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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Physiology and Pharmacology

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MATERNAL NUTRIENT RESTRICTION IN PREGNANT GUINEA PIGS IMPACTS FETAL-PLACENTAL GROWTH AND OXYGENATION

(Thesis format: Integrated Article)

by

Alexander A Elias

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

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ABSTRACT

Maternal nutrient restriction (MNR) in guinea pigs results in placental structural abnormalities that reduce nutrient transport contributing to fetal growth restriction (FGR). However, whether fetal oxygenation is also reduced as a further mediator of altered growth and development remains unknown. Guinea pig sows were fed ad libitum (Controls) or 70% of the control diet pre-pregnant switching to 90% at mid-pregnancy (MNR). Animals were necropsied near term for fetal-placental growth measures, blood metabolites, and markers of tissue hypoxia and oxidative stress. MNR resulted in FGR with brains that are large and livers that are small relative to body weight which suggests a degree of blood flow redistribution. FGR-MNR fetuses showed increased levels of hypoxia related markers in a tissue and sex specific manner, and decreased makers of oxidative stress. Together these results implicate hypoxia as a mechanism for aberrant growth and development and evidence for protective adaptations with MNR induced FGR.

Keywords: Maternal Nutrient Restriction (MNR), Fetal Growth Restriction (FGR), Hypoxia, Oxidative stress, Fetal Programming, Undernutrition

CO-AUTHORSHIP STATEMENT

All work presented in this thesis was performed by Alexander Elias. Animal care and drug administration during necropsy were in part carried out by Brad Matushewski. All Glutathione and malondialdehyde experiments were performed with the assistance of Emily Villeneuve. Hypoxyprobe imaging and analysis performed with the assistance of Karyan Nygard at the Western University Biotron. The pathology core at the Robarts Research Institute, London, Ontario, performed organ tissue blocking.

ACKNOWLEDGEMENTS

I am very grateful for all the support and guidance I have had over the years to help me on my academic journey. First, I wish to express my extreme gratitude to my parents, who never questioned why the heck I was going back to school or what I was doing there. There is no way any of this would have been possible without all of your help and love, and I cannot thank you enough for that! A special thank you to my sister Alyson for putting up with all my shenanigans for the last 24 years. Thanks also to all my extended family and friends who patiently waited as I attempted to respond to the question "so what is your research on?" It is always great to be able to share a passion with those around you, and your interest pushed me to continue to pursue my academic goals!

I was lucky enough to be surrounded by amazing support members throughout my thesis project. Thank you to all of my colleagues in the Richardson and DDT labs, past and present, who helped make my days just a little bit more tolerable and exciting! Specifically to Brad, Lin and Karen, all of whom offered their amazing expert insight towards my project, thank you. My supervisor, Dr. Bryan Richardson deserves so much credit for making this work something to be proud of and always looking to bring the best out of me as a scientist. Thank you to my advisory committee, Dr. Tim Regnault, Dr. Rob Cumming, Dr. Kaiping Yang, and Dr. Lynne Postovit for offering support and guidance and reassuring me my results looked somewhat like what we expected.

There is no way I would be in the position I am today without the overwhelming support and motivation I received from my pal Coach and his friend Hannah. Coach made sure I was up early every morning, and Hannah offered endless guidance and direction as one of the most wonderful human beings and scientists I know. I love you both very much and cannot be thankful enough to have you by side every step of the way throughout this thesis!

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LIST OF ABBREVIATIONS

3NT	3-Nitrotyrosine
4-HNE	4-hydroxynonenal
8-OHdG	8-hydroxy-2'-deoxyguanosine
ABC	Avidin-biotin complex
ACTH	Adrenocorticotropic hormone
aFGR	Asymmetrical fetal growth restriction
AGA	Appropriate for gestational age
AGA-CTL	Appropriate for gestational age from Control cohort
BMI	Body mass index
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of health and disease
EDTA	Ethylenediaminetetraacetic acid
EPO	Erythropoietin
EPOR	Erythropoietin Receptor
ER	Endoplasmic reticulum
EtOH	Ethanol
FGR	Fetal growth restriction
gm(s)	Gram(s)
γ-GCS	Gamma glutamylcysteine synthetase
GSH	Glutathione
GSSG	Glutathione disulfide
HIF-1	Hypoxia inducible factor 1
HIF-1α	Hypoxia inducible factor 1 (α subunit)

HIF-1β	Hypoxia inducible factor 1 (β subunit)
HP-1	Hypoxyprobe 1
HPF	High power fields
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor-binding protein
IUGR	Intrauterine growth restriction
M	Molar
MDA	Malondialdehyde
MNR	Maternal nutrient restriction
MNR-FGR	Maternal nutrient restriction- fetal growth restriction cohort
MPR	Maternal protein restriction
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
PI	Ponderal Index
PLGF	Placental growth factor
PO_2	Partial pressure of oxygen
PRDX	Peroxiredoxin
ROS	Reactive oxygen species
SDF-1	Stromal-derived factor 1
SDS	Sodium dodecyl sulfate
SGA	Small for gestational age
SEM	Standard error of the mean
sFGR	Symmetrical fetal growth restriction
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances

TBST	Tris-buffered saline containing 0.1% Tween
TRX	Thioredoxin
UAL	Uterine artery ligation
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1

LITERATURE REVIEW

1.1 CLINICAL RELEVANCE

1.1.1 Incidence and Classification of FGR

The restriction of growth throughout pregnancy is an area of continuing research interests. This research is aimed at understanding both the etiology of growth restriction and its continued impact on health and disease. Fetal growth restriction (FGR), also known as intrauterine growth restriction (IUGR), and formally fetal growth retardation, is defined clinically and academically as having a weight below the 10th, 5th, or 3rd percentile for a given gestational age. However, not all fetuses that are small for their gestational age (SGA) are pathologically growth restricted and rather are constitutionally small. In Canada, in keeping with the World Health Organization guidelines, FGR is below the 10th percentile for gestation age and less than 2500 gms at term (Kramer 2003; Lackman 2001; Pollack 1992). FGR pregnancies have up to 6x higher fetal mortality. In addition, FGR severity is correlated with 5-30x higher neonatal mortality (Seeds 1998; Piper 1996), along with increased neonatal morbidity (Bernstein 2000; Kramer 1990). Approximately 70% of fetuses with a birthweight below the 10th percentile are considered constitutionally small, with the remaining 30% having a pathologic origin for FGR. Due to this discrepancy, these definitions are not always reliable for being able to distinguish genuine FGR as a failure to obtain proper growth *in utero* from a fetus that is inherently small. Unfortunately, this distinction is often made by clinicians in retrospect after the infant is born, after a medical examination has been performed. This discrimination is very important as misdiagnosed small and healthy babies as FGR will generalize the definition, prevalence and resources of FGR.

These indistinct definitions of FGR have resulted in other measures of fetal growth to be used in conjunction with fetal growth curves and percentile charts. Ponderal Index (PI) is the ratio of weight-to-height^3 and is positively correlated with fetal leanness and nutritional status at term (Brandi 2003). Additionally, a decrease in the weight to length ratio at birth is ofeten used as a measure of fetal leanness and improper growth. A study from London, Ontario found placental weight to have a positive relationship with birth weight, but other studies have suggested that it is the functionality, not the overall size, that is impaired in the placenta with FGR and is another important indicator (Lackman 2001; Pollack 1992). This has led other studies to suggest that placental weight as a ratio for fetal weight will increase with the degree of FGR. Understanding

markers for fetal and placental size and shape is useful as predicators for the health of the fetus and postnatal outcome.

1.1.2 Symmetrical vs. Asymmetrical Growth

FGR phenotypically is classified as either symmetrical (sFGR) or asymmetrical (aFGR) growth restriction, based primarily on body proportion. The sFGR fetus implies that the entire body has undergone proportional restriction, and equally affects all growing parameters. This results in reduced growth measurements in early developing fetuses, thereby limiting brain growth proportionally to reductions in weight. Additionally, the sFGR will present with decreases in length and head circumference, but will have a normal ponderal index at birth (al Riyami 2011). The majority of growth restricted infants have asymmetric inhibition of growth. First, there is a restriction of weight, and then length, with a relative "head sparing" or "brain sparing" effect. The aFGR will present with normal head dimensions but small abdominal circumference (due to decreased liver size), scrawny limbs (due to decreased muscle mass) and a low ponderal index (due to increased leanness reflective of a disproportioned body) (al Riyami 2011; Halliday 2009; Campbell 1997; Lafeber 1984). This asymmetry in growth is more commonly due to extrinsic late-onsetting restriction in the latter half of gestation, which usually occurs in a more severe fashion, and gives rise to extreme concern to the developmental potential and health outcome of the fetus. Unlike aFGR, sFGR can be difficult to distinguish from inherently small fetuses because they are proportionally similar, but extreme deviation from a growth curve infers pathological sFGR. Previous studies have also distinguished between sFGR and aFGR *in utero* using a variety of ultrasound measurements. (Cambell 1977, al Riyami 2011), nevertheless the most reliable method for discriminating the two varieties is the brain-to-liver weight ratio; with aFGR disproportional with a decrease in liver weight, but a relatively normal or less decreased head and brain size, while sFGR presents with small livers and brains but correspondingly similar to appropriate for gestational age (AGA) fetuses (Cox 2009). Accordingly, brain-to-liver weight ratio is the preferred and popular method in animal studies of growth restriction to accurately identify aFGR at necropsy, where in the clinical setting head-to-abdominal circumference is used to determine aFGR in newborn or human fetus.

Asymmetrical fetal growth restriction is the more common variety, and is reported to occur in 70% of growth restricted fetuses (Campbell 1977), although socioeconomic differences have been identified. Studies have reported that aFGR incidence is 60-80% of growth restricted babies in developed countries whereas symmetrical growth occurs more frequently in developing countries constituting 70-80%. This indicates that the causes of FGR, both asymmetrical and symmetrical, comes not from a single anatomical dysfunction or inhibition, and has led many researches to examine the exact combination of conditions that can contribute partially or sufficiently to incomplete fetal growth.

1.1.3 Etiology of FGR

Fetal growth does not rely on any one aspect, but rather is dependent on maternal, placental and genetic factors. Optimal growth is obtained by a balance of the genetic growth potential of the fetus, the efficiency of the placenta in transporting nutrients and oxygen and the state of the material environment. These maternal-placental-fetal factors work in unison to provide an optimal envinroment for the fetus throughout gestation while supporting the physiological changes occurring in the mother. When one or more of these factors becomes dysfunctional, a sub-optimal intrauterine environment is created, and the fetus is unable to sustain normal growth and development. Fetal causes are the rarest and include genetic and chromosomal abnormalities and intrauterine infections, and usually have poor long-term prognosis (Cetin 2004; Han 1993; Pollack 1992). Maternal causes can arise before pregnancy; such as weight, age and parity, or during gestation; which include malnutrition, hypertension, and hypoxic conditions. Environmental conditions such as alcohol and drug use (Feng 2005; Pollack 1992) have also been reported to give rise to growth restriction. Placental factors that can contribute to sub-optimal growth and development include abnormal cord insertions, placenta previa, placental infarcts, multiple gestations, and abruption placentae (Pollack 1992). Although any one of these factors can cause FGR, it is estimated that 60% of FGR is idiopathic deriving from a combination of factors, the majority of which involve some form of abnormal or aberrant placental development, commonly referred to as placental insufficiency (Ghidini 1996; Pallotto 2006; Pollack 1992).

1.1.4 Placental Insufficiency

The human placenta is the organ responsible for nutrient transfer between maternal and fetal tissues throughout gestation. Due to an increase in feto-placental blood vessels around 21 days gestation, nutrient transfer increases, turning an initially hypoxic *in utero* environment to one that is optimal for fetal growth and organ system development during the latter half of pregnancy (Kingdom 2000). This growth and development is achieved by a 10-fold increase in the volume of the vasculature, an increase in the surface area of the placental labyrinth and a decrease in trophoblast thickness, all changes that promote nutrient exchange from maternal to the fetus (Myatt 2006). This transfer of includes nutrients, essential substrates, and oxygen required for proper growth and development.

As mentioned, the majority of conditions affecting fetal growth are placental in origin. The most common placental conditions are abnormal alterations in the uteroplacental and fetal-placental circulations. In the majority of these cases, there is diminished maternal uteroplacental blood flow, caused by insufficient or incomplete trophoblastic invasion of the spiral arteries in the placental bed (Salafia 2006). Placental insufficiency is a general term used to describe any condition whereby the placenta is unable to transfer an appropriate amount of nutrients or oxygen for ultimate fetal growth. As mentioned earlier, this placental dysfunction can arise from numerous different aberrant structural or developmental changes during placentation (Kingdom 2000). These alterations may result in poor fetal development and therefore FGR due to inadequate blood flow and thereby inefficient oxygenation, glucose and amino acid transport to the fetus (Jansson 1990; Mahendran 1993; Jones 1983; Myatt 2006). Under these conditions, the fetus adapts by altering metabolic and developmental processes at the expense of appropriate growth.

1.1.5 FGR and long-term health

Fetal growth restriction is the most common factor in stillborn births (Cosmi 2011). Furthermore, a number of causes of FGR have been associated with an increased risk of FGR and intrauterine death in a mother's subsequent pregnancy (Cox 2009). Behind preterm birth, FGR is the second most common condition in newborns arising from pregnancy complications. Studies have suggested that the risk for spontaneous preterm delivery is increased for FGR fetuses and that

FGR contributed to a 5-6 fold increase in risk for perinatal morbidity (Lackman 2001). Along with morbidity among premature neonates, FGR is associated with a variety of long-term adverse health outcomes, with a risk of hypoxic brain injury; chronic lung disease; retinopathy of prematurity; tissue hypoxia; hypoglycemia; and mortality (Jang 2011; Rodriguez 2011 Feng 2005). Sex differences have been studied in incidences of FGR, although scantly, and it has been reported that females tend to be better protected and have a more favourable chance of surviving complications due to FGR compared to males (Synnes 2010). In a study from China, the incidence of FGR in females was significantly higher than in males, suggesting a female predilection for adaptation to a sub-optimal intrauterine environment relative to male counterparts (Liu 2014).

Modern obstetric care has now targeted early gestation as a critical time point to intervene and attempt to rescue adverse outcomes and promote fetal growth and survival. Mothers will often receive glucocorticoids treatment if there is a risk of delivering preterm with FGR, in an attempt to match the endogenous glucocorticoid burst prenatally that is essential for brain and lung development. Catch-up growth is a phenomenon that has also been studied in cases of FGR, where there is rapid growth after birth to match the growth curves of AGA babies . It has been reporeted that aFGR infants catch up in size within 3 years, while interestingly; sFGR newborns actually have a decreased rate of growth and weight gain for the first 38-40 weeks, followed by an increased weight gain rate (Strauss 1997). Strauss also demonstrated how 70% of sFGR babies reached greater than the 10th percentile by four months, they remained at a lower weight compared to AGA babies up to 3 years old (Strauss 1997). These growth trajectories, along with the myriad of complications and disorders that arise later in life due to FGR, suggest that intrauterine insults have a lasting impact on the health throughout pregnancy and into adulthood.

1.1.6 Developmental Programming

Fetal programming is the theory that organ development during times of sub-optimal substrate transfer is altered through adaptations by the fetus, which then predisposes the outcomes for health in later life (Barker 1998). This became known has the “developmental origins of adult disease” (DOHaD) hypothesis or the “Barker” hypothesis. These adaptations can permanently

change the structure and function of many of the body's systems, and can be cardiovascular, metabolic, or endocrine in nature. Fetal programming can occur in three different ways; 1) direct damage, such as early loss of a limb; 2) induction, deletion, or impaired development of structure resulting from insult during a critical period of development; or 3) physiological re-setting by an early stimulus or insult at a critical period with long term consequences for endocrine axes (Lucas 1994). These critical periods, as Barker and his colleges described, most likely occur in the fetus during phases of rapid cell division, and are usually influenced by the intrauterine environment rather than by genetics (Cosmi 2011). Low birth weight was the most common cause of infant death at the start of the 20th century, and these observations led to the hypothesis that low-birth-weight babies who survived infancy and childhood might be at increased risk of coronary heart disease in later life (Barker 2002; Boo 2006). Low birth weight, caused by either growth restriction or preterm birth, has more recently been associated with increased rates of cardiovascular disease and type-2 diabetes in adult life (Langley-Evans 2009). Therefore, the theory of DOHaD focuses on intrauterine life offers a novel approach to research investigating the long-term outcomes of a sub-optimal intrauterine environment, and the consequences of a fetus that has not matched its full growth potential during pregnancy. Growing evidence has supported the idea that developmental adaptations made *in utero*, may lead to permanent changes or "programming" of the body (Barker 1998). To help characterize this phenomenon, Hales and Barker put forth the "Thrifty phenotype". They proposed that the *in utero* environment provides cues of the postnatal environment for the fetus, which causes the fetus to make thrifty adaptations for survival. However, these once advantageous changes become detrimental if the postnatal environment differs from the cues established *in utero* (Hales 2001). Since the conception of DOHaD, a wide range of adverse intrauterine environments including alterations in oxygenation and hormone levels have been linked to increased incidence of cardiovascular and metabolic disease in adult life (Barker 1999).

1.2 CLINICAL STUDIES OF FGR AND FETAL PROGRAMMING

1.2.1 Clinical Studies of Maternal Nutrition

The most common cause of FGR arising from a maternal insult is malnutrition. Clinical study of maternal undernourishment and the impact on fetal development and adult health is complex, with many compounding factors. However, some studies suggest the impact of sub-optimal maternal nutrition on birth weight and developmental consequences. Early evidence of DOHaD in humans was observed during the Dutch famine, an acute period of exposure to suboptimal nutrition between 1944-1946 in The Netherlands. This cohort has been extensively studied, and reports show an increase in placental weight, but not in birthweight, in infants whose mothers' nutrition was compromised around conception or in the first trimester of pregnancy (Lumey 1998). The increase in placental weight may represent a compensatory response for the reduction in substrate availability, as noted in medical evaluations of human FGR cases (Lumey 1998). Additionally, these children exposed to malnutrition *in utero* experienced decreased glucose tolerance in later life (Ravelli 1998) as further evidence for fetal programming. Similar studies in England found that low birth weight arising from maternal undernourishment was associated with increased mortality from cardiovascular and metabolic disease in later life (Barker 1993a; Barker 1993b).

Maternal undernutrition is the most prominent contributor to FGR in developing countries, and it is estimated that over 200 million children and 11% of current births in these countries fail to reach their full growth potential due to nutritional, social and health factors arising from poverty (Walker 2007; de Onis 1998). These studies in developing countries have linked low birth weight induced by poor nutrition to increases in cognitive impairment, depression, and behavioural problems in children up to three years of age, which primarily remain throughout adulthood compared to normal birth weight groups (Strauss 2000; Walker 2004; Villar 1984; Gardner 2003). It has also been found that food supplementation throughout pregnancy benefitted child motor development at 8 months but not 5 years, and food supplementation given only during the last trimester and throughout infancy did not benefit development from 6 months to 3 years (Adair 1981; Waber 2002).

From the above studies, it appears that the placenta functions as a nutrient sensor regulating nutrient transport according to the ability of the maternal supply line to deliver nutrients. By directly regulating fetal nutrient supply and fetal growth during gestation, the placenta plays a central role in fetal programming and low birth weight FGR (Jansson 2007). Furthermore, it appears that the placenta is "set" early during pregnancy based on the maternal nutritional supply and cannot easily be rescued by supplementation later on in pregnancy. It has also been reported that perturbations in the maternal compartment may affect the methylation status of placental genes and increase placental oxidative stress, resulting in changes in placental function (Jansson 2007). Overall, maternal undernutrition plays a very important role in fetal programming and development, perhaps second only to placental insufficiency arising from placental complications during pregnancy, and constitutes a chronic insult on the mother, placenta and fetus.

1.2.2 Chronic Hypoxia and Oxygen Homeostasis

The mammalian body has many adaptive responses to both acute and chronic decreases in oxygen availability. Oxygen homeostasis is of the upmost importance, not only for proper development during pregnancy, but a continuous supply is necessary for survival. Fluctuating oxygen supply and demand cause premature reactions of electrons with oxygen, which produces the damaging reactive oxygen species (ROS), a mediator of damage in oxidative stress. The mechanism by which our body senses and reacts to hypoxia was brought to light through the discovery of hypoxia inducible factor 1 (HIF-1) and the delineation of its role as a master regulator of chronic oxygen homeostasis (Semenza 2008). HIF-1 is a heterodimeric protein that is composed of a constitutively expressed HIF-1β subunit and an O_2-regulated HIF-1α subunit (Wang 1995). Since HIF-1α is regulated by changes in oxygen, it and the HIF-1 mechanistic pathway has been the primary focus on studies investigating chronic hypoxia in human and animal studies alike. HIF-1α controls a vast amount of protein, transcription factors, and metabolic processes to ensure that every cell receives adequate oxygen perfusion. Specifically, direct HIF-1α target genes help regulate two important processes of oxygen homeostasis; erythropoiesis, or red blood cell production, and angiogenesis, the branching of new blood vessels off existing ones to increase vasculature (Jelkmann 2007; Iyer 1998; Ceradini 2004;

Forsythe 1996; Kelly 2003). During systemic tissue hypoxia, HIF-1 transciptional activity is induced in cells throughout the body, including specialized cells in the kidney that produce erythropoietin (EPO), a glycoprotein hormone that is secreted into the blood and binds to its cognate receptor (EPOR) on erythroid progenitor cells, thereby stimulating red blood cell survival and differentiation (Jelkmann 2007; Iyer 1998). In the developing fetus, EPO is produced not only by the fetal kidney, but also by the fetal liver and placenta until the kidney has developed enough to produce sufficient amounts on its own (Noguchi 2008). In contrast to the systemic role of EPO, angiogenesis represents a local tissue response to decreased oxygenation (Semenza 2008). As cells grow and proliferate, their consumption of O_2 increases and HIF-1 activity is induced, resulting in increases in a variety growth factors, including vascular endothelial growth factors (VEGFs) stromal-derived factor 1 (SDF-1), and placental growth factor (PLGF) (Ceradini 2004; Forsythe 1996; Kelly 2003).

1.2.2.1 Chronic Hypoxia and Growth Restriction

From an extremely early time point in gestation, the fetus is very sensitive and adaptive to changes in nutrient availability. One of the first responses to a decrease in substrate delivery, such as oxygen, is a reduction in fetal growth (Peebles 2004). FGR is one of the major complications of antenatal hypoxic exposure in humans, with FGR arising from both acute and chronic levels of hypoxia at different time points throughout gestation (de Onis 1998). It is believed that restriction in fetal growth appears to be the most important factor in balancing reduced oxygen delivery and consumption. During prolonged oxygen deprivation, brain-sparing adaptations take effect, to ensure appropriate brain and heart development. This means that although cerebral metabolism is preserved and myocardial oxygen consumption may actually increase, oxygen consumption in muscle, kidney, gut, and lung may be decreased markedly (Braems 1991). This represents the adaptive response by the fetus to lowered oxygenation in the intrauterine environment.

