Prenatal Hypoxia and the Impact of Maternal Antioxidant (MitoQ) Treatment on Developmental Programming of Cardiovascular Disease

by

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ABSTRACT

Cardiovascular disease is the primary cause of morbidity and mortality worldwide. Prenatal hypoxia (due to placental or maternal hypoxia) is an established risk factor for cardiovascular disease. Hypoxia in pregnancy can lead to intrauterine growth restriction (IUGR; defined as a fetus who does not reach its genetic growth potential) and to an increase in offspring susceptibility to develop cardiovascular disease later in life, a concept known as developmental programming of cardiovascular disease. This PhD project focusses on assessing the effect of hypoxia in pregnancy on maternal vascular function, fetal development and on offspring cardiovascular function later in life in both males and females separately. I also aimed to propose an intervention to improve offspring cardiovascular health in complicated pregnancy. As placental oxidative stress is important in the pathophysiology of many pregnancy complications including IUGR and developmental programming, the mitochondrial antioxidant MitoQ loaded onto nanoparticles (nMitoQ) was proposed herein as an intervention to prevent placental oxidative stress and optimally prevent IUGR and fetal programming of cardiovascular disease. Nanoparticles were used to prevent MitoQ from crossing the placental barrier to the fetus in order to avoid risking direct fetal exposure to the antioxidant treatment.

In this thesis, an established rat model of maternal hypoxia was used. Maternal vascular function was assessed using both *in vivo* and *ex vivo* techniques. Fetal body weight was measured after euthanizing the dams. Hypoxia in pregnancy increased maternal blood pressure and caused vascular bed-dependent alterations in maternal vascular function (mesenteric artery versus uterine artery). Furthermore, hypoxia in pregnancy caused a reduction in uterine artery resistance index and IUGR in the fetuses.

To assess the effect of maternal hypoxia and nMitoQ treatment on developmental programming, a similar animal model but with a shorter period of maternal hypoxic exposure was used (our laboratory has previously assessed the phenotype of offspring cardiovascular function in this animal model). nMitoQ (125 μM) was intravenously injected on GD 15 just before animal exposure to hypoxia and at the same day for the control (dams that stayed in normoxia all over the pregnancy period). In one set of animals, rats were euthanized near term to assess pregnancy outcome, placental oxidative stress, and fetal cardiac development. Cultured medium was prepared and used to assess the effect of placental secreted factors on normal cardiomyocyte growth in male and female fetuses separately. In another set of animals, dams were allowed to give birth and male and female offspring were assessed at 7 and 13 months of age for *in vivo* cardiac function and *ex vivo* vascular function.

Hypoxia in pregnancy led to IUGR and to an increase in placental oxidative stress and relative heart weight to body weight (cardiac hypertrophy) in both male and female fetuses. However, hypoxia led to an increased cardiomyocyte size in only male but not female fetuses. Factors released from placentas of hypoxic dams did not alter normal cardiomyocyte growth. nMitoQ treatment prevented placental oxidative stress in both male and female fetuses. nMitoQ prevented IUGR and prevented cardiac hypertrophy in only female fetuses. However, nMitoQ rescued cardiomyocyte growth, which was increased in male fetuses due to maternal hypoxia.

In young (7 months) and aged (13 months) male and female offspring, maternal hypoxia led to sex-dependent cardiovascular dysfunction. This includes changes in cardiac morphology, signs of diastolic, and systolic dysfunction and abnormal vascular responses to vasoconstriction and vasorelaxation. nMitoQ treatment partly improved cardiovascular function in offspring of both sexes. For example, in hypoxic dams nMitoQ improved systolic function in aged female offspring

compared to the nMitoQ untreated controls; and increased sensitivity to vasorelaxation in aged male and female offspring from normoxic and hypoxic dams.

In conclusion, this thesis has elucidated factors that play an important role in the pathophysiology of IUGR and developmental programming of cardiovascular disease in male and female offspring. This thesis as well showed that targeting antioxidant treatment to the placenta can impact fetal body weight, fetal cardiac development and offspring cardiovascular function in adult life, suggesting that interventions do not necessarily need to target the fetus to improve fetal outcome. This project helped in setting the stage for future studies about employing nanoparticles to deliver interventions to specific targets during pregnancy to improve offspring cardiovascular function in complicated pregnancy.

DEDICATION

All the hard work and sacrifices I made throughout my PhD are dedicated with respect to my beloved mom and dad.

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TABLE OF CONTENTS

CHAPTER 1: GENERAL INTRODUCTION 1

1.1 DEVELOPMENTAL PROGRAMMING OF CARDIOVASCULAR DISEASE, AN OVERVIEW 2

1.2 CARDIOVASCULAR PHYSIOLOGY IN FETAL AND ADULT LIFE 7

1.2.1 Cardiomyocyte development and mediators in fetal life 7

1.2.1.1 Aortic load pressure 8

1.2.1.2 Growth factors 8

1.2.1.3 Glucocorticoids 10

1.2.1.4 Angiotensin II 12

1.2.2 Fetal cardiac circulation 12

1.2.3 Cardiac physiology in adult life 13

1.2.4 Assessment of cardiac morphology and function using echocardiography in adult life 15

1.2.5 Vascular physiology in adult life 17

1.2.5.1 Arterial function and structure 17

1.2.5.2 Mechanisms of vascular smooth muscle cell relaxation and contraction 17

1.2.5.3 Endothelial mediators of VSMC relaxation 19

1.2.5.4 Endothelial mediators of VSMC contraction 25

1.3 HYPOXIA IN PREGNANCY AND LINKAGES TO DEVELOPMENTAL ORIGINS OF CARDIOVASCULAR DISEASE 27

1.3.1 Effect of hypoxia on fetal cardiomyocyte development 29

1.3.2 Hypoxia in pregnancy and the programming of cardiac dysfunction in adult life 31

1.3.3 Hypoxia in pregnancy and the programming of vascular dysfunction in adult life 32

1.4 MECHANISMS BY WHICH HYPOXIA IN PREGNANCY MIGHT ALTER FETAL DEVELOPMENT AND LEAD TO OFFSPRING CARDIOVASCULAR DYSFUNCTION 34

1.4.1 Alterations in maternal hemodynamics during pregnancy 34

1.4.1.1 Maternal systemic cardiovascular adaptations during pregnancy 34

1.4.1.2 Uterine vascular adaptations during pregnancy 36

1.4.1.3 Effects of hypoxia on maternal vascular adaptations in pregnancy 39

1.4.2 Placental oxidative stress and secreted factors 41

1.4.2.1 Establishment of uteroplacental and fetoplacental blood flow 41

1.4.2.2 Placental development and fetal sex differences 43

1.4.2.3 Trophoblast mitochondria 47

1.4.2.4 Placental secreted factors 49

1.4.2.5 Effect of hypoxia on placental oxidative stress and secreted factors 53

1.5 ANTIOXIDANT INTERVENTIONS 56

1.6 NANOPARTICLE DELIVERY TO TARGET TREATMENT TO THE PLACENTA 58

1.7 CONCLUDING REMARKS 63

1.8 HYPOTHESIS AND AIMS 64

1.8.1 Hypothesis 64

1.8.2 Aims 64

CHAPTER 2: MATERNAL VASCULAR RESPONSES TO HYPOXIA IN A RAT MODEL OF INTRAUTERINE GROWTH RESTRICTION 66

2.1 INTRODUCTION 67

2.2 MATERIALS AND METHODS 68

2.2.1 Animals and treatments 69

2.2.2 Blood pressure and heart rate measurement 71

2.2.3 Ultrasound biomicroscopy 71

2.2.4 Fetal and placental measurements and maternal weight 74

2.2.5 Wire myography for ex vivo vascular function 74

2.2.6 Statistical analyses 75

2.3 RESULTS 76

2.3.1 Maternal blood pressure 76

2.3.2 Uterine and umbilical artery resistance indices 78

2.3.3 Reproductive phenotype and maternal weight gain 79

2.3.4 Uterine artery *ex vivo* vascular function 82

2.3.5 Mesenteric artery *ex vivo* vascular function 86

2.3.6 Internal diameter of uterine and mesenteric arteries 89

2.4 DISCUSSION 90

2.5 PERSPECTIVES 93

CHAPTER 3: MATERNAL TREATMENT WITH A PLACENTAL-TARGETED ANTIOXIDANT MITOQ IMPACTS FETAL CARDIOMYOCYTE DEVELOPMENT IN A RAT MODEL OF MATERNAL HYPOXIA 95

3.1 INTRODUCTION 96

3.2 METHODOLOGY 99

3.2.1 Experimental design 99

3.2.2 Preparation of MitoQ loaded onto nanoparticles 101
3.2.3 Assessment of placental blood flow indices 102
3.2.4 Fetal-placental measurements 102
3.2.5 Assessment of placental oxidative stress 102
3.2.6 Assessment of placental morphology 103
3.2.7 Assessment of cardiomyocyte growth and binucleation 105
3.2.8 Cardiomyocyte development in placental cultured media 106
3.2.9 Assessment of inflammatory factors in placental cultured media 106
3.2.10 Assessment of corticosterone in fetal plasma 107
3.2.11 Statistical analysis 108
3.3 RESULTS 108
3.3.1 Hemodynamic parameters of the uterine and umbilical vasculature 108
3.3.2 Pregnancy phenotype 110
3.3.3 Placental oxidative stress 116
3.3.4 Placental morphology 118
3.3.5 Effect of maternal hypoxia and nMitoQ treatment on the development of cardiomyocytes 123
3.3.6 Effect of placental conditioned medium on cardiomyocyte growth and binucleation 126
3.3.7 Effect of maternal hypoxia and nMitoQ treatment on corticosterone level in fetal plasma 130
3.4 DISCUSSION 132
3.5 PERSPECTIVES 137

CHAPTER 4: MATERNAL TREATMENT WITH A PLACENTAL-TARGETED ANTIOXIDANT (MITOQ) IMPACTS OFFSPRING CARDIOVASCULAR FUNCTION IN A RAT MODEL OF MATERNAL HYPOXIA ... 138
4.1 INTRODUCTION ... 139
4.2 METHODOLOGY ... 140
4.2.1 Experimental model ... 140
4.2.2 Blood pressure measurement ... 142
4.2.3 Echocardiography ... 142
4.2.4 Vascular function assessment ... 142
4.2.5 Statistical analysis ... 143
4.3 RESULTS ... 143
4.3.1 Offspring body weight ... 143
4.3.2 Cardiac morphology ... 144
4.3.3 Diastolic function ... 150
4.3.4 Systolic function ... 153
4.3.5 Pulmonary artery function ... 154
4.3.6 Blood pressure ... 156
4.3.7 *Ex vivo* vascular function ... 158
4.4 DISCUSSION ... 171
4.5 PERSPECTIVES ... 176
CHAPTER 5: GENERAL DISCUSSION AND FUTURE DIRECTIONS ... 178
5.1 SUMMARY OF THE MOST SIGNIFICANT FINDINGS ... 179
5.2 EFFECT OF HYPOXIA ON MATERNAL VASCULAR FUNCTION ... 182

5.3 EFFECT OF HYPOXIA ON PLACENTAL AND FETAL DEVELOPMENT 183

5.4 EFFECT OF MATERNAL HYPOXIA ON OFFSPRING CARDIOVASCULAR FUNCTION......... 185

5.5 PROJECT LIMITATIONS 187

5.6 FUTURE DIRECTIONS 191

5.7 SIGNIFICANCE OF THE THESIS RESULTS......... 193

APPENDICES 195

REFERENCES......... 200

LIST OF TABLES

Table 1.1 Sex differences in placental function and structure ………………….…………..… 45

Table 2.1 Maternal blood pressure and heart rate at GD 5 and GD 20 ………………..…...… 77

Table 2.2 Hemodynamic parameters of the uterine and umbilical vasculature as assessed by ultrasound microscopy at GD 20 ……………………………………………………..….... 78

Table 2.3 Fetal and placental parameters at GD 21 …………………………….....……. 81

Table 2.4 Vascular function of the uterine and mesenteric arteries as assessed by wire myography at GD 21 ……………………………………………………………………...... 83

Table 3.1 Male fetal and placental measurements on GD 21 ………………….………….. 114

Table 3.2 Female fetal and placental measurements on GD 21 ……………….……….…... 115

Table 3.3 Measurements of the labyrinth zone and junctional zone depth and area in placentas of male and female fetuses on GD 21 ……………………………………...….... 119

Table 3.4 Percentage of binucleated cardiomyocytes after *in vitro* incubation with placental conditioned medium ………………………………………………...……….. 128

Table 3.5 Placental inflammatory secreted factors were assessed using rat inflammation ELISA strip. Placentas collected on GD 21 ……………………………………...….... 129

Table 4.1 Body weight (g) at postnatal day (PD) 1 (neonate) & 21 (weaning), 7 months and 13 months of age ………………………………………………………………...….. 144

Table 4.2 Cardiac morphology and systolic function as assessed by echocardiography at 7 months of age in male offspring …………………………………………………….….…. 146

Table 4.3 Cardiac morphology and systolic function as assessed by echocardiography at 13 Months of age in male offspring ……………………………………………………...….... 147

Table 4.4 Cardiac morphology and systolic function as assessed by echocardiography at 7

months of age in female offspring …………………………………………………………. 148

Table 4.5 Cardiac morphology and systolic function as assessed by echocardiography at 13 months of age in female offspring …………………………………………………………. 149

Table 4.6 Diastolic function as assessed by echocardiography at 7 months of age …...…… 151

Table 4.7 Diastolic function as assessed by echocardiography at 13 months of age ………. 152

Table 4.8 Mesenteric artery responses to high K^+ (mN/mm) in male and female offspring . 166

LIST OF FIGURES

Figure 1.1 Perinatal hypoxia and linkages to developmental programming ……………………..… 5

Figure 1.2 Representative image of mitral valve waveforms ………………………………….. 16

Figure 1.3 Endothelium-dependent vasodilation pathways ……………………………………. 20

Figure 1.4 Placental structure …………………………….…………………………………….. 42

Figure 1.5 Role of Coenzyme Q10 in the mitochondrial respiratory chain …………………….. 48

Figure 1.6 Treatment of the placenta using MitoQ loaded nanoparticles ……………………… 61

Figure 2.1 Experimental design of maternal study …………………………………………….. 70

Figure 2.2 Representative images of the uterine and umbilical artery waveforms ……………. 73

Figure 2.3 Fetal and placental weight on gestational day (GD) 21 …………………………….. 80

Figure 2.4 Uterine artery responses to the vasodilator methacholine (MCh) in the absence or presence of Nώ-nitro-L-arginine methyl ester (L-NAME) ………………………………..… 84

Figure 2.5 Uterine artery responses to the vasoconstrictor phenylephrine (PE) in the absence or presence of Nώ-nitro-L-arginine methyl ester (L-NAME) ………………………….……..……85

Figure 2.6 Mesenteric artery responses to the vasodilator methacholine (MCh) in the absence or presence of Nώ-nitro-L-arginine methyl ester (L-NAME) …………………………..……… 87

Figure 2.7 Mesenteric artery responses to the vasoconstrictor phenylephrine (PE) in the absence or presence of Nώ-nitro-L-arginine methyl ester (L-NAME) ………………………………….. 88

Figure 3.1 Experimental design of fetal and placental study ………………………….….… 100

Figure 3.2 Placental morphology as shown after hematoxylin and eosin (H&E) staining …..... 104

Figure 3.3 Effect of maternal hypoxia and nMitoQ treatment on placental vasculature hemodynamics ……………………………………………………………………….…..... 109

Figure 3.4 Effect of maternal hypoxia and nMitoQ treatment on pregnancy phenotype ……………………………………………………………………………………….….…112
Figure 3.5 Effect of maternal hypoxia and nMitoQ treatment on relative fetal heart weight to body weight ……………………………………………………………………………...…….. 113
Figure 3.6 Effect of maternal hypoxia and nMitoQ treatment on placental reactive oxygen species (ROS) level ……………………………………………………………………………… 117
Figure 3.7 Assessment of fetal and maternal blood space area per field of view in placentas of male fetuses ……………………………………………………………………………………. 121
Figure 3.8 Assessment of fetal and maternal blood space area per field of view in placentas of female fetuses …………………………………………………………………………..…… 122
Figure 3.9 Effect of nMitoQ treatment on the size of mononucleated cardiomyocytes ………. 124
Figure 3.10 Effect of nMitoQ treatment on the size of binucleated cardiomyocytes ………… 125
Figure 3.11 Effect of placental secreted factors on cardiomyocyte size ……………………… 127
Figure 3.12 Effect of maternal hypoxia and nMitoQ treatment on corticosterone level in fetal plasma ……………………………………………………………………………………. 131
Figure 4.1 Experimental design of adult offspring study ……………………………….…… 141
Figure 4.2 Pulmonary valve peak velocity (PV Peak Vel) ……………………………………. 155
Figure 4.3 Blood pressure and heart rate …………………………………………………… 157
Figure 4.4 Mesenteric artery responses to phenylephrine (PE) in male offspring ……………... 159
Figure 4.5 Mesenteric artery responses to phenylephrine (PE) in female offspring …………… 160
Figure 4.6 Mesenteric artery sensitivity to phenylephrine (PE) in male and female offspring …162
Figure 4.7 Mesenteric artery responses to big endothelin-1 (bET-1) in male offspring ……..… 164
Figure 4.8 Mesenteric artery responses to big endothelin-1 (bET-1) in female offspring ……. 165

Figure 4.9 Mesenteric artery responses to methacholine (MCh) in male offspring …………... 168

Figure 4.10 Mesenteric artery responses to methacholine (MCh) in female offspring ………… 170

Figure 6.1 Effect of maternal hypoxia and nMitoQ treatment on myocardial susceptibility to ischemia/reperfusion injury in male and female offspring at 7 months of age …………..…… 197

Figure 6.2 Effect of maternal hypoxia and nMitoQ treatment on myocardial susceptibility to ischemia/reperfusion injury in male offspring at 13 months of age …………………..…..…… 198

Figure 6.3 Effect of maternal hypoxia and nMitoQ treatment on myocardial susceptibility to ischemia/reperfusion injury in female offspring at 13 months of age ……………………...… 199

LIST OF ABBREVIATIONS

^{o}C	Degree Celsius
AA	Arachidonic acid
ACE	Enzyme angiotensin-converting enzyme
ACh	Acetylcholine
Ang II	Angiotensin II
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
a.u.	Arbitrary unit
AUC	Area under the curve
AVP	Arginine vasopressin
bET-1	Big endothelin-1
BP	Blood pressure
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
cADP-ribose	Cyclic adenosine diphosphate-ribose
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanidine monophosphate
CIHR	Canadian Institutes of Health Research
CO	Cardiac output
CO_2	Carbon dioxide
COX-1	Cyclooxygenase-1

DAPI	4',6-diamidino-2-phenylindole
DBP	Diastolic blood pressure
DMEM	Delbecco's modified eagles medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of health and disease
EC_{50}	Effective concentration required to produce 50% of the maximal response
ECs	Endothelial cells
ECG	Electrocardiogram
EDH	Endothelium-derived hyperpolarization
EDTA	Ethylenediaminetetraacetic acid
EDV	End diastolic velocity
EETs	Epoxyeicosatrienoic acids
ELISA	Enzyme linked immunoassay
ET	Ejection time
ET-1	Endothelin-1
eNOS	Endothelial nitric oxide synthase
et al	*Et alii* (Latin; and others)
FABP	Fatty acid-binding protein
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FS	Fractional shortening

g	Gram
GD	Gestational day
GR	Glucocorticoid receptor
GPR30	G protein-coupled receptor 30
GTP	Guanosine-5'-triphosphate
GLUT1	Erythrocyte/HepG2-type glucose transporter
h	Hour/s
H2-DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
HIF-1α	Hypoxia-inducible factor 1-alpha
H_2O	Water
H_2O_2	Hydrogen peroxide
HBSS	Hank’s balanced salt solution
H&E	Hematoxylin and eosin
HEPES	Hydroxyethyl-1-1pipeazineethanesulfonic acid
HR	Heart rate
ICT	Isovolumic contraction time
i.e.	*Id est* (Latin; that is)
IGF	Insulin-like growth factor
IGF-1R	Insulin-like growth factor 1 receptor
IGF-2R	Insulin-like growth factor 2 receptor
IK_{Ca}	Intermediate potassium channels
IL-1	Interleukin-1
IL-6	Interleukin-6

INF-r	Interferon receptor
iNOS	Inducible nitric oxide synthase
InsP3R	Inositol trisphosphate receptor
IP_3	Inositol triphosphate
PI3K	Phosphoinositide 3-kinase
I/R	Ischemia/reperfusion
IRT	Isovolumic relaxation time
IUGR	Intrauterine growth restriction
i.v.	Intravenous
IVCT	Isovolumetric contraction time
IVRT	Isovolumetric relaxation time
IVS	Interventricular septum thickness
JZ	Junctional zone
K^+	Potassium ion
KCL	Potassium chloride
K_2PO_4	Dipotassium phosphate
L-NAME	Nώ-nitro-l-arginine methyl ester
LV	Left ventricle
LVPW	Left ventricular posterior wall
LV Vol	Left ventricular volume
LZ	Labyrinth zone
MAP	Mean arterial pressure
MCP-1	Monocyte chemoattractant protein-1

$MgSO_4$	Magnesium sulfate
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
miRNA	Micro RNA
min	Minute(s)
MIP	Macrophage inflammatory protein
mm	Millimeter
mm HG	Millimeters of mercury
MMPS	Matrix metalloproteinases
MR	Mineralocorticoid receptor
MTOR	Mammalian target of rapamycin
MV A	A wave velocity
MV Decel	Mitral deceleration time
MV E	E wave velocity
MV E/A	Mitral e/a index
n	Number of animals or experiment
Na	Sodium
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
nMitoQ	MitoQ loaded onto nanoparticles
L-NNA	N(g)-nitro-l-arginine
NO	Nitric oxide

NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NormQ	Normoxia + nMitoQ
NormS	Normoxia + saline
O_2	Molecular oxygen
O_2^-	Superoxide anion
PBS	Phosphate buffered saline
PBVA	Placental blood vessel area
PE	Phenylephrine
PFA	Paraformaldehyde
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PH	Logarithmic unit measuring acidity
pHypQ	Prenatal hypoxia + nMitoQ
pHypS	Prenatal hypoxia + saline
PKCε	Protein kinase C epsilon
PLA_2	phospholipase a2
PLC	Phospholipase c
PlGF	Placental growth factor
γ-PGA-graft-L-PAE	γ-glutamic acid-*graft*-L-phenylalanine ethyl ester
PGH_2	Prostaglandin H2
PGHS	Prostaglandin H synthase
PGI_2	Prostacyclin
PKA, C, G	Protein kinase A, C, G

PI	Pulsatility index
PI3K	Phosphatidylinositol-3 kinase
PSV	Peak systolic velocity
P value	Probability (of incorrectly rejecting the null hypothesis)
PV Peak Vel	Pulmonary valve peak velocity
R	Receptor
RAHF	Royal Alexandra Hospital Foundation
RANTES	Regulated on activation, normal T cell expressed and secreted
RAS	Renin-angiotensin system
RI	Resistance index
ROS	Reactive oxygen species
s	Second(s)
SBP	Systolic blood pressure
SCHF	Stollery children's hospital foundation
S/D	PSV to EDV ratio
SEM	Standard error of the mean
sFlt-1	Soluble Fms-related tyrosine kinase-1
SK_{Ca}	Small potassium channels
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
STBEVs	Syncytiotrophoblast extracellular vesicles
TAV	Time averaged velocity
Tei index	Myocardial performance index

TIMPs	Metalloproteinases tissue inhibitors
TNF-α	Tumor necrosis factor-alpha
TP	Thromboxane receptor
TXA2	Thromboxane A2
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
WCHRI	Women and Children's Health Research Institute
WHO	World Health Organization
w/w	Weight / Weight

CHAPTER 1: GENERAL INTRODUCTION

Part of this chapter has been published:

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1.1 DEVELOPMENTAL PROGRAMMING OF CARDIOVASCULAR DISEASE, AN OVERVIEW

Cardiovascular disease is the primary cause of death worldwide claiming over 17 million lives per year (4). In Canada, cardiovascular disease claimed over 48,000 lives in 2012. Furthermore, 2.4 million Canadians who are aged 20 and over are suffering from cardiac disease, which makes it one of the costliest diseases (the cost was 21.2 billion Canadian dollars in 2000) (2, 334). In the United States, the annual cost of cardiovascular disease was about 300 billion American dollars in 2011 and projected to exceed 800 billion American dollars in 2020 (220). Therefore, due to the global impact and high cost of cardiovascular disease, there is a need to find an intervention to reduce its incidence and limit its debilitating effects.

Many factors can increase the risk of developing cardiovascular disease including smoking, obesity and low physical activity. More recently, pregnancy complications have been established as risk factors of cardiovascular disease, and the concept was termed as developmental programming of cardiovascular disease (25, 26). Developmental programming is explained by fetal adaptions to a suboptimal environment at the expense of the developing organs (such as the heart), which leaves these organs vulnerable and offspring more susceptible to cardiovascular disease (24).

One of the most established factors that can cause a suboptimal environment in fetal life and offspring cardiovascular dysfunction is maternal and/or fetal hypoxia [reviewed in (11)]. The mechanisms of how hypoxia in pregnancy can increase offspring susceptibility to cardiovascular disease are not fully understood. However, it was suggested that hypoxia in pregnancy can increase oxidative stress level in the placenta and maternal and fetal body. Thus, causing changes in the

normal physiology of pregnancy and the normal surrounding environment of the fetus, resulting in detrimental effects on the offspring [reviewed in (11, 338)].

As oxidative stress is important in the pathophysiology of developmental programming, antioxidant interventions have been used in human clinical trials, and in animal models of pregnancy complications to assess the effect of reducing oxidative stress on offspring health and development (113, 146). These antioxidant interventions showed encouraging results in preventing developmental programming. However, antioxidant treatment in pregnancy has not always been successful in improving pregnancy outcome in complicated pregnancies and in some cases antioxidant therapeutics had detrimental effects on the offspring of control groups in normal pregnancy [(33, 97, 113), and reviewed in (216)]. For example, randomized controlled trials have been performed to assess the potential benefits of antioxidant therapy on women suffering from compromised pregnancies. These trials have failed to demonstrate improved pregnancy outcomes such as intrauterine growth restriction (IUGR; fetus does not reach its genetic growth potential) or neonatal mortality [reviewed in (216)]. Furthermore, while the antioxidant tempol prevented IUGR in endothelial nitric oxide synthase (eNOS) knockout mice, it also caused an increase in fetal body weight of control C57Bl/6J mice (325), which is considered a detrimental effect [increases vulnerability to transgenerational obesity] (103). Maternal supplementation of vitamin C in rats resulted in improved vascular function in offspring of hypoxic pregnancy but led to vascular endothelial dysfunction in the offspring of normal pregnancy (113). Therefore, the safety of applying antioxidants during gestation should be taken into consideration, and a new strategy should be developed to prevent oxidative stress in pregnancy and improve fetal outcome without compromising normal fetal physiology. Consequently, and in order to avoid direct exposure of the therapeutic to the fetus, placental-targeted antioxidant treatment was proposed in pregnancy

complications to improve fetal development (156, 249). Choosing placenta as a target to prevent oxidative stress was proposed because the placenta is essential for supplying nutrients and oxygen to the fetus, and it is an important source of hormones and secreted factors that impact both the maternal body and fetal development. A study in our laboratory showed that scavenging $O_2^{\cdot}$ using superoxide dismutase (SOD) restored endothelium-dependent vasodilation in uterine arteries from pregnant rats that were exposed to plasma collected from women with preeclampsia. Confirming that circulating factors can lead to vascular endothelial dysfunction (147). Furthermore, Phillips *et al*. showed that abnormal placental secreted factors can affect fetal neural development (249). Thus, an abnormal increase in placental oxidative stress can have a detrimental effect on placental function and consequently on fetal development and offspring well-being [reviewed in (11), Figure 1.1].

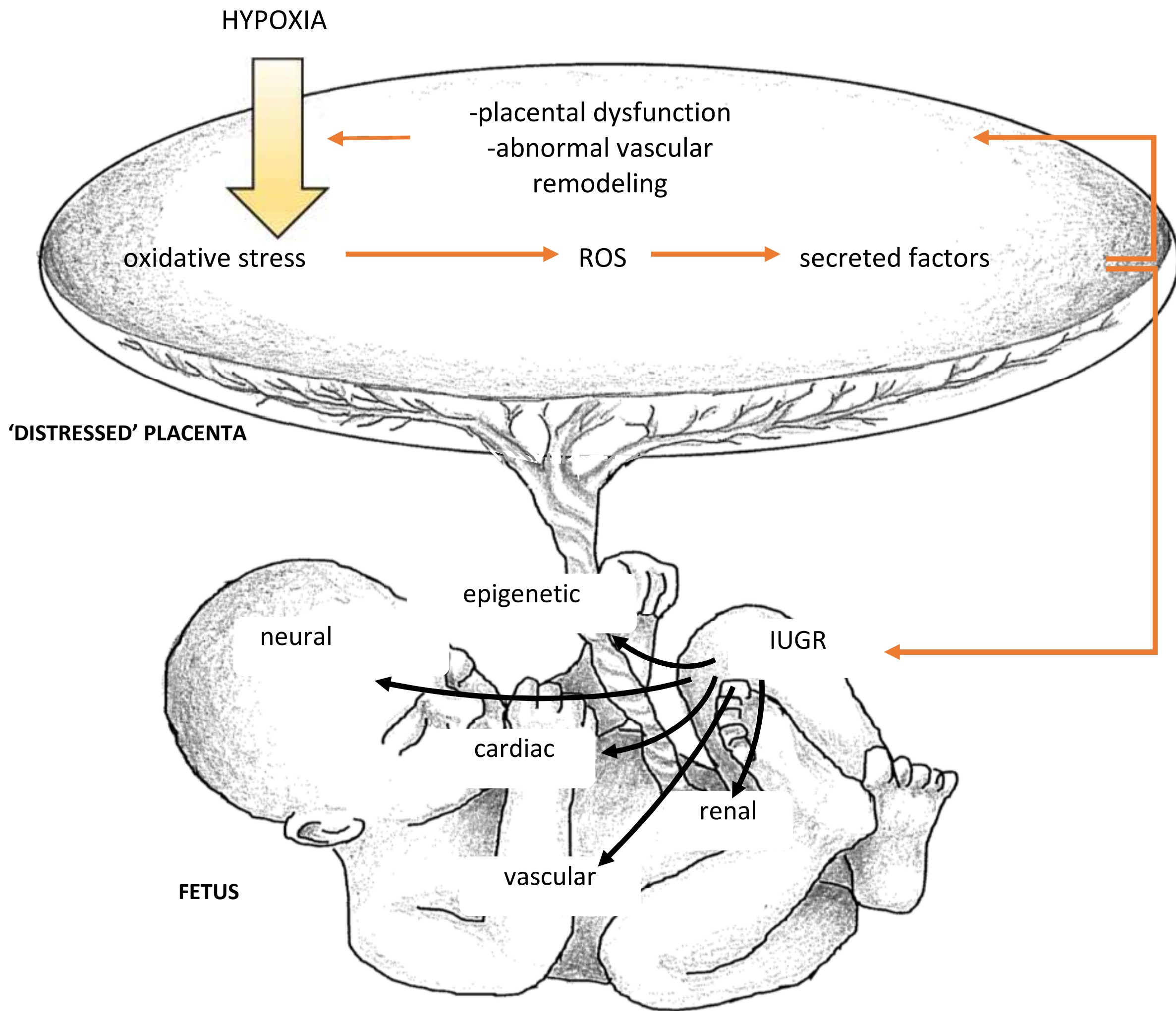

Figure 1.1 Perinatal hypoxia and linkages to developmental programming

Hypoxia in fetal life can lead to increased placental oxidative stress and production of reactive oxygen species (ROS). As a result, the placenta releases factors both into the maternal circulation system, causing abnormal placental function, and into the fetal circulation system, which are associated with intrauterine growth restriction (IUGR) and fetal programming of cardiovascular disease. Mitochondria are a major source of placental oxidative stress and may provide a therapeutic target to mitigate the detrimental effects of placental oxidative stress on pregnancy outcomes. Aljunaidy *et al*. 2017 (11).

Phillips *et al*. showed that loading the mitochondrial antioxidant MitoQ onto nanoparticles can restrict MitoQ treatment to the placenta and prevent it from crossing the placental barrier to the fetus (due to the size and the charge of the nanoparticles) (249). Phillips *et al*. also showed that MitoQ loaded onto nanoparticles (nMitoQ) can prevent placental oxidative stress, rescue neonate growth and prevent abnormal development of fetal neurons in hypoxic pregnancy in rats (249). In this PhD project, the effect of hypoxia in pregnancy on maternal and placental hemodynamics and on offspring cardiovascular function were investigated. Further, the effects of placental-targeted antioxidant treatment (nMitoQ) on fetal cardiomyocyte development and offspring cardiovascular function later in life were assessed. Overall, the current PhD project aimed to advance our knowledge about the possible mechanisms that could be involved in the pathophysiology of developmental programming of cardiovascular disease and to propose a potential target for intervention.

1.2 CARDIOVASCULAR PHYSIOLOGY IN FETAL AND ADULT LIFE

Understanding cardiac development and function is important to understand offspring cardiovascular dysfunction.

1.2.1 Cardiomyocyte development and mediators in fetal life

After cardiogenesis, the fetal heart grows due to an increase in the number of cardiomyocytes (hyperplasia). Near-term and shortly after birth, cardiomyocytes become binucleated, and binucleated cardiomyocytes are mature cells that exit the cell cycle and no longer can proliferate due to the absence of karyokinesis and/or cytokinesis (169, 241). Once the cardiomyocytes are fully developed, the increase in heart size becomes dependent on the cardiomyocyte hypertrophy and damaged cardiomyocytes cannot be replaced by hyperplasia.

Binucleation is considered an indicator of cardiomyocyte maturity (241). In rats, a small percentage of binucleation can be seen in fetal life. Only 5% of cardiomyocytes are binucleated on gestational day (GD) 21 (term is GD 22) in rats. However, the percentage of binucleation becomes 50% between birth and day 7 of life and 80-90% by the third postnatal week (169). Unlike rats, cardiomyocytes in sheep and humans are mostly developed before birth. At GD 77 in sheep, 2% of the myocytes are binucleated (term is 150 days). The percentage of binucleation increases to 50% at GD 135 and becomes 90% at 4-6 weeks after birth (40). In humans, 90% of the cardiomyocytes are binucleated in late gestation while 97% of them become binucleated at week 7 of life (5). There are many factors that contribute to fetal cardiomyocyte development. The most established factors include aortic load pressure, insulin-like growth factors, corticosterone and angiotensin (Ang) II.

1.2.1.1 Aortic load pressure

Cardiomyocytes enlarge in response to an increase in systolic pressure in the fetal heart to the degree needed to normalize wall stress and this is called physiological hypertrophy. In fetal sheep, for example, when the pulmonary artery is mildly constricted near term, the systolic pressure increases leading to an increase in the proliferation and hypertrophy in the ventricle wall of the fetal heart; an increase in the wall thickness and in the number of cardiomyocytes that undergo terminal differentiation and become binucleated (23). Whether the reduction in systolic load can slow the growth and maturation of fetal cardiomyocytes was also investigated in sheep by blocking the angiotensin-converting enzyme which converts Ang I into Ang II. This led to a reduction in fetal arterial pressure and systolic cardiac load and to a reduction in the percentage of cells active in the cell cycle. However, fetuses which experienced a prevention in Ang II production showed no difference in their cardiomyocyte size compared to those from those with normal Ang II production which disproves the suggestion that the reduction in systolic pressure prevents normal cardiomyocyte hypertrophy (230). Diastolic cardiac load beside systolic load can also affect cardiac development. For example, in near-term ovine fetuses, increasing the arterial and venous pressure by infusing sterile sheep plasma led to an increase in the relative heart weight to body weight; and caused an increase in the size and percentage of binucleated cardiomyocytes compared to the control cardiomyocytes from normal pregnancies (139).

1.2.1.2 Growth factors

Growth factors are established mediators of cardiomyocyte development. For instance, insulin-like growth factors-1 and -2 (IGF-1 and IGF-2; proteins that have a similar molecular structure of insulin) are synthesized in the placenta and fetal liver, and play an important role in the placental growth and fetal development (32). Maternal IGFs can affect placental growth and

placental nutrient transporters and thus fetal growth, but they do not cross the placental barrier in physiologically significant quantities (308). IGFs transcripts and peptides can be detected in almost all fetal tissues from very early stages of fetal development (pre-implantation) until the final stages of organ maturation [reviewed in (6)]. IGFs are also present in syncytiotrophoblast and cytotrophoblast throughout pregnancy (32, 67, 341). IGFs work by activating the protein kinase B (PKB) signaling pathways, a stimulator of cell growth and proliferation, and a strong inhibitor of programmed cell death (374). IGFs have two cell-surface receptors; the insulin-like growth factor 1 receptor (IGF-1R) and the insulin-like growth factor 2 receptor (IGF-2R). IGF-1R is a receptor tyrosine kinase which means it signals by causing the addition of a phosphate molecule on specific tyrosines and signals through different pathways. Both IGF-1 and IGF-2 can bind with the IGF-1R. However, only IGF-2 can bind with IGF-2R which lacks signal transduction capacity, and its main role is to make less IGF-2 available for binding with IGF-1R.

Effects of IGF on fetal cardiac development were shown in many studies. Transgenic mice, for example, with enhanced cardiac IGF1/PI3K (phosphoinositide 3-kinase) signaling can develop cardiac hypertrophy (313). On the other hand, mice with reduced cardiac IGF1/PI3K signaling have smaller hearts than the control group (183). Furthermore, IGF-1 plays a role in cardiomyocyte hyperplasia. In transgenic mice, heart over-expression of IGF-1 led to a significant increase in cardiomyocyte hyperplasia (262). IGF-2 signaling can also affect cardiomyocyte hyperplasia, and can activate the ERK (extracellular signal-regulated kinases) proliferation pathway in the developing heart (173). Disruption of the IGF-2 gene in mice, for example, resulted in fetal ventricular wall hypoplasia due to the reduction in cardiomyocyte proliferation (173).

Another regulator of cardiomyocyte proliferation is fibroblast growth factor-2 (FGF-2). FGF-2 mediates its growth effects through its myocardium fibroblast growth factor receptor-1

(FGFR-1) which is the major FGF receptor in the heart. Abnormal function or expression of FGF-2 or FGFR-1 can lead to abnormal cardiomyocyte proliferation. For example, in mouse embryos, deficiency in FGFR-1 caused severe cardiomyocyte hypoplasia (165). In chick embryonic ventricular wall, FGF2 synthesis increased in cardiomyocytes in response to increased stretch due to pressure overload; and the inhibition of FGF2 signaling decreased myocyte proliferation (161). In neonatal rats, an increase in FGFR-1 stimulated an increase in FGF-2-mediated proliferation of cardiac myocyte cultures (311).

1.2.1.3 Glucocorticoids

Glucocorticoids are steroid hormones produced by fetal adrenal cortex, and are essential for fetal tissue and organ maturation (83). Glucocorticoids have two cytoplasmic receptors that are expressed in fetal heart. These receptors are the widely distributed receptor; glucocorticoid receptor (GR, crucial for fetal heart maturation), and the less widely distributed receptor; mineralocorticoid receptor (MR, crucial for adult cardiac remodeling) (279). Both GR and MR mediate their effects through binding to DNA, but rapid non-genomic signaling has been reported as well [reviewed in (39, 80)].

The role of glucocorticoids (cortisol in humans and sheep, and corticosterone in rats) in fetal heart maturation and function has been reported (267, 277, 279). For example, exposing primary mouse fetal cardiomyocytes to physiologically relevant glucocorticoids levels *in vitro*, improved cardiomyocyte contractility, promoted Z-disc assembly and increased mitochondrial activity compared to the control group that was not exposed to glucocorticoids (277). These glucocorticoid effects on cardiomyocytes *in vitro* were partly mediated by genomic transcription of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α, regulates cardiac mitochondrial capacity) (277). Further assessment of glucocorticoids role in

cardiomyocyte development came from *in vivo* studies. For example, disruption of GR in vascular smooth muscle cells and cardiomyocytes in mice, led to a longer initial phase of cardiac contraction and a disrupted myocardium components (disorganized and short myofibrils) in the fetuses (279). Furthermore, exogenous glucocorticoids had various other effects as well on fetal cardiomyocyte size, proliferation and binucleation [reviewed in (278)]. For example, antenatal glucocorticoid administration in pregnant rats [dexamethasone, 0.2 or 0.8 mg/kg/day subcutaneously, GD 17-19; (320)] and sheep [cortisone, fetal coronary artery infusion of 25 mL/min for 72-80 h, GD 128; (287)], led to cardiomyocyte hypertrophy, and to reduced cardiomyocyte proliferation compared to those that did not receive the treatment. In fetal sheep, exogenous glucocorticoid [cortisol, fetal coronary artery infusion of 0.5 microg/kg.min for 7 d starting at GD 122; (112)] led to an increased cardiomyocyte proliferation but did not alter cardiomyocyte size and binucleation compared to fetuses that did not receive glucocorticoid treatment. In pregnant rats, dexamethasone treatment [48 microg/d, GD 17-20; (347)] increased heart weight relative to body weight, increased cardiomyocyte proliferation and reduced ventricular extracellular matrix compared with control pups. Assessment of sex-specific response in the same study (347) showed that in rats treated with dexamethasone, the proportion of proliferated cardiomyocytes in female fetuses was greater than in male fetuses. In addition, ventricles of male fetuses treated with dexamethasone contained lower levels of alpha-MHC mRNA compared to female fetuses, showing a sex-difference in the cardiomyocyte response to glucocorticoids.

