with chondrocyte differentiation. *Bone*. **40**, 1361-9.

Armitage, J. A., P. D. Taylor and L. Poston (2005) Experimental models of developmental programming: consequences of exposure to an energy rich diet during development. *J Physiol*. **565**, 3-8.

Baer, D. J., J. T. Judd, B. A. Clevidence and R. P. Tracy (2004) Dietary fatty acids affect plasma markers of inflammation in healthy men fed controlled diets: a randomized crossover study. *Am J Clin Nutr*. **79**, 969-73.

Bajoria, R., S. R. Sooranna, B. S. Ward and R. Chatterjee (2002) Prospective function of placental leptin at maternal-fetal interface. *Placenta*. **23**, 103-15.

Barker, D. J. (1995) The fetal and infant origins of disease. *Eur J Clin Invest*. **25**, 457-63.

Barker, D. J. (1998) In utero programming of chronic disease. *Clin Sci (Lond)*. **95**, 115-28.

Barker, D. J., C. Osmond, J. Golding, D. Kuh and M. E. Wadsworth (1989) Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *BMJ*. **298**, 564-7.

Barnes, P. J. and M. Karin (1997) Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med*. **336**, 1066-71.

Bastard, J. P., M. Maachi, C. Lagathu, M. J. Kim, M. Caron, H. Vidal, J. Capeau and B. Feve (2006) Recent advances in the relationship between obesity, inflammation, and insulin resistance. *Eur Cytokine Netw*. **17**, 4-12.

Baynes, J. W. (1991) Role of oxidative stress in development of complications in diabetes. *Diabetes*. **40**, 405-12.

Bertoli, S., L. Striuli, G. Testolin, S. Cardinali, P. Veggiotti, G. C. Salvatori and A. Tagliabue (2002) [Nutritional status and bone mineral mass in children treated with ketogenic diet]. *Recenti Prog Med*. **93**, 671-5.

Biswas, P., S. Canosa, J. Schoenfeld, D. Schoenfeld, A. Tucker and J. A. Madri (2003) PECAM-1 promotes beta-catenin accumulation and stimulates endothelial cell proliferation. *Biochem Biophys Res Commun*. **303**, 212-8.

Bloomfield, F. H., M. H. Oliver, C. D. Giannoulias, P. D. Gluckman, J. E. Harding and J. R. Challis (2003) Brief undernutrition in late-gestation sheep programs the hypothalamic-pituitary-adrenal axis in adult offspring. *Endocrinology*. **144**, 2933-40.

Bo, S., G. Menato, A. Lezo, A. Signorile, C. Bardelli, F. De Michieli, M. Massobrio and G. Pagano (2001) Dietary fat and gestational hyperglycaemia. *Diabetologia*. **44**, 972-8.

Briaud, I., C. L. Kelpe, L. M. Johnson, P. O. Tran and V. Poitout (2002) Differential effects of hyperlipidemia on insulin secretion in islets of langerhans from hyperglycemic versus normoglycemic rats. *Diabetes*. **51**, 662-8.

Cao, R., E. Brakenhielm, C. Wahlestedt, J. Thyberg and Y. Cao (2001) Leptin induces vascular permeability and synergistically stimulates angiogenesis with FGF-2 and VEGF. *Proc Natl Acad Sci U S A*. **98**, 6390-5.

Catalano, P. M., J. P. Kirwan, S. Haugel-de Mouzon and J. King (2003) Gestational diabetes and insulin resistance: role in short- and long-term implications for mother and fetus. *J Nutr*. **133**, 1674S-1683S.

Cederberg, J., S. Basu and U. J. Eriksson (2001) Increased rate of lipid peroxidation and protein carbonylation in experimental diabetic pregnancy. *Diabetologia*. **44**, 766-74.

Collins, T. (1993) Endothelial nuclear factor-kappa B and the initiation of the atherosclerotic lesion. *Lab Invest*. **68**, 499-508.

Coskun, O., M. Kanter, A. Korkmaz and S. Oter (2005) Quercetin, a flavonoid antioxidant, prevents and protects streptozotocin-induced oxidative stress and beta-cell damage in rat pancreas. *Pharmacol Res*. **51**, 117-23.

Coughlan, M. T., M. Permezel, H. M. Georgiou and G. E. Rice (2004) Repression of oxidant-induced nuclear factor-kappaB activity mediates placental cytokine responses in gestational diabetes. *J Clin Endocrinol Metab*. **89**, 3585-94.

Dahri, S., A. Snoeck, B. Reusens-Billen, C. Remacle and J. J. Hoet (1991) Islet function in offspring of mothers on low-protein diet during gestation. *Diabetes*. **40 Suppl 2**, 115-20.

Dandrow, R. V. and J. B. O'Sullivan (1966) Obstetric hazards of gestational diabetes. *Am J Obstet Gynecol*. **96**, 1144-7.

Davies, J. H., B. A. Evans and J. W. Gregory (2005) Bone mass acquisition in healthy children. *Arch Dis Child*. **90**, 373-8.

DeLisser, H. M., P. J. Newman and S. M. Albelda (1994) Molecular and functional aspects of PECAM-1/CD31. *Immunol Today*. **15**, 490-5.

Desai, M., N. J. Crowther, A. Lucas and C. N. Hales (1996) Organ-selective growth in the offspring of protein-restricted mothers. *Br J Nutr*. **76**, 591-603.

Dresner, A., D. Laurent, M. Marcucci, M. E. Griffin, S. Dufour, G. W. Cline, L. A. Slezak, D. K. Andersen, R. S. Hundal, D. L. Rothman, K. F. Petersen and G. I. Shulman (1999) Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest*. **103**, 253-9.

Ducy, P., M. Amling, S. Takeda, M. Priemel, A. F. Schilling, F. T. Beil, J. Shen, C. Vinson, J. M. Rueger and G. Karsenty (2000) Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell*. **100**, 197-207.

Elchuri, S., T. D. Oberley, W. Qi, R. S. Eisenstein, L. Jackson Roberts, H. Van Remmen, C. J. Epstein and T. T. Huang (2005) CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life. *Oncogene*. **24**, 367-80.

Erden Inal, M. and A. Kahraman (2000) The protective effect of flavonol quercetin against ultraviolet a induced oxidative stress in rats. *Toxicology*. **154**, 21-9.

Erhardt, J. G., S. S. Lim, J. C. Bode and C. Bode (1997) A diet rich in fat and poor in dietary fiber increases the in vitro formation of reactive oxygen species in human feces. *J Nutr*. **127**, 706-9.

Ford-Hutchinson, A. F., D. M. Cooper, B. Hallgrimsson and F. R. Jirik (2003) Imaging skeletal pathology in mutant mice by microcomputed tomography. *J Rheumatol*. **30**, 2659-65.

Gao, Z., Q. He, B. Peng, P. J. Chiao and J. Ye (2006) Regulation of nuclear translocation of HDAC3 by IkappaBalpha is required for tumor necrosis factor inhibition of peroxisome proliferator-activated receptor gamma function. *J Biol Chem*. **281**, 4540-7.

Garcia-Patterson, A., L. Erdozain, G. Ginovart, J. M. Adelantado, J. M. Cubero, G. Gallo, A. de Leiva and R. Corcoy (2004) In human gestational diabetes mellitus congenital malformations are related to pre-pregnancy body mass index and to severity of diabetes. *Diabetologia*. **47**, 509-14.

Garcia-Saura, M. F., M. Galisteo, I. C. Villar, A. Bermejo, A. Zarzuelo, F. Vargas and J. Duarte (2005) Effects of chronic quercetin treatment in experimental renovascular hypertension. *Mol Cell Biochem*. **270**, 147-55.

Gennser, G., P. Rymark and P. E. Isberg (1988) Low birth weight and risk of high blood pressure in adulthood. *Br Med J (Clin Res Ed)*. **296**, 1498-500.

Gerber, R. T., K. Holemans, I. O'Brien-Coker, A. I. Mallet, R. van Bree, F. A. Van Assche and L. Poston (1999) Cholesterol-independent endothelial dysfunction in virgin and pregnant rats fed a diet high in saturated fat. *J Physiol*. **517 (Pt 2**), 607-16.

Gluckman, P. D. and M. A. Hanson (2004a) The developmental origins of the metabolic syndrome. *Trends Endocrinol Metab*. **15**, 183-7.

Gluckman, P. D. and M. A. Hanson (2004b) Living with the past: evolution, development, and patterns of disease. *Science*. **305**, 1733-6.

Gluckman, P. D., M. A. Hanson and C. Pinal (2005) The developmental origins of adult disease. *Matern Child Nutr*. **1**, 130-41.

Gordeladze, J. O. and J. E. Reseland (2003) A unified model for the action of leptin on bone turnover. *J Cell Biochem*. **88**, 706-12.

Guo, F. and K. L. Jen (1995) High-fat feeding during pregnancy and lactation affects offspring metabolism in rats. *Physiol Behav*. **57**, 681-6.

H.E., L. and M. A. Banerji (2004) Treatment of insulin resistance in diabetes mellitus. *Eur J Pharmacol*. **490**, 135-46.

Haddad, J. J. (2002) Oxygen homeostasis, thiol equilibrium and redox regulation of signalling transcription factors in the alveolar epithelium. *Cell Signal*. **14**, 799-810.

Hales, C. N. and D. J. Barker (1992) Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia*. **35**, 595-601.

Hales, C. N. and D. J. Barker (2001) The thrifty phenotype hypothesis. *Br Med Bull*. **60**, 5-20.

Hales, C. N., D. J. Barker, P. M. Clark, L. J. Cox, C. Fall, C. Osmond and P. D. Winter (1991) Fetal and infant growth and impaired glucose tolerance at age 64. *BMJ*. **303**, 1019-22.

Halliwell, B. (1997) Antioxidants and human disease: a general introduction. *Nutr Rev*. **55**, S44-9; discussion S49-52.

Henson, M. C. and V. D. Castracane (2002) Leptin: roles and regulation in primate pregnancy. *Semin Reprod Med*. **20**, 113-22.

Hertog, M. G. and P. C. Hollman (1996) Potential health effects of the dietary flavonol quercetin. *Eur J Clin Nutr*. **50**, 63-71.

Hill, D. J., J. Petrik and E. Arany (1998) Growth factors and the regulation of fetal growth. *Diabetes Care*. **21 Suppl 2**, B60-9.

Holemans, K., S. Caluwaerts, L. Poston and F. A. Van Assche (2004) Diet-induced obesity in the rat: a model for gestational diabetes mellitus. *Am J Obstet Gynecol*. **190**, 858-65.

Holt, R. I. (2002) Fetal programming of the growth hormone-insulin-like growth factor axis. *Trends Endocrinol Metab*. **13**, 392-7.

Huk, I., V. Brovkovych, J. Nanobash Vili, G. Weigel, C. Neumayer, L. Partyka, S. Patton and T. Malinski (1998) Bioflavonoid quercetin scavenges superoxide and increases nitric oxide concentration in ischaemia-reperfusion injury: an experimental study. *Br J Surg*. **85**, 1080-5.

Ichikawa, Y., M. Sumi, N. Ohwatari, T. Komori, T. Sumi, H. Shibata, T. Furuichi, A. Yamaguchi and T. Nakamura (2004) Evaluation of 9.4-T MR microimaging in assessing normal and defective fetal bone development: comparison of MR imaging and histological findings. *Bone*. **34**, 619-28.

Igura, K., T. Ohta, Y. Kuroda and K. Kaji (2001) Resveratrol and quercetin inhibit angiogenesis in vitro. *Cancer Lett*. **171**, 11-6.

Ilan, N., S. Mahooti, D. L. Rimm and J. A. Madri (1999) PECAM-1 (CD31) functions as a reservoir for and a modulator of tyrosine-phosphorylated beta-catenin. *J Cell Sci*. **112 Pt 18**, 3005-14.

Inoue, N., S. Ramasamy, T. Fukai, R. M. Nerem and D. G. Harrison (1996) Shear stress modulates expression of Cu/Zn superoxide dismutase in human aortic endothelial cells. *Circ Res*. **79**, 32-7.

Inzerillo, A. M. and S. Epstein (2004) Osteoporosis and diabetes mellitus. *Rev Endocr Metab Disord*. **5**, 261-8.

Jansson, T. and T. L. Powell (2007) Role of the placenta in fetal programming: underlying mechanisms and potential interventional approaches. *Clin Sci (Lond)*. **113**, 1-13.

Jauniaux, E., L. Poston and G. J. Burton (2006) Placental-related diseases of pregnancy: Involvement of oxidative stress and implications in human evolution. *Hum Reprod Update*. **12**, 747-55.

Kahn, B. B. (1998) Type 2 diabetes: when insulin secretion fails to compensate for insulin resistance. *Cell*. **92**, 593-6.

Kanter, M., M. F. Altan, S. Donmez, A. Ocakci and M. E. Kartal (2007) The effects of quercetin on bone minerals, biomechanical behavior, and structure in streptozotocin-induced diabetic rats. *Cell Biochem Funct*. **25**, 747-52.

Karnik, H. B., B. R. Sonawane, J. S. Adkins and S. Mohla (1989) High dietary fat feeding during perinatal development of rats alters hepatic drug metabolism of progeny. *Dev Pharmacol Ther*. **14**, 135-40.

Kervran, A., M. Guillaume and A. Jost (1978) The endocrine pancreas of the fetus from diabetic pregnant rat. *Diabetologia*. **15**, 387-93.

Khan, I. Y., V. Dekou, G. Douglas, R. Jensen, M. A. Hanson, L. Poston and P. D. Taylor (2005) A high-fat diet during rat pregnancy or suckling induces cardiovascular dysfunction in adult offspring. *Am J Physiol Regul Integr Comp Physiol*. **288**, R127-33.

Kim, Y. J., Y. C. Bae, K. T. Suh and J. S. Jung (2006) Quercetin, a flavonoid, inhibits proliferation and increases osteogenic differentiation in human adipose stromal cells. *Biochem Pharmacol*. **72**, 1268-78.

Kingdom, J., B. Huppertz, G. Seaward and P. Kaufmann (2000) Development of the placental villous tree and its consequences for fetal growth. *Eur J Obstet Gynecol Reprod Biol*. **92**, 35-43.

Koukkou, E., P. Ghosh, C. Lowy and L. Poston (1998) Offspring of normal and diabetic rats fed saturated fat in pregnancy demonstrate vascular dysfunction. *Circulation*. **98**, 2899-904.

La Cava, A., C. Alviggi and G. Matarese (2004) Unraveling the multiple roles of leptin in inflammation and autoimmunity. *J Mol Med*. **82**, 4-11.

Langley-Evans, S. C. (1996) Intrauterine programming of hypertension in the rat: nutrient interactions. *Comp Biochem Physiol A Physiol*. **114**, 327-33.

Langley, S. C. and A. A. Jackson (1994) Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. *Clin Sci (Lond)*. **86**, 217-22; discussion 121.

Lappas, M., M. Permezel, H. M. Georgiou and G. E. Rice (2002) Nuclear factor kappa B regulation of proinflammatory cytokines in human gestational tissues in vitro. *Biol Reprod*. **67**, 668-73.

Lappas, M., K. Yee, M. Permezel and G. E. Rice (2005) Release and regulation of leptin, resistin and adiponectin from human placenta, fetal membranes, and maternal adipose tissue and skeletal muscle from normal and gestational diabetes mellitus-complicated pregnancies. *J Endocrinol*. **186**, 457-65.

Leatherbury, L., Q. Yu and C. W. Lo (2003) Noninvasive phenotypic analysis of cardiovascular structure and function in fetal mice using ultrasound. *Birth Defects Res C Embryo Today*. **69**, 83-91.

Lecka-Czernik, B., E. J. Moerman, D. F. Grant, J. M. Lehmann, S. C. Manolagas and R. L. Jilka (2002) Divergent effects of selective peroxisome proliferator-activated receptor-gamma 2 ligands on adipocyte versus osteoblast differentiation. *Endocrinology*. **143**, 2376-84.

Lowell, B. B. and G. I. Shulman (2005) Mitochondrial dysfunction and type 2 diabetes. *Science*. **307**, 384-7.

Lumey, L. H., A. C. Ravelli, L. G. Wiessing, J. G. Koppe, P. E. Treffers and Z. A. Stein (1993) The Dutch famine birth cohort study: design, validation of exposure, and selected

characteristics of subjects after 43 years follow-up. *Paediatr Perinat Epidemiol*. **7**, 354-67.

Lyall, F., J. N. Bulmer, E. Duffie, F. Cousins, A. Theriault and S. C. Robson (2001) Human trophoblast invasion and spiral artery transformation: the role of PECAM-1 in normal pregnancy, preeclampsia, and fetal growth restriction. *Am J Pathol*. **158**, 1713-21.

Lyall, F., J. L. Gibson, I. A. Greer, D. E. Brockman, A. L. Eis and L. Myatt (1998) Increased nitrotyrosine in the diabetic placenta: evidence for oxidative stress. *Diabetes Care*. **21**, 1753-8.

Manach, C., C. Morand, C. Demigne, O. Texier, F. Regerat and C. Remesy (1997) Bioavailability of rutin and quercetin in rats. *FEBS letters*. **409**, 12-6.

Manolagas, S. C. and M. Almeida (2007) Gone with the Wnts: beta-catenin, T-cell factor, forkhead box O, and oxidative stress in age-dependent diseases of bone, lipid, and glucose metabolism. *Mol Endocrinol*. **21**, 2605-14.

Mayhew, T. M., D. S. Charnock-Jones and P. Kaufmann (2004) Aspects of human fetoplacental vasculogenesis and angiogenesis. III. Changes in complicated pregnancies. *Placenta*. **25**, 127-39.

Megia, A., L. Gallart, J. M. Fernandez-Real, J. Vendrell, I. Simon, C. Gutierrez and C. Richart (2004) Mannose-binding lectin gene polymorphisms are associated with gestational diabetes mellitus. *J Clin Endocrinol Metab*. **89**, 5081-7.

Middleton, E., Jr. (1998) Effect of plant flavonoids on immune and inflammatory cell function. *Adv Exp Med Biol*. **439**, 175-82.

Moreira, A. J., C. Fraga, M. Alonso, P. S. Collado, C. Zetller, C. Marroni, N. Marroni and J. Gonzalez-Gallego (2004) Quercetin prevents oxidative stress and NF-kappaB activation in gastric mucosa of portal hypertensive rats. *Biochem Pharmacol*. **68**, 1939-46.

Muller, W. A., C. M. Ratti, S. L. McDonnell and Z. A. Cohn (1989) A human endothelial cell-restricted, externally disposed plasmalemmal protein enriched in intercellular junctions. *J Exp Med*. **170**, 399-414.

Muller, W. A., S. A. Weigl, X. Deng and D. M. Phillips (1993) PECAM-1 is required for transendothelial migration of leukocytes. *J Exp Med*. **178**, 449-60.

Myatt, L. (2006) Placental adaptive responses and fetal programming. *J Physiol*. **572**, 25-30.

Myatt, L., W. Kossenjans, R. Sahay, A. Eis and D. Brockman (2000) Oxidative stress causes vascular dysfunction in the placenta. *J Matern Fetal Med*. **9**, 79-82.

Negre-Salvayre, A., C. Hirtz, G. Carrera, R. Cazenave, M. Troly, R. Salvayre, L. Penicaud and L. Casteilla (1997) A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. *FASEB J*. **11**, 809-15.

Newman, P. J., M. C. Berndt, J. Gorski, G. C. White, 2nd, S. Lyman, C. Paddock and W. A. Muller (1990) PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. *Science*. **247**, 1219-22.

Notoya, M., Y. Tsukamoto, H. Nishimura, J. T. Woo, K. Nagai, I. S. Lee and H. Hagiwara (2004) Quercetin, a flavonoid, inhibits the proliferation, differentiation, and mineralization of osteoblasts in vitro. *Eur J Pharmacol*. **485**, 89-96.

Oest, M. E., J. C. Jones, C. Hatfield and M. R. Prater (2008) Micro-CT evaluation of murine fetal skeletal development yields greater morphometric precision over traditional clear-staining methods. *Birth Defects Res A Clin Mol Teratol*. **83**, 582-589.

Oh, W., N. L. Gelardi and C. J. Cha (1988) Maternal hyperglycemia in pregnant rats: its effect on growth and carbohydrate metabolism in the offspring. *Metabolism: clinical and experimental*. **37**, 1146-51.

Oh, W., N. L. Gelardi and C. J. Cha (1991) The cross-generation effect of neonatal macrosomia in rat pups of streptozotocin-induced diabetes. *Pediatr Res*. **29**, 606-10.

Orita, J., E. Sato, S. Saburi, T. Nishida and Y. Toyoda (1996) Magnetic resonance imaging of the internal structure of the mouse fetus. *Experimental animals / Japanese Association for Laboratory Animal Science*. **45**, 171-4.

Ornoy, A. (2007) Embryonic oxidative stress as a mechanism of teratogenesis with special emphasis on diabetic embryopathy. *Reprod Toxicol*. **24**, 31-41.

Ozanne, E. S. (2001) Metabolic programming in animals. *British Medical Bulletin*. **60**, 143-152.

Palinski, W., F. P. D'Armiento, J. L. Witztum, F. de Nigris, F. Casanada, M. Condorelli, M. Silvestre and C. Napoli (2001) Maternal hypercholesterolemia and treatment during pregnancy influence the long-term progression of atherosclerosis in offspring of rabbits. *Circ Res*. **89**, 991-6.

Paravicini, T. M. and R. M. Touyz (2006) Redox signaling in hypertension. *Cardiovasc Res*. **71**, 247-58.

Pawlikowska-Pawlega, B., W. I. Gruszecki, L. E. Misiak and A. Gawron (2003) The study of the quercetin action on human erythrocyte membranes. *Biochem Pharmacol*. **66**, 605-12.

Peres, W., M. J. Tunon, P. S. Collado, S. Herrmann, N. Marroni and J. Gonzalez-Gallego (2000) The flavonoid quercetin ameliorates liver damage in rats with biliary obstruction. *J Hepatol*. **33**, 742-50.

Persson, E. and T. Jansson (1992) Low birth weight is associated with elevated adult blood pressure in the chronically catheterized guinea-pig. *Acta Physiol Scand*. **145**, 195-6.

Poulsen, P., A. A. Vaag, K. O. Kyvik, D. Moller Jensen and H. Beck-Nielsen (1997) Low birth weight is associated with NIDDM in discordant monozygotic and dizygotic twin pairs. *Diabetologia*. **40**, 439-46.

Prater, M. R., C. L. Laudermilch and S. D. Holladay (2006) Does Immune Stimulation or Antioxidant Therapy Reduce MNU-induced Placental Damage Via Activation of Jak-STAT and NFkappaB Signaling Pathways? *Placenta*. **28**, 566-70.

Prouillet, C., J. C. Maziere, C. Maziere, A. Wattel, M. Brazier and S. Kamel (2004) Stimulatory effect of naturally occurring flavonols quercetin and kaempferol on alkaline phosphatase activity in MG-63 human osteoblasts through ERK and estrogen receptor pathway. *Biochem Pharmacol*. **67**, 1307-13.

Pustovrh, M. C., A. Jawerbaum, E. Capobianco, V. White, N. Martinez, J. J. Lopez-Costa and E. Gonzalez (2005) Oxidative stress promotes the increase of matrix metalloproteinases-2 and -9 activities in the feto-placental unit of diabetic rats. *Free Radic Res*. **39**, 1285-93.

Qiu, C., S. Vadachkoria, L. Meryman, I. O. Frederick and M. A. Williams (2005) Maternal plasma concentrations of IGF-1, IGFBP-1, and C-peptide in early pregnancy and subsequent risk of gestational diabetes mellitus. *Am J Obstet Gynecol*. **193**, 1691-7.

Ribatti, D., M. T. Conconi and G. G. Nussdorfer (2007) Nonclassic endogenous novel [corrected] regulators of angiogenesis. *Pharmacol Rev*. **59**, 185-205.

Ribeiro, P. S., H. Cortez-Pinto, S. Sola, R. E. Castro, R. M. Ramalho, A. Baptista, M. C. Moura, M. E. Camilo and C. M. Rodrigues (2004) Hepatocyte apoptosis, expression of death

receptors, and activation of NF-kappaB in the liver of nonalcoholic and alcoholic steatohepatitis patients. *Am J Gastroenterol*. **99**, 1708-17.

Riggs, B. L., H. W. Wahner, E. Seeman, K. P. Offord, W. L. Dunn, R. B. Mazess, K. A. Johnson and L. J. Melton, 3rd (1982) Changes in bone mineral density of the proximal femur and spine with aging. Differences between the postmenopausal and senile osteoporosis syndromes. *J Clin Invest*. **70**, 716-23.

Ritman, E. L. (2004) Micro-computed tomography-current status and developments. *Annu Rev Biomed Eng*. **6**, 185-208.

Rodrigo, R., M. Parra, C. Bosco, V. Fernandez, P. Barja, J. Guajardo and R. Messina (2005) Pathophysiological basis for the prophylaxis of preeclampsia through early supplementation with antioxidant vitamins. *Pharmacol Ther*. **107**, 177-97.

Rolo, A. P. and C. M. Palmeira (2006) Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress. *Toxicol Appl Pharmacol*. **212**, 167-78.

Saltiel, A. R. and J. M. Olefsky (1996) Thiazolidinediones in the treatment of insulin resistance and type II diabetes. *Diabetes*. **45**, 1661-9.

San Martin, R. and L. Sobrevia (2006) Gestational diabetes and the adenosine/L-arginine/nitric oxide (ALANO) pathway in human umbilical vein endothelium. *Placenta*. **27**, 1-10.

Shao, J., H. Yamashita, L. Qiao, B. Draznin and J. E. Friedman (2002) Phosphatidylinositol 3-kinase redistribution is associated with skeletal muscle insulin resistance in gestational diabetes mellitus. *Diabetes*. **51**, 19-29.

Sibley, C. P., M. A. Turner, I. Cetin, P. Ayuk, C. A. Boyd, S. W. D'Souza, J. D. Glazier, S. L. Greenwood, T. Jansson and T. Powell (2005) Placental phenotypes of intrauterine growth. *Pediatr Res*. **58**, 827-32.

Sikkema, J. M., B. B. van Rijn, A. Franx, H. W. Bruinse, R. de Roos, E. S. Stroes and E. E. van Faassen (2001) Placental superoxide is increased in pre-eclampsia. *Placenta*. **22**, 304-8.

Skibola, C. F. and M. T. Smith (2000) Potential health impacts of excessive flavonoid intake. *Free Radic Biol Med*. **29**, 375-83.

Stevenson, D. K., A. O. Hopper, R. S. Cohen, L. R. Bucalo, J. A. Kerner and P. Sunshine (1982) Macrosomia: causes and consequences. *J Pediatr*. **100**, 515-20.

Stoker, A. W. (2005) Protein tyrosine phosphatases and signalling. *J Endocrinol*. **185**, 19-33.

Styrud, J., L. Thunberg, O. Nybacka and U. J. Eriksson (1995) Correlations between maternal metabolism and deranged development in the offspring of normal and diabetic rats. *Pediatr Res*. **37**, 343-53.

Takada, Y., A. Gillenwater, H. Ichikawa and B. B. Aggarwal (2006) Suberoylanilide hydroxamic acid potentiates apoptosis, inhibits invasion, and abolishes osteoclastogenesis by suppressing nuclear factor-kappaB activation. *J Biol Chem*. **281**, 5612-22.

Tasaka, S., L. Qin, A. Saijo, S. M. Albelda, H. M. DeLisser and C. M. Doerschuk (2003) Platelet endothelial cell adhesion molecule-1 in neutrophil emigration during acute bacterial pneumonia in mice and rats. *Am J Respir Crit Care Med*. **167**, 164-70.

Taylor, P. D., I. Y. Khan, M. A. Hanson and L. Poston (2004) Impaired EDHF-mediated vasodilatation in adult offspring of rats exposed to a fat-rich diet in pregnancy. *J Physiol*. **558**, 943-51.

Taylor, P. D., J. McConnell, I. Y. Khan, K. Holemans, K. M. Lawrence, H. Asare-Anane, S. J. Persaud, P. M. Jones, L. Petrie, M. A. Hanson and L. Poston (2005) Impaired glucose homeostasis and mitochondrial abnormalities in offspring of rats fed a fat-rich diet in pregnancy. *Am J Physiol Regul Integr Comp Physiol*. **288**, R134-9.

Tjoa, M. L., T. Cindrova-Davies, O. Spasic-Boskovic, D. W. Bianchi and G. J. Burton (2006) Trophoblastic oxidative stress and the release of cell-free feto-placental DNA. *Am J Pathol*. **169**, 400-4.

Tonks, N. K. (2005) Redox redux: revisiting PTPs and the control of cell signaling. *Cell*. **121**, 667-70.

Trueman, D., S. W. Jackson and B. Trueman (1999) An automated technique for double staining rat and rabbit fetal skeletal specimens to differentiate bone and cartilage. *Biotech Histochem*. **74**, 98-104.

Vickers, M. H., P. D. Gluckman, A. H. Coveny, P. L. Hofman, W. S. Cutfield, A. Gertler, B. H. Breier and M. Harris (2008) The effect of neonatal leptin treatment on postnatal weight gain in male rats is dependent on maternal nutritional status during pregnancy. *Endocrinology*. **149**, 1906-13.

Wadsworth, T. L., T. L. McDonald and D. R. Koop (2001) Effects of Ginkgo biloba extract (EGb 761) and quercetin on lipopolysaccharide-induced signaling pathways involved in the release of tumor necrosis factor-alpha. *Biochem Pharmacol*. **62**, 963-74.

Ward, W. E., S. Kim and W. Robert Bruce (2003) A western-style diet reduces bone mass and biomechanical bone strength to a greater extent in male compared with female rats during development. *Br J Nutr*. **90**, 589-95.

Wattel, A., S. Kamel, R. Mentaverri, F. Lorget, C. Prouillet, J. P. Petit, P. Fardelonne and M. Brazier (2003) Potent inhibitory effect of naturally occurring flavonoids quercetin and kaempferol on in vitro osteoclastic bone resorption. *Biochem Pharmacol*. **65**, 35-42.