Fetal hypoxia is one of the most significant challenges facing obstetric clinical study, as it presents a large ethical concern for human cohorts. A low oxygen intrauterine environment can be generated through variety of adverse conditions, including placental insufficiency (Myatt

2010), preeclampsia (Roberts 2002) high-altitude environments (Moore 2001; Giussani 2001; Niermeyer 2009) and exposure to toxic substances (Julian 2009). Specifically, high-altitude living exposes particular problems and imposes a significant challenge to the pregnant woman and her developing fetus, and often result in low birth weight offspring and preeclampsia during pregnancy (Moore 2001; Giussani 2001; Niermeyer 2009). The risk of small size at birth due to lack of oxygen during fetal life is associated with the increased incidence of high blood pressure and heart disease in the adult offspring (Julian 2009). Similar to that seen with inadequate substrate availability due to placental insufficiency, when a fetus experiences hypoxic stress it redistributes its cardiac output to preferentially perfuse the heart and brain at the expense of other organs (Peeters 1979; Kitanaka 1989; Longo 1993). Recently, our lab has used a patient cohort from St. Joseph's hospital in London, Ontario to show that low pre-pregnancy BMI is another contributor to both FGR with reduced umbilical O_2 levels (Richardson 2014). Together, these studies demonstrate how chronic hypoxia can lead to various disruptions in normal fetal development and eventually to dysfunction and disorders in postnatal life.

1.2.3 Oxidative Stress and Redox Homeostasis

Oxidative stress occurs during an imbalance between ROS and a cells antioxidant capacity. ROS can be produced naturally, as a biological by-product oxidative phosphorylation (the process that produces ATP) and play a major role in cell signalling (Zhang 2011). ROS can additionally be generated from environmental sources such as pollutants, tobacco, smoke, drugs, xenobiotics or radiation (Trachootham 2008). To a lesser extent, ROS can be produced from endoplasmic reticulum (ER) during stress stimuli (Malholtra 2007). It has been suggested that low levels of ROS work in signalling pathways to promote a variety of beneficial activities, including cell proliferation, differentiation, while higher levels of ROS lead to the advantageous programmed cell death via apoptosis (Ye 2015). Therefore, ROS in lower quantities is beneficial and maintenance of this redox homeostasis is essential for proper cell functionality.

A build-up of ROS due to oxygen imbalance can have a variety of damaging effects, including DNA damage, and inactivation of valuable amino acids, enzymes, and fatty acids (Brooker 2011). During states whereby there is a chronic lack of oxygen (ie:hypoxia), this build-up of

ROS occurs from consistent anerobic production by the mitochondria where ROS are a natural by-product. This physiological stress can be demonstrated by measuring levels of cellular antioxidant molecules, enzymes involved in their production or in the reduction of ROS, and/or indexes of cellular damage due to oxidative stress (Hracsko 2008). For example, glutathione (GSH) is an abundent antioxidant and a free radical scavenger that is reduced to GSH after enzymatic interaction with free radicals. The ratio of GSH to its oxidized form (GSSG) is indicative of the antioxidant state of the cell, with a lower ratio signifying a cell undergoing oxidative stress (Schafer 2001; Owen 2010). Malondialdehyde (MDA) is a by-product of ROS lipid peroxidation that represents an index of cellular damage (Rice-Evans 1993). 8-hydroxyguanosine (8-OHdG) has been used as a measure of DNA oxidative stress, and 3-Nitrotyrosine (3NT) is used as an indicator or marker of cell damage due to the buildup of ROS such as peroxynitrite anion and nitrogen dioxide (NO_2) (Mohiuddin 2006; Pacher 2007).

Under normal physiological conditions, when an increase in ROS and oxidative stressors is detected, the endogenous cellular defense system is activated. This consists of a number of antioxidants that protect the cell by maintaining a desirable redox status. Many antioxidants, including peroxiredoxins (PRDX) which are controlled by the thioredoxin (THX) class of redox proteins, the oxidized form of glutathione, superoxide dismutase (SOD) and catalases act directly on ROS to inactivate them (Reddy 2008). These enzymes are at the forefront of a defensive and protective system in place to ensure long-term chronic oxidative stress from exposure to ROS can be avoided.

1.2.3.2 Clinical Studies of Oxidative Stress and Aberrant Development

The role of oxidative stress in fetal programming is supported by epidemiological evidence of oxidant indices and low birth weight in association with type 2 diabetes (Peuchant 2004), cardiovascular disease (Bloc 2002) and preeclampsia (Roberts 2002). Thus, it follows that oxidative stress may be an additional mechanism for intrauterine insult and programming consequences after birth. Studies have shown that oxidative stress plays an important role in FGR pregnancies resulting from placental insufficiency, preeclampsia, and maternal undernourishment. Umbilical cord plasma from FGR pregnancies have been found to have

increased levels of oxidative stress markers, including MDA and xanthine oxidase, while the levels of antioxidant potential were identified to be lower in maternal plasma, umbilical cord plasma, and placental tissues of the patients with FGR (Biri 2007). Other studies have found that FGR pregnancies have increased markers for both oxidative DNA damage and antioxident function, including 8-OHdG, 4-hydroxynonenal (4-HNE), (TRX), and redox factor-1 which are enhanced in cases of FGR with preeclampsia (Takagi 2004; Thompson 2012). Interestingly, some studies such as Hracsko *et al.,* have reported decreases in antioxidant enzyme activity and the levels of antioxidants, which suggest a level of defensive deficiency in cases of FGR. It was found that that both were significantly lower in the FGR group of neonates, while damage of proteins and DNA was slightly, but non-significantly, higher in the FGR group (Hracsko 2008). Human cases of maternal undernourishment-induced FGR have also reported an increase in markers of oxidative stress. In particular, Gupta *et al.*. (2004) showed increased oxidative stress in FGR fetuses born to undernourished mothers through the measure of SOD, catalase, glutathione (GSH), and malondialdehyde (MDA) in cord blood. Together these studies indicate a strong link between sub-optimal fetal growth and oxidative stress, which suggest oxidative stress associated with pregnancy complications may be a contributing factor in postnatal consequences of FGR.

1.3 ANIMAL MODELS OF FGR AND ADVERSE DEVELOPMENT

1.3.1 Animal Studies of FGR with Placental Insufficiency

From the clinical study of FGR arising from placental insufficiency, one of the major contributors is aberrant placental vascularization and associated decreases in umbilical blood flow. These changes have directed research focus to animal models of placental insufficiency induced by a variety of pre-pregnancy, mid-pregnancy and later pregnancy interventions. Pre-pregnancy uterine carunclectomy, the removal of endometrial tissue of the placenta, resulted in decreased fetal weight as well as increased brain weight to fetal weight ratio in the fetal sheep (Robinson 1979). These fetuses also display markers implicating hypoxaemia and hypoglycemia compared to controls with intact placentae (Robinson 1979). Mid-pregnancy exposure to hypothermic environments in ewes resulted in altered placental hormone concentrations most

likely due to impaired trophoblast cell development, which was an additional causative factor for FGR arising from placental insufficiency (Regnault 1999). Models of later pregnancy perturbations include uterine artery ligation (UAL)/ablation, where the uterine artery is ligated midway through gestation, which has been shown to result in aFGR with fetal brain and adrenal weight being less affected then that of controls in the guinea pig (Lafeber 1984; Turner 2009). Furthermore, uterine artery ligation in rodent (Olivier 2007) and sheep (Rees 1999) has been a popular technique to induce FGR, with decreases in fetal brain development and long-term brain function. UAL in guinea pigs has resulted in decreased placental size and placental substrate transfer (Jansson 1990), as well as shown reduced oxygen and nutrient delivery (Jones 1983; Lafeber 1984), which successfully mimics human placental insufficiency. More recently, our lab has demonstrated how UAL in pregnant guinea pigs leads to decreases in synapse formation, myelination and maturation of synapses in the hippocampal brain regions of the fetus (Piorkowska 2014). Finally, placental embolization, whereby the placenta is partially removed midway through gestation, results in aFGR with evidence for chronic hypoxemia in the fetal sheep (Murotsuki 1997; Gagnon 1997). Together, these various techniques and methods primarily used in sheep, rats, and guinea pigs, lead to FGR with variable hypoxemia and nutrient restriction. However, while certainly successful for inducing growth restriction, these UAL/cauterization/embolization models have also reported very high pup mortality relative to the human condition, up to 50% in the guinea pig (Turner 2009). While these models are representative of aberrant placental development as seen with human cases of FGR and placental insufficiency, they involve a one time, invasive procedure that results in variable severity and timing that doesn't always closely parallel the human condition.

1.3.2 Animal Studies of FGR with Maternal Undernourishment

As stated, clinical study of undernutrition is dependent on a variety of factors, most prominently the timing of undernourishment pre-conception and throughout pregnancy as well as the severity of undernutrition. As a result, there have been many animal models targeted at investigating the impact of global or total calorie maternal feed restriction on fetal and placental growth and development. These studies, primarily using sheep and guinea pigs, have used a variety of moderate to severe restriction of feed resulting in a range of different levels growth restriction

and altered development. Typically, moderate feed restriction has been characterized as 70-85% of a control *ad libitum* diet, and severe restriction studies usually use around 50% feed restriction to mimic extreme maternal undernourishment. Overall, growth restriction midway through pregnancy in guinea pigs tends to result in changes in the placenta over the fetus, and maternal nutrient restriction (MNR) starting pre-conception and lasting throughout pregnancy results in altered fetal and placental weights (Roberts 2001a). Severe maternal restriction studies, using a 50% dietary restriction have led to deceased placental weight in the sheep, and while severe restriction midway through pregnancy interestingly did not lead to any changes to overall fetal weight; it did result in altered fetal dimensions indicating an organ sparing effect (Heasman 1999). Also, a 50% feed restriction in the maternal ewe has been shown to increase growth factors, arterial blood pressure, and decrease fetal plasma glucose concentration (Edwards 2001). FGR has been reported when the severe growth restriction is applied starting early on in gestation (Vonnahme 2003). These studies resulted in fetuses that were markedly smaller, had reduced fetal and maternal blood glucose levels, but with unaltered placental weight changes (Vonnahme 2003). Studies of moderate nutrient restriction, usually involving 70-80% of the control feed intake, have also shown to decidedly impact fetal-placental growth during preconception food restriction (MacLaughlin 2005). This study examined how even during preconception and ending on day 7 of gestation, maternal weight due to undernutrition impacts fetal and placental weight at birth, implicating long-term consequences as seen with human FGR as seen with placental insufficiency. Global MNR during early to mid-pregnancy in sheep increased the placental weight:fetal weight ratio by enhancing placental weight at term without altering fetal weight (Heasman 1998) . Conversely, a 50% maternal undernourishment during the second half of rat pregnancy yielded a decrease in the placental weight:fetal weight ratio (Belkacemi 2009). Together, these results suggest that MNR permanently affects placental weight when nutrient deprivation coincides with the time when fetal nutrient demand is maximal.

The guinea pig has also been used extensively to study MNR and fetal growth and development. A group from Australia used moderate MNR in guinea pigs pre-conception and throughout pregnancy lead to changes in growth factors that resulted in altered development (Sohlstrom, 1998). In addition to decreased fetal and placental growth measures, Roberts and colleges found impactful structural and functional changes in the developing placenta when mothers were subjected to a moderate MNR diet pre-conception and throughout pregnancy. These changes

included increased barrier thickness, decreased placenta labyrinth, and therefore decreased availability for oxygen exchange between mother and fetus (Roberts 2001a, Roberts 2001b). These fetal guinea pigs, because of moderate MNR, also had increased glucose tolerance levels, growth factors such as IGFs, and adiposity compared to control counterparts (Roberts 2001b).

1.3.3 Animal Studies of Altered Development with Hypoxia

Due to the drastic effect of maternal or environmental hypoxia fetal growth and development, combined with the complications of studying chronic hypoxia in human FGR, there is considerable interest in using animal models in the study of hypoxia-induced FGR. These studies, primarily in sheep and guinea pigs, have been studied extensively the effects of both chronic and acute hypoxia on fetal development. Experimentally, chronic hypoxia (over days to weeks or even months) can be initiated early or late in gestation in the sheep and can be induced through placental embolization (Boyle 1984; Gagnon 1997), placental insufficiency via caruncletomy (Robinson 1979; Robinson 1994), or by high altitude resulting in moderate continuous hypoxia with normal pregnancy duration and varying degrees of growth restriction (Harvey 1993; Imamura 2004; Kamitomo 1992). Studies of placental embolization have resulted in growth restriction with progressive hypoxemia with reduced fetal plasma adrenocorticotropic hormone (ACTH) but increased prostaglandin E_2 as a marker of fetal stress (Gagnon 1997). Another study, using the caruncletomy model to induce placental insufficiency, reported FGR with fetal arterial PO_2 levels reduced by 30%, and associated increases in cortisol levels (Phillips 1996). Another study found decreased protein synthesis in the fetal sheep with a corresponding decrease in overall oxygen consumption of up to 10% (Richardson 1998). Richardson also reported that growth restriction can so successfully reduce oxygen consumption that the arterial oxygen concentration can be maintained or only minimally decreased until substrate delivery is severely restricted (Richardson 1998). In the guinea pig, exposure to chronic hypoxia demonstrated an over 10-fold increase in expression levels of hypoxia index genes such as EPO and HIF-1α, and an over 40% reduction in neuronal density, implicating prenatal brain damage (Blutstein 2013; Kim 2014). Furthermore, pregnant guinea pigs placed in hypoxic chambers have resulted in fetuses that are FGR, with increased oxidative stress and pro-inflammatory markers in the fetal heart and liver (Evans 2012a, Oh 2008; Al-Hasan 2012; Oh 2007), as well as

differentially altered apoptotic and necrotic factors in the fetal brain (Evans 2012b). These studies in the sheep and guinea pig suggest that chronic hypoxia can induce FGR with additional mediators of damage, including oxidative stress, and combined these factors play a considerable role in long-term fetal programming.

1.3.4 Animal Studies of Oxidative Stress and Aberrant Development

As further support that oxidative stress acts as a mechanism of altered fetal development and consequences later in life, animal models of FGR and hypoxia have reported elevated makers of oxidative stress in both fetal and adult offspring. Prenatal hypoxia has been shown to generate oxidative stress in fetal hearts in a variety of animal species, including sheep (Derks 2010), rat (Giussani 2012; Zhang 2005), and guinea pig (Thompson 2005; Evans 2012a; Dong 2006). Taken together, these studies suggest that oxidative stress influences the fetal heart and initiates cardiovascular alterations in offspring. Further work done suggests other fetal and placental tissues impacted by hypoxia and oxidative stress are causative of adverse developmental programming. One such study, by Oh *et al.,* found that late-onsetting, chronic maternal hypoxia increased total glutathione levels in the fetal liver, and also increased gamma glutamylcysteine synthetase (γ-GCS) protein levels in the fetal liver, kidney and lung (Oh 2008). Prenatal hypoxia increased DNA fragmentation and lipid peroxidation in fetal guinea pig livers, which was reversed by the antioxidant, N-acetylcysteine (Hashimoto 2012). Hypoxia during gestation decreases insulin-signaling proteins in livers of rat offspring (Rueda-Clausen 2011) Overall, reduced fetal oxygenation from hypoxia may be responsible for organ-specific damage via oxidative stress.

1.4 SUMMARY

Fetal growth restriction is failure to attain proper developmental growth during intrauterine life. It can be present as both symmetrical or an asymmetrical growth restriction, without or with a brain sparing effect. FGR can arise from a combination of maternal, placental and genetic factors, the most common being abnormal placental development in developed countries or

undernourishment in developing countries. Overall, placental dysfunction leads to the inability to transport nutrients and oxygen to the fetus. This growth restriction leads to an increased risk for many adverse outcomes throughout life, including cardiovascular, metabolic and cognitive disorders, which has spurred investigative interests towards the Developmental Origins of Health and Disease. Clinical study of the potential primary mechanisms behind FGR; placental insufficiency, hypoxia and oxidative stress, are complicated and relies on the ability to control many variables. This has led to many animals models of FGR, using a variety of techniques to mimic the human condition. Although the connection between placental insufficiency and increased hypoxia and oxidative stress are known, little work has been done to study if similar results are found in models of maternal nutrient restriction. Accordingly, it will be important to determine the extent to which fetal hypoxia and related oxidative stress also occur with growth restriction resulting from maternal undernourishment, and thereby the potential for treatment aimed at decreasing placental-fetal oxidative stress.

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CHAPTER 2

RATIONALE, HYPOTHESIS, AND RESEARCH OBJECTIVES

2.1 RATIONALE

Fetal growth restriction (FGR) with birth weight < 3rd, 5th, or 10th percentile for gestational age, denotes a decrease in growth rate that prevents a newborn from obtaining complete growth potential (Lackman 2001). It is also the second most antenatal complication in developed countries, and increases the risk for morbidity and mortality in newborns (Seeds 1998; Piper 1996). Further, FGR offspring are at increased risk for later adverse health outcomes including heart disease, diabetes and neurodevelopmental disability, with the greatest risk observed in those with severe and early-onsetting FGR (Barker 2004). This has led to the notion that the intrauterine environment during fetal life can 'programme' aberrant organ system development leading to adverse outcomes, and there has been a plethora of animal-based studies examining mechanisms underlying this relationship (Barker 2004; Zaw 2003).

Fetal growth restriction is an idiopathic condition, where unknown combinations of maternal and fetal factors give rise to incomplete growth, although the majority of FGR is associated with placental insufficiency. Placental insufficiency is the inefficiency in the transport of oxygen and substrates at the maternal-placental-fetal border and results in a sub-optimal intrauterine environment for growth. Despite advances in obstetric care, FGR remains prevalent in developed countries where placental insufficiency is the major cause and in developing countries where inadequate maternal nutrition plays a greater role (Lumey 1998). Clinical studies of placental insufficiency with FGR demonstrate aberrant placental vascularization (Wang 1992) and associated decreases in umbilical blood flow (Roberts 1998), leading to chronic fetal hypoxemia as a primary signalling mechanism for the degree of growth restriction (Hracsko 2008). This has led to animal models of placental insufficiency induced by uterine artery ligation, placental embolization, or carunclectomy, primarily in sheep and guinea pigs, and leading to FGR with variable hypoxemia and nutrient restriction (Belkacemi 2010). While relevant for representing human FGR and developmental programming, these models of placental insufficiency mainly target the latter part of pregnancy and limit the ability to study placental responses with the artifactual blood flow manipulations. Clinical study of maternal nutrition and FGR is complex, and while maternal undernourishment is undoubtedly causative for FGR, this will depend upon severity and timing both pre-conception and throughout pregnancy. This has led to animal models of maternal nutrient restriction (MNR) induced by global nutrient restriction or protein

restriction, pre-conception, peri-conception, and through pregnancy, in a number of animal species and leading to variable degrees of FGR (Lumey 1998). These models of MNR are also relevant for representing human FGR and developmental programming, and have the added advantage of targeting the insult throughout pregnancy paralleling the human situation with severe FGR (Low 1992), and of studying placental responses which has increased in the past few years (Stevens-Simon 1995).

These studies of MNR beginning pre-conception in guinea pigs (Sohlstrom 1998; Roberts 2001) and early in pregnancy in sheep (Thompson 2011) have generated placentas with reduced surface area for exchange, increased barrier thickness, and altered vascular development. These histopathological changes are similar to that seen in human FGR with maternal undernourishment (Soothill 1987) and should result in reduced nutrient transport to the fetus which has been shown for glucose, amino acids and lipids (Crozier 2009). However, whether oxygen transfer to the fetus is also limited in MNR FGR fetuses either globally or selectively with blood flow redistribution (Cox 1988), or is increased with decreased oxygen utilization, has yet to be determined.

In human pregnancies complicated by placental insufficiency with FGR, placental and cord blood markers for oxidative stress are increased (Heasman 1999). Likewise, prenatal hypoxia in pregnant guinea pigs leads to increased oxidative stress with lipid peroxidation and DNA fragmentation in fetal livers which can be reversed by the antioxidant, N-acetylcysteine (Brodsky 2004). As such, chronic hypoxemia in the FGR fetus with placental insufficiency can lead to an excess of reactive oxygen species that surpasses antioxidant capacity with damaging effects to plasma membranes via lipid peroxidation and to nuclear integrity via DNA fragmentation, which may be mechanistic for fetal programming (Brodsky 2004). Accordingly, it will be important to determine the extent to which fetal hypoxia and related oxidative stress also occur with growth restriction resulting from the guinea pig model of maternal nutrient restriction, and thereby the potential for treatment aimed at decreasing placental-fetal oxidative stress. Overall, it is hypothesized that MNR in pregnant guinea pigs will result in fetal tissue growth restriction with lowered tissue oxygenation and increased tissue oxidative stress.

2.2 HYPOTHESES

1. Maternal nutrient restriction in guinea pigs beginning pre-conception and continuing throughout pregnancy will result in aberrant placental growth and fetal growth restriction in near term pups; with the growth restriction being asymmetrical compared to control pups.

2. Maternal nutrient restriction will alter levels of tissue oxygenation in the near-term guinea pig fetus separated into cohorts based on body weight. Compared to control and appropriate for gestational age fetuses, MNR-FGR fetuses will have decreased levels of oxygenation resulting in increased levels of hypoxia related markers.

3. Maternal nutrient restriction will alter oxidative stress and antioxidant responses in the near-term guinea pig fetus separated into cohorts based on body weight. Compared to control and appropriate for gestational age fetuses, MNR-FGR fetuses will have increased levels of oxidative stress markers resulting in increased levels of antioxidants.

2.3 OBJECTIVES

1. To apply a MNR (70% of ad lib diet at least 28 days prior to pregnancy and continuing throughout and increased to 90% of ad lib diet at mid gestation until near term put-down) diet regime to guinea pig sows to create an adverse *in utero* environment for fetal development, that will lead to a degree of growth restriction in guinea pig fetuses, compare growth and placental development characteristics, and compare organ system development in relation to asymmetrical or symmetrical growth patterns.

2. To determine differences in oxygenation related protein expression between AGA-Control fetuses and MNR-FGR guinea pig fetuses. Specifically, examining Hypoxyprobe 1 (HP-1) expression using immunohistochemistry in the placenta and fetal tissues. Also, to substantiate the histology using Western Blot techniques in these organs to determine changes in EPO, EPOR, and VEGF protein levels.

3. To determine differences in oxidative stress and stress response related protein expression between AGA-Control and MNR-FGR guinea pig fetuses. Specifically, examining MDA and

GSSG/GSH expression using a thibarbituric acid reactive substances and cholometric assays, respectively, in placental and various fetal tissues. Also, to substantiate the assays using Western Blot techniques in these organs to determine changes in Prdx protein levels.

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CHAPTER 3

MATERNAL NUTRIENT RESTRICTION IN GUINEA PIGS AS AN ANIMAL MODEL FOR INDUCING FETAL GROWTH RESTRICTION

3.1 INTRODUCTION

Fetal growth restriction (FGR) with infants small for their gestational age when born, is a major contributor to perinatal morbidity and mortality and for later adverse health outcomes, including heart disease, diabetes, and neurodevelopmental disability (Kramer 1990; Piper 1996; Lackman 2001; Barker 2004; Pryor 1995). This has led to the notion that the intrauterine environment during fetal life can "programme" the development of risk factors for these later adverse outcomes and an increasing number of human and animal based studies examining mechanisms underlying this relationship support this concept (Barker 2004; Godfrey 2000; Armitage 2004; Fowden 2006).