In addition to affecting fetal cardiomyocyte development, using glucocorticoids in pregnancy can also have long-term consequences on offspring cardiovascular function. For example, adult offspring of rats treated with dexamethasone in pregnancy had a higher blood pressure when compared to offspring of rats that did not receive the dexamethasone treatment (31).

Furthermore, using corticosteroids during pregnancy in women have moderately increased systolic and diastolic blood pressures of the offspring at 7 (366) and 14 (81) years of age compared to those children of women who did not receive corticosteroids during pregnancy.

1.2.1.4 Angiotensin II

Ang II is also important for fetal cardiac growth (228, 376). The Ang II effect on the fetal heart is mainly mediated via Ang II receptor subtype I (AT1-R) through the activation of ERK1/2 pathway (376). For example, in fetal sheep, infusion of Ang II led to an increase in the fetal cardiac mass by increasing the cardiomyocyte size, number, and differentiation (298). The same study showed that the effect of Ang II on increasing cardiomyocyte size was mechanical-load dependent (due to the increase in the fetal blood pressure) and the effect of Ang II on increasing the cardiomyocyte number was load-independent and resulted from a direct effect of Ang II on the heart (298). Furthermore, Ang II can lead to hypertrophy in cultured neonatal rat cardiomyocytes (52). In neonatal rat as well, Ang II induced an increase in cADP-ribose (cyclic adenosine diphosphate-ribose) synthesis in the cardiomyocytes during days 2–4 after birth. cADP-ribose works as a second messenger downstream of Ang II receptors, providing additional proof of Ang II involvement in cardiac development (126).

1.2.2 Fetal cardiac circulation

After the initial cardiac development, fetal heart starts beating and becomes functional as early as 4 weeks of pregnancy in humans [term is gestational week 40; (212)] and GD12 in rats (377). Blood is pumped by the heart through the vascular system. In fetal life, lungs are not used to obtain oxygen; rather, oxygen is taken from the mother through the placenta and carried through the umbilical vein and inferior vena cava to the right atrium of the fetal heart. Blood, then, goes through the foramen ovale to the left atrium and is pumped through the aorta to the body. Some

blood which enters the right atrium does not pass to the left atrium but is pumped through the right ventricle to the pulmonary artery and passes through ductus arteriosus to the aorta, thus bypassing pulmonary circulation. At birth, resistance in the pulmonary vasculature decreases and blood circulation becomes divided into the systemic and pulmonary circulations. The pulmonary circulation collects oxygen from the lungs while systemic circulation transports oxygen to the body and returns deoxygenated blood to the pulmonary circulation. Systemic and pulmonary circulations in the newborn continue to function in the same way throughout life.

1.2.3 Cardiac physiology in adult life

In adult life in humans, the cardiac cycle is known as the period that starts with contraction of the atria and ends with ventricular relaxation. The period of cardiac contraction is named systole, and the period of cardiac relaxation is named diastole. Atrial wall depolarization is followed by atrial contraction. When this contraction happens, the ventricles are already filled with 80% of their normal capacity of blood due to inflow in diastole. Atrial contraction contributes to the remaining 20% of ventricle filling and ends prior to ventricular systole. Ventricle wall depolarization then starts, leading into ventricular systole, and the blood is pumped from the heart through the pulmonary and aortic valves. The volume of the blood pumped is referred to as stroke volume which is about 70–80 mL in humans. Ventricles normally contain about 130 mL of blood at the end of atrial systole which is referred to as end-diastolic volume (EDV) or preload. This means, following ventricle systole there is still about 50–60 mL of blood remaining in the ventricle, which is referred to as the end systolic volume (ESV). Ventricular repolarization is followed by ventricular diastole. Ventricles at this point are relaxed and pressure of the blood inside them drops below the pressure in the atria, leading to blood flow from the atria to the ventricles, followed by diastole of the atria and the end of the cardiac cycle (363).

The amount of blood pumped by each ventricle in one minute is defined as cardiac output which is calculated using the equation: cardiac output = heart rate X stroke volume. This means that factors which affect heart rate and stroke volume, also affect cardiac output. Cardiac nerves (sympathetic and parasympathetic) are major factors that can affect heart rate. There are other factors that slow or increase the heart rate such as, hormones (epinephrine), body temperature, metabolites produced by myocardial cells (adenosine) and plasma electrolyte concentrations. Stroke volume, on the other hand, is affected by three major factors, which are preload, afterload and contractility. Preload reflects EDV which means when EDV increases, preload increases too. The key determinant of EDV value is ventricular filling time. The faster the systole, the shorter the filling time and the lower the EDV and preload. Afterload, on the other hand, is tension inside the ventricles which is important for pumping blood throughout the vascular system. When there is an increase in resistance due to factors such as valve stenosis or high blood pressure, the afterload becomes higher. Contractility of the heart also determines stroke volume by affecting ESV. The higher the myocardium contraction the greater the stroke volume is and the lower the ESV. Contractility is monitored by sympathetic stimulation via the release of norepinephrine from cardiac nerves leading to an increase in cardiac contractility; and parasympathetic stimulation which stimulates the release of acetylcholine (ACh) from the vagus nerve leading to a reduction in contractility (363).

In clinical and animal research, echocardiography is widely used to assess cardiac morphology and function (245). Thus, the next paragraph will briefly discuss using echocardiography for cardiac morphology and function assessment in adults.

1.2.4 Assessment of cardiac morphology and function using echocardiography in adult life

Echocardiography provides information about the internal chamber size of the heart, pumping capacity and other estimates of heart function, such as a calculation of the cardiac output, stroke volume, ejection fraction, and diastolic function. Echocardiography helps in detecting cardiomyopathies, such as cardiac hypertrophy (an increase in the left ventricular mass) and cardiac dilation (an increase in the anterior-posterior diameter or the volume of the left ventricle) (367). For example, echocardiography can measure the ejection fraction which is defined as the percentage of the end-diastolic volume ejected in the systole. A low ejection fraction is an indication of left ventricular systolic dysfunction. Diastolic function, on the other hand, can be assessed using the E/A ratio. Normally, when blood flow across the mitral valve is assessed, two flow waves can be seen. The first one represents passive filling of the ventricle and it is called the E wave. The second wave represents active filling derived from atrial systole and this wave is called the A wave. In normal conditions, the E wave is slightly higher than the A wave. When the A wave is higher than the E wave this could be due to a slow filling of the left ventricle, due to old age, diastolic dysfunction, left ventricle hypertrophy or hypertension. A small or absent A wave is an indication of restrictive cardiomyopathy (the heart pumps normally but it does not relax normally). Generally, diastolic dysfunction as demonstrated by echocardiography is presented as a normal left ventricle cavity size, thickened ventricle wall, and reversed E/A ratio (A wave is higher than E wave) (Figure 1.2) (1).

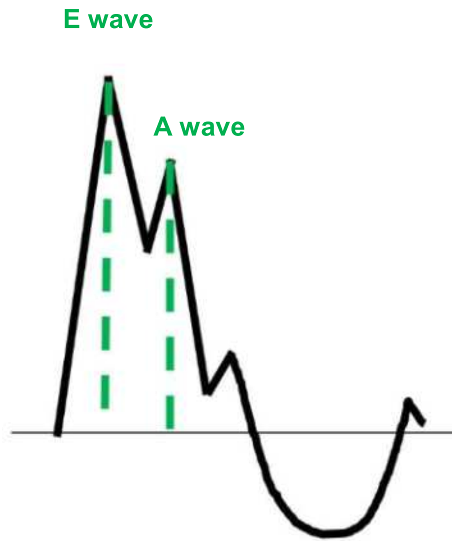

Figure 1.2 Representative image of mitral valve waveforms

A schematic image represents mitral valve waveforms in an adult rat as assessed using echocardiography. Waveforms show both E mitral wave (E wave) and A mitral wave (A wave).

Echocardiography can also be used to assess the shape and function of cardiac valves (1). These include an assessment of the valves' area, calcification, and blood flow velocity. For example, valvular stenosis could be associated with an increase in blood flow velocity, while, valve regurgitation could be associated with a decline in the pressure gradient or with an increase in the regurgitant jet (1).

1.2.5 Vascular physiology in adult life

As developmental programming can affect the whole cardiovascular system, understanding systemic vascular physiology of the circulatory system is as important as understanding cardiac physiology. Thus, normal systemic vascular physiology in adult life will be discussed herein.

1.2.5.1 Arterial function and structure

The vascular system is responsible for delivering an adequate amount of blood throughout the body. Small resistance-sized arterioles have the greatest influence on systemic vascular resistance and on local blood flow, which are crucial determinants of the overall peripheral blood pressure. The vascular wall consists of many layers of vascular smooth muscle cells (VSMC) surrounding one layer of endothelial cells (endothelium; ECs) and covered by adventitia (collagen fibres).

The vascular endothelium layer is important in controlling VSMC relaxation or contraction which alters the vascular tone. Thus, effects of vascular endothelium on vascular relaxation and contraction are discussed below [reviewed in (91, 158)].

1.2.5.2 Mechanisms of vascular smooth muscle cell relaxation and contraction

An increase or decrease in the calcium ion (Ca^{+2}) level in VSMC cytosol plays a predominant role in vascular contraction or relaxation, respectively [reviewed in (127)].

Excitation-contraction coupling is defined as a physiological process of turning an electrical stimulus into a mechanical response. Vasodilators and vasoconstrictors (such as endothelium-derived factors, neurotransmitters, and circulating hormones) change the level of Ca^{+2} in VSMC cytosol through electrochemical and pharmacomechanical coupling (8).

The electrochemical coupling is initiated by altering the membrane potential, which is defined as a voltage created by the difference in concentrations of ions on opposite sides of a cellular membrane. Membrane potential is regulated by ion channels. The opening of VSMC potassium channels, for instance, results in the diffusion of potassium cation from outside the cell, where the electrochemical gradient is higher, to the inside of the cell which leads, in turn, to hyperpolarization. This hyperpolarization then closes voltage-gated calcium channels and causes vasorelaxation.

Pharmacomechanical coupling, however, does not necessarily happen due to changes in the membrane potential. Rather, pharmacomechanical coupling occurs through targeting intracellular calcium stores. This happens when vasoconstrictors (such as endothelin-1 and noradrenaline) stimulate VSMC by activating phospholipase C to split the phospholipid component of the plasma membrane phosphatidyl 1, 5 inositol diphosphate into two messenger factors inositol triphosphate (IP3) and diacylglycerol (DAG) [reviewed in (330)]. IP3 binds to the inositol trisphosphate receptor (InsP3R) on the sarcoplasmic reticulum and releases Ca^{+2} into the cytosol. DAG, however, works by activating protein kinase C (PKC) which is involved in the phosphorylation and activation of the contractile machinery (214).

An increase in intracellular calcium derived from cellular stores can also interact directly with the receptor-activated calcium channels, and lead to an increase in the level of Ca^{+2} in the

VSMC cytosol through an extracellular calcium influx [reviewed in (127)]. The increase in intracellular calcium levels also activates myosin light chain kinase (MLCK), which phosphorylates the myosin light chain (MLC). Phosphorylation of the MLC activates myosin ATPase by actin, causing the formation of actin-myosin cross-bridges and consequently VSMC contraction. Myosin light chain phosphatase (MLCP) de-phosphorylates the phosphorylated light chains, returning myosin to its inactive state and resulting in VSMC relaxation [reviewed in (248)].

To allow VSMC relaxation and maintenance of vascular homeostasis, Ca^{+2} must be removed from the cytosol. Ca^{+2} extrusion from the cytoplasm happens by activating the plasma membrane calcium ATPase pump, which pumps Ca^{+2} out of the cytosol into the extracellular milieu, and also by activating the sarcoendoplasmic reticulum calcium ATPase pump, which returns calcium to its intracellular stores [reviewed in (127)]. The vascular endothelium produces factors that initiate VSMC relaxation and thus is crucial for fine-tuning VSMC responses.

1.2.5.3 Endothelial mediators of VSMC relaxation

The vascular endothelium is strategically located in direct contact with the circulating blood. Thus, the vascular endothelium interacts directly with physical factors such as an increase in shear stress. Vascular endothelium as well interacts with endogenous mediators such as, bradykinin, substance P, acetylcholine, adrenomedullin and histamine which are produced by different sources, but they can arrive to the endothelium through circulation or neurotransmission. These physical and endogenous factors activate specific receptors on endothelial cells resulting in the production and release of vasodilator molecules.

There are three major pathways that control endothelium-dependent arterial vasodilation. These are nitric oxide, prostacyclin and endothelium-derived hyperpolarization (Figure 1.3).

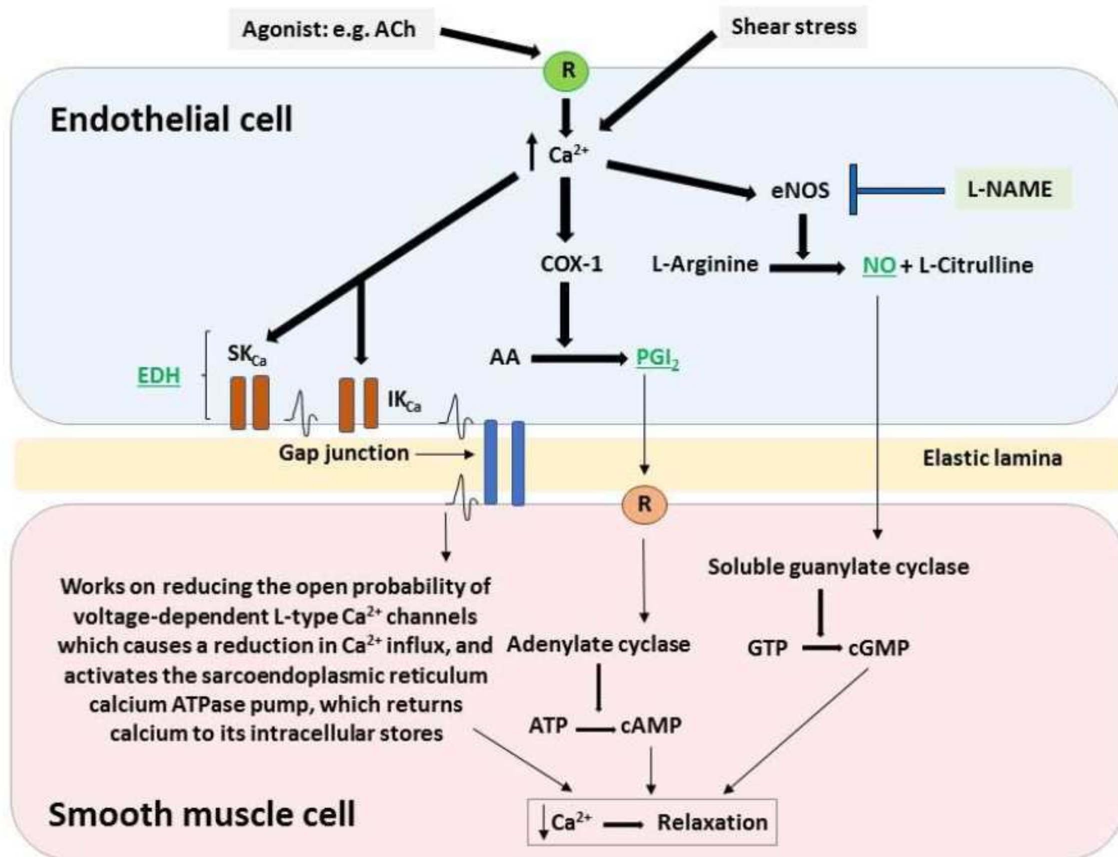

Figure 1.3 Endothelium-dependent vasodilation pathways

The vascular endothelium initiates vascular smooth muscle cell relaxation through three endothelium-dependent vasorelaxation pathways [nitric oxide (NO), prostacyclin (PGI2) and endothelium-derived hyperpolarization (EDH)]. ACh: acetylcholine, Ca^{2+}: calcium ion, R: receptor, eNOS: endothelial nitric oxide synthesis, COX-1: cyclooxygenase-1, AA: arachidonic acid, SK_{Ca}: small potassium channels, IK_{Ca}: intermediate potassium channels, L-NAME: N^G-nitro-L-arginine methyl ester, GTP: guanosine triphosphate, cGMP: cyclic guanosine-3,5-monophosphate, ATP: adenosine triphosphate, cAMP: cyclic adenosine monophosphate, schematic waves: hyperpolarization.

The relative contribution of each one of these endothelium-dependent vasodilation pathways depends on the species and vascular bed, but generally nitric oxide is dominant in larger conduit arteries such as aorta whereas endothelium-derived hyperpolarization is dominant in smaller resistance vessels, whereas prostacyclin (PGI_2) contribution increases when nitric oxide is reduced in pathological conditions (312).

Nitric oxide is a free radical and its half-life is very short (seconds) due to its rapid interaction with free radicals such as superoxide ($O_2^{\cdot}$). Nitric oxide acts as a very potent vasodilator. Along with its function as a vasodilator, nitric oxide has many biological actions that involve endogenous defenses against vascular inflammation and thrombosis, such as the inhibition of smooth muscle proliferation, platelet aggregation and leukocyte adhesion (90).

Stimulation of the nitric oxide vasorelaxation pathway requires an increase in endothelial Ca^{2+} levels. For instance, shear stress or agonists such as ACh or bradykinin can activate endothelial receptors leading to an increase in IP_3 generation which then releases Ca^{2+} from stores in the cytosol and increases the intracellular Ca^{2+} level. This increase in the Ca^{2+} level stimulates binding of calmodulin to eNOS and results in the production of nitric oxide from L-arginine. Nitric oxide is a gas that diffuses to the VSMC and activates soluble guanylate cyclase, a heme-dependent enzyme which converts guanosine-5'-triphosphate (GTP) into cyclic guanosine-3,5-monophosphate (cGMP). Subsequent activation of protein kinase G leads to VSMC relaxation through several mechanisms, namely by decreasing the binding of vasodilatory-stimulated phosphoprotein to actin filaments, decreasing Ca^{2+} sensitivity of contractile proteins, activation of large-conductance Ca^{2+}-activated K^+ channels to release K^+ outside of the cell, and reducing the Ca^{2+} inflow through L-type Ca^{2+} channels all of which result in a fall in Ca^{2+} levels and consequently dephosphorylation of myosin light chain (contractile proteins) [reviewed in (177)].

The short-term increase in nitric oxide is intracellular Ca^{2+}-dependent. However, once Ca^{2+} levels decrease additional mechanism can regulate nitric oxide production. This Ca^{2+}-independent mechanism works by phosphorylation of eNOS *via* protein kinases (such as PKA and cGMP protein kinase dependent II) [reviewed in (299)].

The nitric oxide synthase (NOS) is responsible for nitric oxide production, has three isoforms. Neuronal NOS (nNOS) and eNOS, both which are calcium-dependent enzymes; and inducible NOS (iNOS) which is regulated by pro-inflammatory cytokines and is calcium-independent [due to its tight non-covalent interaction with calmodulin and Ca^{2+} (73)]. All three NOS isoforms are expressed in the endothelial cells and smooth muscle cells. Similarly to the endothelium-derived nitric oxide, VSMC-derived nitric oxide can enhance vasorelaxation and regulate platelet reactivity (304). The expression of NOS in VSMC is mediated by factors coming from the blood and the vascular endothelial cells. Thrombin, transforming growth factor beta and insulin-like growth factor inhibit the NOS expression in the VSMC while plasmin, epidermal growth factor and fibroblast growth factor enhance the VSMC-derived NOS expression (304). Additionally, to the endothelium and VSMC expression, NOS isoforms are also expressed in many other cell types. For example, nNOS is expressed in perivascular nerves; iNOS has been detected in nucleated cells in the cardiovascular system, leukocytes, and mast cells; and eNOS is expressed in cardiomyocytes and platelets (90).

Nitric oxide opposes tonic adrenergic stimulation continuously. Thus, nitric oxide maintains a basal state of vasorelaxation in the vascular wall. Furthermore, NOS inhibition [by using N^G-nitro-L-arginine methyl ester (L-NAME) for example] can induce vasoconstriction, which shows the important role of nitric oxide in the general maintenance of normal vascular homeostasis. The role of nitric oxide in opposing adrenergic effects has been established and

shown when intravenous bolus administration of L-NAME in normal rats *in vivo* led to an acute increase in hepatic arterial vascular resistance, suggesting that nitric oxide serves as a potent vasodilator in the hepatic arterial circulation (239). Another example of nitric oxide is importance in maintaining normal vascular homeostasis was shown when eNOS knockout in mice led to hypertension (high blood pressure) (133).

Alongside enhancing endothelium-dependent vasorelaxation by stimulating NOS, the increase in endothelial Ca^{2+} also stimulates the cyclooxygenase pathway leading to the formation of PGI_2. Although PGI_2 works as a vasodilator, it is often thought that the overall contribution of endothelium-derived PGI_2 to basal systemic vascular tone and blood pressure, may depend on whether the release of the other vasodilator, nitric oxide, is intact or not [reviewed in (329)]. For example, when nitric oxide bioavailability is low, an increase in the role of endothelium-derived PGI_2 has been shown to prevent the increase in vascular resistance due to loss of nitric oxide. This compensatory role was observed *in vivo* in gracilis muscle arterioles of eNOS knockout mice (329), and in mesenteric arteries of rats (125) and coronary arteries of dogs (255) undergoing chronic inhibition of eNOS. Furthermore, unlike nitric oxide which is released continuously, prostacyclin is released in a transient manner (206).

PGI_2 is produced in the endothelial cells as a result of arachidonic acid metabolism. Arachidonic acid is normally released from a phospholipid molecule in the plasma membrane by the enzyme phospholipase A2 (PLA_2), that is responsible for splitting fatty acids. Arachidonic acid is then converted into prostaglandin H2 (PGH_2) through Ca^{2+} activated cyclooxygenases 1 and 2 (COX-1 and -2), which results in the formation of PGI2 and thromboxane A2 (TXA2).TXA2 binds to thromboxane receptor (TP) on the VSMC and causes vasoconstriction. PGI2, however, binds to either TP receptor and causes vasoconstriction, or to the prostacyclin receptor (IP, higher affinity)

and causes vasorelaxation. PGI_2 causes VSMC relaxation by activating adenylate cyclase to form adenosine monophosphate (AMP). In its turn, AMP enhances protein kinase A to stimulate plasmalemmal and sarcoplasmic reticulum Ca-ATPases to increase Ca^{2+} extrusion and reuptake, causing VSMC relaxation [reviewed in (190)].

The nitric oxide and PGI_2 pathways can affect each other. For example, nitric oxide reacts with $O_2^{\cdot}$ and forms the oxidant peroxynitrite. In turn, peroxynitrite can selectively inhibit PGI_2 in the endothelium by causing a catalytic reaction of the iron-thiolate center of PGI_2. Additionally, peroxynitrite can activate both TXA2-synthase and COX, causing a reduction in PGI_2 production and thus an inhibition in PGI_2-dependent vasorelaxation (385). Therefore, the link between peroxynitrite and PGI_2 shows that the PGI_2 pathway can be affected by physiopathological actions of nitric oxide.

Alongside nitric oxide and PGI2 pathways, endothelium-derived hyperpolarization (EDH) is an established mediator of endothelium-dependent vasorelaxation. EDH has a potent vasodilator effect on small resistance arteries, and thus is considered as an important regulator of blood pressure. EDH stimulates its vasorelaxation effect by enhancing VSMC hyperpolarization. Therefore, EDH pathway is insensitive to nitric oxide and PGI_2 pathways inhibitor but the EDH effect can be abolished by applying depolarizing concentrations of potassium (92).

An increase in endothelial Ca^{2+}, however, is still required for EDH pathway stimulation. Cytosolic Ca^{2+} activates endothelial small and intermediate potassium channels (SK_{Ca} and IK_{Ca} respectively; EDH pathway). The opening of these channels [SK_{Ca} (molecular identity SK3) and IK_{Ca} (molecular identity IK1); (84, 158)] causes potassium efflux and hyperpolarization in the endothelial cell membrane. Hyperpolarization spreads to the underlying VSMC through myo-

endothelial gap junctions and reduces the open probability of voltage-dependent L-type Ca^{2+} channels, and therefore decreases the Ca^{2+} influx [reviewed in (236)].

EDH-stimulated hyperpolarization in the endothelial cells spreads between endothelial cells themselves, and between endothelial cells and VSMC. This means that focal hyperpolarization can spread widely in the vascular wall of resistance arteries, and enough to influence the systemic blood flow (105).

1.2.5.4 Endothelial mediators of VSMC contraction

Beside mediating vasorelaxation, the vascular endothelium can also mediate VSMC contraction through secreted factors, and through responding to the nervous system and myogenic tone. Vascular endothelium is a source of many agonists that stimulate vasoconstriction including endothelin-1 (ET-1), thromboxane A_2, prostaglandin H_2, $O_2^{\cdot}$ anions, and endoperoxides [reviewed in (352)]. However, ET-1 has been identified as one of the most potent known vasoconstrictors (375). Production of ET-1 is stimulated by hormonal factors such as angiotensin and vasopressin (87).In the vascular endothelial cells, ET-1 is synthesized as inactive pre-proET-1 which is turned into proET-1 by a signal peptidase, ProET-1, which is then cleaved by furin convertase to inactive big-ET-1 (bET-1) converted by the action of endothelin-converting enzymes (ECEs) and matrix metalloproteinases (MMPs) into the active 21-amino acid ET-1(69, 93). ET-1 can interact with two types of G-protein-coupled receptors (ET_A and ET_B) on the VSMC. In arteries, the main vasoconstrictor receptor is ET_A (284). Stimulating ET_A receptors enhances the increase of Ca^{2+} levels in VSMC by increasing extracellular Ca^{2+} influx, and by activating phospholipase C (PLC) to form 1,4,5-triphosphate (IP3) which stimulates the release of intracellular Ca^{2+} from sarcoplasmic reticulum stores. Ca^{2+} then binds to calmodulin and activates myosin light chain kinase phosphorylation which leads to vasoconstriction.

The contractions provoked by ET_A receptor activation develop slowly and last even after washing out the peptide due to the almost irreversible binding of ET-1 to ET_A receptors (157). To counterbalance the effects of ET_A receptor stimulation on the VSMC, ET_B receptors work as clearance receptors for ET-1. Furthermore, In the endothelium, eNOS and ET-1 are distributed throughout the cell cytoplasm and in association with the intracellular organelle membranes (76, 179). This co-localization of eNOS and ET-1 distribution facilitates eNOS fine tuning of ET-1 effects so normally any overproduction of ET-1 would be balanced by the increased release of nitric oxide, which opposes ET-1 vasoconstrictor effect (142). It has been suggested that activation of ET-1 receptors in endothelial cells stimulates an increase in intracellular calcium through activation of PLC and PKC-dependent pathways which leads to an increase in the activity of eNOS (331, 351). Additional to the upregulation of eNOS, ET-1-enhanced vasoconstriction is blunted by both exogenous and endothelium-derived nitric oxide (via the activation of cGMP), also by the release of prostacyclin (via the activation of adenylyl cyclase) and by the initiation of EDH-mediated responses (90).

Alongside endothelium-derived agonists that stimulate vasoconstriction, the sympathetic and parasympathetic nervous systems can control vascular tone as well. Normally, under resting conditions, the parasympathetic system has a stronger effect than the sympathetic system. The parasympathetic system works mainly by releasing ACh that binds to its muscarinic receptors on the endothelium and stimulates vasorelaxation. The neuromuscular junctions, which release ACh from neurons are not directly hooked to the endothelium and how ACh arrives to the vascular endothelial cells is still not clear. Under stress and increased activity conditions, the sympathetic system becomes more active and works by shifting sufficient blood flow to important organs such

as the heart and brain. The sympathetic system works mainly through the release of noradrenaline that binds to α-adrenoreceptors 1 and 2 receptors on the VSMC causing vasoconstriction (101).

Together with endothelial secreted factors and the nervous system, the myogenic response is a key determinant of vascular control as well. The myogenic response is referred to changes of the vascular wall constriction in response to changes in intravascular pressure. An increase in the myogenic response leads to vasoconstriction, while a reduction in the myogenic response leads to vasorelaxation. Intravascular pressure initiates myogenic vasoconstriction by increasing the stretch on the vascular wall. This results in a stretch in the VSMC membrane and in membrane depolarization, which activates the voltage-gated Ca^{2+} channel and causes Ca^{2+} influx (143). The increase in the level of Ca^{2+} in VSMC cytosol stimulates Ca^{2+} release from sarcoplasmic reticulum through phospholipase C activation and IP3 formation (202), effects that cause vasoconstriction as detailed previously (section 1.2.5.2).

1.3 HYPOXIA IN PREGNANCY AND LINKAGES TO DEVELOPMENTAL ORIGINS OF CARDIOVASCULAR DISEASE

There are many factors (environmental, genetic, maternal lifestyle...etc.) that can alter normal cardiovascular physiology in fetal and adult life and increase offspring vulnerability to cardiovascular disease. This thesis will focus on the impact of maternal hypoxia on fetal cardiac development and adult cardiovascular health in male and female offspring separately. It will also focus on maternal vascular dysfunction and placental oxidative stress as possible mechanisms by which maternal hypoxia might impair fetal development.

Normally, the placenta develops under a low oxygen environment as new blood vessels in the placenta are still developing and because cytotrophoblast cells plug spiral arteries that are

important for oxygen delivery to the placenta [reviewed in (17)]. Early in human pregnancy, the partial oxygen pressure in the placental bed is 17.9 mmHg compared to that in the endometrium, which is 39.6 mmHg. Partial oxygen pressure then starts to increase in the placental bed around weeks 8 to 10 of pregnancy. By week 13 of gestation, the partial oxygen pressure in the placental bed becomes similar to that in the endometrium (275). In pathological conditions, poor implantation and abnormal vascular remodeling can lead to a lower than normal level of oxygen supply to the placenta. This is defined as uteroplacental hypoxia which means the maternal oxygenation is normal but the uteroplacental circulation is impaired (i.e., due to placental insufficiency), so the oxygen supply relative to the oxygen demand of the tissue is reduced (339). However, hypoxia can be preplacental as well which means that both the mother and her fetus are suffering from hypoxia (e.g. due to high altitude). Both preplacental and uteroplacental hypoxia can affect maternal and fetal health and lead to pregnancy complications. including IUGR and preeclampsia (high maternal blood pressure and proteinuria or other signs of systemic involvement) [(215, 261, 292, 355); reviewed in (260)]. Furthermore, hypoxia in pregnancy can lead to abnormal development of the fetal heart, and abnormal cardiovascular function later in adult life (21, 268, 289).

In both human and animal models, hypoxia in the placenta can cause changes in the blood flow velocities of the uteroplacental circulation when assessed by ultrasound biomicroscopy *in vivo*. A reduction in uterine artery blood flow might reflect a reduction in oxygen delivery to the placenta in humans and rodents (240, 325). More recently, photoacoustic imaging system coupled to high-resolution ultrasound imaging is used *in vivo* to measure the level of blood oxygen saturation in placentas. This was shown in a rat model during hypoxic exposure stimulated by reducing the oxygen concentration in inspired air (20).

As discussed herein, hypoxia in pregnancy can affect fetal development. This includes alterations in fetal cardiomyocyte development which I will focus on in the next section.

1.3.1 Effect of hypoxia on fetal cardiomyocyte development

The effect of hypoxia on the development of fetal heart has been studied in different animal models. In sheep, placental hypoxia [placental embolization; GD 110-130; (38)] led to delay in cardiomyocyte maturation (reduced the percentage of binucleated cardiomyocytes). In sheep as well, placental hypoxia [created by umbilicoplacental embolization; GD 116-136 or caruncle removal; GD 110-125, (181, 215) respectively] led to a reduction in the proliferation and cell cycle activity, and to a higher cardiomyocyte size relative to heart weight when compared to controls normoxic pregnancy. In chicks, cardiomyocyte number was reduced in response to hypoxia (14% O_2, incubation days 0-19), but cardiomyocyte maturation was not affected (234). In near term fetal rats, hypoxia in pregnancy (10.5% O_2, GD 15-21) increased the heart to body weight ratio (cardiac hypertrophy), increased apoptosis and caused a premature transition into maturity (increased the percentage of binucleated cardiomyocytes) (346).

Cardiomyocytes express micro RNAs (miRNAs) which are non-coding RNAs and major epigenetic regulators of gene expression, including those genes that regulate cardiovascular development and function. miRNAs can be beneficial in hypoxia. For example, a combination of miRNA-1 and miRNA-21 can prevent apoptosis induced by hypoxia (1% O_2) in cultured cardiomyocytes of neonatal rats (373). Furthermore, hypoxic pregnancies can be associated with alterations in placental miRNAs expression (79, 249, 373). For example, miRNA-93 (involved in DNA damage response) was up-regulated and miRNA-424 (stimulates trophoblast differentiation) was down-regulated in cultured primary term human trophoblasts when exposed to a hypoxic environment ($<1\%$ O_2) compared to those trophoblasts which were cultured in normal air

(79). However, it is still unknown whether there is a link between placental miRNA and fetal cardiomyocyte responses to hypoxia. It was suggested that placental miRNA can reach the fetal developing organs through extracellular vesicles that are released from the placenta to the fetal circulation, and affect fetal development in hypoxic pregnancies (249). To assess this, Phillips *et al*. showed that placental conditioned medium taken from rats exposed to hypoxia in pregnancy (11% O_2, GD 15-21) had an increased and decreased levels of individual miRNAs, including miRNA-30c, 150, 96 and 15b [regulates placental growth factor function, promotes angiogenesis, regulates progesterone receptors and enhances regulatory T cell induction respectively (172, 178, 244, 317)] compared to conditioned medium from normoxic pregnancies. Exposure to hypoxic placental media *in vitro* affected fetal neural development, including alterations in dendrite length and glutamate receptors. However, the exact involved factors were not determined (249).

Hypoxia in pregnancy induces not only changes in fetal cardiomyocytes, but also in the gross structure and function of the heart. For example, maternal hypoxia in rats increased the heart to body weight ratio, suggesting a reduction in the body weight and/or cardiac enlargement (21, 370). Hypoxic exposure during fetal life led to epicardium detachment, myocardial thinning and ventricle dilation in mice [8% O_2 for 24 h, GD 11.5; (259)] and to reduced ventricular wall mass in chicks [15% O_2, GD 10-20; (343)]. A previous study in humans assessed the cardiac function of fetuses suffering from IUGR due to placental insufficiency. Using color and Pulsed Doppler echocardiography, they found a reduction in cardiac output and aortic and pulmonary peak blood flow velocities in IUGR fetuses compared to controls normal pregnancy, suggesting an abnormal cardiac function in IUGR fetuses (269). Furthermore, sheep fetuses at high altitude (3820 m, GD 30-140), had a lower cardiac output and contractility than control ,low altitude animals (111, 145). Possible mechanisms involved in hypoxia-induced cardiac dysfunction of fetal sheep include

changes in calcium homeostasis and a reduction in ATP availability as a result of decreased magnesium-activated myofibrillar ATPase activity (37, 145). In chick embryos, hypoxia led to signs of systolic dysfunction as well left ventricular dilatation and a reduction in left ventricular ejection fraction. These alterations in chick embryo cardiac function were linked to an increase in the vascular endothelial growth factor (VEGF) in their hearts due to hypoxia [15% O_2, GD 10-20; (343)]. Thus, as discussed in this section, hypoxia in pregnancy can cause abnormal cardiac development in fetal life. However, hypoxia in pregnancy can also cause cardiac dysfunction in adult life.

1.3.2 Hypoxia in pregnancy and the programming of cardiac dysfunction in adult life

Our laboratory has focused on the effect of hypoxia in pregnancy on developmental programming for many years. The results of this work are part of the rationale for my PhD project. It has been established by our laboratory and other laboratories that hypoxia in pregnancy can increase offspring susceptibility to cardiovascular disease in adulthood. For example, maternal exposure to hypoxia can lead to an increased vulnerability to ischemia/reperfusion (I/R) insult in the hearts of both male and female offspring, which becomes clearer as animals age or are exposed to a high-fat diet in postnatal life (113, 123, 170, 288-290). This altered cardiac function is mediated by a reduction in antioxidant pathways, thus increasing oxidative stress in the heart (288), and to uncoupling of glucose metabolism (lowers energy production and contributes to the development of heart failure), (290). Maternal hypoxia as well can lead to abnormal cardiac function as shown in rats by using echocardiography imaging system. This included ventricular diastolic dysfunction in both adult male and female offspring, as demonstrated by restrictive filling (77, 225, 289). Further, aged male, but not female, offspring of rats exposed to hypoxia in pregnancy suffered from signs of ventricular hypertrophy (289).

Mechanisms of how hypoxia in pregnancy can cause cardiac dysfunction were assessed in rats (291). Data showed that hypoxia in pregnancy can cause an increase in myocardial markers of oxidative stress such as malondialdehyde and the oxidized/reduced glutathione ratio in male offspring of dams exposed to hypoxia in pregnancy compared to controls normal pregnancy. Furthermore, hypoxia in pregnancy can lead to interstitial myocardial remodeling characterized by myocyte loss and disrupted extracellular matrix, suggesting that hypoxic insults before birth have long-term effects on cardiac oxidative stress levels and cause extracellular matrix distribution in male but not female offspring (291). Alongside cardiac dysfunction, hypoxia in pregnancy can also cause systemic vascular dysfunction in adult offspring.

1.3.3 Hypoxia in pregnancy and the programming of vascular dysfunction in adult life

Hypoxia in pregnancy is an established factor in the pathophysiology of IUGR and fetal programming of vascular dysfunction in adult life. For example, in humans, IUGR has been associated with endothelial dysfunction in the first decade of life and in early adulthood as demonstrated by a reduction in flow-mediated dilation of the brachial artery (167, 168). In rats, maternal hypoxia led to vascular endothelial dysfunction in adult offspring seen as an increase in vasoconstriction and a reduction in flow-mediated and in nitric oxide-dependent vasorelaxation (12, 113, 217, 218, 364).

Pulmonary artery function in rats was also affected in hypoxic IUGR male and female offspring. This was demonstrated by an abnormal reduction in pulmonary artery acceleration time as assessed by echocardiography. The abnormal cardiopulmonary function was also further assessed by performing pulmonary histology and morphometry on the lungs of 12 months old animals. This revealed a significant increase in the arterial media thickness both in male and female hypoxic

offspring compared to the normoxic controls (289). The increase in arterial media thickness is considered a marker of atherosclerotic risk (210, 315).

One proposed mechanism for vascular dysfunction is oxidative stress in the vascular wall which can scavenge the endothelial-derived nitric oxide and reduce nitric oxide-mediated vasorelaxation. Also, when nitric oxide reacts with $O_2^{\cdot}$ anions, it forms the powerful oxidant, peroxynitrite, that may lead to further impairment of vascular function (30). However, decreased nitric oxide-mediated vasorelaxation is not the only reason for the vascular dysfunction resulting from prenatal hypoxia. Prostaglandin and EDH pathways are also important, and may compensate for a lack of nitric oxide-mediated relaxation (217, 218). Furthermore, the increased vasoconstriction in mesenteric arteries of adult offspring exposed to prenatal hypoxia is stimulated, at least in part, by ET-1 pathway via enhanced conversion of big ET-1 to ET-1 (34).

Some of the mechanisms by which prenatal hypoxia can lead to cardiovascular dysfunction in the offspring are not fully clear. However, prenatal hypoxia causes abnormal maternal vascular adaptations to pregnancy which can alter placental oxygen and nutrient supply and consequently affect fetal development (71). Prenatal hypoxia, as well, can cause placental oxidative stress and abnormal changes in placental secreted factors which are linked to developmental programming (11). Therefore, I will discuss maternal vascular adaptations to pregnancy and placental function as it relates to oxidative stress and secreted factors. I will as well discuss how hypoxia in pregnancy can impair maternal vascular adaptations, increase placental oxidative stress and affect the normal secretion of placental-derived factors.

1.4 MECHANISMS BY WHICH HYPOXIA IN PREGNANCY MIGHT ALTER FETAL DEVELOPMENT AND LEAD TO OFFSPRING CARDIOVASCULAR DYSFUNCTION

1.4.1 Alterations in maternal hemodynamics during pregnancy

During pregnancy, maternal cardiovascular adaptations happen which accommodate the increase of blood flow going to the uterus to meet the needs of fetal growth. Failure in these adaptations has been shown to accompany many pregnancy complications such as IUGR, preeclampsia and developmental programming. The theory is that incomplete maternal vascular adaptations will negatively affect nutrients and oxygen supply to the fetus resulting in a suboptimal intrauterine environment and pregnancy complications (71). Understanding normal changes in the maternal circulation in pregnancy and the normal development and function of the placenta will help in understanding the pathological alterations in placental and maternal vascular function that might affect fetal growth and development; and lead to an increased vulnerability to develop cardiovascular disease later in life.