Wender-Ozegowska, E., K. Wroblewska, A. Zawiejska, M. Pietryga, J. Szczapa and R. Biczysko (2005) Threshold values of maternal blood glucose in early diabetic pregnancy--prediction of fetal malformations. *Acta Obstet Gynecol Scand*. **84**, 17-25.

White, V., A. Jawerbaum, D. Sinner, C. Pustovrh, E. Capobianco and E. Gonzalez (2002) Oxidative stress and altered prostanoid production in the placenta of streptozotocin-induced diabetic rats. *Reprod Fertil Dev*. **14**, 117-23.

Widdowson, P. S. (1997) Obesity in diabetes and the impact of leptin. *Diabetes Rev Int*. **6**, 2-5.

Wilson, B. D., M. M. Ollmann and G. S. Barsh (1999) The role of agouti-related protein in regulating body weight. *Molecular medicine today*. **5**, 250-6.

Woodall, S. M., B. M. Johnston, B. H. Breier and P. D. Gluckman (1996) Chronic maternal undernutrition in the rat leads to delayed postnatal growth and elevated blood pressure of offspring. *Pediatr Res*. **40**, 438-43.

Yang, K., S. A. Lamprecht, Y. Liu, H. Shinozaki, K. Fan, D. Leung, H. Newmark, V. E. Steele, G. J. Kelloff and M. Lipkin (2000) Chemoprevention studies of the flavonoids quercetin and rutin in normal and azoxymethane-treated mouse colon. *Carcinogenesis*. **21**, 1655-60.

Zernicke, R. F., G. J. Salem, R. J. Barnard and E. Schramm (1995) Long-term, high-fat-sucrose diet alters rat femoral neck and vertebral morphology, bone mineral content, and mechanical properties. *Bone*. **16**, 25-31.

Zhou, Y., S. J. Fisher, M. Janatpour, O. Genbacev, E. Dejana, M. Wheelock and C. H. Damsky (1997) Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? *J Clin Invest*. **99**, 2139-51.

CHAPTER II

Experimental Methods and Protocols

Methods for both *in vitro and in vivo* experiments were utilized in this dissertation. Animals were humanly handled. Experiments were all approved by the Virginia Tech Animal Care and Use Committee and not initiated until approval was granted.

Chapter III:

1) Mouse body weight was recorded on a top-loading balance.

2) Maternal blood samples were obtained via tail venipuncture to determine insulin and glucose levels. Non-fasting blood glucose levels were determined by glucometer.

3) Non-fasting plasma insulin levels were quantified by enzyme-linked immunosorbent assay (ELISA)

4) Placental weights were recorded on a top-loading balance.

5) Placental histopathology was evaluated by hematoxylin-eosin (H & E) staining for morphological analysis under light microscopy.

6) Placental OS were assessed by malondialdehyde (MDA) analysis

7) Placental CD 31 expression was examined by immunofluorescence staining and confocal microscopy.

Chapter IV:

1) Fetal weights were recorded on a top-loading balance.

2) Fetal gross measurements of crown-to- rump length was measured by an Olympus SZX7 Stereomicroscope and Image J software was used for pixel-to-mm conversion.

3) Fetal BV, BMD and skeletal structural lengths were measured by micro-CT.

Chapter V:

1) Six and twelve month old offspring body weight was recorded on a top-loading balance.

2) Adult mouse blood samples were obtained via tail venipuncture to determine insulin and glucose levels. Non-fasting blood glucose levels were determined by glucometer.

3) Adult mouse non-fasting plasma insulin levels were quantified by enzyme-linked immunosorbent assay (ELISA)

4) Systolic and diastolic blood pressure (BP) were determined by noninvasive tail cuff method, using the SC1000 blood pressure analysis system.

5) Adult offspring bone development was evaluated by micro-CT.

CHAPTER III

High Saturated Fat Diet Induces Gestational Diabetes and Placental Vasculopathy in C57BL/6 Mice

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Abstract

Gestational diabetes mellitus (GDM), which is described as impaired glucose tolerance during mid- to late-pregnancy, is associated with fetal malformation and increased risk for type 2 diabetes mellitus later in life. The etiology of GDM is thought to be multifactorial in origin, including both genetic predisposition and contribution of environmental factors. However, mechanisms are incompletely understood, and current GDM animal models do not appear to closely mimic the clinical situation in humans. In the present study, we used environmental exposure to high saturated fat diet (HFD) to develop a GDM mouse model, which closely simulates the metabolic abnormalities seen in human GDM. This model was then used to determine the relative contributions of HFD-induced insulin resistance, elevated oxidative stress (OS), and placental vascular dysregulation to quantify pathologic manifestations of the disease. This model was also used to explore potential preventive intervention with dietary antioxidant to reduce incidence and severity of GDM, which could be applied to improved human health. In this study, female C57BL/6 mice were fed "cafeteria-style" HFD (including 32.1% saturated fat to mimic a typical fast food menu) for one month prior to conception, and throughout gestation. Dam body weight increased from gestation day 0 (GD 0) to GD 19 by 41% with HFD, as compared to 23% in control dams; HFD dams also developed insulin resistance (66% increase in plasma insulin and 27% increase in plasma glucose levels by GD 10) as compared to control dams. GD 19 placental weight was 19% lower in HFD vs. control mice, which was attributed to decreased vascular and cellular viability and OS-mediated cellular loss. Our findings suggested that a diet high in saturated fat prior to and during pregnancy alters glucose metabolism, characteristic of GDM, and increases placental vasculopathy that may impair successful

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pregnancy outcome. Further, we determined that concurrent dietary antioxidant supplementation may provide partial protection against HFD-induced adverse effects during pregnancy.

Introduction

GDM is characterized by hyperglycemia, hyperinsulinemia, and insulin resistance, as well as placental vascular maldevelopment and fetal malformation (Shao *et al.* 2002). GDM affects 2–8% of all pregnancies with increased risk of development of diabetes and obesity in offspring, and elevated long-term risk of development of type 2 diabetes in the mother (Shao *et al.* 2002). It is believed that hormones produced in pregnancy increase insulin resistance, which is then followed by beta cell dysfunction and resistance to insulin-mediated glucose disposal. Beta cells normally compensate for insulin resistance by secreting more insulin to maintain the glucose homeostasis, which results in a compensatory hyperinsulinemia. Progressive impairment of beta cell function leads to deterioration of glucose homeostasis and subsequent development of hyperglycemia, hyperinsulinemia, impaired glucose tolerance, and frank diabetes (Saltiel and Olefsky 1996; Lebovitz and Banerji 2004).

During recent years, scientists have recognized that genetic factors are unable to fully explain the development of GDM. Environmental factors, such as a high saturated fat intake, may directly promote glucose intolerance during pregnancy (Bo *et al.* 2001). Recent changes in Western lifestyles include increased intake of fast food, which is high in saturated fat. Consumption of such a diet increases OS and adversely affects endocrine, cardiovascular and bone health, and causes poorly-controlled hyperglycemia, hyperinsulinemia, and insulin resistance (Bo *et al.* 2001). Consumption of HFD during pregnancy may also contribute to

hyperglycemia, hyperinsulinemia, and insulin resistance through similar mechanisms, and may elevate risk of GDM.

Human GDM is associated with placental vascular dysfunction that reduces blood flow, gas and nutrient supply from placenta to fetuses (Garcia-Patterson *et al.* 2004), and these changes are thought to be mediated by elevated OS. HFD and hyperglycemia increase OS either by elevating production of reactive oxygen species (ROS) or by decreasing antioxidant capacity. Fatty acid consumption is thought to decrease superoxide dismutase (SOD) and increase production of superoxide by the mitochondrial electron-transport chain, which together generate excess ROS (Rolo and Palmeira 2006). SOD is considered to be an important component of placental health in pregnancy: SOD aids placental trophoblast differentiation, and diminished placental SOD increases trophoblast apoptosis and syncitialization, as is seen in preeclampsia (Myatt *et al.* 2000; Sikkema *et al.* 2001). Diabetic women demonstrate placental vascular damage due to increased superoxide generation, and this underscores the importance of mitigating OS to protect placental health during pregnancy and improve birth outcome (Lyall *et al.* 1998).

Quercetin is one of the most potent antioxidants in the flavonoid family, and is found in apples, grapes, citrus fruits, onions, and many green vegetables. Quercetin contains two antioxidant pharmacophores within the molecule that deliver the optimal configuration for free radical scavenging (Paravicini and Touyz 2006). Quercetin can readily pass through the placenta, so that several components of the conceptus, including fetus and placenta, can benefit from maternal quercetin ingestion (Skibola and Smith 2000). In this study, the protective antioxidant effects of quercetin against HFD-induced hyperglycemia, hyperinsulinemia, insulin resistance, and placental vasculopathy were examined.

Animal models of diabetes during pregnancy are crucial to explore the pathophysiologic aspects of human GDM. Although there are several animal models (genetic as well as chemically-induced) available for the study of GDM, none closely mimic the pattern of disease initiation and development seen in the clinical situation in humans. The classical use of streptozocin (STZ) during pregnancy to induce hyperglycemia results in almost complete destruction of pancreatic beta cells. The suitability of this model has been questioned because destruction of pancreatic beta cells typifies type 1 rather than type 2 diabetes mellitus or GDM: mean glucose levels often exceed 350mg/dl (Holemans *et al.* 2004) and beta cell destruction causes insulin deficiency rather than insulin resistance and reactive hyperinsulinemia (Rerup 1970). Consequently, low dose STZ has been widely used to induce hyperglycemia, but its use in the study of GDM is not ideal due to its unpredictable effects on pancreatic insulin secretion (Aerts *et al.* 1988; Oh *et al.* 1988; Oh *et al.* 1991). Transgenic GDM animal models are also available; however, these genetically altered animals do not tend to develop hyperglycemia similarly to human GDM (Luo *et al.* 1998). Moreover, the observation derived from these genetic strains may not always be satisfactorily extended to the human population as a whole because GDM patients have not been shown to express the gene alterations that cause hyperglycemia in these genetically altered animal models (Widdowson 1997; Wilson *et al.* 1999).

The present study endeavors to develop a GDM mouse model using HFD that closely simulates the common metabolic abnormalities seen in human GDM, and to explore the pathophysiologic mechanisms linking gestational HFD to elevated OS, insulin resistance and placental vascular damage.

Materials and Methods

Animals and dietary protocol

Male and female C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 6 weeks of age. Mice were housed in groups of five in a temperature (22.0 $\pm$ 1ºC), humidity (40%-60%), and light-controlled (12/12 h light/dark) room for a 2-week acclimation period. Mice were given food and fresh water *ad libitum*. Experiments were all approved by the Virginia Tech Animal Care and Use Committee and not initiated until approval was granted. Female mice were arbitrarily assigned to one of three treatment groups: control, HFD, or HFD + quercetin, in a generalized randomized complete block design, with 16 mice per group. Mice were fed control rodent diet (Harlan Teklad Global Diet 2018: 18% protein and 5% fat, Madison, WI), HFD (20% protein, 60% total fat, 32.1% saturated fat, Research Diets Lab, New Brunswick, NJ) (Table 1) or HFD supplemented with 66mg/kg quercetin (HFD/Q). A typical daily human consumption is 700mg/ day (200-400mg 3x/day) (Manach *et al.* 1997). Following one month dietary intervention, breeding studies were initiated. For breeding, male mice were housed individually, and then bred to females overnight in a 1:2 ratio; mating was confirmed by the presence of a vaginal mucous plug the next morning. The presence of the vaginal plug was designated day 0 of gestation.

Body weight, blood glucose and plasma insulin measurements

Female body weight, blood glucose, and plasma insulin were recorded prior to dietary intervention, after four weeks HFD, and at GD 0, 10, and 19. Body weight was recorded on a top-loading balance (Accu-622, Fisher Scientific, Suwanee, GA), and maternal blood samples were obtained via tail venipuncture to determine insulin and glucose levels. Non-fasting blood

glucose levels were determined by glucometer (Lifescan Surestep, Johnson & Johnson, Langhorne, PA) and non-fasting plasma insulin levels were quantified by enzyme-linked immunosorbent assay (ELISA) (Alpco Diagnostics, Windham, NH), according to the manufacturer's instructions.

Placental weight

Dams were euthanized on GD 19 by intraperitoneal injection of sodium pentobarbital (150-200mg/kg). Uteri were excised and fetuses were separated from placentas. Placental weights were recorded on a top-loading balance and reserved for histopathological analysis, quantification of OS, and immunofluorescent staining of endothelial cells. *n*=16.

Histopathology

GD 19 placentas from control and treatment groups were collected at the time of euthanasia and some of these placentas were preserved in Bouin's fixative for 18 hr, washed with 10 volumes ddH_2O, and stored in 70% EtOH. Placentas were transected perpendicular to the long axis of the disc, processed, paraffin-embedded, and 5µm sections were stained with hematoxylin-eosin (H & E) for morphological analysis under light microscopy. Trophoblast health was evaluated by enumeration of viable trophoblasts per 10-1000x fields. Additionally, non-viable labyrinthine endothelial cells from placentas of control and treatment dams were enumerated per 10-400x fields. Both trophoblast and labyrinthine placentas from all groups were also observed for evidence of necrosis, inflammation, hemorrhage, and fibrosis. *n*=5.

Measurement of placental OS

GD 19 placentas from control and treatment groups were collected at the time of euthanasia and some of these placentas were immediately stored at -70°C until measurement of malondialdehyde (MDA), a common marker for OS. Quantification of lipid peroxidation per mg total protein (using BCA protein assay kit; PIERCE, Rockford, IL) was performed to assess the extent of OS of placentas using a commercially available MDA kit (Bioxytech MDA-586, Oxis Research, Portland, OR). Briefly, placentas in each treatment group (*n*=5) were homogenized in assay buffer (0.1 M phosphate buffered saline (PBS) with 0.5 M butylated hydroxytoluene (BHT). Using 200µl samples, 10µl of 0.5M BHT and 640µl of N-methyl-2-phenylindole were added. The samples were mixed by briefly vortexing each tube and then 150µl of concentrated hydrochloric acid were added to the tubes. The tubes were vortexed and samples were then incubated at 45°C for 60 min. Then the turbid samples were centrifuged for 10 min at 10,000 x g. Absorbance of the clear supernatant was measured at 586 nm (Beckman DU-640 spectrophotometer, International MI-SS, Corona CA).

Immunofluorescence

GD 19 Bouins-fixed placentas from each treatment group were processed, paraffin-embedded, and sectioned at 5µm for CD31 immunofluorescence and confocal microscopic evaluation. Placental tissues were deparaffinized in xylene and rehydrated in serial alcohol solutions. Tissues were dipped in unmasking solution (Vector Laboratories, Burlingame, CA) at 120°C in a pressure cooker for 5 min and washed three times in 0.1 M PBS for 5 min each time. Placental sections were then blocked in 10% rabbit serum (Fisher Scientific) for 30 min followed by incubation in CD31 primary antibody (M-20, Santa Cruz Biotechnology, Santa Cruz, CA)

diluted 1:300 in 1.5 % serum overnight at 4°C. The following day, the slides were washed three times for 10 min each in 0.1 M PBS followed by incubation for 30 min in Alexa Fluor 594 rabbit anti-goat antibody (Invitrogen Corporation, Camarillo, CA) diluted 1:200 in 0.1M PBS. After several washes in 0.1 M PBS, the slides were mounted using mounting solution with DAPI (Vector Laboratories) and visualized under confocal laser scanning microscopy (Zeiss CLSM 510 META, Carl Zeiss, Germany).

Statistical analysis

All data in this study were presented as mean ± standard error. One-way analysis of variance (ANOVA) was used with Tukey-Kramer HSD test (JMP, SAS Institute Inc., Cary, NC) to establish significant differences between groups. Data were determined to be statistically different when *P*<0.05.

Results

Effects of HFD on maternal body weight

Maternal body weight was determined before HFD feeding, at the end of four weeks' HFD feeding, and during pregnancy at GD 0, GD 10, and GD 19. After exposure to the respective diet for 4 weeks, as shown in Fig. 1, the control maternal body weight moderately increased throughout the pregnancy. In contrast, HFD dams rapidly increased in body weight. At GD 0 and 10, HFD dam weight was significantly increased as compared to controls. At GD 10, HFD feeding increased maternal body weight by 89.9% compared with 37% for control dams. Body weights of mice from HFD/Q group were not significantly different from control group,

which suggested protective effects against developing obesity by dietary supplementation with quercetin.

Effects of HFD on blood glucose

Blood glucose levels were determined before HFD feeding, at the end of HFD feeding and at GD 0, 10, and 19 for control, HFD, and HFD/Q groups. As shown in Fig. 2, HFD dam blood glucose levels progressively increased at the end of HFD feeding and during pregnancy, and were significantly higher than control glucose levels. Blood glucose level reached its highest point of 229.3mg/dl on GD10 and decreased at the advanced GD19 in the HFD group, as compared to a high of 172.4mg/dl at GD 10 in controls. The blood glucose level from HFD/Q group was not significantly different from control.

Effects of HFD on plasma insulin

Plasma insulin levels were measured by ELISA before HFD feeding, at the end of HFD feeding and at GD 0, 10, and 19 for control, HFD, and HFD/Q groups. As shown in Fig. 3, plasma insulin levels at the end of HFD feeding increased 58.3% in the HFD group, which was significantly higher than the controls that did not increase during this period. Plasma insulin levels of HFD dams continued to rapidly increase throughout the pregnancy. Mice from HFD group showed significantly greater plasma insulin level from GD 0 to GD 10 compared with controls. Quercetin supplementation was not significantly protective against HFD-induced hyperinsulinemia.

Effect of HFD on insulin resistance

Data from Fig. 2 and Fig. 3 together demonstrated a close correlation between blood glucose levels and plasma insulin levels during pregnancy. The HFD group developed concurrent hyperglycemia and hyperinsulinemia, which defines insulin resistance. This change was not observed in the control group. Concurrent quercetin supplementation did not significantly protect against HFD-induced insulin resistance in mid-gestation.

GD 19 mouse placental weight

As shown in Fig. 4, GD 19 placental weight was numerically decreased to 78% of control by HFD, which was statistically insignificant, but showed a trend that may have resulted from OS-mediated cellular loss. HFD/Q placental weight was not different from control.

Histopathologic examination of GD 19 placenta

Placentas from GD 19 dams of each treatment group were evaluated under light microscopy for alterations in architecture across treatment groups, using 5 μm H & E sections (Fig. 5). Placentas from HFD treated dams (Fig. 5B) showed multifocal areas of necrosis that predominately targeted endothelium in the labyrinthine layer, with karyolysis and pyknosis, cellular fragmentation, and hypereosinophilia, and mixed fibrinous inflammation which are key indicators of cellular necrosis. The trophoblast cell layer also contained mild mixed inflammation and loss of trophoblasts (Fig. 5B) which may be attributable to OS-mediated trophoblast apoptosis. Control (Fig. 5A) and HFD/Q (Fig. 5C) placentas were highly cellular, with minimal evidence of necrosis, inflammation, or hemorrhage. HFD significantly reduced

viable trophoblast cell numbers to 53.40 per 1000x magnification field compared to 9.73/field in control placental trophoblast (Fig. 6), but quercetin did not significant protect viability of trophoblast against HFD-induced cell loss. Gestational HFD significantly increased necrosis of labyrinthine endothelial cells (14.48 necrotic cells/400x magnification field, as compared to 1.87/field in control placentas; Fig. 7). Quercetin was protective against HFD-induced endothelial cell loss, and significantly decreased endothelial necrosis to 5 per 400x magnification field (Fig. 7).

Lipid peroxidation of GD 19 mouse placenta

Placentas from GD 19 HFD dams showed significantly increased MDA levels compared to controls, indicating increased lipid peroxidation and elevated OS due to gestational dietary HFD (Fig. 8). Quercetin supplementation significantly decreased placental MDA levels, which suggested beneficial antioxidative effects of quercetin against HFD-induced placental labyrinthine vascular damage. These data supported cellular changes observed via histopathologic examination of GD19 placentas in this study.

CD 31 immunofluorescence

Placenta from HFD dams (Fig. 9B) showed decreased levels of CD31 immunostaining intensity of labyrinthine endothelial cells and altered vascular integrity compared to control (Fig. 9A), which indicated endothelial necrosis and placental cellular and vascular pathology secondary to gestational HFD. Placenta from HFD/Q dams showed highly improved level of CD31 immunostaining intensity compared to HFD group (Fig. 9C), which was comparable to the staining intensity of control placentas. Fig. 10 displays CD31 immunostaining intensity of

labyrinthine layer capillaries from control, HFD, HFD/Q placentas (Figs 10A, B, and C, respectively). Control placental labyrinthine layer capillaries showed dramatically stronger CD31 staining intensity compared to HFD placenta capillaries, which showed minimal scattered staining. HFD/Q placental labyrinthine layer capillary CD31 immunofluorescence staining was comparable to controls, indicating the protective effects of quercetin against GDM-induced placental vascular damage.

Discussion

The present study demonstrated that administration of a diet rich in saturated fats to mice prior to and during pregnancy results in pathologic manifestations similar to the human condition of GDM. This dietary intervention elevated midgestational body weight, insulin resistance, placental OS, and placental vasculopathy, that together are thought to contribute to adverse consequences of fetal development and impaired birth outcome.

Human GDM is associated with hyperglycemia, hyperinsulinemia, insulin resistance, and a greater risk for producing type 2 diabetes in the offspring (Bo *et al.* 2001; Rolo and Palmeira 2006). Maternal hyperglycemia, hyperinsulinemia, and insulin resistance were observed in this murine study by feeding C57BL/6J mice HFD for 4 weeks before breeding and throughout pregnancy, suggesting that HFD feeding increases plasma free fatty acid (FFA) concentrations and induces an insulin-resistant state that results in obesity and non-insulin-dependent diabetes (Steiner *et al.* 1980; Reaven *et al.* 1988; Frayne 1993). Elevations in plasma FFA levels in humans cause insulin resistance by initial inhibition of glucose transport and/or phosphorylation activity that is concurrently followed by a reduction in the rate of both muscle glycogen synthesis and glucose oxidation (Dresner *et al.* 1999). Studies show that the preferential use of

increased fatty acids for oxidation blunts the insulin-mediated reduction of hepatic glucose output and reduces the glucose uptake or utilization in skeletal muscle leading to compensatory hyperinsulinemia, a common feature of insulin resistance (Iwanishi and Kobayashi 1993; Rosholt *et al.* 1994; Belfiore and Iannello 1998). Our study suggested that the dynamic relationship between insulin resistance and compensatory increases in beta cell glucose metabolism were disrupted by alteration in dietary intake of fat. When the compensatory process is adequate, normal glucose tolerance is maintained. When beta cell compensation fails, glucose levels rise, leading to either impaired glucose tolerance or overt diabetes.

Our discovery of a link between HFD and insulin production offers new information that may aid in the understanding of the etiopathogenic mechanism that triggers the early stages of GDM. Ohtsubo *et al.* (2005) found that HFD suppresses the activity of pancreatic *Mgat4a*-encoded GlcNAcT-IV glycosyltransferase (GnT-4a) and leads to type 2 diabetes in mice due to failure of pancreatic beta cells (Ohtsubo *et al.* 2005). GnT-4a normally maintains glucose transporters on the surface of beta cells in the pancreas, such as glucose transporter 2 (Glut-2). Glut-2 plays a crucial role in allowing the beta cell to sense circulating glucose levels, and transport of glucose across the cell membrane into pancreatic beta cells then triggers insulin secretion. The study from Ohtsubo *et al.* (2005) shows that in the absence of sufficient GnT-4a enzyme, Glut-2 lacks an attached glycan which is required for it to be expressed at the cell membrane. Without that glycan, Glut-2 leaves the cell surface and becomes internalized, where it can no longer transport glucose into the cell. Consequently, this failure impairs insulin secretion, causing insulin-resistant diabetes in the mice. These researchers also revealed that HFD attenuates GnT-4a mRNA levels (Ohtsubo *et al.* 2005). Results of the present study may suggest

GnT-4a dysfunction; additional studies are required to explore this pathophysiologic mechanism of HFD-induced insulin resistance.

The induction of HFD-induced alterations in glucose metabolism and insulin pathways in this study may also be associated with increased insulin-like growth factor (IGF) levels during pregnancy. IGFs are major endocrine and paracrine regulators of metabolism and may potentially be important regulatory factors in insulin-sensitive diabetes (Park *et al.* 2006) as well as insulin-resistant diabetes. Studies indicate that transgenic mice over-expressing IGF-2 in beta cells show increased insulin mRNA levels and enhanced glucose-stimulated insulin secretion. These mice develop hyperinsulinemia, mild hyperglycemia, and about 30% of these animals develop type 2 diabetes when they are fed HFD (Devedjian *et al.* 2000). IGFs share a high degree of structural homology with insulin and both of them belong to the protein tyrosine kinase receptor family (Devedjian *et al.* 2000; van Haeften and Twickler 2004), and as such, share common signaling pathways with insulin (van Haeften and Twickler 2004); therefore, reciprocal interference during pregnancy between IGFs and insulin could mechanistically explain alterations in glucose homeostasis during pregnancy. Maternal circulating IGFs increase during early pregnancy, and their activity, along with that of insulin, may be partially blocked by the variety of hormones that are produced by the placenta during pregnancy (Sferruzzi-Perri *et al.* 2006). This may be an important factor to consider in the phenomenon of insulin resistance and GDM. Human placental lactogen is suggested to be one of the hormones primarily responsible for decreased insulin sensitivity with advanced gestation (Yamashita *et al.* 2000). Other research reveals that insulin receptor tyrosine phosphorylation is significantly decreased in subjects with GDM compared with pregnant and non-pregnant control subjects (Shao *et al.* 2000). These findings correspond well with our results, which demonstrated that HFD feeding during

pregnancy induced a suitable mouse model of hyperglycemia, hyperinsulinemia, and insulin resistance, similar to human GDM.

In this study, placental OS and vascular damage were increased with HFD feeding prior to and throughout gestation, similar to human GDM. In human GDM, increased blood glucose is accompanied by either reduced antioxidant superoxide dismutase (SOD) production (Djordjevic *et al.* 2004) or overproduction of ROS such as superoxide by the mitochondrial electron-transport chain, which results in vascular complications and DNA damage associated with embryonic dysmorphogenesis (Little and Gladen 1999). This serves as a plausible etiopathological mechanism of placental vascular damage in human GDM and our GDM mouse model. The elevation of placental OS, blood glucose, and plasma insulin, due to increased plasma FFA concentration secondary to HFD, likely resulted in diminished placental blood flow, placental trophoblast loss, disruption of endothelium, and vascular dysfunction, similar to what has been reported in the human literature (Myatt *et al.* 2000).

Our results of placental histopathology, MDA measurement and CD31 immunoflourescence staining data further demonstrated that OS-induced vasculopathy of placenta is associated with symptoms of GDM of C57BL/6 mice. CD31, also called platelet-endothelial cell adhesion molecule-1 (PECAM-1) and endothelial cell adhesion molecule (endoCAM), is secreted by trophoblasts and is found in the embryonic vasculature of the placental labyrinth (Ottersbach and Dzierzak 2005) and is well recognized as one of the important endothelial cell markers (Lyall *et al.* 2001). CD31 is important in mediating the adhesive interaction between trophoblast and vascular endothelium, and as such is instrumental in placental development (Coukos *et al.* 1998). Our research suggested that HFD treatment decreased CD31 staining intensity of placental vessels, which supported histological evidence of

labyrinthine endothelial necrosis and vascular damage caused by HFD during pregnancy. Supporting studies also show that CD31 is involved in controlling the localization and levels of tyrosine phosphorylated beta-catenin by both recruiting it to the plasma membrane and by its dephosphorylation (Ilan *et al.* 1999). Moreover, CD31 has the ability to prevent beta-catenin nuclear translocation and to enable cell border localization upon stable expression. This suggests that endothelial cells regulate changes in the free cytoplasmic pools of beta-catenin and maintain their stability. Therefore, CD31 functions as a reservoir for beta-catenin (Ilan *et al.* 1999). Beta-catenin is a key downstream component of the Wnt/ beta-catenin signaling pathway. It plays a very profound role in embryonic development and glucose and lipid metabolism, as well as bone formation. Attenuation of Wnt/ beta-catenin signaling due to beta-catenin deficiency leads to hyperglycemia, hyperlipidemia, and osteoporosis (Manolagas and Almeida 2007). Therefore, our results may suggest an additional parallel mechanism by which HFD-induced vascular endothelial damage during pregnancy decreased CD31 expression, and reduced availability and stability of beta-catenin for the proper function of Wnt/ beta-catenin pathway. Therefore, disturbed glucose and lipid metabolism due to attenuation of Wnt/ beta-catenin signaling may exacerbate development of GDM.

In this study, the antioxidant effects of dietary supplementation of quercetin were noteworthy. Our results showed that the GDM-induced placental OS and endothelial necrosis were significantly mitigated by quercetin supplementation. Results from this report also revealed that quercetin supplementation partially decreased maternal hyperglycemia and hyperinsulinemia. These findings may provide a therapeutic method for improved management of GDM mothers.

In conclusion, our data suggested that a diet high in saturated fat prior to and during pregnancy alters glucose metabolism and results in gestational hyperglycemia, development of insulin resistance, and placental OS. The phenotype is similar to that observed in humans with GDM. Because of its simplicity and its resemblance to GDM in women, this novel method of GDM development in rodents may provide a useful tool for improving mechanistic understanding of OS and metabolic effects of HFD in GDM. Further study of maternal, fetal, and neonatal sequelae of this dietary intervention will offer more complete avenues for novel treatments and preventions of adverse consequences of GDM.

References

Aerts, L., F. Sodoyez-Goffaux, J. C. Sodoyez, W. J. Malaisse and F. A. Van Assche (1988) The diabetic intrauterine milieu has a long-lasting effect on insulin secretion by B cells and on insulin uptake by target tissues. *Am J Obstet Gynecol*. **159**, 1287-92.

Belfiore, F. and S. Iannello (1998) Insulin resistance in obesity: metabokic mechanisms and measurement methods. *Mol Gen Metab*. **65**, 121-8.

Bo, S., G. Menato, A. Lezo, A. Signorile, C. Bardelli, F. De Michieli, M. Massobrio and G. Pagano (2001) Dietary fat and gestational hyperglycaemia. *Diabetologia*. **44**, 972-8.