Fetal growth restriction remains prevalent in developed countries where aberrant placental development or "placental insufficiency" is a major cause, and in developing countries where maternal undernourishment plays a greater role (Jansson 2007; Walker 2007). Clinical study of placental insufficiency with FGR demonstrates aberrant placental vascularization (Kingdom 1997) and associated decreases in umbilical blood flow (Ferrazzi 2000). This has led to animal models of placental insufficiency induced by pre-pregnancy uterine carunclectomy (Robinson 1979), mid-pregnancy exposure to hyperthermic environments (Regnault 1999), or later pregnancy uterine artery ligation (UAL)/ablation (Lafeber 1984; Jansson 1990; Detmer 1992; Turner 2009), or placental embolization (Murotsuki 1997), primarily in sheep, rats, and guinea pigs, and leading to FGR with variable hypoxemia and nutrient restriction. While relevant for representing human FGR and developmental programming, these vascular models are variable in their timing and severity through pregnancy and can limit the study of placental responses with the artifactual blood flow manipulations.

Clinical study of maternal nutrition and FGR is complex, and while maternal undernourishment is undoubtedly causative for FGR, this will depend upon severity and timing pre-conception and through pregnancy (Jansson 2007; Walker 2007; Godfrey 1998; Lumey 1998). This has led to animal models of maternal nutrient restriction (MNR) induced by global nutrient restriction or protein restriction, pre-conception, peri-conception, and through pregnancy, in a number of animal species and leading to variable degrees of FGR (Jansson 2007; Sohlstrom 1998; Heasman 1999; Edwards 2001; Vonnahme 2003; Redmer 2004; MacLaughlin 2005; Belkacemi 2010; Soo

2012). These models of MNR are also relevant for representing human FGR and developmental programming, and have the advantage of targeting the insult throughout pregnancy analogous to the human situation with moderate to severe FGR where intrauterine deprivation is likely to be early in onset (Kingdom 1997; MacLaughlin 2005; Sung 1993; Wienerroither 2001).

Guinea pigs deliver precocial young after a relatively long pregnancy with many developmental events occurring during fetal life similar to that in humans (Carter 2007). They have therefore proved useful for modeling human FGR with UAL and ablation, but with high fetal loss rates and variable occurrence of growth restriction (Lafeber 1984; Jansson 1990; Detmer 1992; Turner 2009). Moderate MNR in guinea pigs at 70% of an *ad libitum* diet from 4 weeks pre-conception until mid-pregnancy increasing to 90% thereafter, has also been well studied for modeling human FGR (Sohlstrom 1998; Roberts 2001; Kind 2003; Kind 2005). Here the capacity of the mother to deliver nutrients to the fetus is further impaired since her own fuel reserves are depleted prior to conception (Roberts 2001). This should better reflect the human situation with undernourishment where pre-pregnant weight is a better determinant of fetal growth and development than weight gain during pregnancy (Stevens-Simon 1995) and most women do not improve their dietary and lifestyle patterns in pregnancy (Crozier 2009). Study with moderate MNR in guinea pigs has shown fetal weights to be decreased as much as 40% in animals near term in association with maternal insulin-like growth factor (IGF) and insulin-like growth factor-binding protein (IGFBP) alterations (Sohlstrom1998; Roberts 2001), and leading to insulin resistance in male offspring as also seen in humans born growth restricted (Barker 2004; Kind 2003). Of note, placental weights are decreased less than fetal weights suggesting compensatory growth, but with reduced surface area for nutrient exchange, increased barrier thickness, and altered vascular development (Sung 1993; Roberts 2001). These structural alterations in the placenta indicate functional impairment and can be seen in human FGR with maternal undernourishment (Belkacemi 2010; Aherne 1966) and with pre-eclampsia associated placental insufficiency (Aherne 1966; Teasdale 1988). Accordingly, moderate MNR in guinea pigs may result in adverse *in utero* conditions similar to that seen with maternal undernourishment or placental insufficiency during human pregnancy and prove useful for modeling FGR in both of these.

Moderate MNR in guinea pigs has proved useful for inducing FGR and studying maternal, placental and fetal growth characteristics, and offspring outcomes (Sohlstrom 1998; Roberts 2001; Kind 2003; Kind 2006. However, there has been little or no study of breeding and pregnancy success, the distribution of fetal weights and means by which FGR might be denoted, and the impact on fetal organ weights and blood metabolites. We have therefore studied moderate MNR in guinea pigs and report on our breeding and pregnancy success, and distribution of fetal weights near term and means for denoting FGR to further characterize the utility of this model. We have also determined the impact on fetal crown rump length and organ weights, and blood hemoglobin and glucose, hypothesizing that MNR-FGR animals will be lean with asymmetrical growth restriction, and polycythemic and hypoglycemic, as often seen in human pregnancy with moderate to severe FGR (Kramer 1990; Godfrey 1998; Piorkowska 2014).

3.2 MATERIALS AND METHODS

3.2.1 Animal Feeding, Breeding and Pregnancy

A previously established model of moderate MNR in guinea pigs (Sohlstrom 1998) was used with all experimental procedures approved by The University of Western Ontario Animal Use Subcommittee and followed the guidelines of the Canadian Council on Animal Care. Nulliparous female guinea pigs (Dunkin-Hartley, from Charles River Laboratories, Sherbrooke, Que, Canada) were housed in individual cages in a dedicated small animal care facility with a 12 hour light/dark cycle and temperature at 25ºC. Animals were fed a guinea pig ration diet (Guinea Pig Diet 5025, LabDiet, St. Louis, MO) and after a two week period of acclimatization, daily food consumption was monitored and estrous cycles were tracked (Lilley 1997).

Thirty guinea pig sows were randomly assigned to either a Control group fed *ad libitum* or an MNR group fed 70% of the average food intake per kilogram of body weight of the *ad libitum* fed animals as described by Sohlstrom *et al.*. (1998). After 4 weeks of adaptation to respective feeding regimens, animals were mated. A female found to be in estrous was placed in a cage with a male for 48-72 hours and removed when the vaginal membrane was again closed. Animal

pregnancies were confirmed by ultrasound 14-21 days later with conception taken to be the day prior to membrane closure and thereby day zero of gestation. Animals that were not pregnant were rebred at their next estrous cycle. During the first 34 days of pregnancy, the MNR animals continued at 70% average food intake of the Control animals per kilogram body weight, and from 35 days onward this was increased to 90% average food intake of the Control animals per kilogram body weight. Throughout the experiment, daily food intake and body weight of the animals were monitored 3-4 times per week and the dietary intake of the MNR animals adjusted as needed to maintain their food intake at 70% or 90% of the average food intake per kilogram of body weight of the *ad libitum* fed animals.

3.2.2 Necropsy, Tissue Collection and Blood Analytes

On day 60-61 of pregnancy (term = ~ 68 days), guinea pig sows were weighed and then sedated with an intramuscular injection of Versed (midazolam, 5 mg/kg; Sandoz Canada Inc., Boucherville, Que., Canada) and after 10 minutes an intramuscular injection of Vetalar (ketamine, 50 mg/kg; Bioniche Animal Health, Belleville, Ont., Canada) and Rompun (xylazine, 3 mg/kg; Bayer Inc., Toronto, Ont., Canada). A sub-umbilical midline incision was made after local infiltration of the abdominal skin with lidocaine (2%; Pfizer Animal Health, Kirkland, Que., Canada) followed by uterine incision and delivery of each of the fetuses. Approximately 1cc of amniotic fluid was obtained from each gestational sac prior to delivery, which was cold-centrifuged and stored at -80ºC for later analysis. All liveborn fetuses were treated with Vetalar as above with body and placental weights then obtained along with crown rump length measurements. The number of live and demised fetuses in each uterine horn was also noted. Fetuses were considered to be appropriate for gestational age (AGA) if $\geq$ 80 gms and FGR if $<$ 80 gms, which is in accord with the criteria we (Piorkowska 2014) and others (Jansson 1990) have used for categorizing AGA and FGR fetal weights in guinea pigs near-term. Subsequently, only AGA fetuses from Control group litters and FGR fetuses from MNR group litters were subjected to full necropsy, with priority given to the medial fetuses in each uterine horn meeting these criteria and with no more than three full necropsies per litter to ensure rapid tissue collection. Full necropsy consisted of an initial cardiac puncture to obtain ~ 1cc of blood in a

heparinized syringe which was then placed on ice until analysis for glucose and hemoglobin using an ABL 725 Blood Gas Analyzer (Radiometer, Copenhagen, Denmark). This was followed by dissection and weighing of the brain, heart, liver and kidneys, extraction of the gonads for determining fetal sex, and extraction of skeletal muscle and peri-renal adipose tissue. These organs/tissues along with the placenta were partitioned and both fixed in 4% paraformaldehyde and frozen in liquid nitrogen for later analysis.

3.2.3 Data Acquisition and Statistical Analysis

Litter size was based on the number of liveborn and demised fetuses noted at necropsy. Fetal body weight (gms)/crown rump length (cm) was calculated as a measure of leaness. Overall Control and MNR Population characteristics included data from all Control sows and their liveborn fetuses, and all MNR sows and their liveborn fetuses, excluding data from animals who failed to conceive, and those delivering prior to necropsy. Select AGA-Control and FGR-MNR Population characteristics included data from all AGA-Control and FGR-MNR fetuses who were liveborn, met the weight criteria noted, and underwent full necropsy. Maternal and fetal characteristic findings are presented as group means ± SEM. Overall Control and MNR Population characteristics and select AGA-Control and FGR-MNR Population characteristics were compared using analysis of variance and non-paired student`s t-test which were also nested for litter size (Graphpad Software, San Diego, CA). For all analysis, statistical significance was assumed for $p<0.05$.

3.3 RESULTS

3.3.1 Breeding and Pregnancy Outcomes

Of the thirty guinea pig sows, 12 were bred under *ad libitum* feeding conditions and 18 under MNR feeding conditions assuming breeding and pregnancy outcomes would be more adverse for the MNR animals and with the smaller litter size reported for MNR pregnancies. Of the 12 animals bred under *ad libitum* feeding conditions, three or 25% failed to become pregnant

despite up to four breeding attempts while the remaining animals took 2.4 breeding attempts on average to conceive. All nine of these control animals had continuing pregnancies to necropsy at 60/61 days gestation with 31 liveborn fetuses and one fetal demise which formed the overall Control Population. Of the 18 animals bred under MNR feeding conditions, three or 17% failed to become pregnant despite up to four breeding attempts while the remaining animals took 2.6 attempts on average to conceive. However, three of these pregnant MNR sows delivered preterm and prior to necropsy at 60/61 days gestation; one at 54 days with 3 fetuses weighing 21, 27, and 35 gms, one at 56 days with 3 fetuses weighing 50, 60, and 75 gms, and one at 57 days with 4 fetuses weighing 22, 33, 51, and 66 gms. The remaining 12 MNR animals had continuing pregnancies to the time of necropsy with 42 liveborn fetuses and one fetal demise which formed the overall MNR Population.

3.3.2 Maternal and Fetal Population Characteristics

The overall maternal and fetal population characteristics from all *ad libitum* fed control pregnancies and all maternal nutrient restriction pregnancies excepting the three MNR sows delivering preterm, are shown in Table 3.1. These data are presented for all fetuses to indicate the population variance and allow for comparison with past studies. While maternal weights were not different at conception averaging ~ 800 gms, by 60/61 days gestation MNR sows were ~ 17% lighter at 1046 ± 25 gms than Control sows at 1253 ± 60 gms ($p<0.01$). Food consumption for both animal groups increased through pregnancy as maternal weight increased, with the actual food consumption of MNR sows at conception and at 60/61 days gestation being ~ 65 % and 70 %, respectively, of that consumed by the Control sows. While litter size did not differ between the two study groups averaging 3-4, the combined fetal weight per litter was less for the MNR animals at 242 ± 21 gms than that of the Control animals at 331 ± 30 gms ($p<0.05$). This was due to fetal weights at necropsy being ~ 28% less in the MNR pregnancies at 69 ± 2 gms than in the Control pregnancies at 96 ± 2 gms ($p<0.01$). The 31 liveborn Control fetuses ranged in weight from 119 gms to 70 gms with the 50^{th} and 10^{th} percentiles being ~ 96 gms and 78 gms, respectively, while the 42 liveborn MNR fetuses ranged in weight from 102 to 41 gms with the 50^{th} and 10^{th} percentiles being ~ 69 gms and 50 gms, respectively (Figure 3.1). Placental

weights were also decreased in the MNR pregnancies by ~ 23% at 5.1 ± 0.2 gms vs that of the Control pregnancies at 6.6 ± 0.3 gms ($p<0.01$). As such, placental weights were decreased less than the fetal weights with the placental/fetal weights thereby increased in the MNR pregnancies at 7.6 ± 0.3% vs that of the Control pregnancies at 6.8 ± 0.3% ($p<0.05$). Fetal crown rump lengths were also decreased in the MNR pregnancies by ~ 15% at 10.6 ± 0.1 cm vs that of the Control pregnancies at 12.3 ± 0.2 cm ($p<0.01$), but again less than the decrease in fetal weights. As such, fetal weight/length as a measure of leaness was also decreased in the MNR pregnancies by ~ 15% at 6.4 ± 0.1 gm/cm vs that of the Control pregnancies at 7.8 ± 0.1 gm/cm ($p<0.01$).

While MNR fetuses were smaller than Control fetuses on average, there was considerable overlap in the population weight distributions as seen in Figure 3.1. This is not surprising since litter size, number of fetuses per uterine horn, and fetal position within the horn are all known to impact fetal growth (Piorkowska 2014; Turner 2000). We therefore chose to establish a cohort of AGA fetuses from the Control group pregnancies and a cohort of FGR fetuses from the MNR group pregnancies to allow for more in depth comparative study of growth related parameters. As noted, we used 80 gms as our threshold for categorizing AGA and FGR fetal weights at 60/61 days gestation and determining which Control and MNR fetuses were to be subjected to full necropsy with priority given to the medial fetuses in each uterine horn and with no more than three full necropsies per litter. This resulted in 20 AGA-Control fetuses and 25 FGR-MNR fetuses with the select population characteristics from these animals shown in Table 3.2 and Figure 3.1. After examining the gonads for sexing animals, it was determined there were 10 AGA-Control males and 10 AGA-Control females, and 11 FGR-MNR males and 14 FGR-MNR females with no sex differences evident for any of the select fetal population characteristics as assessed using analysis of variance. Accordingly, these data are presented for all males and females combined as a measure of the population variance and to allow for comparison with past studies. While all fetal weights in MNR pregnancies were decreased ~ 28% on average compared to Control pregnancies, FGR-MNR fetal weights were decreased by ~ 37% at 64 ± 2 gms compared to that of the AGA-Control fetuses at 101 ± 2 gms ($p<0.01$). FGR-MNR brain weights were also decreased, but less so, by ~ 12% at 2.39 ± 0.04 gms vs that of the AGA-Controls at 2.73 ± 0.05 gms ($p<0.01$) while FGR-MNR liver weights were markedly decreased by ~ 40% at 2.8 ± 0.1 gms vs that of the AGA-Controls at 4.7 ±0.2 gms ($p<0.01$). Accordingly, the brain/fetal weights and brain/liver weight ratio as measures of asymmetrical growth were increased 40-50%

in the FGR-MNR fetuses at 3.8 ± 0.1% and 0.90 ± 0.03 compared to that of the AGA-Control fetuses at 2.7 ± 0.1% and 0.61 ± 0.03, respectively (both $p<0.01$). FGR-MNR heart weights also showed a smaller decrease than the corresponding decrease in fetal weights, by ~ 23% at 0.46 ± 0.03 gms vs that of AGA-Controls at 0.60 ± 0.03 ($p<0.01$). As such, the heart/fetal weights was increased ~ 22% in the FGR-MNR fetuses at 0.72 ± 0.05% compared to that of the AGA-Controls at 0.59 ± 0.03% ($p<0.05$). It is also of note that the threshold of 80 gms here used at 60/61 days gestation for denoting AGA and FGR fetal weights, is close to the 10^{th} percentile for the population weight distribution of the 31 liveborn Control fetuses at ~ 78 gms and thereby in accord with the FGR definition often used for human pregnancies (Lackman 2001; Resnik 2014). Using this 80 gms threshold, 26 of the 31 Control fetuses or ~ 85% met weight criteria for AGA study, while 32 of the 42 MNR fetuses or ~ 75% met weight criteria for FGR study (Figure 3.1).

3.3.3 Blood Analytes

Blood glucose and hemoglobin values obtained by cardiac puncture at the time of full necropsy in the select AGA-Control and FGR-MNR animals are shown in Table 3.2. FGR-MNR glucose values were decreased by ~ 27% at 4.3 ± 0.2 mmol/l vs that of the AGA-Control values at 5.9 ± 0.4 mmol/l ($p<0.01$). Conversely, FGR-MNR hemoglobin values were increased by ~ 9% at 15.9 ± 0.2 gm/dl vs that of the AGA-Control values at 14.6 ± 0.4 gm/dl ($p<0.05$).

Table 3.1 Overall Maternal and Fetal Population Characteristics

	Control (9/31)*	MNR (12/42)*	Control (9/9)*	MNR (12/12)*
Maternal wt (gms)				
Conception	816±33	778±9		
GA 60/61	1253±60	1046±25‡		
Food (gm/day)				
Conception	38±2	28±1‡		
GA 60/61	54±4	37±1‡		
Litter size	3.4±0.3	3.5±0.3		
Total litter wt (gms)	331±30	242±21†		
Fetal wt (gms)	96±2	69±2‡	96±3	71±4‡
Placental wt (gms)	6.6±0.3	5.1±0.2‡	6.5±0.4	5.3±0.2‡
Placental/fetal wt (%)	6.8±0.3	7.6±0.3†	6.8±0.4	7.7±0.4
Crown rump length (cm)	12.3±0.2	10.6±0.1‡	12.3±0.2	10.8±0.2‡
Fetal wt /length (gm/cm)	7.8±0.1	6.4±0.1‡	7.8±0.2	6.5±0.2‡

Data presented as means ± SEM; † $p < 0.05$, ‡ $p < 0.01$ vs corresponding Control group value analyzed using non-paired Student's t-test, both for the overall populations and nested for litter size; * n values were 9 and 12 for maternal and 31 and 42 (overall populations) or 9 and 12 (nested) for fetal Control and Maternal Nutrient Restricted characteristics, respectively, except for litter size where demised fetuses were also counted; MNR = maternal nutrient restriction; GA = gestational age.

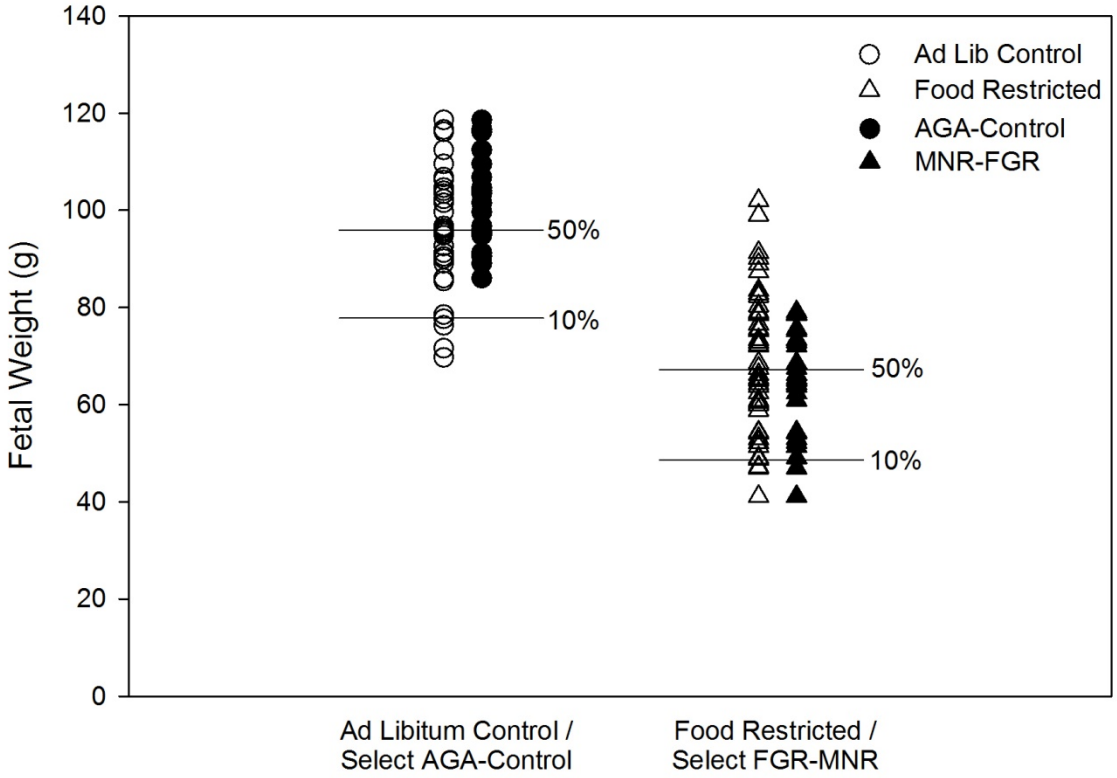

Figure 3.1 Scatter plot showing the fetal weights for all 31 liveborn Control fetuses (open circles) and all 42 liveborn maternal nutrient restricted (MNR) fetuses (open triangles) along with the 50th and 10th percentiles for each of these cohort populations. Additionally shown are the distribution of fetal weights for the select 20 AGA-Control fetuses (closed circles) and 25 FGR-MNR fetuses (closed triangles).

Table 3.2 Select Fetal Population Characteristics

	AGA-Control (20)*	FGR-MNR (25)*	AGA-Control (9)*	FGR-MNR (11)*
Fetal wt (gms)	101±2	64±2‡	99±2	65±3‡
Brain wt (gms)	2.73±0.05	2.39±0.04‡	2.72±0.06	2.42±0.05‡
Heart wt (gms)	0.60±0.03	0.46±0.03‡	0.59±0.04	0.47±0.03†
Liver wt (gms)	4.7±0.2	2.8±0.1‡	4.6±0.2	2.9±0.2‡
Brain/fetal wt (%)	2.7±0.1	3.8±0.1‡	2.8±0.1	3.8±0.1‡
Brain/liver wt ratio	0.61±0.03	0.90±0.03‡	0.61±0.03	0.89±0.05‡
Heart/fetal wt (%)	0.59±0.03	0.72±0.05†	0.60±0.04	0.73±0.07
Blood glucose (mmol/l)	5.9±0.4	4.3±0.2‡	5.9±0.5	4.4±0.3†
Blood Hb (gm/dl)	14.6±0.4	15.9±0.2†	14.6±0.4	15.8±0.3†

Data presented as means ± SEM; † $p < 0.05$, ‡ $p < 0.01$vs corresponding AGA-Control group value analyzed using non-paired Student's t-test, both for the select populations and nested for litter size; *n values were 20 and 25 (select populations) or 9 and 11 (nested) for fetal AGA-Control and FGR-MNR characteristics, respectively, except for blood glucose and hemoglobin where these were instead 15 and 19 (select populations) or 8 and 11 (nested), respectively; AGA = appropriate for gestational age, FGR = fetal growth restricted, MNR = maternal nutrient restricted, Hb = hemoglobin.