1.4.1.1 Maternal systemic cardiovascular adaptations during pregnancy

Maternal systemic cardiovascular changes during pregnancy include an increase in cardiac output, heart rate, myocardial contractility and blood volume; and a reduction in systemic vascular resistance and blood pressure. The maternal heart rate, for instance, increases 20% by week 32 of pregnancy (365). Furthermore, stroke volume increases to a maximum of 30% by week 20 of pregnancy (110, 185, 342), which leads to a 50% increase in the cardiac output by the third trimester (47, 57, 185). Blood volume also increases during pregnancy due to systemic vasodilation in the first trimester that leads, in turn, to a reduction in the arterial filling and stimulates the RAS and the release of arginine vasopressin (AVP). Both RAS and AVP cause water retention and increase the plasma volume (48). Despite the increase in blood volume and

cardiac output, maternal blood pressure falls gradually especially between weeks 24 and 32 of pregnancy. This happens because of reduced peripheral vascular resistance that is due to hormonal vasodilators such as estrogens, and because of maternal refractoriness to arterial vasopressors such as Ang II and norepinephrine (56, 365).

Estrogens' effects on increasing peripheral vasculature vasodilation in pregnancy are mediated by enhancing endothelium-dependent vasodilators [NOS and prostaglandin H synthase (PGHS)] (53, 188, 294, 324). This was shown when estradiol-17 beta was injected to non-pregnant ewes which led into similar hemodynamic changes observed in pregnancy (189). These changes included a fall in mean arterial pressure (MAP) and systemic vascular resistance, and an increase in heart rate and cardiac output (189). Furthermore, pregnant mice had an enhanced endothelial-dependent vasodilation of mesenteric arteries compared to non-pregnant mice, and this pregnancy-mediated effect on vasodilation was due to the enhancement of NOS and PGHS activity (62). Involvement of EDH pathway in maternal peripheral artery vasorelaxation has been controversial and varies among species. For example, in mesenteric arteries, EDH-like relaxation did not differ between nonpregnant and pregnant mice (62). In pregnant rats, however, mesenteric arteries demonstrated an increased vascular relaxation to ACh compared to those of control nonpregnant rats, an effect which was enhanced by nitric oxide, prostanoid synthesis and hyperpolarizing factors (108).

Beside the increase in systemic vasodilation, pregnancy also reduces peripheral vasculature contractile response. For example, in wild-type mice, pregnancy was associated with a reduction in contractile responses to ET-1, Ang II and phenylephrine (PE) in mesenteric arteries (194). In rat mesenteric arteries as well, sensitivity to PE vasoconstriction was lower in pregnant than non-pregnant rats, confirming a reduced vasoconstriction sensitivity in pregnancy (70).

Peripheral vascular adaptations in pregnancy are essential to prepare for the demand of increased uterine blood flow, which is essential to supply the growing fetus with oxygen and nutrients. Therefore, to prepare for the increase in the blood flow to the uterus, uterine artery needs to respond to pregnancy by changing its size (an increase in its length and diameter) and function (an increase in vasorelaxation and a reduction in vasoconstriction).

1.4.1.2 Uterine vascular adaptations during pregnancy

During pregnancy, the uterine artery undergoes vascular remodeling resulting in significant functional and structural changes in the uterine artery and in facilitating the creation of the placenta (233). When uterine arteries remodel, their diameter doubles without changes in their wall thickness, and their axial length becomes four times bigger (232, 238). This happens due to an increase in the number and size of endothelial and VSMC, and due to matrix remodeling (16, 54, 55, 152). The increase in the diameter of a vessel, however, is more important in regulating vascular resistance compared to the increase in the length of a vessel [reviewed in (193)]. This is because the relation between vessel length and flow resistance is linear (a two time increase in length will reduce the resistance by half), while the relation between vessel diameter and flow resistance is quadratic (a two time increase in diameter will lead to a 16 times reduction in resistance; Poiseiulle's law: [resistance = (viscosity X length)/inner radius^4]). Spiral arteries, which supply blood to the endometrium of the uterus undergo vascular remodeling as well [reviewed in (251)]. They lose their spiral shape and become wider. However, discussion of spiral artery remodeling is outside the scope of this thesis.

Vascular remodeling does not only involve an alteration in vascular shape, but also a stretching of the elastic components of the vascular wall (55, 196, 232, 237). Vascular distensibility increases as well, due to changes in the volume of extracellular matrix and alterations

in the composition and orientation of collagen and elastin. For example, collagen content decreases in the vascular wall of the uterine artery in pregnant pigs and sheep (115, 117); and both collagen and elastin content decreases in the uterine artery of pregnant women compared to those who are not pregnant (272).

Factors which stimulate uterine vascular remodeling during pregnancy include hormonal factors, VEGF and shear stress. In early stages of pregnancy, steroids are major stimulators of uterine vascular remodeling. Higher circulating levels of estrogen are, at least in part, responsible for the higher expression of eNOS in the uterine artery (187, 281, 360). eNOS affects vascular remodeling through nitric oxide which has an impact on the expression of MMPs and their tissue inhibitors (TIMPs) (256). In later stages of pregnancy, uterine vascular remodeling is enhanced mainly by local influences such as fetoplacental secreted factors and myometrial stretch [reviewed in (193)]. This is supported by previous studies using rodents. These studies showed that using ligation to prevent implantation in one of the two uterine horns resulted in prevention of the massive vascular remodeling rather than allowing the massive remodeling in the horn that was not ligated. The minor changes which were seen in the ligated horn were due to the elevated levels of estradiol-17 beta which still have some effect on vascular remodeling, while the massive vascular changes in the none-ligated horn were mainly due to the presence of the fetuses (94, 100). Furthermore, in ovariectomized guinea pigs, injections of estradiol benzoate (7–11 µg/day for 3 weeks) doubled the internal diameter of segmental mesometrial arteries (209). Exogenous estradiol also stimulated DNA synthesis within the uterine artery of guinea pigs (191).

Along with the hormonal factors, syncytiotrophoblast cells in the placenta secrete VEGF which plays an important role in vascular remodeling. VEGF induces vasodilation, vascular enlargement, endomitosis and hypervascularization (200, 231). In early stages of spiral artery

remodeling in humans, trophoblast cells invade the spiral arteries and prevent the flow of maternal blood, leading to physiological hypoxia in the placental tissue. Physiological hypoxia induces hypoxia-inducible factor 1-alpha (HIF-1α) which stimulates the secretion of VEGF [reviewed in (136, 148)]. Shear stress is another factor which plays a role in uterine vascular remodeling in pregnancy (238). Shear stress increases uterine circumferential arterial growth; an effect which is likely mediated by increasing eNOS expression, but the exact mechanism is still unknown (285, 348).

Along with the changes in the uterine vasculature length and diameter in pregnancy, uterine vascular resistance decreases as well. This includes an increase in the uterine artery's ability to vasodilate and a reduction in its ability to constrict. Reduction in uterine vascular resistance in pregnancy is mainly mediated by effects of estrogen which enhance the endothelium-dependent vasodilation enzymes [NOS and prostaglandin H synthase (PGHS)] (53, 188, 294, 324). Progesterone, besides estrogen, can induce eNOS protein expression in the uterine artery endothelium (95, 96). Progesterone can influence α1- adrenergic/calmodulin system by increasing the α1- adrenergic receptor expression in uterine VSMC. α1- Adrenergic/calmodulin system is responsible for uterine artery phasic contractility [increases short-term contractility; (95)]. Along with the increase in the phasic contractility, progesterone decreases long-term contractility (tonic contraction) by decreasing PKC mediated contractility, and uterine vessels' myogenic tone (372). Thereby raising uterine artery blood flow. On the other hand, the increase in the phasic contractility works as a maternal protection mechanism by reducing the uterine blood flow and directing the blood to maternal organs that demand more blood flow under stress conditions such as the heart and brain.

Reduction in uterine vasculature contractile occurs in normal pregnancy as well and contributes to the increased blood flow to the uterus. For example, in the uterine artery of pregnant guinea pigs, vascular sensitivity to the vasoconstrictor PE was opposed by an increase in nitric oxide activity (362). Similarly, in pregnant ewes, uterine artery constrictor response to ephedrine was hindered due to the increase in the release of the vasorelaxant nitric oxide (174). In wild-type mice, pregnancy was associated with a reduction in contractile sensitivity to ET-1 in uterine arteries (194). Furthermore, the abnormal increase in uterine artery sensitivity to vasoconstrictors (i.e. Ang II) has been considered as a marker of hypertensive pregnancy in humans and rats (254, 301).

1.4.1.3 Effects of hypoxia on maternal vascular adaptations in pregnancy

Hypoxia in pregnancy can affect the maternal cardiovascular system and lead to pregnancy complications such as preeclampsia and IUGR. In pregnant guinea pigs, for instance, hypoxia increased maternal blood pressure (340), and impaired proliferation of uterine artery VSMC and uterine vascular growth (273, 274). Furthermore, hypoxia increased maternal blood pressure and was associated with an increased ET-1 plasma levels in pregnant rats (383). In pregnant sheep, hypoxia increased uterine artery myogenic tone (371). Hypoxia in pregnancy also prevented the reduction in vasorelaxation response, which can be normally enhanced by NO inhibition in uterine arteries of pregnant guinea pigs (361, 362). While in pregnant mice, hypoxia increased uterine artery vasodilator responses to adenosine monophosphate-activated protein kinase through NO-dependent and -independent mechanisms (318).

Hypoxia in pregnancy can also affect the placental vasculature development and alter uteroplacental and fetoplacental blood flow, thereby altering oxygen and nutrient supply to the fetus and leading to IUGR. For example, umbilical artery RI was increased *in vivo* in pregnant

wild-type mice exposed to hypoxia and associated with IUGR (292). Further, uterine artery diameter and blood flow were lower in normotensive pregnant women living at high altitude compared to those women living at low altitude; resulting in lower birth weight offspring (140, 380). However, in pregnant Andeans, high altitude increased the uterine artery blood flow and oxygen delivery to the fetus which prevented the altitude-associated IUGR (141).

Defective uterine vasculature remodeling causes cellular ischemia in the placenta which can alter placental function and affects fetal development. Incomplete vascular remodeling of upstream maternal vessels and spiral arteries can be initiated by many factors such as abnormal migration of trophoblasts into the spiral arteries, growth factors, cytokines and local immune cells in the decidua (macrophages and uterine natural killer cells). As a result of a hypoxic placenta, oxygen and nitric oxide levels become lower than normal, which increases placental anti-angiogenic factors [such as soluble Fms-related tyrosine kinase-1 (sFlt-1)] resulting in attenuation of VEGF signaling, widespread endothelial dysfunction, abnormal oxygen delivery to the fetus, and pregnancy complications including preeclampsia and IUGR [reviewed in (106)]. For example, in rats, reduction in uterine artery blood flow was associated with placental hypoxia, IUGR and preeclampsia-like symptoms (171). Applying artificial oxygen carriers successfully treated the placental hypoxia and prevented both IUGR and preeclampsia (171). In women living at high altitude, uterine arteries showed an inadequate increase in their diameter during pregnancy, which led to a reduction in blood supply to the placenta and associated IUGR (380). .

As disruption of normal placental perfusion can disrupt placental function, I will discuss herein the establishment of uteroplacental and fetoplacental blood flow, changes in placental secreted factors and placental oxidant production with special focus on mitochondria as a source of oxidative stress in the placenta.

1.4.2 Placental oxidative stress and secreted factors

The placenta is the key organ where nutrients, wastes, and gas (O_2 and CO_2) exchange happens between mother and fetus. Placenta as well is a major source of hormones and growth factors that have a big impact on maternal adaptation to pregnancy and on fetal development. Therefore, understanding placental physiology is essential to understand the pathophysiology of pregnancy complications and develop interventions to prevent them.

1.4.2.1 Establishment of uteroplacental and fetoplacental blood flow

During pregnancy, the fertilized egg implants in the uterine wall initiating the formation of the placenta, which is important for establishing gas and nutrients transfer between the maternal and fetal circulations. Syncytiotrophoblast and cytotrophoblast cells form the main structure of the human placental villi; and they are developed from labyrinth trophoblast cells which invade the uterine wall as the implantation starts. These labyrinth trophoblast cells begin to fold creating a path for the fetal vasculature to grow. By the end of the placental development, syncytiotrophoblast and cytotrophoblast cells create a placental barrier that separates fetal blood capillaries inside the villi from maternal blood on the surface of the villi. The placental barrier is where the transfer of electrolytes, O_2, carbon dioxide (CO_2) and nutrients occurs between the mother and fetus. Because maternal and fetal blood flow systems do not mix, uteroplacental, and fetoplacental blood flow are discussed separately below (Figure 1.4A & B).

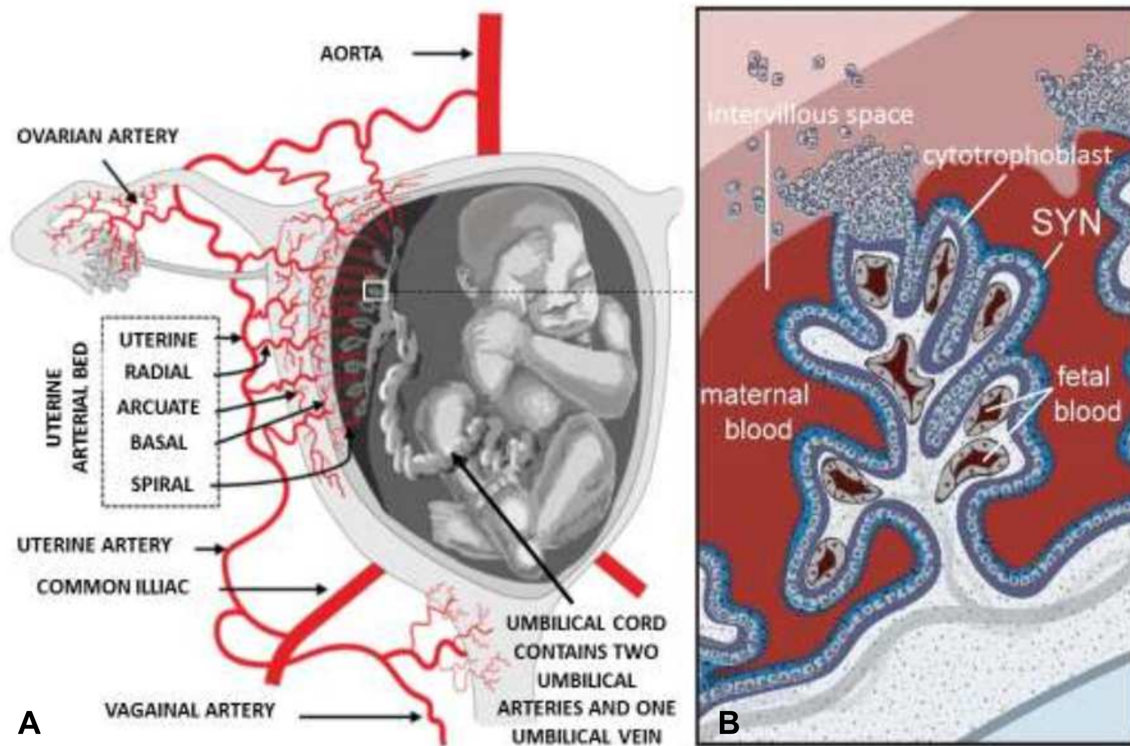

Figure 1.4 Placental structure

A: An illustration of uterine artery vasculature and umbilical cord. **B**: Syncytiotrophoblast and cytotrophoblast cells form the main structure of the placenta and separate maternal circulation from fetal circulation. SYN: syncytiotrophoblast. A: Morton *et al.* 2017 (219), B: Zeldovich *et al*. 2011 (382).

The exchange of oxygen and nutrients between maternal and fetal blood happens in the intervillous space. There, maternal blood flow is called uteroplacental blood flow and fetal blood flow is called fetoplacental blood flow (Figure 1.4B). The in-flowing maternal arterial blood pushes the deoxygenated blood into the endometrial uterine veins and back to the maternal circulation. Blood comes from the placenta to the fetus through the umbilical cord which contains one vein (the umbilical vein) and two arteries (the umbilical arteries). The umbilical vein brings oxygen and nutrients from the placenta to the fetus, and the umbilical arteries carry CO_2 and wastes from the fetus to the placenta.

In normal pregnancy, uteroplacental blood flow increases throughout pregnancy. At term in humans, the volume of the uterine blood flow becomes about 342 ml/minute compared to the non pregnant level of 94.5 ml/minute (337). During the transition from the uterine arteries to the intervillous space, a dramatic reduction in pressure happens, decreasing in humans from about 80–100 mmHg to 70 mmHg in spiral arteries and only 10 mmHg within intervillous space. This reduction in the pressure happens due to uterine vascular remodeling and due to a reduction in uterine vascular resistance, and allows the increased uterine blood flow to arrive gently to the intervillous space [reviewed in (358)].

Placentas, however, develop differently in male and female fetuses and sex-specific differences occur in fetal growth and fetal and neonatal morbidity and mortality [reviewed in (59)]. Thus, sex differences in placental development will be discussed below.

1.4.2.2 Placental development and fetal sex differences

Males are generally larger than females when they are born, a fact which has been established long ago (58). However, males have greater disease vulnerability and less chance to

survive a pregnancy complication than females [reviewed in (59)]. It was suggested that sex differences in development and adaptation are the central causes of the differences in fetal growth and survival. One possible mechanism may be a sex difference in the placenta, for which there is a considerable evidence to support that there are sex influences in placental function and development (Table 1.1), reviewed in (59).

Table 1.1 Sex differences in placental function and structure

Placental characters	Physiological differences	Species	Reference
Genetics			
Genes related to immune pathways (JAK1, IL2RB, Clusterin, LTBP, CXCL1, IL1RL1 and TNF receptor)	Expressed at higher levels in placentas of female fetuses than placentas of male fetuses	Human beings	Sood *et al.* 2006 (322)
Sex specific	Y linked genes are only in male but not female placentas	Human beings	Sood *et al.* 2006 (322)
Hormonal			
Steroids Profile including cortisol, cortisone, estriol, 17b-estradiol, and progesterone	Not sex specific	Human beings	Clifton *et al.* 2007 (60)
Testosterone	- Expression of 5a-reductase (converts testosterone to its bioactive form dihydro-testosterone) was greater in placentas of females compared to male fetuses at term. - Testosterone was a more potent inhibitor of explant cytokine production in placentas of females compared to placentas of male fetuses.	Human beings	Vu *et al.* 2009 (353) Stark *et al.* 2009 (326)
Growth hormones	In cord blood: - IGF-1 and IGFBP-3 concentrations were higher in females than males. - Growth hormone concentrations were higher in males than females.	Human beings	Geary *et al.* 2003 (107)
Structural			
Invasion and surface differentiation	Placentas of male fetuses invaded more deeply into the spiral arteries compared to placentas of female fetuses, exhibited a larger placental size	Human beings	Alwasel *et al.* 2014 (13)
Labyrinth and junctional zones	Placentas of female fetuses contained a bigger labyrinth but smaller junctional zone than placentas of male fetuses	Mice	O'Connell *et al.* 2013 (229)
Blood space area	On GD 15 placentas of female fetuses had a lower fetal blood space surface area and fetal and maternal blood space volume compared to placentas of male fetuses. On GD 20 these differences in the placental structure between male and female disappeared	Rats	Kalisch-Smith *et al.* 2017 (144)
Oxidative stress and antioxidant status			
Placental biological oxidation and antioxidant enzyme activity	No sex differences in the biological increase of placental oxidant generation as gestation progress, and placental antioxidant enzyme activity in normal pregnancy	Human beings	Clifton *et al.* 2005 (61)
Oxidant production and antioxidant defenses in response to betamethasone exposure	A higher level of prooxidant production and a lower level of antioxidant defenses in placentas of male than female neonates delivered within 72 h of antenatal betamethasone exposure	Human beings	Stark *et al.* 2011 (327)

GD: gestational age, TNF: tumor necrosis factor, IGF: insulin growth factor, IGFBP-3: Insulin-like growth factor-binding protein-3.

In humans, for example, assessment of the fetal-placental steroid profile (cortisone, cortisol, 17b-estradiol, estriol, and progesterone) shows no sex-specific differences (60). However, testosterone activity in human placenta and levels of growth factors in umbilical vein plasma were different in male and female fetuses (107, 326, 353). Autosomal and sex-specific gene expression also differed in placentas of male and female human fetuses (322). Furthermore, placental structure and development differ between male and female fetuses, which might impact placental function in a sex-dependent manner [reviewed in (59, 282)]. For example, an epidemiological study pregnant women showed that extravillous trophoblasts in placentas of male fetuses invaded more deeply into the spiral arteries compared to female placentas, which exhibited a larger placental size (13). In mice, female placentas contain a bigger labyrinth zone (responsible for nutrient and gas exchange between the maternal and fetal circulations) but smaller a junctional zone (responsible for placental hormonal secretion) than male placentas (229). Additionally, a study by Kalisch-Smith JI *et al*. showed a positive link between sex-differences in placental development and placental and fetal body weight (144). Kalisch-Smith JI *et al*. collected placentas on gestational day (GD) 13 from rats and showed that these placentas had similar placental structures in both sexes. However, on GD15 female placentas had a lower labyrinthine volume, fetal blood space surface area, and fetal and placental blood space volume compared to male placentas. By GD20 these differences in structure between male and female placentas disappeared but female body and placental weights were lower than male body and placental weights, suggesting that the difference in placental development between male and female likely contributed to the lower fetal body weight and placental weight in females compared to males at GD20 (144).

1.4.2.3 Trophoblast mitochondria

Oxidants such as $O_2^{\cdot}$ and peroxynitrite ($ONOO^{-}$) are important for trophoblast proliferation, differentiation and placental vascular responses during normal pregnancy [reviewed in (247, 369)]. However, an abnormal increase in the production of ROS can alter in trophoblast function.

Mitochondria are the major source of cellular energy production [adenosine triphosphate (ATP)], in the trophoblasts, a process which normally leads to $O_2^{\cdot}$ production. This process of ATP production causes the release of free radicals in the mitochondria via the electron transport chain. Of the four mitochondrial redox carriers, complex I (NADH-ubiquinone oxidoreductase) is the main site where premature electron leakage occurs, thus being one of the main sites of $O_2^{\cdot}$ production. Complex I passes electrons to CoQ10, a coenzyme, which acts as an electron and proton donor between complexes I and II and complex III in the chain (Figure 1.5) (369).

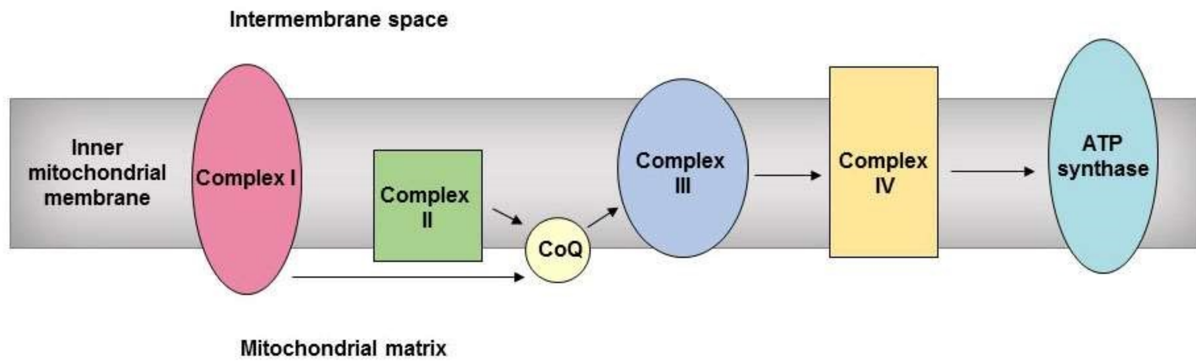

Figure 1.5 Role of Coenzyme Q10 in the mitochondrial respiratory chain

Coenzyme Q10 (CoQ10) is located within the inner mitochondrial membrane and is a necessary component of the mitochondrial electron transport chain. CoQ10 can accept electrons from complexes I (NADH-ubiquinone oxidoreductase) and II (succinate dehydrogenase), and then transfer these electrons to complex III (ubiquinol-cytochrome c reductase). ATP: adenosine triphosphate.

As CoQ10 has the ability to mediate electron transport, it acts as an antioxidant by accepting the free electrons from free radicals, which afterward converted to the harmless compound ubiquinol. Consequently, this prevents damage to metabolic processes, cellular DNA and cell architecture (207). Under normal conditions, production of $O_2^{\cdot}$ by complex I is negligible and can be scavenged by CoQ10. However, under suboptimal conditions (i.e. hypoxia) and when damage to the respiratory chain occurs, NADH levels increase leading to an increased production of $O_2^{\cdot}$ to the extent that cannot be prevented by CoQ10 (369).

In the trophoblasts, mitochondria have a different shape and function, which depends on the type of the cell. In syncytiotrophoblast, mitochondria are smaller and have a non-classical vesicular cristae structure and dense matrix compared to those of the cytotrophoblast. Furthermore, syncytiotrophoblast mitochondria have reduced coupling control of redox and phosphorylation reactions during oxidative phosphorylation in comparison to cytotrophoblast mitochondria, which is considered important for efficient ATP production (44). In syncytiotrophoblast mitochondria, complex IV activity (involved in $O_2^{\cdot}$ generation) is present at high levels in the inner mitochondrial membrane which makes the syncytiotrophoblast mitochondria a bigger source of oxygen radicals than the cytotrophoblast mitochondria [(118), reviewed in (129)].

1.4.2.4 Placental secreted factors

Together with normal placental ROS production, placental secreted factors are essential for fetal development. Hormones that support pregnancy (such as estrogen, progesterone and human chorionic gonadotropin) can be secreted from the placenta. The placental barrier consists of basement membranes and many types of cells (trophoblasts and fetal capillary endothelial cells). In humans, the placental barrier has two trophoblast layers: the syncytiotrophoblast layer which faces the maternal blood, and the cytotrophoblast layer which is separated from the fetal connective

tissue by a basement membrane. Fetal connective tissue contains fetal blood capillaries. Each one of these capillaries has a basement membrane and one layer of endothelial cells. As pregnancy progresses, the syncytiotrophoblast becomes the predominant layer, whereas the cytotrophoblast becomes discontinuous with interruption of the cytotrophoblast layer being seen below the still-intact syncytiotrophoblast layer. In rodents, however, there are three continuous trophoblast layers (two layers of syncytiotrophoblast facing the fetal side and one layer of giant trophoblast cells facing the maternal blood sinus). These trophoblast layers are separated from the fetal connective tissue by a basement membrane [reviewed in (162)].

Fetal capillary endothelium and the underlying basement membrane are present in all mammals. Among all these cell types that form the placenta, syncytiotrophoblast cells are the source of many hormones that have large effects on maternal and fetal physiology, such as progestins (support the endometrium and prevent the contractility of uterine smooth muscle), lactogens (mobilize energy substrates for fetal use) and relaxin (acts with progesterone to maintain pregnancy). Pro-angiogenic factors are among secreted factors from the syncytiotrophoblast cells as well, these include VEGF and placental growth factor (PlGF), which normally increase during the first two trimesters of pregnancy and decrease afterward (due to the elevation of the placental anti-angiogenic factor sFlt-1) to prevent the progression of angiogenesis in the uterine wall which could cause serious pregnancy complications (i.e. placental accrete) and heavy bleeding at birth (150). Pro-angiogenic factors are proteins and can pass from syncytiotrophoblast to cytotrophoblast and across the basement membrane to fetal circulation through active transportation and protein transporters (192). In the fetal body, VEGF can enhance the formation of embryonic circulatory system which is referred to as (vasculogenesis) (386).

VEGF production is normally induced due to physiological hypoxia in placenta (130). When a cell is hypoxic, it produces hypoxia-inducible factor 1- alpha (HIF1-α) which stimulates the release of VEGF. Circulating VEGF then binds to tyrosine kinase receptors (VEGFRs) on the endothelial cell surface and activates these receptors through transphosphorylation leading to tyrosine kinase pathway stimulation and angiogenesis (119). PlGF also stimulates angiogenesis through tyrosine kinase pathways; and is presented in the syncytiotrophoblast and in the media of blood vessels in the placenta (153).

Beside hormonal and growth factor secretion, syncytiotrophoblast can release syncytiotrophoblast extracellular vesicles (STBEVs) to the maternal and fetal circulation. The structure of STBEVs is basically a plasma membrane surrounding a small amount of cytosol which contains growth and apoptotic factors, proteins, microRNAs (miRNAs), messenger RNAs (mRNAs), DNA fragments, calcium, phosphate, lipids and cell surface factors such as binding proteins. Therefore, STBEVs work as regulators of cell to cell communication [(349), and reviewed in (250)] and stimulate many cellular actions including invasion, proliferation, migration, angiogenesis or apoptosis (89, 314), indicating that STBEVs can play a role in inflammation and vascular dysfunction.

There are three types of STBEVs depending on their size and mechanism of release. Exosomes [30–100 nm; released from endosomal compartments (74)], microparticles [100 nm–1 µm; released from cell surface plasma membrane (302)] and apoptotic bodies [1–5 µm released from blebs of apoptotic cells (132)]. When STBEVs are released, they interact with target cells through a receptor-mediated mechanism or they enter the cells by endocytosis through the cellular plasma membrane where they release their content (137, 307).

miRNAs are an important compartment of STBEVs. miRNAs are delivered via STBEVs to the blood circulation to target cells where they bind to the three prime untranslated region (3'UTR) of target messenger RNAs (mRNAs) and work as negative regulators of target mRNAs expression at the post-transcriptional level, which leads to cleavage or translation inhibition of the target mRNAs (14). Through their effect on the translation of mRNA, miRNAs can control and interfere with processes of cell growth, differentiation, proliferation and apoptosis, and can therefore they can affect placental and fetal development (88, 213). For example, down-regulation of miRNAs, such as miRNA-378a-5p (184) and miRNA-376c (99) promotes trophoblast cell proliferation and invasion. Intravenous administration of miRNA-145 and miRNA-675 inhibitors to pregnant C57BL/6J mice significantly increases fetal and placental weights compared to controls; showing that miRNA-145 and miRNA675 are negative regulators of placental and fetal growth (29). STBEVs also contain miRNA, which is important in placental function and fetal development. Alterations in miRNA expression are used to predict pregnancy complications. For example, miRNA-16 and miRNA-21 expression in human placenta is linked to fetal growth, with their reductions being used as a predictor of IUGR (186).

The placenta also is a source of cytokines such as monocyte chemotactic peptide-1 (MCP-1), interleukin-8 (IL-8) and interleukin-10 (IL-10). Cytokines are messengers usually secreted by immune cells and found in the fetal circulation and tissues (72). However, some cytokines such as tumor necrosis factor-α (TNF-α) and interferon (IFN) can also be secreted from any structural cells such as syncytiotrophoblast, cytotrophoblast, and fibroblasts (333). Moreover, maternal cytokines can cross the placental barrier to the fetus (381). Cytokines play pivotal roles in immune recognition, acceptance of the fetal allograft, placental angiogenesis (the growth of new blood vessels) and gene transcription (197, 208, 271). Furthermore, the cytokine TNF-α works in the

placenta as a cell signaling protein which induces MMPs synthesis and facilitates cytotrophoblast migration to the spiral arteries within the uterine decidua (203).

1.4.2.5 Effect of hypoxia on placental oxidative stress and secreted factors

[Part of the section is adapted from the review: Aljunaidy *et al*. 2017, (11)]

Hypoxia in pregnancy can affect the level of secreted factors and oxidants in the placenta, which contributes to the pathophysiology of developmental programming. The placenta normally develops in hypoxic conditions. However, lower than normal exposure to hypoxia can lead to placental mitochondrial dysfunction and an abnormal increase in ROS (43, 357). A hypoxia-induced elevation in placental ROS happens via various mechanisms including a reduction in oxygen availability which works as a terminal receptor of electrons in the mitochondria (201), activation of NADPH oxidase [through the mitochondrial ROS-protein kinase Cε (PKCε) signaling axis] or activation of xanthine oxidase leading to the production of $O_2^{\cdot}$ (258). Hypoxia can also increase in cytosolic calcium (Ca^{2+}) levels by increasing ROS which can directly cause extracellular Ca^{2+} influx through the inhibition of voltage-dependent potassium channels and by activation of ryanodine receptors which causes a release in intracellular Ca^{2+} (19, 176). The increase in cytosolic Ca^{2+} due to hypoxia activates NOS function and increases nitric oxide production. Further, mitochondria are a major source of ROS and ATP production in the cell. In the placenta, mitochondrial oxygen consumption is downregulated under hypoxic conditions causing, altered metabolic substrate availability and lactated production (344). In addition, there is a greater reliance on glycolytic ATP production and, therefore, a higher production of ROS [reviewed in (224)].

Abnormal increases of placental oxidants are harmful due to their ability to cause DNA damage, apoptosis, lipid and protein peroxidation; all of which can affect placental function

adversely and lead to reduced delivery of oxygen and nutrients to the fetus as well as to subsequent effects on fetal development. Notably, oxidative stress was shown to have a key role in the vascular dysfunction observed in placental chorionic vessels from women with IUGR (305). Furthermore, placental oxidative stress has been described in many pregnancy complications such as IUGR, preeclampsia, and diabetes (18, 42); which are known to be associated with fetal programming of cardiovascular disease [reviewed in (10)].

The pathophysiological mechanisms by which placental oxidative stress can lead to pregnancy complications and fetal programming of cardiovascular disease remain unclear. However, it has been suggested that oxidative stress can alter placental function and trophoblast secreted factors which, in turn might adversely affect the maternal body, and fetal development via pregnancy complications and developmental programming [(71, 98), and reviewed in (300)]. For example, placental STBEVs in pregnant women express number of death messengers that control cytokine release in the maternal body, which impedes maternal immunity and promote fetal survival (266, 295). A study in our laboratory showed that exposing uterine artery of pregnant rats to plasma collected from women suffering from preeclampsia led to a reduction in endothelium-dependent vasorelaxation via altering the level of oxidative stress in the vascular wall (147). Furthermore, our laboratory showed that STBEVs (200 μg/ml) can increase uterine artery constriction in pregnant mice via alterations in the vascular low-density lipoprotein receptor-1 (323).

Some placental miRNAs (such as miRNA-516-5p, -518b, -520h and -526a, which work as post-transcriptional regulators of gene expression) were detected in maternal plasma of normal pregnancies and categorized as pregnancy-associated miRNAs (159). Abnormal miRNA levels in placenta or maternal plasma, however, were detected in complicated pregnancies. For

instance, miRNA-20a and miRNA-17 (important for fetal growth) were up-regulated in placentas from preeclampsia (356) and miRNA-378a-5p (perturbs mitotic fidelity) was down-regulated in preterm birth (184). Furthermore, placental exosomal miRNA-486-1-5p and hsa-miRNA-486-2-5p (post-transcriptional regulators of gene expression) were higher in maternal plasma of women suffering from preeclampsia than those from normal pregnancies (296).

A normal balance between pro-angiogenic and anti-angiogenic factors is also important in pregnancy. Hypoxia can stimulate an increase in the levels of the anti-angiogenic sFlt-1, which binds to VEGF and PlGF in the maternal circulation, and reduces their beneficial pro-angiogenic effects (150, 199). This inhibition of the pro-angiogenic effects happens early in pregnancy when angiogenesis is still needed for placental vascular development. Therefore, the abnormal balance of pro-angiogenic and anti-angiogenic factors contributes to the pathophysiology of pregnancy complications such as preeclampsia. For example, in women with preeclampsia and IUGR) maternal concentration of sFlt1 was higher than in normal pregnancies (328). In pregnant or nonpregnant rats, injection of an adenovirus expressing sFlt-1 led to preeclampsia signs, including hypertension and proteinuria (199). In a mouse model of preeclampsia (created by lentiviral transduction), pravastatin (1 mg/mL/day intraperitoneally, GD7.5 to GD18.5) increased PlGF in maternal circulation and prevented preeclampsia (163).

Placental factors are also released into the fetal compartment and, therefore, may be involved in mediating the effects of placental oxidative stress on fetal programming of adult onset disease. For example, Curtis *et al*. demonstrated that exposure of fetal neurons to factors secreted from hypoxic placentas caused abnormal development *in vitro* (66); providing a potential link between a hypoxic/oxidative placenta and developmental programming. Recently, in a rat model of maternal hypoxia, we demonstrated altered placental secretions which was associated with

impaired fetal neural development *in vivo,* supporting this hypothesis (249). However, whether these placental secretions impacted the fetal cardiovascular system during early development into adulthood, and the identity of the specific circulating factors remains to be determined.

Previous studies using animal models showed that hypoxia in pregnancy can lead to a phenotype of abnormal fetal cardiomyocyte development and cardiovascular dysfunction in the adult offspring. For example, prenatal hypoxia caused cardiac hypertrophy and cardiomyocyte premature transition into maturity in rats (346), led to a delay in cardiomyocyte maturation in sheep (38), and reduced cardiomyocyte number in chicks (234). Furthermore, maternal hypoxia caused aortic thickening, impaired endothelium-dependent vasorelaxation in femoral and mesenteric arteries, increased myocardial contractility with sympathetic dominance, and increased the low frequency to high frequency heart variability ratio in adult offspring (113, 146, 217). Therefore, signs of developmental programming of cardiovascular disease can be enhanced by prenatal exposure to hypoxia in animals and thus antioxidant interventions can be assessed using these animal models.

As placental dysfunction is the hallmark of many pregnancy complications (including IUGR, preeclampsia and developmental programming), I will discuss targeting placental oxidative stress as an intervention for developmental programming of cardiovascular disease.

1.5 ANTIOXIDANT INTERVENTIONS

[Part of this section is adapted from the review: Aljunaidy *et al*. 2017, (11)]

Given the abundant literature concerning the involvement of prenatal hypoxia in pregnancy complications, the ability of hypoxia to initiate placental oxidative stress, the release of circulating factors from a dysfunctional placenta, and ultimate fetal programming outcomes, the use of an antioxidant to interrupt this cascade of events seems logical. Animal studies have shown that using

antioxidant treatments in either hypoxic or complicated pregnancies can prevent fetal programming of cardiovascular disease. For example, perinatal treatment with the antioxidant resveratrol [4 g/kg diet, gestational day (GD) 0.5-21] mitigated the development of hypertension in spontaneously hypertensive rats (49). Maternal exposure to hypoxia in rats led to cardiovascular disease in the offspring in adult life and maternal antioxidant treatment using ascorbic acid (5 mg/ml in drinking water, GD 6-20) prevented placental oxidative stress associated with maternal exposure to hypoxia (268) and prevented fetal programming of cardiovascular disease in rats (113). In rats exposed to maternal low protein diet, maternal treatment with a lipid peroxidation inhibitor (lazaroid, 10 mg/kg/day, gavage, GD 0-term) attenuated the increase in mean arterial pressure and the abnormal constrictive vascular phenotype in the adult offspring (46). Also, maternal treatment with a SOD mimetic (tempol, 10 μM in drinking water, GD 7-term) prevented right ventricular hypertrophy in the adult offspring from malnourished pregnant mice (264).

However, antioxidant treatment in pregnancy has not always been successful in preventing fetal programming. In some cases, antioxidant treatment had unbeneficial or even detrimental outcomes. For example, Stanley *et al*. showed that tempol (1 mmol/l in drinking water, GD 12.5-18.5) increased fetal weight in eNOS knockout mice (a model of IUGR) but also increased fetal weight of control C57Bl/6J mice which can have detrimental effects (325). Maternal supplementation of vitamin C in normoxic rats led to cardiovascular dysfunction in the offspring as demonstrated by an increase in myocardial contractility, and impairment in endothelium-dependent vasorelaxation of femoral arteries (113). A study by Franco *et al*. showed that combined antioxidant treatment with vitamins C and E, selenium and folate (150 mg/kg/day, 250 mg/kg/day, 0.3 mg/kg/day, 4 mg/kg/day respectively, gavage, GD 1-term) in undernourished pregnant rats prevented programming of vascular dysfunction, but was unable to prevent the reduction in

glomerular number or glomerular filtration rate (97). Furthermore, maternal resveratrol (chow final concentration of 0.37%, initiated 3 months before breeding) can impair fetal pancreatic development in macaques (270). Randomized controlled trials in women with compromised pregnancies have also been performed to assess the potential benefits of antioxidant therapy but have failed to demonstrate improved pregnancy outcomes such as reducing IUGR or neonatal mortality [reviewed in (216)].

Together, the studies discussed above suggest that, while antioxidant treatment may provide a potential strategy to prevent fetal programming of cardiovascular disease, the safety and efficacy of applying antioxidants during pregnancy should be considered to avoid undesirable possible outcomes. Thus, there is a need to find a new strategy to improve fetal growth (via enhanced oxygen delivery and/or prevention of placental oxidative stress) without directly interfering with fetal development, as drugs which do not cross the placental barrier to the fetus are generally safer than the drugs which cross the placental barrier to the fetus (235).