Coukos, G., A. Makrigiannakis, K. Amin, S. M. Albelda and C. Coutifaris (1998) Platelet-endothelial cell adhesion molecule-1 is expressed by a subpopulation of human trophoblasts: a possible mechanism for trophoblast-endothelial interaction during haemochorial placentation. *Mol Hum Reprod*. **4**, 357-67.

Devedjian, J. C., M. George, A. Casellas, A. Pujol, J. Visa, M. Pelegrin, L. Gros and F. Bosch (2000) Transgenic mice overexpressing insulin-like growth factor-II in beta cells develop type 2 diabetes. *J Clin Invest*. **105**, 731-40.

Djordjevic, A., S. Spasic, A. Jovanovic-Galovic, R. Djordjevic and G. Grubor-Lajsic (2004) Oxidative stress in diabetic pregnancy: SOD, CAT and GSH-Px activity and lipid peroxidation products. *J Matern Fetal Neonatal Med*. **16**, 367-72.

Dresner, A., D. Laurent, M. Marcucci, M. E. Griffin, S. Dufour, G. W. Cline, L. A. Slezak, D. K. Andersen, R. S. Hundal, D. L. Rothman, K. F. Petersen and G. I. Shulman (1999) Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest*. **103**, 253-9.

Frayne, K. N. (1993) Insulin resistance and lipid metabolism. *Curr. Opin. Lipidol*. **4**, 197-204.

Garcia-Patterson, A., L. Erdozain, G. Ginovart, J. M. Adelantado, J. M. Cubero, G. Gallo, A. de Leiva and R. Corcoy (2004) In human gestational diabetes mellitus congenital malformations are related to pre-pregnancy body mass index and to severity of diabetes. *Diabetologia*. **47**, 509-14.

Holemans, K., S. Caluwaerts, L. Poston and F. A. Van Assche (2004) Diet-induced obesity in the rat: a model for gestational diabetes mellitus. *Am J Obstet Gynecol*. **190**, 858-65.

Ilan, N., S. Mahooti, D. L. Rimm and J. A. Madri (1999) PECAM-1 (CD31) functions as a reservoir for and a modulator of tyrosine-phosphorylated beta-catenin. *J Cell Sci*. **112 Pt 18**, 3005-14.

Iwanishi, M. and M. Kobayashi (1993) Effect of pioglitazone on insulin receptors of skeletal muscles from high-fat-fed rats. *Metabolism: clinical and experimental*. **42**, 1017-21.

Lebovitz, H. E. and M. A. Banerji (2004) Treatment of insulin resistance in diabetes mellitus. *Eur J Pharmacol*. **490**, 135-46.

Little, R. E. and B. C. Gladen (1999) Levels of lipid peroxides in uncomplicated pregnancy: a review of the literature. *Reprod Toxicol*. **13**, 347-52.

Luo, J., J. Quan, J. Tsai, C. K. Hobensack, C. Sullivan, R. Hector and G. M. Reaven (1998) Nongenetic mouse models of non-insulin-dependent diabetes mellitus. *Metabolism: clinical and experimental*. **47**, 663-8.

Lyall, F., J. N. Bulmer, E. Duffie, F. Cousins, A. Theriault and S. C. Robson (2001) Human trophoblast invasion and spiral artery transformation: the role of PECAM-1 in normal pregnancy, preeclampsia, and fetal growth restriction. *Am J Pathol*. **158**, 1713-21.

Lyall, F., J. L. Gibson, I. A. Greer, D. E. Brockman, A. L. Eis and L. Myatt (1998) Increased nitrotyrosine in the diabetic placenta: evidence for oxidative stress. *Diabetes Care*. **21**, 1753-8.

Manach, C., C. Morand, C. Demigne, O. Texier, F. Regerat and C. Remesy (1997) Bioavailability of rutin and quercetin in rats. *FEBS letters*. **409**, 12-6.

Manolagas, S. C. and M. Almeida (2007) Gone with the Wnts: beta-catenin, T-cell factor, forkhead box O, and oxidative stress in age-dependent diseases of bone, lipid, and glucose metabolism. *Mol Endocrinol*. **21**, 2605-14.

Myatt, L., W. Kossenjans, R. Sahay, A. Eis and D. Brockman (2000) Oxidative stress causes vascular dysfunction in the placenta. *J Matern Fetal Med*. **9**, 79-82.

Oh, W., N. L. Gelardi and C. J. Cha (1988) Maternal hyperglycemia in pregnant rats: its effect on growth and carbohydrate metabolism in the offspring. *Metabolism: clinical and experimental*. **37**, 1146-51.

Oh, W., N. L. Gelardi and C. J. Cha (1991) The cross-generation effect of neonatal macrosomia in rat pups of streptozotocin-induced diabetes. *Pediatr Res*. **29**, 606-10.

Ohtsubo, K., S. Takamatsu, M. T. Minowa, A. Yoshida, M. Takeuchi and J. D. Marth (2005) Dietary and genetic control of glucose transporter 2 glycosylation promotes insulin secretion in suppressing diabetes. *Cell*. **123**, 1307-21.

Ottersbach, K. and E. Dzierzak (2005) The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Dev Cell*. **8**, 377-87.

Paravicini, T. M. and R. M. Touyz (2006) Redox signaling in hypertension. *Cardiovasc Res*. **71**, 247-58.

Park, S. H., S. K. Ko, J. G. Choi and S. H. Chung (2006) Salicornia herbacea prevents high fat diet-induced hyperglycemia and hyperlipidemia in ICR mice. *Arch Pharm Res*. **29**, 256-64.

Reaven, G. M., C. Hollenbeck, C. Y. Jeng, M. S. Wu and Y. D. Chen (1988) Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM. *Diabetes*. **37**, 1020-4.

Rerup, C. C. (1970) Drugs producing diabetes through damage of the insulin secreting cells. *Pharmacol Rev*. **22**, 485-518.

Rolo, A. P. and C. M. Palmeira (2006) Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress. *Toxicol Appl Pharmacol*. **212**, 167-78.

Rosholt, M. N., P. A. King and E. S. Horton (1994) High-fat diet reduces glucose transporter responses to both insulin and exercise. *Am J Physiol*. **266**, R95-101.

Saltiel, A. R. and J. M. Olefsky (1996) Thiazolidinediones in the treatment of insulin resistance and type II diabetes. *Diabetes*. **45**, 1661-9.

Sferruzzi-Perri, A. N., J. A. Owens, K. G. Pringle, J. S. Robinson and C. T. Roberts (2006) Maternal insulin-like growth factors-I and -II act via different pathways to promote fetal growth. *Endocrinology*. **147**, 3344-55.

Shao, J., P. M. Catalano, H. Yamashita, I. Ruyter, S. Smith, J. Youngren and J. E. Friedman (2000) Decreased insulin receptor tyrosine kinase activity and plasma cell membrane glycoprotein-1 overexpression in skeletal muscle from obese women with gestational diabetes mellitus (GDM): evidence for increased serine/threonine phosphorylation in pregnancy and GDM. *Diabetes*. **49**, 603-10.

Shao, J., H. Yamashita, L. Qiao, B. Draznin and J. E. Friedman (2002) Phosphatidylinositol 3-kinase redistribution is associated with skeletal muscle insulin resistance in gestational diabetes mellitus. *Diabetes*. **51**, 19-29.

Sikkema, J. M., B. B. van Rijn, A. Franx, H. W. Bruinse, R. de Roos, E. S. Stroes and E. E. van Faassen (2001) Placental superoxide is increased in pre-eclampsia. *Placenta*. **22**, 304-8.

Skibola, C. F. and M. T. Smith (2000) Potential health impacts of excessive flavonoid intake. *Free Radic Biol Med*. **29**, 375-83.

Steiner, G., S. Morita and M. Vranic (1980) Resistance to insulin but not to glucagon in lean human hypertriglyceridemics. *Diabetes*. **29**, 899-905.

van Haeften, T. W. and T. B. Twickler (2004) Insulin-like growth factors and pancreas beta cells. *Eur J Clin Invest*. **34**, 249-55.

Widdowson, P. S. (1997) Obesity in diabetes and the impact of leptin. *Diabetes Rev Int*. **6**, 2-5.

Wilson, B. D., M. M. Ollmann and G. S. Barsh (1999) The role of agouti-related protein in regulating body weight. *Molecular medicine today*. **5**, 250-6.

Yamashita, H., J. Shao and J. E. Friedman (2000) Physiologic and molecular alterations in carbohydrate metabolism during pregnancy and gestational diabetes mellitus. *Clin Obstet Gynecol*. **43**, 87-98.

Table 1. High Fat Diet (HFD) Composition

High Fat Diet	gm%	Kcal%
Protein	26.2	20
Carbohydrate	26.3	20
Fat	34.9	60
Fat composition:		%
Saturated		32.1
Monounsaturated		46
Polyunsaturated		16.9
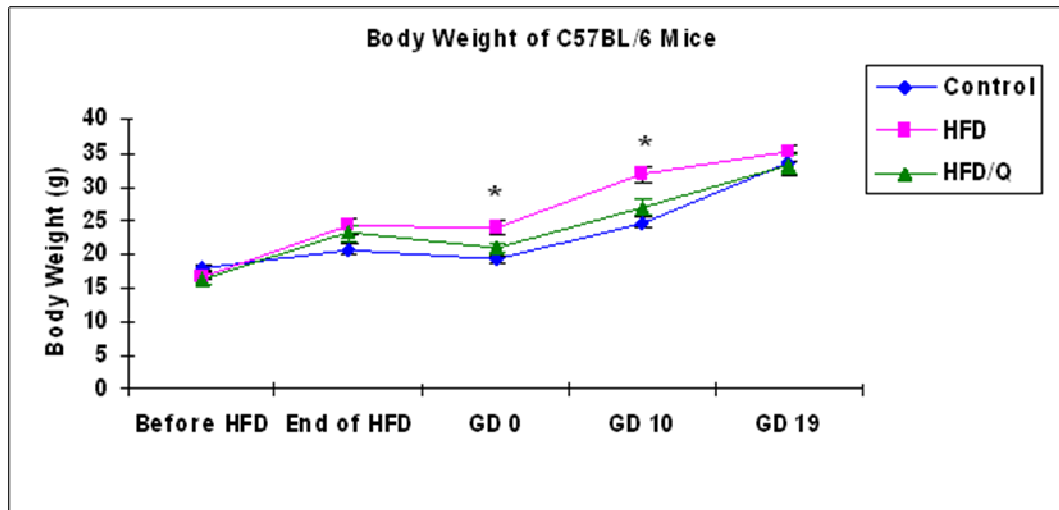
Fig. 1. Effect of HFD on Maternal Body Weight. Female body weight was measured before HFD, at the end of four weeks' HFD and during pregnancy at GD 0, 10, 19. HFD-treated mice weighed significantly more than controls on the GD 0 and GD 10. Body weight of mice in the HFD/Q group was not significantly different compared to HFD. Values are given as means ± SEM. * denotes significant difference, $P < 0.05$. n=16.
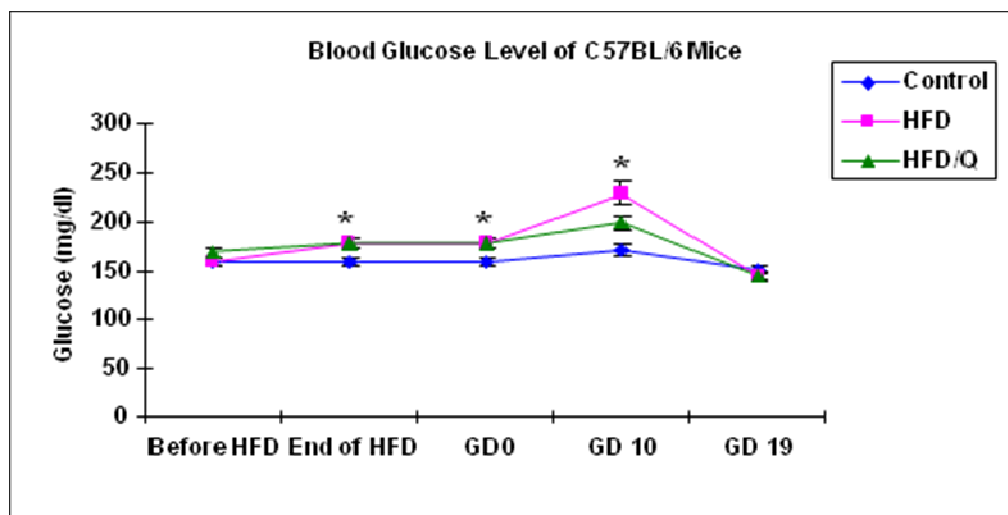
Fig. 2. Blood Glucose Level of C57BL/6 Mice. Blood glucose concentrations were measured before HFD, at the end of four weeks' HFD, and during pregnancy (GD 0, 10, and 19) in control, HFD and HFD/Q groups. The HFD group showed significantly higher blood glucose level compared to control dams. The HFD/Q group was not significantly different with HFD. Values are given as means ± SEM. * denotes significant difference, $P < 0.05$. n=16.
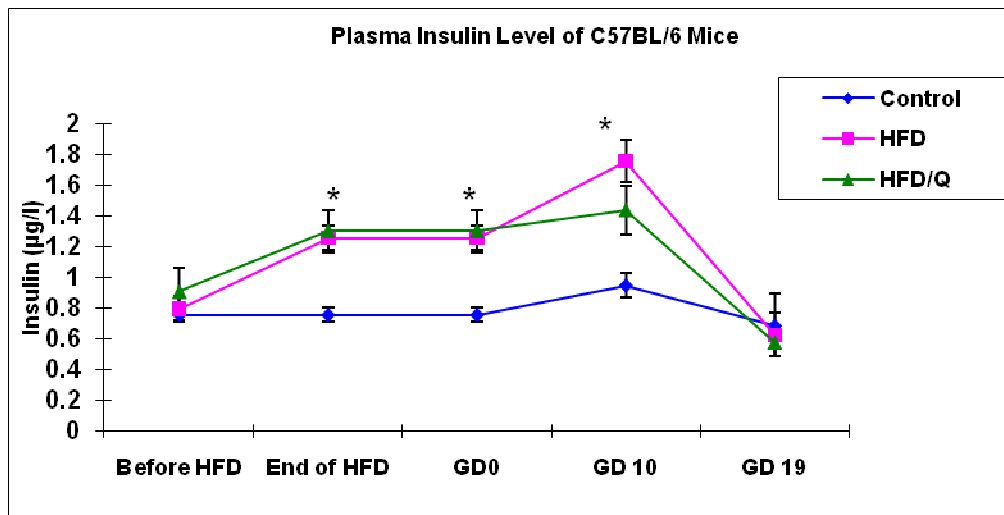
Fig. 3. Plasma Insulin Level of C57BL/6 Mice. Plasma insulin concentrations were measured by ELISA before, after four weeks' HFD, and during pregnancy (GD 0, 10, and 19). Mice in HFD group showed significantly increased plasma insulin level compared to control. Quercetin supplementation was not significantly protective against HFD-induced hyperinsulinemia. Values are given as means ± SEM. * denotes significant difference, $P < 0.05$. n=16.
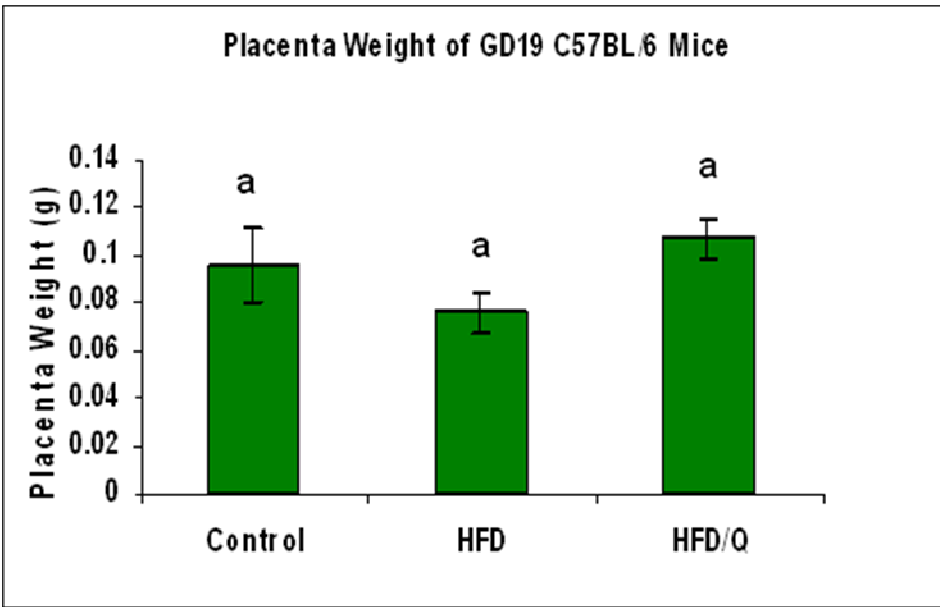
Fig. 4. Placental Weight of GD 19 Mice. Placental weights were measured immediately after euthanasia. Neither HFD nor quercetin affected placental wet weight. Values are given as means ± SEM. Bars with same letters are not significantly different, $P > 0.05$. n=16.
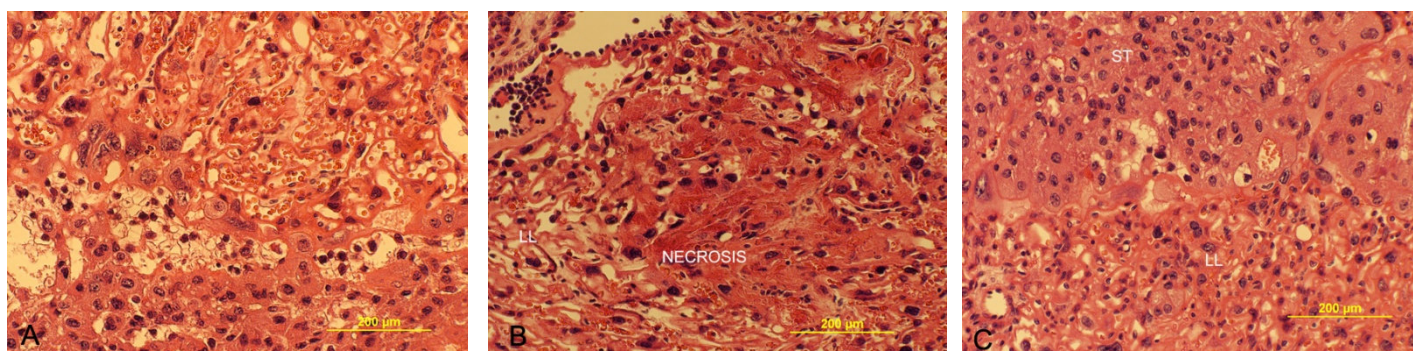
Fig. 5. Histopathologic Images of GD 19 Mouse Placentas. GD 19 placentas were stained with hematoxylin-eosin for morphological analysis under light microscopy. Control GD 19 placenta (5A) was highly cellular, with minimal evidence of necrosis, inflammation or hemorrhage. HFD GD 19 placenta (5B) showed multifocal areas of necrosis, cellular fragmentation, and hypereosinophilia, which predominately targeted endothelium in the labyrinthine layer. The trophoblast cell layer also contained marked mixed inflammation with necrosis and hemorrhage, and displayed reduced cellularity as compared to controls. HFD/Q GD 19 placenta (5C) was highly cellular, with minimal evidence of necrosis, inflammation or hemorrhage, which was comparable to control. LL (labyrinthine layer), ST (syncitiotrophoblast).
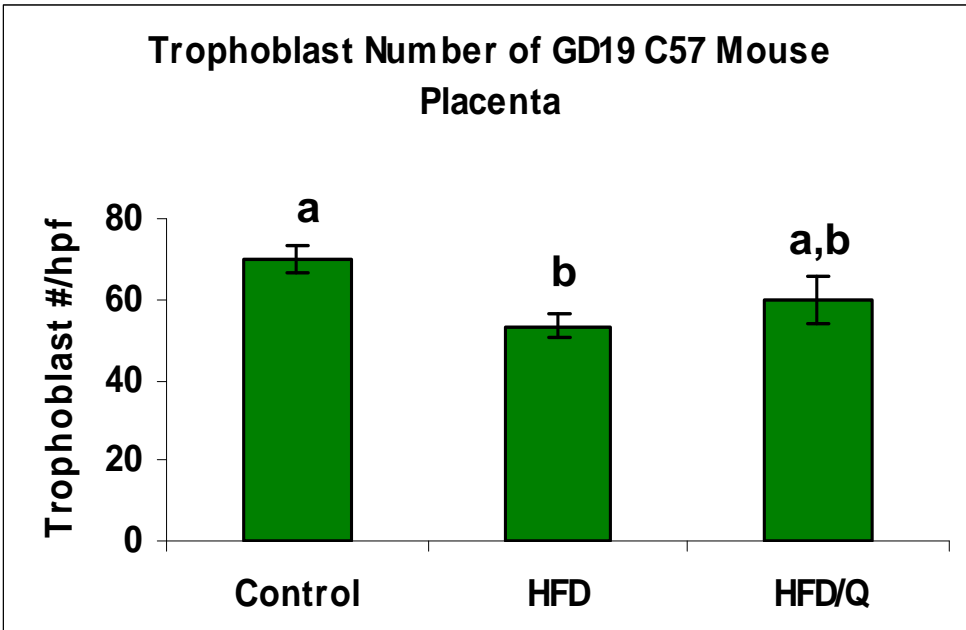
Fig. 6. Trophoblast Numbers in GD 19 C57BL/6 Mouse Placentas. Trophoblast health was evaluated by enumeration of viable trophoblasts per 10-1000x fields. The HFD group showed significantly fewer viable trophoblast cells than controls. Quercetin partially protected against trophoblast loss. Values are given as means ± SEM. Bars with different letters are significantly different, $P < 0.05$. n=5.

Dead Endothelial Cells of GD19 C57 Mouse Placenta
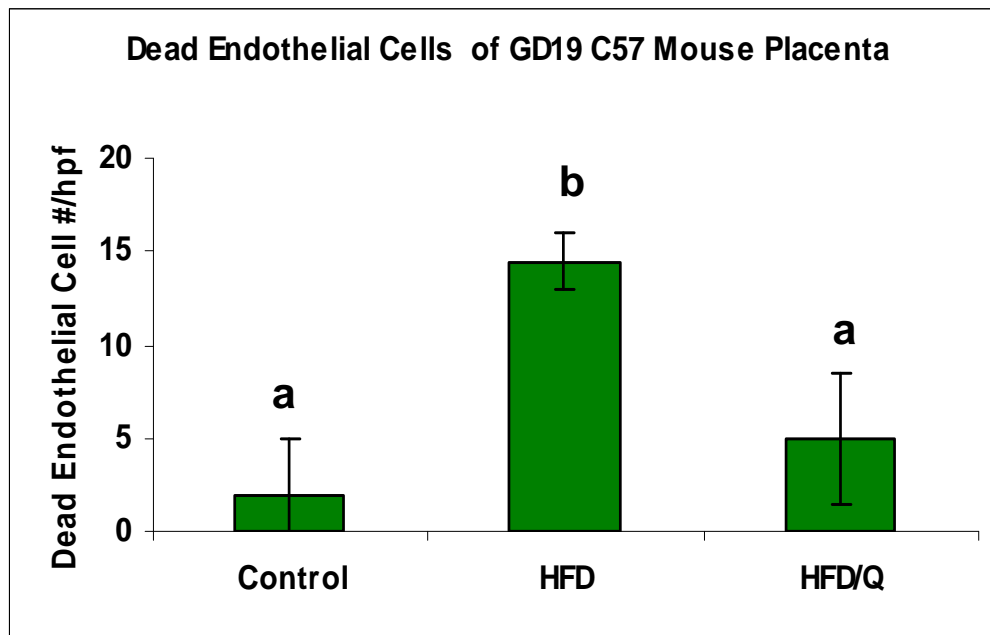
Fig. 7. Endothelial Cell Necrosis in GD 19 C57BL/6 Mouse Placentas. Non-viable labyrinthine endothelial cells from different dams were enumerated in 10 fields of 400x. The HFD group had significantly increased necrotic endothelial cells as compared to controls. Quercetin supplementation showed significant protective effects against endothelial cell loss. Values are given as means ± SEM. Bars with different letters are significantly different, $P < 0.05$. n=5.
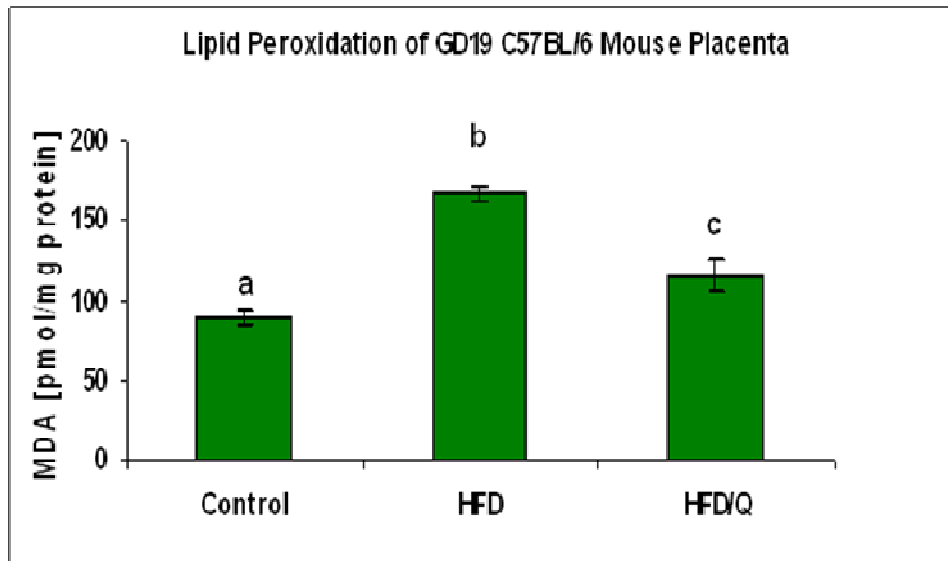
Fig. 8. Lipid Peroxidation of GD 19 C57BL/6 Mouse Placentas. Malondialdehyde (MDA) levels were evaluated in GD 19 placenta from each group, as an indicator of relative OS induced by HFD or quercetin dietary interventions. The HFD group showed significantly higher MDA level compared to controls, indicating HFD-induced elevated placental OS. Quercetin supplementation significantly reduced placental lipid peroxidation. Values are given as means ± SEM. Bars with different letters are significantly different, *P* < 0.05. n=5.
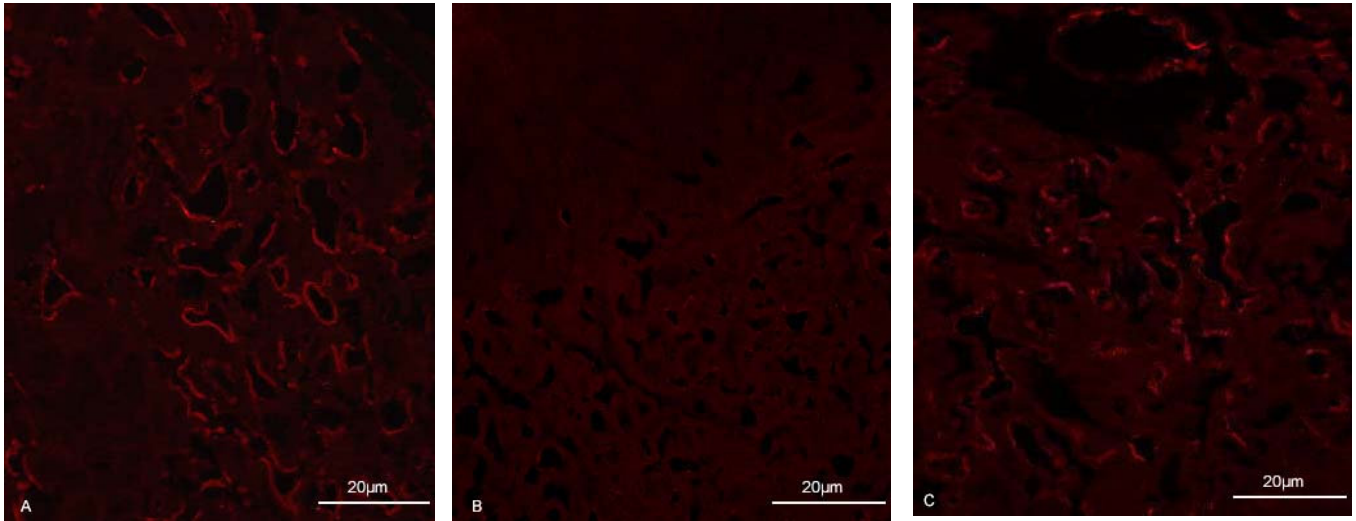
Fig. 9. CD31 Immunofluorescence Staining of GD 19 Mouse Placenta. CD31 immunofluorescently stained placentas from control, HFD, and HFD/Q groups were observed via confocal microscopy. Placentas from control dams showed much stronger CD31 staining intensity (9A) compared to HFD dams placenta,which showed much less CD31 staining (9B). HFD/Q (9C) dams placenta also showed strong CD31 staining intensity, which was comparable to control.
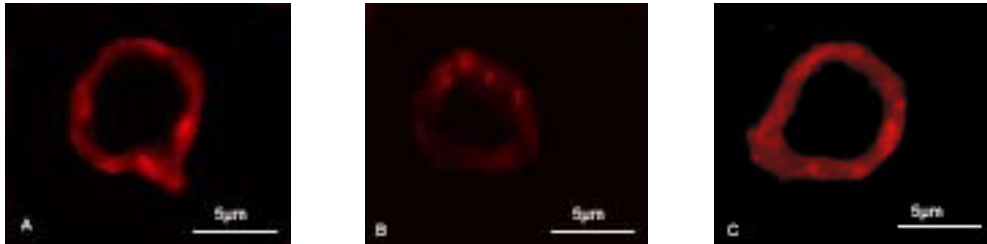
Fig. 10. CD31 Immunofluorescence Staining of GD 19 Mouse Placenta Labyrinthine Capillary Endothelium. Control placental labyrinthine capillary endothelium showed much stronger CD31 staining intensity (10A) compared to HFD placental labyrinthine capillary endothelium (10B). HFD placental labyrinthine capillary endothelial cells showed dramatically decreased CD31 staining intensity, and concurrent quercetin supplementation returned CD31 immunofluorescence staining to control-level staining, suggesting the protective effects of antioxidant supplementation against HFD-induced placental vasculopathy.