3.4 DISCUSSION

In the present study, we have characterized pregnancy outcomes in guinea pigs subjected to moderate nutrient restriction both before and through pregnancy as a useful model for inducing FGR with similarities to that seen in humans with maternal undernourishment and idiopathic placental insufficiency. Both Control and MNR sows had comparable fertility with successful pregnancies in ~ 80% of the animals, and requiring ~ 2.5 breeding attempts on average. Maternal age did not have an impact on pregnancy success, although no animals were bred prior to four months which is presumed adulthood for guinea pigs. However, only two animals, one control and one MNR, became pregnant with a conception weight less than 750 grams, suggesting an effect of maternal weight on pregnancy success which has also been noted in guinea pig studies with UAL (Detmer 1992). Since MNR animals took anywhere from 1 to 4 breeding attempts before becoming pregnant, the duration of preconception undernourishment was also impacted, and could be from 4 weeks up to 12 weeks. However, there was no evidence that an increased duration of MNR here impacted maternal weight at conception, litter size, fetal weights, or the risk of preterm delivery. As such, 4 weeks of moderate MNR prior to breeding in guinea pigs is sufficient for inducing FGR, while longer periods of moderate MNR as studied did not appear to worsen pregnancy outcomes. The fetal demise rate was low at 1 in 32 control fetuses and 1 in 43 MNR fetuses and much lower than that reported with uterine artery ligation (UAL) or ablation models, where demise rates upwards of 70 – 80% have been noted (Lafeber 1964; Turner 2009). This undoubtedly reflects the insidious nature of MNR-FGR with early and gradual growth restriction in response and matched to early nutritional deficiency (Godfrey 1998; Sohlstrom 1998; Redmer 2004; Belkacemi 2010; Roberts 2001) versus the relatively abrupt nature of UAL/ablation-FGR with normal fetal growth then a sudden reduction in placental blood flow and a variable mismatch between metabolic needs and nutrient delivery. However, 3 of 15 MNR sows delivered preterm and prior to the planned necropsy at 60/61 days gestation, with 2 of these mothers having the lowest maternal weights adjusted for gestational age and with low fetal weights at delivery. This finding has also been reported in guinea pigs by Kind *et al.*. (2003) with moderate MNR-FGR and by Palliser *et al.*. with uterine artery ablation-FGR and likely involves FGR associated increases in inflammatory processes with a shift in prostaglandin production over metabolism (Palliser 2014). In the present study the overall fetal loss rate whether from demise or preterm birth was therefore 1 of 32 or 3% for the control group and 11 of 53 or 20%

for the MNR group which is still considerably less than that reported for UAL/ablation models as noted. Additionally, some of the MNR fetuses delivering preterm were liveborn and did survive offering the opportunity for study of longer term outcomes with FGR and preterm birth which is also well known to occur in humans (Lackman 2001).

Moderate MNR at 70% of the *ad libitum* diet beginning at least 4 weeks pre-pregnancy and increasing to 90% of the *ad libitum* diet at mid-pregnancy, resulted in a decrease in maternal weights by ~ 5% and 17% at conception and 60/61 days gestation, respectively. This decrease in maternal weight is somewhat less than that previously reported by Sohlstrom *et al.* and Roberts *et al.* at ~ 10% and 28% using the same moderate MNR dietary regime, but their animals were smaller to begin with averaging 550 gms at mating and likely indicating strain differences to the guinea pigs presently used. Likewise, the actual decrease in food consumption at ~ 26% and 32% in MNR sows at conception and 60/61 days gestation, respectively, was somewhat less than that reported by Roberts *et al.* at ~ 37% and 36% in their MNR sows which may be attributable to their smaller animals. Interestingly, litter size was unchanged in the present study which differs from the findings of Sohlstrom *et al.* where moderate MNR decreased litter number from ~3 to 2 when necropsied at 60 days. This may again be attributable to their smaller animals and indicates that moderate MNR as outlined may adversely affect early embryonic/fetal developmental events leading to failure pending initial maternal weight and thereby fuel reserves for mobilization (Godfrey 1998; Heasman 1999; Belkacemi 2010; Kramer 1987; Abrams 1991). Fetal weights were decreased by 28% on average for all MNR pregnancies necropsied near term at 60/61 days gestation, which not surprisingly is less than that reported by Sohlstrom *et al.* at ~ 40% given their smaller animals. This again emphasizes the importance of maternal pre-pregnancy weight as a measure of nutritional reserve for fetal/placental growth and development when nutrient intake and thereby weight gain become compromised during pregnancy (Godfrey 1998; Heasman 1999; Redmer 2004; Belkacemi 2010; Kramer 1987; Abrams 1991). Of interest, the fetal weight variation in these animal cohort populations can be assessed using the coefficient of variation (CV) and calculated as the standard deviation divided by the population mean. In the present study and that of Sohlstrom *et al.*, this was comparable at ~ 12% for all fetal weights from control animals fed *ad libitum*, and somewhat less than that at ~ 18% for control fetuses in the untreated uterine horn of animals subjected to UAL/ablation and suggesting an impact of the surgical procedure here. In both the present and Sohlstrom studies, the CV for all fetal weights

from MNR animals was substantially increased at ~ 22%, and now similar to that in the treated horn of UAL/ablation animals (Turner 2009) and indicating an increase in growth variance with these animal models for inducing FGR. Placental weights were decreased by 23% on average for all MNR pregnancies and less than the corresponding decrease in fetal weights thereby resulting in an ~ 12% increase in the placental/fetal weight ratio which is similar to that noted by Sohlstrom *et al.* with moderate MNR (Sohlstrom 1998). Notably this increase in placental to bodyweight ratio is also seen in human pregnancies leading to FGR both with maternal undernourishment and presumed placental insufficiency and taken to indicate a degree of compensatory growth by the placenta to minimize FGR (Jansson 2007; Kingdom 1997; Godfrey 1998; Lumey 1998; Redmer 2004; Belkacemi 2010; Lackman 2001). Fetal crown rump lengths were also decreased in MNR pregnancies, but again less than the corresponding decrease in fetal weights resulting in an ~ 15% decrease in fetal weight/length and indicating leaner animals which was also noted by Kind *et al.* with moderate MNR in guinea pigs (Kind 2005). Likewise, leanness is often a characteristic in human infants with moderate growth restriction whether resulting from maternal undernourishment or idiopathic placental insufficiency (Godfrey 1998; Kramer 1990; Fall 1999).

We set a threshold of ≥ 80 gms or < 80 gms for categorizing AGA-Control and FGR-MNR fetal cohorts, respectively, which is in accord with the criteria we (Piorkowska 2014) and others (Jansson 1990) have used for categorizing AGA and FGR fetal weights in guinea pigs near-term. Of note, this threshold of 80 gms was close to the 10^{th} percentile for the population weight distribution of the liveborn Control fetuses at ~ 78 gms further justifying its use. We did not include the requirement for an increased brain/liver weight ratio for the FGR cohort as in other studies with UAL/ablation (Detmer 1992; Piorkowska 2014), since we did not want to presume how this would be impacted by MNR induced FGR. This establishment of AGA-Control and FGR-MNR cohort groups has the advantage of avoiding any confounding effects of tissue/metabolite study in AGA fetuses from MNR pregnancies and FGR fetuses from Control pregnancies; further reduces the fetal weight variation with the CV in these cohort groups now decreased to ~ 9% and 16%, respectively; and better reflects the human situation where AGA and FGR birth weight distributions are separate and often delineated by the 10^{th} percentile adjusted for gestational age (Resnik 2014; Lackman 2001). As expected, the decrease in FGR-MNR fetal weights vs the AGA-Controls at ~ 37% was considerably more than the decrease in

overall MNR fetal weights versus the overall Controls at ~ 28%. Moreover, growth restriction in MNR pregnancies was asymmetrical with the mean decrease in liver weights at ~ 40% much more than that of the brain and heart at ~ 12% and 23%, respectively. Accordingly, the brain/liver weight ratio was increased by almost 50% in FGR-MNR fetuses which was similar to that noted by Kind *et al.* in their MNR fetuses, while the heart/fetal weight ratio was increased by almost 25% in FGR-MNR fetuses (Kind 2005). This asymmetrical growth restriction is likely due in part to chronic blood flow redistribution favouring the vital organs, including the brain and heart at the expense of the liver and carcass tissues (Richardson 1989), and altered gluconeogenic capacity and/or protein synthesis with a greater impact on the liver and muscle than other tissues (Thorn 2009). Of note, asymmetrical FGR is also seen in guinea pigs with mid-gestation UAL/ablation (Lafeber 1984; Turner 2009; Piorkowska 2014), and in humans with placental insufficiency leading to growth restriction (Resnik 2014; Kramer 1987; Abrams 1991), with both of these likely to involve chronic fetal hypoxemia as a primary signaling mechanism (Lafeber 1984; Turner 2009; Lackman 2001). It is also of note that MNR induced FGR in sheep leads to ventricular hypertrophy which is thought to reflect increased afterload due to increased placental vascular resistance (Vonnahme 2003). Accordingly, the present increase in heart/fetal weight ratio in FGR-MNR fetuses may also indicate a degree of ventricular hypertrophy secondary to increased placental vascular resistance. This is in fact likely with the altered vascular development and structural changes reported in the placenta of guinea pigs subjected to moderate MNR (Roberts 2001).

Blood sampling at necropsy revealed FGR-MNR fetuses to be relatively polycythemic and hypoglycemic compared to AGA-Control fetuses. Likewise, an increase in hemoglobin and decrease in glucose are well associated with FGR in several animal models and human clinical studies. These include those in guinea pigs after UAL (Lafeber 1984; Jansson 1990) and sheep after MNR (Vonnahme 2003) or carunclectomy (Robinson 1979), and in human pregnancies with suspected placental insufficiency subjected to cordocentesis (Soothill 1987; Cox 1988; Economides 1989). These studies indicate that the basis for the hypoglycemia with FGR is likely multifactorial including lowered maternal glucose (Vonnahme 2003), reduced placental glucose transport and/or fetal glucose delivery (Robinson 1979; Lafeber 1984; Economides 1989), and reduced fetal gluconeogenesis (Jansson 1990), and dependent on the underlying etiology with maternal hypoglycemia likely to play a greater role with undernourishment. However, the basis

for the polycythemia with FGR is likely singularly due to stimulated erythropoiesis attempting to maintain oxygen carrying capacity in response to chronic hypoxemia as variably shown in these studies(Robinson 1979; Lafeber 1984; Soothill 1987; Cox 1988; Economides 1989).

Moderate MNR in guinea pigs has been well studied for modeling human FGR including maternal, placental, and fetal growth characteristics, associated IGF and IGFBP alterations, and mechanisms for programming longer-term adverse outcomes in offspring (Sohlstrom 1998; Roberts 2001; Kind 2003; Kind 2005). We now add to these findings further characterizing breeding and pregnancy success in MNR animals and showing low fetal demise rates in contrast to that seen with uterine ligation/ablation models (Lafeber 1984; Turner 2009), albeit with increased preterm delivery as is seen with FGR in humans (Lackman 2001). Comparative study to that using the same moderate MNR dietary regime and impact on maternal/fetal weights and litter size (Sohlstrom 1998; Roberts 2001) highlight the importance of maternal pre-pregnancy weight as a measure of nutritional reserve for fetal/placental growth when nutrient intake is compromised during pregnancy (Godfrey 1998; Heasman 1999; Redmer 2004; Belkacemi 2010; Kramer 1987; Abrams 1991). As previously shown (Sohlstrom 1998; Kind 2005), we confirm that MNR fetuses are leaner and have increased placental/fetal weight ratios as is often seen in human infants with moderate growth restriction whether resulting from maternal undernourishment or placental insufficiency (Kramer 1990; Godfrey 1998; Lumey 1998; Belkacemi 2010; Lackman 2001; Fall 1999). We also provide justification for using a fetal weight threshold for categorizing AGA-Control and FGR-MNR cohorts which ~ the 10^{th} percentile in our *ad libitum* fed animals, and serves to reduce the population variance in these groups. Of note, these FGR-MNR fetuses show asymmetrical growth restriction, and are polycythemic and hypoglycemic which are well associated with moderate growth restriction during human pregnancy (Kramer 1990; Resnk 2014; Kramer 1991; Abrams 1991; Soothill 1987; Cox 1988; Economides1989). These findings along with the altered vascular development and structural changes reported in the placenta of guinea pigs subjected to moderate MNR (Roberts 2001), also raise the possibility of chronic hypoxemia as a primary signaling mechanism for the decreased fetal growth in these pregnancies which requires further study. As such, the present and past studies of moderate MNR in guinea pigs (Sohlstrom 1998; Roberts 2001; Kind 2003; Kind 2005) support the utility of this model for inducing FGR with many similarities to that in humans with moderate growth restriction whether resulting from maternal

undernourishment or placental insufficiency. While there will be differences in FGR outcomes resulting from maternal undernourishment vs placental insufficiency including the impact on fetal gene expression (Nüsken 2011), it is likely that these will also depend on the timing, severity and duration of the nutrient deprivation as much as the cause (Heasman 1999; McMillen 2001).

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CHAPTER 4

MATERNAL NUTRIENT RESTRICTION IN GUINEA PIGS LEADS TO FETAL GROWTH RESTRICTION WITH EVIDENCE FOR CHRONIC HYPOXIA

4.1 INTRODUCTION

Fetal growth restriction (FGR) with infants small for their gestational age when born, is an important risk factor for later adverse health outcomes, including heart disease, diabetes, and neurodevelopmental disability, with the greatest risk observed in those with more severe and early-onsetting FGR (Barker 2004; Sung 1993). This has led to the notion that the intrauterine environment during fetal life can "programme" the development of risk factors for these later adverse outcomes and extensive animal-based study examining mechanisms underlying this relationship (Barker 2004; Armitage 2004; Fowden 2006).

Fetal growth is dependent on maternal nourishment and placental development which impact substrate availability for regulating fetal growth processes with maternal undernourishment and idiopathic placental insufficiency thereby primary causations for FGR (Jansson 2007; Resnik 2014). Clinical study of placental insufficiency-related FGR demonstrates aberrant placental vascularization and decreases in umbilical blood flow (Kingdom 1997; Ferrazzi 2000), leading to chronic fetal hypoxemia as a primary signaling mechanism for the degree of growth restriction (Soothill 1987; Lackman 2001). This has led to animal models of placental insufficiency induced by restricting placental blood flow, mostly through the latter part of pregnancy, and leading to FGR with variable hypoxemia and nutrient restriction (Jansson 1986; Murotsuki 1997; Turner 2009). Clinical study in populations subjected to food restriction (Lumey 1998) and in mothers deemed underweight (Godfrey 1998; Sebire 2001; Ehrenberg 2003) support the notion that maternal undernourishment is undoubtedly causative for FGR dependent upon severity and timing pre-conception and through pregnancy. This has led to animal models of maternal nutrient restriction (MNR) induced by global nutrient restriction or protein restriction, pre-conception, peri-conception, and through pregnancy, and leading to variable degrees of FGR (Jansson 2007; Sohlstrom 1998; Roberts 2001; Vonnahme 2003; Redmer 2004; Belkacemi 2010). MNR pre-conception continuing through pregnancy also parallels the human situation with moderate to severe FGR where intrauterine deprivation is likely to be early in onset (Sung 1993; Resnik 2014; Kingdom 1997). These studies in guinea pigs and sheep have generated placentas with reduced surface area for nutrient exchange, increased barrier thickness, and altered vascular development (Roberts 2001; Redmer 2004; Belkacemi 2010), which have also been reported in human FGR with maternal undernourishment (Aherne 1966). This should result in reduced

nutrient transport to the fetus which has been shown for glucose, amino acids, and lipids (Belkacemi 2010; Gaccioli 2013). However, whether fetal oxygenation is also limited as a signaling mechanism, or is even increased with decreased utilization, has yet to be determined.

Guinea pigs deliver precocial young after a relatively long pregnancy with many developmental events occurring during fetal life similar to that in humans (Carter 2007). Moderate MNR in guinea pigs at 70% of an ad libitum diet from 4 weeks pre-conception until mid-pregnancy increasing to 90% thereafter, has therefore been well studied for modeling human FGR and longer term outcomes (Sohlstrom 1998; Roberts 2001; Kind 2005). Here the capacity of the mother to deliver nutrients to the fetus is further impaired since her own fuel reserves are depleted prior to conception (Sohlstrom 1998; Roberts 2001) and reflects the human situation where pre-pregnant weight is a better determinant of fetal growth and development than weight gain during pregnancy (Stevens-Simon 1995). This study has shown fetal weights to be decreased as much as 40% in animals near term, but with placental weights less decreased (Sohlstrom 1998; Roberts 2001) suggesting compensatory growth as often seen in human FGR infants (Lackman 2001; Belkacemi 2010). Additionally, these MNR-FGR fetuses have asymmetrical organ growth (Kind 2005), and as outlined in Chapter 3, are polycythemic and hypoglycemic (Table 3.2) which are also well associated with FGR in humans (Soothill 1987). These findings along with the structural changes and altered vascular development in the placentas of these animals (Roberts 2001), also make it likely that chronic hypoxemia is occurring as a primary signaling mechanism for the decreased fetal growth in these pregnancies.

In the present study, we have determined the extent to which moderate MNR in guinea pigs as a causative factor for FGR also impacts markers for tissue hypoxia to test the hypothesis that these will be increased further implicating chronic hypoxia as a primary signaling mechanism here. Protein levels for erythropoietin (EPO) and its receptor (EPOR) have been assessed since these are up-regulated to promote erythropoiesis in response to systemic hypoxia, and protein levels for vascular endothelial growth factor (VEGF) have been assessed since this is up-regulated to promote angiogenesis in response to local tissue hypoxia (Semenza 2009). Immunoreactivity for hypoxyprobe-1 (HP-1), a pimonidazole hydrochloride that is reduced in hypoxic cells, has additionally been assessed as a widely used marker of tissue hypoxia (Raleigh 1999; Lee 2001). Moreover, these hypoxic markers have been assessed in the fetal liver and kidney as well as the

placenta, in both male and female fetuses, given the likelihood of tissue and/or sex specific changes with differences in production rates for these proteins and basal oxygenation.

4.2 MATERIALS AND METHODS\

4.2.1 Animal Cohorts and Tissue Collection

A previously established model of moderate MNR in guinea pigs (Sohlstrom 1998; Roberts 2001; Kind 2005) was used with all experimental procedures approved by The University of Western Ontario Animal Use Subcommittee and followed the guidelines of the Canadian Council on Animal Care. Animal feeding, breeding and pregnancy outcomes have previously been reported in Chapter 3. Briefly, nulliparous female guinea pigs (Dunkin-Hartley, from Charles River Laboratories, Sherbrooke, Que, Canada) were fed a guinea pig ration diet (Guinea Pig Diet 5025, LabDiet, St. Louis, MO) and after a two week period of acclimatization, daily food consumption was monitored and estrous cycles were tracked.

Guinea pig sows were assigned to either a Control group fed ad libitum or an MNR group fed 70% of the average food intake per kilogram of body weight of the ad libitum fed animals. After 4 weeks of adaptation to respective feeding regimens, animals were mated. During the first 34 days of pregnancy, the MNR animals continued at 70% average food intake of the Control animals per kilogram body weight, and from 35 days onward this was increased to 90% average food intake of the Control animals per kilogram body weight.

On day 60-61 of pregnancy (term = ~ 68 days), the hypoxia marker pimonidazole hydrochloride (Hypoxyprobe-1, 60 mg/kg, Chemicon, Temecula, CA) was injected intraperitoneally into Control and MNR sows and allowed to circulate for 90 minutes. Animals were then sedated followed by laparotomy and delivery of each of the fetuses. Body and placental weights were obtained from all liveborn fetuses along with crown rump length measurements. Fetuses were considered to be appropriate for gestational age (AGA) if $\geq$ 80 gms and FGR if < 80 gms, which is in accord with the criteria we (Piorkowska 2014) and others (Jansson 1990) have used for categorizing AGA and FGR fetal weights in guinea pigs near-term. Moreover, this threshold of 80 gms is close to the 10^{th} percentile for the population weight distribution of the liveborn Control fetuses at ~ 78 gms and thereby in accord with the FGR definition often used for human

pregnancies (Resnik 2014, Lackman 2001). Subsequently, only AGA fetuses from Control group litters and FGR fetuses from MNR group litters were subjected to full necropsy which consisted of an initial cardiac puncture to obtain ~ 1cc of blood for glucose and hemoglobin analysis using an ABL 725 Blood Gas Analyzer (Radiometer, Copenhagen, Denmark). This was followed by dissection and weighing of the brain, heart, liver and kidneys, extraction of the gonads for determining fetal sex, and extraction of skeletal muscle and peri-renal adipose tissue. These organs/tissues along with the placenta were partitioned and both frozen in liquid nitrogen and fixed in 4% paraformaldehyde for later analysis. Liver (right lobe), kidney and placental tissues were assessed for hypoxia markers with that for protein analysis stored at -80^{0}C until protein quantification, and that for histology kept in 4% paraformaldehyde for 72 hours, then washed in phosphate buffered saline (PBS) daily for 3 days, and then placed in 70% ethanol for 7-14 days, prior to blocking in paraffin. Tissue sections were subsequently cut at 5um on a rotary microtome and mounted on superfrost Plus slides (VWR Scientific, West Chester, PA).

Sixteen AGA-Control fetuses (8 male and 8 female) and 16 FGR-MNR fetuses (8 male and 8 female) were selected for hypoxic tissue analysis. These animals were representative of the mean fetal weights for their respective cohort groups and were from either the first or second medial position in the uterine horn, and with no more than one male and one female fetus from each litter.

4.2.2 EPO, EPOR and VEGF Protein Analysis

Approximately 30-50 mg of liver, kidney or placental tissue were homogenized with 10uL of RIPA buffer (50mM Tris-HCL, pH 7.4, 150mM NaCl, 1mM EDTA, 1% Nonidet P40, 0.25% C24H39Na04, supplemented with 1mM NaV, 50mM NaF, and 25nM C3H7O6PNa2.XH2O) per mg of kidney and placental tissue and 15uL of RIPA buffer per mg of liver tissue in the presence of a protease inhibitor (Roche, Mississauga, Ontario, Canada). Homogenates were sonicated using two pulses at 30% amplitude and centrifuged at 15000g for 10 minutes at 4^{0}C, with 300uL of the supernatant then extracted and retained as the total protein fraction. Equal amounts of the tissue extracts were normalized by colorimetric BCA Protein Assay (Pierce Corp., Madison, WI). Samples were stored at -20^{0}C until protein analysis.

The extracted protein samples were used to prepare loading samples for gel electrophoresis at a voltage of 180V in gradient NuPAGE 4-12% Bis-Tris polyacrylamide gels (Invitrogen Life Technologies Co., Burlington ON, Canada) for one hour. AGA-Control and FGR-MNR male samples were loaded and run together on one gel, while AGA-Control and FGR-MNR female samples were run together on a separate gel and each tissue analyzed was run on a separate gel. This was followed by a gel transfer onto a polyvinyliden difluoride membrane (Millipore, Etobicoe, ON, Canada) at a voltage of 100V for two hours. Following transfer, membranes were thoroughly washed and then blocked for two hours with 5% albumin in Tris-buffered saline containing 0.1% Tween (TBST). Membranes were then incubated overnight at 4^0C using primary antibodies specific for the protein targets, EPO (1:500, Novus Biologicals, Oakville ON, Canada), EPOR (1:500, Santa Cruz Biotechnology Inc. Dallas, TX) and VEGF (1:500 for liver and kidney; 1:300 for placenta, Santa Cruz Biotechnology Inc.) diluted in 5% milk 1xTris-buffered saline-Tween 20 buffer. The following day, membranes were washed with PBS and then probed for one hour at room temperature with Horseradish peroxidase conjugated donkey anti-rabbit IgG (1:10000, Jackson ImmunoResearch Laboratories, West Grove, PA), diluted in 5% milk. Immunoreactive bands were visualized using a Luminata Forte Western HRP enhanced chemiluminesence detection system (Thermo Scientific, Nepean, ON, Canada) and VersaDoc Imaging System (BioRad Laboratories, Mississauga, ON, Canada). Densitometry analysis of protein bands was performed using ImageLab with signal saturation software (BioRad Laboratories) normalized to the protein levels of β-actin as a loading control.