1.6 NANOPARTICLE DELIVERY TO TARGET TREATMENT TO THE PLACENTA

[Part of this section is adapted from the review: Aljunaidy *et al*. 2017, (11)]

MitoQ is a commercially available antioxidant which targets oxidative stress in mitochondria, a major source of oxidative stress in the placenta (43). The principal mechanism by which MitoQ acts as an antioxidant is related to its location on the inner mitochondrial membrane (135). MitoQ is produced by linking ubiquinone to a positively charged lipophilic cation. This positive charge restricts its extensive accumulation to within the negatively charged mitochondrial inner membrane inside cells. MitoQ accumulates thousand-fold greater within mitochondria compared to untargeted antioxidants such as exogenous Coenzyme Q,, which makes MitoQ more effective in targeting mitochondrial oxidative damage (283). In Complex II, the ubiquinone of

MitoQ, identical to the active antioxidant in endogenous Coenzyme Q, can be rapidly activated to antioxidant ubiquinol. Ubiquinol donates a hydrogen atom to a free radical, which is ROS formed during the mitochondrial respiratory chain and, in doing so, is recycled back into its ubiquinone form. Evidence exists for a potential therapeutic benefit for Coenzyme Q10 in the developmental origins of cardiovascular disease. In a rat model of maternal malnutrition, early postnatal supplementation with Coenzyme Q10 reduced signs of cardiac aging including oxidative stress, cellular senescence and telomere shortening (335). These promising findings illustrate the importance of further research into mitochondrial antioxidants as a pharmacological target to prevent adverse long-term outcomes following an impoverished *in utero* environment.

Although MitoQ can normally pass to the fetus through the placental barrier (321), attaching MitoQ to nanoparticles is possible and can restrict delivery of the antioxidant to maternal-placental compartments and prevent direct exposure of the fetus to the treatment (249). In addition, nanoparticle-mediated drug delivery may also lower the required dosage by increasing drug bioavailability (149). How nanoparticles prevent MitoQ from crossing the placental barrier to the fetus is not fully understood. One of the suggestions is that the nanoparticles might release MitoQ in the trophoblast cells, which are rich in mitochondria. MitoQ would then accumulate in the mitochondria and, therefore, would not cross the placental barrier to the fetus. The second suggestion is that nanoparticles release MitoQ inside the trophoblast mitochondria, which means that MitoQ is trapped inside the mitochondria and cannot cross the placental barrier to the fetal circulation. Future studies are necessary to determine cellular localization of the nanoparticles and release of MitoQ.

Attaching interventions to nanoparticles to target treatment to the placenta was used previously and showed encouraging results. For example, a study showed that attaching a growth

factor (IGF-2) or onto nanoparticles can be beneficial in targeting the treatment to the placenta and prevent it from crossing to the fetus (156). IGF-2 (approximately 0.3 mg/kg maternal body weight) was attached to liposome nanoparticles and injected to healthy pregnant mice (100 μl, once on GD11.5). The IGF-2 loaded onto nanoparticles treatment led to an increase in mouse placental weight compared to those placentas from dams that received an injection of liposomes nanoparticles without a growth factor (156).

Our recent study utilized biodegradable polymeric nanoparticles composed of a poly γ-glutamic acid (γ-PGA) hydrophilic outer shell and a phenylalanine ethyl ester (L-PAE) hydrophobic inner core (249). Nanoparticles can be taken by endocytosis into the placental syncytium to release their content (i.e. MitoQ) within the trophoblast cells. Due to the size (180 nm diameter, most substances that are bigger than 25 nm can not cross the placental barrier), charge (non-ionized substances usually cross the placental barrier easier than ionized substances) and surface composition of these nanoparticles (nanoparticles with hydrophilic surface have less advantage of crossing the basement membrane than nanoparticles with lipophilic surface) they do not cross the placental basement membrane barrier to reach the fetus [(128, 249), and reviewed in (162)]. Once delivered to the placental tissue through maternal circulation, these nanoparticles enter trophoblast cells by endocytosis where they are recognized by γ-glutamyl transpeptidase (γ-GTP) on the cell membrane (332). Inside the trophoblasts, nanoparticles undergo endosomal escape and are released into the cytosol (Figure 1.6) (249).

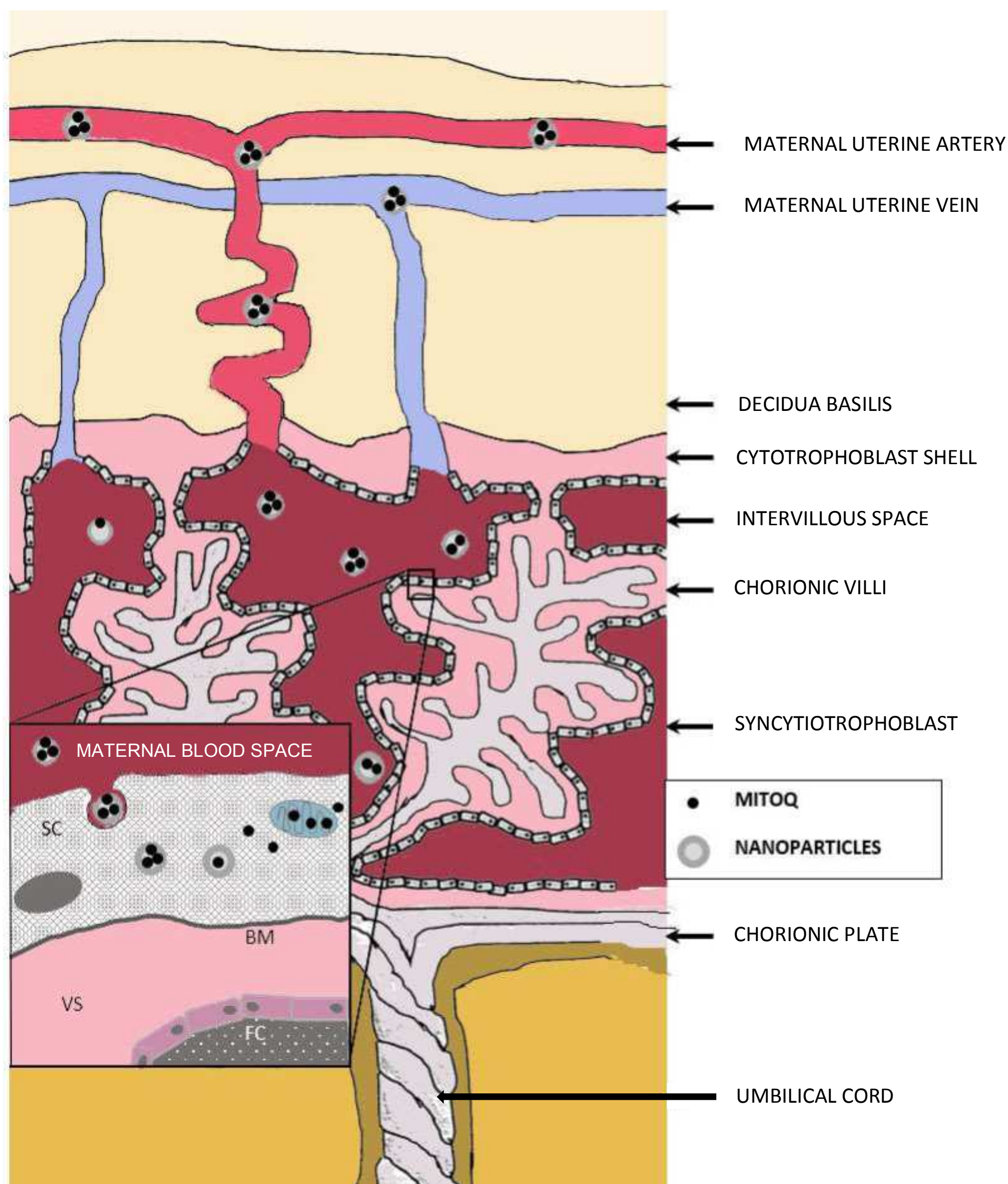

Figure 1.6 Treatment of the placenta using MitoQ loaded nanoparticles

Due to the size (180 nm diameter), charge (Zeta potential -20 mV) and surface composition of the polymeric nanoparticles, (poly (γ-glutamic acid)-*graft*-l-phenylalanine ethyl ester [γ-PGA-graft-L-PAE; NPs]). The nanoparticles can undergo endocytotic uptake from the maternal blood into the placental syncytium (SC) to release their content (MitoQ treatment) within the trophoblast cells without crossing the placental basement membrane (BM) barrier to reach the fetus. VS: villous stroma, FC: fetal capillary. Aljunaidy *et al*. 2017 (11)

It is not clear whether nanoparticles accumulate inside mitochondria and lysosomes due to their positive charge; thus, allowing drug activity at this specific site within the trophoblast cells, or if they release their content (MitoQ) inside the cytosol where MitoQ accumulates fast inside the mitochondria. As the delivery of nMitoQ is through an intravenous tail injection in the dam, it is possible that nMitoQ could be taken up by maternal tissues. However, due to the size of the nanoparticles, passage across the vascular endothelium is limited thus preventing uptake in maternal tissues. Indeed, preliminary data from our laboratory showed that the polymeric nanoparticles are not taken up by maternal organs. However, MitoQ levels in the maternal tissue has not yet been assessed. nMitoQ might prevent maternal vascular oxidative stress and optimally attenuate maternal vascular dysfunction in complicated pregnancies. Indeed maternal vascular dysfunction in pregnancy complications such as preeclampsia is enhanced at least in part by an increase in the normal level of vascular oxidants (147). This possible effect of nMitoQ on maternal vascular function could further contribute to its impact on placental and fetal development.

Furthermore, depending on the chemical properties of nanoparticle surface, drugs can either be attached to the surface of the nanoparticles or get encapsulated inside the nanoparticle mass [reviewed in (316)]. The surface properties of γ-PGA-graft-L-PAE nanoparticles (their zeta potential and hydrophilicity) facilitate the encapsulation of MitoQ inside the nanoparticle mass (7, 253). Encapsulation of drugs inside the nanoparticle mass allows more consistent prediction of the interaction and behavior of nanoparticles inside cells compared to having different types of drugs attached to the surface of nanoparticles every time, which could change the chemical properties of the nanoparticle surface and its interactions with the surroundings in the animal or human body [reviewed in (316)].

Recent data show that in a rat model of maternal hypoxia, nanoparticle-linked delivery of MitoQ (final dose: 0.5 μM) improved fetal outcomes, including increased birth weight and prevention of adverse alterations in miRNA and neuronal gene expression, which are effects that may be linked to the developmental programming of psychiatric disease (249). Therefore, in light of the large number of pregnancies complicated by gestational hypoxia, these data are important to develop a targeted therapy method to treat placental oxidative stress; which could achieve a significant advance in therapeutic interventions to reduce offspring susceptibility to cardiovascular disease.

1.7 CONCLUDING REMARKS

[This section is adapted from the review: Aljunaidy *et al*. 2017, (11)]

Intrauterine growth restriction is a leading cause of fetal and newborn morbidity and mortality and may lead to cardiovascular diseases in adult life (developmental programming). IUGR is associated with low oxygen levels in the placenta, which in turn, can lead to placental oxidative stress and abnormal placental function. Mitochondria are a major source of oxidative stress in the placenta and may provide a target for placental treatment that will mitigate the detrimental effects of placental oxidative stress in pregnancy outcomes and fetal programming of cardiovascular disease. A nanoparticle-mediated delivery system of mitochondrial antioxidants to the placenta is potentially novel approach that may avoid unwanted off-target effects on the developing offspring. Both short-term effects on the developing feto-placental unit and long-term developmental programming outcomes of this treatment require further investigation.

1.8 HYPOTHESIS AND AIMS

1.8.1 Hypothesis

Hypoxia in pregnancy is an established cause of developmental programming of cardiovascular disease and is associated with increased placental oxidative stress. I propose to use nanoparticles to deliver mitochondrial antioxidant (MitoQ) treatment to the placenta to avoid risking direct exposure of the therapeutic to the fetus. I hypothesize that maternal hypoxia will lead to placental oxidative stress and abnormal offspring cardiovascular development and function. I hypothesize as well that maternal treatment with placental-targeted antioxidant using nMitoQ will improve fetal cardiomyocyte growth and maturation and improve offspring cardiovascular function and morphology in rats exposed to hypoxia in pregnancy.

1.8.2 Aims

Using established rat models of maternal hypoxia, I aimed to:

1- Assess the effect of hypoxia in pregnancy on maternal peripheral and uterine vascular function (using wire myography) and uteroplacental and fetoplacental blood flow velocities (using ultrasound biomicroscopy).
2- Assess the effect of hypoxia in pregnancy on fetal growth, cardiomyocyte growth and maturation *ex vivo* (by performing cell culture) and, cardiovascular function and morphology both *in vivo* (using echocardiography and tail-cuff plethysmography) and *ex vivo* (using wire myograph) in adult male and female offspring (separately).
3- Assess whether placental antioxidant treatment using the antioxidant MitoQ loaded onto nanoparticles can improve uteroplacental and fetoplacental blood flow velocities, fetal growth, cardiomyocyte growth and maturation, and cardiovascular function and morphology in male and female offspring of dams exposed to hypoxia in pregnancy.

This rat model of maternal hypoxia has been chosen as previous studies in our laboratory and other laboratories have shown that exposing pregnant rats to hypoxia can lead to maternal vascular dysfunction (383), placental oxidative stress (268), intrauterine growth restriction (218, 289), abnormal fetal cardiomyocyte development (21) and cardiovascular dysfunction in adult offspring (218, 289). All these phenotypes are important to assess our hypothesis.

CHAPTER 2: MATERNAL VASCULAR RESPONSES TO HYPOXIA IN A RAT MODEL OF INTRAUTERINE GROWTH RESTRICTION

A version of this chapter has been published:

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Author contributions: *M.M.A. experimental design, data acquisition, data analysis, draft preparation and critical revision of manuscript. J.S.M. and S.T.D. experimental design, draft preparation and critical revision of manuscript. C.L.C. draft preparation and critical revision of manuscript.*

2.1 INTRODUCTION

Intrauterine growth restriction (IUGR) is a common pregnancy complication that occurs when a fetus does not reach its genetic growth potential during gestation. IUGR prevalence is approximately 15% for all pregnancies and it is one of the leading causes of neonatal morbidity and mortality worldwide (166, 359). Further, IUGR offspring are at increased risk of cardiovascular disease later in life (26, 216). A common feature of IUGR is low oxygen availability/hypoxia which can affect normal maternal vascular adaptations to pregnancy leading to disruption in placental blood supply and ultimately restricting fetal growth and development (36, 45, 265, 361, 362). In order to develop therapeutic strategies, there is a critical need to understand the factors involved in the pathophysiology of IUGR and developmental programming. Therefore, I will assess herein the effect of hypoxia on maternal peripheral and uterine vascular function both *in vivo* and *ex vivo* by using an established rat model of maternal hypoxia and IUGR (383).

Studies in our laboratory have shown that exposing pregnant rats to hypoxia [11-12% O_2, gestational day (GD) 15-21] led to fetal growth restriction (288, 309, 364). Other laboratories have also demonstrated fetal growth restriction resulting from maternal hypoxia in rats (9% O_2, GD 14.5-17.5), mice (12% O_2, GD 15.5-18.5), as well as in guinea pigs, sheep and women at high altitude (138, 151, 155, 243, 274, 345). Furthermore, hypoxia during pregnancy has been shown to affect the maternal cardiovascular system. For example, hypoxia increased maternal blood pressure by increasing plasma levels of endothelin-1 in pregnant rats (371), increased uterine artery myogenic tone in pregnant sheep (371), impaired proliferation of uterine artery vascular smooth muscle cells and impaired uterine vascular growth in pregnant guinea pigs (273, 274). Gestational hypoxia can also increase uterine artery vasodilator responses to adenosine monophosphate-

activated protein kinase through both nitric oxide (NO)-dependent and -independent mechanisms in pregnant mice (318). Further, hypoxia during pregnancy diminished the effects of NO inhibition in uterine arteries and enhanced basal NO activity in mesenteric arteries in pregnant guinea pigs (361, 362). The effect of hypoxia on uterine artery vascular function in response to hypoxia may also involve alterations in utero-placental blood flow. For example, the umbilical artery showed an increased RI in pregnant wild-type mice exposed to hypoxia (292). Further, in normotensive pregnant women living at high altitude, uterine artery diameter and blood flow were lower than those of women living at low altitude; resulting in lower birth weight offspring (140, 380). In Andean women, however, increased uterine artery blood flow and oxygen delivery to the fetus prevented altitude-associated reduction in birth body weight (141).

Due to the complexity of maternal vascular adaptations to hypoxia and some conflicting results in the literature, further assessment of *in vivo* and *ex vivo* maternal vascular function is warranted. We hypothesize that exposing pregnant rats to hypoxia will cause maternal systemic vascular dysfunction, increase the uterine artery RI, and IUGR. In order to fully evaluate the effects of disrupted maternal vascular function, we assessed maternal blood pressure, fetal growth, and performed *in vivo* uterine and umbilical vasculature Doppler ultrasonography. In addition, we assessed uterine artery (important for placental blood supply) and mesenteric artery (a representative resistance-sized systemic artery) vascular function *ex vivo*.

2.2 MATERIALS AND METHODS

All procedures used were approved by the University of Alberta Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care and conformed to the Guide for the Care and Use of Laboratory Animals.

2.2.1 Animals and treatments

Female and male Sprague Dawley (SD) rats were obtained from Charles River, Quebec, Canada at 12 weeks of age and were acclimatized for one week within the animal facilities of the University of Alberta. Females were then mated with a young male overnight and pregnancy was confirmed the following morning via the presence of sperm in a vaginal smear; designated as GD 0 (term 22 days). Throughout the pregnancy, rats were single housed in standard rat cages under a 10:14 hr light: dark cycle and fed standard rat chow *ad libitum*. Rats were randomly divided into two groups; in one group of rats were kept in normal atmospheric conditions (21% O_2; normoxia) throughout pregnancy. In the second group, rats were exposed to hypoxia [11% O_2; prenatal hypoxia (p-hypoxia)] by placing them in an acrylic chamber (Animal Chamber for Disease Modeling type A, Biospherix, Lacona, New York) which maintained the concentration of oxygen at 11% by regulating nitrogen infusion. Soda lime was placed inside the chamber to absorb excess CO_2. The hypoxic exposure was started on GD 6 based on a previous study which showed that chronic hypoxia prior to GD 5 can considerably increase the rate of pregnancy loss in rats (268). Both normoxia and hypoxia rats were housed in the same room. On GD 14, rats' cages were rapidly replaced with clean cages, fresh feed and water which caused the O_2 levels to rise to $15.0 \pm 0.6\%$ for less than 5 minutes. Rats were removed from the hypoxia chamber on GD 20 to perform experimental procedures (Figure 2.1).

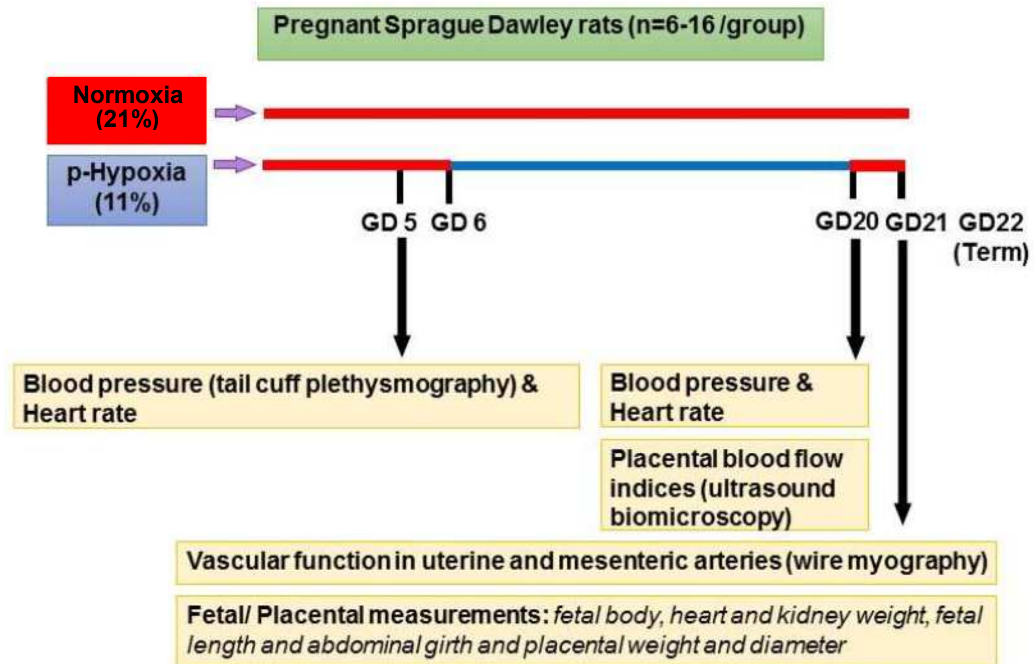


Figure 2.1 Experimental design of maternal study
Maternal blood pressure and heart rate were assessed before and after exposure to hypoxia. Dams were taken out of the hypoxic chamber on gestational day (GD) 20 to assess placental blood flow indices. Then, dams were left in normoxia till the next day, when it was euthanized to assess vascular function and perform fetal and placental measurements. p-Hypoxia: prenatal hypoxia.

2.2.2 Blood pressure and heart rate measurement

Blood pressure was measured on GD 5 and GD 20 (before and after hypoxia) using tail-cuff plethysmography (CODA, Kent Scientific Corporation, Torrington, Connecticut, USA). Rats were placed in restraint tubes and left for 20 minutes to warm (tail skin surface temperature ~30 °C). An average was taken of at least ten blood pressure measurements performed over a period of 10 minutes.

2.2.3 Ultrasound biomicroscopy

Fetal heart rate and uterine artery, umbilical artery and umbilical vein blood flow velocities and RI were assessed on GD 20 using an ultrasound biomicroscope (model Vevo 2100, VisualSonics, Toronto, Ontario, Canada) and a previously established protocol (221). Ultrasound was performed on GD 20 when structural vascular remodeling is complete and therefore any uterine artery changes that may have occurred with hypoxia would be expected to persist in the normoxic environment (233). One hour of re-acclimatization was used prior to performing ultrasound to avoid acute responses to changing oxygen levels. Rats were anesthetized with inhaled isoflurane (3% induction, 1-3% maintenance). Doppler waveforms were obtained from the uterine artery near the utero-cervical junction close to the iliac artery. The highest point of the systolic waveform was considered as the peak systolic velocity (PSV) and the end point of the diastolic waveform was considered as the end diastolic velocity (EDV). Both PSV and EDV were measured from at least three consecutive cardiac cycles that were not affected by maternal breathing motion, and the results were averaged per dam. Waveforms were obtained from umbilical arteries and veins near the placental surface of at least three randomly selected fetuses per dam and the values were averaged to generate a single value per dam. RI was calculated using the equation (PSV−EDV)/PSV (Figure 2.2). The pulsatility index [PI, (PSV−EDV)/TAV (time

averaged velocity per cardiac cycle)] and the ratio of PSV to EDV (S/D) were calculated. Fetal heart rate was calculated by averaging the measured distances between the peaks of three consecutive umbilical artery waveforms. In order to increase the power of our *in vivo* studies of blood pressure and Doppler ultrasound measurements, data from the current study were merged with data from a pilot study in which rats experienced exactly the same normoxia and hypoxia environments but were given Ensure as a vehicle control (5 ml orally once/day, GD 0-20; Abbott Laboratories, QC). In both normoxia and hypoxia groups, subgroup analysis demonstrated that there were no significant differences in blood pressure or ultrasound parameters between rats which received Ensure and rats that did not; hence these data could be merged.

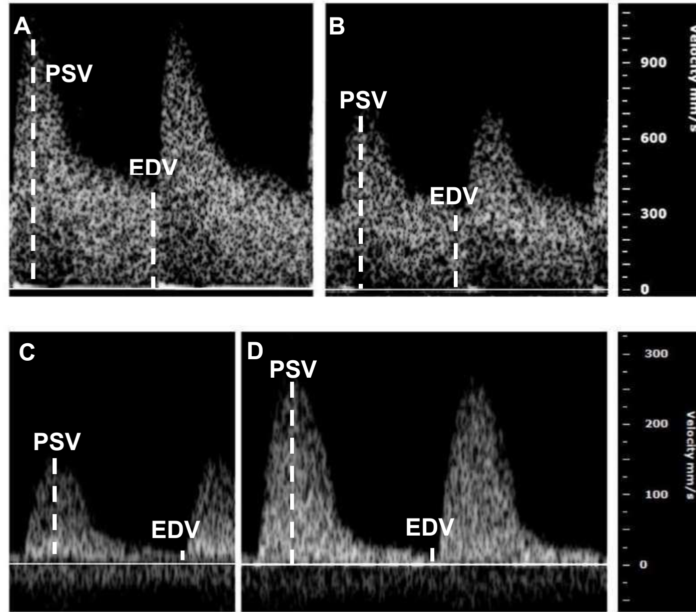


Figure 2.2 Representative images of the uterine and umbilical artery waveforms
Measurements were done using ultrasound biomicroscopy in normoxia and hypoxia groups at gestational day 20. Uterine artery waveforms (**A**: normoxia, **B**: p-hypoxia). Umbilical artery waveforms (**C**: normoxia, **D**: p-hypoxia). Peak systolic velocity (PSV) and end diastolic velocity (EDV) (dotted lines).

2.2.4 Fetal and placental measurements and maternal weight

On GD 21, rats were euthanized by exsanguination under isoflurane anesthesia. The uterus containing pups was removed, and its weight measured and subtracted from the maternal weight taken prior to euthanasia. After dissecting the pups away from the placentas, fetal weight, crown to rump length, and abdominal circumference, were measured, and averaged per litter. Heart and kidney weights of three, randomly chosen, pups per dam were taken and averaged. The gross anatomy of the pups was also examined. All placentas were removed from the uterus and blotted dry, their diameter and wet weight recorded, then dried at 40 °C overnight and their dry weight measured. The ponderal index was calculated as 1000 X (body weight X $length^{-3}$).

2.2.5 Wire myography for ex vivo vascular function

Main branch uterine arteries and second-order mesenteric arteries were collected on GD 21 and cleaned of surrounding adipose tissue in ice-cold fresh PSS (in mmol/L: NaCl 142, KCl 4.7, $MgSO_4$ 1.17, $CaCl_2$ 4.7, K_2PO_4 1.18, HEPES 10 and glucose 5.5; pH 7.4). Arteries were then mounted on two 40 µm tungsten wires in a wire myograph system (DMT, Copenhagen, Denmark) to allow isometric tension recordings. Vessels were normalized through a series of stepwise increases in diameter to determine their optimal resting tension. After a 30-minute equilibration, arteries were twice exposed to a single dose of phenylephrine (PE, 5 µmol/L, Sigma), followed by a single dose of methacholine (MCh, 15 µmol/L, Sigma) to evaluate the functional integrity of the smooth muscle and vascular endothelium respectively. Constrictor responses were determined using PE (0.01 to 100 µmol/L). Vascular responses to PE were used to calculate EC_{80} (PE concentration required to produce 80% of the maximal vascular response). Then, EC_{80} for each vessel was used for vascular constriction that is needed to initiate MCh and SNP vasodilation curves. Cumulative concentration response curves to MCh (0.0001 to 100 µmol/L) and sodium

nitroprusside (SNP, 0.0001 to 10 μmol/L) were performed to assess endothelium-dependent and -independent vasodilation, respectively. To study the role of NO in MCh-induced vasodilation and PE-induced constriction, responses were performed in the absence or presence of the NO synthase (NOS) inhibitor, L-NAME (100 μmol/L, Sigma, preincubation for 30 min). Finally, vessels were exposed to high K^+ solution (124 μmol/L). Vessels segments were chosen randomly from at least two mesenteric or from both uterine arteries of each animal. Each vascular response assessment was duplicated in two random vessel segments and the responses were averaged per dam. Data for vessels whose constriction decreased following the initial response to the second PE dose (the second vascular wake up by PE, which was done before initiating the cumulative concentration response curves), or that failed to relax by more than 30% in response to MCh, were excluded.

Vascular diameter (n= 8 dams/group) was measured by taking micrometer readings under a dissection microscope with the mounting wires close together (a1) and then with the wires gently touching the vessel walls but not under tension (a2). The values were subtracted and the diameter of the mounting wires (2 X 40 μm) was added to obtain the final internal vascular diameter using the equation: internal diameter = (a2-a1) + 80.

2.2.6 Statistical analyses

GraphPad Prism 5.0 software was used for statistical analyses. Sigmoidal curve fitting was performed on wire myography data for each vessel. From these curves, a pEC_{50} value was calculated (the effective concentration required to produce 50% of the maximal response values). Area under the curve (AUC) was calculated as the change from 0% of vasodilation using GraphPad Prism. AUC with and without inhibitors were subtracted to obtain the contribution of a specific pathway to vasodilation or vasoconstriction expressed as delta AUC. Data which were normally distributed were expressed as mean ± SEM and compared using the Student *t*-test. Non-parametric

data (litter size) were presented as median (range) and compared using the Mann-Whitney test. Where more than one conceptus (pup or placenta) was measured per dam, an average was taken to provide a single value per dam. A P value < 0.05 was considered statistically significant.

2.3 RESULTS

2.3.1 Maternal blood pressure

Mean blood pressure and heart rate increased after 2 weeks of hypoxic exposure with both systolic and diastolic arterial blood pressure tending to increase (Table 2.1). Maternal blood pressure was lower at the third week compared to the first week of pregnancy (mean maternal blood pressure at GD 5 vs. GD 20: $P < 0.01$).

Table 2.1 Maternal blood pressure and heart rate at GD 5 and GD 20

Blood pressure and heart rate	Normoxia	p-Hypoxia	P value
GD 5			
Systolic blood pressure (mmHg)	140 ± 3	146 ± 5	
Diastolic blood pressure (mmHg)	104 ± 3	109 ± 5	
Mean blood pressure (mmHg)	116 ± 3	121 ± 5	
Heart rate (bpm)	411 ± 8	405 ± 13	
GD 20			
Systolic blood pressure (mmHg)	**125 ± 3**	**133 ± 3**	**0.058**
Diastolic blood pressure (mmHg)	**90 ± 3**	**98 ± 3**	**0.055**
Mean blood pressure (mmHg)	**101 ± 3**	**109 ± 3**	*
Heart rate (bpm)	**403 ± 6**	**431 ± 9**	*

GD: gestational day. Data presented as mean ± SEM (n=10-16/group). *P < 0.05. **P < 0.01.

2.3.2 Uterine and umbilical artery resistance indices

In the uterine artery, RI, S/D, and PI decreased in the hypoxic group compared to the normoxic control. In contrast, in the umbilical artery there were no significant changes in RI, S/D or PI, but the umbilical vein RI was increased (Table 2.2). Fetal heart rate was higher following hypoxia (normoxia 245 ± 4 bpm vs. p-hypoxia: 263 ± 7 bpm, $P < 0.05$).

Table 2.2 Hemodynamic parameters of the uterine and umbilical vasculature as assessed by ultrasound biomicroscopy at GD 20

Parameter	Normoxia	p-Hypoxia	P value
Uterine artery			
RI	**0.64 ± 0.02**	**0.54 ± 0.01**	***
PI	**1.08 ± 0.09**	**0.75 ± 0.03**	**
S/D	**2.93 ± 0.22**	**2.19 ± 0.06**	**
TAV (mm/s)	**541 ± 33**	**386 ± 29**	***
Umbilical artery			
RI	0.92 ± 0.01	0.91 ± 0.01	
PI	1.69 ± 0.03	1.73 ± 0.11	
S/D	14.58 ± 1.17	12.69 ± 1.76	
TAV (mm/s)	113 ± 7	124 ± 7	
Umbilical vein			
RI	**0.35 ± 0.02**	**0.45 ± 0.05**	*

GD: gestational day; RI: resistance index; PI: pulsatility index; S/D: PSV to EDV ratio; TAV: time averaged velocity. Data presented as mean ± SEM (n=13-17/group). *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

2.3.3 Reproductive phenotype and maternal weight gain

Placental parameters including wet weight, dry weight and diameter did not differ between the normoxic and hypoxic groups (Figure 2.3A and Table 2.3). Litter size was not significantly different between the groups (normoxia: 15 (14-17) pups vs. p-hypoxia: 15 (5-16) pups, P=0.22), but the variance of litter size was greater in the hypoxic group. In the hypoxic group, the average pup weight was significantly reduced (Figure 2.3B). Crown-rump length and abdominal girth were significantly decreased by hypoxia while ponderal index was not altered; demonstrating symmetrical growth restriction with hypoxia (Table 2.3). Neither absolute nor relative (normalized to body weight) fetal heart weights were altered by hypoxia. The absolute fetal kidney weight was decreased in the hypoxic group; however, the relative kidney weight (normalized to body weight) did not change (Table 2.3).

While total maternal body weight decreased at GD 21 in dams exposed to hypoxia compared to their normoxic counterparts (normoxia: 461 ± 13 g vs. p-hypoxia: 424 ± 11 g, $P < 0.0001$), maternal body weight without the utero-placental unit was unaltered between groups (normoxia: 347 ± 11 g vs. p-hypoxia: 344 ± 10 g, P=0.63); suggesting that the difference was due to the reduced fetal weights.

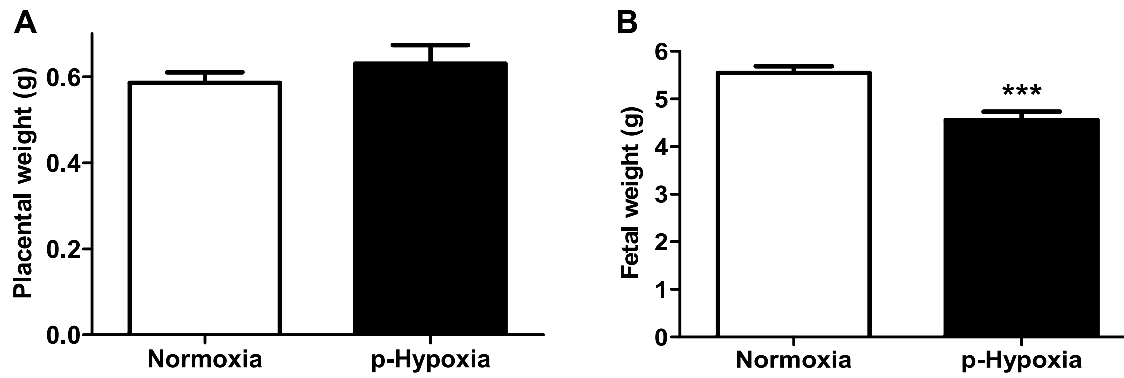


Figure 2.3 Fetal and placental weight on gestational day (GD) 21

A: Placental wet weight (g) was not significantly different (P=0.30), while **B**: fetal body weight (g) was lower in p-hypoxia compared to the normoxia group on GD 21. Data are presented as mean ± SEM (n= average weights from pups born from 6-8 dams/group). ***P < 0.001.

Table 2.3 Fetal and placental parameters at GD 21

Parameter	Normoxia	p-Hypoxia	P value
Crown-rump length (cm)	**4.58 ± 0.08**	**4.15 ± 0.07**	**
Abdominal girth (cm)	**4.60 ± 0.14**	**3.99 ± 0.10**	**
Ponderal index ($g.cm^{-3}$)	58 ± 2	63 ± 2	
Heart weight (mg)	45 ± 1	41 ± 3	
Heart weight /body weight (%)	0.80 ± 0.02	0.88 ± 0.05	
Kidney weight (mg)	**48 ± 2**	**38 ± 3**	**
Kidney weight /body weight (%)	0.87 ± 0.03	0.82 ± 0.04	
Placental dry weight (mg)	107 ± 4	106 ± 7	
Placental diameter (cm)	1.42 ± 0.03	1.49 ± 0.04	
Placental wet weight /body weight (%)	**10.54 ± 0.25**	**14.03 ± 0.98**	**

GD: gestational day. Data presented as mean ± SEM. (n=6-8/group). **$P < 0.01$.

2.3.4 Uterine artery *ex vivo* vascular function

Uterine artery dilation to MCh demonstrated decreased sensitivity in the hypoxic group (Table 2.4). L-NAME significantly decreased responses to MCh in uterine arteries from normoxic dams (Figure 2.4A, $P < 0.01$) but not from the hypoxic dams (Figure 2.4B). Assessment of the delta AUC of the vasodilator response to MCh in the presence or absence of L-NAME demonstrated that exposure to hypoxia caused a reduction in the contribution of NO to vasodilation (Figure 2.4C). Sensitivity to the endothelium-independent agonist SNP was not altered following exposure to hypoxia (Table 2.4).

Uterine artery vasoconstriction in response to high K^+ or PE was not altered by exposure to hypoxia (Table 2.4). L-NAME did not alter vasoconstriction to PE in either the normoxic or hypoxic groups (Figure 2.5). Delta AUC of the vasoconstrictor response to PE in the presence or absence of L-NAME was not altered between the groups (normoxia: 6.02 ± 1.58 vs. p-hypoxia: 5.25 ± 0.99, $P=0.68$).

Table 2.4 Vascular function of the uterine and mesenteric arteries as assessed by wire myography at GD 21

Vascular bed/agonist	Normoxia	p-Hypoxia	P value
Uterine artery			
MCh (pEC_{50})	**6.56 ± 0.24**	**5.03 ± 0 .36**	**
SNP (pEC_{50})	7.10 ± 0.11	6.89 ± 0.08	
High K^{+} (mN/mm)	8.99 ± 0.66	9.14 ± 0.74	
PE (pEC_{50})	5.95 ± 0.10	5.98 ± 0.09	
Mesenteric artery			
MCh (pEC_{50})	8.36 ± 0.12	8.08 ± 0.05	
SNP (pEC_{50})	9.02 ± 0.07	9.09 ± 0.07	
High K^{+} (mN/mm)	5.66 ± 0.58	5.29 ± 0.55	
PE (pEC_{50})	5.80 ± 0.11	5.52 ± 0.08	

GD: gestational day; MCh: methacholine; SNP: sodium nitroprusside; K^{+}: potassium ion; PE: phenylephrine; pEC_{50}: the effective concentration required to produce 50% of the maximal response. Data presented as mean ± SEM. (n=4-8/group). **$P < 0.01$.

Uterine artery

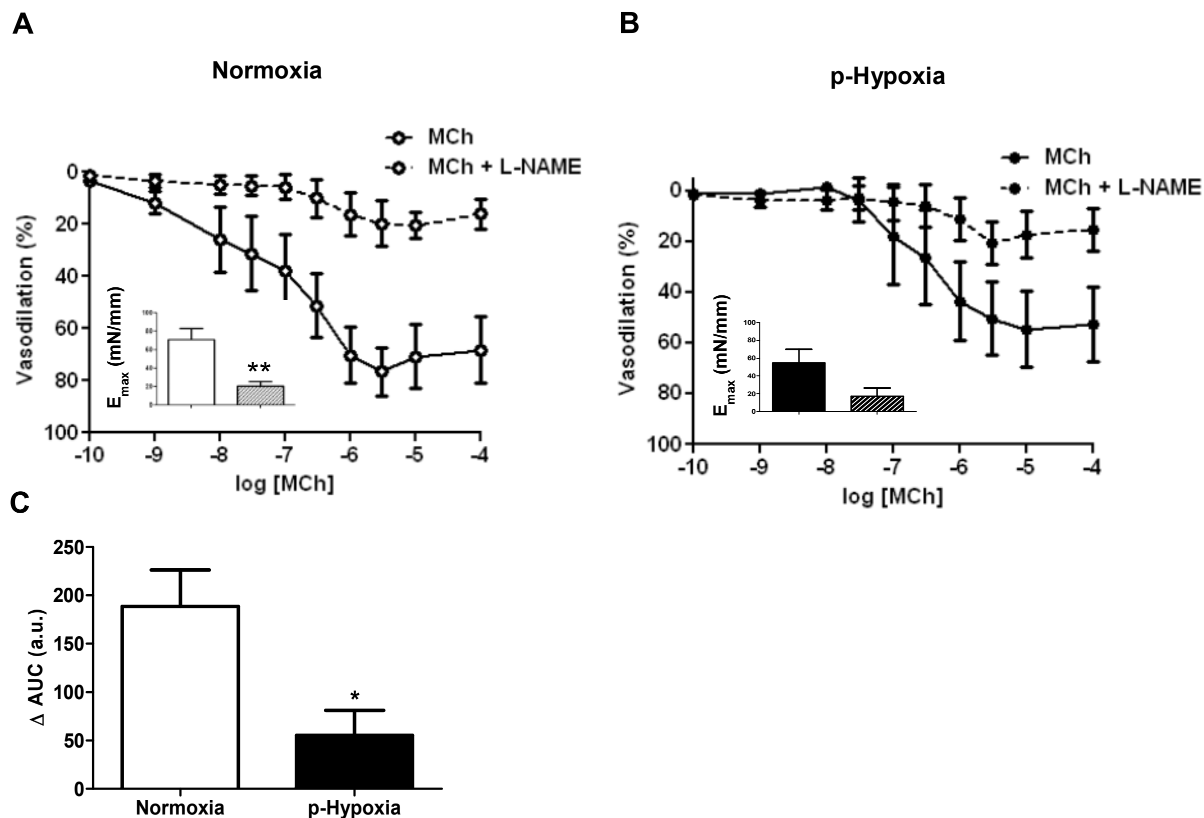


Figure 2.4 Uterine artery responses to the vasodilator methacholine (MCh) in the absence or presence of Nώ-nitro-L-arginine methyl ester (L-NAME)

Vasodilation in normoxia (**A**) and p-hypoxia (**B**) groups. Inserted graphs in A & B show Emax (mN/mm). **C**: summary delta area under the curve (△ AUC, the difference between MCh-induced vasodilation ± L-NAME) in normoxia and p-hypoxia groups showing a reduction in nitric oxide contribution to vasodilation. Data are presented as mean ± SEM (n=4-6/group). *P < 0.05. **P < 0.01.