CHAPTER IV

Gestational High Saturated Fat Diet Alters C57BL/6 Mouse Perinatal Skeletal Formation

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Abstract

Recent studies have focused on the effects of fetal environment (maternal nutrition, gestational health, and oxidative stress) on prenatal development. Our previous study showed that gestational high saturated fat diet (HFD) consumption increases placental oxidative stress (OS) and vascular damage, which are thought to reduce maternal-fetal gas and nutrient exchange and impair fetal development. This study also demonstrated that concurrent dietary supplementation with the antioxidant quercetin largely ameliorates HFD-induced placental vasculopathy, and as such, is considered to likely protect against HFD-induced fetal malformation. In the present study, C57BL/6J mouse offspring from dams exposed to HFD or HFD with dietary antioxidant quercetin supplementation (HFD/Q) during gestation were examined for quality of skeletal formation. We observed a marked reduction in GD19 limb length, bone mineral density (BMD; 20%), total bone volume (BV; 45%), and crown-to-rump length (12%) in progeny of dams who consumed HFD, as compared to controls. Our findings demonstrated that placental damage from HFD-induced reactive oxygen species (ROS) may contribute to improper perinatal skeletal formation, and concurrent antioxidant supplementation during pregnancy may significantly improve placental integrity and function, and protect fetal development. Improved understanding of the role of HFD and elevated OS on fetal skeletal development will help to determine the contribution of the fetal environment to long-term risk of adult-onset disease.

Introduction

Environmental stress, particularly at critical periods of development, has recently been recognized as a powerful determinant of long-term health (Barker *et al.* 2002). To understand the etiopathological mechanisms by which improper maternal diet during gestation alters fetal skeletal formation, we used a mouse model to study the effects of chronic fast food consumption on placental and fetal development. Previous studies demonstrated a marked increase in placental OS and reduced viability of trophoblastic and labyrinthine endothelial cells following maternal consumption of a diet that mimics the macronutrient content of fast food, or a cafeteria-style diet (Liang *et al.* 2009a), and the protective effects of concurrent dietary supplementation with the antioxidant quercetin against placental OS and vasculopathy. The present study was designed to extend these previous studies to determine the impact of gestational HFD and placental vascular dysregulation on fetal skeletal development, using gross measurements and three-dimensionally (3-D) reconstructed micro-computed tomographic image analysis (micro-CT) of the developing fetus.

Quantifiable measures of bone formation such as bone volume (BV) and bone mineral density (BMD) are influenced by both genetic and environmental factors (Horlick *et al.* 2004). Dietary composition, particularly saturated fat content, has been shown to negatively impact bone formation: children consuming a high fat and low carbohydrate diet developed a poor bone mineral status (Bertoli *et al.* 2002). In animal models, a western - style diet, consisting of high saturated fat content, results in low bone mass, poor bone quality (Ward *et al.* 2003), diminished whole bone mineralization (Smith *et al.* 2000), and decreased calcium absorption and accretion, which collectively negatively impact BMD (Lac *et al.* 2008).

Neonatal bone mass and osteogenesis are positively correlated with placental health (Jansson and Powell 2007). The placenta is a multifunctional organ and the interface between the fetus and mother. It is essential for fetal growth as it supplies the developing fetus with oxygen and nutrients, and it may play a crucial role in protecting the fetus from adverse effects of maternal environmental exposures. Abnormalities in placental structural development impair placental function, reduce substrate supply to the fetus, or contribute to intrauterine growth restriction (Khong *et al.* 1986). Previous studies in our laboratory demonstrated the effect of environmental chemical exposures on placental and fetal development. Gestational administration of the teratogenic alkylating agent methylnitrosourea (MNU), which is found in tobacco smoke, and is produced endogenously in the acidic gastric environment following ingestion of a nitrite-rich protein meal, causes fetal and placental pathology due to elevated OS (Prater *et al.* 2006a). Increased OS during pregnancy, either due to environmental chemical exposure or pre-existing disease (e.g. maternal diet, diabetes mellitus or preeclampsia) causes placental vascular pathology, increased blood pressure, decreased fetal-maternal gas and nutrient exchange, increased fetal malformation, and intrauterine growth restriction (Prater *et al.* 2006a).

Reactive oxygen species (ROS) play important roles in fetal development, and are instrumental early in development during implantation, and in late gestation related to parturition (Fantel 1996). Therefore, appropriate levels of ROS are important regulators of normal fetal development. However, altered ROS levels may underlie fetal dysmorphogenesis. For instance, excessive OS secondary to maternal diabetes has been shown to increase incidence of neural tube defects (NTDs) in the rat fetus and embryo (Yang *et al.* 1997; Wentzel and Eriksson 1998). Embryonic development is exquisitely sensitive to the effects of oxidative stress, perhaps because of the immaturity of the free radical scavenging pathway (Neubert *et al.* 1971; Clough

and Whittingham 1983; el-Hage and Singh 1990), especially at the early stages of organogenesis, where excessive ROS at inappropriate times of development result in severe embryonic damage, embryonic death, and /or congenital anomalies (Ornoy 2007). It is therefore not surprising that environmental agents or maternal disease that produce increased levels of free radicals (free oxygen or nitrogen radicals) might affect the developing embryo by increasing lipid peroxidation, protein carbonylation, and DNA or RNA damage (Ornoy 2007) .

Quercetin, a member of flavonoid family, is ubiquitously present in foods including vegetables, fruit, tea and wine as well as countless food supplements. It has been recognized for its wide range of clinical properties, including anti-inflammatory, antibacterial, and antioxidant activities (Boots *et al.* 2008). Quercetin is also thought to effect changes in development due to its capacity to alter key signaling pathways such as NFkB, JakSTAT, and protein kinase C. These signaling pathways regulate cell proliferation, differentiation, apoptosis, transcription, inflammation, and immunity, and as such, are important determinants of proper fetal development (Dias *et al.* 2005; Kempuraj *et al.* 2005). Quercetin is highly efficient in reducing lipid peroxidation, protecting proper osteogenic differentiation of mesenchymal stem cells, and normalizing developing bone tensile strength and trabecular bone density (Prouillet *et al.* 2004; Kim *et al.* 2006; Kanter *et al.* 2007). Additional studies show that quercetin treatment may ameliorate some hematological values (blood glucose, insulin, Ca^{2+}, and Mg^{2+}) and bone histomorphometry in streptozocin-induced diabetic rats (Kanter *et al.* 2007). Prior studies in our lab indicated an association between quercetin-associated reduction of chemical-induced OS, placental improvement, and reduction of distal limb defects (Prater *et al.* 2008). To date, mechanisms linking quercetin to protection of fetal skeletal formation are poorly understood. In

the present study, we investigated the antioxidant effects of dietary supplementation with quercetin against maternal HFD-induced fetal skeletal malformation.

Methods

Animals and dietary protocol

Six-week-old male and female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were acclimated for 2 weeks at $22.0 \pm 1°C$, 40%-60% humidity, and on a 12/12 h light/dark cycle. Mice were given standard rodent chow (Harlan Teklad Global Diet 2018: 18% protein and 5 % fat, Madison, WI) and fresh water *ad libitum*. Following two-week acclimation, female mice were arbitrarily assigned to one of three groups: control, HFD, or HFD + quercetin. Mice were fed either with a HFD (20% protein and 60% total fat, 32.1% saturated fat, Research Diets Lab, New Brunswick, NJ) (Table 1) or with the standard rodent diet (Harlan Teklad Global Diet 2018: 18% protein and 5% fat), or the high fat diet supplemented with 66mg/kg quercetin. Average human consumption is 700mg/day (200-400mg 3x/day) (Manach *et al.* 1997)). Breeding was initiated following a four-week period of dietary intervention. Males and females (1:2 ratio) were bred overnight, and mating was confirmed by the presence of a vaginal mucous plug in females the next morning. The presence of vaginal plug was designated as GD0. Females and males were then separated after pregnancy was confirmed. Pregnant female were sacrificed on GD19 by pentobarbital overdose (150mg/kg intraperitoneal injection, IP; Schering-Plough Animal Health Corp, Kenilworth, NJ). Fetuses were immediately harvested, preserved in 70% ethanol (EtOH), and submitted for gross and micro-CT image analysis. Each group *n*=16. Experiments were all approved by the Virginia Tech Animal Care and Use Committee and not initiated until approval was granted.

Fetal weight

GD 19 Pregnant dams from control, HFD and HFD/Q groups were euthanized and fetuses were harvested as described above. Fetal weights were recorded on a top-loading balance (Accu-622, Fisher Scientific, Suwanee, GA). Fetal offspring were then fixed in 70% EtOH for later evaluation.

Gross measurements

Fetuses were harvested as described above. After fixation in 70% EtOH, fetuses were imaged using an Olympus SZX7 Stereomicroscope (Olympus Europa GmbH, Hamburg, Germany) with a DBX microscope-to-video camera coupler for direct image projection (Diagnostic Instruments, Sterling Heights, MI) and a Scion digital camera to acquire the image (Scion CFW-1310C 1394, Scion Corporation, Frederick MD). The image was captured on the computer via a Scion firewire camera image acquisition application (version 1.0, Scion Corporation, Frederick, MA). Whole body measurement of crown-to-rump length was thus measured. Limb lengths were obtained by Image J software for pixel-to-mm conversion (v1.30, NIH, Bethesda, MD).

Micro-CT assessments of total bone volume and bone mineral density

Fixed fetuses were placed in an acrylic tube and a high-speed *in vivo* micro-CT system (VivaCT 40, Scanco Medical, Bassersdorf, Switzerland) was used to obtain 3-D images of GD19 fetal skeletal structures. Fetuses were imaged using the following settings: 55kVp, 145µA, 500 projections, 21µm voxel size. A microfocus X-ray source with a 5-7µm focal spot was used in

the VivaCT and a voxel resolution of 38µm was employed. Using a global threshold corresponding to 54.25 mg hydroxylapatite (HA)/cm^3, and a Gaussian filter to partially smooth and suppress noise in the images (sigma = 1.5 support = 3), the fully mineralized skeletal structures were isolated from the surrounding soft tissue of each fetus. Total bone volume (BV) (expressed as mm^3) and average bone mineral density (BMD) (expressed as mg HA/cm^3) were then calculated. Component labeling was applied to reduce any image artifacts not contiguous with the fetal skeleton.

Micro-CT assessments of skeletal structural lengths

Using micro-CT techniques described above, reconstructed 3-D grayscale images of each GD 19 fetus were converted to DICOM (Digital Imaging and Communications in Medicine) format. The DICOM files were then exported to a local workstation customized to be able to handle large image datasets (Mac Pro, Apple Inc., Cupertino, CA). Measurement of the fetal skeletal structural lengths was accomplished using OsiriX, a DICOM-compatible freeware program (OsiriX, version 2.6, Medical Imaging Software, Los Angeles, CA). For all measurements, uniform image display factors were used. Three-dimensional images were rotated to display the maximum length (single-plane orientation) for the structure of interest. Skeletal structural lengths were determined using the image analysis software's length measurement tool. Overlying structures were removed as needed in order to clearly visualize the bone of interest. Skeletal structure measurements of each fetus included mandible, maxilla, humerus, radius, ulna, femur, and tibia.

All data in this study were presented as mean ± standard error. One-way analysis of variance (ANOVA) was performed, and Tukey-Kramer HSD test (JMP, SAS Institute Inc., Cary, NC) was used to establish significant differences between groups. Differences were considered statistically significant at *P<0.05.*

Results

Fetal size

As showed in Fig. 1, Fetal weight from HFD dams was numerically but not significantly decreased compare to control. Fetal weight from HFD/Q dams was not different from control. Measurement of crown-to-rump lengths on fetuses provided comparisons of overall fetal size and was used as a general indicator for fetal development. As shown in Fig. 2, fetuses from HFD dams were significantly shorter than controls. Concurrent dietary quercetin supplementation significantly protected against HFD-induced shortened crown-to-rump length.

3-D Micro-CT image analysis of fetal skeletal development

Using reconstructed, 3-D micro-CT imaging techniques, fetal skeletal structures were evaluated to determine the contribution of gestational HFD and elevated OS to fetal skeletal formation. Fig. 3 displays the visible changes in mineralized tissue between treatment groups using 3-D micro-CT image analysis of GD 19 mouse fetuses from control, HFD, HFD/Q groups. Fetuses from HFD group showed dramatically lowered total mineralized tissue associated with maternal HFD due to delayed ossification, as compared to control. Fetuses from HFD/Q group showed numerically improved skeletal ossification, compared to HFD fetuses.

Micro-CT assessments of BV and BMD

Fetal BV and BMD were calculated using information from micro-CT images. Fig. 4 demonstrates that the BV of the HFD group was significantly reduced by 45% as compared to controls. Quercetin supplementation significantly protected against HFD-induced fetal low bone mass. We also observed a marked reduction in GD 19 BMD of 20% (Fig. 5) in HFD group compared to controls. However, quercetin did not protect against HFD-induced BMD loss.

Micro-CT assessments of skeletal structural lengths

Micro-CT image analysis represents a precise, repeatable, and non-destructive method to quantify both axial and appendicular skeletal development in developing murine fetuses (Oest 2008). In the present study, we measured several ossified structures of fetuses from control and treatment groups, to determine the role of gestational HFD on fetal endochondral as well as intramembranous skeletal development. Structures measured included mandible, maxilla, humerus, radius, ulna, femur and tibia. Figures 6-8 demonstrate that fetuses from the HFD group had significantly shortened mandibular and maxillary skeletal structure lengths compared to controls (Fig. 6), which indicates fetal skeletal malformation and developmental delay associated with maternal HFD. Maternal dietary quercetin supplementation significantly protected against HFD-induced mandibular and maxillary maldevelopment. Similarly, gestational HFD was associated with significantly shortened humeral, radial and ulnar lengths compared to controls (Fig. 7). Maternal dietary quercetin supplementation significantly protected against HFD-induced skeletal limb length loss in all structures except the radius. Similar results were observed in the measurement of skeletal lengths of femur and tibia, where gestational HFD significantly

impaired femoral and tibial lengths as compared to controls (Fig. 8). Quercetin supplementation significantly protected against shortened femoral and tibial lengths.

Discussion

Recent studies suggest that both maternal environment and genetic background significantly contribute to the trajectory of fetal development (Eriksson and Borg 1993). Maternal factors such as OS, malnutrition, and placental vasculopathy during pregnancy may collectively contribute to fetal dysmorphogenesis. Maternal nutrition is an important factor in determining fetal growth. Our previous study showed that maternal consumption of HFD elevates OS and is associated with placental vascular impairment and cellular loss, which is likely related to fetal skeletal maldevelopment. Others have demonstrated fetal malformation subsequent to inflammation-induced placental damage (Sharova *et al.* 2003). In this study, we further investigated the effects of HFD on fetal skeletal development. Our data demonstrated that fetuses from HFD dams showed a numerically decreased body weight and a significantly reduced fetal crown-rump length compared to controls, indicating poor fetal growth. These findings may be due to elevated OS and placental vasculopathy, leading to growth retardation. The placenta functions as a nutritional intermediary between mother and child and it plays a central role in fetal programming by regulating fetal nutrient supply and fetal growth (Jansson and Powell 2007). Appropriate development of the placenta is crucial to normal fetal development. Damage of placental trophoblast and endothelium adversely affects the transplacental transport of nutrients (Jansson and Powell 2007). Our previous study showed severe placental vascular damage in HFD-fed subjects with decreased viable placental trophoblast and labyrinthine cell numbers (Liang *et al.* 2009a). Since trophoblasts direct fetal

development through liberation of cytokines and growth factors, trophoblast cell loss is considered to be detrimental to fetal development as demonstrated in this research. We observed for the first time a marked reduction in GD19 crown-rump length, BV, BMD, and skeletal limb lengths in offspring of HFD-fed dams. These maternal dietary-induced changes affected both intramembranous and endochondral ossification processes, and resulted in significant developmental delay that is not likely to be repaired postnatally and may result in long-term skeletal deformities.

Placental vascular damage and cellular loss in our laboratory and others was determined to result from increased OS (Myatt *et al.* 2000), as quantified by placental malondialdehyde levels, a stable lipid peroxidation product of elevated OS (Liang *et al.* 2009a). This study extends previous studies suggesting that elevated OS diminishes vascular integrity in the placenta, leading to fetal compromise (Myatt *et al.* 2000). It is thought that increased ROS up-regulates *p53* (pro-apoptotic) genes, and down-regulates bcl-2 (anti-apoptotic) gene expression in proliferating conceptus tissues (Bianchi *et al.* 2003; Cicchillitti *et al.* 2003), which adversely affects fetal and placental development. The relative immaturity of the fetal free radical scavenging system (el-Hage and Singh 1990) may also contribute to the developing embryonic and fetal sensitivity to OS.

As demonstrated in our studies (Liang *et al.* 2009a), maternal HFD, increased OS and placental vascular damage are believed to be the causes of fetal malformation. It is noted that in our previous study HFD dams also developed gestational hyperglycemia with blood glucose levels of 229.3mg/dl at GD 10 (Liang *et al.* 2009a). Maternal hyperglycemia exceeding 200mg/dl is thought to cause mitochondrial dysfunction, altered cell signaling, and adversely

affect fetal development (Heinze and Vetter 1987). So, maternal hyperglycemia may also contribute to the fetal dysmorphogenesis observed in the present study.

One quantitative method of assessing the fetal dysmorphogenesis in animal models is by measurement of specific skeletal parameters, such as limb lengths, crown-to-rump length, and cranial size (Oest *et al.* 2008). Dual energy x-ray absorptiometry (DEXA) has been a widely used general method to evaluate overall skeletal health; however, this technique relies on 2-D estimates for bone mineral assessment and yields relatively poor resolution as compared to newer tomographic image technology. DEXA analysis also does not permit detailed analysis of bone architecture by excluding key information on structural morphology such as architectural assessment of trabecular bone, which renders this a suboptimal technology with which to fully evaluate bone development, architectural soundness, and long-term risk of chronic disease such as osteoporosis (Schreiweis *et al.* 2003; Hester *et al.* 2004). Other traditional techniques, such as gross morphometry and clearing staining are also appropriate for crude determination skeletal structures and lengths, however, the time-consuming and unpredictable outcome of these tedious processes often do not accurately represent skeletal formations due to variability in findings, and result in unacceptable specimen fragility, dissociation, and destruction of fetal tissues (Oest *et al.* 2008).

In this study, results from micro-CT image analysis were consistent with those obtained using traditional technique (i.e. clear-staining) while decreasing variability within and between samples, and simultaneously providing additional information including total skeletal BV and BMD for each fetus, as we had previously reported (Oest *et al.* 2008). Micro-CT is a rigorous tool for measurement of murine fetal skeletal structure parameters, and because of high signal contrast between bone and soft tissue (Holdsworth and Thornton 2002), it is especially suited for

measurements of bone density and evaluation of fine bone structures. Using micro-CT technique, we observed a significant reduction on fetal BV and a marked reduction on fetal BMD in HFD group compared to controls. Bones are formed by two distinct mechanisms—intramembranous ossification and endochondral ossification, both starting from mesenchymal condensations. Skeletal structural lengths of bones from both intramembranous ossification (mandible, maxilla) and endochondral ossification (humerus, radius, ulna, femur and tibia) were measured by micro-CT in this study. Our data revealed that both fetal intramembranous ossification and endochondral ossification processes were adversely impacted by maternal HFD. Fetuses from HFD dams had significantly shortened limb lengths, and changes in mineralized structure development were clearly visible by micro-CT image analysis. HFD fetuses showed dramatically lowered total mineralized tissue and delayed skeletal ossification compared to control, suggesting skeletal maldevelopment by HFD. Also, for long bones, the central part of the shaft is the embryonic site of the primary ossification center and secondary ossification centers would develop at either or both ends of the bone in the later course of maturation (Hochberg 2002). Therefore, our findings of fetal long bone maldevelopment also suggested that maternal malnutrition of HFD seems to predominantly disturb the primary ossification pathway.

There have been studies showing that maternal dietary supplementation can effectively protect against fetal teratogenesis of NTDs (Fleming and Copp 1998), indicating possible transplacental activity of soluble mediators. Many studies using rodent embryos have shown that elevated maternal ROS increases fetal malformation (Chang *et al.* 2003) and administration of antioxidants, such as butylated hydroxytoluene, vitamin E, or flavones can mitigate TNF-alpha-induced adhesion of monocytes to endothelium, inhibit the NFkB pathway, and protect against ROS-mediated placental and fetal malformation (Eriksson and Siman 1996; Sivan *et al.* 1996;

Choi *et al.* 2004; Prater *et al.* 2006a; Prater *et al.* 2006b). Other methods to reduce excessive OS, such as transgenic overexpression of Cu^{2+}/Zn^{2+} superoxide dismutase, have also been shown to protect mouse embryos from developmental defects cause by excessive OS (Hagay *et al.* 1995). Our previous study showed quercetin significantly decreased OS, increased viable placental trophoblast and labyrinthine cell numbers, and improved placental vascular integrity and placental function in HFD-fed mice (Liang *et al.* 2009a). The present study further demonstrated that fetuses from HFD/Q showed significantly improved skeletal development compared to those from HFD dams. These effects may be due to the antioxidant effects of quercetin on reduction of placental OS. Alternatively, this may represent a direct, transplacental effect of gestational dietary antioxidants on fetal formation; clearly, additional studies are required to more completely elucidate the mechanisms surrounding protective effects of quercetin on fetal formation. Quercetin prevents oxidant injury and cell death by several mechanisms, which include ROS scavenging and protection against lipid peroxidation (Boots *et al.* 2008). There is also evidence that quercetin has bone-building effects, which suggest that quercetin may exert some direct effect on stimulation of alkaline phosphatase activity, activation of extracellular-regulated kinases in osteoblasts by an estrogen receptor-dependent pathway (Prouillet *et al.* 2004), or by influencing other signaling pathways involved in osteoblastic proliferation, differentiation, and mineralization. Studies demonstrate that treatment with quercetin dramatically increases bone formation rate in streptozocin-induced osteopenia (Kanter *et al.* 2007). Quercetin can both decrease osteoclastic activity and stimulate osteoblastic activity and therefore may represent a new pharmacological tool for the treatment of osteoporosis (Kanter *et al.* 2007). In this study, fetuses from HFD/Q dams showed significantly improved crown-to-rump lengths, BV and skeletal structural lengths compared to fetuses from HFD dams. 3-D

micro-CT images showed dietary quercetin supplementation dramatically reduced HFD induced low bone bass and improved overall bone quality. Also, dietary quercetin did not cause externally visible morphologic defects in C57BL/6 fetuses in this research. These findings provided evidence for a contributing role of placental OS in fetal skeletal malformation and a rationale that antioxidants might have a positive impact on the outcome of human pregnancy.

In summary, our study demonstrated that elevated gestational OS by maternal HFD plays a causal role in the incidence of congenital defects and fetal skeletal dysmorphogenesis. Dietary quercetin supplementation improved developmental outcome in HFD-exposed mice simply by reducing OS and ROS-related placental damage. It has been suspected for many years that control of OS lowers the risk of congenital anomalies (Mills *et al.* 1988). Thus, our finding offers one more supporting clue toward understanding the complex etiology of fetal malformation.

References

Barker, D. J., J. G. Eriksson, T. Forsen and C. Osmond (2002) Fetal origins of adult disease: strength of effects and biological basis. *Int J Epidemiol*. **31**, 1235-9.

Bertoli, S., L. Striuli, G. Testolin, S. Cardinali, P. Veggiotti, G. C. Salvatori and A. Tagliabue (2002) [Nutritional status and bone mineral mass in children treated with ketogenic diet]. *Recenti Prog Med*. **93**, 671-5.

Bianchi, P., M. H. Seguelas, A. Parini and C. Cambon (2003) Activation of pro-apoptotic cascade by dopamine in renal epithelial cells is fully dependent on hydrogen peroxide generation by monoamine oxidases. *J Am Soc Nephrol*. **14**, 855-62.

Boots, A. W., G. R. M. M. Haenen and A. Bast (2008) Health effects of quercetin: from antioxidant to nutraceutical. *Eur J Pharmacol*. **585**, 325-337.

Chang, T. I., M. Horal, S. K. Jain, F. Wang, R. Patel and M. R. Loeken (2003) Oxidant regulation of gene expression and neural tube development: Insights gained from diabetic pregnancy on molecular causes of neural tube defects. *Diabetologia*. **46**, 538-45.

Choi, J. S., Y. J. Choi, S. H. Park, J. S. Kang and Y. H. Kang (2004) Flavones mitigate tumor necrosis factor-alpha-induced adhesion molecule upregulation in cultured human endothelial cells: role of nuclear factor-kappa B. *J Nutr*. **134**, 1013-9.

Cicchillitti, L., P. Fasanaro, P. Biglioli, M. C. Capogrossi and F. Martelli (2003) Oxidative stress induces protein phosphatase 2A-dependent dephosphorylation of the pocket proteins pRb, p107, and p130. *J Biol Chem*. **278**, 19509-17.

Clough, J. R. and D. G. Whittingham (1983) Metabolism of [14C]glucose by postimplantation mouse embryos in vitro. *J Embryol Exp Morphol*. **74**, 133-42.

Dias, A. S., M. Porawski, M. Alonso, N. Marroni, P. S. Collado and J. Gonzalez-Gallego (2005) Quercetin decreases oxidative stress, NF-kappaB activation, and iNOS overexpression in liver of streptozotocin-induced diabetic rats. *J Nutr*. **135**, 2299-304.

el-Hage, S. and S. M. Singh (1990) Temporal expression of genes encoding free radical-metabolizing enzymes is associated with higher mRNA levels during in utero development in mice. *Dev Genet*. **11**, 149-59.

Eriksson, U. J. and L. A. Borg (1993) Diabetes and embryonic malformations. Role of substrate-induced free-oxygen radical production for dysmorphogenesis in cultured rat embryos. *Diabetes*. **42**, 411-9.

Eriksson, U. J. and C. M. Siman (1996) Pregnant diabetic rats fed the antioxidant butylated hydroxytoluene show decreased occurrence of malformations in offspring. *Diabetes*. **45**, 1497-502.

Fantel, A. G. (1996) Reactive oxygen species in developmental toxicity: review and hypothesis. *Teratology*. **53**, 196-217.

Fleming, A. and A. J. Copp (1998) Embryonic folate metabolism and mouse neural tube defects. *Science*. **280**, 2107-9.

Hagay, Z. J., Y. Weiss, I. Zusman, M. Peled-Kamar, E. A. Reece, U. J. Eriksson and Y. Groner (1995) Prevention of diabetes-associated embryopathy by overexpression of the free radical scavenger copper zinc superoxide dismutase in transgenic mouse embryos. *Am J Obstet Gynecol*. **173**, 1036-41.

Heinze, E. and U. Vetter (1987) Skeletal growth of fetuses from streptozotocin diabetic rat mothers: in vivo and in vitro studies. *Diabetologia*. **30**, 100-3.

Hester, P. Y., M. A. Schreiweis, J. I. Orban, H. Mazzuco, M. N. Kopka, M. C. Ledur and D. E. Moody (2004) Assessing bone mineral density in vivo: dual energy X-ray absorptiometry. *Poult Sci*. **83**, 215-21.

Hochberg, Z. (2002) *Endocrine control of skeletal maturation*, Karger, Basel

Holdsworth, D. W. and M. M. Thornton (2002) Micro-CT in small animal and specimen imaging. *Trends Biotechnol*. **20**, S34-39.

Horlick, M., J. Wang, R. N. Pierson, Jr. and J. C. Thornton (2004) Prediction models for evaluation of total-body bone mass with dual-energy X-ray absorptiometry among children and adolescents. *Pediatrics*. **114**, e337-45.

Jansson, T. and T. L. Powell (2007) Role of the placenta in fetal programming: underlying mechanisms and potential interventional approaches. *Clin Sci (Lond)*. **113**, 1-13.

Kanter, M., M. F. Altan, S. Donmez, A. Ocakci and M. E. Kartal (2007) The effects of quercetin on bone minerals, biomechanical behavior, and structure in streptozotocin-induced diabetic rats. *Cell Biochem Funct*. **25**, 747-52.

Kempuraj, D., B. Madhappan, S. Christodoulou, W. Boucher, J. Cao, N. Papadopoulou, C. L. Cetrulo and T. C. Theoharides (2005) Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells. *Br J Pharmacol*. **145**, 934-44.

Khong, T. Y., F. De Wolf, W. B. Robertson and I. Brosens (1986) Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants. *Br J Obstet Gynaecol*. **93**, 1049-59.

Kim, Y. J., Y. C. Bae, K. T. Suh and J. S. Jung (2006) Quercetin, a flavonoid, inhibits proliferation and increases osteogenic differentiation in human adipose stromal cells. *Biochem Pharmacol*. **72**, 1268-78.

Lac, G., H. Cavalie, E. Ebal and O. Michaux (2008) Effects of a high fat diet on bone of growing rats. Correlations between visceral fat, adiponectin and bone mass density. *Lipids Health Dis*. **7**, 16.

Liang, C., K. DeCourcy and M. R. Prater (2009) High saturated fat diet induces gestational diabetes and placental vasculopathy. (in review). *Placenta*.

Manach, C., C. Morand, C. Demigne, O. Texier, F. Regerat and C. Remesy (1997) Bioavailability of rutin and quercetin in rats. *FEBS letters*. **409**, 12-6.

Mills, J. L., R. H. Knopp, J. L. Simpson, L. Jovanovic-Peterson, B. E. Metzger, L. B. Holmes, J. H. Aarons, Z. Brown, G. F. Reed, F. R. Bieber and et al. (1988) Lack of relation of

increased malformation rates in infants of diabetic mothers to glycemic control during organogenesis. *N Engl J Med*. **318**, 671-6.

Myatt, L., W. Kossenjans, R. Sahay, A. Eis and D. Brockman (2000) Oxidative stress causes vascular dysfunction in the placenta. *J Matern Fetal Med*. **9**, 79-82.