4.2.3 Hypoxyprobe 1 Immunohistochemistry

Hypoxyprobe-1 (HP-1) immunoreactivity was assessed by avidin-biotin complex enhanced immunohistochemistry (Vectastain; Vector Laboratories, Burlingame, CA). Tissue sections were deparaffinized in 3 sequential xylene baths for 5 minutes and immediately rehydrated in baths of 100% (2x2min), 90% (2x2min), and 70% (1x2min) ethanol and placed under running tap water for 5 minutes. After a PBS rinse, slides were incubated in boiling 10mM citrate buffer (pH 6.0) in a vegetable steamer for 20 minutes, followed by 5 minutes in boiling H_2O to enhance antigen target retrieval. After cooling, endogenous peroxidase was quenched by a 1% hydrogen peroxide bath for 10 minutes. Endogenous biotin activity was then blocked using an avidin and biotin kit

(Vector Laboratories) for 40 minutes followed by Background SNIPER (Biocare Medical, Concord, CA) for 5 minutes to reduce non-specific binding.

Tissue sections were incubated overnight with primary antibody, mouse-anti rabbit monoclonal hypoxyprobe-1, at 1:400 (liver and placenta) or 1:200 (kidney), (Hypoxyprobe Inc, Burlington, MA) diluted with Universal Antibody Dilutent (Dako Canada, Burlington, ON, Canada) at room temperature in a covered humidity chamber. The following day sections were rinsed and then incubated with secondary antibody (biotinylated goat anti-rabbit immunoglobulin G, 1:200; Vector Laboratories) at room temperature for 40 minutes in a covered humidity chamber and again rinsed. Sections were then incubated with an avidin-biotin complex (ABC) solution (avidin-biotin peroxidase complex 1:50, Vector Laboratories) at room temperature for 30 minutes in a covered humidity chamber. The bound antibody was visualized with Cardassian D.A.B. (3,3-diaminobenzadine) chromogen (Biocare Medical) at room temperature for 2 minutes. Sections were again rinsed with PBS and then counterstained by immersing in 1:2 Harris` Hematoxylin (Fisher Scientific, Ottawa, Ontario) for 30 seconds and immediately rinsing in running tap water until clear. Lastly, sections were rinsed with PBS and then dehydrated in alcohol baths of increasing concentration (70% 2x30 sec; 90% 2x1 min; 100% 2x3 min) followed by 3x5 minute rinses in xylene before being cover slipped using Permount. Slides were left 24 hours to dry in a fume hood before being visualized. All slides for the primary antibody were stained on the same day using the same solutions to minimize variation in intensity of stain. Negative controls included substitution of the primary antiserum with nonimmune serum to rule out nonspecific binding and confirmed absence of staining.

Hypoxyprobe-1 IR was imaged in 4 randomly selected high power fields (HPF, 20X magnification) from comparable areas in the liver, kidney and placenta for each of the AGA-Control and FGR-MNR fetuses using a Zeiss upright light microscope (Carl Zeiss Microimagin, Thornwood, NY). The percentage area of positive cell staining (brown staining) was determined using Image Pro Premier 9.2 software (Meyer Instruments, Houston, TX). To insure consistency and impartial evaluation, a threshold macro was set up for each of the three tissues examined based on testing of a random sample of images including screening against negative controls to select and count only cell bodies deemed to be positively stained. While a different threshold macro was thereby established for each of the three tissues, once set, the same macro was used

for each image of that tissue. The scoring of images for a given tissue was completed within the same day and all analyses were performed with the investigator blinded to the animal cohort grouping. Hypoxia intensity was expressed as the percentage area of positive cell staining meaned for the 4 HPFs for each of the 3 tissues examined for each of the AGA-Control and FGR-MNR fetuses.

4.2.4 Data Acquisition and Statistical Analysis

Overall Control and MNR Population characteristics included data from all Control sows and their liveborn fetuses, and all MNR sows and their liveborn fetuses undergoing necropsy at 60/61 days gestation. Select AGA-Control and FGR-MNR Population characteristics included data from all AGA-Control and FGR-MNR fetuses who were liveborn, met the weight criteria noted, and underwent full necropsy. Maternal and fetal characteristic findings, hypoxia-related protein levels, and HP-1 immunoreactvity are presented as group means ± SEM. Control and MNR Population characteristics, and AGA-Control and FGR-MNR Population characteristics, hypoxia-related protein levels, and HP-1 immunoreactvity were compared using non-paired student`s t-test (Graphpad Software, San Diego, CA). For all analysis, statistical significance was assumed for $p<0.05$.

4.3 RESULTS

4.3.1 Fetal Population Characteristics

As previously outlined in Chapter 3, nine control animals and 12 MNR animals had continuing pregnancies out to necropsy at 60/61 days gestation with 31 and 42 liveborn fetuses, respectively, which formed the overall Control and MNR Populations. The fetal growth characteristics from these animals are shown in Table 4.1 and have previously been reported in Chapter 3. Briefly, fetal weights were ~ 28% less in the MNR pregnancies than in the Control pregnancies ($p<0.001$). Placental weights were also decreased in the MNR pregnancies by ~ 23% ($p<0.01$), but less so than the fetal weights and accordingly the placental/fetal weight ratios were increased by ~ 12% ($p<0.05$). Fetal crown rump lengths were also decreased in the MNR

pregnancies by ~ 15% ($p<0.001$), but again less than the decrease in fetal weights such that fetal weight/length as a measure of leaness was also decreased by ~ 15% ($p<0.001$).

As noted, we used 80 gms as our threshold for categorizing AGA and FGR fetal weights and determining which Control and MNR fetuses were to be subjected to full necropsy. This resulted in 20 AGA-Control fetuses and 25 FGR-MNR fetuses with the growth characteristics from these select animals shown in Table 4.2, which have also been outlined in Chapter 3 results. Briefly, FGR-MNR fetal weights were decreased by ~ 37% compared to that of the AGA-Control fetuses ($p<0.001$). FGR-MNR brain weights were also decreased, but less so, by ~ 12% ($p<0.001$) while FGR-MNR liver weights were markedly decreased by ~ 40% ($p<0.001$). Accordingly, the brain/liver weight ratio as a measure of asymmetrical growth was increased almost 50% in the FGR-MNR fetuses compared to that of the AGA-Control fetuses ($p<0.001$). FGR-MNR glucose values were decreased by ~ 27% ($p<0.01$) while FGR-MNR hemoglobin values were increased by ~ 9% ($p<0.05$) vs that of the AGA-Control values.

4.3.2 EPO, EPOR, and VEGF Protein Levels

EPO, EPOR, and VEGF protein levels normalized to the protein levels of β-actin are presented as the mean change for that of the FGR-MNR fetuses from that of the AGA-Control fetuses. For all 16 FGR-MNR animals, EPO was increased in the liver ~ 1.5 fold, in the kidney ~ 1.6 fold, and in the placenta ~ 1.4 fold when compared to that of the 16 AGA-Control animals (all $p<0.01$) (Figure 4.1). However, this was largely due to even greater fold increases in the FGR-MNR females (all $p<0.01$ or $p<0.001$) with little change in the FGR-MNR males (all NS). EPOR showed no overall change for any of the tissues studied when assessed for all FGR-MNR animals (Figure 4.2). However, sex differences were again evident for the liver with FGR-MNR females showing an ~ 1.7 fold increase ($p<0.01$), while FGR-MNR males showed an ~ 50% decrease ($p<0.05$) from respective AGA-Controls. VEGF was increased in the liver ~ 1.5 fold ($p<0.05$) and in the placenta ~ 1.7 fold ($p<0.05$) when assessed for all FGR-MNR animals (Figure 4.3); but this again involved sex differences with that for the liver largely due to an even greater fold increase in FGR-MNR females ($p<0.01$) and that for the placenta largely due to an even greater fold increase in FGR-MNR males ($p<0.05$).

4.3.3 Hypoxyprobe-1 Immunoreactivity

Pimonidazole hydrochloride crosses the placenta and is reduced by nitroreductases in relatively hypoxic cells in the fetus ($pO2 < 10$ mmHg) to form covalent protein adducts which can then be detected immunohistochemically using the Hypoxyprobe-1 kit (Raleigh 1999; Lee 2001). Accordingly, immunostaining for Hypoxyprobe-1 can be used to assess differences in local tissue hypoxia as we have done in the present study in the fetal liver, kidney and placenta. Since the EPO, EPOR, and VEGF protein changes were primarily observed between the FGR-MNR and AGA-Control female groups, the Hypoxyprobe-1 study was limited to these animals.

Representative images are shown in Figure 4.4 and illustrate the presence of positive staining in both the FGR-MNR and AGA-Control animals for each of the liver, kidney and placenta, indicating some level of local tissue hypoxia even under normal physiologic conditions in the fetus. In the liver, Hypoxyprobe-1 staining was widely seen within the hepatocytes, but the percent area of positive cell staining as an index of local tissue hypoxia was increased in the FGR-MNR fetuses by ~ 3 fold compared to the AGA-Control fetuses ($p<0.05$)(Figure 4.4). In the kidney, Hypoxyprobe-1 was widely seen within the cortex in the proximal convoluted tubule although subjectively less so in the glomerulae, and within the medulla in the distal convoluted tubule, but with the percent area of positive cell staining increased in the FGR-MNR fetuses by ~ 4.5 fold ($p<0.01$)(Figure 4.4) In the placenta, image analysis was confined to the lobulated labyrinth region since this is the largest structural region near term and where nutrient and gas exchange primarily occur (Miglino 2004). Hypoxyprobe-1 was again widely seen, and subjectively moreso peripherally and less so centrally within the labyrinth lobules. However, in contrast to the liver and kidney there was no increase in the percent area of positive cell staining for Hypoxyprobe-1 in the placentas of the FGR-MNR fetuses as studied (Figure 4.4).

Table 4.1 Overall Fetal Population Characteristics

	Ad Libitum Control	Maternal Nutrient Restricted
Fetal weight (gms)	96±2	69±2***
Placental weight (gms)	6.6±0.3	5.1±0.2**
Placental/fetal weight ratio	0.068±0.003	0.076±0.003*
Crown rump length (cm)	12.3±0.2	10.6±0.1***
Fetal weight/length (gm/cm)	7.8±0.1	6.4±0.1***

Data presented as means ± SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs corresponding Control group value; n values were 31 and 42 for fetal Control and Maternal Nutrient Restricted characteristics, respectively; GA = gestational age.

Table 4.2 Select Fetal Population Characteristics

	AGA-Control	FGR-MNR
Fetal weight (gms)	101±2	64±2***
Brain weight (gms)	2.73±0.05	2.39±0.04***
Liver weight (gms)	4.7±0.2	2.8±0.1***
Brain/liver weight ratio	0.61±0.03	0.90±0.03***
Blood glucose (mmol/l)	5.9±0.4	4.3±0.2**
Blood hemoglobin (gm/dl)	14.6±0.4	15.9±0.2*

Data presented as means ± SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs corresponding AGA-Control group value; n values were 20 and 25 for fetal AGA-Control and FGR-MNR characteristics, respectively, except for blood glucose and hemoglobin where these were instead 15 and 19, respectively; AGA = appropriate for gestational age, FGR = fetal growth restricted, MNR = maternal nutrient restricted.

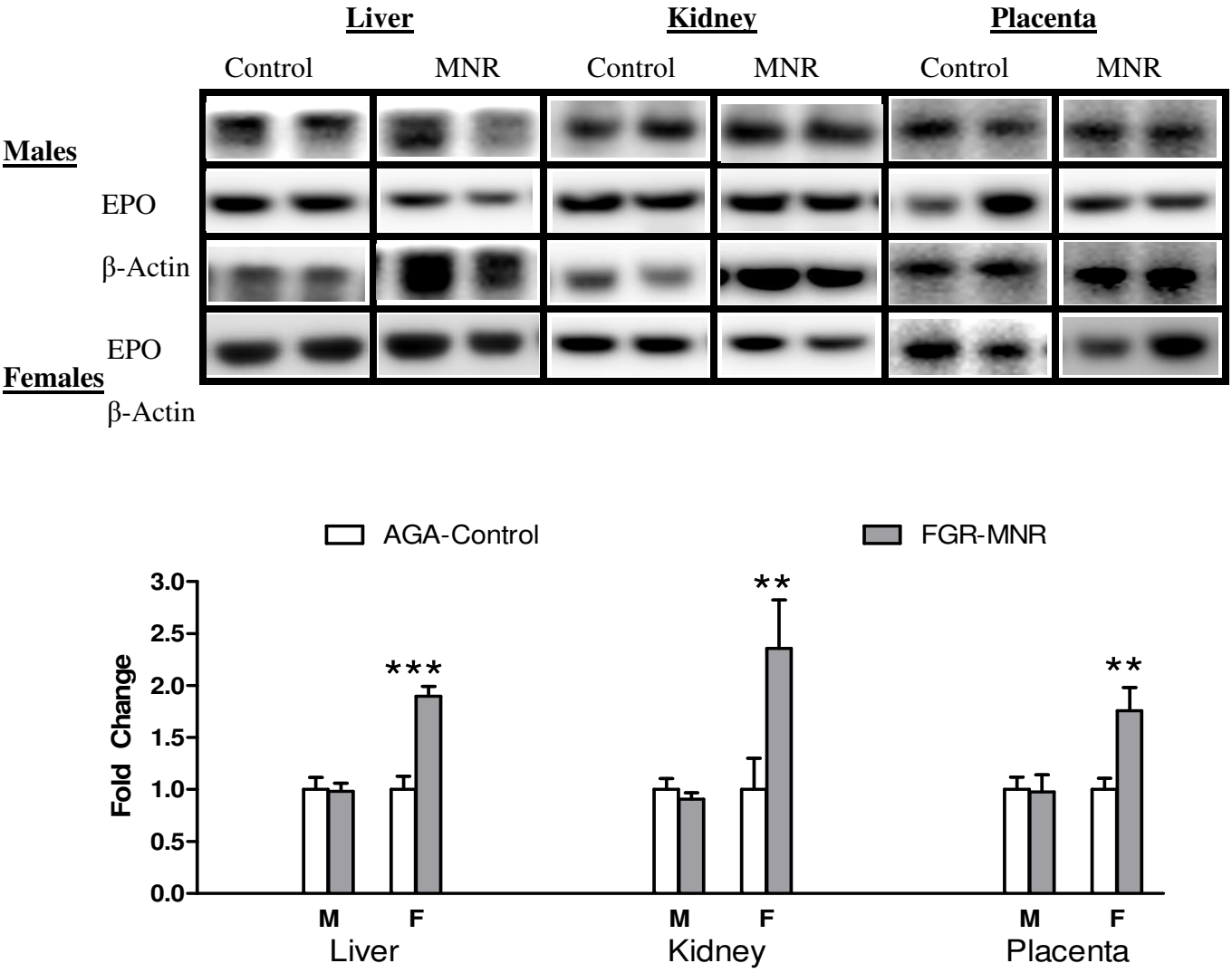

Figure 4.1 Western blot analysis of EPO from fetal guinea pig liver, kidney and placenta. Representative immunoblots (top) of EPO (28 kDa) and ß-actin (42 kDa) from male and female AGA-Control and FGR-MNR animals. Density of EPO (bottom) normalized to ß-actin and presented as the mean fold change ±SEM for FGR-MNR fetuses (grey bars, n=16) from that of the AGA-Control fetuses (open bars, n=16), separated for the male (M) (n= 8 + 8) and female (F) (n= 8 + 8) subgroupings. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; EPO=erythropoietin; MNR=maternal nutrient restriction; AGA=appropriate for gestational age; FGR=fetal growth restriction.

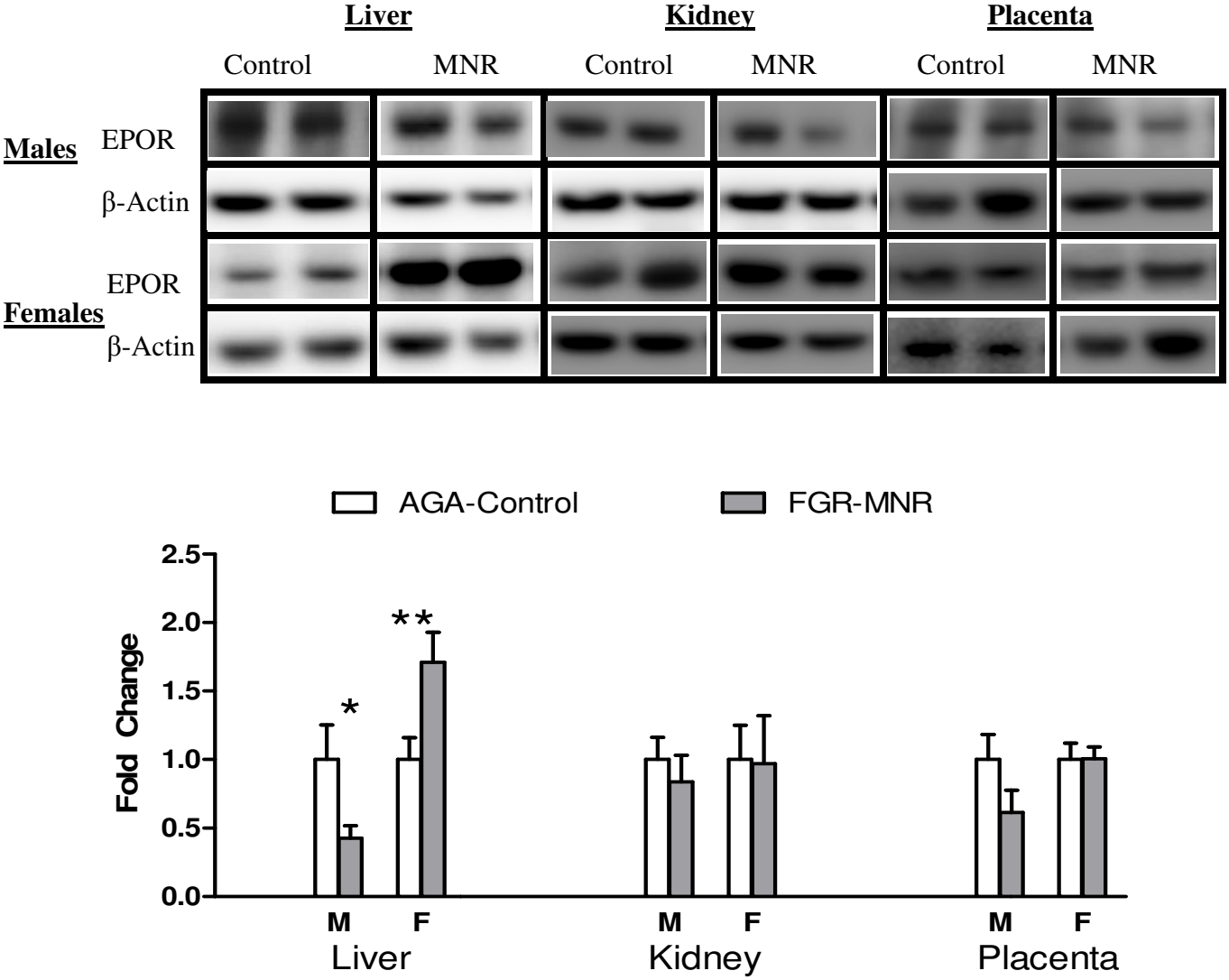

Figure 4.2 Western blot analysis of EPOR from fetal guinea pig liver, kidney and placenta. Representative immunoblots (top) of EPOR (55 kDa)and ß-actin (42 kDa) from male and female AGA-Control and FGR-MNR animals. Density of EPOR (bottom) normalized to ß-actin and presented as the mean fold change ±SEM for FGR-MNR fetuses (grey bars, n=16) from that of the AGA-Control fetuses (open bars, n=16), separated for the male (M) (n= 8 + 8) and female (F) (n= 8 + 8) subgroupings. *p<0.05, **p<0.01; EPOR=erythropoietin receptor; MNR=maternal nutrient restriction; AGA=appropriate for gestational age; FGR=fetal growth restriction.

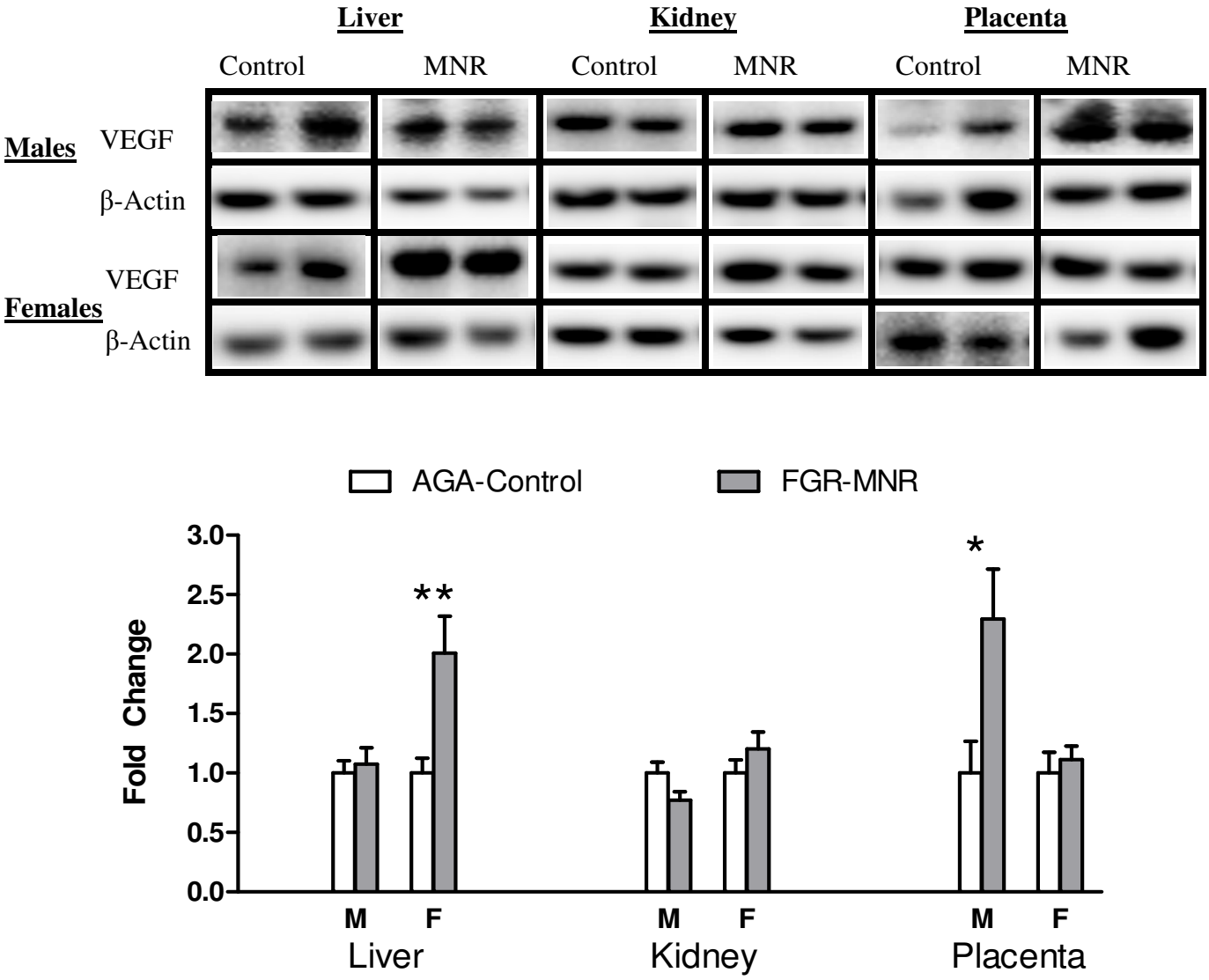

Figure 4.3 Western blot analysis of VEGF from fetal guinea pig liver, kidney and placenta. Representative immunoblots (top) of VEGF (42 kDa) and ß-actin (42 kDa) from male and female AGA-Control and FGR-MNR animals. Density of VEGF (bottom) normalized to ß-actin and presented as the mean fold change ±SEM for FGR-MNR fetuses (grey bars, n=16) from that of the AGA-Control fetuses (open bars, n=16), separated for the male (M) (n= 8 + 8) and female (F) (n= 8 + 8) subgroupings. *p<0.05, **p<0.01; VEGF=vascular endothelial growth factor; MNR=maternal nutrient restriction; AGA=appropriate for gestational age; FGR=fetal growth restriction.