Uterine artery

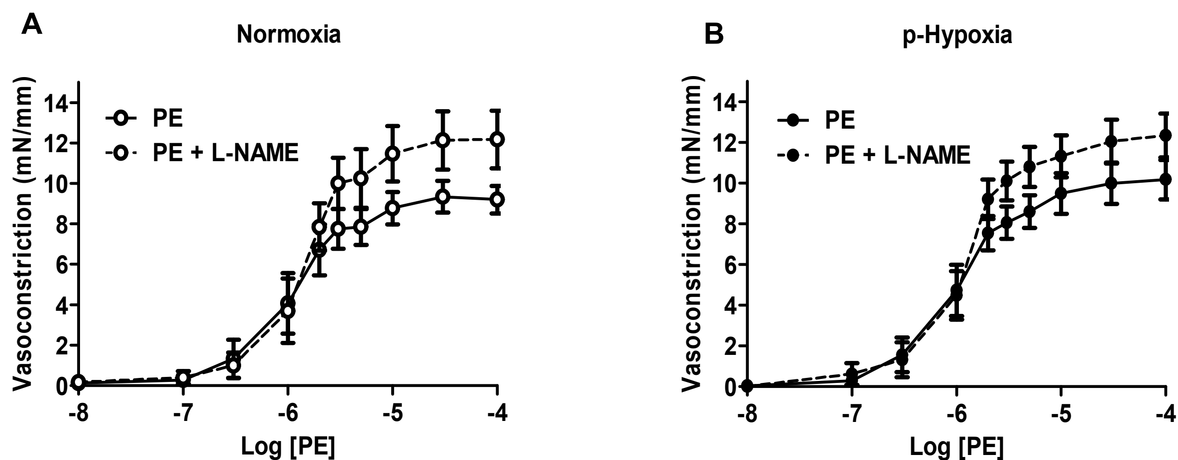


Figure 2.5 Uterine artery responses to the vasoconstrictor phenylephrine (PE) in the absence or presence of Nώ-nitro-L-arginine methyl ester (L-NAME)
Vasoconstriction in normoxia (**A**) and hypoxia (**B**) groups. Vasoconstriction in response to PE was not altered by exposure to prenatal hypoxia (p-hypoxia). Data are presented as mean ± SEM (n=8/group).

2.3.5 Mesenteric artery *ex vivo* vascular function

Mesenteric artery dilation to MCh demonstrated no difference in sensitivity following exposure to hypoxia (Table 2.4). L-NAME significantly decreased sensitivity to MCh in both the normoxic and hypoxic groups (Figure 2.6A and B, $P < 0.05$ and $P < 0.001$ respectively). However, hypoxia led to a significant increase in the contribution of NO to the dilation of mesenteric arteries as assessed by the delta AUC (Figure 2.6C). Sensitivity to SNP was not altered between the groups (Table 2.4).

Mesenteric artery constriction in response to high K^+ or PE was not altered between the hypoxia and normoxia groups (Table 2.4). L-NAME did not increase PE-induced vasoconstriction in either group (Figure 2.7); therefore, the delta AUC for the vasoconstrictor response to PE in the presence or absence of L-NAME was also not altered by hypoxic exposure (normoxia: 1.94 ± 0.64 vs. hypoxia: 0.84 ± 0.09, $P=0.21$).

Mesenteric artery

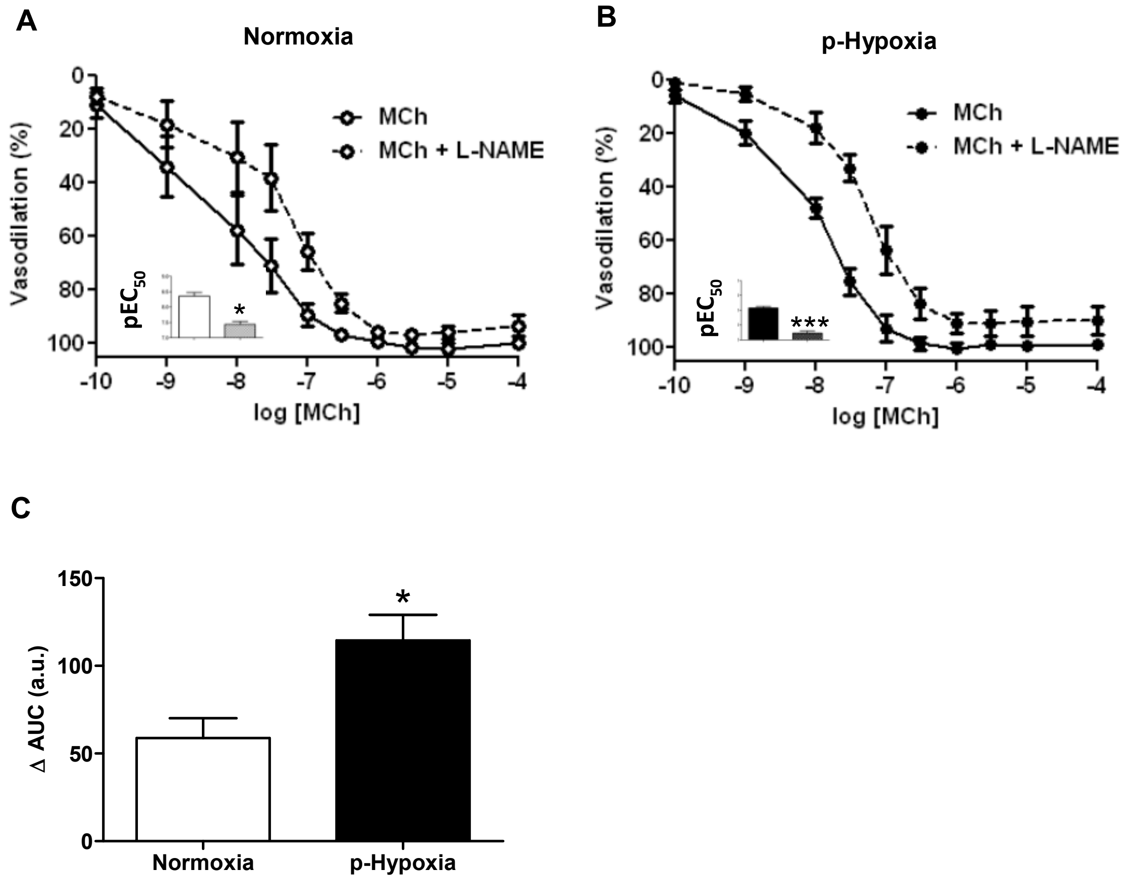


Figure 2.6 Mesenteric artery responses to the vasodilator methacholine (MCh) in the absence or presence of Nώ-nitro-L-arginine methyl ester (L-NAME)

Vasodilation in normoxia (**A**) and p-hypoxia (**B**) groups. Inserted graphs in A & B show pEC_{50}. **C**: summary delta area under the curve (△ AUC, the difference between MCh-induced vasodilation ± L-NAME) in normoxia and p-hypoxia groups showing an increase in nitric oxide contribution to vasodilation. Data are presented as mean ± SEM (n=5/group). $*P < 0.05$. $***P < 0.001$.

Mesenteric artery

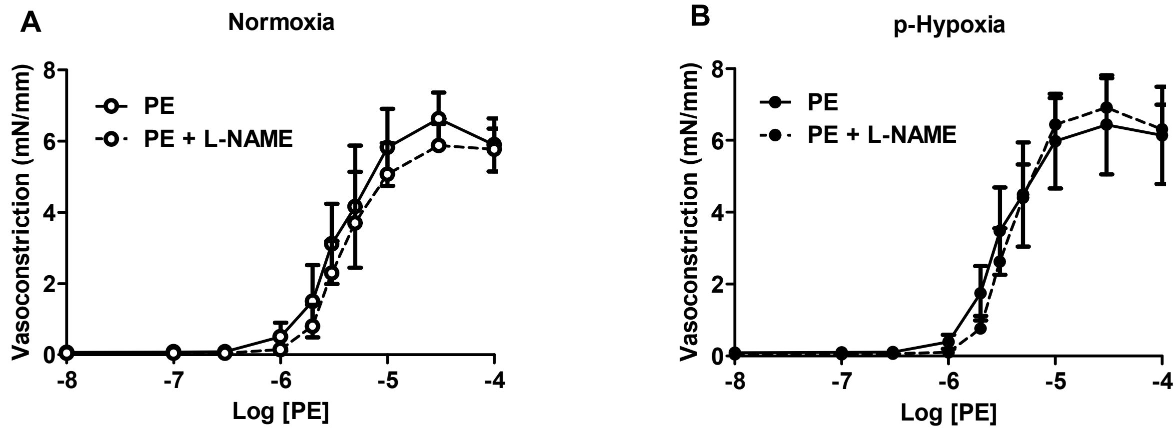


Figure 2.7 Mesenteric artery responses to the vasoconstrictor phenylephrine (PE) in the absence or presence of Nώ-nitro-L-arginine methyl ester (L-NAME)
Vasoconstriction in normoxia (**A**) and hypoxia (**B**) groups. Vasoconstriction in response to PE was not altered by exposure to p-hypoxia. Data are presented as mean ± SEM (n=4-6/group).

2.3.6 Internal diameter of uterine and mesenteric arteries

The internal diameter of both uterine and mesenteric arteries was not altered between the groups. Internal diameters were as follows: uterine arteries: (normoxia: 176 ± 9 µm vs. p-hypoxia: 200 ± 17 µm, P= 0.23) and mesenteric arteries: (normoxia: 121 ± 4 µm vs. p-hypoxia: 128 ± 4 µm, P= 0.22).

2.4 DISCUSSION

The current study demonstrates that hypoxia during rat pregnancy is a valid model of IUGR, and associated with increased maternal blood pressure and altered vascular reactivity using both *in vivo* and *ex vivo* assessments. Our data show that gestational hypoxia decreased uterine artery RI, as well as reduced the uterine artery sensitivity to the vasodilator MCh. No effect on vasoconstriction in response to PE or high K^+ was noted. Hypoxia did not lead to a difference in the vascular dilation or constriction of the mesenteric artery but increased the contribution of NO to vasodilation. Fetal consequences of gestational hypoxia included symmetric growth restriction, an increase in the umbilical vein RI, but no change in the umbilical artery RI. Thus, although our data demonstrated what appear to be a compensatory reduction in uterine artery RI, this was not sufficient to prevent IUGR.

Our results *in vivo* demonstrated a reduction in the uterine artery RI in late gestation in animals exposed to hypoxia compared to those in normoxic conditions. Interestingly, and in contrast to these results, a previous study in our laboratory showed unchanged uterine artery RI in IUGR mouse model created by maternal exposure to gestational hypoxia (10.5% O_2, GD 10.5-18.5), demonstrating different maternal vascular responses to hypoxia between rat and mouse (292). However, our current results in rats parallel recent study observations in mice exposed to hypoxia in pregnancy (11% O_2, GD 14.5-17.5), which showed a preserved placental weight, IUGR and significant decrease in the total uteroplacental resistance compared to normoxic controls (45). By assessing the possible mechanisms, hypoxic placentas showed an increase in the diameter of the radial arteries, expansion of the capillary network and a reduction in the interhaemal membrane thickness compared to the normoxic placentas (45). These mechanisms may have contributed to the reduction in uteroplacental resistance in hypoxic dams and the preservation of placental but

not fetal weight. Alongside animal studies, human observations showed a reduction in uterine artery RI associated with reduction in birth weight in women living at high altitude (4300 m altitude), compared to women living at low altitude (sea level) (160). Together, these findings support that reduction in uterine artery RI in response to perinatal hypoxia is not capable of rescuing fetal growth.

In the fetal umbilical vessels, our study showed no changes in the umbilical artery RI but an increase in the umbilical vein RI following exposure to hypoxia, suggesting that different mechanisms may regulate the maternal and fetal vascular responses to hypoxia. Studies conducted in human pregnancies and animal models complicated with IUGR show variable data regarding the umbilical artery RI. For example, in pregnancies complicated with IUGR in women, umbilical artery RI was either increased (9, 116, 120, 180); or did not show a difference (306) compared to those with a normal pregnancy. In mice, umbilical artery RI was higher in hypoxia compared to normoxia in wild-type, but was not altered in catechol-O-methyl transferase ($COMT^{-/-}$) mice (292). Studies that assessed umbilical vein responses to hypoxia have shown variable data as well. In one study of both wild-type and $COMT^{-/-}$ mice, umbilical vein RI was not different after exposure to hypoxia (292) while, in human pregnancies complicated by IUGR, umbilical vein blood flow was decreased compared to normal pregnancy (242). Thus, based on our data which suggest a hypoxia-induced increase in umbilical vein RI, the relationship and mechanisms behind umbilical artery and vein blood flow parameters in animal models of IUGR warrant further investigation. One of the possible mechanisms that could enhance the increase in umbilical vein RI in hypoxia was suggested by an *in vitro* study that showed an increase in the secretion of the vasoconstrictor endothelin-1 in human umbilical vein endothelial cells (HUVEC) in hypoxia compared to the HUVEC that stayed in normoxia (109).

Our data showed a reduction in maternal blood pressure near term compared to the blood pressure early in pregnancy. This has been shown in previous studies in humans (22) and rats (319) and could be mediated by a reduction in parasympathetic responsiveness early in pregnancy which returns to prepregnant levels later in pregnancy (85, 86). The relative vascular underfill in maternal peripheral vasculature near term does not practically associate with a reduction in the blood supply to the uterus due the rise that happens in maternal blood volume, cardiac output and stroke volume (319).

Our study also investigated mechanisms that could influence maternal vascular responses following exposure to hypoxia. Our data demonstrated that the uterine artery sensitivity to MCh was decreased due to reduced NO-dependent modulation in the hypoxic group. This is in agreement with a previous study that showed a reduction in the effect of NOS inhibition in the uterine artery of pregnant guinea pigs at high- vs. low-altitude (361). However, in the current study, uterine artery RI decreased *in vivo* in the hypoxia group. This suggests the presence of pathophysiological changes *ex vivo*, which did not translate into increased uterine vascular resistance when measured *in vivo* with ultrasonography. However, these subclinical changes may manifest further in the presence of co-morbidities such as obesity, diabetes or maternal aging, thus affecting blood flow through the uterine vasculature. In addition, other factors regulate uterine vascular function *in vivo* such as neuronal pathways, vascular remodeling and shear stress [reviewed in (193)], which were not assessed in our wire myograph system.

Interestingly, a previous study by Mateev *et al.* showed that hypoxia in guinea pigs prevented the normal pregnancy-associated increase in uterine artery vasodilator responses to flow. However, the addition of the nitric oxide synthase inhibitor N(G)-nitro-l-arginine (l-NNA) increased uterine artery flow-mediated vasodilation in hypoxia animals (195). Nitric oxide

inhibition would be expected to impair vasodilation, and this unanticipated result indicates that in guinea pigs, hypoxia disrupted the normal NO-dependent vascular responses. We speculate, in this case, that the activity of other endothelium-dependent vasodilation pathways (such as EDH and PGI2) could be enhanced due to hypoxia in the guinea pig model. These data highlight the complex regulation of endothelium-dependent vasodilator pathways in pregnancy and the effects of hypoxia. Indeed, in our current study, maternal exposure to hypoxia differentially affected the contribution of NO to uterine and mesenteric artery vasodilation. We demonstrate that the NO contribution to vasodilation was decreased in uterine arteries but increased in mesenteric arteries. This may be due to differences in their primary vasodilatory mechanisms; namely, whereby uterine arteries relied almost exclusively on NO, whereas mesenteric arteries demonstrated a greater contribution of non-NO mediated vasodilator mechanisms. Therefore, it is important to consider differences in vascular beds when interpreting impact of hypoxia.

Our data show that hypoxia exposure did not cause significant changes in placental size or weight compared to the control animals; however, there was a significant reduction in the fetal body weight. A similar study in which pregnant rats were exposed to a similar duration of hypoxia from day 6-20 but at 13% O_2 showed an increase in placental weight in the hypoxia group, but without inducing an IUGR phenotype (268). This suggests that the increase in placental size might "compensate" for the suboptimal environment caused by hypoxia and prevent IUGR, whereas the lower oxygen levels (11% O_2) used in our study attenuated this compensation.

2.5 PERSPECTIVES

In summary, this study illustrates how a hypoxic environment in pregnancy may affect both maternal and fetal vascular function. These data show that vascular responses to hypoxia are complex and vascular bed specific. Therefore, developing therapeutic interventions to benefit

uteroplacental function must consider all of these (sometimes conflicting) vascular effects. Only through this holistic approach, will there be hope to improve neonatal outcomes.

CHAPTER 3: MATERNAL TREATMENT WITH A PLACENTAL-TARGETED ANTIOXIDANT MITOQ IMPACTS FETAL CARDIOMYOCYTE DEVELOPMENT IN A RAT MODEL OF MATERNAL HYPOXIA

Part of this chapter (placental oxidative stress, placental weight, and fetal body weight) has been submitted and accepted for publication:

***MM Aljunaidy**, JS Morton, R Kirschenman, T Phillips, CP Case, CL Cooke and ST Davidge. Maternal Treatment with a Placental-Targeted Antioxidant (MitoQ) Impacts Offspring Cardiovascular Function in a Rat Model of Prenatal Hypoxia. Pharmacological research 134: 332-342. 2018.*

Author contributions (for this chapter): *M.M.A. experimental design, data acquisition, data analysis, draft preparation and critical revision of manuscript, J.S.M., C.L.C.* and *S.T.D. experimental design, draft preparation and critical revision of manuscript, E.G. data acquisition and analysis (level of corticosterone), T.P.* and *P.C. experimental design, provision of study materials, critical revision of manuscript*.

3.1 INTRODUCTION

As discussed in Chapter 2, intrauterine growth restriction (IUGR) is a common pregnancy complication that leads to a greater susceptibility to cardiovascular disease in adult life [reviewed in (216)]. We illustrated in the previous chapter how maternal hypoxia can cause alterations in maternal vascular function and placental hemodynamics. These changes might negatively impact placental function and consequently fetal growth and organ development (e.g. fetal heart) (10). Therefore, in the current chapter I assessed the effect of maternal hypoxia on fetal cardiomyocyte development. Furthermore, we assessed the effect of placental-targeted therapeutics on the fetal heart in hypoxic pregnancy.

Hypoxia is a major stress to the fetus and linked to IUGR and abnormal fetal cardiomyocyte development [reviewed in (10, 11)]. For example, maternal hypoxia [10.5% O_2, gestational day (GD) 15-21] in rats led to IUGR and abnormal fetal cardiac development including a higher than normal heart to body weight ratio, an increase in the size of binucleated myocytes, and a premature transition into maturity (increased percentage of binucleated cardiomyocytes) (21). In fetal sheep, placental insufficiency and hypoxia led to a delay in cardiomyocyte maturation (reduced percentage of binucleated cardiomyocytes) (38), a reduction in proliferation and cell cycle activity and a higher cardiomyocyte size relative to heart weight compared to fetal sheep from normal pregnancies (181, 215).

The mechanisms by which hypoxia can alter fetal development are not fully understood. We have suggested that maternal hypoxia can lead to an increase in the placental reactive oxygen species (ROS), which were linked to abnormal development of fetal organs including the cardiovascular system (11, 249). Mitochondria are one of the major sources of ROS in the placenta (129). Data from our laboratory showed that hypoxia can increase the mitochondrial source of

ROS in the placenta (104). Normally, ROS in the placenta plays a beneficial role in cellular functions such as inflammatory responses and cell signaling. However, higher production than normal of placental ROS caused by hypoxia can lead to placental dysfunction and over or underproduction of placental-derived circulating factors (66, 249). These placental factors can be released into the maternal and fetal circulation and affect fetal development [reviewed in (11)]. A study by Curtis *et al*. showed that abnormal development of fetal neurons *in vitro* after exposure to factors secreted from hypoxic placentas (66). We recently showed that placental secreted factors from hypoxic pregnancies can lead to abnormal fetal neuronal development in rats (249). However, the effects of placental oxidative stress and placental-secreted factors on fetal cardiomyocyte development in hypoxic pregnancy are still unknown.

As placental oxidative stress is likely key in the pathophysiology of IUGR and developmental programming, the effects of antioxidant intervention in pregnancy on offspring cardiovascular function have been assessed in complicated pregnancies and shown to have beneficial effects [reviewed in (216)]. For example, providing antioxidant treatment during pregnancy (ascorbic acid) prevented an increase in placental oxidative stress and abnormal cardiovascular function in offspring of rats exposed to hypoxia in pregnancy (97, 264). However, maternal antioxidant treatment has not been always beneficial in improving pregnancy outcome as shown in a clinical trial (Cochrane Pregnancy and Childbirth Group's Trials) on human pregnancy using vitamin C and E (293). Furthermore, in some cases, antioxidant treatment in pregnancy has had detrimental outcomes on the dams and offspring of control uncomplicated pregnancy. For example, although antioxidant treatments improved the fetal outcome of dams exposed to hypoxia in pregnancy, control rats from normal pregnancy also had insufficient placental growth after exposure to resveratrol (33). Maternal ascorbic acid supplementation as well led to vascular

endothelial dysfunction later in life in offspring from normal pregnancies (113). Therefore, in order to assess the effect of placental oxidative stress treatment on fetal cardiomyocyte development in hypoxic pregnancies, and to avoid risking direct fetal exposure to the antioxidant treatment, a new strategy was developed. This involved the attachment of the mitochondrial antioxidant (MitoQ) to nanoparticles to deliver the antioxidant treatment to the placenta and prevent it from crossing the placental barrier to the fetus [polymeric nanoparticles are poly γ-glutamic acid-*graft*-L-phenylalanine ethyl ester (γ-PGA-graft-L-PAE), NPs size ~ 180 nm, Zeta potential -20 mV; (249), and reviewed in (11)]. We showed previously that MitoQ loaded onto nanoparticles (MitoQ final dose: 0.5 μM) prevented placental oxidative stress, IUGR and the adverse effects of placental secreted factors on fetal neural development in a rat model of hypoxia, which included an improved dendritic shortening and decreased glutamate receptor expression. The abnormal fetal neural development due to maternal hypoxia could be related to the pathophysiology of fetal programming of psychiatric disease and provides a link between abnormal placental function and developmental programming (249). However, the effect of placental targeted MitoQ treatment on fetal programming of cardiovascular disease still needs investigation.

We hypothesize that hypoxia in pregnancy will lead to placental oxidative stress, which will cause abnormal fetal cardiomyocyte development mediated by alterations in placental secreted factors. Placental oxidative stress will also alter placental morphology which can cause a reduction in fetal growth. Furthermore, we hypothesize that maternal MitoQ treatment will prevent placental oxidative stress and ultimately improve fetal growth and cardiomyocyte development in hypoxic pregnancy.

3.2 METHODOLOGY

3.2.1 Experimental design

Female and male SD rats were obtained at 12 weeks of age from Charles River, Quebec, Canada and left for 1 week to acclimatize within the animal facilities of the University of Alberta. Females were then mated with a male overnight and the presence of sperm in a vaginal smear taken the following morning designated as GD 0 (term is on GD 22). Pregnant rats then were randomly divided into two groups. Each group was tail-vein injected once with either 100 µl MitoQ loaded into nanoparticles (nMitoQ; 125 µM) or with saline on GD 15. At this dose, maternal blood had a MitoQ weight/weight (w/w) 27.71 µg/kg (332.5 ng/ml blood concentration), nanoparticle vehicle w/w 62.37 µg/kg (748.5 ng/ml blood volume) and a combined weight of (nMitoQ) 90.08 µg/kg (1081 ng/ml), which is effective in preventing placental oxidative stress *in vivo* (249). nMitoQ or saline injected rats were exposed to either hypoxia (11% O_2) from GD 15-21 by placing them in a hypoxic chamber or being kept in the same room at atmospheric oxygen (21% O_2) throughout their pregnancy (Figure 3.1).

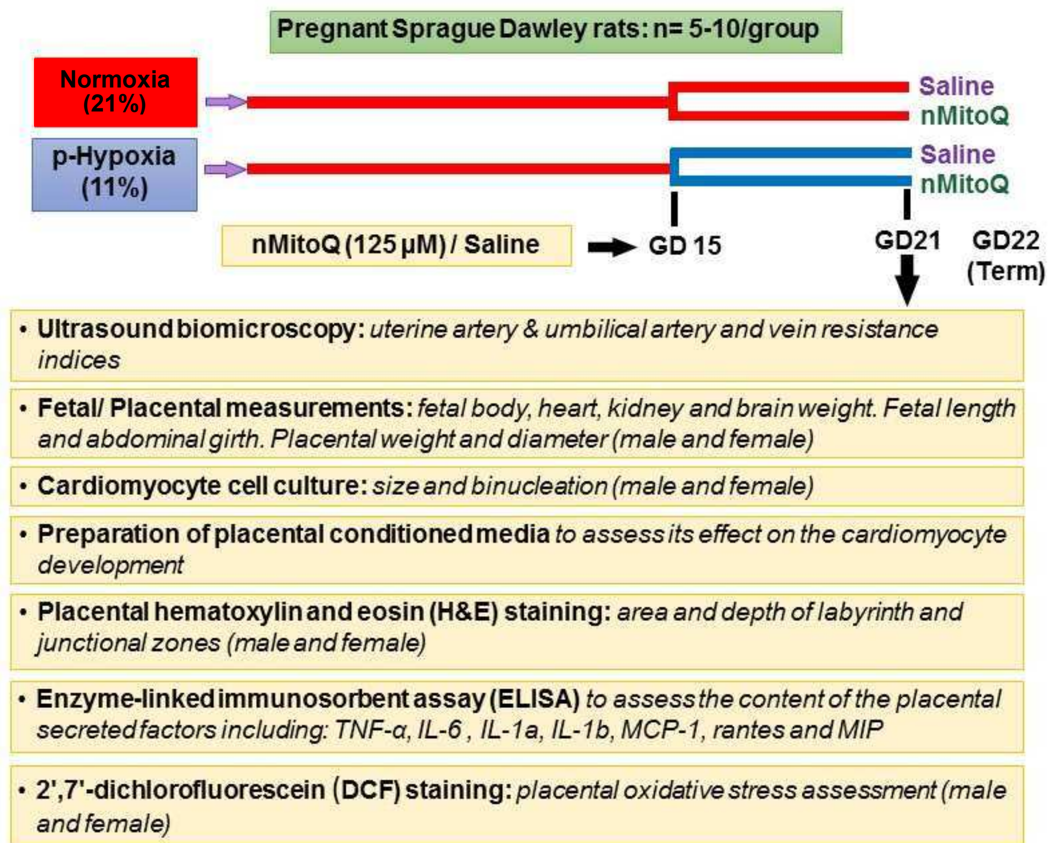


Figure 3.1 Experimental design of fetal and placental study
Pregnant rats were exposed to hypoxia between gestational day (GD) 15 and 21. Tail-vein injection of nMitoQ (125 µM) or saline for the control was performed on GD 15. On GD 21, ultrasound biomicroscopy was done to assess placental blood flow indices. Then, dams were euthanized on the same day to perform cardiomyocyte cell culture and collect placental and fetal tissues. p-Hypoxia: prenatal hypoxia.

Compared to what has been reported in Chapter 2, dams were exposed to a shorter period of hypoxia in pregnancy. This is because that shorter period of maternal hypoxia has previously been shown to cause a phenotype of IUGR, abnormal fetal cardiomyocyte development, and offspring cardiovascular dysfunction (21, 289). The final experimental groups consisted of saline-treated dams exposed to either normoxia (NormS) or hypoxia in pregnancy (pHypS) and nMitoQ-treated dams were exposed to either normoxia (NormQ) or hypoxia in pregnancy (pHypQ). Using pimonidazole staining (oxygenation marker), preliminary data from our laboratory showed a reduction in the fetal liver and heart oxygenation in the hypoxic dams of this animal model. Hence, herein we were able to use the term pHyp (prenatal hypoxia) to refer to the offspring of dams exposed to hypoxia in pregnancy.

3.2.2 Preparation of MitoQ loaded onto nanoparticles

An amphiphilic copolymer of poly (γ-glutamic acid) and L-phenylalanine ethyl ester (γ-PGA-graft-L-PAE; size ~ 180 nm) was synthesized by a coupling reaction. Using a previously established method (154, 249), 10 mg/ml of γ-PGA-Phe was dissolved in dimethyl sulfoxide (DMSO), added to an equivalent volume of 0.15 M NaCl, dialyzed against distilled water, freeze-dried and resuspended in phosphate buffered saline (PBS; 10 mg/ml). Nanoparticles were assessed by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK) as 180 nm diameter, Zeta potential -20 mV and polydispersity index 0.12. In 0.2 M NaCl, γ-PGA-Phe (nanoparticles, 10 mg/ml) were mixed with an equivalent volume of MitoQ (2 mg/ml) and left at 4°C for 12 h. MitoQ loaded nanoparticles were then isolated by centrifugation, washed with PBS, and resuspended in PBS to 10 mg/ mL (249).

3.2.3 Assessment of placental blood flow indices

As described previously in section 2.2.3, blood flow velocities of the uterine artery, umbilical artery and vein were assessed on GD 21 using an ultrasound biomicroscope (Vevo 2100). RI, PI and S/D then were calculated.

3.2.4 Fetal-placental measurements

Fetuses and placentas were obtained after euthanizing the rats on GD 21. Fetal body, heart, kidney, and brain weight were measured in addition to the fetal crown-rump length, fetal abdominal girth, placental weight and diameter. The fetal ponderal index was calculated using the same equation in section 2.2.4.

3.2.5 Assessment of placental oxidative stress

Placentas of male and female fetuses (one/sex/litter) were collected randomly from the uterine horn and cut transversely into halves, separately embedded in optimal cutting medium (OCT) and snap frozen in liquid nitrogen. Placental sections (20 µm, one/placenta) were prepared using cryostat LEICA 3050s, mounted on glass slides, washed with Hank's balanced salt solution (HBSS) and incubated with HBSS for 10 min at 37°C. The sections were then incubated with H2-DCF-DA dye (100 µM, 2',7'-dichlorodihydrofluorescein diacetate) for 20 min at 37°C. H2-DCF-DA is a non-fluorescent probe that turns into a fluorescent dye after oxidation inside the cell. The intensity of the fluorescence gives a sensitive and rapid quantitation of oxidative products (ROS) in the placenta. The choice of DCF staining was based on the postulation that superoxide is converted to hydrogen peroxide inside the mitochondria and then hydrogen peroxide leaves the mitochondria to the cytosol (114). Therefore, detection of superoxide, for example, in the cytosol might not detect any change in the level of total cellular oxidative stress. Thus, looking at the general intensity of ROS in the placenta was chosen rather than focusing on specific oxidants or

the activity of specific antioxidant enzymes. To visualize the nuclei, sections were washed and incubated for 5 min with 3.63% DAPI (4',6-diamidino-2-phenylindole, Thermo Fisher Scientific, MA, USA). Placental sections were imaged [2 images (with or without DAPI) per section, per zone] using an IX81 Olympus fluorescence microscope. Image J was used to quantify the intensity of ROS generation per field of view for both the placental labyrinth zone (important for nutrient and gas exchange between maternal and fetal circulations) and junctional zone (important for placental hormone secretion).

3.2.6 Assessment of placental morphology

Placentas (one/sex/litter) were fixed in 4% paraformaldehyde (PFA) overnight and stored in 70% ethanol at 4°C. Placentas, then, were cut transversely into halves and sent to the Alberta Diabetes Institute Histology Core for hematoxylin and eosin (H&E) staining. Placentas were embedded in wax, cross-sectioned (5μ) using a LEICA RM 2245 microtome in a vertical plane parallel to the block cutting. Using an established H&E staining protocol (3), the sections were dewaxed in histoclear, rehydrated and stained with filtered Harris's hematoxylin for 3 min, washed with distilled water and then put into filtered eosin for 30 sec. The placental sections were washed in cold water and left overnight to dry. Images were taken with a digital camera mounted on a brightfield microscope (EVOS XL Core Imaging System, Thermo Fisher Scientific, Canada) at a magnification of X2. Image J image analysis system was used to measure the depth and area of the labyrinth and junctional zones. The depth of the labyrinth zone was measured by drawing a vertical line from the center of the fetal surface to the peripheral edge. The depth of the junctional zone, however, was assessed by drawing a perpendicular line from the end of labyrinth depth line to the edge of the junctional zone. Cross-sectional areas of the labyrinth and junctional zones were outlined and measured (Figure 3.2).

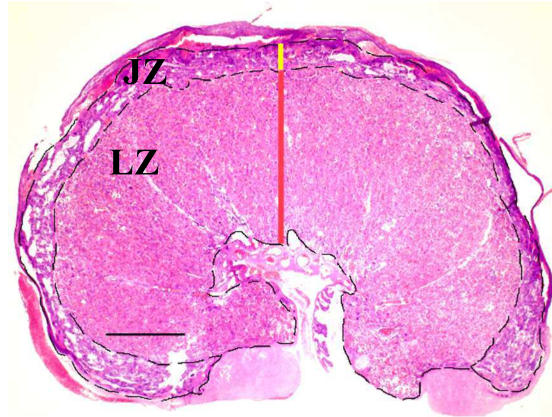


Figure 3.2 Placental morphology as shown after hematoxylin and eosin (H&E) staining

Representative image of labyrinth zone (LZ) area, junctional zone (JZ) area, LZ depth (red line) and JZ depth (yellow line). Placentas were collected on gestational day 21, cut transversely into halves and H&E stained. Scale bar = 1 mm.

Three randomly selected fields of each placenta were imaged at a magnification of X40. Then, using Image J software placental blood space area in each field of view was converted into black and remaining placental tissue into white for quantification. Total area of fetal and maternal blood space per field of view was calculated using Image J software, and the values were averaged per dam.

3.2.7 Assessment of cardiomyocyte growth and binucleation

At GD 21, hearts were collected from five fetuses of each sex per dam. Male and female cardiomyocytes were cultured separately as previously described (297). Briefly, the atria were removed, and the ventricles minced with scissors. A series of tissue digestions were performed using an enzyme buffer [collagenase (134.4 u/ml) and pancreatin ($6x10^{-4}$ mg/ml)]. Cardiomyocytes were then resuspended in cardiomyocyte media (Medium-199: 2% albumin, $2x10^{-3}$ M L-carnitine, $5x10^{-3}$ M creatine, $5x10^{-3}$ M taurine, 1:100 penicillin-streptomycin, 1:10.000 gentamicin) and incubated at 21% O_2, 37°C for 24 h.

Cultured cardiomyocytes were stained for actin as previously described (378). Briefly, cardiomyocytes were fixed in 3.5% PFA, permeabilized using 0.1% Triton-X-100 and incubated for 20 min with 2.5% rhodamine phalloidin (Thermo Fisher Scientific, MA, USA; fluorescently-labeled phalloidin binds selectively to F-actin which forms the cytoskeletal structure of the cardiomyocyte cell). Cells were then incubated for 5 min with 3.63% DAPI to visualize the nuclei. Cardiomyocytes were rinsed in 1% PBS before visualization by fluorescence microscopy. From each dam, 25 randomly-selected fields of isolated male and female cardiomyocytes were separately imaged using IX81 Olympus fluorescence microscope as previously described (252). The length, width and area of regularly-striated mononucleated and binucleated cardiomyocytes

were assessed at X40 magnification using CellSens Dimension software. Mononucleated and binucleated cardiomyocytes were counted/field and the percent binucleation was calculated.

3.2.8 Cardiomyocyte development in placental cultured media

Using a previously established method (249), placentas collected from NormS, NormQ, pHypS and pHypQ rats were cut into halves (to expose all the placental layers to the media), incubated overnight (at 8% O_2: physiological oxygen level in the placenta, 37°C) in culture plates containing 2 ml/well of warmed placental media [44% DMEM (Dulbecco's Modified Eagle's Media), 44% F12, 1% gentamicin, 0.4% penicillin-streptomycin, 0.6% L-glutamine and 10% FBS (fetal bovine serum)]. The placental media was then disposed of (to remove artifacts of a stressed placenta), replaced with warmed optimal cardiomyocyte growing media and incubated at 8% O_2, 37°C for 24h. The placental cultured media was then collected and frozen at -80 °C. Media from each study group were pooled and used to assess the effect of placental secreted factors on cardiomyocyte growth. Briefly, fetal cardiomyocytes were collected on GD 21 from normal pregnant rats (did not undergo any intervention such as hypoxia or nMitoQ exposure). These cardiomyocytes were isolated from male and female fetuses and cultured separately for 24h in one of the placental cultured media. Cardiomyocytes, then, were fixed, stained, imaged and their length, width, area and binucleation assessed. The assessment was done by merging placental conditioned medium from both male and female fetuses and applying it on male and female cardiomyocytes separately.

3.2.9 Assessment of inflammatory factors in placental cultured media

Changes in placental inflammatory factors (an increase or decrease) is associated with a reduction in neonatal body weight (15, 205). Therefore, placental cultured medium from the four groups (NormS, NormQ, pHypS and pHypQ) were assessed for the presence of inflammatory

factors using a rat inflammation ELISA (enzyme-linked immunosorbent assay; Signosis, CA, USA) strip. Briefly, ELISA plates were coated by the manufacturer with 8 different antibodies against rat inflammation cytokines; namely, TNF-α (tumor necrosis factor alpha), IL-6 (interleukin 6), INF-r (interferon receptor), IL-1α (interleukin 1α), IL-1β (interleukin 1β), MCP-1 (monocyte chemoattractant protein-1), RANTES (regulated on activation, normal T cell expressed and secreted), and MIP (macrophage inflammatory protein). Each well was already covered with one specific antibody for a specific cytokine. Samples (placental cultured medium) were added (100μl per well) and left for 2h at room temperature with gentle shaking. Then, wells were washed, a diluted biotin-labeled antibody mixture (1:50) was added and incubated for 1h at room temperature. This process allows test samples to react simultaneously with pairs of two antibodies, and consequently inflammatory cytokines become sandwiched between the solid phase and enzyme-linked antibodies. After incubation, the wells were washed to remove unbound-labeled antibodies. Then, streptavidin–HRP conjugate (1:200, 45 min) followed by substrate (100 μl per well, 30 min) were added to result in the development of a blue color. To stop color development, stop solution (50 μl per well, 10 min) was used. After that, absorbances were immediately read at wavelength of 450nm using a Bio-TEK plate reader (EL808, USA).

3.2.10 Assessment of corticosterone in fetal plasma

Plasma from male and female fetuses was collected on GD 21, snap frozen in liquid nitrogen and stored at -80 °C. Corticosterone levels in fetal plasma were then assessed following the manufacturer instructions of corticosterone ELISA kit (abcam, Canada). Briefly, diluted samples (fetal plasma, 1:100, 25 μl per well) were added, followed by biotinylated corticosterone applied on the top the samples (25 μl per well). The plate was covered and left at room temperature for 2 hours. Streptavidin-peroxidase conjugate (1:100), was added (50 μl per well, 30 minute),

followed by chromogen substrate (50 µl per well) for 20 minutes. Color development was stopped by stop solution (50 µl per well, 5 minutes), and absorbances were read immediately at wavelength of 450nm using a Bio-TEK plate reader (EL808, USA).

3.2.11 Statistical analysis

GraphPad Prism 5.0 software was used for statistical analyses, data were compared using a two-way ANOVA and expressed as mean ± SEM, and Tukey's post hoc test was performed to confirm where the differences occurred between groups. Non-parametric data (litter size) were shown as median (range) and compared using a Mann-Whitney test. A P value < 0.05 was considered statistically significant.

3.3 RESULTS

3.3.1 Hemodynamic parameters of the uterine and umbilical vasculature

Maternal hypoxia did not affect RI, PI and S/D ratio in the umbilical artery. Unlike what was observed in Chapter 2, that a shorter period of perinatal hypoxic exposure did not cause an elevation in umbilical vein RI. However, hypoxia in the last week of pregnancy was still able to cause a reduction in the uterine artery RI, PI and S/D ratio on GD 21. Maternal nMitoQ treatment did not affect the RI, PI or S/D ratio of the uterine and umbilical vasculature (Figure 3.3), showing that nMitoQ did not prevent the phenotype which was caused by hypoxia.