Neubert, D., H. Peters, S. Teske, E. Kohler and H. J. Barrach (1971) Studis on the problem of "aerobic glycolysis" occuring in mammalian embryos. *Naunyn Schmiedebergs Arch Pharmakol*. **268**, 235-41.

Oest, M. E., J. C. Jones, C. Hatfield and M. R. Prater (2008) Micro-CT evaluation of murine fetal skeletal development yields greater morphometric precision over traditional clear-staining methods. *Birth Defects Res A Clin Mol Teratol*. **83**, 582-589.

Ornoy, A. (2007) Embryonic oxidative stress as a mechanism of teratogenesis with special emphasis on diabetic embryopathy. *Reprod Toxicol*. **24**, 31-41.

Prater, M. R., C. L. Laudermilch and S. D. Holladay (2006a) Does Immune Stimulation or Antioxidant Therapy Reduce MNU-induced Placental Damage Via Activation of Jak-STAT and NFkappaB Signaling Pathways? *Placenta*. **28**, 566-70.

Prater, M. R., C. L. Laudermilch, C. Liang and S. D. Holladay (2008) Placental oxidative stress alters expression of murine osteogenic genes and impairs fetal skeletal formation. *Placenta*. **29**, 802-8.

Prater, M. R., K. L. Zimmerman, L. C. Pinn, J. M. Keay, C. L. Laudermilch and S. D. Holladay (2006b) Role of maternal dietary antioxidant supplementation in murine placental and fetal limb development. *Placenta*. **27**, 502-9.

Prouillet, C., J. C. Maziere, C. Maziere, A. Wattel, M. Brazier and S. Kamel (2004) Stimulatory effect of naturally occurring flavonols quercetin and kaempferol on alkaline phosphatase

activity in MG-63 human osteoblasts through ERK and estrogen receptor pathway. *Biochem Pharmacol*. **67**, 1307-13.

Schreiweis, M. A., J. I. Orban, M. C. Ledur and P. Y. Hester (2003) The use of densitometry to detect differences in bone mineral density and content of live White Leghorns fed varying levels of dietary calcium. *Poult Sci*. **82**, 1292-301.

Sharova, L. V., A. A. Sharov, P. Sura, R. M. Gogal, B. J. Smith and S. D. Holladay (2003) Maternal immune stimulation reduces both placental morphologic damage and down-regulated placental growth-factor and cell cycle gene expression caused by urethane: are these events related to reduced teratogenesis? *Int Immunopharmacol*. **3**, 945-55.

Sivan, E., E. A. Reece, Y. K. Wu, C. J. Homko, M. Polansky and M. Borenstein (1996) Dietary vitamin E prophylaxis and diabetic embryopathy: morphologic and biochemical analysis. *Am J Obstet Gynecol*. **175**, 793-9.

Smith, E. E., V. L. Ferguson, S. J. Simske, E. C. Gayles and M. J. Pagliassotti (2000) Effects of high fat or high sucrose diets on rat femora mechanical and compositional properties. *Biomed Sci Instrum*. **36**, 385-90.

Ward, W. E., S. Kim and W. Robert Bruce (2003) A western-style diet reduces bone mass and biomechanical bone strength to a greater extent in male compared with female rats during development. *Br J Nutr*. **90**, 589-95.

Wentzel, P. and U. J. Eriksson (1998) Antioxidants diminish developmental damage induced by high glucose and cyclooxygenase inhibitors in rat embryos in vitro. *Diabetes*. **47**, 677-84.

Yang, X., L. A. Borg and U. J. Eriksson (1997) Altered metabolism and superoxide generation in neural tissue of rat embryos exposed to high glucose. *Am J Physiol*. **272**, E173-80.

Table 1. High fat diet (HFD) composition

High Fat Diet	gm%	Kcal%
Protein	26.2	20
Carbohydrate	26.3	20
Fat	34.9	60

Fat composition:		%
Saturated		32.1
Monounsaturated		46
Polyunsaturated		16.9
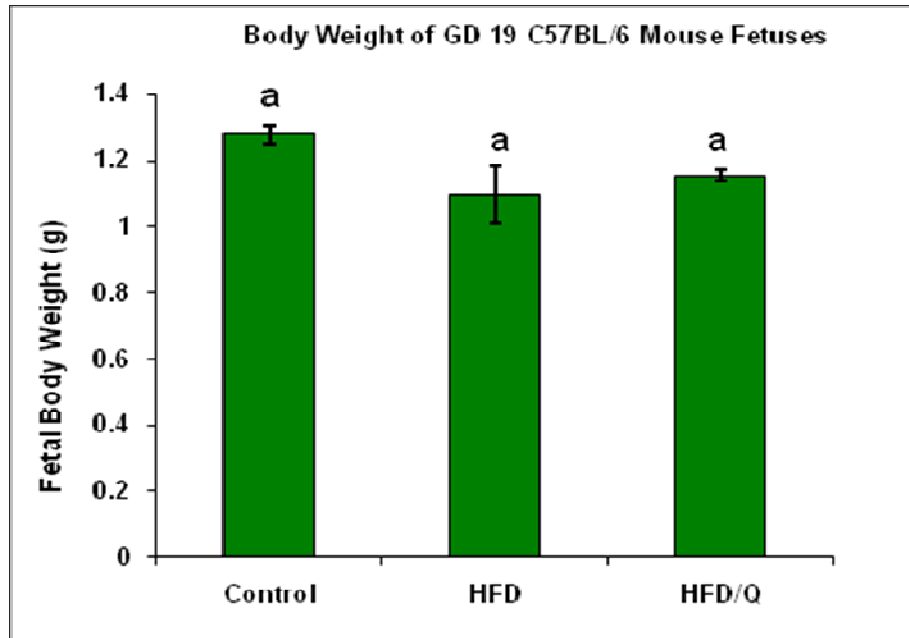
Fig. 1. Measurement of GD19 Mouse Fetal Body Weight. Dams were sacrificed on GD 19. Fetal weights from control, HFD and HFD/Q dams were recorded. Fetuses from HFD dams showed a numerically decreased body weight by 15% compared to control, but this was not statistically significant. Fetal body weight from HFD/Q group was not significantly different from HFD. Values are given as means ± SEM. Bars with same letters indicate values are not significantly different, $P > 0.05$. $n=16$.
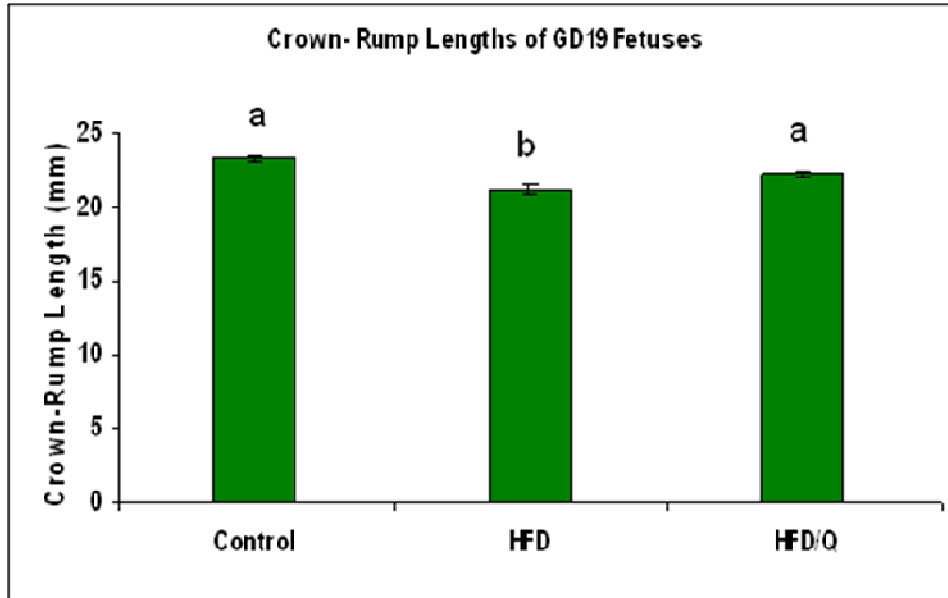
Fig. 2. Crown- Rump Lengths of GD19 Fetuses. Gross measurement was used to quantify the crown-rump lengths of GD 19 fetuses from control, HFD and HFD/Q dams. The HFD group showed significantly shortened crown-rump lengths compared to control. Quercetin significantly protected against fetal crown-to-rump length loss by HFD. Values are given as means ± SEM. Bars with different letters represent significantly different values, $P < 0.05$. $n=16$.
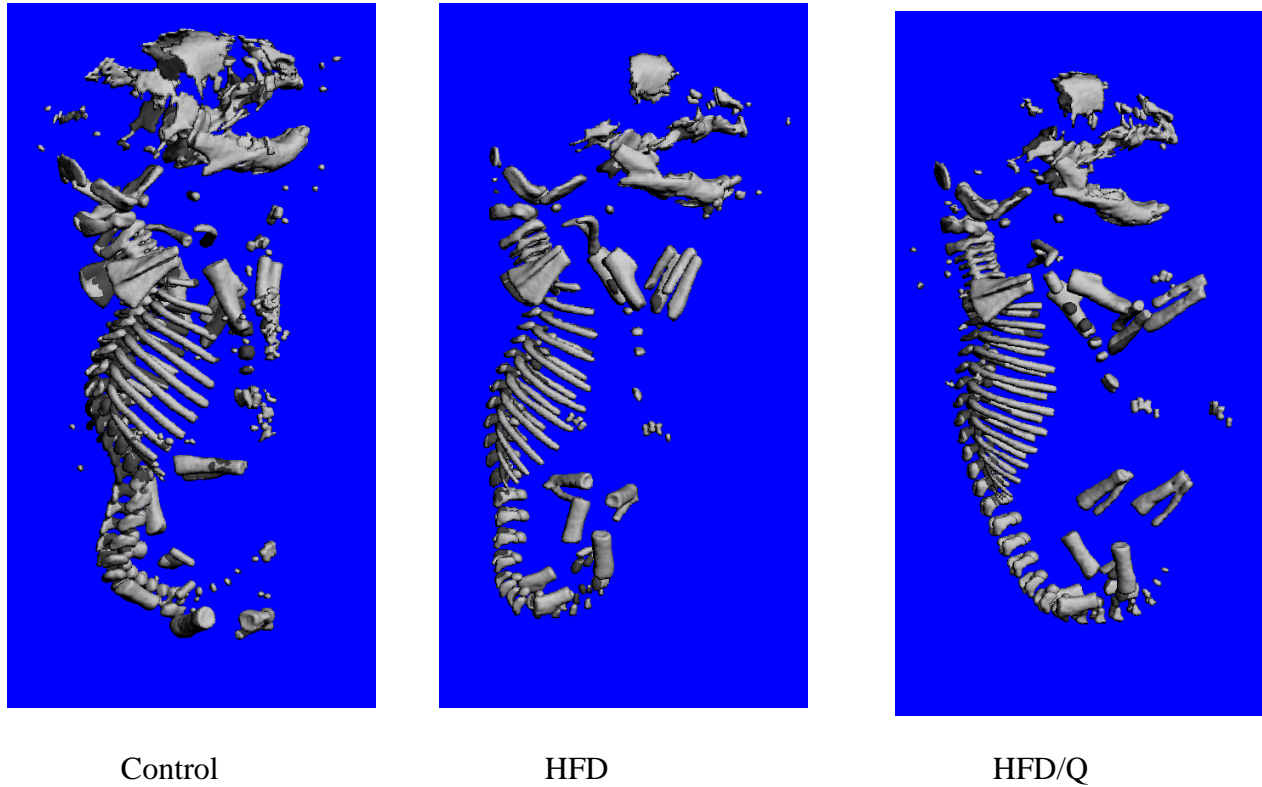
Control	HFD	HFD/Q

Fig. 3. 3-D Micro-CT Imaging of GD 19 Fetuses. Fetal skeletal structures were generated using reconstructed 3-D micro-CT imaging technique. From left to right: control, HFD and HFD/Q. Fetuses from HFD dams showed dramatically lowered total mineralized tissue, poor bone quality and delayed ossification process, while fetuses from HFD/Q dams showed numerically improved total mineralized tissue, bone quality and ossification process compared to HFD fetuses.

Total Bone Volume of GD19 C57BL/6 Mouses Fetuses
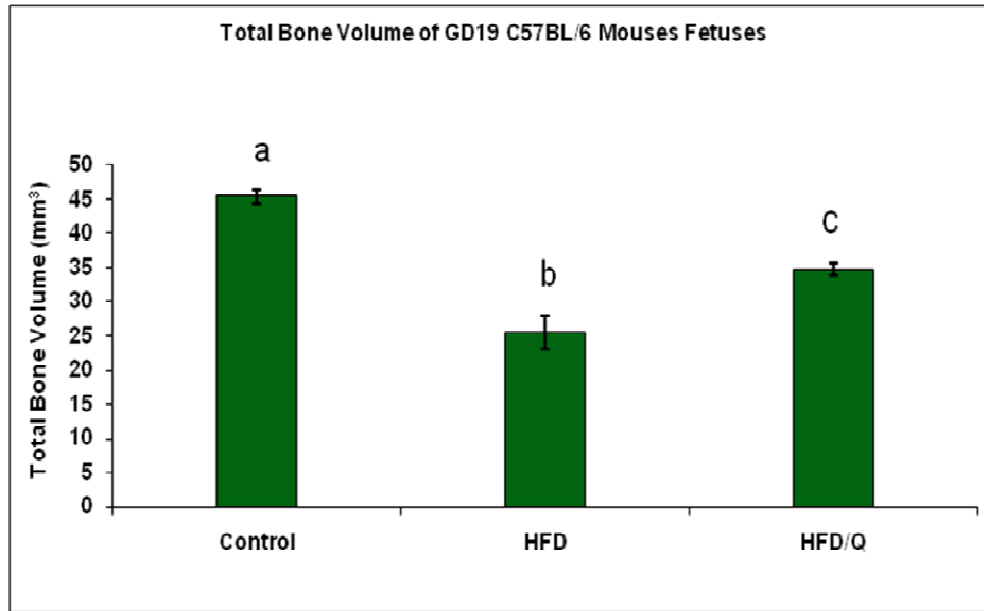
Fig. 4. Measurement of Total Bone Volume of GD 19 Mouse Fetuses. Fetal BV from control, HFD and HFD/Q dams were measured by micro-CT. Fetuses from HFD group showed significantly less fetal BV compared to control. Quercetin significantly protected against HFD induced total bone loss. Values are given as means ± SEM. Bars with different letters represent significantly different values, $P < 0.05$. $n=16$.
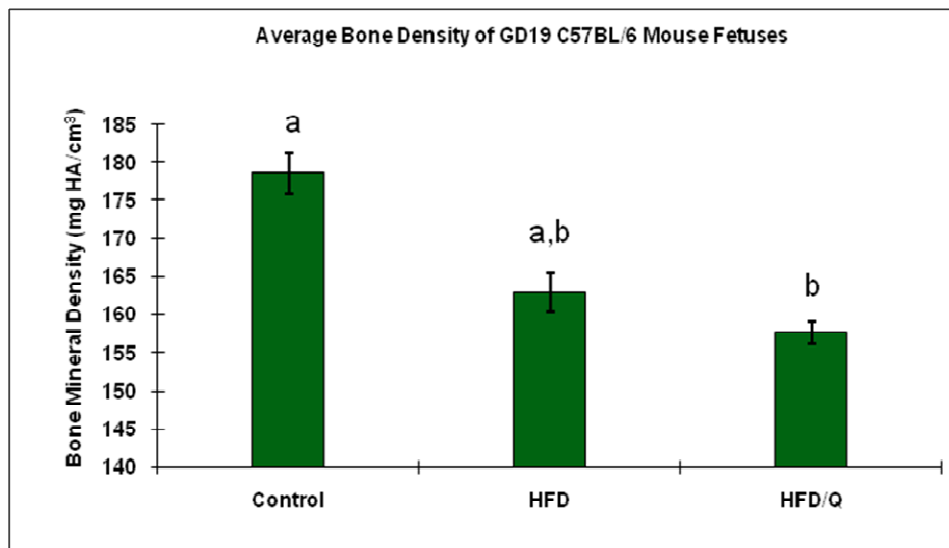
Fig. 5. Measurement of Average Bone Mineral Density of GD 19 Mouse Fetuses. Fetal BMD from control, HFD and HFD/Q dams were measured by micro-CT. Fetuses from HFD dams showed numerically decreased BMD as compared to control. Quercetin did not show protective effects against fetal BMD loss by HFD. Values are given as means ± SEM. Bars with different letters represent significantly different values, $P < 0.05$. $n=16$.
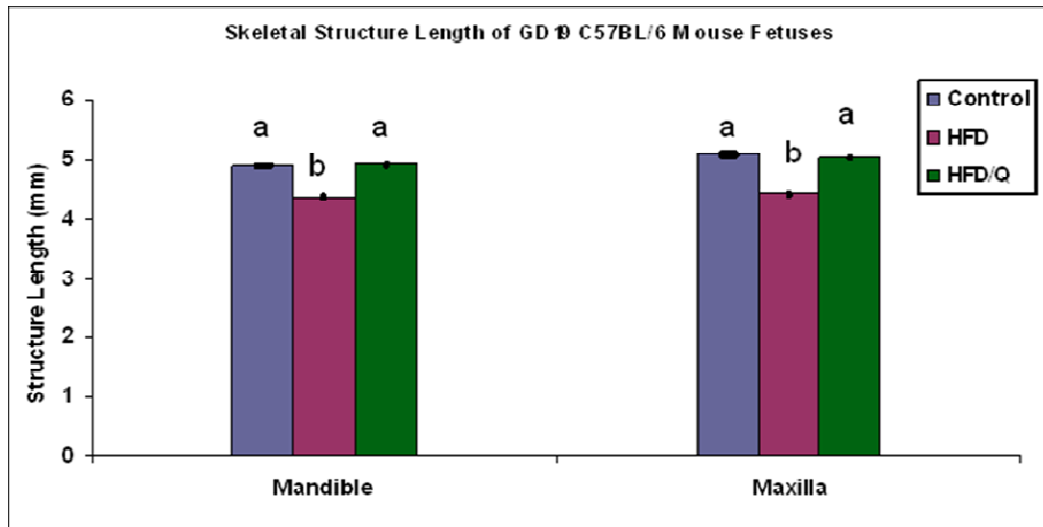
Fig. 6. Measurement of Mandibular and Maxillary Skeletal Structure Lengths of GD 19 Mouse Fetuses. Axial skeletal lengths (mandible and maxilla) of fetuses from control, HFD and HFD/Q were measured by micro-CT. Fetuses from HFD dams showed significantly shortened mandibular and maxillary structural lengths compared to control. Quercetin significantly protected against HFD-induced mandible and maxilla length loss. Values are given as means ± SEM. Bars with different letters represent significantly different values, $P < 0.05$. $n=16$.
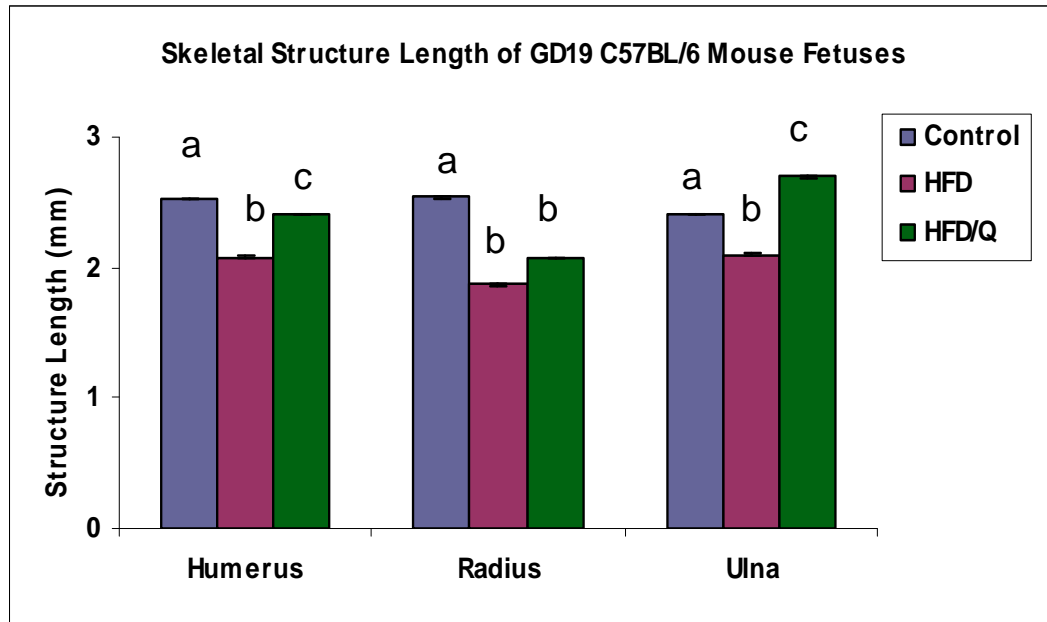
Fig. 7. Measurement of Humeral, Radial and Ulnar Skeletal Structure Lengths of GD 19 Mouse Fetuses. Appendicular skeletal lengths (humerus, radius and ulna) of fetuses from control, HFD and HFD/Q dams were measured by micro-CT. Fetuses from HFD dams showed significantly shortened humerus, radius and ulna structural lengths compared to control. Quercetin significantly protected against HFD-induced skeletal structural length loss in the measurement of humerus and ulna, but not in radius. Values are given as means ± SEM. Bars with different letters represent significantly different values, *P* < 0.05. *n*=16.
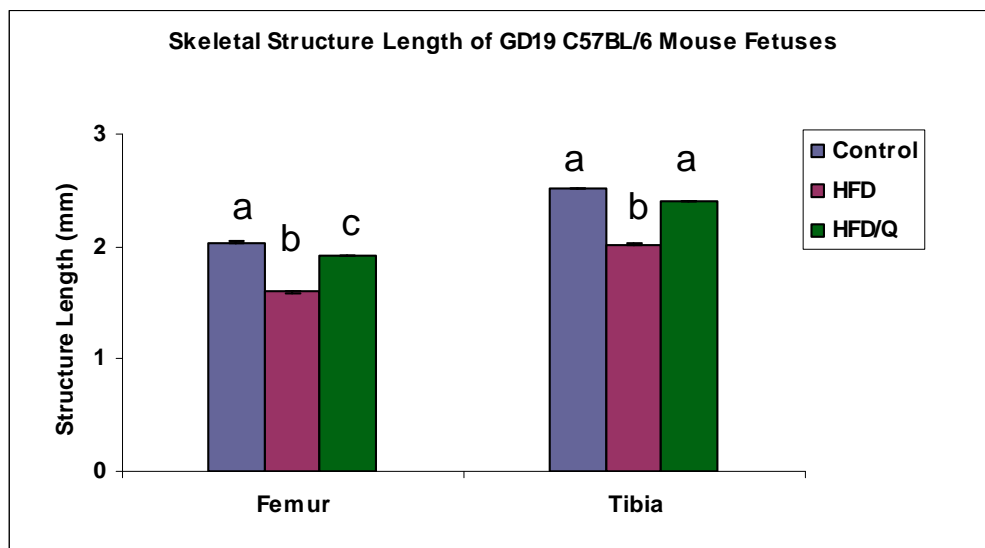
Fig. 8. Measurement of Femoral and Tibial Skeletal Structure Lengths of GD 19 Mouse Fetuses. Appendicular skeletal lengths (femur and tibia) of fetuses from control, HFD, HFD/Q dams were measured by micro-CT. Fetuses from HFD dams showed significantly shortened femoral and tibial structural lengths compared to control. Quercetin significantly protected against HFD-induced femoral and tibial length loss. Values are given as means ± SEM. Bars with different letters represent significantly different values, $P < 0.05$. $n=16$.

CHAPTER V

Intrauterine Exposure to High Saturated Fat Diet Elevates Risk of Adult-Onset Chronic Diseases in C57BL/6 Mice

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Abstract

A large body of human epidemiological and animal studies has demonstrated a close relationship between poor fetal growth and increased risk of diseases in adult life; this concept is referred to as the Developmental Origins of Health and Disease (DOHaD). The developmental environment is thought to strongly influence fetal programming and determine, in part, lifelong metabolic parameters and risk of adult diseases. The effects of maternal malnutrition on fetal growth and adult offspring health have been studied extensively. However, the contribution of gestational high saturated fat diet (HFD) and oxidative stress (OS) to adult-onset metabolic disease and skeletal dysfunction is incompletely understood. Animal models show deleterious effects of maternal HFD during pregnancy, including excessive OS, placental and vascular dysfunction, and fetal maldevelopment. Human children born of mothers consuming HFD during pregnancy are smaller at birth. In the present study, pathophysiologic mechanisms linking gestational HFD and elevated OS to perinatal development and adult-onset skeletal, cardiovascular and metabolic dysfunction were explored. Results of this study demonstrated that adult offspring of dams fed HFD during pregnancy exhibited life-long adverse effects, including hyperglycemia, insulin resistance, obesity, and hypertension, despite being fed healthy standard rodent chow throughout postnatal life. These offspring also showed significant lower femoral epiphyseal bone mineral density (BMD) at 6 months of age, and dysregulation of distal femoral trabecular architecture at 12 months of age, characteristics of osteoporosis, as measured by micro-computed tomographic image analysis. We were able to reduce incidence of these adverse skeletal and metabolic effects with maternal antioxidant (quercetin) supplementation during pregnancy. This effect may be related to improved placental integrity and function. Collectively, these data suggest that offspring of dams who consume a diet rich in saturated fat during

pregnancy are at increased risk of type 2 diabetes, hypertension and osteoporosis in adult life. Maternal quercetin supplementation may be considered as a possible preventive strategy to reduce altered fetal programming caused by gestational HFD consumption.

Introduction

Chronic diseases such as cardiovascular diseases, obesity, diabetes mellitus, and osteoporosis are leading causes of death and disability in the United States. These diseases account for 7 of every 10 deaths and affect the quality of life of 90 million Americans. An estimated 10 million Americans over age 50 have osteoporosis and 1.5 million people suffer an osteoporotic-related fracture each year (www.cdc.gov). Results of recent studies suggest that many adult diseases may originate from suboptimal conditions in fetal life. Low birth weight and short body length at birth are associated with elevated risk of obesity, cardiovascular disease and non-insulin dependent diabetes in adult life (Risbridger *et al.* 2005). This phenomenon is referred to as the fetal basis of adult disease, or 'DOHaD', the developmental origins of health and disease. These studies link fetal environment (maternal nutrition, gestational health, and OS) with permanently altered fetal programming and elevated risk of adult-onset diseases (Rolo and Palmeira 2006).

Optimal fetal development relies on an intricately orchestrated availability of nutrients and oxygen. Stresses to the fetus such as malnutrition, maternal diseases and exposure to environmental chemicals lead to inadequate fetal development (Hales and Barker 2001). Studies in experimental animals have demonstrated that maternal nutrition also plays an important role in lifelong health status, as poor nutrition during pregnancy is related to adulthood cardiovascular and metabolic disorders (Barker 1998; Barker *et al.* 2002). Low protein nutrition results in

significant growth restriction, and offspring of moms consuming insufficient protein are born 15% lighter than controls. These babies also demonstrate impaired glucose tolerance (Ozanne *et al.* 2003) and are at increased risk of developing frank diabetes and hypertension in adulthood (Petry *et al.* 1997; Petry *et al.* 2001). Maternal iron restriction has also been documented to induce low birth weight, increased systolic blood pressure, and insulin resistance in adult life (Lewis *et al.* 2001). A large body of epidemiological studies has also demonstrated a strong relationship between early growth restriction and the subsequent development of type 2 diabetes and metabolic syndrome (Ozanne 2001). However, little information is available regarding the role of maternal HFD in adult-onset multisystemic chronic diseases.

Exposure to environmental toxins during childhood, or even during gestation, is thought to also cause improper skeletal development and mineralization (Andrade *et al.* 2007), and may be an important contributors to adult bone pathology (Bianchi 2007). Bony defects such as malformation or disorganized long bone trabecular architecture that arise during gestation are not typically repaired postnatally, and are thought to increase the risk for bone fragility fractures in adulthood (Gluckman *et al.* 2005). Worldwide epidemiological studies have shown that poor growth in fetal life and infancy is associated with decreased bone mass in adulthood due to altered fetal skeletal growth trajectory during critical periods of early development (Mehta *et al.* 2002). However, few animal studies have been conducted, to date, that mechanistically explain the observations from human populations regarding development original of adult-onset osteoporosis. We previously reported that maternal HFD during gestation induces placental damage and fetal malformation in the mouse model (Liang *et al.* 2009a; Liang *et al.* 2009b). In an attempt to understand mechanisms relating fetal environment to development of chronic adult diseases, the present study is intended to extend the results of our prenatal research of maternal

HFD-induced fetal malformation into adulthood. This study was designed to determine the role of maternal gestational HFD and fetal environment in elevating risk of cardiovascular, metabolic, and skeletal disease in 6 and 12-month offspring of C57BL/6 mouse dams. We hypothesized that maternal HFD-induced placental OS and vascular dysregulation, which interfere with fetal formation, will elevate risk of adult-onset type 2 diabetes, hypertension, and osteoporosis.

Quercetin is among the most common and potent flavonoid antioxidants available that can be administered orally to reduce dietary-induced lipid peroxidation. It is commonly found in fruits and vegetables, and has been shown to exert beneficial health effects, including protection against osteoporosis, cancer, cardiovascular diseases and aging (Boots *et al.* 2008). In addition to its antioxidant effects, quercetin is also thought to reduce bone resorption in animals by inhibiting osteoclast differentiation and activation (Woo *et al.* 2004). Therefore, the present study also examined the effect of maternal gestational dietary antioxidant supplementation with quercetin on development of HFD-induced adult-onset type 2 diabetes, hypertension, and osteoporosis. To our knowledge, few studies have been conducted that examine possible preventive strategies to reduce fetal origins of adult diseases. Our study demonstrating protective effects of quercetin against gestational HFD induced adult-onset multi-systemic chronic diseases may provide new insight into treatment or preventive strategies of these diseases.