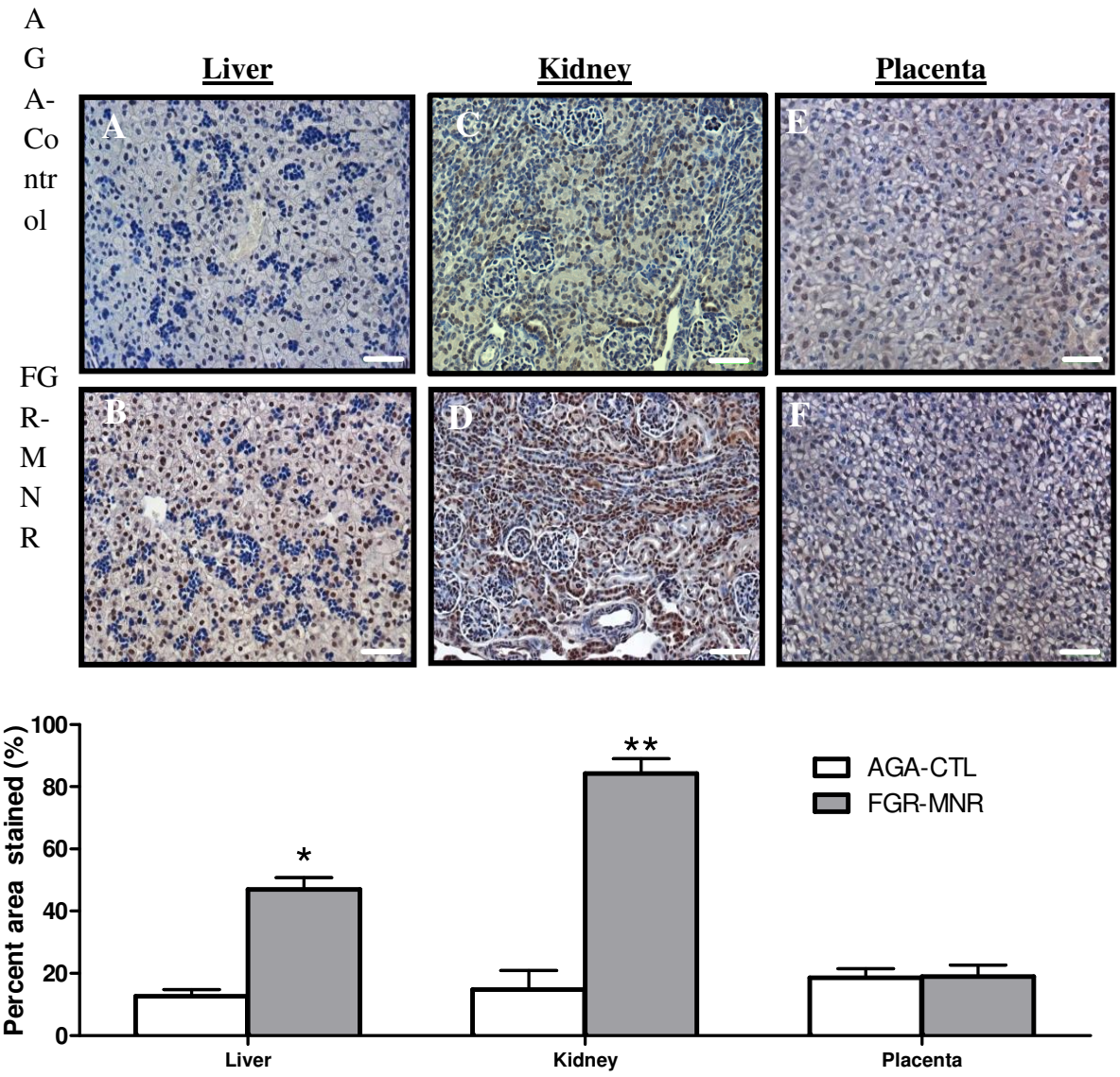

Figure 4.5 Hypoxyprobe-1 immunohistochemical staining and quantification in the fetal guinea pig. Representative photomicrographs illustrating positively stained cells for Hypoxyprobe-1 (brown) in the liver of an AGA-Control (A) and FGR-MNR (B) fetus showing the staining to be widely dispersed within the hepatocytes but increased in FGR-MNR fetuses; in the renal cortex of an AGA-Control (C) and FGR-MNR (D) fetus showing the staining moreso in the proximal convoluted tubule and increased in FGR-MNR fetuses; and in the labyrinthine zone of the placenta of an AGA-Control (E) and FGR-MNR (F) fetus showing the staining moreso peripherally than centrally within the labyrinthine lobules, but with no difference between FGR-MNR and AGA-Control fetuses. Sections were also counterstained with hematoxylin. Scale bar = 50µm. AGA=appropriate for gestational age; FGR=fetal growth restriction; MNR=maternal nutrient restriction.

4.4 DISCUSSION

In the present study, we have examined markers of tissue hypoxia in guinea pig fetuses subjected to moderate MNR both before and throughout pregnancy as a useful model for inducing FGR with similarities to that seen in humans with maternal undernourishment and idiopathic placental insufficiency. As outlined in Chapter 3, and as others (Sohlstrom 1998; Roberts 2001) have reported, moderate MNR at 70% of the ad libitum diet beginning at least 4 weeks pre-pregnancy and increasing to 90% of the ad libitum diet mid-pregnancy at day 35, results in a decrease in fetal weights by 30 to 40% on average for all MNR pregnancies necropsied near term at 60/61 days gestation. These fetuses have increased placental/fetal weight ratios as also seen in human pregnancies leading to FGR both with maternal undernourishment and presumed placental insufficiency and taken to indicate a degree of compensatory growth by the placenta to minimize FGR (Resnik 2014; Kramer 1987; Abrams 1991). These fetuses also have a decrease in fetal weight/length and indicating leaner animals. Likewise, leanness is often a characteristic in human infants with moderate growth restriction whether resulting from maternal undernourishment or idiopathic placental insufficiency (Godfrey 1998; Kramer 1990; Fall 1999).

We set a threshold of ≥ 80 gms or < 80 gms for categorizing AGA-Control and FGR-MNR fetal cohorts, respectively, which was close to the 10th percentile for the population weight distribution of the liveborn Control fetuses at ~ 78 gms. This establishment of AGA-Control and FGR-MNR cohort groups has the advantage of avoiding any confounding effects of tissue/metabolite study in AGA fetuses from MNR pregnancies and FGR fetuses from Control pregnancies and better reflects the human situation where AGA and FGR birth weight distributions are separate and often delineated by the 10th percentile adjusted for gestational age (Resnik 2014; Lackman 2001). These FGR-MNR fetuses show asymmetrical growth restriction with the mean decrease in liver weights much more than that of the brain and heart, and are polycythemic and hypoglycemic which are also well associated with moderate growth restriction during human pregnancy (Lafeber 1984; Turner 2009; Lackman 2001). Of note, asymmetrical FGR and polycythemia and/or hypoglycemia are also seen in guinea pigs with mid-gestation uterine artery ligation/ablation (Lafeber 1984; Jansson 1990) and in humans with placental insufficiency leading to growth restriction (Soothill 1987; Cox 1988; Economides 1989), with both of these likely to involve chronic fetal hypoxemia as a primary signaling mechanism. These

findings along with the altered vascular development and structural changes reported in the placenta of guinea pigs subjected to moderate MNR (Sohlstrom 1998; Roberts 2001) also raise the possibility of chronic hypoxemia as a primary signaling mechanism for the decreased fetal growth in these pregnancies.

Erythropoietin or EPO, is a glycoprotein hormone that binds to its cognate receptor on erythroid progenitor cells stimulating their survival and differentiation, with the rate of red blood cell production thereby normally determined by blood/tissue EPO levels (Semenza 2009; Eckardt 2005, Wardrop 1996). EPO synthesis is inversely related to oxygen availability with the main determinant the transcriptional activity of its gene by hypoxia-inducible factor 1 in tissues which is related to local oxygen tensions (Eckardt 2005, Wardrop 199641,42). During fetal development, EPO synthesis gradually changes from hepatocytes stimulating erythropoiesis in the liver in a paracrine manner, to renal EPO-producing cells in the cortex and outer medulla stimulating erythropoiesis in the bone marrow in an endocrine manner, with the timing for this transition species dependent (Wardrop 1996; Teramo 2009; Kowalska-Kańka 2013; Suzuki 2015) 42-45). However, secretion of small amounts of EPO have also been identified in other fetal tissues and for the placenta this can become substantial in response to fetal hypoxemia (Teramo 2009; Kowalska-Kańka 2013). Cord blood/amniotic fluid EPO levels and erythroblastosis have been well studied in high-risk pregnancies where fetal hypoxemia is suspected including FGR, and shown to be increased as an adaptive response and variably relating to the degree of hypoxemia (Teramo 2009; Snijders 1993) 43,44,46). In the present study, the female FGR-MNR fetuses exhibited increased EPO levels in the three tissues studied, namely the liver, kidney and placenta. To our knowledge this is the first study of tissue EPO levels in the guinea pig fetus and indicates that all three of these tissues contribute to the production of EPO near term. Moreover, the increase in EPO levels as a measure of related gene activity is in keeping with a degree of local hypoxia in these tissues supporting our conjecture of relative fetal hypoxemia in FGR-MNR fetuses. Surprisingly, this increase in EPO levels was confined to the female FGR-MNR fetuses where tissue levels were similarly increased ~ 2 fold, while that for the male FGR-MNR fetuses showed little change, although both males and females showed similar increases in their hemoglobin values. Mechanisms underlying these differences are not readily apparent and Clemons *et al.* found no sexual dimorphism in the EPO response of fetal and neonatal rats to induced hypoxia (Clemons 1986). However, there are

known to be sex-related differences in physiologic responses to hypoxia with females showing better adaptation to hypoxia than males, which includes the ventilatory and erythropoietic system responses to hypoxia which are tightly linked in females but not in males (Soliz 2012 48). Additionally, the kinetics of EPO synthesis are relatively insensitive to the degree of hypoxia due to the "sigmoidal-like" binding curve by the sensor for O2, and the complex relationship between blood PO2 and tissue PO2 which is dependent on a number of factors including blood flow distribution, blood volume, hemoglobin concentration, hemoglobin-O2 affinity, and O2 consumption (Wardrop 1996 42). While these different physiologic processes are interrelated, they result in individual variation in the relationship between EPO synthesis, tissue oxygenation and hemoglobin levels, which could also have a sex-related bias contributing to the present EPO findings.

EPOR is a cell surface transmembrane protein to which EPO must bind in order to achieve signal transduction in both erythroid and non-erythroid tissues (Beleslin-Cokic 2004; David 2006). As such, EPOR is found in both the liver and bone marrow during fetal development in relation to the ontogeny for erythropoiesis (David 2006; David 2002). However, EPO has also been reported to have non-hematologic functions including the promotion of angiogenesis and cellular proliferation/differentiation, as well as cytoprotection (Beleslin-Cokic 2004; Kowalska-Kańka 2013 44,49). Accordingly, EPOR has been found in other fetal tissues including the kidney and placenta (Kowalska-Kańka 2013; David 200244,51). While the signaling for EPOR expression in tissues may variably involve HIF-1, nitric oxide, or even EPO itself, a primary stimulus for all of these will still be tissue hypoxia (Beleslin-Cokic 2004; Cokic 2014). Similar to the EPO findings, EPOR levels were increased ~ 1.5 fold in female FGR-MNR liver tissues compared to that of the female AGA-Controls consistent with some degree of local hypoxic stimulus and the adaptive need for increased erythropoiesis. However, there was no evident change in EPOR levels in the kidney or placenta for either the male or female FGR-MNR fetuses suggesting that non-hematologic EPO-EPOR responses to not occur with chronic hypoxemia, at least for the kidney and placenta. Surprisingly, EPOR levels were actually decreased in the male FGR-MNR liver tissues indicating sexual dimorphism in this response which may relate to the same issues discussed for the dimorphic EPO response or possibly a direct facilitative/inhibitory impact of sex hormones on this response.

Vascular endothelial growth factor or VEGF includes a family of glycoprotein hormones of which VEGF-A is the most abundant, which bind to cognate receptors on endothelial progenitor and mesenchymal stem cells and on vascular endothelial and smooth muscle cells, thereby stimulating vasculogenesis and angiogenesis (Semenza 2009; Rehn 2014,53). As such, VEGF plays a prominent role during fetal development as a major regulator of vascular development and has been reported on in several fetal tissues including the liver, kidney and placenta (Rehn 2013; Baserga 2009; Rehn 201453-55). Although the expression of VEGF is influenced by several factors including other growth factors, transcriptional factors and steroidogenic activity, a major stimulus for the upregulation of VEGF is local tissue hypoxia mediated by HIF-1 (Semenza 2009; Rehn 2014; Shwelki 1993; Trollmann 2003)29,53,56,57). Similar to the EPO and EPOR findings, VEGF levels were increased in female FGR-MNR liver tissues compared to that of the female AGA-Controls and again consistent with some degree of local hypoxic stimulus. However, VEGF levels in the kidney and placenta of the female FGR-MNR fetuses remained unchanged suggesting that the increased liver values might somehow relate to increased erythropoiesis here, rather than as an angiogenic stimulus. It is thus of note that in mice erythropoiesis in the fetal liver is impaired when hypoxic induction of VEGF is lacking and indicating a role for VEGF with erythropoiesis in the fetal liver (Rehn 2014). The unchanged VEGF levels in the liver and kidney of the male FGR-MNR fetuses likewise support a VEGF-erythropoiesis linkage rather than an angiogenic linkage. Here, the absence of change in the liver might be due to an earlier transition in erythropoeisis from the liver to the bone marrow in FGR males which might also explain the lower EPOR levels seen here. However, VEGF levels were increased more than two-fold in male FGR-MNR placentas and likely now as an angiogenic response to tissue hypoxia. While the mechanisms underlying this differential response from that of the female FGR-MNR fetuses remain to be determined, altered vascular development and increased VEGF expression are well described in human FGR placentas where chronic hypoxemia is known to be a contributing factor (Economides 1989; Trollmann 2003; Bosco 2010).

Pimonidazole, a 2-nitroimidazole compound, is reduced at low oxygen concentrations forming covalent protein adducts and binding to thiol-containing molecules that can then be detected immunohistochemically as markers for intracellular hypoxia (Raleigh 1999; Lee 2001). Accordingly, Hypoxyprobe-1 or pimonidazole hydrochloride and associated antibodies that bind

to pimonidazole adducts in hypoxic tissues, has been widely used as a hypoxia marker including study in fetal and placental tissues (Oh 2008; Kulandavelu 2013; Schaffer 2006 59-61). In the present study, Hypoxyprobe-1 staining was seen in the AGA-Control animals for each of the liver, kidney and placenta, indicating some level of local tissue hypoxia even under normal physiologic conditions in the fetus which was also noted by Oh *et al.* in near term fetal guinea pigs (Oh 2008). This is to be expected as oxygen consumption in cells close to blood vessels creates oxygen gradients for more distal cells, which is then amplified by the lower partial pressure for oxygen normally seen during fetal development. Hypoxyprobe-1 staining in the liver was widely seen within the hepatocytes, while in the kidney this was subjectively less in the glomeruli than the surrounding proximal convoluted tubule area. This is consistent with renal blood flow initially to the glomeruli and thereby better oxygenation, prior to passing through the surrounding cortical tissue as reported for the adult kidney (Suzuki 2015). Hypoxyprobe-1 was substantially increased in both the liver and kidney tissues of the female FGR-MNR fetuses indicating lower levels of oxygenation in these tissues relative to that of the female AGA-Control fetuses. Of note, chronic exposure to lower environmental oxygen in pregnant guinea pigs and leading to FGR, has also been shown to increase Hypoxyprobe-1 staining in fetal livers attributable to relative tissue hypoxia (Oh 2008). Hypoxyprobe-1 staining in the placenta was again widely seen, but subjectively moreso peripherally and less so centrally within the labyrinth lobules. This is then consistent with the countercurrent blood flow described for the guinea pig placenta whereby maternal blood flows from arterial channels at the centre of the lobule to venous channels in the interlobium, while fetal blood flows from fetal arteries in the interlobium to fetal veins at the center of the lobule, with the centre lobule tissue thereby better oxygenated (Miglino 2004). Despite the substantial increases in Hypoxyprobe-1 staining in the liver and kidney of the FGR-MNR fetuses, there was no evident change in the placental tissues of these animals. Since utero-placental blood flow supplies oxygen to the placenta prior to the fetus, placental tissue will normally be better oxygenated than fetal tissue (Kingdom 1997). This may be further exaggerated under hypoxic conditions by nitric-oxide mediated vasodilation within the placenta`s central region as a mechanism to maintain oxygen homeostasis, albeit at the expense of fetal oxygenation (Kulandavelu 2013; Schaffer 2006 61,62). As such, it is likely that tissue PO2 values in the FGR-MNR placentas were either unchanged or not lowered sufficiently for

differences to be detectable using Hypoxyprobe-1 where marked increases in staining are only seen with PO2 values < 10 mmHg (Raleigh 1999; Lee 2001).

The present study examined the impact of MNR on FGR and the resulting levels of tissue hypoxia in FGR-MNR guinea pigs. There was a significant increase in the amount of HP-1 immunostaining as a direct measure of lowered oxygen in FGR-MNR fetuses compared to their AGA-CTL counterparts in the fetal kidney and liver. Additionally, FGR-MNR animals showed a significant increase in EPO protein levels across all three tissues examined. Together, these findings suggest that FGR resulting from MNR leads to a degree of hypoxia in the guinea pig fetus. Furthermore, the fetal liver also displayed a significant increase in EPOR and VEGF protein levels, suggesting an increase in erythropoetic and angiogenic factors resulting from blood redistribution, and suggesting that the liver may be undergoing the most adaptive and protective mechanisms to allow for proper function in the postnatal environment. These results may be explained in part by a greater degree of growth restriction in the liver and therefore a greater compensation by the fetus to redirect blood flow to increase nutrient exchange. Many of these changes were seen in a sex dependent manner, with females showing greater increases than males, therefore underlying mechanisms of these differences have not been fully investigated. It should be of continuing interest to examine why females and males undergo different degrees of response with respect to MNR and chronic hypoxia. In keeping with an increase hemoglobin levels, this provides further evidence for altered oxygen distribution due to maternal undernourishment. Overall, these findings implicate chronic hypoxia as a mediator of growth restriction and altered development arising from MNR.

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CHAPTER 5

MATERNAL NUTRIENT RESTRICTION IN GUINEA PIGS ALTERS OXIDATIVE STRESS AND AN ANTIOXIDANT RESPONSE

5.1 INTRODUCTION

A variety of maternal, placental, and fetal factors can lead to fetal growth restriction (FGR), where fetuses do not meet their full growth-potential. Placental insufficiency is the major cause of FGR in developed countries (Jansson 2007). In these cases, aberrant placental vascularization results in fetal hypoxemia, which signals the fetus to restrict its growth in response to the decreased nutrient transport (Ferrazzi 2000; Cox 1988). This resulted in many animal models that mimic the human FGR condition, using a variety of methodology, including uterine artery ligation (UAL), placental embolization, or hypoxia, which all decrease the transport of oxygen to the fetus (Kingdom 1997; Jansson 1986). Furthermore, animal models and human cases of FGR have elevated makers of oxidative stress in both fetal and placental tissues (Hracsko 2008; Oh 2008; Evans 2012), which may trigger fetal programming (Luo 2006; Thompson 2012). Although these animal models mimic several of the physiological parameters of human FGR with placental insufficiency, they insult pregnancy with a one time, acute stressor, which prevents the direct translation to the human condition of placental insufficiency seen constant throughout pregnancy,

Maternal undernutrition plays a more prominent role in FGR pregnancies in developing countries (Walker 2007). Maternal nutrient restriction (MNR) pregnancies show similar pathophysiological characteristics as placental insufficiency and hypoxic pregnancies, such as decreased placental vascularization and decreased oxygen transport (Redmer 2004). Although not as extensively studied as other FGR animal models, MNR animal models may offer more insight into chronic gestational insults that cannot be extrapolated from acute hypoxia or blood flow occlusion models since MNR is a chronic *in utero* stress similar to that with placental insufficiency. Studies in guinea pigs have already shown the adverse effects of MNR on placental development leading to "placental insufficiency" (Roberts 2001), and recently, as described in Chapters 3 and 4, MNR gives rise to severe asymmetrical FGR (aFGR) with brain and heart sparing, and fetal and placental tissues show evidence for chronic hypoxia. Together these results implicate fetal hypoxemia as a further mechanism of injury from maternal undernourishment. Of interest, Roberts (2001) reported placentas with reduced surface area, increased barrier thickness, and aberrant vascular development in pregnant guinea pigs subject to a restricted diet. This has led to the hypothesis that the delivery of nutrients in these

underdeveloped placentas is decreased as a function of the smaller area for substrate exchange along with the increased diffusional distance. Moreover, human cases of MNR-induced FGR have reported an increase in markers of oxidative stress. In particular, Gupta *et al.*. (2004) showed increased oxidative stress in FGR fetuses born to undernourished mothers through the measure of superoxide dismutase, catalase, glutathione (GSH), and malondialdehyde (MDA) in cord blood. The main objective of this study focuses on the unknown effects of MNR on markers of oxidative stress in FGR fetuses.