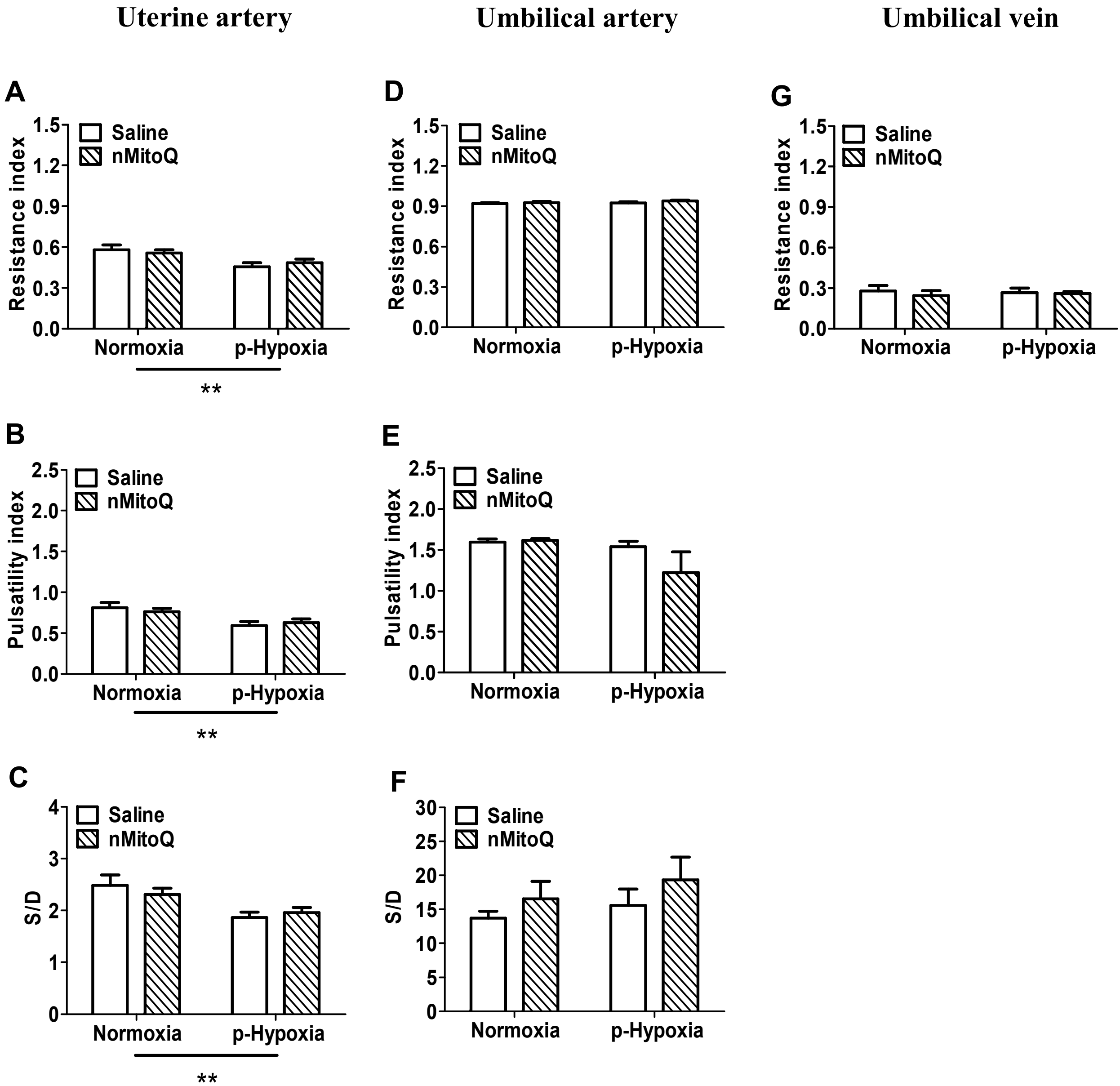


Figure 3.3 Effect of hypoxia in pregnancy and nMitoQ treatment on placental vasculature hemodynamics

Resistance index, pulsatility index and S/D [ratio of peak systolic velocity (PSV) to end diastolic velocity (EDV)] of the uterine artery (**A, B & C**), umbilical artery (**D, E & F**) and umbilical vein (**G**); assessed using ultrasound biomicroscopy on gestational day 21. Data are presented as mean ± SEM. **P < 0.01 group effect of maternal environment. n= 4-7/group.

3.3.2 Pregnancy phenotype

Litter size was not different between the groups [NormS: 15-16 (16) pups; NormQ: 8-20 (14) pups; pHypS: 10-17 (15) pups; pHypQ: 13-16 (15) pups].

Maternal hypoxia led to intrauterine growth restriction (IUGR) in both male and female fetuses (Figure 3.4A & D). nMitoQ treatment prevented IUGR only in female but not male fetuses (Figure 3.4A & D). Absolute heart weight did not change between all the groups in male and female fetuses (Table 3.1 & Table 3.2). However, relative heart weight (normalized to the body weight) increased in the male and female fetuses of hypoxic dams, indicating cardiac hypertrophy stimulated by hypoxia; this was prevented by nMitoQ treatment in the female but not the male fetuses (Figure 3.5A & B).

Both absolute and relative brain weight were not altered between the groups in male and female fetuses (Table3.1 & Table 3.2). In male and female fetuses, absolute kidney weight was lower in hypoxic than normoxic groups. However, when the kidney weight was normalized to the body weight, there was no difference between the groups (Table 3.1 & Table 3.2).

Hypoxic exposure did not alter male and female ponderal index (indicating symmetrical growth restriction), fetal crown-rump length, abdominal girth, or their ratio (Table 3.1 & Table 3.2). nMitoQ increased the crown-rump length of male fetuses in both normoxic and hypoxic groups (Table 3.1). This effect of nMitoQ on the crown-rump length was not observed in female fetuses (Table 3.2). nMitoQ did not alter abdominal girth, crown-rump length to abdominal girth ratio or ponderal index in male and female fetuses (Table 3.1 & Table 3.2).

Absolute placental weight increased in female fetuses of normoxic and hypoxic dams treated with nMitoQ (Figure 3.4E). This increase in the absolute placental weight was not observed

in male fetuses (Figure 3.4B). Placental weight to fetal body weight ratio was not altered between the groups due to hypoxia or nMitoQ treatment in male and female fetuses (Figure 3.4C & F). Placental dry weight was not altered between the groups (Table 3.1 & Table 3.2). Hypoxia did not alter male placental diameter. However, in male fetuses of hypoxic dams nMitoQ treatment caused a higher placental diameter than saline treatment, which could reflect an attempt to increase placental efficiency in hypoxia (Table 3.1). Hypoxia did not alter placental diameter in female fetuses, but similarly to the male fetuses, in female fetuses of hypoxic dams nMitoQ treatment caused a higher placental diameter compared to saline treatment (Table 3.2).

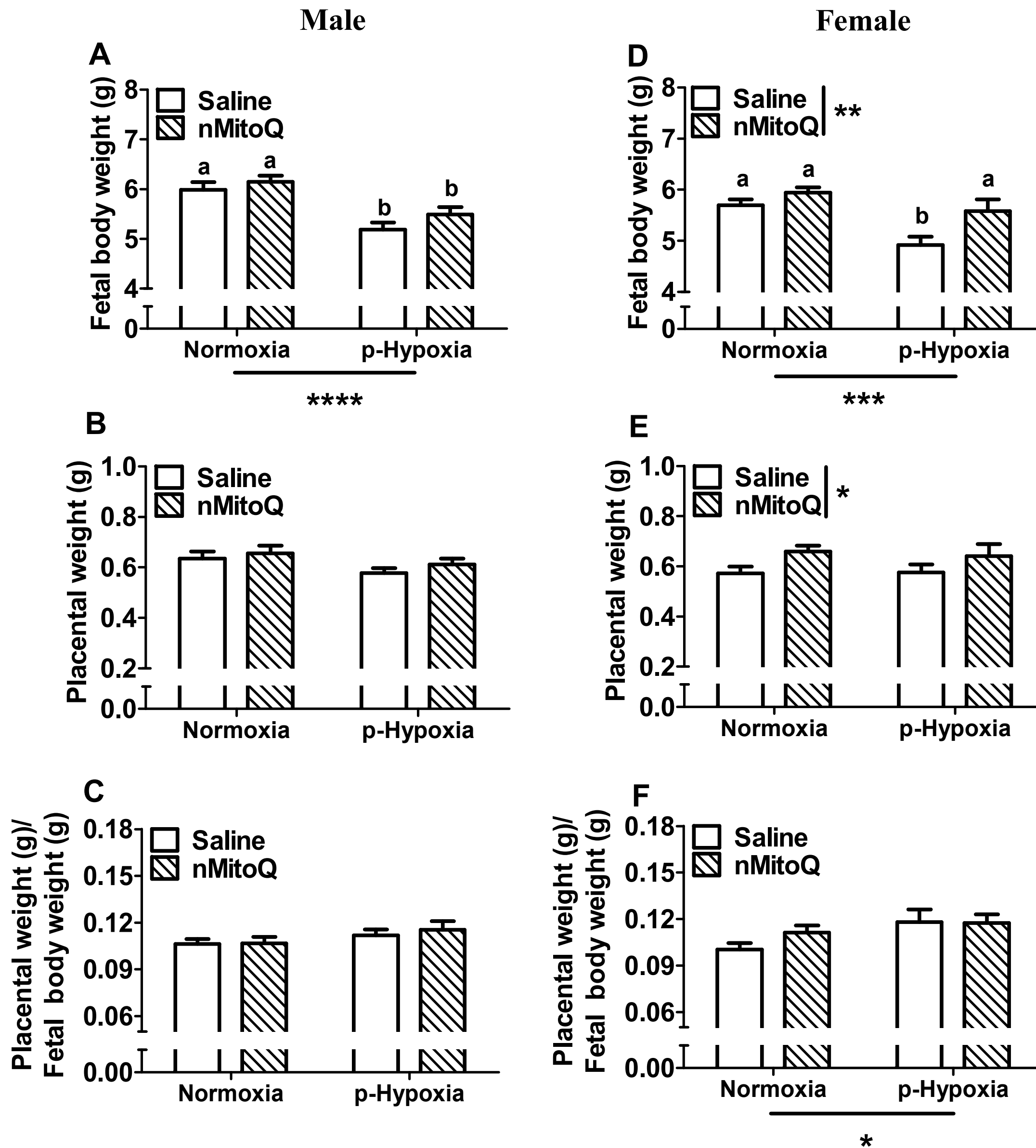


Figure 3.4 Effect of maternal hypoxia and nMitoQ treatment on pregnancy phenotype

A & **D**: Hypoxia in pregnancy led to IUGR in both male and female fetuses. However, nMitoQ treatment rescued only female body weight. **B** & **E**: Maternal hypoxia did not affect placental weight in male and female fetuses. nMitoQ also did not affect male placental weight but caused an increase in female placental weight. Placental weight to fetal body weight ratio was not altered between the groups due to hypoxia or nMitoQ treatment in male fetuses (**C**) but was increased due to maternal hypoxia in female fetuses (**F**). Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 group effect of maternal environment or maternal treatment. Different letters denote significant differences (P < 0.05) between groups using the Tukeys post-hoc test. n= 8-10/group.

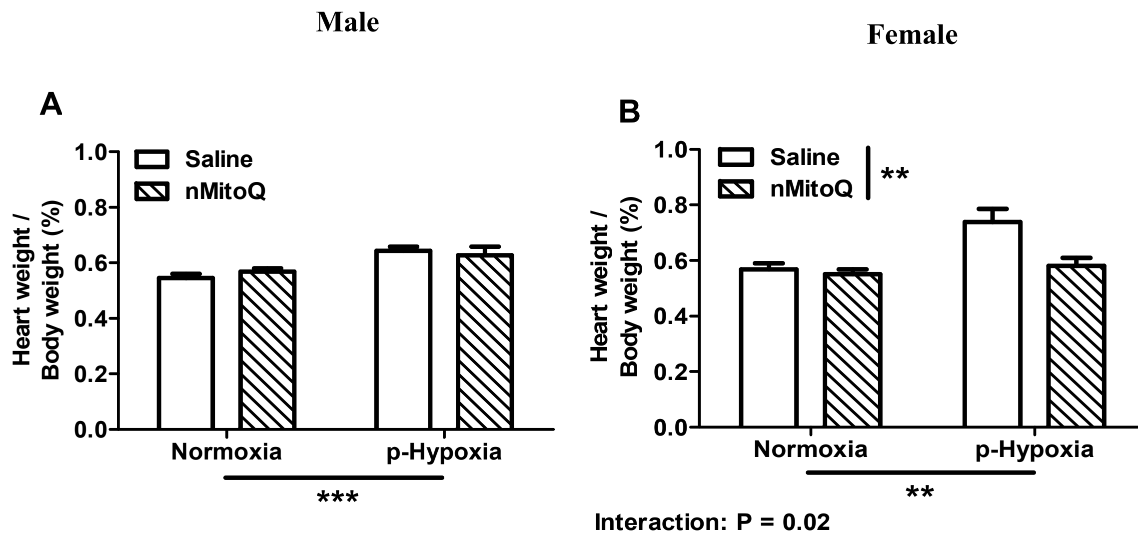


Figure 3.5 Effect of maternal hypoxia and nMitoQ treatment on relative fetal heart weight

Maternal hypoxia led to cardiac hypertrophy and nMitoQ did not prevent this in male (**A**) but prevented it in female (**B**) fetuses. Data collected on gestational day 21. Data are presented as mean ± SEM. $**P < 0.01$, $***P < 0.001$ group effect of maternal environment or maternal treatment. n= 8-10/group.

Table 3.1 Male fetal and placental measurements on GD21

	Normoxia		p-Hypoxia		2way ANOVA		
Male reproductive phenotype	**Saline**	**nMitoQ**	**Saline**	**nMitoQ**	**p-Hypoxia**	**nMitoQ**	**Int**
Fetal parameters							
Heart weight (mg)	32 ± 1	35 ± 1	34 ± 2	34 ± 1			
Brain weight (mg)	216 ± 12	214 ± 7	179 ± 24	204 ± 10			
Brain weight / Fetal body weight (%)	3.6 ± 0.2	3.5 ± 0.1	3.3 ± 0.4	3.8 ± 0.2			
Kidney weight (mg)	**55 ± 2**	**59 ± 3**	**47 ± 3**	**48 ± 3**	**		
Kidney weight / Fetal body weight (%)	0.91 ± 0.04	0.96 ± 0.04	0.88 ± 0.05	0.88 ± 0.04			
Crown-rump length (cm)	**4.18 ± 0.13**	**4.36 ± 0.13**	**4.17 ± 0.08**	**4.45 ± 0.09**		*	
Abdominal girth (cm)	4.40 ± 0.10	4.45 ± 0.07	4.22 ± 0.09	4.33 ± 0.10			
Ponderal index (PI)	87 ± 11	77 ± 7	72 ± 3	64 ± 5			
Crown-rump length / Abdominal girth (%)	95 ± 3	98 ± 2	99 ± 2	103 ± 3			
Placental parameters							
Dry weight (g)	0.113 ± 0.007	0.114 ± 0.008	0.097 ± 0.004	0.110 ± 0.007			
Diameter (cm)	**1.53 ± 0.03**	**1.53 ± 0.02**	**1.44 ± 0.04**	**1.56 ± 0.01**			*

GD: gestational day. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01 group or interaction effect of maternal environment or maternal treatment. n= 5-10/group.

Table 3.2 Female fetal and placental measurements on GD21

Female reproductive phenotype	Normoxia		p-Hypoxia		2way ANOVA		
	Saline	nMitoQ	Saline	nMitoQ	p-Hypoxia	nMitoQ	Int
Fetal parameters							
Heart weight (mg)	32 ± 1	33 ± 1	36 ± 3	33 ± 2			
Brain weight (mg)	186 ± 12	191 ± 10	188 ± 12	190 ± 10			
Brain weight / Fetal body weight (%)	3.5 ± 0.2	3.2 ± 0.2	3.8 ± 0.2	3.6 ± 0.2			
Kidney weight (mg)	**49 ± 2**	**58 ± 2**	**47 ± 2**	**46 ± 3**	*		
Kidney weight / Fetal body weight (%)	0.91 ± 0.06	0.99 ± 0.05	0.95 ± 0.03	0.87 ± 0.04			
Crown-rump length (cm)	4.07 ± 0.13	4.20 ± 0.13	4.18 ± 0.10	4.15 ± 0.08			
Abdominal girth (cm)	4.31 ± 0.16	4.42 ± 0.05	4.14 ± 0.09	4.34 ± 0.08			
Ponderal index (PI)	87 ± 9	84 ± 7	69 ± 6	79 ± 5			
Crown-rump length / Abdominal girth (%)	95 ± 2	95 ± 3	101 ± 3	96 ± 3			
Placental parameters							
Dry weight (g)	0.101 ± 0.004	0.112 ± 0.006	0.098 ± 0.006	0.243 ± 0.126			
Diameter (cm)	**1.56 ± 0.05**	**1.54 ± 0.05**	**1.40 ± 0.03**	**1.66 ± 0.06**		*	*

GD: gestational day. Data are presented as mean ± SEM. *P < 0.05 group or interaction effect of maternal environment or maternal treatment. n= 5-10/group.

3.3.3 Placental oxidative stress

Maternal exposure to hypoxia led to an increase in the general ROS levels (such as superoxide and hydrogen peroxide) in both labyrinth and junctional zones of the placenta from both male and female fetuses (Figure 3.6). nMitoQ treatment prevented this increase in placental ROS in both labyrinth and junctional zones (Figure 3.6).

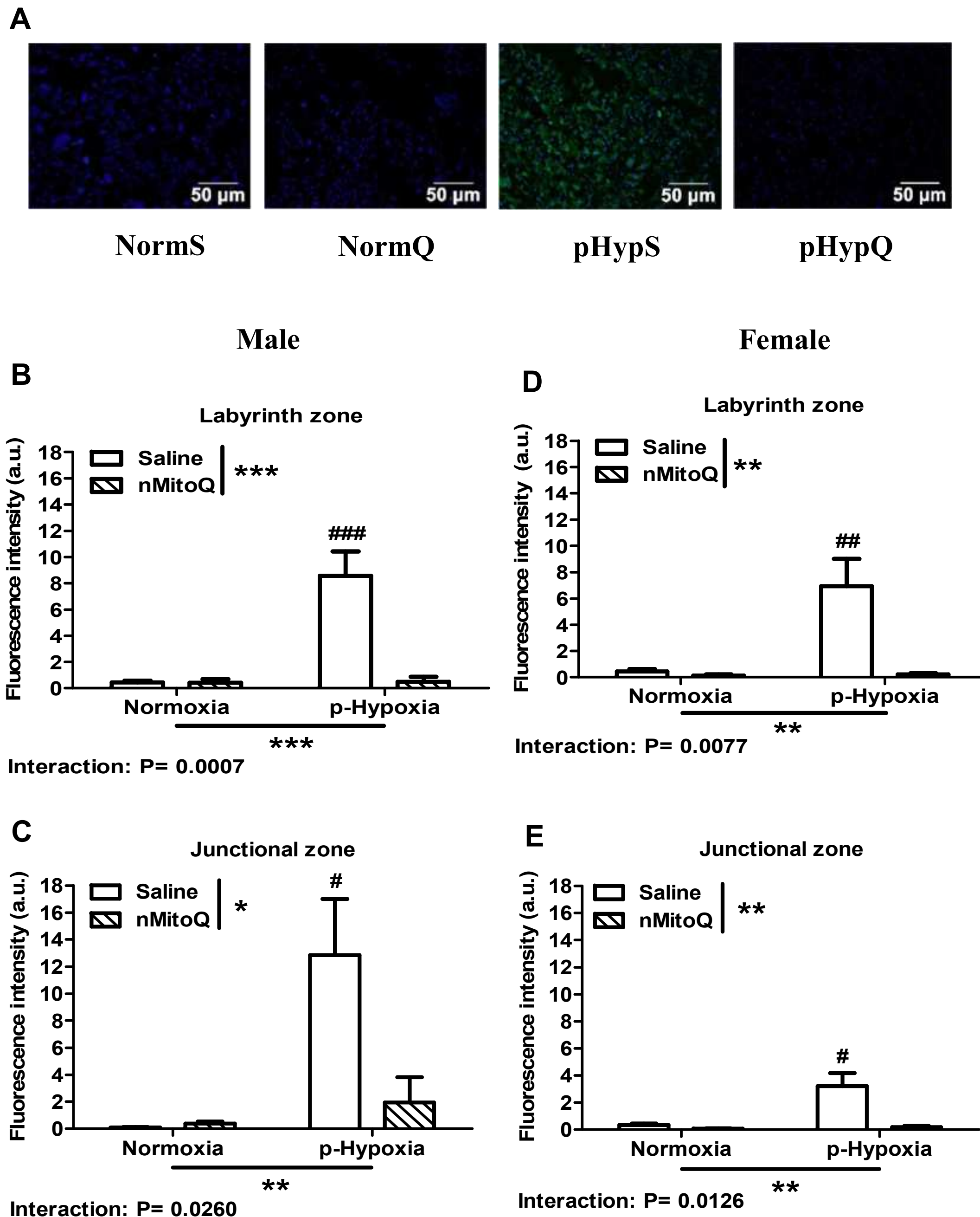


Figure 3.6 Effect of maternal hypoxia and nMitoQ treatment on placental reactive oxygen species (ROS) level

A: Representative images of fluorescence intensity in the labyrinth zone of placentas collected from male fetuses on gestational day 21. Green: DCF (dihydrodichlorofluorescein; ROS level). Blue: DAPI (4',6-diamidino-2-phenylindole; nuclei). Level of ROS in placentas taken from male (**B & C**) and female (**D & E**) fetuses. NormS: normoxia + saline, NormQ: normoxia + nMitoQ, pHypS: prenatal hypoxia + saline and pHypQ: prenatal hypoxia + nMitoQ. a.u.: arbitrary unit. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 group effect of maternal environment or maternal treatment. # P < 0.05, ## P < 0.01, ### P < 0.001 Tukeys post-hoc effect vs. all other groups. n= 5/group.

3.3.4 Placental morphology

Hypoxia did not affect the area of labyrinth and junctional zones of placentas obtained from male and female fetuses (Table 3.3). However, hypoxia reduced labyrinth depth in placentas of female fetuses, reflecting a reduction in the exchange surface. Hypoxia did not affect labyrinth depth in placentas of male fetuses (Table 3.3).

nMitoQ treatment did not alter area or depth of the labyrinth and junctional zones in placentas from either male or female fetuses (Table 3.3). nMitoQ also, did not prevent phenotype caused by maternal hypoxia in placentas of female fetuses (Table 3.3).

Table 3.3 Measurements of the labyrinth zone and junctional zone depth and area in placentas of male and female fetuses on GD21

Placental morphology	Normoxia		p-Hypoxia		2way ANOVA		
	Saline	**nMitoQ**	**Saline**	**nMitoQ**	**p-Hypoxia**	**nMitoQ**	**Int**
Male							
Labyrinth area (mm^2)	21.45 ± 1.03	21.62 ± 1.03	20.29 ± 1.22	22.07 ± 0.74			
Junctional area (mm^2)	4.73 ± 0.64	4.59 ± 0.71	4.88 ± 0.59	4.67 ± 0.56			
Labyrinth depth (mm)	2.56 ± 0.06	2.47 ± 0.12	2.54 ± 0.11	2.66 ± 0.09			
Junctional depth (mm)	0.53 ± 0.02	0.57 ± 0.04	0.60 ± 0.06	0.39 ± 0.10			
Female							
Labyrinth area (mm^2)	21.91 ± 0.75	19.66 ± 0.73	18.77 ± 1.36	20.13 ± 0.43			
Junctional area (mm^2)	5.28 ± 0.49	7.23 ± 2.32	4.70 ± 0.81	5.67 ± 0.29			
Labyrinth depth (mm)	**2.61 ± 0.04**	**2.38 ± 0.12**	**2.23 ± 0.12**	**2.26 ± 0.12**	*		
Junctional depth (mm)	0.58 ± 0.08	0.72 ± 0.11	0.51 ± 0.05	0.59 ± 0.10			

GD: gestational day. Data are presented as mean ± SEM. *P < 0.05 group effect of maternal environment. n= 5/group.

Fetal and maternal blood space in the placenta is important for sufficient nutrient and oxygen exchange between the maternal and fetal circulations (41). Hypoxia reduced fetal and maternal blood space area per field of view in male and female fetuses (Figure 3.7 & Figure 3.8). nMitoQ did not prevent the hypoxic effect on fetal and maternal blood space area in male fetuses (Figure 3.7). However, in female fetuses, nMitoQ caused a reduction in fetal and maternal blood space area in placentas of normoxic dams and an increase in fetal and maternal blood space area in placentas of hypoxic dams (Figure 3.8).

Male

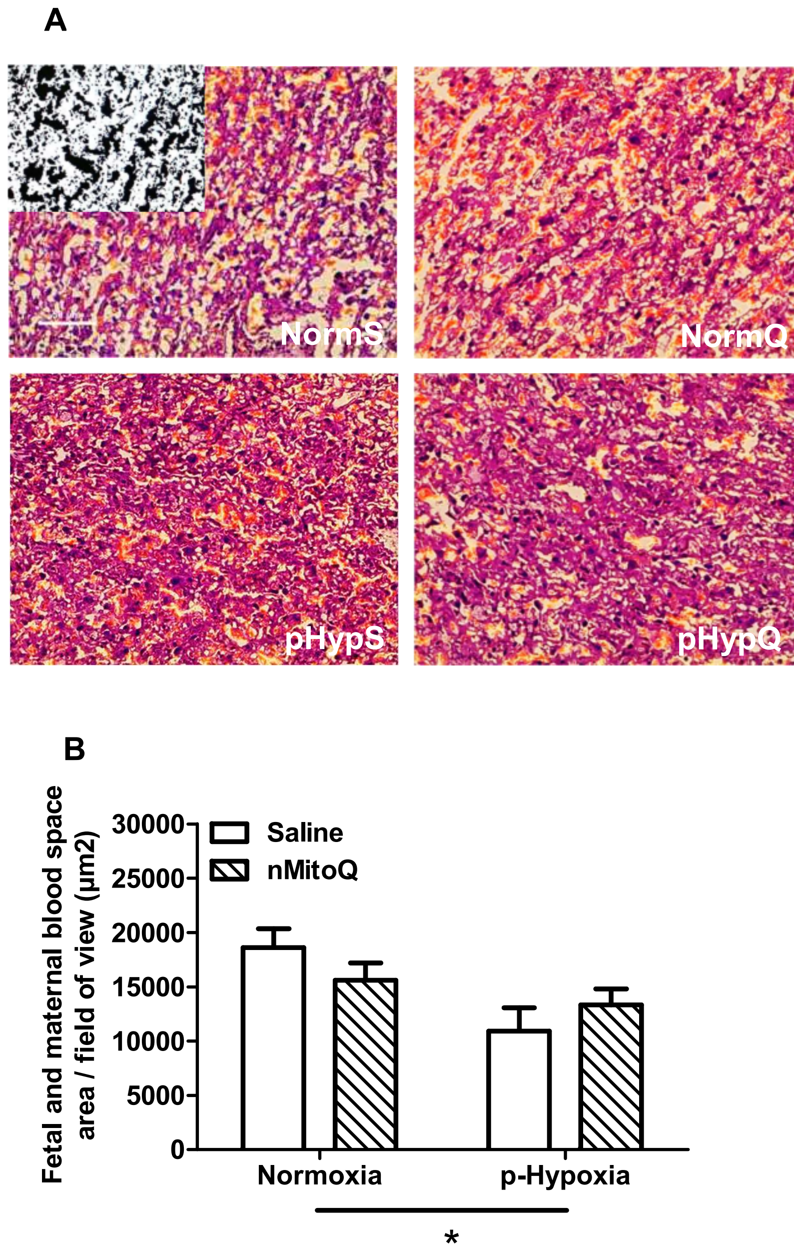


Figure 3.7 Assessment of fetal and maternal blood space area per field of view in placentas of male fetuses

A: Representative images of placental morphology in the labyrinth zone. Inset: Image J was used to convert fetal and maternal blood space into black and remaining placental tissue white for quantification. **B**: Fetal and maternal blood space area per field of view on gestational day 21. NormS: normoxia + saline, NormQ: normoxia + nMitoQ, pHypS: prenatal hypoxia + saline and pHypQ: prenatal hypoxia + nMitoQ. Data are presented as mean ± SEM. Scale bar = 50 µm. *P < 0.05 group effect of maternal environment. n= 5/group.

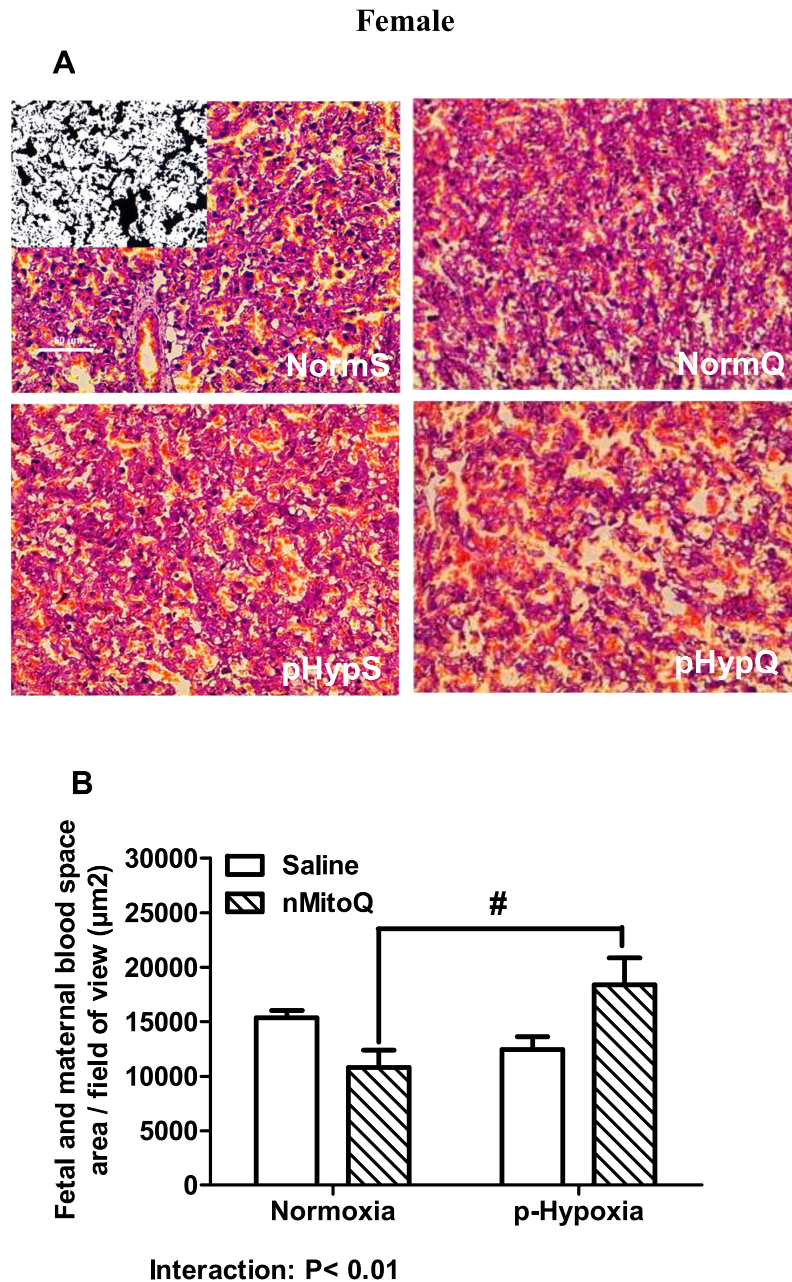
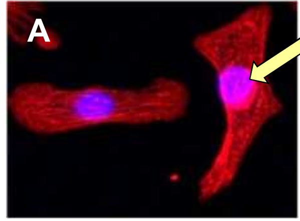


Figure 3.8 Assessment of fetal and maternal blood space area per field of view in placentas of female fetuses

A: Representative images of placental morphology in the labyrinth zone. Inset: Image J was used to convert fetal and maternal blood space into black and remaining placental tissue white for quantification. **B**: Fetal and maternal blood space area per field of view on gestational day 21. NormS: normoxia + saline, NormQ: normoxia + nMitoQ, pHypS: prenatal hypoxia + saline and pHypQ: prenatal hypoxia + nMitoQ. Data are presented as mean ± SEM. Scale bar = 50 µm. # $P < 0.05$ Tukeys post-hoc effect vs. all other groups. n= 5/group.

3.3.5 Effect of maternal hypoxia and nMitoQ treatment on the development of cardiomyocytes

Hypoxia led to cardiomyocyte hypertrophy in male fetuses illustrated by an increase in the length and area of mononucleated cardiomyocytes. nMitoQ treatment prevented this cardiomyocyte hypertrophy in male fetuses of hypoxic dams (Figure 3.9B & D). In males of normoxic and hypoxic dams, however, the width of mononucleated cardiomyocytes; the length, width and area of binucleated cardiomyocytes; and the percentage of binucleation were not altered between the groups (Figure 3.9C & Figure 3.10A-G). In female fetuses, the growth of mononucleated and binucleated cardiomyocytes was not affected by hypoxia or nMitoQ treatment (Figure 3.9E-G & Figure 3.10E-G). The percentage of cardiomyocyte binucleation was not altered by hypoxic exposure or drug treatment in either males (NormS: 11 ± 2%, NormQ: 9 ± 1%, pHypS: 9 ± 1%, pHypQ: 10 ± 1%) or females (NormS: 11 ± 3%, NormQ: 6 ± 2%, pHypS: 6 ± 2%, pHypQ:10 ± 3%) fetuses.



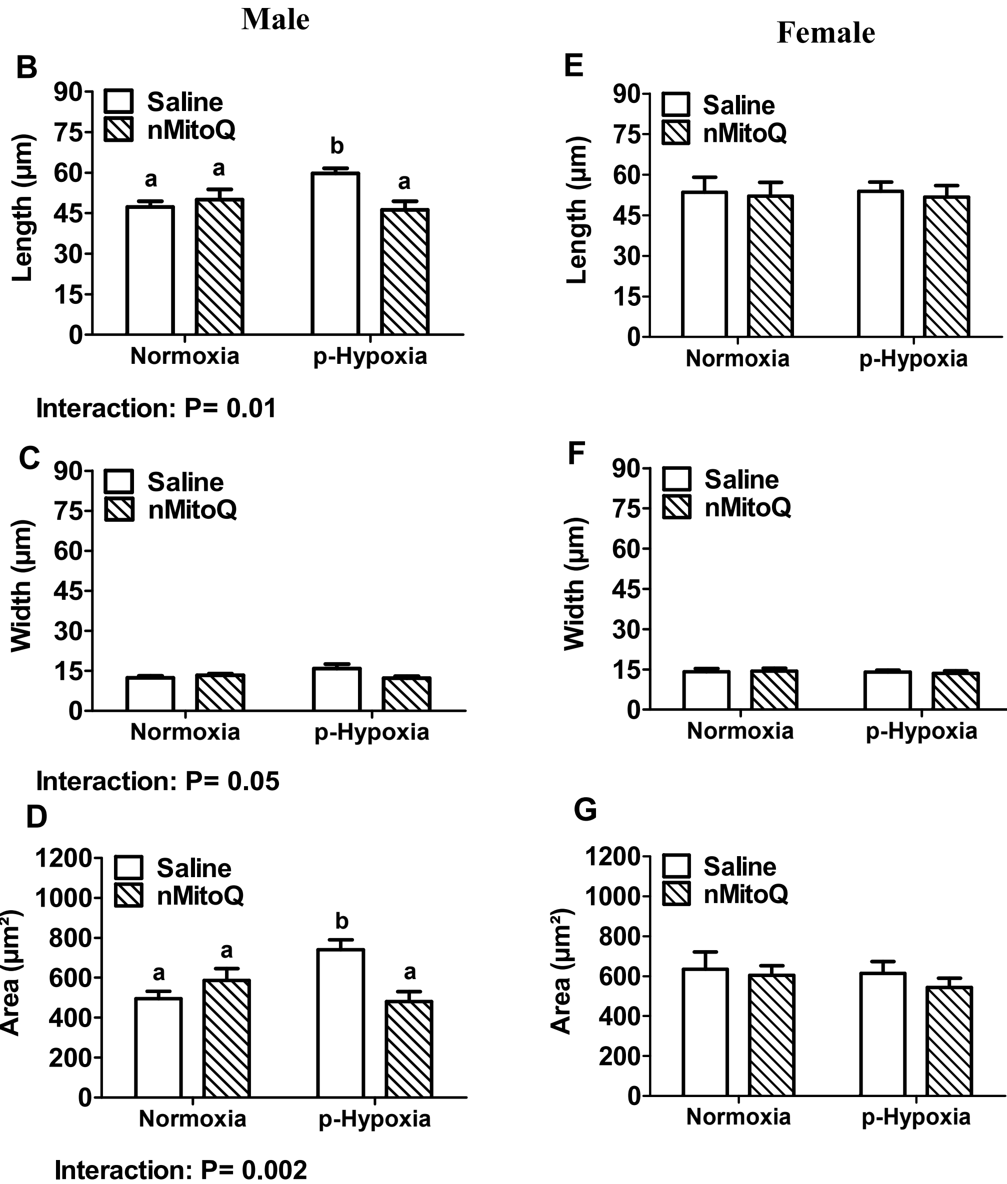
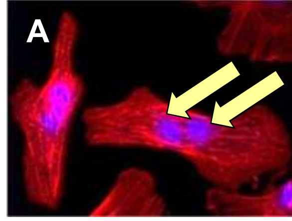


Figure 3.9 Effect of nMitoQ treatment on the size of mononucleated cardiomyocytes

A: Mononucleated cardiomyocyte (yellow arrow). Cardiomyocyte size in male (**B-D**) and female (**E-G**) fetuses on gestational day 21. Data are presented as mean ± SEM. Different letters denote significant differences ($P < 0.05$) between groups in the Tukeys post-hoc test. n=5-8/group.



Male

B

90 75 60 45 30 15 0

Length (μm)

Saline

nMitoQ

Normoxia p-Hypoxia

C

90 75 60 45 30 15 0

Width (μm)

Saline

nMitoQ

Normoxia p-Hypoxia

D

1200 1000 800 600 400 200 0

Area (μm²)

Saline

nMitoQ

Normoxia p-Hypoxia

Female

E

90 75 60 45 30 15 0

Length (μm)

Saline

nMitoQ

Normoxia p-Hypoxia

F

90 75 60 45 30 15 0

Width (μm)

Saline

nMitoQ

Normoxia p-Hypoxia

G

1200 1000 800 600 400 200 0

Area (μm²)

Saline

nMitoQ

Normoxia p-Hypoxia

Figure 3.10 Effect of nMitoQ treatment on the size of binucleated cardiomyocytes

A: Binucleated cardiomyocyte (yellow arrow). Binucleated cardiomyocyte size in male (**B-D**) and female (**E-G**) fetuses on gestational day 21 was not affected by maternal hypoxia or nMitoQ treatment. Data are presented as mean ± SEM. n=5-8/group.

3.3.6 Effect of placental conditioned medium on cardiomyocyte growth and binucleation

Cardiomyocyte growth (including length, width and area) and percentage of binucleation were not altered between the groups after cardiomyocytes from male or female fetuses were cultured in placental conditioned medium (Figure 3.11A-F) and (Table 3.4). Secretion of placental inflammatory factors was not altered as the result of hypoxia or nMitoQ treatment (Table 3.5).

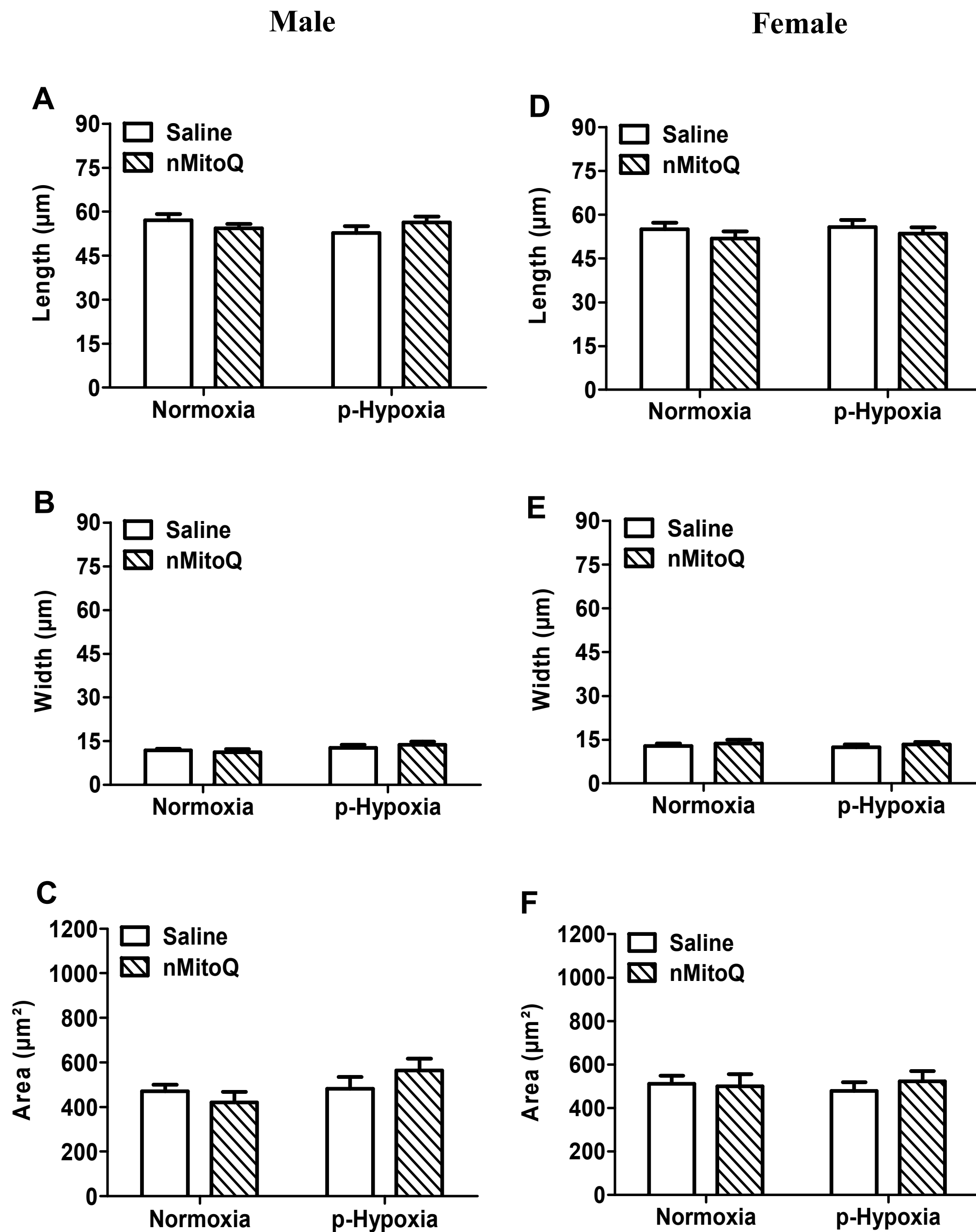


Figure 3.11 Effect of placental conditioned medium on cardiomyocyte size

Placental conditioned medium did not affect the size of cultured cardiomyocytes from male (**A-C**) and female (**D-F**) fetuses. The assessment was done by merging placental conditioned medium from both male and female fetuses and applying it on male and female cardiomyocytes separately. Data are presented as mean ± SEM. n= 5/group.