Methods

Animals and dietary protocol

Male and female C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 6 weeks of age. Mice were housed in groups of five and were acclimated for 2 weeks at 22.0 ± 1ºC, 40%-60% humidity, and on a 12/12 h light/dark cycle. Mice were given food and

fresh water *ad libitum*. Experiments were all approved by the Virginia Tech Animal Care and Use Committee and not initiated until approval was granted. Following two-week acclimation, female mice were arbitrarily assigned to one of three groups: control, HFD, or HFD + quercetin. Mice were then fed either with a standard rodent diet (Harlan Teklad Global Diet 2018: 18% protein and 5% fat; SD) or with the HFD (20% protein and 60% total fat, 32.1% saturated fat, Research Diets Lab, New Brunswick, NJ) (Table 1), or with HFD supplemented with 66mg/kg quercetin, which is equivalent to 700mg/day (average human consumption is 200-400mg 3x/day; (Manach *et al.* 1997) for four weeks prior to breeding. Breeding was initiated following the four-week period of dietary intervention as such: males and females (1:2 ratio) were bred overnight, and mating was confirmed by the presence of a vaginal mucous plug in females the following morning. The presence of a vaginal plug was designated day 0 of gestation (GD 0). Female were permitted to give birth, with an expected gestation length of 20 days. Littermates were housed together with their dam until weaning at 21 days of age. After weaning, dams and male offspring were euthanized by pentobarbital overdose (150mg/kg intraperitoneal injection, IP; Schering-Plough Animal Health Corp, Kenilworth, NJ). Female offspring were housed in groups of ≤5, and fed conventional rodent chow (SD) *ad libitum* for 12 months. Six month old offspring of all groups have *n*=5. Twelve month old offspring of control and HFD/Q groups have *n*=5, HFD group has *n*=4 (due to premature death of one mouse).

Measurements of blood glucose, plasma insulin and body weight

Female blood glucose, plasma insulin, and body weight were recorded at 6 and 12 months of age. Body weight was recorded on a top-loading balance (Accu-622, Fisher Scientific, Suwanee, GA), and maternal blood samples were obtained via tail venipuncture to determine

non-fasting insulin and glucose levels. Blood glucose levels were determined by glucometer (Lifescan Surestep, Johnson & Johnson, Langhorne, PA) and plasma insulin levels were quantified by enzyme-linked immunosorbent assay (ELISA) (Alpco Diagnostics, Windham, NH), according to the manufacturer's instructions.

Measurements of blood pressure

Systolic and diastolic blood pressure (BP) were determined by noninvasive tail cuff method, using the SC1000 blood pressure analysis system (Hatteras Instruments, Cary, NC) in adult female offspring of control, HFD, and HFD/Q dams, at 6 and 12 months of age. Mice were comfortably restrained and constantly observed in a pre-warmed dark chamber, to allow consistent BP reading. Real-time data (systolic, diastolic, and mean arterial pressures) were displayed using volume pressure recording. Tail systolic and diastolic BP were then recorded following the manufacturer's instructions.

Micro-CT assessments of offspring bone development

The present study also endeavored to improve understanding of the contribution of the intrauterine environment to the bone architecture through adulthood. We hypothesized that gestational HFD would dysregulate skeletal programming, increase bony malformation, diminish bone density, and disrupt trabecular architecture of adult offspring. The effect of maternal HFD on the bone mass of the adult offspring was assessed by high-resolution micro-computed tomographic image analysis (micro-CT). Trabecular bone density and bony trabecular microarchitecture were measured postnatally at ages 6 and 12 months, to document gestational HFD-induced fetal skeletal alterations that persist throughout adulthood. A high-speed *in vivo*

micro-CT system (VivaCT 40, Scanco Medical, Bassersdorf, Switzerland) was used to assess adult mouse skeletal structures, following mild sedation with isoflurane. At six months of age, the female offspring skeletal structures were examined as such: the scanning chamber was pre-warmed using flexible tubing attached to a Bair hugger warm air machine (Arizant Healthcare Inc., Eden Prairie, MN). Mice were positioned in an acrylic container in sternal recumbency with hindlimbs extended caudally, with head supported with gauze pads to maintain neck extension and open airway, and the tail was taped to the side of the tube. Skeletal image data were archived and manipulated using OsiriX, a DICOM-compatible freeware program (OsiriX, version 2.6, Medical Imaging Software, Los Angeles, CA) to calculate bone mineral density (BMD) and to evaluate metaphyseal and epiphyseal trabecular microarchitecture. At 12 months of age, offspring were euthanized by intraperitoneal pentobarbital overdose (150mg/kg), and skeletal structure scanning and evaluation were performed using the same protocol as previously described for 6-month old offspring.

Statistical analysis

All data in this study were presented as mean ± standard error. One-way analysis of variance (ANOVA) was used with Tukey-Kramer HSD test (JMP, SAS Institute Inc., Cary, NC) to establish significant differences between groups. Data were determined to be statistically different when *P*<0.05.

Results

Effects of HFD on blood glucose

Blood glucose levels of mouse offspring were determined at 6 and 12 months of age. As shown in Fig.1, blood glucose levels at 6 months and 12 months from the HFD offspring increased 31.2% and 78.8% respectively, which were significantly different from control, indicating the development of hyperglycemia in the HFD group. We also observed a progressively increased blood glucose level from 6 to 12 months, indicating an age-dependant effect of gestational HFD on postnatal female mice. Maternal quercetin supplementation significantly protected against gestational HFD-induced increased of blood glucose of offspring at the ages of 6 and 12 months.

Effects of HFD on plasma insulin

Six-month old HFD offspring showed numerically increased of insulin levels, but this did not become significant until 12 months of age, when HFD offspring showed significantly higher plasma insulin levels compared to controls. Quercetin supplementation significantly protected against the development of hyperinsulinemia in adult offspring at 12 months of age.

Effects of HFD on insulin resistance

Taken together, data of Fig. 1 and Fig. 2 demonstrated a close association between blood glucose levels and plasma insulin levels in 6 and 12 month old HFD offspring. These offspring developed hyperglycemia and hyperinsulinemia concurrently, which suggested development of insulin resistance and type 2 diabetes following gestational exposure to HFD. These changes were not observed in the control group, and were not observed in offspring of dams fed concurrently with quercetin during pregnancy. These studies demonstrated that gestational HFD significantly increases risk of insulin resistance and type 2 diabetes in female offspring, and that

antioxidant supplementation effectively reduces gestational HFD-induced insulin resistance and development of type 2 diabetes in 6- and 12- month old offspring.

Effects of HFD on body weight

Gestational HFD also altered female offspring body weight in adulthood. As shown in Fig. 3, at the age of 6 months, HFD offspring showed significantly increased body weight compared to control. At 12 months of age, HFD offspring showed numerically increased of body weight compared to control, but this number was no longer significantly different than controls. At this point, the female offspring appeared to be hunched and exhibited an unthrifty hair coat, which gave the appearance of accelerated aging. Maternal quercetin supplementation significantly protected against HFD-induced weight gain at 6 months of age.

Effects of HFD on blood pressure

At 6 months of age, offspring from the HFD group showed numeric but insignificantly increased systolic and diastolic BP compared to control (Fig. 4). By the age of 12 months, BP of HFD offspring increased significantly as compared to controls, which demonstrated that gestational exposure to HFD caused female offspring to develop hypertension over time (Fig. 5). A progressively increased systolic and diastolic BP from 6 to 12 months was again observed, indicating an age-dependant association. Maternal quercetin supplementation showed significant protection against HFD-induced hypertension in 12-month old offspring.

Micro-CT assessments of 6- month offspring bone development

Using reconstructed, 3-D micro-CT imaging techniques, 6 and 12 month old offspring bone health was evaluated to determine the contribution of gestational HFD and elevated OS to offspring skeletal development. Trabecular bone from both metaphyseal and epiphyseal femur was examined in order to more completely understand the effects of gestational HFD, with or without concurrent antioxidant supplementation, on adult bone health. In the epiphyseal region, the HFD offspring at 6 months of age showed significantly lower BMD compared to control (HFD offspring were 87% of control), which suggested development of early osteoporosis (Fig. 6). Maternal quercetin supplementation significantly protected against HFD-induced BMD loss. In metaphyseal region, there was no difference observed regarding BMD among groups. Regarding 6-month femoral trabecular microarchitecture, femoral trabecular number, thickness, spacing, anisotropy and connectivity density were examined. As shown in Fig. 7 and Fig. 8, there was no difference observed regarding trabecular microarchitecture changes among groups at this time point.

Micro-CT assessments of 12- month offspring bone development

Femoral trabecular microarchitecture was similarly evaluated by micro-CT in offspring at 12 months of age. Femoral trabecular number, thickness, spacing, anisotropy, and connectivity density were examined. The trabecular spacing in metaphyseal region significantly increased in offspring from HFD dams compared to control (Fig. 9). Maternal quercetin supplementation did not protect against the increasing of trabecular spacing of HFD offspring. Fig. 10 displays the measurement of 12-month offspring trabecular connectivity density. At epiphyseal region, trabecular connectivity density of offspring from HFD dams was significantly deceased

compared to control. Concurrent maternal quercetin supplementation significantly protected against HFD-induced reduction of trabecular connectivity density of 12 month old offspring. There was no difference observed among groups in the metaphyseal region.

Discussion

Evidence from animal and human studies demonstrates that many adult chronic diseases have their origins in fetal life (Barker 1998). The present study demonstrates the important role of gestational exposure to HFD in increased risk of type 2 diabetes, hypertension and osteoporosis in adult female offspring. An important factor that makes this study particularly relevant is that all the offspring from control, HFD and HFD/Q dams were fed standard rodent chow and fresh water *ad libitum* after weaning. This experimental design permitted the researchers to specifically isolate the effects of maternal HFD +/- quercetin consumption, and removed post-weaning diet as a confounding variable. To our knowledge, this is the first time maternal HFD intervention was used to explore the contribution of gestational diet on long-term elevated risk of cardiovascular, endocrine and skeletal diseases.

The terms 'developmental origins of health and disease' and 'fetal programming' were coined on the basis of the inverse association between birth weight and adult-onset hypertension, diabetes, coronary heart disease, and stroke seen in numerous epidemiological studies (Barker 1998). The importance of environmental factors in perinatal development has been demonstrated by a number of human studies. A link between intrauterine environment and adult disease was pioneered by Barker and co-workers, who demonstrated association between low birth weight and adverse profile of adult glucose and insulin metabolism (Hales *et al.* 1991b) and cardiovascular disease (Barker *et al.* 1989). These studies suggest that fetal environment is in

large part responsible for permanent modification of gene pathways that influence birth size and neonatal health, as well as relative risk of adult-onset diseases. Identical twins studies also suggest that that fetal as well as postnatal environments are important determinants of programming and risk of adult-onset chronic disease (Hales and Barker 2001). However, the mechanistic basis of this relationship between genes and the environment remains a subject of debate. Studies of extensive genomic scans to identify universal diabetes susceptibility genes/ polymorphisms have been unsuccessful in isolating genetic alterations as the sole predictor of chronic disease (Ozanne 2001).

The biological basis of the associations observed between poor fetal growth and the subsequent development of diseases in adult life in the epidemiological study was explained by the *'thrifty phenotype hypothesis'*. When the fetal environment is poor, the fetus adapts to an adverse intrauterine environment by optimizing the use of a reduced nutrient supply to ensure survival. Therefore, blood flow is redistributed in favor of key body organs at the expense of non-essential organs, and this results in altered production of fetal and placental hormones which control fetal growth, and leads to an altered postnatal metabolism (Hales *et al.* 1991a; Barker *et al.* 1993). It is believed that early life responses to nutritional insufficiency may enhance survival in the short run, but may be maladaptive in later life when the organism no longer is faced with nutritional insufficiency (Gluckman and Hanson 2004b).

Many studies demonstrate the close relationship between low birth weight and adult-onset diseases (Gennser *et al.* 1988; Hales *et al.* 1991b; Gale *et al.* 2001; Ozanne 2001). However, few studies have been performed regarding birth size other than birth weight. This is a major limitation because birth weight only provides a crude summary index of growth (Barker 1998). Our research evaluated both fetal birth weight as well as various indicators of fetal size to

determine its effects of adult diseases from development origin. We previously reported (Liang *et al.* 2009b) fetuses from HFD dams have a numerically decreased birth weight, significantly decreased crown-to-rump length and skeletal lengths compared to control, which is a more comprehensive method to characterize small birth size. HFD fetuses also showed decreased BV and BMD, indicating fetal skeletal malformation and developmental delay. All these changes are believed to contribute to adult-onset chronic multi-organ diseases demonstrated in the present study, as these skeletal changes observed in fetal life are not repaired postnatally, and persist into adulthood. Therefore, our study provides very convincing evidence to demonstrate a strong relationship between poor fetal growth and elevated incidence of adult-onset bone disease.

Over recent decades the incidence of diabetes and metabolic syndrome has dramatically increased in Western populations and has been attributed in part to environmental factors, notably diet (Holemans *et al.* 2000). Studies of individuals exposed *in utero* to famine during the Dutch hunger winter have revealed that maternal nutrition leads to restriction of the fetus and is associated with poor glucose tolerance and insulin resistance (Kahn 2001). Other studies also show that subjecting the pregnant dams to protein malnutrition results in disturbance of glucose homeostasis and insulin metabolism (Dahri *et al.* 1991). Males and females with low birth weight from mothers suffering malnutrition during pregnancy have a high prevalence of a collection of symptoms referred to as 'insulin resistance syndrome' (Barker *et al.* 1993) in which impaired glucose tolerance, hyperinsulinemia and raised serum triacylglycerol concentrations occur simultaneously, and increase lifetime risk of metabolic and cardiovascular disease (Barker 1998). Our previous studies determined that maternal HFD intake induces smaller fetal size and is associated with fetal malformation (Liang *et al.* 2009b). The present study extended the fetal study to adulthood and showed 6 and 12-month old offspring from HFD dams developed

hyperglycemia, hyperinsulinemia and insulin resistance, indicating development of type 2 diabetes in adulthood. HFD offspring also accrued more body weight by 6 months of age. We also observed an age-dependent increase of blood glucose and plasma insulin from 6 to12 months of age, which provides strong evidence that fetal environment and poor fetal growth are a major contributor to adult-onset metabolic disorder and development of type 2 diabetes.

Hypertension represents a major public health challenge worldwide due to its high prevalence and importance as a risk factor for cardiovascular disease mortality (Adair and Dahly 2005). Although a great deal is known about modifiable risk factors for hypertension, including obesity, smoking, alcohol consumption, diet, and physical activity, these factors do not fully explain differences in its occurrence (Adair and Dahly 2005). Several recent studies have examined the role of protein restriction, fetal development, birth weight, and birth size in lifelong regulation of BP, and risk of cardiovascular disease mortality (Barker 1998; Ozanne and Hales 1999; Adair and Dahly 2005). Gardiner and others reported that offspring of mothers fed a HFD have altered fatty acid content and reduced endothelial-dependent vascular dilation and endothelial dysfunction in small mesenteric arteries (Khan *et al.* 2005; Gardiner 2007). Based on these findings, we investigated the effects of maternal HFD on BP of 6-and 12-month old adult offspring of C57BL/6 mice. Our data demonstrated that offspring from HFD dams showed increases of both systolic BP and diastolic BP. We also observed an age-dependent increase of systolic and diastolic BP from 6 month to 12 months. This hypertension may be related to changes in fetal kidney structure and the activity of the renin-angiotensin system (Langley-Evans *et al.* 1999) or fetal hypothalamic-pituitary-adrenal axis which in turn resets homoeostatic mechanisms controlling BP (Edwards *et al.* 1993).

Associations between low birth weight and cardiovascular and metabolic disorders have been repeatedly demonstrated, however, there is less information about developmental origins of osteoporosis. Osteoporosis and osteoporotic fracture are the leading causes of disability in the United States (Rakel *et al.* 2008). Although the most widely recognized risk factor for development of osteoporosis is middle-aged estrogen fluctuation, other contributions of genetic and environmental factors to the development of osteoporosis are not fully understood. Recent studies suggest that environmental influences such as maternal OS and malnutrition during fetal development may play a causal role in the pathogenesis of osteoporosis by permanently altering fetal skeletal gene programming (Cooper *et al.* 2002). Impaired bone density and architecture result when the fetal environment is sub-optimal, and requires the infant to adapt for survival. Perinatal adaptation of fetal growth restriction is often manifested as 'catch-up growth', which favors differentiation of immature cells into fat cells (adipocytes) rather than bone cells (osteoblasts) (Hales and Barker 1992). Studies also show the bone mass at any stage in later life depends upon the peak level attained during skeletal growth (Fall *et al.* 1998) and growth during prenatal and early postnatal life may be a determinant of peak adult bone mass (Cooper *et al.* 1995).

Recent studies in our laboratory have shown that maternal gestational HFD induces placental vascular damage and increased OS, and results in fetal skeletal growth restriction and increased risk of permanent bony malformation (Liang *et al.* 2009a). The mechanisms of ossification of metaphyseal and epiphyseal regions are different. Metaphyseal femur undergoes endochondral ossification, and the epiphyseal region undergoes intramembranous ossification. In order to determine the contribution of gestational HFD to long-term bone health and relative risk of adult-onset osteoporosis, in the present study, we measured BMD from both regions in order

to completely define the effects of HFD on the process of osteoporosis. In additional to bone mass, the trabecular structure is a critical factor contributing to bone strength. Three-dimensionally reconstructed micro-CT image analysis permits the quantitative assessment of trabecular bone architecture and yields an overall characterization of both bone quantity (total bone volume) and quality (trabecular structure), which leads to a more comprehensive and accurate understanding of skeletal integrity (Judex *et al.* 2003). In the present study, in addition to observe BMD changes, we also examined changes of trabecular microarchitecture such as trabecular spacing and connectivity density among groups. Six-month old offspring from HFD dams showed significantly decreased BMD in epiphyseal femur compared to control, which demonstrated development of early stage of osteoporosis. However, in metaphyseal region, no difference observed among groups suggested that in the development of osteoporosis, the epiphyseal region responds differently to environmental challenges than the metaphyseal region. This could be due to the differences in bone ossification mechanisms of these two regions. However, neither trabecular spacing nor trabecular connectivity were affected at this stage. Our 12 months measurement data revealed that HFD offspring showed significantly increased trabecular spacing in metaphyseal region and decreased trabecular connectivity density in epiphyseal region, indicating trabecular microarchitectural changes and development of overt osteoporosis. These results also suggested the age-dependent manner of the progress of osteoporosis. These observations in mice mirror those made in epidemiological studies among human populations and suggest that HFD-induced fetal skeletal malformation is associated with permanent dysregulation of skeletal programming during gestation and increased skeletal osteoporosis in adult life. There was no difference observed in trabecular spacing of epiphyseal

region and trabecular connectivity density of metaphyseal region among groups. This could be due to low sample number of HFD group caused by premature death of mice.

The DOHaD hypothesis proposes that developmental plasticity and fetal adaptation underlie the links between maternal nutrition, size at birth and coronary heart disease and type 2 diabetes in adult life (Godfrey 2002). There is evidence that the placenta may play an important role in determining these adaptations and developmental changes (Godfrey 2002). The placenta functions as a nutrient sensor, which coordinates nutrient transport functions with maternal nutrient availability and serves as a key player in the regulation of fetal growth and, as a consequence, fetal programming. Epidemiological studies suggest that placental health appears to provide information on the long-term outcome for the baby (Godfrey 2002). Studies show a possible link between placental structure changes and elevated risk of adult diseases, which is particularly evident for the fetal programming of cardiovascular disease (Jansson and Powell 2007). The placental pathologies that are associated with fetal programming are also associated with increased OS in the placenta (Wang *et al.* 1992; Giugliano *et al.* 1996). These findings correspond well with our research. We previously reported that placenta from HFD dams showed significantly decreased viable trophoblast and endothelial cell number, increased OS, severe placental vasculopathy and poor placental health (Liang *et al.* 2009a). These further result in poor fetal growth and, as a consequence, increased incidence in adult-onset type 2 diabetes, hypertension and osteoporosis in surviving offspring, as demonstrated in the present study. The relationship between placental damage, fetal malformation and adult-onset diseases explored in our study may provide a better understanding of the mechanisms underlying fetal programming.

A number of studies have suggested potential mechanisms underlying the developmental origins of adult disease. However, few studies have been completed which explore potential

therapeutic or preventive therapies to reduce adverse effects of maternal malnutrition on poor fetal growth and their roles in adult-onset type 2 diabetes, hypertensive vascular diseases and osteoporosis. In our study, concurrent maternal quercetin supplementation was used as the preventive strategy to protect against HFD-induced fetal malformation and adult-onset chronic diseases. Quercetin, a potent antioxidant in the flavonoid family, has been shown to exert beneficial health effects, which includes protection against various diseases such as osteoporosis, cancer, and cardiovascular diseases and also against aging (Boots *et al.* 2008). We previously reported that quercetin significantly decreases maternal HFD-induced placental OS and improves placental vascular health (Liang *et al.* 2009a). Thus, fetuses from HFD/Q dams show significantly improved birth size and skeletal development as well as protection against development origin of type 2 diabetes, hypertension and osteoporosis, as demonstrated in the present study. Quercetin significantly decreased maternal HFD induced hyperglycemia, hyperinsulinemia, insulin resistance and BP of 6 and 12 month HFD offspring. Quercetin also showed significant protection of skeletal programming during fetal development and result in significantly improved trabecular connectivity density, therefore, improved trabecular microarchitecture and partially protected against osteoporosis in adult offspring of HFD treated C57BL/6 dams.

In conclusion, our study demonstrated that maternal malnutrition due to gestational HFD perturbed fetal environment and resulted in fetal maldevelopment, and therefore appeared to program the fetus towards development of adult-onset type 2 diabetes, hypertension and osteoporosis. The observations presented in this study provide strong support for the developmental origins of adult disease. Our findings of the effects of fetal environment on adult offspring multi-organ chronic diseases, the role of the placenta in fetal programming and the

therapeutic effects of quercetin on adult-onset diseases not only improve the mechanistic understanding of the development origins of adult disease, but may also provide possible effective preventive strategies against fetal programming throughout the life course.

References

Adair, L. and D. Dahly (2005) Developmental determinants of blood pressure in adults. *Annu Rev Nutr*. **25**, 407-34.

Andrade, A. C., O. Nilsson, K. M. Barnes and J. Baron (2007) Wnt gene expression in the post-natal growth plate: regulation with chondrocyte differentiation. *Bone*. **40**, 1361-9.

Barker, D. J. (1998) In utero programming of chronic disease. *Clin Sci (Lond)*. **95**, 115-28.

Barker, D. J., J. G. Eriksson, T. Forsen and C. Osmond (2002) Fetal origins of adult disease: strength of effects and biological basis. *Int J Epidemiol*. **31**, 1235-9.

Barker, D. J., C. N. Hales, C. H. Fall, C. Osmond, K. Phipps and P. M. Clark (1993) Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. *Diabetologia*. **36**, 62-7.

Barker, D. J., C. Osmond, J. Golding, D. Kuh and M. E. Wadsworth (1989) Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *BMJ*. **298**, 564-7.

Bianchi, M. L. (2007) Osteoporosis in children and adolescents. *Bone*. **41**, 486-95.

Boots, A. W., G. R. M. M. Haenen and A. Bast (2008) Health effects of quercetin: from antioxidant to nutraceutical. *Eur J Pharmacol*. **585**, 325-337.

Cooper, C., M. Cawley, A. Bhalla, P. Egger, F. Ring, L. Morton and D. Barker (1995) Childhood growth, physical activity, and peak bone mass in women. *J Bone Miner Res*. **10**, 940-7.

Cooper, C., M. K. Javaid, P. Taylor, K. Walker-Bone, E. Dennison and N. Arden (2002) The fetal origins of osteoporotic fracture. *Calcif Tissue Int*. **70**, 391-4.

Dahri, S., A. Snoeck, B. Reusens-Billen, C. Remacle and J. J. Hoet (1991) Islet function in offspring of mothers on low-protein diet during gestation. *Diabetes*. **40 Suppl 2**, 115-20.

Edwards, C. R., R. Benediktsson, R. S. Lindsay and J. R. Seckl (1993) Dysfunction of placental glucocorticoid barrier: link between fetal environment and adult hypertension? *Lancet*. **341**, 355-7.

Fall, C., P. Hindmarsh, E. Dennison, S. Kellingray, D. Barker and C. Cooper (1998) Programming of growth hormone secretion and bone mineral density in elderly men: a hypothesis. *J Clin Endocrinol Metab*. **83**, 135-9.

Gale, C. R., C. N. Martyn, S. Kellingray, R. Eastell and C. Cooper (2001) Intrauterine programming of adult body composition. *J Clin Endocrinol Metab*. **86**, 267-72.

Gardiner, H. M. (2007) Early environmental influences on vascular development. *Early Hum Dev*. **83**, 819-23.

Gennser, G., P. Rymark and P. E. Isberg (1988) Low birth weight and risk of high blood pressure in adulthood. *Br Med J (Clin Res Ed)*. **296**, 1498-500.

Giugliano, D., A. Ceriello and G. Paolisso (1996) Oxidative stress and diabetic vascular complications. *Diabetes Care*. **19**, 257-67.

Gluckman, P. D. and M. A. Hanson (2004) Living with the past: evolution, development, and patterns of disease. *Science*. **305**, 1733-6.

Gluckman, P. D., M. A. Hanson and C. Pinal (2005) The developmental origins of adult disease. *Matern Child Nutr*. **1**, 130-41.

Godfrey, K. M. (2002) The role of the placenta in fetal programming-a review. *Placenta*. **23 Suppl A**, S20-7.

Hales, C., D. Barker and C. PMS (1991a) Fetal and infant growth and impaired glucose tolerance. *BMJ*. **303**, 1474-5.

Hales, C. N. and D. J. Barker (1992) Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia*. **35**, 595-601.

Hales, C. N. and D. J. Barker (2001) The thrifty phenotype hypothesis. *Br Med Bull*. **60**, 5-20.

Hales, C. N., D. J. Barker, P. M. Clark, L. J. Cox, C. Fall, C. Osmond and P. D. Winter (1991b) Fetal and infant growth and impaired glucose tolerance at age 64. *BMJ*. **303**, 1019-22.

Holemans, K., R. Gerber, I. O'Brien-Coker, A. Mallet, R. van Bree, F. A. van Assche and L. Poston (2000) Raised saturated-fat intake worsens vascular function in virgin and pregnant offspring of streptozotocin-diabetic rats. *Br J Nutr*. **84**, 285-96.

Jansson, T. and T. L. Powell (2007) Role of the placenta in fetal programming: underlying mechanisms and potential interventional approaches. *Clin Sci (Lond)*. **113**, 1-13.

Judex, S., S. Boyd, Y. X. Qin, L. Miller, R. Muller and C. Rubin (2003) Combining high-resolution micro-computed tomography with material composition to define the quality of bone tissue. *Curr Osteoporos Rep*. **1**, 11-9.

Kahn, H. S. (2001) Glucose tolerance in adults after prenatal exposure to famine. *Lancet*. **357**, 1798-9.

Khan, I. Y., V. Dekou, G. Douglas, R. Jensen, M. A. Hanson, L. Poston and P. D. Taylor (2005) A high-fat diet during rat pregnancy or suckling induces cardiovascular dysfunction in adult offspring. *Am J Physiol Regul Integr Comp Physiol*. **288**, R127-33.

Langley-Evans, S. C., R. C. Sherman, S. J. Welham, M. O. Nwagwu, D. S. Gardner and A. A. Jackson (1999) Intrauterine programming of hypertension: the role of the renin-angiotensin system. *Biochem Soc Trans*. **27**, 88-93.

Lewis, R. M., C. J. Petry, S. E. Ozanne and C. N. Hales (2001) Effects of maternal iron restriction in the rat on blood pressure, glucose tolerance, and serum lipids in the 3-month-old offspring. *Metabolism: clinical and experimental*. **50**, 562-7.

Liang, C., K. DeCourcy and M. R. Prater (2009a) High saturated fat diet induces gestational diabetes and placental vasculopathy. (in review). *Placenta*.

Liang, C., M. E. Oest, J. C. Jones and M. R. Prater (2009b) Gestational high saturated fat diet alters C57BL/6 mouse perinatal skeletal formation. (in review). *Birth Defects Res A Clin Mol Teratol*.

Manach, C., C. Morand, C. Demigne, O. Texier, F. Regerat and C. Remesy (1997) Bioavailability of rutin and quercetin in rats. *FEBS letters*. **409**, 12-6.

Mehta, G., H. I. Roach, S. Langley-Evans, P. Taylor, I. Reading, R. O. Oreffo, A. Aihie-Sayer, N. M. Clarke and C. Cooper (2002) Intrauterine exposure to a maternal low protein diet reduces adult bone mass and alters growth plate morphology in rats. *Calcif Tissue Int*. **71**, 493-8.

Ozanne, E. S. (2001) Metabolic programming in animals. *British Medical Bulletin*. **60**, 143-152.

Ozanne, S. E. and C. N. Hales (1999) The long-term consequences of intra-uterine protein malnutrition for glucose metabolism. *Proc Nutr Soc*. **58**, 615-9.

Ozanne, S. E., G. S. Olsen, L. L. Hansen, K. J. Tingey, B. T. Nave, C. L. Wang, K. Hartil, C. J. Petry, A. J. Buckley and L. Mosthaf-Seedorf (2003) Early growth restriction leads to

down regulation of protein kinase C zeta and insulin resistance in skeletal muscle. *J Endocrinol*. **177**, 235-41.

Petry, C. J., M. W. Dorling, D. B. Pawlak, S. E. Ozanne and C. N. Hales (2001) Diabetes in old male offspring of rat dams fed a reduced protein diet. *Int J Exp Diabetes Res*. **2**, 139-43.

Petry, C. J., S. E. Ozanne, C. L. Wang and C. N. Hales (1997) Early protein restriction and obesity independently induce hypertension in 1-year-old rats. *Clin Sci (Lond)*. **93**, 147-52.

Rakel, A., O. Sheehy, E. Rahme and J. LeLorier (2008) Osteoporosis among patients with type 1 and type 2 diabetes. *Diabetes Metab*. **34**, 193-205.