Previously, hypoxic and blood occlusion animal models resulting in FGR have demonstrated that FGR fetuses become hypoxemic and undergo significant oxidative stress due to decreased oxygen transport from the placenta. In particular, prenatal hypoxia in pregnant guinea pigs leads to increases in MDA, total GSH, and □-glutamyl cysteine synthetase (γ-GCS) (Hasimoto 2012; Oh 2008). Further, clinical studies have collected placental and cord blood and determined that fetal hypoxemia and oxidative stress are present in FGR pregnancies (Hracsko 2008; Gupta 2004). The present study will use GSH/GSSG and MDA as markers of oxidative stress in the MNR guinea pig model to assess both the antioxidant state of the cell and the subsequent oxidative stress-induced lipid damage. To further understand the extent to which oxidative stress is occurring as a result of MNR in the fetal guinea pig, this study will assess peroxiredoxin antioxidant levels and would expect them to be increased in the FGR-MNR animals compared to their AGA-CTL counterparts.

The guinea pig has been frequently used as an animal model to study human pregnancy and the pathophysiological effects of gestational stress. The guinea pig is subject to a longer gestation (68 days) than other rodents, which allows for a more thorough examination of chronic *in utero* stresses (Dyson 2012). It also possesses a placenta similar to the human's placenta in terms of the single layer of trophoblasts that separate maternal and fetal blood (Roberts 2001). Lastly, similar to human perinatal brain development, guinea pigs are prenatal developers born with full brain function. This is in contrast to other post-natal developer rodents, whose central nervous system development reaches completion postpartum (Dyson 2012). This distinction is important in studying the effects of fetal development in response to gestational insults, such as MNR and oxidative stress.

Following these findings, it is of importance to determine if the MNR guinea pig model also demonstrates similar increases in markers of oxidative stress, further validating the MNR model as an indicator of placental insufficiency seen with human FGR. The present study tested the hypothesis that MNR in near term fetal guinea pigs will result in tissue specific oxidative stress as a mechanism for damage from MNR induced placental insufficiency, with an increase in oxidative stress makers and antioxidant levels, compared to AGA (appropriate for gestational age)-CTL animals. Identifying MNR induces oxidative stress in a tissue-specific manner will further our understanding of fetal development and associated organ-specific sparing effects that occur during FGR. This will also underline the importance of developing preventative antioxidant treatments for identified MNR/FGR human pregnancies.

5.2 MATERIALS AND METHODS

5.2.1 Animals Cohorts and Tissue Collection

Guinea pig sows were subjected to either the control or MNR diet regime as outlined in Chapter 3. At 60 days gestation pregnant guinea pigs underwent a laproectomy as previously described in Chapter 3. The fetuses were delivered and body weight and placental weights were obtained for all fetuses. All fetuses that met weight characteristics as previously described (Chapter 3) underwent a full necropsy, where sections from liver, kidney and placenta tissues were both fixed in 4% paraformaldehyde and frozen in liquid nitrogen. During necropsies, guinea pig sex was determined by anatomical analysis of the reproductive organs, for presence or absence of testis.

Briefly, and as extensively outlined in Chapter 3, select animals were chosen for protein and histological analysis based on sex and position in the uterine horn, in order to be consistent for growth factors related to position in horn. In this study, 16 AGA-CTL animals (8 males and 8 females) that were from either the first or second medial position in the uterine horn and 16 FGR-MNR animals (8 males and 8 females) that were from the first or second most medial position in the horn were used. For all subsequent analysis, AGA-CTL males were compared

only to FGR-MNR males and similar analysis was performed for female fetuses. Tissues were stored in -80^{0}C freezer until used for glutathione, malondialdehyde and western blot analysis.

5.2.2 Quantification of Glutathione

Placenta, along with fetal liver and kidney total GSH and oxidized GSSG were measured using a commercially available colorimetric assay kit (Cayman Chemical Company, MI). Frozen tissue samples were homogenized with 10 mL cold buffer per gm of tissue (50mM MES, 1mM EDTA, pH 6.7). Samples were then centrifuged at 10 000g for 15 minutes at 4^{0}C and supernatant separated in two two aliquots; one for protein quantification and the other for deproteniziation and GSH/GSSG measurement. Protein concentrations were quantified for each sample using a BCA protein assay kit (Pierce Crop, WI).

Supernatant to be deproteinzied was mixed with equal volumes of metaphosphoric acid (5g /50 ml water) then centrifuged at 2000g for 3 minutes. Resulting supernatant was exctracted and was frozen at -20^{0}C until ready for GSH quantification. For total GSH analysis, all samples were diluted to 20x in MES buffer. For GSSG quantification, liver samples were diluted to 20x and kidney and liver samples were diluted 11x in MES. All samples were prepared with 50 µl triethanolamine reagent (531 µl triethanolamine and 469 µl water) per ml of supernatant. AGA-CTL and FGR-MNR males and females were plated in duplicates on a single 96-well plate and measured at 405 nm wavelength.

To exclusively measure just the oxidized form of GSH (GSSG), 10 µl of 1M 2-vinylpyridine solution per ml of sample was added to each well. Samples were incubated in the dark at room temperature for 60 minutes prior to colorimetric quantification. GSH was calculated by taking GSSG from the total GSH. Reduced GSH and oxidized GSSG levels were normalized to protein concentrations. Data is presented as total mg for total GSH and as a ratio of reduced GSH/oxidized GSSG.

5.2.3 Quantification of Malondialdehyde

Extracted frozen guinea pig placentas and fetal liver and kidney tissues were homogenized with 10 µL per mg tissue (placenta and kidney) or 15µL per mg tissue (liver) RIPA buffer (50mM Tris-HCL, pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Nonidet P40, 0.25% C24H39Na04, supplemented with 1mM NaV, 50mM NaF, and 25nM C3H7O6PNa2.XH2O) in the presence of an added protease inhibitor (Roche, Mississauga, Ontario, Canada). Homogenized samples were sonicated and then centrifuged at 10 000g for 20 minutes at 4^0C . Supernatant was extracted and stored at -20 C until quantification.

Quantification of MDA was carried out using the commercially available thiobarbituric acid reactive substances (TBARS) assay (Cayman Chemical Company, Ann Arbor, MI), and was plated in duplicate. All samples were normalized to mg protein (as previously described by BCA protein assay). Samples and MDA standards were heated with colour reagents (thiobarbituric acid, acetic acid, and sodium hydroxide) and sodium dodecyl sulfate in boiling water for 60 minutes, removed and immediately placed on ice for 10 seconds, centrifuged and pipetted into a 96 well plate. MDA levels were measured at 530 nm.

5.2.4 Peroxiredoxin Protein Analysis

The extracted protein samples from the fetal and placental tissues were used to prepare loading samples along with NuPAGE SDS Sample Buffer (4x), Reducing Agent (10x), and dionized water. These samples were heated at 70^0C for 10 minutes to allow for protein denaturation. Gel electrophoresis at a voltage of 180V was used to fractionate proteins in NuPage, 4-12% gradient polyacrylamide gels (Invitrogen Life Technologies Co., Burlington ON, Canada) for 1 hour. This was followed by a gel transfer onto polyvinyliden difuoride membrane (Millipore, Etobicoe, ON, Canada) at a voltage of 100V for 2 hours.

Immunoblotting was performed using primary antibodies specific peroxiredoxin 1 (1:2000; (Lab-Frontier, Seoul, Korea) and diluted in TBST buffer. Blots were subsequently probed with secondary antibody Horseradish peroxidase conjugated donkey anti-rabbit IgG (1:10000, Jackson ImmunoResearch Laboratories, West Grove, PA), diluted in 5% milk. Immunoreactive

bands were visualized using an Luminata Forte Western HRP enhanced chemilumnesnsece detection system (Thermo Scientific, MA, USA) and VersaDoc Imaging System (BioRad Laboratories, Mississauga, ON, Canada). Densitometry analysis of bands was performed using ImageLab, with signal saturation software (BioRad Laboratories) and normalized to the protein levels of β-actin as a loading control.

5.2.5 Data Acquisition and Statistical Analysis

Data is reported as mean ± standard error of the mean (SEM). Western blot and population data was analyzed using Student's T-test. Oxidative stress marker data was analysed using 2-way ANOVA followed by Tukey's HSD post-hoc test ($p < 0.05$) to compare means within groups. All statistical analysis was run using Graphpad Prism software (Graphpad Software, California, USA).

5.3 RESULTS

5.3.1 Impaired Fetal Growth Measures and Organ Development

As outlined in Chapter 4, and shown in Table 4.1, MNR resulted in a significant decrease in maternal ($p<0.01$) and fetal weight ($p<0.001$) at necropsy. MNR fetuses were 28% lighter (96±2.3 g) than control fetuses (69.1±2.3 g). Placental weight also decreased significantly in response to MNR ($p<0.01$), but relative placental/fetal weight was increased significantly in MNR pups ($p<0.05$). Ponderal index increased significantly in MNR fetuses ($p<0.01$), indicating increased leanness as a measure of weight/height^3.

As outlined in Chapter 4, and shown in Table 4.2, both fetal brain (2.73±0.05 vs. 2.39±0.04 g) and liver weight (4.79±0.24 vs. 2.80±0.14 g; AGA-control vs. FGR-MNR respectively) decreased significantly in select FGR-MNR offspring ($p<0.001$). Brain/liver weight increased in FGR-MNR offspring ($p<0.001$) which indicates brain-sparing as a measure blood flow redistribution. FGR-MNR offspring also had significantly lower blood glucose levels (5.9±0.4

vs. 4.2±0.2 mmol/L; AGA-Control vs. FGR-MNR respectively) and had significantly elevated hemoglobin and lactate content ($p<0.01$). Together, these results indicate FGR-MNR fetuses underwent asymmetric FGR, were hypoglycemic, and hypoxemic.

5.3.2 Markers of Oxidative Stress and Antioxidants

There were no significant changes in total glutathione content between groups, (Figure 5.1) although there was an overall trend for decreased glutathione content in the liver, kidney and placenta in FGR-MNR fetuses compared to AGA-CTL. Figure 5.2 shows the effect of MNR on fetal liver and placental GSH/GSSG ratio. Diet had a significant effect on both liver and placental GSH/GSSG ratio ($p<0.01$). When controlling for sex, only FGR-MNR female liver and FGR-MNR male placental GSH/GSSG ratio increased significantly ($p<0.01$). Two-way ANOVA further pointed to a significant interaction ($p<0.05$) between sex and diet on placental GSSG/GSH.

Figure 5.3 shows the significant effect of both diet ($p<0.001$) and sex ($p<0.0001$) on liver MDA content which was not significant in the placenta (diet $p=0.06$). Tukey's HSD Post-hoc analysis demonstrated significantly reduced MDA in the female FGR-MNR offspring only ($p<0.001$).

PRDX protein levels from Western Blot analysis are displayed in Figure 5.4, There was a significant increase in both male ($p<0.05$) (A) and female ($p<0.001$) (B) FGR-MNR PRDX protein levels in the liver, by 93% and 192%, respectively, as compared to AGA-CTL fetuses. There were no significant changes in the kidney or placenta PRDX protein levels.

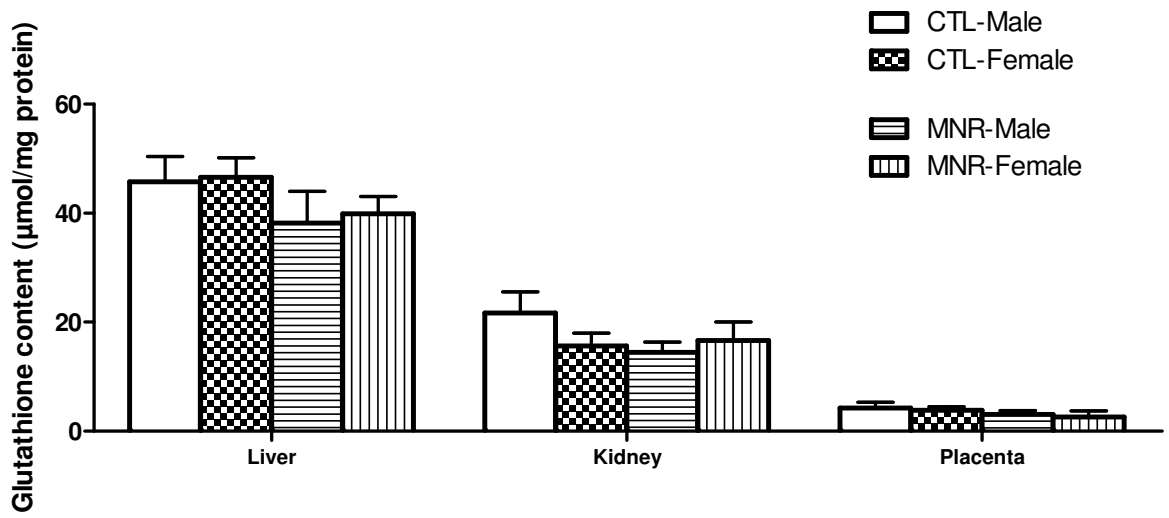

Figure 5.1 MNR does not influence total Glutathione Content. Total glutathione content for AGA-CTL males and females; and FGR-MNR males and females. N=8 in each group. Data presented as mean glutathione ± SEM. Data between MNR and Control groups was analyzed using 2-way ANOVA, followed by Tukey's HSD test. Fetal kidney, liver, and placenta all had decreased GSH, although it was not significant in any tissue.

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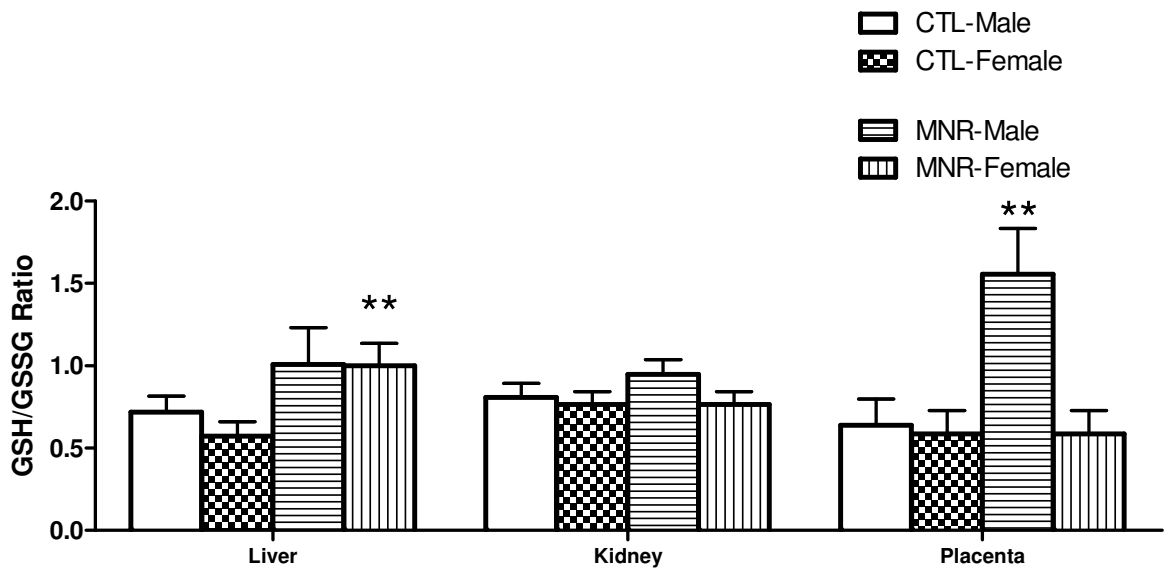

Figure 5.2 MNR selectively increases the ratio of the reduced form of glutathione (GSH) compared to oxidized glutathione (GSSG). Ratio of GSH/GSSG for AGA-CTL and FGR-MNR fetuses, both males and females. N=8 in each group. Data presented as mean GSH/GSSG ± SEM. Data between MNR and Control groups was analyzed using 2-way ANOVA, followed by Tukey's HSD test. FGR-MNR fetal kidney, liver, and placenta all had increased GSH/GSSG, although only significant in the female liver, and male placenta compared to AGA-CTL fetuses. ** Denotes significant difference between groups, $P<0.01$.

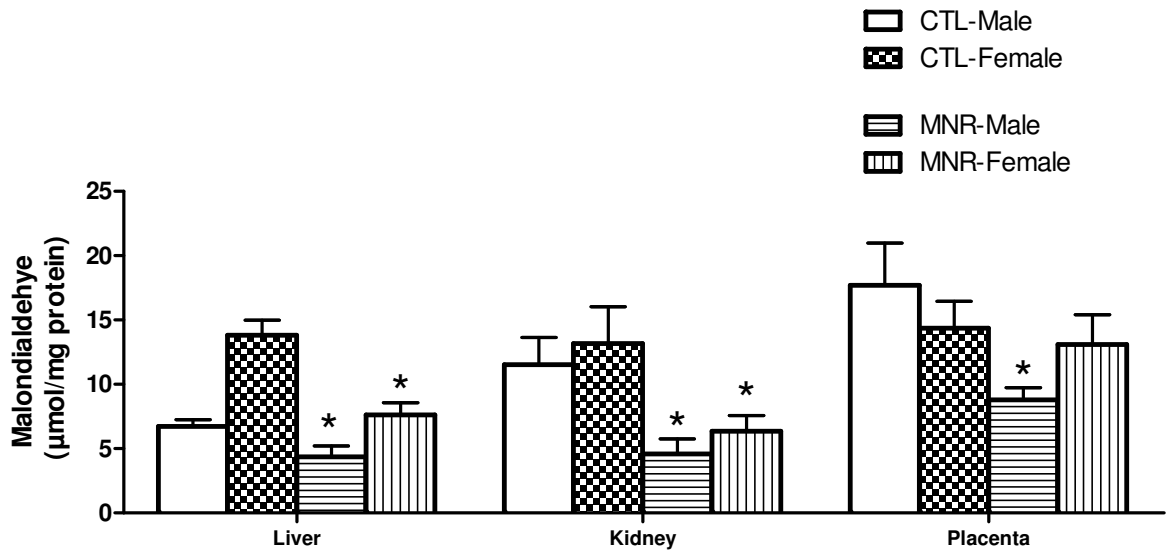

Figure 5.3 MNR decreases malondialdehye content as a maker of lipid peroxidation in the fetal guinea pig. Total malondialdehyde content (µmol/mgprotein) for AGA-CTL and FGR-MNR fetuses, both males and females. N=8 in each group. Data presented as mean malondialdehyde content (µmol/mgprotein ± SEM. Data between MNR and Control groups was analyzed using 2-way ANOVA, followed by Tukey's HSD test. FGR-MNR fetal kidney, liver, and placenta all had decreased malondialdehyde in both sexes, and it was significant in all tissues with the exception of the MNR-female placenta.

* Denotes significant difference between groups, $P<0.05$.

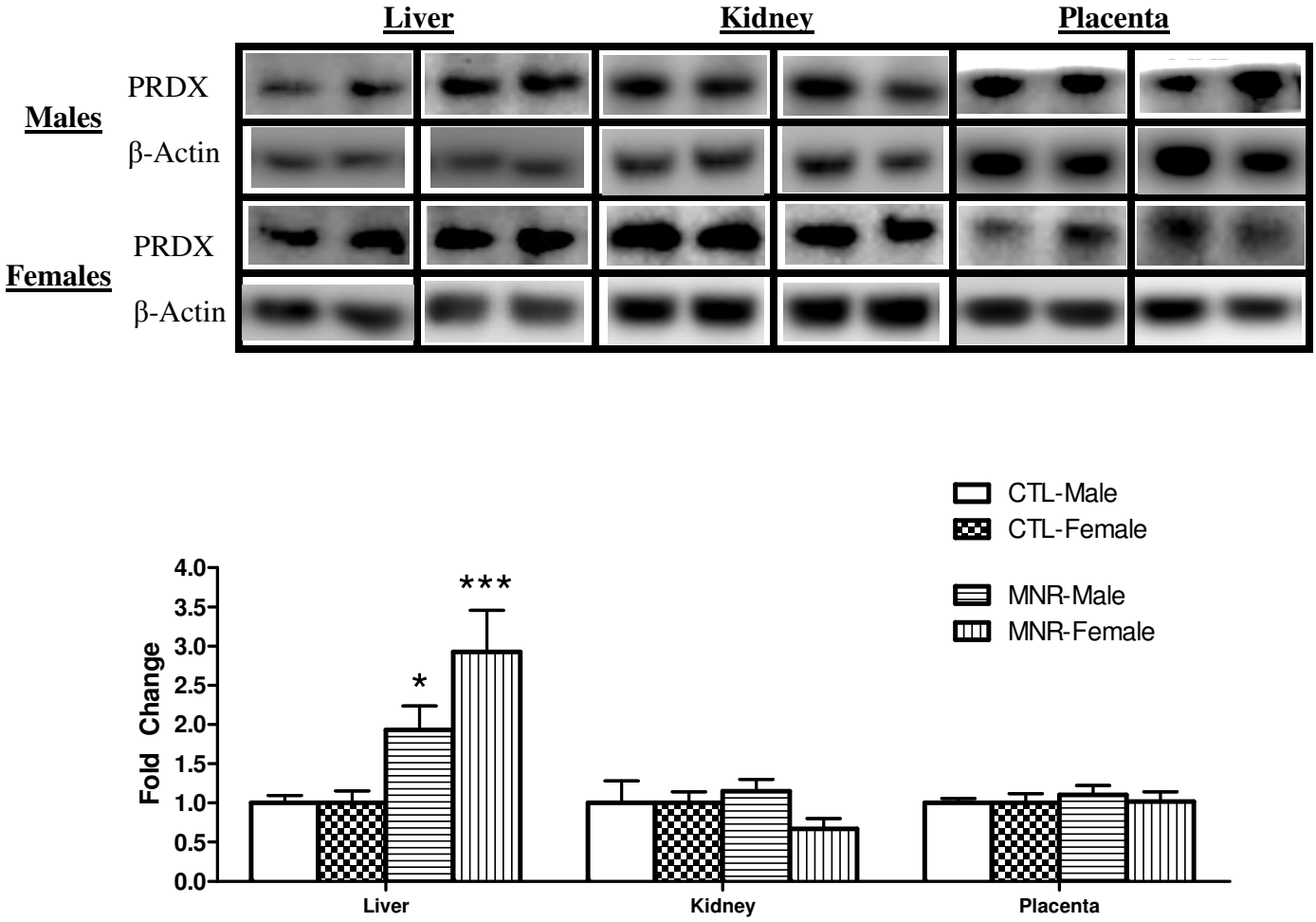

Figure 5.4 MNR results in increased liver PRDX protein in the fetal guinea pig. Peroxiredoxin protein levels (normalized to B-Actin protein for each sample) for AGA-CTL and FGR-MNR fetuses, both males and females. N=8 in each group. Data presented as mean fold change from AGA-CTL groups ± SEM. Data between MNR and Control groups was analyzed by Western Blot using Student's T-Test. FGR-MNR fetal liver had significantly increased PRDX protein in both the male and female groups, compared to their AGA-CTL counterparts.

* Denotes significant difference between groups, $P<0.05$.