Table 3.4 Percentage of binucleated cardiomyocytes after *in vitro* incubation with placental conditioned medium

Percentage of binucleated cardiomyocytes	Normoxia		p-Hypoxia	
	Saline	nMitoQ	Saline	nMitoQ
Male				
Percentage of binucleation (%)	8 ± 1	8 ± 2	7 ± 1	6 ± 1
Female				
Percentage of binucleation (%)	8 ± 1	9 ± 1	10 ± 1	9 ± 1

NS: not significant. Data are presented as mean ± SEM. The assessment was done by merging placental conditioned medium from both male and female fetuses and applying it for 24 hours on male and female cultured cardiomyocytes separately.

Table 3.5 Placental inflammatory secreted factors were assessed using rat inflammation ELISA strip. Placentas collected on GD21

Inflammatory factors (unit: OD 450nM)	**Normoxia**		**p-Hypoxia**	
	Saline	**nMitoQ**	**Saline**	**nMitoQ**
IL-6	0.162 ± 0.013	0.162 ± 0.015	0.164 ± 0.011	0.178 ± 0.006
INF-r	0.052 ± 0.005	0.053 ± 0.007	0.068 ± 0.012	0.073 ± 0.014
IL-1α	0.365 ± 0.076	0.426 ± 0.107	0.395 ± 0.095	0.375 ± 0.049
TNF-α	0.137 ± 0.038	0.164 ± 0.051	0.138 ± 0.035	0.215 ± 0.008
IL-1β	0.315 ± 0.046	0.301 ± 0.036	0.300 ± 0.038	0.301 ± 0.041
MCP-1	0.052 ± 0.002	0.063 ± 0.008	0.055 ± 0.003	0.094 ± 0.031
Rantes	0.062 ± 0.003	0.068 ± 0.003	0.068 ± 0.005	0.083 ± 0.012
MIP	0.185 ± 0.040	0.278 ± 0.064	0.184 ± 0.045	0.261 ± 0.055

GD: gestational day. NS: not significant. ELISA: enzyme-linked immunosorbent assay. OD: optical density. TNF-α: tumor necrosis factor alpha. IL-6: interleukin 6. INFr: interferon receptor. IL-1α: interleukin 1α. IL-1β: interleukin 1β. MCP-1: Monocyte chemoattractant protein-1. rantes: regulated on activation, normal T cell expressed and secreted. MIP: macrophage inflammatory protein. The assessment was done on merged placental conditioned medium collected from both male and female fetuses. Data are presented as mean ± SEM. n= 5/group.

3.3.7 Effect of maternal hypoxia and nMitoQ treatment on corticosterone level in fetal plasma

In order to investigate possible mechanisms that led to different cardiomyocyte responses to hypoxia and nMitoQ treatment in both sexes, level of corticosterone in fetal plasma was assessed in male and female fetuses separately. Preliminary data from our laboratory (n=5) showed that maternal hypoxia did not alter corticosterone level in the plasma of male fetuses but reduced it in the plasma of female fetuses (Figure 3.12A-B). nMitoQ treatment did not have an effect on the plasma level of corticosterone in male and female fetuses (Figure 3.12A-B).

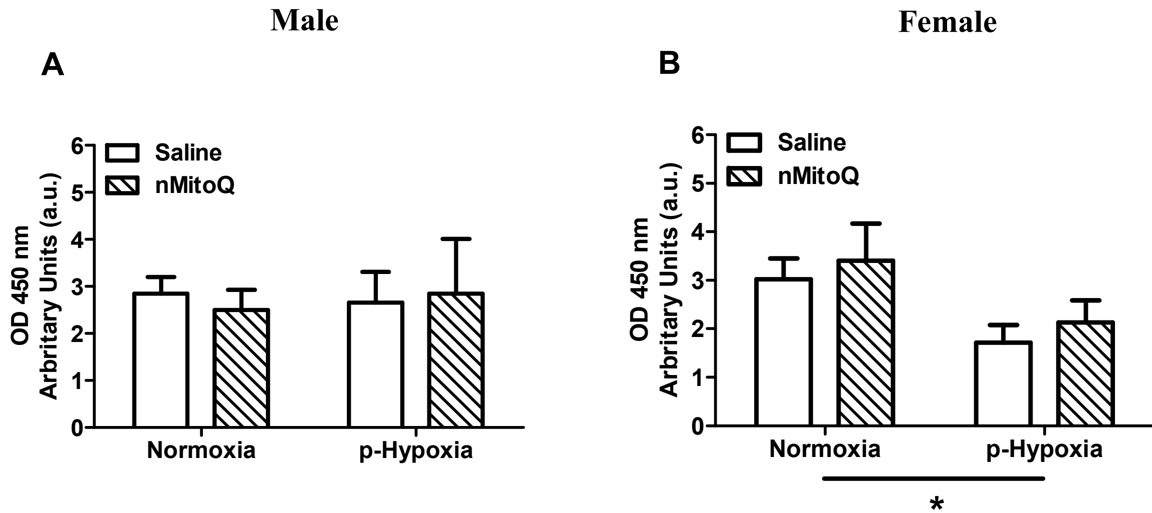


Figure 3.12 Effect of maternal hypoxia and nMitoQ treatment on corticosterone level in fetal plasma

Corticosterone level in the plasma of male (**A**) and female (**B**) fetuses at gestational day 21. Data are presented as mean ± SEM. *P < 0.05 group effect of maternal environment. n= 5/group. Data acquisition: Esha Ganguly and Mais Aljunaidy. Data analysis: Esha Ganguly.

3.4 DISCUSSION

The current study was conducted to assess the effect of targeting placental oxidative stress on fetal growth and cardiomyocyte development in a rat model of maternal hypoxia. We found that hypoxia led to placental oxidative stress in male and female fetuses and nMitoQ treatment prevented oxidative stress in the placentas of both sexes. Furthermore, hypoxia led to IUGR, but did not alter placental weight in male and female fetuses. Interestingly, nMitoQ treatment prevented IUGR in female but not male fetuses but this was associated with an increase in the placental weight in female but not male fetuses suggesting more placental efficiency in female fetuses. Assessment of placental morphology showed that hypoxia reduced fetal and maternal blood space area (area of fetal capillaries and maternal blood space per field of view) in placentas of male and female fetuses. However, nMitoQ treatment had a sex-dependent effect on fetal and maternal blood space area. nMitoQ did not affect fetal and maternal blood space area in males but reduced it in females of dams stayed in normoxia and increased it in females of dams exposed to hypoxia.

Our study also showed that hypoxia led to cardiac hypertrophy (an increase in relative cardiac to body weight) in both sexes and nMitoQ treatment was protective, but only preventing cardiac hypertrophy in female fetuses. Hypoxia, as well, led to an increase in cardiomyocyte size in male fetuses, which was prevented by nMitoQ treatment, but there were no such effects on female relative heart weights. Assessing the effect of placental secreted factors on fetal cardiomyocytes showed that placental conditioned medium did not affect cardiomyocyte growth or binucleation nor was the secretion of placental inflammatory factors altered by hypoxia or nMitoQ treatment. The current study provides evidence that placental antioxidant treatment can

rescue fetal growth and cardiac development in complicated pregnancy in a sex-dependent manner, but that such effects are not due to placental secreted factors.

A previous study showed that nMitoQ treatment prevented placental oxidative stress and rescued neonate body weight after maternal exposure to hypoxia in rats; however, sex was not taken into account (249). In the current study, both placental oxidative stress and fetal body weight were assessed in male and female fetuses separately, showing that a sex difference in the response to the treatment as IUGR was prevented in female but not male fetuses despite the prevention of placental oxidative stress in both sexes. Furthermore, normal levels of ROS are important for placental function, thus, scavenging placental ROS completely or to levels lower than normal could lead to harmful effects on the placental function. Phillips *et al*. showed that maternal nMitoQ treatment reduced placental ROS level in normoxic dams when compared to the level of ROS in placentas of normoxic dams that did not receive nMitoQ treatment (249). Whether this effect has a significant physiological impact on placental function is still unknown and should be assessed in the future.

Assessment of placental weight showed that female fetuses from dams who received nMitoQ treatment had a higher placental weight compared to female fetuses from dams that did not receive nMitoQ treatment. This effect of nMitoQ on placental weight was not observed in male fetuses. It has been established that normal placental structure and function differ between male and female fetuses, and that pregnancy complications and intrauterine environment can impact placental function in a sex-dependent manner [reviewed in (59, 282)]. For example, placentas of murine female fetuses contain a bigger labyrinth but smaller junctional zone than placentas of male fetuses (229). Further, the reduction in the area of sectioned placentas (cut into halves transversely) was larger in male than female newborns of women experiencing famine in

early gestation (280). Thus, as placentas of male and female fetuses develop differently, it was not surprising to see different placental responses to nMitoQ treatment between the sexes. However, the increase in female placental weight was also associated with an increase in female body weight. Indeed, the relation between placental structure and fetal growth has been established. For example, Kalisch-Smith JI *et al*. showed that labyrinth volume, labyrinth surface area and fetal and maternal blood space area can positively affect fetal and placental weight (144). Furthermore, Cahill *et al*. showed that hypoxia in pregnant mice can cause a reduction in the volume and number of vessel segments in feto-placental vascular tree and that the fetal body weight had a positive dependence on the total number of placental vessel segments (45). Therefore, to assess the mechanisms that could be involved in the increase of female body weight after nMitoQ treatment and to compare with male fetuses, fetal and maternal blood space area per field of view in the placenta was assessed in both sexes. Fetal and maternal blood space area is important for fetal-maternal nutrient and gas exchange. Thus, the reduction in fetal and maternal blood space area might contribute to the reduction in fetal body weight that we observed in placentas of hypoxic male and female fetuses. Furthermore, the increase in fetal and maternal blood space area in hypoxic female placentas of dams treated with nMitoQ could explain the increase in the body weight of these female fetuses compared to their hypoxic control from dams that did not receive nMitoQ treatment. However, nMitoQ reduced fetal and maternal blood space area in placentas of female fetuses from normoxic dams, but an increase in the body weight of these female fetuses compared to their normoxic control was detected. We, therefore, speculate that this adaptation in normoxia/MitoQ female fetuses might come at the expense of their development, and the overall impact of these adaptations on normoxia/MitoQ female cardiovascular health later in life was assessed in Chapter 4.

Our study also showed that hypoxia can lead to cardiac hypertrophy in male and female fetuses. nMitoQ treatment prevented cardiac hypertrophy in female but not male fetuses, showing a better ability of nMitoQ to preserve normal cardiac weight in females. Only a few studies here reported the effect of maternal antioxidant treatment on fetal heart weight. For example, Itani *et al*. showed that hypoxia reduced heart weight in chick embryos; and antioxidant treatment (melatonin) prevented this effect of hypoxia on the heart weight (134). In a reduced utero-placental perfusion pressure (RUPP) rat model, antioxidant treatment using pravastatin prevented the abnormal increase in the heart weight in IUGR fetuses (28). However, fetal sex was not taken into account in these studies and therefore we could not compare their findings with ours with respect to the sex differences observed.

In rats (unlike human and sheep), cardiomyocytes continue to develop until day 14 after birth. At term in rats, most cardiomyocytes are mononucleated while few only are binucleated (~ 6%, mature cells) (169). By assessing cardiomyocyte growth, we found that hypoxia led to mononucleated cardiomyocyte hypertrophy in only male but not female fetuses which was prevented by nMitoQ treatment. We properly were not able to observe an effect of nMitoQ on female fetal cardiomyocytes because maternal hypoxia did not cause a phenotype. Previous studies using animal models of a suboptimal environment in fetal life, such as hypoxia (234) or maternal protein restriction (63), showed a reduction in the number of cardiomyocytes in IUGR fetuses. Because of that, we speculate that the increase in relative heart weight in hypoxic female fetuses were not due to an increase in the number of cardiomyocytes. Instead, it could have been due to other factors such as an increase in the volume of the extracellular matrix or in the number of other cell types in the fetal heart such as fibroblast cells. However, confirming that warrants further investigation.

In order to assess the mechanisms that could be involved in cardiomyocyte response to maternal hypoxia and nMitoQ treatment, corticosterone levels were assessed in male and female fetal plasma. Maternal hypoxia is considered a stress that stimulates an increase in the expression of fetal glucocorticoids [cortisol in humans and corticosterone in rodents; reviewed in (64)]. Furthermore, it was established that glucocorticoids can stimulate fetal cardiomyocyte growth, an effect which happens normally in healthy pregnancy near term or when exogenous corticosteroids are applied *in vivo* or directly on cardiomyocytes *in vitro*; [(164, 222, 263), and reviewed in (286)].

Female fetuses might be adapted to the suboptimal environment (hypoxia) by reducing the level of corticosterone, which could explain why we did not observe cardiomyocyte hypertrophy in female fetuses. Male fetuses, as demonstrated by the absence of a reduction in their corticosterone level did not appear to adapt. nMitoQ did not reduce the corticosterone level in male fetal plasma, though nMitoQ prevented cardiomyocyte hypertrophy in hypoxic male fetuses. Overall, we speculate that the reduction in corticosterone in female was an adaptive mechanism that contributed, at least in part, in preventing cardiomyocyte hypertrophy in hypoxic female fetuses. In males, however, this adaptive mechanism did not occur which might contribute to the cardiomyocyte hypertrophy that was observed in hypoxic male fetuses.

Further investigation was done to assess possible mechanisms for mediating cardiomyocyte response to maternal hypoxia and nMitoQ treatment. A previous study (using our same animal model) showed that placental secreted factors from rats exposed to hypoxia can affect fetal neuronal development, and that maternal nMitoQ treatment prevented some of the adverse maternal effects of hypoxia on fetal neurons, including an improved dendritic shortening and decreased glutamate receptor expression (249). Therefore, the effect of placental secreted factors on fetal cardiomyocyte development was assessed. The assessment was done by combining

placental conditioned medium from male and female fetuses and applying it on male and female cardiomyocytes separately. The merged placental media did not affect cardiomyocyte growth or maturation. However, since placentas of male and female fetuses might respond to pregnancy complications in different ways [reviewed in (59)], one of the weaknesses of the current study was not separating male and female placental conditioned medium. Also, we only examined the effect of placental conditioned medium on cardiomyocyte growth and maturation but not other aspects of fetal cardiomyocyte development. Therefore, future studies are needed to examine their influences on metabolic function, apoptosis, and proliferation.

3.5 PERSPECTIVES

In summary, targeting placental oxidative stress using MitoQ loaded onto nanoparticles, had beneficial effects on fetal growth and cardiomyocyte development. Further, sex-differences were observed in response to maternal hypoxia and nMitoQ treatment which could have a different impact on male and female vulnerability to develop cardiovascular disease in adult life.

CHAPTER 4: MATERNAL TREATMENT WITH A PLACENTAL-TARGETED ANTIOXIDANT (MITOQ) IMPACTS OFFSPRING CARDIOVASCULAR FUNCTION IN A RAT MODEL OF MATERNAL HYPOXIA

A version of this chapter has been published:

***MM Aljunaidy**, JS Morton, R Kirschenman, T Phillips, CP Case, CL Cooke and ST Davidge. Maternal Treatment with a Placental-Targeted Antioxidant (MitoQ) Impacts Offspring Cardiovascular Function in a Rat Model of Prenatal Hypoxia. Pharmacological research 134: 332-342. 2018.*

***Author contributions:** M.M.A. experimental design, data acquisition (measurement of offspring body weight, echocardiography and wire myography), data analysis, draft preparation and critical revision of manuscript, R.V. data acquisition (tail-cuff plethysmography), J.S.M., C.L.C. and S.T.D. experimental design, draft preparation and critical revision of manuscript, T.P. and P.C. experimental design, provision of study materials, critical revision of manuscript.*

4.1 INTRODUCTION

I have shown in the previous chapter that hypoxia in pregnancy can cause abnormal fetal cardiomyocyte development. These abnormal changes in the fetal heart due to maternal hypoxia could be a precursor to cardiovascular disease in adult life. Therefore, susceptibility to cardiovascular disease in adult offspring of dams exposed to hypoxia in pregnancy was investigated in the current chapter. It is well established that pregnancy complications such as intrauterine growth restriction (IUGR) and hypoxia in pregnancy can increase offspring vulnerability to cardiovascular disease [reviewed in (10, 11)]. In humans, for example, IUGR was associated with endothelial dysfunction in early adulthood, as demonstrated by a reduction in flow-mediated dilation of the brachial artery (167). Furthermore, maternal exposure to hypoxia in rats (12% O_2, GD 15-21) led to abnormal cardiovascular function in both male and female hypoxic offspring later in life; illustrated by an increased cardiac susceptibility to ischemia/reperfusion (I/R) injury and by a restrictive ventricular diastolic phenotype, assessed *in vivo* by echocardiography (289). In rats, adult offspring of hypoxic pregnancies (13% O_2, GD 6-20) had markedly impaired NO-dependent relaxation of femoral resistance arteries (113). Maternal hypoxia in rats also led to vascular endothelial dysfunction in mesenteric arteries of adult offspring, involving an increase in vasoconstriction and a reduction in both flow-mediated and NO-dependent vasorelaxation (217, 364).

It is not fully understood how hypoxia before birth can lead to a higher incidence of cardiovascular dysfunction in the offspring later in life. However, evidence suggests that hypoxia in pregnancy can lead to an increase in the production of placental reactive oxygen species (ROS), which were linked to abnormality in the fetal cardiovascular function. For instance, in catechol-O-methyltransferase-deficient ($COMT^{-/-}$) mice, maternal exposure to hypoxia led to an increase in

the placental formation of the oxidant $ONOO^-$ which was associated with IUGR (292). Further, maternal exposure to hypoxia in rats led to placental oxidative stress (268) and fetal programming of cardiovascular disease (113, 146), both of which were prevented by maternal antioxidant treatment with ascorbic acid (113, 146, 268). Oxidative stress, and in particular placental oxidative stress, therefore, represents a potential link between hypoxia in pregnancy and programming of cardiovascular disease in adult offspring. As such, the ability of nMitoQ treatment to prevent the long-term effects of fetal programming of cardiovascular disease was investigated herein. We hypothesize that nMitoQ treatment of placental oxidative stress will improve cardiac function and morphology *in vivo* and vascular function *ex vivo* in the offspring of rats exposed to hypoxia during pregnancy.

4.2 METHODOLOGY

4.2.1 Experimental model

Female SD rats were mated with young males [number and motility of sperms reduce with aging in males which can lead to smaller litters (182)] within the animal facilities and treated as described in section 3.2.1. Dams in hypoxic chamber, however, were taken outside the chambers on GD 21, and dams of both normoxia and hypoxia were allowed to give birth under normal atmospheric conditions (term: GD 22). On the same day of birth, the number of neonates was documented and then reduced to four male and four female offspring to equalize postnatal conditions. Male and female offspring were weaned at 3 weeks of age and housed under normoxic conditions in same sex pairs until 7 or 13 months of age; at which point cardiovascular assessments were performed (Figure 4.1). Offspring body weight was monitored at day 1, 3 weeks, 7 and 13 months of life.

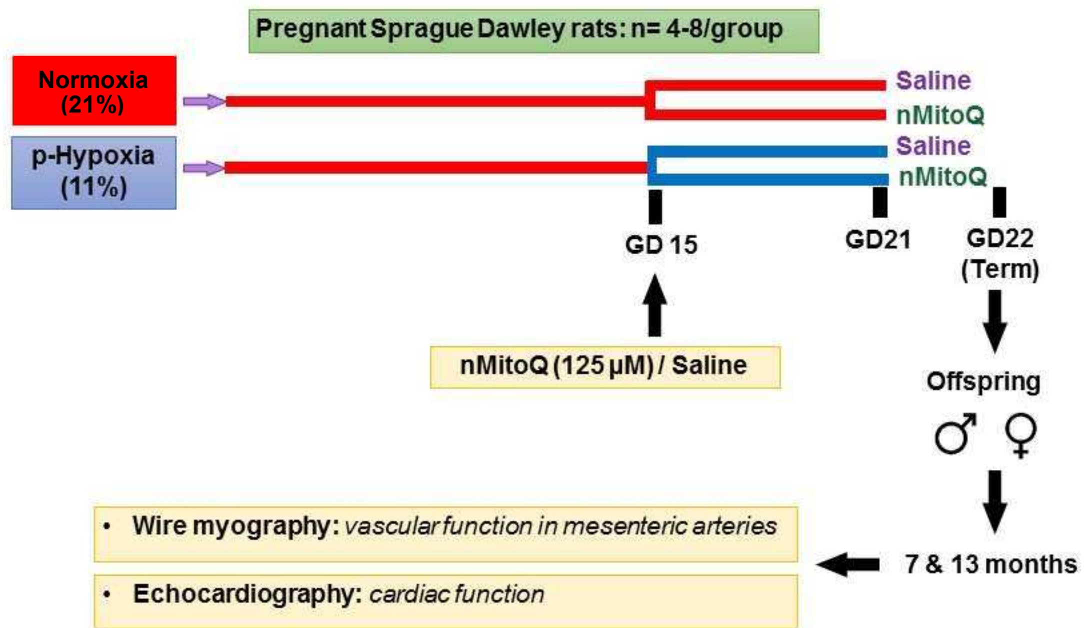


Figure 4.1 Experimental design of adult offspring study
Pregnant rats were exposed to hypoxia between gestational day (GD) 15 and 21. Tail-vein injection of nMitoQ (125 µM) or saline for the control was performed on GD 15. Dams were left to give birth on GD 22, and male and female offspring were aged to 7- or 13-month-old to perform experimental procedures. p-Hypoxia: prenatal hypoxia.

4.2.2 Blood pressure measurement

Blood pressure was measured using tail-cuff plethysmography as previously described in section 2.2.2. Briefly, offspring were trained one day before the actual blood pressure measurements were taken. Rats were placed in restraint tubes and the average of at least ten blood pressure measurements was taken.

4.2.3 Echocardiography

Echocardiographic images were obtained (one to two days following measurement of blood pressure) using an ultrasound biomicroscope (Vevo 2100, VisualSonics, Toronto, ON, Canada) (78, 289). Briefly, rats were anaesthetized using inhaled isoflurane (4% for induction and 2-3% for maintenance). M-mode two-dimensional echocardiography images were obtained to assess cardiovascular function. Left ventricle (LV) systolic function was assessed by estimating ejection fraction, cardiac output and shortening fraction from images obtained in M-mode of the LV long-axis. The transmitral Doppler signal was recorded to assess ventricular diastolic function and to calculate the mitral E wave/mitral A wave index and myocardial performance index: Tei index = [isovolumic relaxation time (IRT) + isovolumic contraction time (ICT)] / ejection time (ET). Pulmonary artery Doppler was measured in long-axis to assess vascular function.

4.2.4 Vascular function assessment

Following euthanization at 7 or 13 months of age, second-order mesenteric arteries were dissected and mounted in a wire myograph system as described previously in section 2.2.5. Constrictor responses to phenylephrine (PE, 0.01 to 100 μmol/L) and big endothelin-1 (bET-1, 0.1 to 100 μmol/L) were determined. To assess endothelium-dependent vasorelaxation, cumulative concentration response curves to methacholine (MCh, 0.0001 to 100 μmol/L) were performed after constricting the vessels with phenylephrine (15 X 0.001 μmol/L). Responses were assessed in the

absence or presence of L-NAME (100 µmol/L). Finally, vessels were exposed to a high potassium solution (K^+, 124 µmol/L) to confirm non-receptor mediated constrictor capacity.

4.2.5 Statistical analysis

GraphPad Prism 5.0 software was used for statistical analyses. All data were expressed as mean ± SEM. Data was compared using a two-way ANOVA or Student *t*-test. Sigmoidal curve fitting was performed on wire myography data and responses were summarized using pEC_{50} values (the effective concentration required to produce 50% of the maximal response) or delta area under curve (ΔAUC; difference in the area under curve of responses with and without inhibitor). A P value < 0.05 was considered statistically significant.

4.3 RESULTS

4.3.1 Offspring body weight

In early neonatal life, both male and female offspring were IUGR following maternal exposure to hypoxia. At the time of weaning and at 7 months of age, there were no differences in the body weights of offspring of dams exposed to hypoxia in pregnancy compared to control offspring in each sex (Table 4.1). However, at 13 months of age maternal exposure to hypoxia resulted in lower body weight in male offspring compared to normoxic offspring; an effect that was not observed in females (Table 4.1). The effect of maternal nMitoQ treatment on birth weight was subtle. nMitoQ did not improve male and female neonatal body weight after maternal exposure to hypoxia. Maternal nMitoQ treatment did not affect offspring body weight at weaning, 7 months, and 13 months of age in male and female offspring (Table 4.1).

Table 4.1 Body weight (g) at postnatal day (PD) 1 (neonate) & 21 (weaning), 7 months and 13 months of age

	Normoxia		**p-Hypoxia**		**2-way ANOVA**		
	Saline	**nMitoQ**	**Saline**	**nMitoQ**	**p-Hypoxia**	**nMitoQ**	**Int**
Male							
PD 1	**7.49 ± 0.26**	**7.45 ± 0.11**	**6.42 ± 0.23**	**6. 81 ± 0.29**	**		
PD 21	64 ± 2	61 ± 3	61 ± 2	57 ± 4			
7 months	744 ± 29	719 ± 28	752 ± 32	682 ± 20			
13 months	**884 ± 27**	**862 ± 30**	**831 ± 26**	**794 ± 18**	*		
Female							
PD 1	**7.48 ± 0.12**	**7.03 ± 0.11**	**6.18 ± 0.31**	**6.50 ± 0.25**	**		
PD 21	62 ± 2	59 ± 2	57 ± 1	58 ± 4			
7 months	411 ± 15	395 ± 17	380 ± 11	404 ± 16			
13 months	518 ± 29	483 ± 19	510 ± 22	471 ± 19			

Data are presented as mean ± SEM. PD: postnatal day. Int: interaction. *P < 0.05, **P < 0.01 group effect of maternal environment, n = 4-8/group.

4.3.2 Cardiac morphology

In male offspring, neither maternal hypoxia nor nMitoQ treatment affected cardiac morphology at 7 months of age (Table 4.2). However, at 13 months of age, exposure to hypoxia *in utero* reduced intraventricular septum thickness in diastole (IVS;d) and left ventricular posterior wall thickness in systole (LVPW;s). Maternal nMitoQ treatment did not prevent either of these effects of *in utero* hypoxia on cardiac morphology in 13-month-old pHypQ male offspring (Table 4.3).

In female offspring neither hypoxia nor nMitoQ significantly altered cardiac morphology at 7 months of age (Table 4.4). However, in 13-month-old females, nMitoQ had opposing effects on the left ventricular internal diameter in systole (LVID;s) in NormQ and pHypQ groups. While LVID;s was increased in NormQ offspring, it was reduced in pHypQ offspring (Table 4.5).

Table 4.2 Cardiac morphology and systolic function as assessed by echocardiography at 7 months of age in male offspring

	Normoxia		**p-Hypoxia**	
	Saline	**nMitoQ**	**Saline**	**nMitoQ**
Cardiac Morphology				
IVS;d (mm)	2.13 ± 0.19	2.16 ± 0.12	1.96 ± 0.12	2.17 ± 0.20
IVS;s (mm)	3.21 ± 0.30	3.42 ± 0.15	3.14 ± 0.19	3.59 ± 0.32
LVID;d (mm)	9.01 ± 0.30	8.60 ± 0.28	8.76 ± 0.29	8.60 ± 0.33
LVID;s (mm)	5.22 ± 0.25	4.94 ± 0.30	5.53 ± 0.18	4.92 ± 0.45
LVPW;d (mm)	2.92 ± 0.53	2.27 ± 0.14	3.00 ± 0.50	2.74 ± 0.37
LVPW;s (mm)	3.58 ± 0.46	3.33 ± 0.22	3.68 ± 0.46	3.29 ± 0.38
Systolic Function				
EF (%)	72.41 ± 2.36	72.88 ± 1.93	67.91 ± 2.44	73.73 ± 4.72
FS (%)	43.65 ± 2.13	43.95 ± 1.74	39.78 ± 1.95	45.52 ± 4.34
CO (ml/min/kg)	130 ± 15	139 ± 20	117 ± 14	122 ± 8
LV Vol;d (ul)	455 ± 39	395 ± 17	429 ± 34	408 ± 40
LV Vol;s (ul)	130 ± 13	107 ± 11	145 ± 15	97 ± 25

Data are presented as mean ± SEM. IVS;d: intraventricular septum, diastole. IVS;s: intraventricular septum, systole. LVID;d: left ventricular internal diameter, diastole. LVID;s: left ventricular internal diameter, systole. LVPW;d: left ventricular posterior wall, diastole. LVPW;s: left ventricular posterior wall, systole. EF: ejection fraction. FS: fractional shortening. CO: cardiac output. LV Vol;d: left ventricular volume in diastole. LV Vol;s: left ventricular volume in systole. No significant differences were found, n = 4-7/group.

Table 4.3 Cardiac morphology and systolic function as assessed by echocardiography at 13 months of age in male offspring

	Normoxia		p-Hypoxia		2-way ANOVA		
	Saline	nMitoQ	Saline	nMitoQ	p-Hypoxia	nMitoQ	Int
Cardiac Morphology							
IVS;d (mm)	**2.13 ± 0.19**	**2.15 ± 0.12**	**1.83 ± 0.13**	**1.85 ± 0.06**	*		
IVS;s (mm)	3.54 ± 0.26	3.68 ± 0.18	3.24 ± 0.12	3.38 ± 0.11			
LVID;d (mm)	9.31 ± 0.32	9.56 ± 0.25	9.91 ± 0.32	9.29 ± 0.28			
LVID;s (mm)	5.18 ± 0.54	5.34 ± 0.24	5.52 ± 0.43	5.42 ± 0.27			
LVPW;d (mm)	2.98 ± 0.24	2.36 ± 0.22	2.33 ± 0.15	2.21 ± 0.25			
LVPW;s (mm)	**4.42 ± 0.12**	**3.83 ± 0.22**	**3.70 ± 0.17**	**3.50 ± 0.31**	*		
Systolic Function							
EF (%)	76 ± 6	76 ± 2	75 ± 3	73 ± 2			
FS (%)	49 ± 5	47 ± 2	47 ± 3	49 ± 4			
CO (ml/min/kg)	295 ± 90	293 ± 77	219 ± 19	204 ± 24			
LV Vol;d (ul)	356 ± 112	506 ± 32	567 ± 37	498 ± 30			
LV Vol;s (ul)	122 ± 35	125 ± 18	145 ± 29	136 ± 14			

Data are presented as mean ± SEM. Int: interaction. IVS;d: intraventricular septum, diastole. IVS;s: intraventricular septum, systole. LVID;d: left ventricular internal diameter, diastole. LVID;s: left ventricular internal diameter, systole. LVPW;d: left ventricular posterior wall, diastole. LVPW;s: left ventricular posterior wall, systole. EF: ejection fraction. FS: fractional shortening. CO: cardiac output. LV Vol;d: left ventricular volume in diastole. LV Vol;s: left ventricular volume in systole. * $P < 0.05$ group effect of maternal environment, n = 5-6/group.

Table 4.4 Cardiac morphology and systolic function as assessed by echocardiography at 7 months of age in female offspring

	Normoxia		p-Hypoxia	
	Saline	**nMitoQ**	**Saline**	**nMitoQ**
Cardiac Morphology				
IVS;d (mm)	1.88 ± 0.20	1.98 ± 0.23	1.68 ± 0.12	1.83 ± 0.11
IVS;s (mm)	3.10 ± 0.18	3.10 ± 0.23	2.97 ± 0.08	3.16 ± 0.14
LVID;d (mm)	7.27 ± 0.52	6.19 ± 0.96	6.71 ± 0.40	7.35 ± 0.18
LVID;s (mm)	4.04 ± 0.40	3.97 ± 0.46	3.77 ± 0.21	3.87 ± 0.22
LVPW;d (mm)	2.36 ± 0.29	2.41 ± 0.58	3.54 ± 0.49	2.74 ± 0.43
LVPW;s (mm)	3.59 ± 0.19	3.07 ± 0.39	3.78 ± 0.37	3.56 ± 0.23
Systolic Function				
EF (%)	81.64 ± 2.15	70.74 ± 4.40	90.47 ± 15.84	79.86 ± 2.04
FS (%)	52.56 ± 2.49	41.98 ± 3.60	51.92 ± 7.15	50.23 ± 2.07
CO (ml/min/kg)	183 ± 13	172 ± 18	155 ± 35	174 ± 17
LV Vol;d (ul)	311 ± 19	269 ± 41	235 ± 39	298 ± 14
LV Vol;s (ul)	62 ± 7	93 ± 29	58 ± 12	63 ± 9

Data are presented as mean ± SEM. IVS;d: intraventricular septum, diastole. IVS;s: intraventricular septum, systole. LVID;d: left ventricular internal diameter, diastole. LVID;s: left ventricular internal diameter, systole. LVPW;d: left ventricular posterior wall, diastole. LVPW;s: left ventricular posterior wall, systole. EF: ejection fraction. FS: fractional shortening. CO: cardiac output. LV Vol;d: left ventricular volume in diastole. LV Vol;s: left ventricular volume in systole. No significant differences were found, n = 4-8/group.

Table 4.5 Cardiac morphology and systolic function as assessed by echocardiography at 13 months of age in female offspring

	Normoxia		p-Hypoxia		2-way ANOVA		
	Saline	**nMitoQ**	**Saline**	**nMitoQ**	**p-Hypoxia**	**nMitoQ**	**Int**
Cardiac Morphology							
IVS;d (mm)	1.95 ± 0.12	1.79 ± 0.09	1.68 ± 0.12	1.59 ± 0.18			
IVS;s (mm)	3.50 ± 0.19	3.11 ± 0.21	2.87 ± 0.17	3.00 ± 0.18			
LVID;d (mm)	7.62 ± 0.28	8.03 ± 0.35	8.54 ± 0.36	7.92 ± 0.34			
LVID;s (mm)	**3.65 ± 0.39**	**4.51 ± 0.38**	**4.98 ± 0.28**	**3.85 ± 0.40**			*
LVPW;d (mm)	2.56 ± 0.27	2.33 ± 0.42	2.29 ± 0.23	1.99 ± 0.22			
LVPW;s (mm)	3.96 ± 0.34	3.12 ± 0.28	3.11 ± 0.19	3.41 ± 0.43			
Systolic Function							
EF (%)	**83 ± 3**	**75 ± 4**	**74 ± 3**	**81 ± 4**			*
FS (%)	**55 ± 3**	**46 ± 4**	**45 ± 3**	**53 ± 5**			*
CO (ml/min/kg)	181 ± 32	164 ± 13	222 ± 43	176 ± 14			
LV Vol;d (ul)	338 ± 29	362 ± 33	425 ± 50	364 ± 27			
LV Vol;s (ul)	**64 ± 9**	**92 ± 17**	**109 ± 14**	**79 ± 14**			*

Data are presented as mean ± SEM. Int: interaction. IVS;d: intraventricular septum, diastole. IVS;s: intraventricular septum, systole. LVID;d: left ventricular internal diameter, diastole. LVID;s: left ventricular internal diameter, systole. LVPW;d: left ventricular posterior wall, diastole. LVPW;s: left ventricular posterior wall, systole. EF: ejection fraction. FS: fractional shortening. CO: cardiac output. LV Vol;d: left ventricular volume in diastole. LV Vol;s: left ventricular volume in systole. * $P < 0.05$ interaction effect of maternal environment and maternal treatment, n = 5-7/group.

4.3.3 Diastolic function

The MV E/A ratio, a marker that is used to assess left ventricular diastolic filling, represents the ratio of the peak velocity of flow in early diastole (E wave) to the peak velocity of flow in late diastole that is produced by atrial contraction (A wave). Assessment of cardiac function demonstrated that hypoxia altered left ventricular diastolic function parameters in male offspring at 7 months of age; namely reducing the mitral A wave and increasing in the E/A wave ratio (MV A and E/A wave index) (Table 4.6). Treatment with nMitoQ did not prevent the effects of hypoxia on diastolic function in male offspring at 7 months of age (Table 4.6). In 7-month-old female offspring, hypoxia also tended to increase the E/A wave ratio; however, nMitoQ treatment prevented the increase in MV E/A ratio in offspring of rats exposed to hypoxia (Table 4.6).

At 13 months of age, maternal hypoxia similarly increased the MV E/A ratio in male offspring without any effect of nMitoQ treatment on this parameter (Table 4.7). In female offspring at 13 months of age, hypoxia *in utero* led to a reduced MV A wave which was not improved by nMitoQ treatment. In addition, maternal treatment with nMitoQ increased mitral deceleration time (MV Decel) in male and increased MV E wave in female 13-month-old offspring (Table 4.7).

Table 4.6 Diastolic function as assessed by echocardiography at 7 months of age

	Normoxia		p-Hypoxia		2-way ANOVA		
	Saline	nMitoQ	Saline	nMitoQ	p-Hypoxia	nMitoQ	Int
Male (7 months)							
MV A (mm/s)	**709 ± 58**	**725 ± 38**	**519 ± 93**	**616 ± 78**	*		
MV Decel (ms)	42 ± 4	36 ± 8	36 ± 5	43 ± 5			
MV E (mm/s)	975 ± 80	965 ± 39	929 ± 44	1086 ± 39			
MV E/A	**1.35 ± 0.12**	**1.35 ± 0.08**	**1.85 ± 0.29**	**1.89 ± 0.21**	*		
Tei index	0.86 ± 0.14	0.81 ± 0.04	0.80 ± 0.11	0.80 ± 0.09			
Female (7 months)							
MV A (mm/s)	584 ± 46	714 ± 60	505 ± 111	602 ± 76			
MV Decel (ms)	42 ± 3	32 ± 4	37 ± 1	39 ± 4			
MV E (mm/s)	868 ± 45	895 ± 131	940 ± 51	879 ± 81			
MV E/A	**1.52 ± 0.10**	**1.23 ± 0.09**	**2.19 ± 0.40**	**1.29 ± 0.08**	**p=0.07**	**	
Tei index	0.76 ± 0.09	0.65 ± 0.02	0.61 ± 0.04	0.65 ± 0.05			

Data are presented as mean ± SEM. Int: interaction. MV A: mitral valve A wave velocity. MV Decel: mitral valve deceleration time. MV E: mitral valve E wave velocity. MV E/A: mitral valve E/A index. Tei index: myocardial performance index. * $P < 0.05$ group effect of prenatal environment, ** $P < 0.01$ group effect of maternal treatment, n=5-8/group.