Risbridger, G. P., A. E. Drummond and M. Wlodek (2005) Reproduction, development, and the early origins of adult disease. *Cell Tissue Res*. **322**, 3.

Rolo, A. P. and C. M. Palmeira (2006) Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress. *Toxicol Appl Pharmacol*. **212**, 167-78.

Wang, Y., S. W. Walsh and H. H. Kay (1992) Placental lipid peroxides and thromboxane are increased and prostacyclin is decreased in women with preeclampsia. *Am J Obstet Gynecol*. **167**, 946-9.

Woo, J. T., H. Nakagawa, M. Notoya, T. Yonezawa, N. Udagawa, I. S. Lee, M. Ohnishi, H. Hagiwara and K. Nagai (2004) Quercetin suppresses bone resorption by inhibiting the differentiation and activation of osteoclasts. *Biol Pharm Bull*. **27**, 504-9.

Table 1. High Fat Diet (HFD) Composition

High Fat Diet	gm%	Kcal%
Protein	26.2	20
Carbohydrate	26.3	20
Fat	34.9	60
Fat composition:		%
Saturated		32.1
Monounsaturated		46
Polyunsaturated		16.9
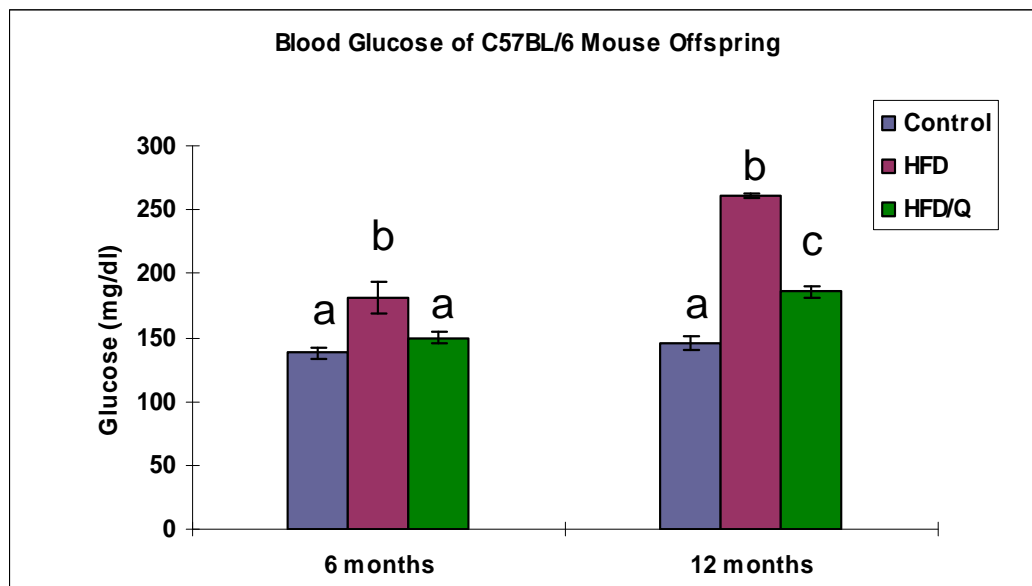
Fig. 1. Measurement of Blood Glucose Levels of 6-and 12-month Offspring. Six month and 12 month old HFD offspring showed significantly increased glucose levels compared to control. Maternal quercetin supplementation significantly protected against HFD-induced hyperglycemia. Values are given as means ± SEM. Bars with different letters represent significantly different values, $P < 0.05$. Six month old offspring of each group n=5. Twelve month old offspring of control and HFD/Q groups n=5, HFD group n=4.
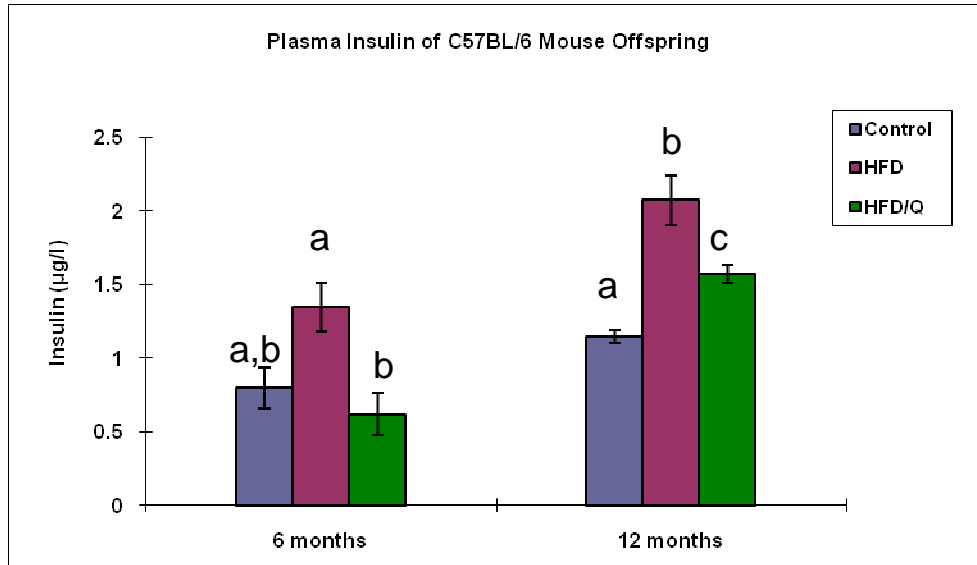
Fig. 2. Measurement of Plasma Insulin Levels of 6-and 12-Month Offspring. Six month old HFD offspring showed numerically increased insulin levels compared to control, but this was not statistically significant. At 12 months of age, a significantly increased insulin level was observed in the HFD offspring compared to controls. Maternal quercetin supplementation significantly protected against HFD induced hyperinsulinemia. Values are given as means ± SEM. Bars with different letters represent significantly different values, *P* < 0.05. Six month old offspring of each group n=5. Twelve month old offspring of control and HFD/Q groups n=5, HFD group n=4.
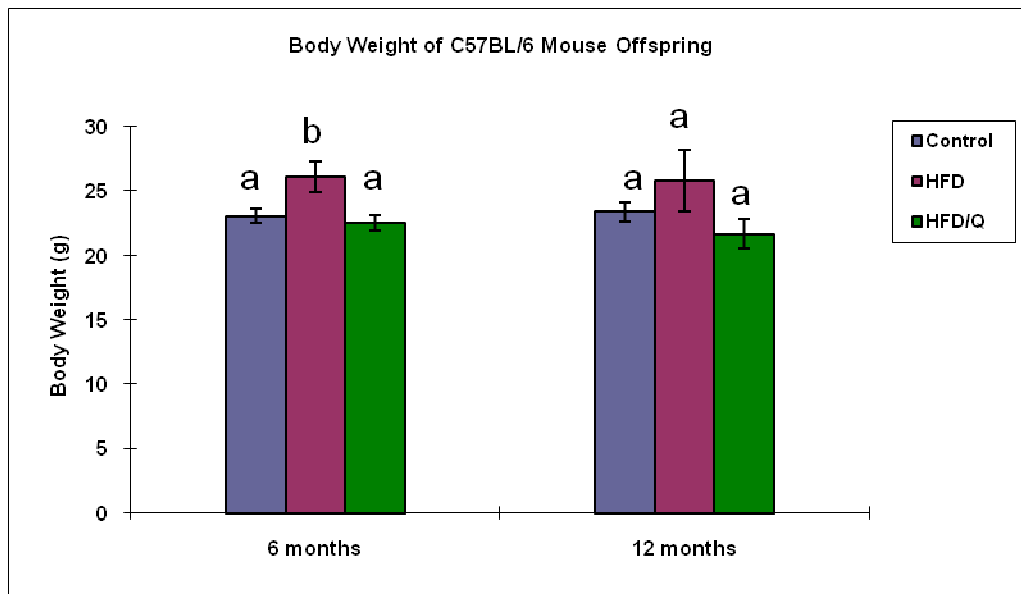
Fig. 3.   Measurement of Body Weight of 6-and 12-Month Offspring. Six month old HFD offspring showed significantly increased body weight compared to controls. Maternal quercetin supplementation significantly protected against body weight gain of 6 month HFD offspring. There was no significant difference observed among groups of 12 month old offspring. Values are given as means ± SEM. Bars with different letters represent significantly different values, *P* < 0.05. Six month old offspring of each group n=5. Twelve month old offspring of control and HFD/Q groups n=5, HFD group n=4.
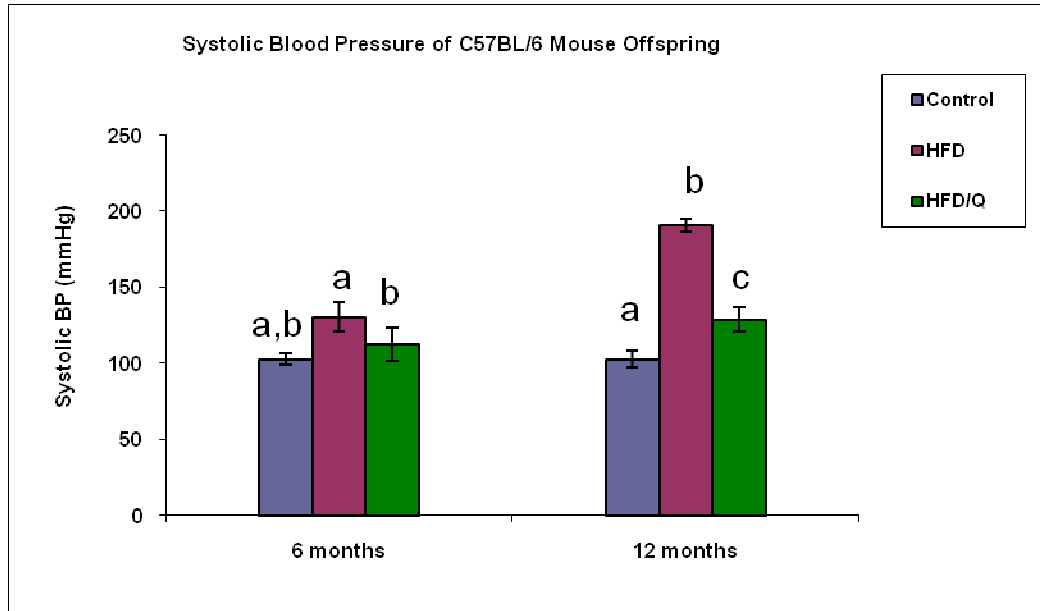
Fig. 4. Measurement of Systolic Blood Pressure of 6-and 12-Month Offspring. Six month old HFD offspring showed numerically increased systolic BP compared to control, but this was not statistically different. Twelve month old HFD offspring showed significantly increased systolic BP compared to controls. Maternal quercetin supplementation significantly protected against HFD-induced high systolic BP. Values are given as means ± SEM. Bars with different letters represent significantly different values, $P < 0.05$. Six month old offspring of each group n=5. Twelve month old offspring of control and HFD/Q groups n=5, HFD group n=4.
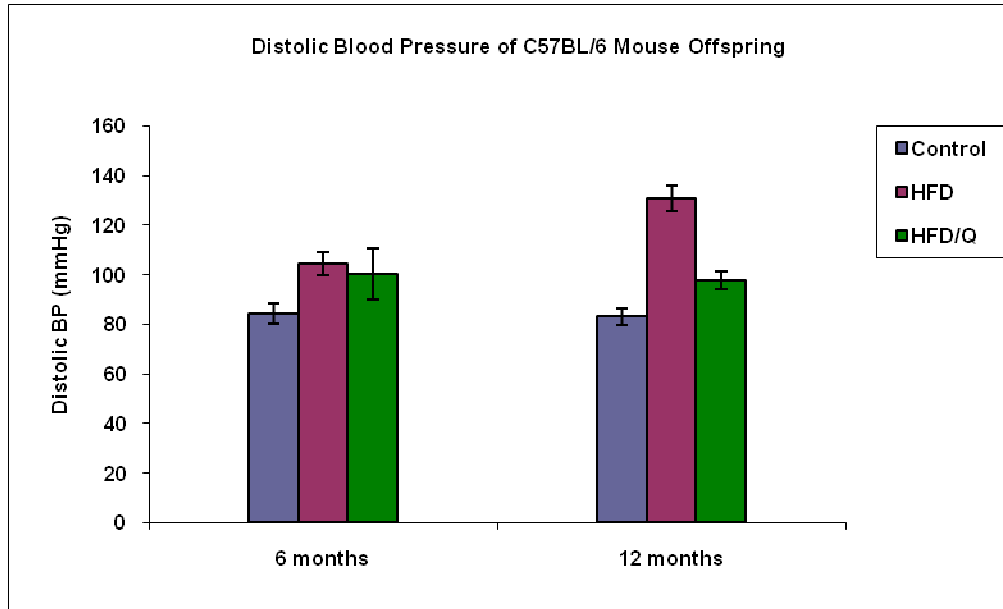
Fig. 5. Measurement of Diastolic Blood Pressure of 6-and 12-Month Offspring. No difference of diastolic BP was observed among the groups of 6-month old offspring. At 12 months of age, the HFD offspring showed significantly increased diastolic BP compared to control and maternal quercetin supplementation significantly protected against HFD-induced high diastolic BP of 12 month old offspring. Values are given as means ± SEM. Bars with different letters represent significantly different values, $P < 0.05$. Six month old offspring of each group n=5. Twelve month old offspring of control and HFD/Q groups n=5, HFD group n=4.
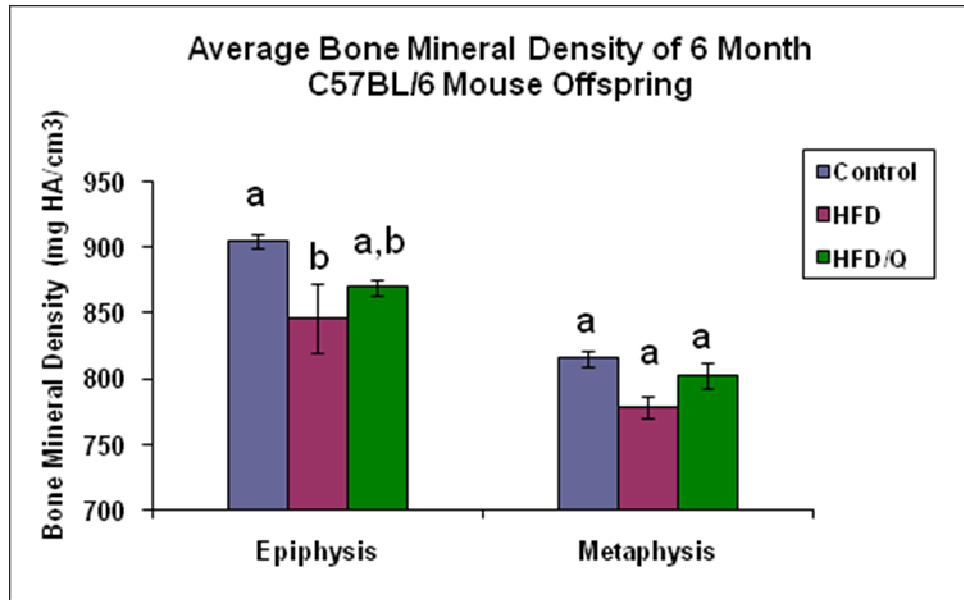
Fig. 6. Measurement of Average Bone Mineral Density of 6-Month Offspring. Average BMD was measured by micro-CT. At epiphyseal femur, the HFD offspring showed significantly less BMD than control. Maternal quercetin supplementation did not show significant protection against the loss of BMD. At metaphyseal femur, there was no difference of BMD observed among groups. Values are given as means ± SEM. Bars with different letters represent significantly different values, $P < 0.05$. n=5.
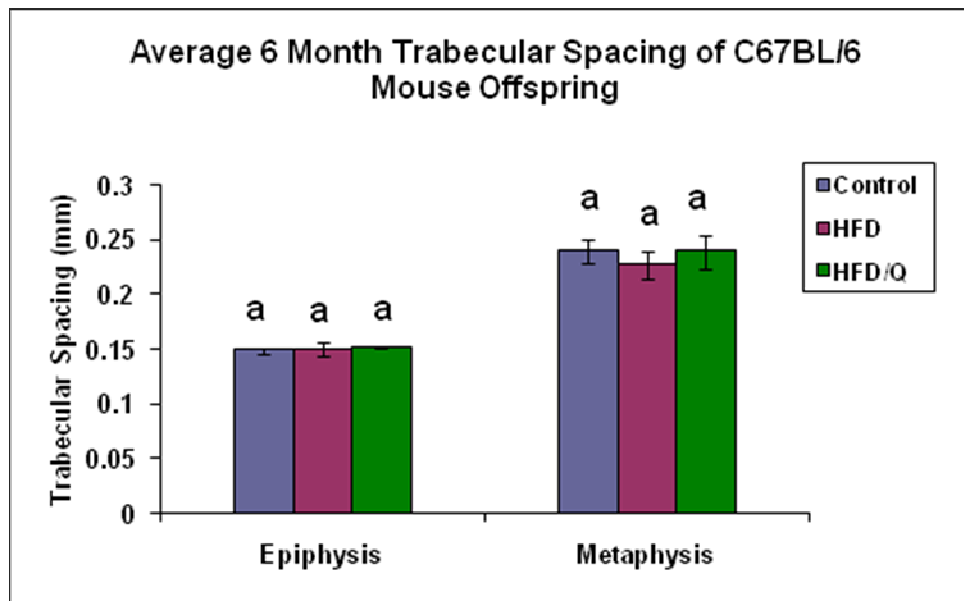
Fig. 7. Measurement of Trabecular Spacing of 6-Month Offspring. Epiphyseal and metaphyseal trabecular spacing of 6-month old offspring from control, HFD and HFD/Q were measured by micro-CT. No difference of trabecular spacing was observed among all groups. Values are given as means ± SEM. Bars with same letters indicates values are not significantly different, *P* >0.05. n=5.
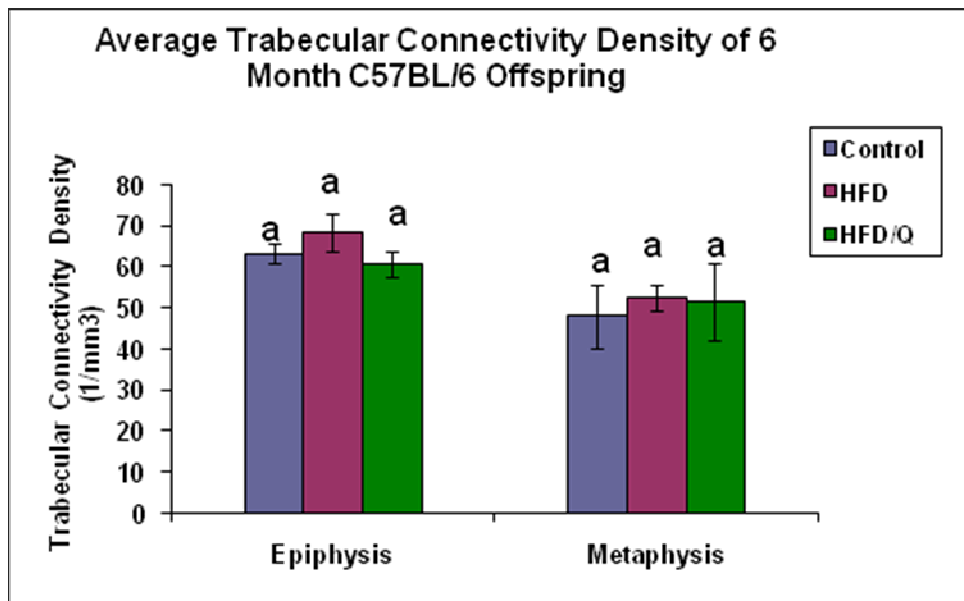
Fig. 8. Measurement of Trabecular Connectivity Density of 6-Month Offspring. Epiphyseal and metaphyseal trabecular connectivity density of 6-month old offspring from control, HFD and HFD/Q were measured by micro-CT. No difference was observed of trabecular spacing among all groups. Values are given as means ± SEM. Bars with same letters represent values are not significantly different, *P* >0.05. n=5.
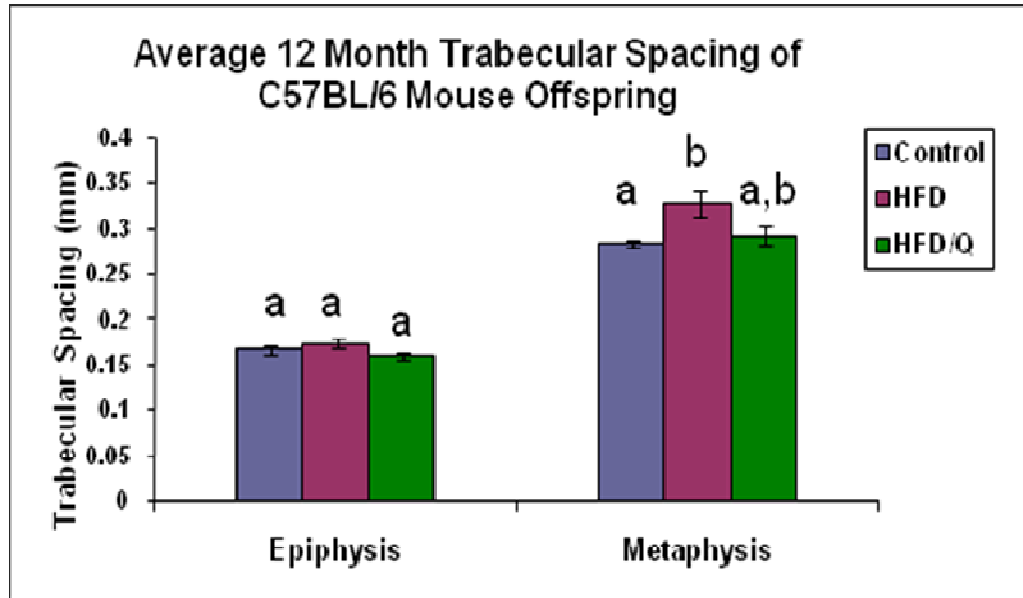
Fig. 9. Measurement of Trabecular Spacing of 12-Month Offspring. Epiphyseal and metaphyseal trabecular spacing of 12-month offspring from control, HFD and HFD/Q were measured by micro-CT. No difference was observed in trabecular spacing in epiphyseal region among groups. In metaphyseal region, a significant increase of trabecular spacing of offspring from HFD group compared to control was observed. Maternal quercetin supplementation did not show protective effects against increased trabecular spacing by HFD. Values are given as means ± SEM. Bars with different letters represent significantly different values, $P < 0.05$. Control and HFD/Q groups n=5, HFD group n=4.
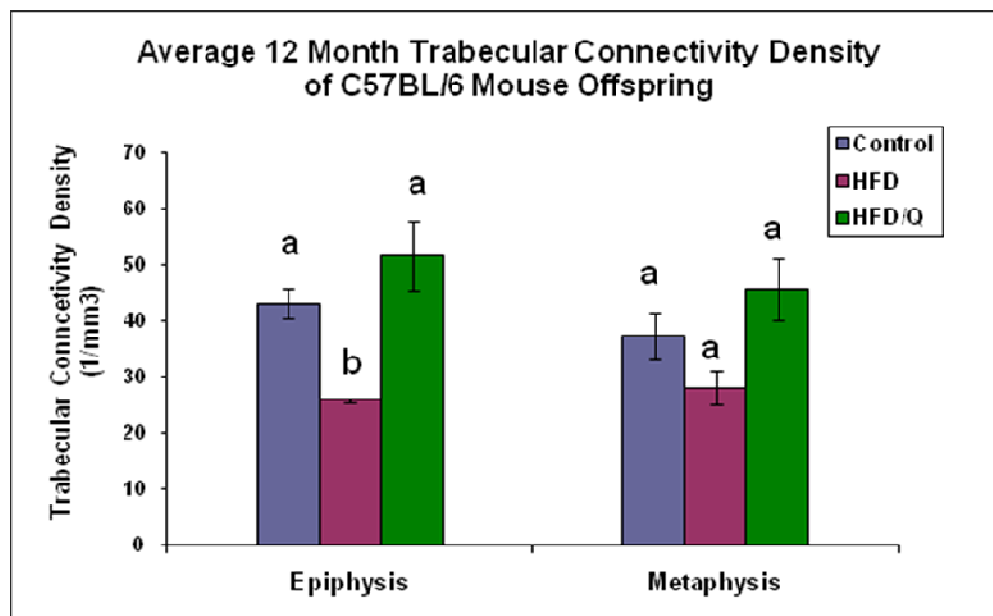
Fig. 10. Measurement of Trabecular Connectivity Density of 12-Month Offspring. Epiphyseal and metaphyseal trabecular connectivity density of 12-month old offspring from control, HFD and HFD/Q were measured by micro-CT. In metaphyseal region, a significant reduction of trabecular connectivity density of HFD offspring was observed. Maternal quercetin supplementation significantly protected against HFD-induced reduction of trabecular connectivity density. No difference of trabecular connectivity density was observed in metaphyseal region among groups. Values are given as means ± SEM. Bars with different letters represent significantly different values, $P < 0.05$. Control and HFD/Q groups n=5, HFD group n=4.

CHAPTER VI

General Conclusions and Future Work

General Conclusions

This study was designed to test the hypotheses that 1) maternal HFD diet induces GDM, placental OS and vasculopathy; maternal qucertin supplementation could at least partially reduce maternal hyperglycemia and hyperinsulinemia induced by HFD; 2) maternal HFD induces perinatal malformation and quercetin could partially protect it; 3) maternal HFD elevates adult-onset cardiovascular, endocrine and skeletal diseases and quercetin could show therapeutic effects on adult-onset chronic diseases. Results from this study proved the above hypotheses.

A large body of epidemiological studies has demonstrated the important role of fetal programming in the risk of adult-onset diseases. These studies have shown that there is a close relationship between prenatal environmental factors, early growth restriction and the subsequent development of adult diseases, such as type 2 diabetes, ischemic heart disease and hypertension (Phillips 1996). However, the mechanistic basis of this relationship has not yet been fully explored. The present study provided a reproducible laboratory animal model in C57BL/6 mice to explore the pathophysiological mechanisms linking prenatal environmental factors, fetal development, and relative risk of adult diseases. This research study included critical time periods throughout perinatal and adult life, and evaluated multiple systems to establish a more comprehensive observation over time, and over many organ systems. Multisystemic effects of maternal HFD consumption were investigated in adulthood, including endocrine, cardiovascular and skeletal systems. To our knowledge, this is the first time maternal HFD intervention was used to explore the contribution of gestational diet to fetal maldevelopment and long-term

elevated risk of multisystemic chronic diseases. Our study demonstrated that maternal HFD induced hyperglycemia, hyperinsulinemia and insulin resistance during pregnancy and contributed to increased placental OS and vasculopathy and fetal skeletal malformation. Fetal developmental delay was associated with significantly increased incidence of adult-onset type 2 diabetes, hypertension and osteoporosis, which is likely attributable to permanently altered fetal gene programming as a result of poor gestational diet. Therefore, maternal HFD exposure had its outcome in mothers and their offspring both as fetuses and adults. Both animal and human studies have supported the theory of the developmental origins of health and disease. These studies were extended in the present study to include potential therapeutic or preventive management to improve fetal development and reduce risk of adult diseases. In this study, maternal antioxidant quercetin was used as a method of dietary intervention to protect against HFD-induced gestational hyperglycemia, placental OS and vasculopathy, fetal malformation, as well as adult-onset type 2 diabetes, hypertension and osteoporosis. Thus, our studies proved the hypothesis and provided not only a potential mechanism linking maternal malnutrition, fetal development and adult-onset diseases, but also provided new insights into preventive and therapeutic strategies.

The precise mechanisms behind fetal malformations and subsequent adult diseases are presently incompletely understood. There is an increasing awareness that the placenta responds to perturbations in the maternal environment, thereby playing a key role in transmitting programming stimuli to the fetus (Godfrey 2002; Myatt 2006). The placenta is the active interface between maternal and fetal blood circulations, that regulate physiological changes in pregnancy and fetal growth (Jansson and Powell 2007). Thus, the placenta is central to human development and in particular to fetal nutrition (Sibley *et al.* 2005). Human studies suggest that

both birth weight and placental morphology are indicators of an adverse intrauterine environment, but placental morphology is a better representation of the intrauterine environment since not all fetuses subjected to an abnormal intrauterine environment have altered fetal birth weight (Myatt 2006). Maternal HFD-induced placental damage was explored in the present studies and provided convincing evidence that placental vasculopathy and elevated OS play a pivotal role in the fetal developmental process.

Mechanisms of placental effects on fetal programming include changes in placental growth and vascular resistance, disturbed nutrient and hormone metabolism, and altered nutrient transfer and partitioning between mother, placenta and fetus (Jansson and Powell 2007). Studies showing a correlation between impaired placental glycine-serine metabolism and altered lean and bone mass (Geddie *et al.* 1996). Experimental restriction of placental growth in animal studies results in low IGF concentration and disturbed 'glucose-insulin-IGF-1 axis' which plays a major physiological role in matching fetal growth to the materno-placental supply of nutrients; the same is true in human pregnancy (Gluckman 1995). Recent work on a knockout mouse model which deleted the placental-specific transcript of IGF-2 gene (a major modulator of placental and fetal growth) shows changes in placental phenotype, reduced placental weight, and diminished fetal growth (Constancia *et al.* 2002). Fetal adaptations commonly occur in response to failure of the materno-placental supply of nutrients to match fetal requirements. Studies show that the placenta may play an important role in determining these fetal adaptations that result in permanent developmental changes (Godfrey 2002). Furthermore, maternal diet influences nutrient supply through direct effects on substrate availability to the fetus and indirectly through changes in placental function and structures (Jansson and Powell 2007). In our study, placentas from the HFD group showed significantly increased OS and decreased viability of trophoblast

and endothelial cells, as demonstrated by reduced CD31 immunofluorescence staining intensity. These placental changes lead to poor fetal growth, smaller fetal size and fetal skeletal malformation, which is associated with development of type 2 diabetes, hypertension and osteoporosis at 6 and 12 months of life. On the other hand, placentas from HFD/Q group show significantly decreased OS and improved vascular health, which was associated with dramatically improved fetal growth and skeletal development as well as decreased risks of adult-onset chronic diseases. Therefore, the present study demonstrated that optimizing placental structure and function by maternal antioxidant quercetin likely has lifelong time health benefits for the offspring. The placenta thus assumes an active role in fetal programming which in part determines susceptibility to diseases in adult life.