*** Denotes significant difference between groups, $P<0.001$.

5.4 DISCUSSION

Fetal growth restriction and oxidative stress during fetal development both contribute to long lasting and severe adverse consequences on post-natal health and are at increased risk for adult disease (Biri 2007; Takagi 2004; Thompson 2012). As previously described in Chapter 3, MNR prior to and continuing throughout pregnancy results in fetuses that are not only smaller in size, but upon further characterization of these FGR-MNR cohort, the growth restriction found was of the vital organ sparing, asymmetrical variety. This finding is consistent with FGR resulting from placental insufficiency where the brain to liver weight ratio is significantly increased in FGR fetuses. These findings indicate brain-sparing and hepatic underdevelopment that is similar to that seen with human cases placental insufficiency and chronic hypoxia (Haugen 2005).

The most pertinent finding in this study was the significant increase in the antioxidant peroxiredoxin in FGR-MNR fetuses as it may indicate that a protective response mechanism has been triggered due lowered oxygen. PRDX acts as a defense against oxidative stress by removal of harmful hydrogen peroxide (Rhee 2005). The increase in PRDX protein levels in FGR-MNR fetuses may indicate that an antioxidant response has been triggered by increased cellular stress due to a build-up of ROS from MNR (Burton 2004; Cumming 2007; Rhee 2005). The increases were limited to the male and female liver FGR-MNR fetuses, which add the findings of Chapters 3 and 4 that the liver is undergoing extensive adaptations due to undernourishment. The increased PRDX combined with a decrease in MDA and GSH/GSSG may suggest an oxidative stress insult has already occurred early on in gestation, perhaps at the onset of improper placental development resulting from MNR, and in turn, the antioxidant levels have increased to further protect the fetus from ROS damage (Burton 2004). These findings also suggest a protective role as PRDX was increased in the organ that was most susciptile to growth restriction and hypoxia as outlined in Chapters 3 and 4.

In conjunction with increased antioxidant activity, the main goal of this study was to assess the levels of oxidative stress markers resulting from MNR during pregnancy. The ratio of reduced to oxidized glutathione is used as a marker of the redox state of a cell and cellular toxicity (Pastore 2003). A decrease in GSH/GSSG relative to physiological normal is considered indicative of oxidative stress (Halprin 1967). In this study, both total and GSH/GSSG ratio was assessed. The most surprising finding of this study was a significant increase in GSH/GSSG ratio in the male

placenta and female liver. These results are contrary to other animal models whereby environmental hypoxia resulted in increased glutathione content in fetal tissues (Hashimoto 2012; Oh 2008). Given that these animals do show evidence for chronic hypoxia resulting from placental insufficiently, these counter intuitive results may be the consequence of a variety of factors from this MNR study, including the model of nutrient restriction, as well as the time point of tissue collection and analysis. Previous studies have indicated that the total GSH content may not be indicative of oxidative stress due to the lack of substrates delivered to the MNR fetuses throughout pregnancy resulting from placental insufficiency (Langley-Evans 1995). Considering the evidence for increased antioxidants in FGR-MNR animals, the near term fetus may also exhibit changes in oxidative stress markers that are markedly different from an early time point in gestation. During exposure to an adverse *in utero* environment, a fetus is able to cater their growth to accommodate and defend against such detrimental insults (Calkins 2011; Luo 2006). FGR-MNR fetuses were subject to lack of nutrition from the point of conception, and fetal organ development was continuously being restricted by the limited nutrient supply arising from improper placental growth in a lowered oxygen environment, as discussed in Chapter 3. The fetus then adapts and may grow more slowly and conservatively as a survival mechanism, reducing substrate demand from an already depleted supply (Roberts 2001). It is possible that such adaptations occurred early on in the undernourished fetal life, and an early oxidative stress response may have triggered altered metabolic activity to support low nutrient demand. The increases in GSH/GSSG ratio also may further illustrate the protective mechanism that occurs in the FGR animal during chronic insults. These adaptations would allow for increased resistance to cellular oxidative stress and by near term the levels of such oxidative stress markers would be lowered. It would be of interest to examine FGR-MNR fetuses at mid-to-early gestation and see if the oxidative stress marker differences remain similar. These studies may point to the differences seen in the ratio of GSH/GSSG.

Another pertinent finding was the difference in levels of MDA as a marker of lipid peroxidation, which is increased during oxidative stress events (Janero 1990). MNR-FGR fetuses showed lower levels of MDA compared to their AGA-CTL counter parts, which again suggests a decreased level of lipid peroxidation in these animals. Similarly to GSH, MDA has been previously reported to increase in animal and human cases of FGR (Hasimoto 2012; Evans 2012; Gupta 2004). This decrease in lipid peroxidation may be partially explained not by an oxidative

stress response, but also by the effect of MNR on lipid availability. MNR leads to placental insufficiency, which in turn decreases the placentas efficiency in transporting glucose, amino acids, and lipids (Belkaceemi 2010). This suggests that the decrease in MDA levels in the FGR-MNR fetuses may be the result of an overall reduction in lipid content of the liver and the placenta. Further studies should aim at assessing lipid content levels in tissues from both control and MNR tissues. Additionally, previous studies have linked undernutrition during adult life with decreased oxidative stress makers with decreased MDA (Bokov 2004). Together with GSH/GSSG, these results point towards a much more impactful role of nutrition itself, rather than the resulting placental insufficiency and hypoxia, on fetal levels of oxidative stress markers.

In addition to differences between the FGR-MNR and AGA-CTL cohorts, differences in males and females were also analyzed for both GSH and MDA levels. Although the trends for both the liver and placenta were similar in each sex in this study, in the AGA-CTL animals there were significant effects of sex in the liver and the placenta in MDA and GSH/GSSG results, respectively. Sexual dimorphisms are prevalent in development (Clifton 2010; Muralimanoharan 2010), and MNR has been previously demonstrated to affect gene expression differently dependant on the sex (Guo 2013; Mao 2010). It has also been suggested that male fetuses are more sensitive to maternal diet than females (Eriksson 2010). This may be due to a female defense mechanism that naturally programmes them to be more resilient to adverse events such as MNR, due to the presence of an increase in post-natal stresses such as pregnancy (Valle 2007). Males also tend to dispense less energy to placental development, and more towards brain and heart development, which may also contribute to the differences seen in AGA-CTL animals. Together, some of these sex differences could account for the changes in oxidative stress markers between males and females.

In summary, there was a reduced amount of MDA as a measure of lipid peroxidation in FGR-MNR fetuses compared to their AGA-CTL counterparts. Additionally, FGR-MNR animals showed an overall increase in GSH/GSSG ratio as a measure of the redox state of the tissues. Both of these results are opposite with previously studied animal models of placental insufficiency and a degree of hypoxia. These results may be explained in part by the lack of nutrients available due to the model of MNR and therefore may not represent the clearest idea of oxidative stress occurring in the near term fetus. However, the increase in PRDX protein levels

as a marker of antioxidants and therefore a defensive mechanism against an oxidative stress response may also explain these decreases. Due to constant state of nutrient restriction, it is likely that an oxidative stress response occurred in these animals at a much earlier time point in gestation, and that by the near-term necropsy, these FGR-MNR fetuses were already combating the stresses by deploying antioxidant which may contribute to the overall decrease in oxidative stress at near-term. This antioxidant response, in combination with the nutritional effects, helps to identify important underlying mechanisms behind improper maternal nutrition and the oxidative stress response.

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CHAPTER 6

GENERAL DISCUSSION

6.1 GENERAL DISCUSSION

Through the advances of the developmental origins of health and disease, the intrauterine environment has been of extreme interest to investigators. Through these studies, evidence now suggests that sub-optimal growth during gestation, resulting in low birth weight, is associated with a number of long-term adverse outcomes, including neurological, cardiovascular, and metabolic disabilities. Although many factors contribute to FGR, improper placental growth and therefore impaired nutritional transport to the fetus plays a major role in many human cases of FGR. Although many animal models use techniques to induce placental insufficiency, this study was designed to further characterize how nutrient restriction model would act as a more representative model of human aFGR, and determine if there were any changes in oxygenation and oxidative stress that occurred because of MNR.

In recent studies, moderate MNR in guinea pigs has been utilized for modeling human FGR including maternal, placental, and fetal growth characteristics. These studies have reported associated IGF and IGFBP alterations, giving insight into mechanisms for programming long-term adverse outcomes in offspring (Sohlstrom 1998; Roberts 2001a; Kind 2003; Kind 2005). This study has further characterized this model by adding breeding success and pregnancy outcomes in MNR animals as outlined in Chapter 3. This study also exhibited the utility of the MNR model to induce FGR, resulting in dramatically lower fetal death rates compared to that seen with uterine ligation/ablation models (Lafeber 1984; Turner 2009), although an increased preterm delivery was reported as is seen with FGR in humans (Lackman 2001). As previously investigated, this study has confirmed that MNR guinea pig fetuses have increased placental/fetal weight ratios that are often seen in human cases of FGR. (Kramer 1990; Godfrey 1998; Lumey 1998; Belkacemi 2010; Lackman 2001; Fall 1999).

This study provided justification for using a fetal weight threshold of 80 gms for categorizing AGA-Control and FGR-MNR cohorts, with 80 gms being representative of the 10^{th} percentile in our ad libitum fed animals. An important finding among the FGR-MNR cohort is that fetuses show aFGR, and are polycythemic and hypoglycemic which are well associated with moderate growth restriction during human pregnancy (Kramer 1990; Resnk 2014; Kramer 1991; Abrams 1991; Soothill 1987; Cox 1988; Economides1989).

These findings, combined with previous work in MNR guinea pigs showing altered vascular development and structural changes in the placentas (Roberts 2001a; Roberts 2001b), also make it likely that chronic hypoxemia is occurring as a primary signaling mechanism for the decreased fetal growth in these pregnancies. As presented in Chapter 4, the impact of MNR on FGR and the resulting levels of tissue hypoxia in FGR-MNR guinea pigs were investigated. There was a significant increase in HP-1 immunostaining as a direct measure of low oxygen environments in female FGR-MNR fetuses in the kidney and liver. HP-1 has been previously used to directly confirm hypoxia in the guinea pig fetus (Oh 2008), suggesting that MNR results in decreased levels of oxygenation in the growth restricted fetus. Additionally, protein markers of the HIF-1α pathway were used to determine changes in response to altered oxygenation. FGR-MNR animals showed a significant increase in EPO protein levels across all three tissues examined. Furthermore, the fetal liver also displayed a significant increase in EPOR and VEGF protein levels, suggesting an increase in erythropoeitic and angiogenic factors resulting from blood redistribution. Together these protein levels suggest an adaptive and systematic response to lowered oxygenation through the HIF pathway, which has been seen with human cases of FGR (Ostlund 2000; Jazaveri 1999). Overall, these findings implicate chronic hypoxia as a mediator of growth restriction and altered development arising from MNR.

Following the changes tissue oxygenation and evidence for chronic hypoxia, this study focused on determining if there were also associated changes in oxidative stress and antioxidant markers in FGR-MNR animals (Chapter 5). There was a reduced amount of MDA as a measure of lipid peroxidation in FGR-MNR fetuses compared to their AGA-CTL counterparts. Additionally, FGR-MNR animals showed an overall increase in GSH/GSSG ratio as a measure of the redox state of the tissues. Both of these results are opposite to previously studied animal models of placental insufficiency with environmental hypoxia (Hracsko 2008; Oh 2008; Evans 2012; Schafer 2001). These results may be explained in part by the lack of nutrients available due to the model of MNR and therefore may not represent the clearest idea of oxidative stress occurring in the near term fetus. However, the increase in PRDX protein levels as a marker of antioxidants and therefore a protective mechanism against oxidative stress due to increased ROS may also explain these decreases (Reddy 2008; Neumann 2009). Due to the chronic nature of nutrient restriction, it is likely that an oxidative stress response occurred in these animals early in gestation, and that by the near-term necropsy, these FGR-MNR fetuses were already combating

the stresses by deploying antioxidants which may contribute to the overall decrease in oxidative stress at near-term. This antioxidant response, in combination with the nutritional effects, helps to identify important underlying mechanisms behind improper maternal nutrition and the oxidative stress response.

Together, the present and past studies of moderate MNR in guinea pigs support the utility of this model for inducing FGR with many similarities to that in human FGR whether resulting from maternal undernourishment or placental insufficiency. This was the first time to date that oxidative stress and hypoxia markers, as mechanisms of damage, have been reported using the MNR guinea pig model. While there are undoubtedly differences between the mechanisms behind FGR resulting from undernourishment vs. placental insufficiency, this study has exhibited the merit of using a translatable chronic perturbation to induce growth restriction. These results are similar to that seen with human cases, and provided further insight into the mechanisms surrounding developmental programming.

6.2 FUTURE STUDIES

Based on the current findings, future studies should focus on the long-term outcomes and health impact of the maternal undernourishment model. Any behavioural and/or neurological changes in the growth restricted fetus could be associated with MNR, and give further insight into the mechanisms behind developmental programming and the health of the newborn.

Defining developmental changes in animal models has been done with a variety of different tests that can indicate changes in cognition, learning, memory, and behaviour. Tests such as the open field test, the T-arm maze, Morris Water maze, and forced swim test could help to better define the extent to which MNR and thus FGR is associated with cognitive changes in the guinea pig. Correlating these behavioral characteristics with the severity of FGR resulting from MNR could provide useful comprehension into the most impactful and advantageous type of therapy, dependant of the duration and severity of undernutrition during gestation

Historically human FGR has been known to cause cardiovascular issues as well as metabolic syndrome. More recently, FGR has been shown to have poor neurodevelopmental and

neurological outcomes later in adult life (Fattal-Valevski 2009; Løhaugen 2013). Specifically, cognitive impairment, lack of behavioral and attention skills, and physical or neurological impact later in life of fetuses who were born FGR. Brain disorders like attention deficit hyperactive disorder, autism, cerebral palsy, and schizophrenia have been directly correlated with the severity of FGR (Dahlseng 2014; Haglund 2011; Mallard 1999; StrangKarlsson 2008). Whether or not MNR acts as a causative factor and indirect contributor to such brain impairment is currently unknown and should be the aim of future investigators.

Neurological changes in the FGR fetus has been well documented in other animal models of placental insufficiency, most notably, uterine artery ligation. Since our study has shown to that MNR leads to FGR, with a preference for the aFGR phenotype, it is reasonable to believe that there are also some similar changes in the brain that are comparable to the FGR human, as well as the UAL animal model. Initiation of myelination, axonal and dendritic growth, synaptogenesis, and proliferation of microglia and astrocytes are all examples of developmental events that can be disrupted or altered if nutrients and oxygen are not available, such as in a growth restricted fetus (Kind 2005) For prenatal brain developers such as guinea pigs, and for humans as perinatal brain developers, the most rapid phase of synapse formation and neuronal myelination onsets during the latter half of pregnancy (McIntosh 1999). As such, FGR with MNR may disrupt synapse formation and neuronal myelination, as they are energy demanding growth processes (Jiang 2002), this may lead to alterations in brain development and underlie risk for later cognitive impairment and neuropathology. Studies done by Piorkowska *et al.* have shown reduced synapse formation in FGR animals, while aFGR animals maintain hippocampal maturation of synapse compared to that of the sFGR animals (Piorkowska 2014). Further research should focus on whether these changes seen with the UAL model of FGR are also present in this studies MNR model of FGR to elucidate MNR as a mechanism for aberrant neurological development.

During exposure to hypoxic environments, neuronal loss has been reported in developing brain cells and results in apoptotic/necrotic cell death dependant on the severity of the insult (Falkowski, 2002). In some cases of severe nutritional impairment, it is possible that protective compensatory mechanisms may become limited and brain energy levels may be sufficiently impacted, resulting in an increase in necrotic cell injury and/or changes in apoptotic regulators

(Rocha 2004). A better understanding of the extent to which developmental processes in the brain are impacted by FGR with maternal undernourishment will allow for the potential for early detection and therapies aimed at mechanistic pathways.

6.3 CONCLUSIONS

In conclusion, the thesis was focused on determining the relationship between MNR and the changes in fetal-placental growth, tissue oxygenation and oxidative stress in the near term guinea pig. The major findings of this study were:

1) MNR leads to aFGR fetuses with high brain to liver weight ratio.
2) Markers of oxygenation are selectively reduced in the MNR-FGR fetus.
3) Markers of oxidative stress are decreased with an increase in antioxidant levels in the MNR-FGR fetus.

These studies suggest a significant role in using MNR to induce aFGR in the fetal guinea pig, with changes in oxygen distribution and antioxidant capacity. This model may better mimic the human condition of FGR as seen with placental insufficiency, and undernutrition during pregnancy may lead to long-term development disorders in the newborn.

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APPENDIX A– ETHICS APPROVAL

Submit - Animal Use Protocol - AUP Form

1. Investigator Contact Information

PI_FULL_NAME	Richardson, Bryan S
AUP NUMBER	2012-060
AUP TYPE	
Primary Role	Principal Investigator
1. PI Full Name	Richardson, Bryan S
2. Primary Institution &Department	Schulich School Of Medicine &Dentistry / Obstetrics &Gynaecolo
3. Office Location - Building &Room #	Westminster Tower (E-Zone) Room E2-640G
4. Weekday Phone #	685-8500 X 64926
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6. Pager - Phone &Pager #	685-8500 X 10393
7. Primary Email	brichar1@uwo.ca
8. Other Email	
9. Lab Campus Location, if different from Q.3	Medical &Dental Sciences Buildings
10. Lab Phone #, if different from Q.4	DSB 5006

2. Protocol Title &Project Type

1. *Animal Use Protocol* Title
Maternal undernourishment and the impact on fetal and placental development
2. Application Type, *Pick One*
New
3. If 'Full Renewal' or 'Post-Pilot Full Protocol' provide Associated Previous Protocol Number
4. If **Post-Pilot Full Protocol** or **Full Renewal**, Provide a 3 R'S PROGRESS REPORT SUMMARY that outlines progress relating to the REPLACEMENT of animals, REDUCTION of animal use numbers AND REFINEMENT of experimental technique. E.g. What did you previously learn that has resulted in a change in study design based upon application of the 3 R's. Link to CCAC's 3 R's Microsite for more information.
5. If **Post-Pilot Full Protocol** or **Full Renewal**, provide previous Protocol Year's animal use number.
6. Proposed Start Date *(mm/dd/yy)*
12/01/2012

3. Lay Summary &Glossary

1. Using non-scientific language, please describe the project's purpose, expected benefit, and a brief summary of your work with the animal model(s). *Please be aware that in the event of communications with Western Media Relations and the PI is not available, this summary will be sent to Western Media Relations.*
Fetal growth restriction (FGR) is a major contributor to perinatal morbidity and mortality and for risk of later adverse health outcomes with heart disease, diabetes and neurodevelopment disability. The studies herein proposed seek to determine the extent to which maternal undernourishment as a causative factor for FGR also results in fetal hypoxia and oxidative stress as potential mediators of injury, both short and longer term. While there is considerable evidence supporting this conjecture, this has not been proven to date. If maternal undernourishment is found to result in fetal hypoxia and oxidative stress in relation to the degree of FGR, then treatment strategies aimed at decreasing placental-fetal oxidative stress can be tested. In summary, we intend to utilize previously developed nutritent restriction models whereby a female guinea pig's nutrition is restricted prior to conception and throughout gestation to create growth-restricted fetal pups.
2. GLOSSARY OF TERMS - Identify each individual scientific term and abbreviation using *CAPITAL LETTERS*, and then briefly define each term to be referenced in any section of this protocol. e.g. ALLELE - The genetic variant of a gene responsible for the different traits of certain characteristics and genetic diseases.
perinatal morbidity: is a diseased state, disability, or poor health due to any cause, around the time of birth. fetal hypoxia: inadequate oxygen supply to the fetus oxidative stress: is an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage placental-fetal oxidative stress: oxidative stress, as defined above due to an abnormality at the placental-fetal level parturition: the act of giving birth

4. CCAC Animal Procedural Outline

CCAC PROCEDURAL OVERVIEW - Use this field to convey in simple terms using approximately 40 words or less the nature of the procedures conducted on the animals. ***Please use KEY WORDS provided through the above link.***
Female guinea pigs will be placed on a nutrient-restricted diet prior to breeding and remain on an adjusted nutrient restricted diet throughout gestation. Guinea pigs will then be sacrificed prior to parturition to remove fetuses for tissue extraction.

CURRICULUM VITAE

Alexander Elias

EDUCATION

2012-2015 **Master of Science** in Physiology and Pharmacology-Developmental Biology
University of Western Ontario
London, Ontario, Canada

2007-2011 **Honors Bachelor of Medical Science** with a specialization in Medical Science
University of Western Ontario
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RESEARCH PRESENTATIONS

Elias AA, Matushewski B, Zhao L, Regnault TRH, Richardson, BS. Maternal Nutrient Restriction (MNR) in Pregnant Guinea Pigs Impacts Fetal-Placental Growth with Evidence for Chronic Hypoxia. **Poster presentation** at the *Department of Physiology and Pharmacology Research Day November 2014,* ***London*** *Ontario.*

Elias AA, Matushewski B, Zhao L, Regnault TRH, Richardson, BS. Maternal Nutrient Restriction (MNR) in Pregnant Guinea Pigs Impacts Fetal-Placental Growth and Tissue Oxygenation. **Oral presentation** at the *Paul Harding Obstetrics and Gynaecology Research Day May 2014,* ***London*** *Ontario.*

Elias AA, Matushewski B, Zhao L, Regnault TRH, Richardson, BS. Maternal Nutrient Restriction (MNR) in Pregnant Guinea Pigs Impacts Fetal-Placental Growth and Erythropoietin (EPO): Implications for Regulatory Mechanisms. **Poster presentation** at the *Society for Gynecologic Investigation Annual Meeting March 2014,* ***Florence, Italy****.*

Elias AA, Matushewski B, Zhao L, Regnault TRH, Richardson, BS. Maternal Nutrient Restriction (MNR) in Pregnant Guinea Pigs Impacts Fetal-Placental Growth and Tissue Hypoxia: Implications for Regulatory Mechanisms. **Poster presentation** at the *London Health Research Day March 2013,* ***London*** *Ontario.*

Elias AA, Matushewski B, Zhao L, Regnault TRH, Richardson, BS. Maternal Nutrient Restriction (MNR) in Pregnant Guinea Pigs Impacts Fetal-Placental Growth and Erythropoietin (EPO): Implications for Regulatory Mechanisms. **Oral presentation** at the *Canadian National Perinatal Research Meeting February 2014,* ***Banff, Alberta****.*

Elias AA, Matushewski B, Zhao L, Regnault TRH, Richardson, BS. Maternal Nutrient Restriction (MNR) in Pregnant Guinea Pigs Impacts Fetal-Placental Growth and Tissue Hypoxia: Implications for Regulatory Mechanisms. **Poster presentation** at the *Department of Physiology and Pharmacology Research Day November 2013, **London** Ontario.*

AWARDS AND FUNDING

2013-2014	Obstetrics and Gynaecology Graduate Scholarship, $15,000
2013	Canadian Institute of Health Research Travel Award, $1,000
2013,2015	Children's Health Research Institute Travel Fund, $750
2012-2013	Physiology and Pharmacology Graduate Research Scholarship, $9,000
2013	Graduate Student Teaching Award (nominee) The University of Western Ontario
2010-2012	Dean's Honour List. The University of Western Ontario

RELATED WORK EXPERIENCE

Sept 2013 – May 2014	**Let's Talk Science** Classroom Leader, The University of Western Ontario Chapter
Sept 2012 – May 2014	**Teaching Assistant**, Department of Physiology and Pharmacology The University of Western Ontario