The main tenet of the developmental origins of adult health and disease (DOHaD) hypothesis is that nutritional insufficiency in early life programs disease risk through persistent effects on structure and metabolic function of organ systems (Barker 1998). Therefore, maternal nutrition has been identified as a very powerful regulator of fetal gene programming, and causes long-lasting consequences for phenotypic changes and chronic dysfunction of immune, metabolic, central nervous, cardiovascular, and skeletal systems in the offspring (McCance and Widdowson 1974; Caballero 2001; Everitt *et al.* 2006). *The 'thrifty phenotype hypothesis'* was proposed as an accurate explanation for this phenomenon (Barker 1998; Jones and Ozanne 2009). This hypothesis indicates that in cases of gestational nutrient paucity, fetal adaptations result in diversion of nutrient-rich, highly oxygenated blood to the brain and other critical organs, at the expense of other non-vital organ systems (Hales and Barker 2001). As demonstrated in our study, HFD fetuses were small at birth with developmental delay of the musculoskeletal apparatus. When offspring from HFD dams were weaned onto a normal diet, they exhibited the

phenomenon of 'catch up' growth where they experienced rapid juvenile weight gain, and obesity in adult life, similar to what has been observed in human studies. Such an adaptation may be beneficial in the short-term, but is believed to elevate risk of cardiovascular disease, metabolic syndrome and hypertension postnatally because, while the offspring eventually approximate postnatal body weight of controls, the weight gain tends to be attributable to fat mass rather than lean mass, which leads to obesity, cardiovascular disease, and metabolic abnormalities later in life (Leon *et al.* 1996; Jansson and Powell 2007). Our studies are also supported by the theory of 'predictive adaptive response', which proposes that the degree of mismatch between the pre- and postnatal environments is an important determinant of subsequent diseases (Armitage *et al.* 2005). According to the 'predictive adaptive response' theory, mouse offspring of dams fed a HFD during pregnancy, then suckled and weaned on a control diet showed increased serum triglyceride levels, elevated plasma glucose concentration, and blunted endothelial-dependant dilatation (Armitage *et al.* 2005), which play a major role in some of most common diseases in adults such as hypertension, type 2 diabetes and metabolic syndrome (Gardiner 2007). All these findings support our data that maternal HFD has a profound effect on fetal malformation, which is associated with hyperglycemia, hyperinsulinemia, insulin resistance and hypertension at 6 and 12 months of age.

There are several possible pathophysiologic mechanisms by which fetal malnutrition and growth may result in increased cardiovascular disease and hypertension in later life. It is thought that the arterial wall is permanently altered by sub-optimal fetal nutrition resulting in reduced arterial impedance and increased loading conditions on the heart (Gardiner 2007). Secondly, abnormal fetal flow patterns may alter vascular sheer stress and endothelial function, which resets baroreceptors, therefore permanently altering an individual's response to changes in flow.

Additionally, altered response of the renin angiotensin system may cause an alteration in vascular response accompanied by myocardial hypertrophy and fibrosis (Gardiner 2007). Renal and vascular mechanisms may work collectively with altered neuroendocrine response to determine an individual's later cardiovascular response (Gardiner 2007). These mechanisms may help to explain HFD-induced alterations in cardiovascular function observed in the present study; additional investigation into renal and cardiovascular alterations in our rodent model will help to further elucidate the pathophysiological mechanisms linking maternal HFD to elevated risk of cardiovascular and metabolic disease.

Compared to adult-onset cardiovascular and metabolic diseases, fetal programming of osteoporosis is less well studied. Osteoporosis is typically diagnosed after middle age in women, when declining perimenopausal estrogen levels increase osteoclastic activity and cause an imbalance favoring bone degradation over bone formation. However, recent studies are beginning to recognize early symptoms of osteoporosis in the young adult population. Many young adult women in their 20's suffer substantial loss of trabecular bone long before perimenopausal hormone fluctuations are observed, suggesting the presence of additional mechanisms contributing to poor bone health. The present study and others demonstrated that the risk of osteoporosis might be modified by environmental influences of malnutrition during early life (Cooper *et al.* 2000). Maternal malnutrition is a global issue that can program human skeletal development (Hales and Barker 2001; Cooper *et al.* 2002), as epidemiological studies have confirmed that infants who are light at birth due to maternal malnutrition have lower adult bone mineral content (Cooper *et al.* 1997). Our study proved the hypothesis and provided evidence of a strong link between maternal malnutrition and fetal environmental influences on adult-onset osteoporosis. We were able to demonstrate that gestational maternal HFD is associated with

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decreased fetal BV, reduced BMD, shortened distal limb lengths, and poor growth which persists into adulthood as decreased BMD at 6 months of age and damaged trabecular microarchitecture at 12 months of age. However, the mechanism of how perinatal changes affect adult bone structure and function remains inconclusive (Hales and Barker 2001). Studies suggest that abnormalities in growth hormone (GH) secretion or sensitivity may play a role, since GH is the major regulator of skeletal growth. It is thought that environmental stressors during intrauterine or early post-natal life may alter the sensitivity of the growth plate to GH and cortisol (which are important skeletally active hormones and strong predictors of future rate of bone loss). Potential consequence of such endocrine programming would be reduced peak skeletal size, decreased mineralization, and accelerated rate of bone loss during adult life (Fall *et al.* 1998; Phillips *et al.* 1998; Dennison *et al.* 1999).

Preventive strategies against osteoporosis may be targeted at either reducing the rates of bone loss or increasing the peak bone mass attained (Cooper *et al.* 2002). Evidence suggests that peak bone mass is in part inherited, but current genetic markers are able to explain only a small portion of the variation in individual bone mass or fracture risk (Ralston 1998). It is likely that environmental influences during early life interact with the genome in establishing the functional level of a variety of metabolic processes involved in skeletal growth (Cooper *et al.* 2002). In this respect, dietary intervention might play a fundamental role in the prevention of these diseases (Wattel *et al.* 2003). Recent data show potential role of tea and vegetable consumption (which are important sources of antioxidants such as quercetin) in prevention of osteoporosis (White and Compston ; Hegarty *et al.* 2000). Dietary quercetin supplementation has been shown to suppress bone resorption and enhance osteogenic differentiation, and as such is proposed as an effective dietary supplement to reduce long-term risk of osteoporosis (Woo *et al.* 2004; Kim *et al.* 2006).

Findings of the present studies demonstrated that maternal quercetin supplementation improve fetal skeletal development and further decreased the risk of osteoporosis in 12-month old C57BL/6 offspring and thus provides a potential novel therapeutic management to reduce risk of adult-onset osteoporosis.

The present studies in C57BL/6 mice demonstrated that intrauterine exposure of HFD induces fetal growth retardation and skeletal malformation, which further leads to development of osteoporosis in adulthood. However, the cellular mechanisms linking gestational exposures to fetal skeletal malformation are incompletely characterized. Identifying the mechanistic basis for dysregulated *in utero* osteogenesis will be a significant step toward designing novel strategies to lower risks of abnormal bone development, and improve life-long bone health. The theory, *'the developmental origins of health and disease'*, suggests that maternal environment affects fetal stem cell development during times of genetic plasticity. Fetuses exposed to suboptimal prenatal environments respond by choosing pathways of gene expression and cell differentiation to suit the prevailing conditions. This altered stem cell differentiation modifies the trajectory of tissue and organ formation, and affects phenotypic outcome into adulthood (Barker 1995; Gluckman *et al.* 2005; Scheideler *et al.* 2008). Initial efforts to model environmental effects on bone development using cell culture showed that human mesenchymal stem cells (MSC) that were intended to become osteoblasts preferentially differentiate into adipocytes in response to presence of poor nutrition, chemicals, etc. (Ahdjoudj *et al.* 2004; Scheideler *et al.* 2008). This *in-vitro* theory was recapitulated *in-vivo* by exposing adult roosters to high dietary fat (e.g., from a cafeteria-style or fast food diet), which negatively impacted bony trabecular composition and mechanical properties (Wohl *et al.* 1998). Based on these *in-vitro* and *in-vivo* studies, we believe that *in-utero* exposure to maternal HFD will alter fetal osteogenic cell signaling pathways, impair

trabecular bone formation, increase cancellous bone adiposity, and compromise skeletal strength in perinates.

Future work

Fetal MSCs receive critical information regarding differentiation and proliferation from numerous complex cell-signaling pathways. The precise nature of these signals dictate whether MSCs undergo differentiation towards osteoblasts, adipocytes, myocytes, or neurons, and the ultimate fate of these multipotent cells relies heavily upon which environmental signals and exposures the cell receives during the times of greatest genetic plasticity (Westendorf *et al.* 2004; Andrade *et al.* 2007; Hurson *et al.* 2007). One of the key signaling pathways driving MSC maturation is the wingless-type integration site/beta-catenin signaling or Wnt pathway. Studies show exposure to environmental toxins during childhood, or even during gestation, may cause improper skeletal development and mineralization due to dysregulation of the Wnt/beta-catenin pathway, otherwise known as canonical Wnt (Andrade *et al.* 2007), and may be important contributors to adult bone pathology (Bianchi 2007). Future work in our laboratory will use an *in-vitro* mouse MSC model to characterize the specific molecular mechanisms by which altered nutrition changes Wnt signaling and alters osteogenic differentiation.

The Wnts are a large family of highly conserved secreted lipid-modified glycoproteins that actively regulate MSC differentiation, proliferation, migration, and gene expression. Binding of the Wnt protein to its cell membrane receptor frizzled (Fzd) activates one of three Wnt intracellular signaling cascades. The most notable of these pathways is the canonical Wnt/beta-catenin pathway, which requires additional activation of the Wnt co-receptor lipoprotein receptor-related protein (Lrp) that increases the efficiency of Wnt signaling in

osteoblasts (Westendorf *et al.* 2004; Andrade *et al.* 2007; Hurson *et al.* 2007). In the canonical Wnt pathway, the intracytoplasmic tail of the Wnt3a/Fzd/Lrp complex functions to stabilize beta-catenin, and permits its accumulation in the cytoplasm and activates downstream osteogenic gene targets. Regulating beta-catenin protein stabilization by control its phosphorylation state is a key step in the canonical Wnt pathway. In the absence of Wnt, beta-catenin is phosphorylated and targeted for ubiquitin/proteasome pathway degradation. Absence of beta-catenin signaling leaves MSCs to differentiate along the default pathway of adipogenesis (Westendorf *et al.* 2004; Ni *et al.* 2007). During embryonic development, beta-catenin levels are increased in differentiating osteoblasts. Active Wnt signaling increases bone mass by renewal of stem cells, stimulation of preosteoblast replication, induction of osteoblastogenesis, and inhibition of osteoblast and osteocyte apoptosis (Kato *et al.* 2002; Bodine *et al.* 2004; Day *et al.* 2005; Reya and Clevers 2005). As such, canonical Wnt signaling is thought to play a central role in skeletal development, osteoblast maturation, and protection against osteoblast apoptosis.

MSCs are able to differentiate along several lineages, and their growth and differentiation are tightly regulated via interactions with specific extracellular mediators. It is now generally believed that osteoblasts and adipocytes share a somewhat reciprocal differentiation from MSCs, based on cues received at key differentiation points. The extent of MSC osteogenic differentiation can be verified based on expression of certain cell surface proteins. For example, multipotent MSCs that differentiate to osteoprogenitors in the lineage commitment stage of development are characterized by expression of Lrp5 and Runx2, and this step is largely driven by Wnt3a; further differentiation to the pre-osteoblast stage during the proliferation/expansion stage is characterized by expression of Low-density lipoprotein receptor-related protein 5 (Lrp5), run-related transcription factor 2 (Runx2), and bone-specific alkaline phosphatase (ALP); mature

osteoblasts gain additional markers including osteocalcin and osterix, and this differentiation is driven largely by vitamin D3 and other key growth factors; terminal differentiation to osteocyte is characterized by mineralization and loss of ALP and Lrp expression (Westendorf *et al.* 2004). These and other studies show that these members of the Wnt pathway, including Wnt3a, Wnt10b, Lrp5, Runx2, and Dickkopf-1 (Dkk1) play important roles in MSC differentiation.

Osteoblastic differentiation of MSCs is a complex process that requires communication and cross-talk between multiple signaling pathways for appropriate osteogenic differentiation. Signaling networks such as Wnt ligand-receptor interactions, altered function of transcription factors such as beta-catenin or Runx2, or altered expression of growth factors such as GH or transforming growth factor-beta, can influence the trajectory of MSC differentiation, that could result in life-long changes in bony integrity (Huang *et al.* 2007). Recent advances using novel molecular techniques and gene targeting in the mouse MSC model have improved our understanding of how these pathways communicate, and how environmental signals may affect downstream changes in gene expression. To study these pathways in the heterogeneous population of osteoblasts, chondrocytes, myocytes, neurons, vessel, skin, and adnexal cells that make up the limb bud of a developing embryo lends itself to confounding factors beyond the control of the investigator. Future study in our laboratory will use a homogeneous population of cells such as the murine MSCs, so that we can ensure a controlled, *in-vitro* environment to quantify the cellular response and identify the specific targets of maternal malnutrition damage to osteoprogenitor cells during key steps in cell proliferation and differentiation. The murine MSC line (C3H10T1/2) has been used extensively in related studies demonstrating that the upregulation of beta-catenin positively influences osteogenic differentiation and activity of alkaline phosphatase (marker of osteoblastic differentiation) in cell culture and that interference

of osteoblastic differentiation (via knockdown of Runx2 transcription, translation or function) reduces osteoblastic differentiation (Gordeladze *et al.* 2008; Qu *et al.* 2008). The homogeneity of response expected from the C3H10T1/2 cells will likely yield a high degree of data precision and will provide a suitable *in-vitro* model for the study of osteoblastic differentiation in stem cells, that can later be used to advance knowledge of *in-vivo* osteogenic systems. We believe that through the discovery of the mechanistic basis for alteration of Wnt signaling pathway in murine stem cells, novel therapies may be developed that are aimed at reducing the risk of skeletal birth defects, and improving lifelong bone health.

Recent studies show that vitamin D3 actively influences osteoblastic proliferation, differentiation, and mineral production via modification of Wnt signaling (Shi *et al.* 2007). The strength of this response is thought to be dependent on the stage of mesenchymal cellular differentiation at treatment initiation, and is osteoinductive in osteoblast lineage MSCs until the point of mineralization, at which time it becomes inhibitory (Shi *et al.* 2007). For this reason, vitamin D3 has been used experimentally to facilitate fracture healing in patients with non-union or delayed union fractures, and in reparative osteogenesis associated with osteoporosis, via stimulation of periosteal or bone marrow-derived osteoblast precursors (Gigante *et al.* 2008). Vitamin D3 is thought to alter the cell cycle in developing osteoblasts, induce expression of the Wnt co-receptor Lrp5, and facilitate matrix mineralization and increased bone mass via binding to the vitamin D3 receptor (VDR) (Fretz *et al.* 2007). The vitamin D-VDR complex binds directly to the regulatory region of a target gene in the Wnt pathway, Lrp5, altering its transcriptional output and facilitating osteoblastic proliferation and differentiation, and bone formation (Fretz *et al.* 2007; Jurutka *et al.* 2007). Vitamin D3 also works in conjunction with other factors such as antioxidants, insulin growth factors, transforming growth factors, and bone

morphogenic proteins to increase osteoblastic differentiation, upregulate endochondral ossification, and enhance bone mineralization (Andrade *et al.* 2007; Byers and Shah 2007; Fretz *et al.* 2007; zur Nieden *et al.* 2007). A protective effect of vitamin D3 against cancer development following MNU exposure has also been demonstrated, by regulating levels of beta-catenin (Murillo and Mehta 2005). Vitamin D3 has been shown to potentially exert two effects in the pathogenesis of carcinoma: induction of Dickkopf-1, which inhibits Wnt/beta-catenin signaling by binding to the VDR and promoting beta-catenin nuclear transport in human colon and mammary tumors of epithelial origin (Aguilera *et al.* 2007; Larriba *et al.* 2007), which is thought to both protect epithelial origin cells from malignant transformation. And although the mechanism of action of vitamin D3 in colonic epithelium varies from its behavior in osteoprogenitor cells, these data further strengthen the hypothesis that vitamin D3 plays an active role in regulation of Wnt signaling and cell fate, and as such, may represent a novel nutritional supplement with which to normalize alterations in osteoprogenitor differentiation and further prevent fetal skeletal maldevelopment and adult-onset osteoporosis. Future studies will also explore the role of vitamin D3 in reducing gestational HFD-induced skeletal malformations.

In summary, results from present studies demonstrated that maternal HFD-induced placental OS and vascular damage alters perinatal skeletal development and elevates risks of multisystemic chronic diseases in adulthood. Further work in our laboratory investigating the role of signaling pathways in fetal stem cell differentiation, and the use of concurrent macro- and micronutrient supplementation to reduce fetal malformation and risk of adult-onset disease, will provide us with both cellular mechanisms responsible for fetal growth and adult health, and will open new avenues for therapeutic strategies for improved lifelong health.

References

Aguilera, O., C. Pena, J. M. Garcia, M. J. Larriba, P. Ordonez-Moran, D. Navarro, A. Barbachano, I. Lopez de Silanes, E. Ballestar, M. F. Fraga, M. Esteller, C. Gamallo, F. Bonilla, J. M. Gonzalez-Sancho and A. Munoz (2007) The Wnt antagonist DICKKOPF-1 gene is induced by 1{alpha},25-dihydroxyvitamin D3 associated to the differentiation of human colon cancer cells. *Carcinogenesis*. **28**, 1877-1884.

Ahdjoudj, S., O. Fromigue and P. J. Marie (2004) Plasticity and regulation of human bone marrow stromal osteoprogenitor cells: potential implication in the treatment of age-related bone loss. *Histol Histopathol*. **19**, 151-7.

Andrade, A. C., O. Nilsson, K. M. Barnes and J. Baron (2007) Wnt gene expression in the post-natal growth plate: regulation with chondrocyte differentiation. *Bone*. **40**, 1361-9.

Armitage, J. A., P. D. Taylor and L. Poston (2005) Experimental models of developmental programming: consequences of exposure to an energy rich diet during development. *J Physiol*. **565**, 3-8.

Barker, D. J. (1995) The fetal and infant origins of disease. *Eur J Clin Invest*. **25**, 457-63.

Barker, D. J. (1998) In utero programming of chronic disease. *Clin Sci (Lond)*. **95**, 115-28.

Bianchi, M. L. (2007) Osteoporosis in children and adolescents. *Bone*. **41**, 486-95.

Bodine, P. V., W. Zhao, Y. P. Kharode, F. J. Bex, A. J. Lambert, M. B. Goad, T. Gaur, G. S. Stein, J. B. Lian and B. S. Komm (2004) The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice. *Mol Endocrinol*. **18**, 1222-37.

Byers, S. and S. Shah (2007) Vitamin D and the regulation of Wnt/beta-catenin signaling and innate immunity in colorectal cancer. *Nutr Rev*. **65**, S118-20.

Caballero, B. (2001) Early nutrition and risk of disease in the adult. *Public Health Nutr*. **4**, 1335-6.

Constancia, M., M. Hemberger, J. Hughes, W. Dean, A. Ferguson-Smith, R. Fundele, F. Stewart, G. Kelsey, A. Fowden, C. Sibley and W. Reik (2002) Placental-specific IGF-II is a major modulator of placental and fetal growth. *Nature*. **417**, 945-948.

Cooper, C., C. Fall, P. Egger, R. Hobbs, R. Eastell and D. Barker (1997) Growth in infancy and bone mass in later life. *Ann Rheum Dis*. **56**, 17-21.

Cooper, C., M. K. Javaid, P. Taylor, K. Walker-Bone, E. Dennison and N. Arden (2002) The fetal origins of osteoporotic fracture. *Calcif Tissue Int*. **70**, 391-4.

Cooper, C., K. Walker-Bone, N. Arden and E. Dennison (2000) Novel insights into the pathogenesis of osteoporosis: the role of intrauterine programming. *Rheumatology (Oxford)*. **39**, 1312-5.

Day, T. F., X. Guo, L. Garrett-Beal and Y. Yang (2005) Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. *Dev Cell*. **8**, 739-50.

Dennison, E., P. Hindmarsh, C. Fall, S. Kellingray, D. Barker, D. Phillips and C. Cooper (1999) Profiles of endogenous circulating cortisol and bone mineral density in healthy elderly men. *J Clin Endocrinol Metab*. **84**, 3058-63.

Everitt, A. V., S. N. Hilmer, J. C. Brand-Miller, H. A. Jamieson, A. S. Truswell, A. P. Sharma, R. S. Mason, B. J. Morris and D. G. Le Couteur (2006) Dietary approaches that delay age-related diseases. *Clin Interv Aging*. **1**, 11-31.

Fall, C., P. Hindmarsh, E. Dennison, S. Kellingray, D. Barker and C. Cooper (1998) Programming of growth hormone secretion and bone mineral density in elderly men: a hypothesis. *J Clin Endocrinol Metab*. **83**, 135-9.

Fretz, J. A., L. A. Zella, S. Kim, N. K. Shevde and J. W. Pike (2007) 1,25-Dihydroxyvitamin D3 induces expression of the Wnt signaling co-regulator LRP5 via regulatory elements located significantly downstream of the gene's transcriptional start site. *J Steroid Biochem Mol Biol*. **103**, 440-5.

Gardiner, H. M. (2007) Early environmental influences on vascular development. *Early Hum Dev*. **83**, 819-23.

Geddie, G., R. Moores, G. Meschia, P. Fennessey, R. Wilkening and F. C. Battaglia (1996) Comparison of leucine, serine and glycine transport across the ovine placenta. *Placenta*. **17**, 619-27.

Gigante, A., M. Torcianti, E. Boldrini, S. Manzotti, G. Falcone, F. Greco and M. Mattioli-Belmonte (2008) Vitamin K and D association stimulates in vitro osteoblast differentiation of fracture site derived human mesenchymal stem cells. *J Biol Regul Homeost Agents*. **22**, 35-44.

Gluckman, P. D. (1995) The endocrine regulation of fetal growth in late gestation: the role of insulin-like growth factors. *J Clin Endocrinol Metab*. **80**, 1047-1050.

Gluckman, P. D., M. A. Hanson and C. Pinal (2005) The developmental origins of adult disease. *Matern Child Nutr*. **1**, 130-41.

Godfrey, K. M. (2002) The role of the placenta in fetal programming-a review. *Placenta*. **23 Suppl A**, S20-7.

Gordeladze, J. O., D. Noel, C. Bony, F. Apparailly, P. Louis-Plence and C. Jorgensen (2008) Transient down-regulation of cbfa1/Runx2 by RNA interference in murine C3H10T1/2 mesenchymal stromal cells delays in vitro and in vivo osteogenesis, but does not overtly affect chondrogenesis. *Exp Cell Res*. **314**, 1495-506.

Hales, C. N. and D. J. Barker (2001) The thrifty phenotype hypothesis. *Br Med Bull*. **60**, 5-20.

Hegarty, V. M., H. M. May and K. T. Khaw (2000) Tea drinking and bone mineral density in older women. *Am J Clin Nutr*. **71**, 1003-7.

Huang, W., S. Yang, J. Shao and Y. P. Li (2007) Signaling and transcriptional regulation in osteoblast commitment and differentiation. *Front Biosci*. **12**, 3068-92.

Hurson, C. J., J. S. Butler, D. T. Keating, D. W. Murray, D. M. Sadlier, J. M. O'Byrne and P. P. Doran (2007) Gene expression analysis in human osteoblasts exposed to dexamethasone identifies altered developmental pathways as putative drivers of osteoporosis. *BMC Musculoskelet Disord*. **8**, 12.

Jansson, T. and T. L. Powell (2007) Role of the placenta in fetal programming: underlying mechanisms and potential interventional approaches. *Clin Sci (Lond)*. **113**, 1-13.

Jones, R. H. and S. E. Ozanne (2009) Fetal programming of glucose-insulin metabolism. *Mol Cell Endocrinol*. **297**, 4-9.

Jurutka, P. W., L. Bartik, G. K. Whitfield, D. R. Mathern, T. K. Barthel, M. Gurevich, J. C. Hsieh, M. Kaczmarska, C. A. Haussler and M. R. Haussler (2007) Vitamin D receptor: key roles in bone mineral pathophysiology, molecular mechanism of action, and novel nutritional ligands. *J Bone Miner Res*. **22 Suppl 2**, V2-10.

Kato, M., M. S. Patel, R. Levasseur, I. Lobov, B. H. Chang, D. A. Glass, 2nd, C. Hartmann, L. Li, T. H. Hwang, C. F. Brayton, R. A. Lang, G. Karsenty and L. Chan (2002) Cbfa1-

independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. *J Cell Biol*. **157**, 303-14.

Kim, Y. J., Y. C. Bae, K. T. Suh and J. S. Jung (2006) Quercetin, a flavonoid, inhibits proliferation and increases osteogenic differentiation in human adipose stromal cells. *Biochem Pharmacol*. **72**, 1268-78.

Larriba, M. J., N. Valle, H. G. Palmer, P. Ordonez-Moran, S. Alvarez-Diaz, K.-F. Becker, C. Gamallo, A. G. de Herreros, J. M. Gonzalez-Sancho and A. Munoz (2007) The inhibition of Wnt/{beta}-catenin signalling by 1{alpha},25-dihydroxyvitamin D3 is abrogated by Snail1 in human colon cancer cells. *Endocr Relat Cancer*. **14**, 141-151.

Leon, D. A., I. Koupilova, H. O. Lithell, L. Berglund, R. Mohsen, D. Vagero, U. B. Lithell and P. M. McKeigue (1996) Failure to realise growth potential in utero and adult obesity in relation to blood pressure in 50 year old Swedish men. *BMJ*. **312**, 401-6.

McCance, R. A. and E. M. Widdowson (1974) The determinants of growth and form. *Proc R Soc Lond B Biol Sci*. **185**, 1-17.

Murillo, G. and R. G. Mehta (2005) Chemoprevention of chemically-induced mammary and colon carcinogenesis by 1alpha-hydroxyvitamin D5. *J Steroid Biochem Mol Biol*. **97**, 129-36.

Myatt, L. (2006) Placental adaptive responses and fetal programming. *J Physiol*. **572**, 25-30.

Ni, Y. G., N. Wang, D. J. Cao, N. Sachan, D. J. Morris, R. D. Gerard, O. M. Kuro, B. A. Rothermel and J. A. Hill (2007) FoxO transcription factors activate Akt and attenuate insulin signaling in heart by inhibiting protein phosphatases. *Proc Natl Acad Sci U S A*. **104**, 20517-22.

Phillips, D. I. (1996) Insulin resistance as a programmed response to fetal undernutrition. *Diabetologia*. **39**, 1119-22.

Phillips, D. I., D. J. Barker, C. H. Fall, J. R. Seckl, C. B. Whorwood, P. J. Wood and B. R. Walker (1998) Elevated plasma cortisol concentrations: a link between low birth weight and the insulin resistance syndrome? *J Clin Endocrinol Metab*. **83**, 757-60.

Qu, Z. H., X. L. Zhang, T. T. Tang and K. R. Dai (2008) Promotion of osteogenesis through beta-catenin signaling by desferrioxamine. *Biochem Biophys Res Commun*. **370**, 332-7.

Ralston, S. H. (1998) Do genetic markers aid in risk assessment? *Osteoporos Int*. **8 Suppl 1**, S37-42.

Reya, T. and H. Clevers (2005) Wnt signalling in stem cells and cancer. *Nature*. **434**, 843-850.

Scheideler, M., C. Elabd, L. E. Zaragosi, C. Chiellini, H. Hackl, F. Sanchez-Cabo, S. Yadav, K. Duszka, G. Friedl, C. Papak, A. Prokesch, R. Windhager, G. Ailhaud, C. Dani, E. Z. Amri and Z. Trajanoski (2008) Comparative transcriptomics of human multipotent stem cells during adipogenesis and osteoblastogenesis. *BMC Genomics*. **9**, 340.

Shi, Y. C., L. Worton, L. Esteban, P. Baldock, C. Fong, J. A. Eisman and E. M. Gardiner (2007) Effects of continuous activation of vitamin D and Wnt response pathways on osteoblastic proliferation and differentiation. *Bone*. **41**, 87-96.

Sibley, C. P., M. A. Turner, I. Cetin, P. Ayuk, C. A. Boyd, S. W. D'Souza, J. D. Glazier, S. L. Greenwood, T. Jansson and T. Powell (2005) Placental phenotypes of intrauterine growth. *Pediatr Res*. **58**, 827-32.

Wattel, A., S. Kamel, R. Mentaverri, F. Lorget, C. Prouillet, J. P. Petit, P. Fardelonne and M. Brazier (2003) Potent inhibitory effect of naturally occurring flavonoids quercetin and kaempferol on in vitro osteoclastic bone resorption. *Biochem Pharmacol*. **65**, 35-42.

Westendorf, J. J., R. A. Kahler and T. M. Schroeder (2004) Wnt signaling in osteoblasts and bone diseases. *Gene*. **341**, 19-39.

White, P. and J. Compston Osteoporosis: Clinical and Commercial Perspectives

Wohl, G. R., L. Loehrke, B. A. Watkins and R. F. Zernicke (1998) Effects of high-fat diet on mature bone mineral content, structure, and mechanical properties. *Calcif Tissue Int*. **63**, 74-9.

Woo, J. T., H. Nakagawa, M. Notoya, T. Yonezawa, N. Udagawa, I. S. Lee, M. Ohnishi, H. Hagiwara and K. Nagai (2004) Quercetin suppresses bone resorption by inhibiting the differentiation and activation of osteoclasts. *Biol Pharm Bull*. **27**, 504-9.

zur Nieden, N. I., F. D. Price, L. A. Davis, R. E. Everitt and D. E. Rancourt (2007) Gene profiling on mixed embryonic stem cell populations reveals a biphasic role for beta-catenin in osteogenic differentiation. *Mol Endocrinol*. **21**, 674-85.