Table 4.7 Diastolic function as assessed by echocardiography at 13 months of age

	Normoxia		p-Hypoxia		2-way ANOVA		
	Saline	nMitoQ	Saline	nMitoQ	p-Hypoxia	nMitoQ	Int
Male (13 months)							
MV A (mm/s)	625 ± 25	661 ± 81	559 ± 49	557 ± 83			
MV Decel (ms)	**22 ± 3**	**46 ± 6**	**25 ± 3**	**30 ± 7**		*	
MV E (mm/s)	879 ± 61	859 ±43	957 ± 61	835 ± 63			
MV E/A	**1.41 ±0.11**	**1.34 ± 0.13**	**1.73 ± 0.08**	**1.51 ± 0.09**	*		
Tei index	0.81 ±0.05	0.76 ± 0.07	0.85 ± 0.17	0.58 ± 0.05			
Female (13 months)							
MV A (mm/s)	**608 ± 70**	**854 ± 173**	**483 ± 43**	**533 ± 49**	*		
MV Decel (ms)	44 ± 3	31 ± 9	33 ± 5	31 ± 6			
MV E (mm/s)	**847 ± 60**	**1204 ± 162**	**844 ± 76**	**930 ± 88**		*	
MV E/A	1.40 ± 0.13	1.46 ± 0.08	1.81 ± 0.24	1.76 ± 0.21			
Tei index	0.61 ± 0.05	0.55 ± 0.07	0.63 ± 0.06	0.58 ± 0.04			

Data are presented as mean ± SEM. MV A: mitral valve A wave velocity. MV Decel: mitral valve deceleration time. MV E: mitral valve E wave velocity. MV E/A: mitral valve E/A index. Tei index: myocardial performance index. * $P < 0.05$ group effect of prenatal environment or maternal treatment, n=4-7/group.

4.3.4 Systolic function

Neither maternal hypoxia nor nMitoQ treatment affected systolic function parameters in male offspring at either 7 (Table 4.2) or 13 (Table 4.3) months of age.

In female offspring at 7 (Table 4.4) and 13 (Table 4.5) months of age, systolic function parameters were unaffected by hypoxia. However, there was a significant interaction effect of nMitoQ treatment in 7-month-old female offspring whereby stroke volume decreased in normoxic offspring and increased in offspring of dams exposed to hypoxia (NormS: 254 ± 19 ml, NormQ: 199 ± 17 ml, pHypS: 191 ± 27 ml, pHypQ: 236 ± 7 ml, interaction: P=0.01). Further, in 13 month-old female offspring, maternal nMitoQ treatment had a differential effect on ejection fraction and fractional shortening; namely nMitoQ treatment decreased ejection fraction and fractional shortening in offspring of dams stayed in normoxia and increased them in offspring of dams exposed to hypoxia, and nMitoQ had a differential effect on left ventricular volume in systole (LV Vol;s) as well, in which the opposite trend was seen (Table 4.5).

4.3.5 Pulmonary artery function

Maternal hypoxia reduced pulmonary valve peak velocity (PV Peak Vel) in male 7-month-old offspring, which was prevented by nMitoQ treatment (Figure 4.2A). In 7-month-old female offspring, PV Peak Vel was unaffected by hypoxia (Figure 4.2C). However, there was an interaction effect of hypoxia and nMitoQ treatment demonstrated by an increase in PV Peak Vel in offspring of dams exposed to normoxia/nMitoQ, and also in offspring of dams exposed to hypoxia/saline. Neither maternal hypoxia nor nMitoQ treatment altered pulmonary artery function in 13-month-old male or female offspring (Figure 4.2B & D).

In a previous study we demonstrated that aging can alter the cardiovascular phenotype of IUGR offspring (218). In order to assess the effect of aging on offspring cardiovascular function and understand the link between aging and maternal nMitoQ treatment, a sub-analysis was performed (2-way ANOVA for age and maternal exposure to hypoxia). In male offspring, there was a significant interaction effect of age and maternal environment which demonstrated that PV Peak Vel was reduced with aging in the normoxia group, was already lowered in the 7-month hypoxia group and was not further reduced with aging to 13 months (PV Peak Vel: aging P=0.12, hypoxia P=0.01, interaction P=0.01); potentially illustrating an accelerated aging phenotype in offspring of dams exposed to hypoxia in pregnancy. Aging, however, did not affect PV Peak Vel in female offspring (PV Peak Vel: aging P=0.25, hypoxia P=0.19, interaction P=0.57).

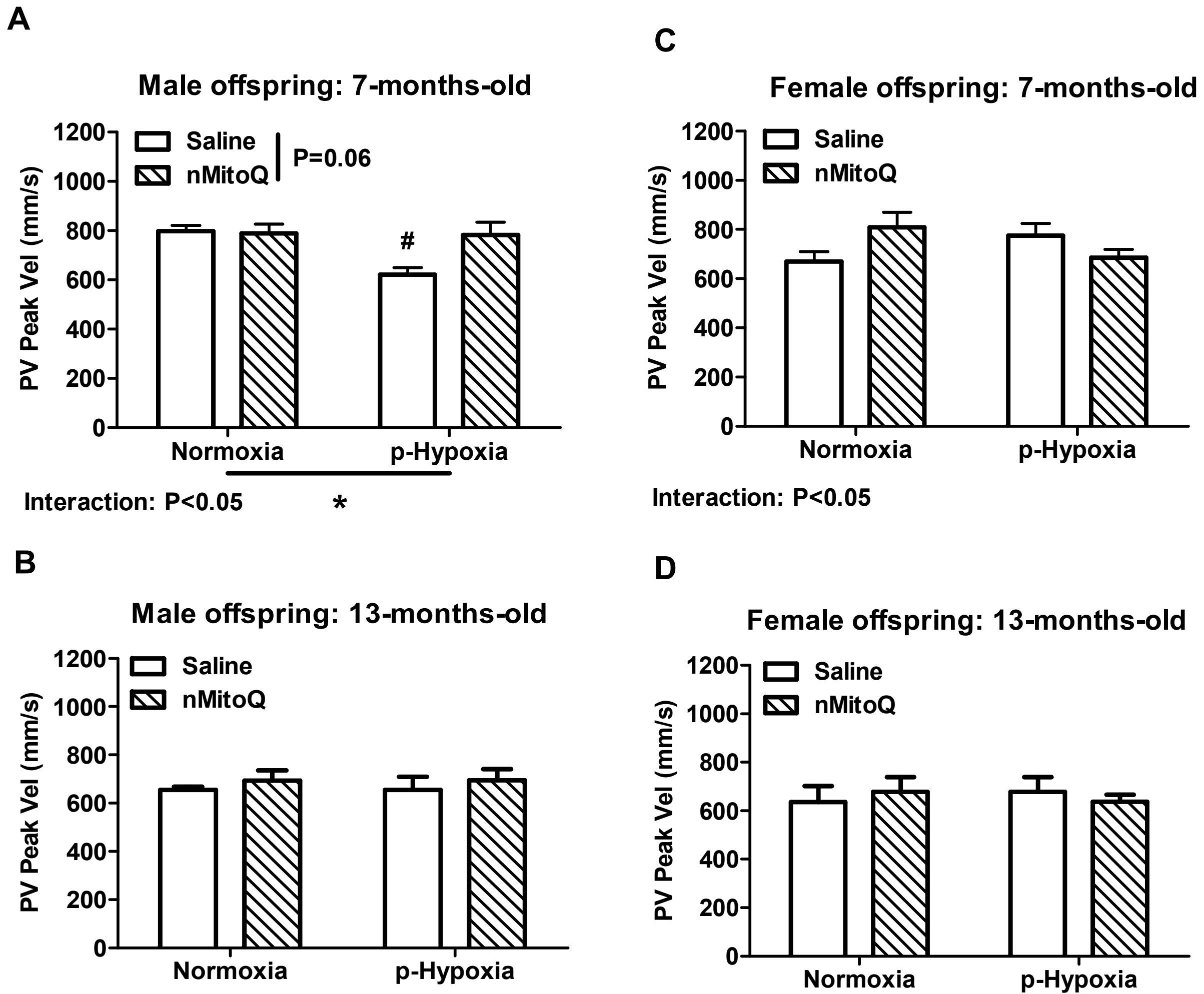


Figure 4.2 Pulmonary valve peak velocity (PV Peak Vel)

Echocardiography was used to assess PV Peak Vel at 7 and 13 months of age in male (**A & B**) and female (**C & D**) offspring. Data are presented as mean ± SEM. * $P < 0.05$ group effect of prenatal environment, # $P < 0.05$ Tukeys post-hoc effect vs. all other groups, n= 5-7/group.

4.3.6 Blood pressure

Neither hypoxia nor nMitoQ affected offspring blood pressure or heart rate in adult (7 month) and aged (13 month) offspring (Figure 4.3).

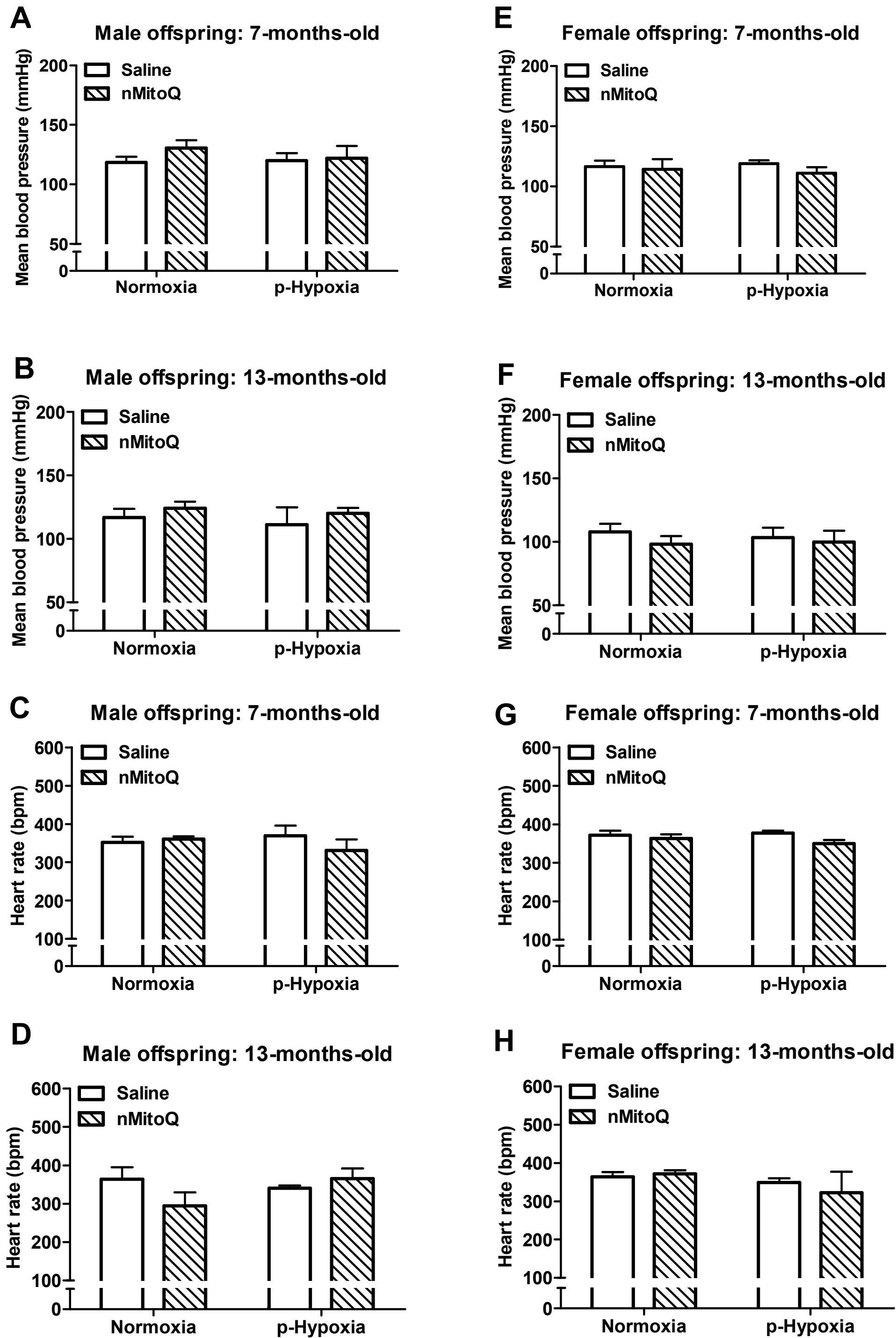


Figure 4.3 Blood pressure and heart rate

Arterial blood pressure and heart rate as assessed using tail-cuff plethysmography at 7 and 13 months of age in male (**A-D**) and female (**E-H**) offspring. Data are presented as mean ± SEM. n= 4-8/group. Data acquisition: Ms. Raven Kirschenman.

4.3.7 *Ex vivo* vascular function

The contribution of basal activation of the NO pathway to reducing mesenteric artery vasoconstrictor responses to PE was assessed by analyzing PE responses in the presence or absence of L-NAME (delta AUC). NO modulation of PE was abolished by exposure to maternal exposure to hypoxia in 7-month-old male, but not female, offspring (Figure 4.4C) and (Figure 4.5C). This reduction in basal NO contribution in males was restored by maternal nMitoQ treatment and became similar to the normoxic control levels (Figure 4.4C). At 13 months of age, NO modulation of PE constriction was unaltered in both male and female offspring and was unaffected by nMitoQ treatment (Figure 4.4D-F) and (Figure 4.5D-F).

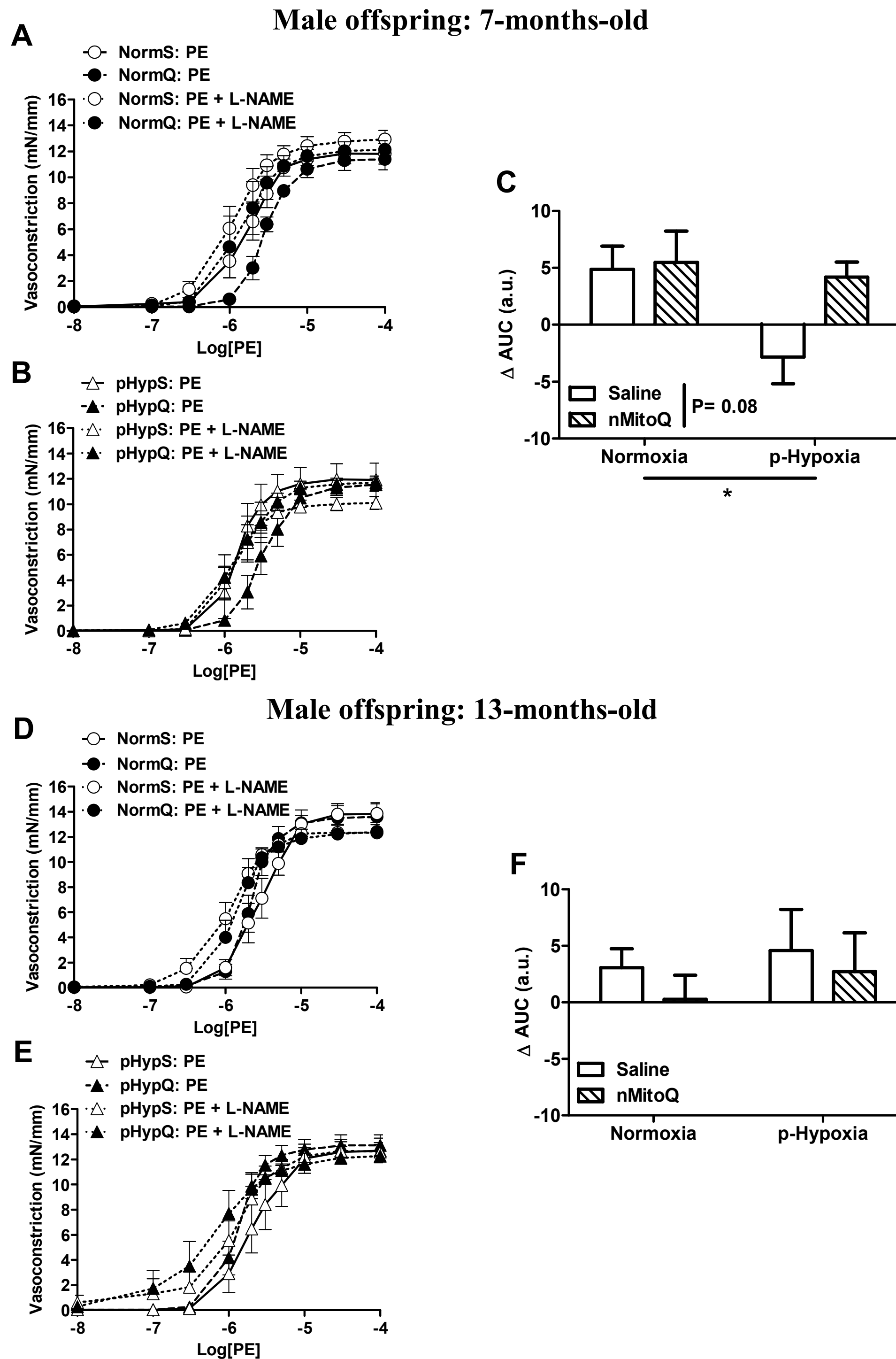


Figure 4.4 Mesenteric artery responses to phenylephrine (PE) in male offspring

Vascular responses to PE were assessed in male offspring at 7 (**A** - **C**) and 13 (**D - F**) months of age. △AUC: delta area under the curve [the difference between phenylephrine (PE)-induced vasoconstriction ± Nώ-nitro-L-arginine methyl ester (L-NAME)]. a.u.: arbitrary unit. The contribution of nitric oxide was reduced by maternal exposure to hypoxia in only 7-month-old male offspring and this effect was reversed by maternal treatment with nMitoQ. Data are presented as mean ± SEM. * $P < 0.05$ group effect of prenatal environment, n=4-7/group.

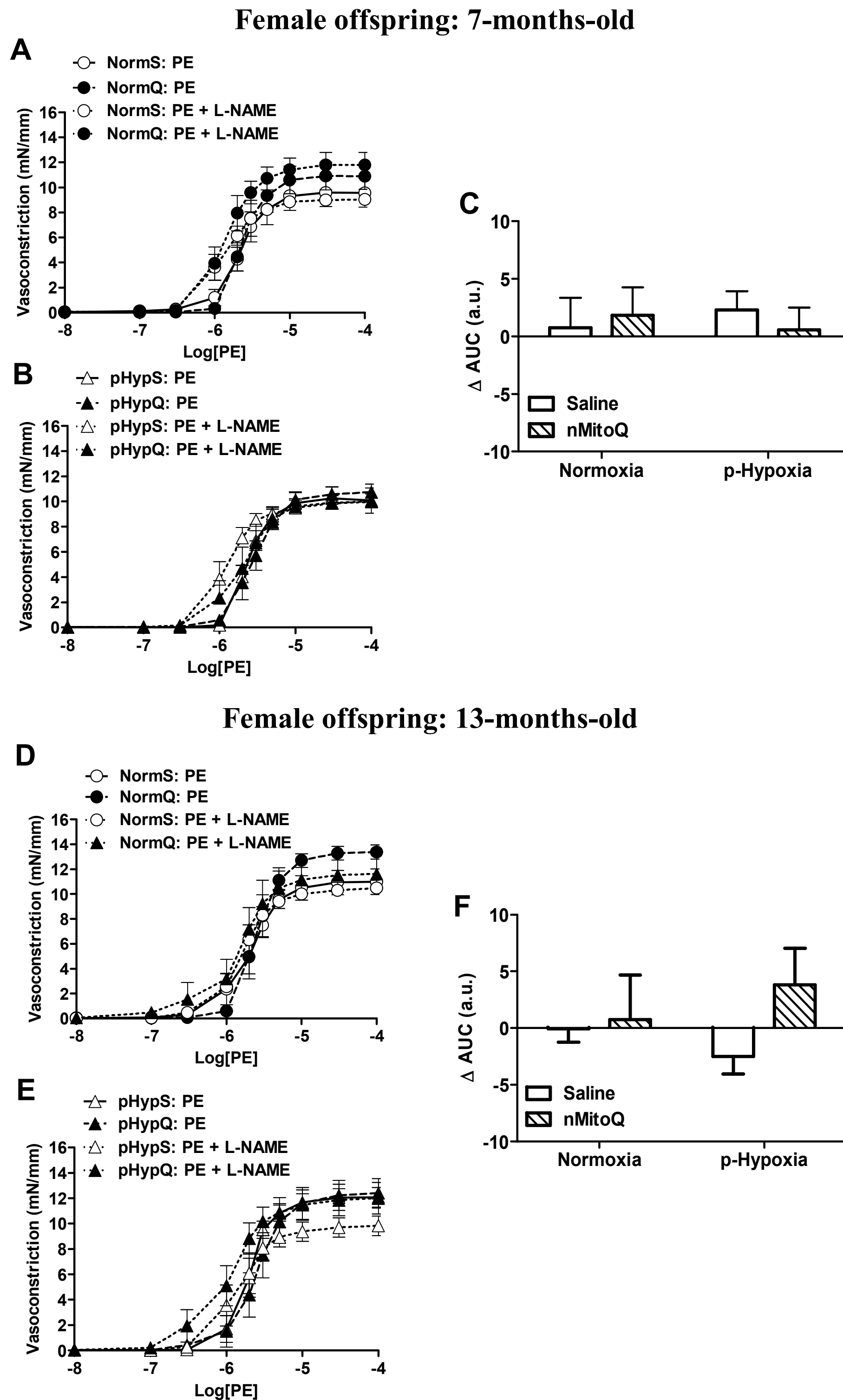


Figure 4.5 Mesenteric artery responses to phenylephrine (PE) in female offspring

Vascular responses to PE were assessed in female offspring at 7 (**A - C**) and 13 (**D - F**) months of age. △ AUC: delta area under the curve [the difference between phenylephrine (PE)-induced vasoconstriction ± Nώ-nitro-L-arginine methyl ester (L-NAME)]. a.u.: arbitrary unit. Data are presented as mean ± SEM. n=4-6/group.

Maternal hypoxia did not affect mesenteric artery sensitivity to PE in 7-month-old male offspring while nMitoQ treatment reduced sensitivity in both groups (Figure 4.6A). However, in 13-month-old male offspring, maternal hypoxia increased vascular sensitivity to PE. nMitoQ treatment further increased sensitivity to PE, particularly in pHypQ offspring (Figure 4.6C). Neither, hypoxia nor nMitoQ treatment affected mesenteric artery sensitivity to PE in 7 and 13-month-old female offspring (Figure 4.6B & D). In order to investigate the dichotomous effect of nMitoQ treatment, which was age dependent, we further compared vascular sensitivity to PE between NormS at 7 and 13 months of age. In male offspring, vascular sensitivity to PE was decreased at 13 months of age (PE pEC_{50}: 7- vs. 13-month-old male: 5.77 ± 0.06 vs. 5.54 ± 0.05, $P=0.01$). Neither maternal hypoxia nor nMitoQ affected vasoconstriction to PE in female offspring (7-month-old PE pEC_{50}: NormS: 5.66 ± 0.06, NormQ: 5.64 ± 0.04, pHypS: 5.63 ± 0.03, pHypQ: 5.50 ± 0.04, 13-month-old PE pEC_{50}: NormS: 5.68 ± 0.06, NormQ: 5.60 ± 0.04, pHypS: 5.70 ± 0.04, pHypQ: 5.59 ± 0.06).

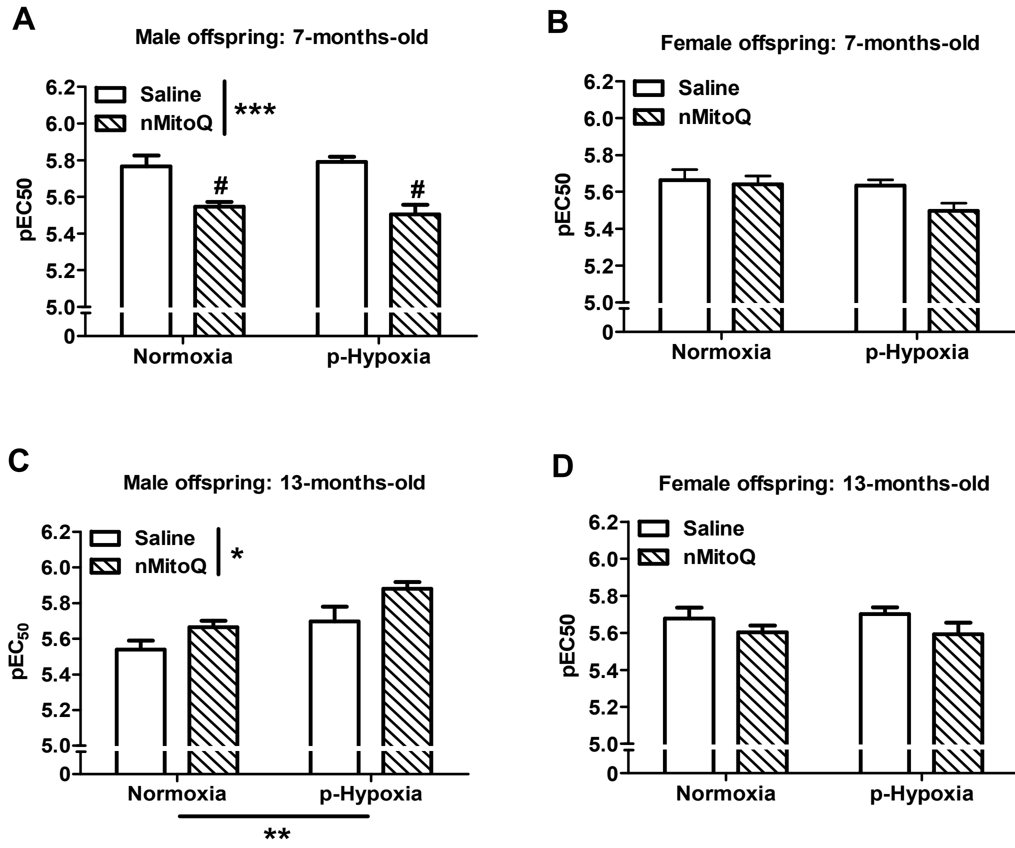
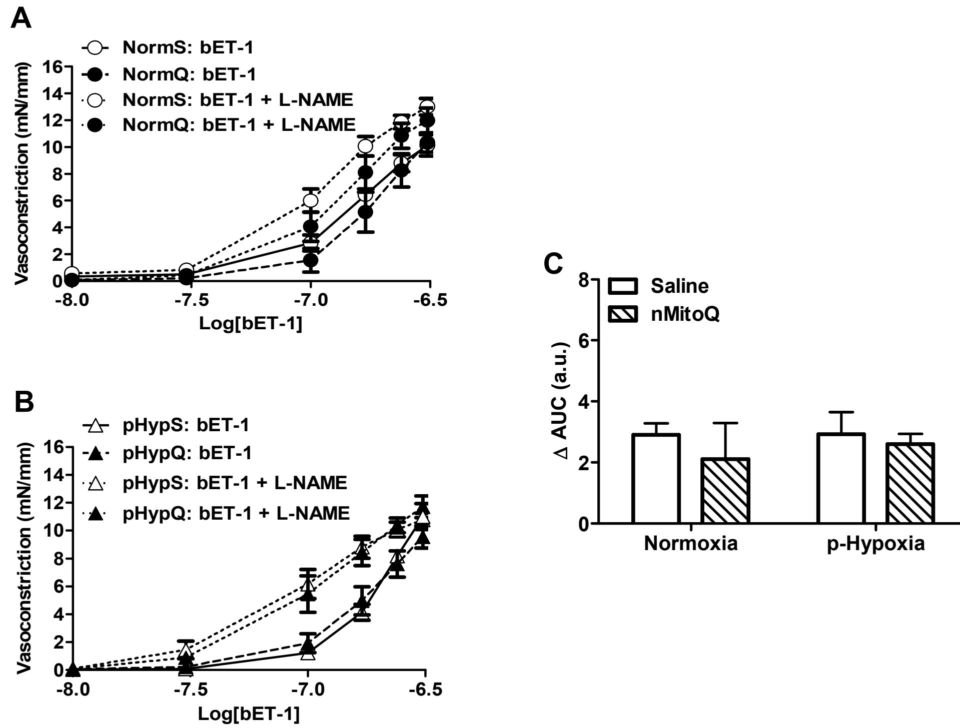


Figure 4.6 Mesenteric artery sensitivity to phenylephrine (PE) in male and female offspring

Summary data of vascular sensitivity (pEC_{50}) to the vasoconstrictor phenylephrine in male (**A** & **C**) and female (**B** & **D**) offspring. At 7 months of age, nMitoQ reduced vascular sensitivity to PE. At 13 months of age, maternal hypoxia increased sensitivity to PE and this was further increased by nMitoQ treatment. Vascular sensitivity to PE was not altered between the groups in female offspring at 7 and 13 months of age. Data are presented as mean ± SEM. * $P < 0.05$ and *** $P < 0.01$ group effect of maternal treatment, ** $P < 0.01$ group effect of maternal environment. # $P < 0.05$ Tukeys post-hoc effects vs. corresponding NormS or pHypS group, n=4-7/group.

Vasoconstriction to either bET-1 (with or without NOS inhibition; Figures 4.7 & 4.8) or high K^+ (Table 4.8) was unaltered by maternal hypoxia or nMitoQ treatment in any group studied.

Male offspring: 7-months-old



Male offspring: 13-months-old

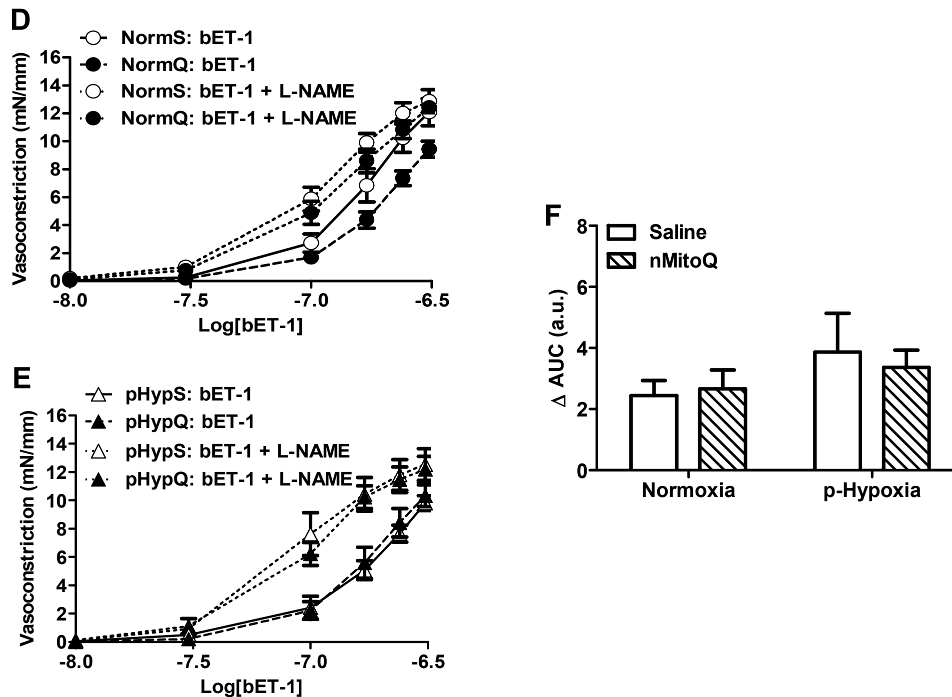
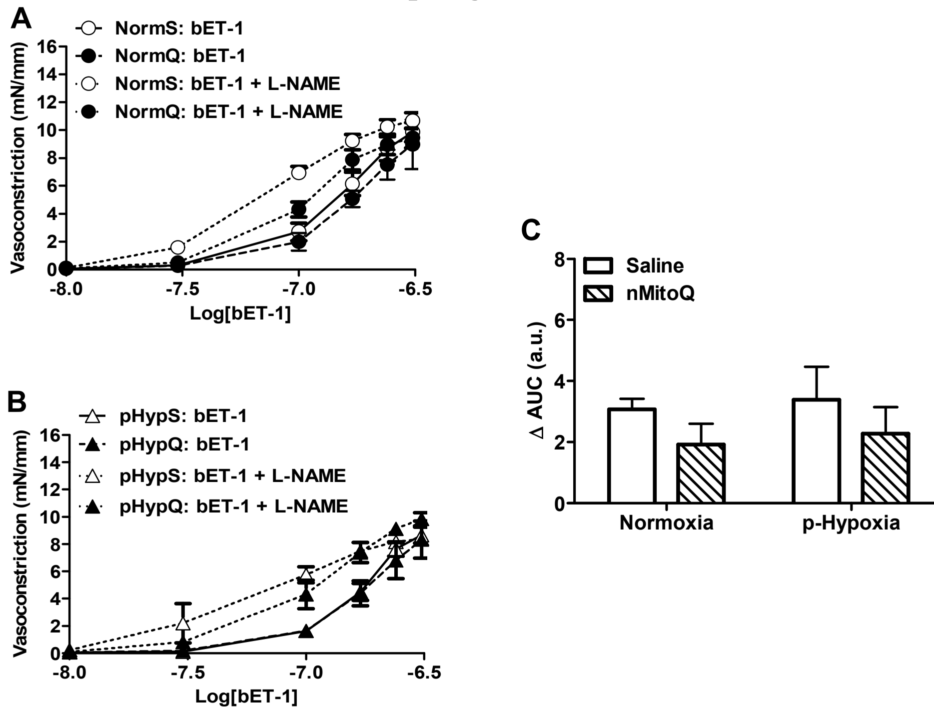


Figure 4.7 Mesenteric artery responses to big endothelin-1 (bET-1) in male offspring

Vascular responses to big endothelin-1 (bET-1) before and after adding Nώ-nitro-L-arginine methyl ester (L-NAME) were assessed in male offspring at 7 (**A-C**) and 13 (**D-F**) months of age.△AUC: delta area under the curve. a.u.: arbitrary unit. Data are presented as mean ± SEM. n=4-7/group.

Female offspring: 7-months-old



Female offspring: 13-months-old

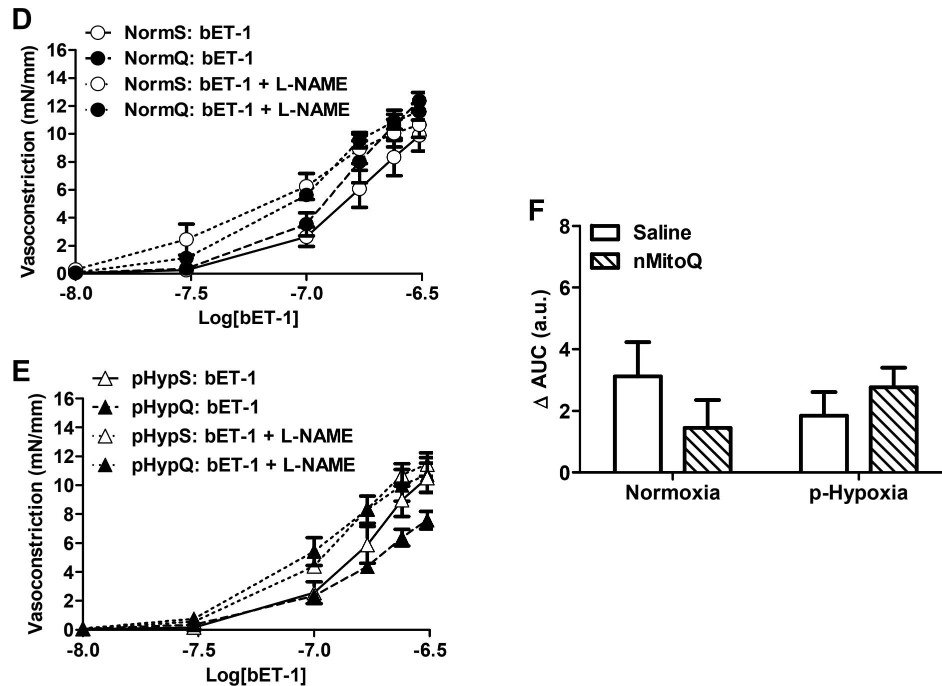


Figure 4.8 Mesenteric artery responses to big endothelin-1 (bET-1) in female offspring

Vascular responses to big endothelin-1 (bET-1) before and after adding Nώ-nitro-L-arginine methyl ester (L-NAME) were assessed in female offspring at 7 (**A-C**) and 13 (**D-F**) months of age. △ AUC: delta area under the curve. a.u.: arbitrary unit. Data are presented as mean ± SEM. n=3-7/group.

Table 4.8 Mesenteric artery responses to high K^+ (mN/mm) in male and female offspring

	Normoxia		p-Hypoxia	
	Saline	**nMitoQ**	**Saline**	**nMitoQ**
7 months				
Male	10.19 ± 0.52	11.19 ± 0.34	9.99 ± 0.87	9.52 ± 0.29
Female	8.28 ± 0.47	8.78 ± 0.89	8.72 ± 0.44	8.29 ± 0.43
13 months				
Male	11.21 ± 0.56	10.47 ± 0.54	10.50 ± 0.63	11.01 ± 0.60
Female	9.05 ± 0.47	9.33 ± 0.32	8.66 ± 0.73	9.11 ± 0.36

Data are presented as mean ± SEM. n=4-8/group.

Neither maternal hypoxia nor nMitoQ treatment altered vasorelaxation to MCh in 7-month-old male offspring (Figure 4.9A & B). While there was also no effect of maternal hypoxia in 13-month-old male offspring, nMitoQ treatment increased sensitivity to MCh in both maternally normoxic and hypoxic groups (Figure 4.9C & D). Analysis of responses in the absence or presence of NOS inhibition in 7-month-old male offspring demonstrated an interaction effect, indicating a reduced contribution of NO to vasorelaxation in offspring of dams exposed to hypoxia in pregnancy following nMitoQ treatment (delta AUC of MCh ± L-NAME (a.u.): NormS: 142 ± 37, NormQ: 194 ± 41, pHypS: 239 ± 45, pHypQ: 88 ± 28, interaction effect: $P = 0.02$). The contribution of NO to MCh-induced vasorelaxation was unaltered by hypoxia or nMitoQ treatment in 13-month-old male offspring (delta AUC of MCh ± L-NAME (a.u.): NormS: 217 ± 50, NormQ: 147 ± 29, pHypS: 195 ± 32, pHypQ: 217 ± 36).

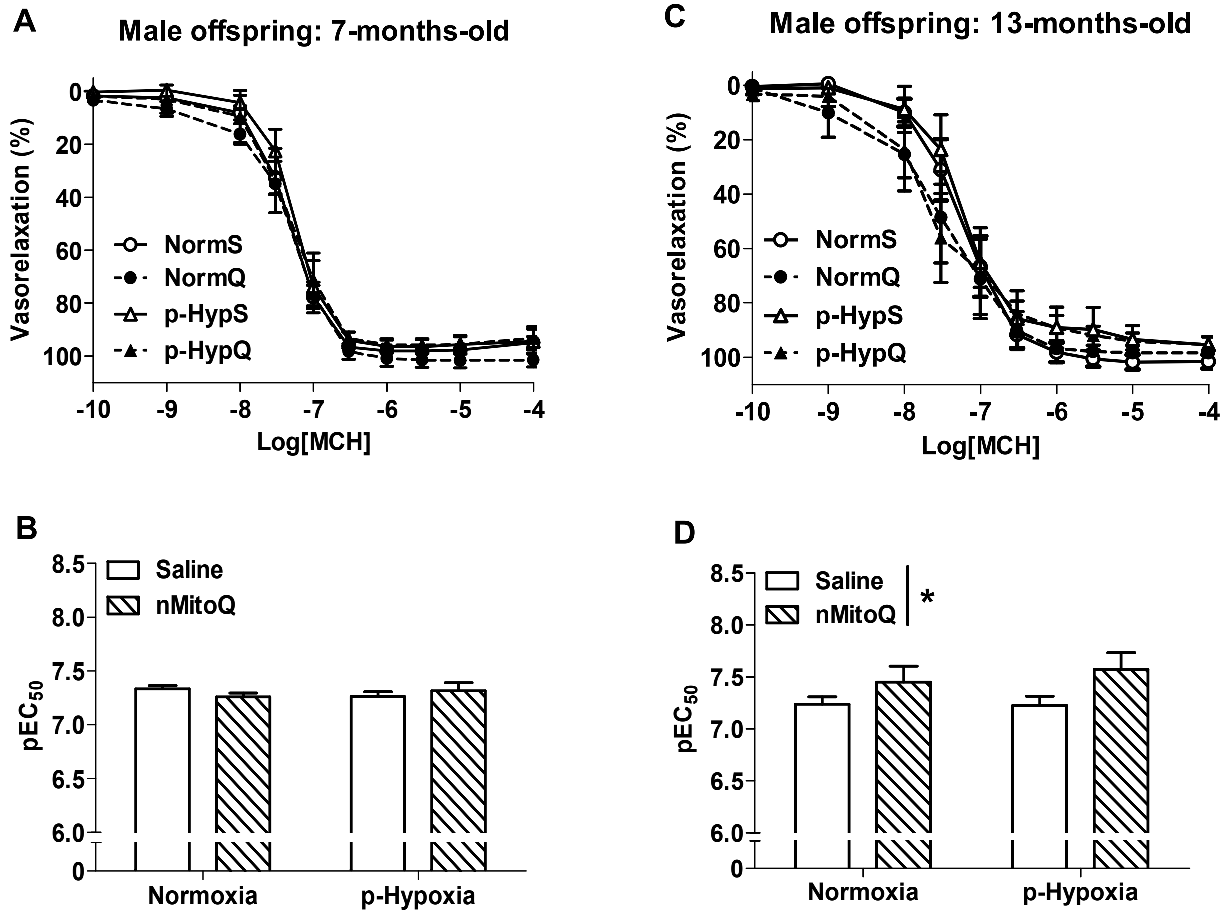


Figure 4.9 Mesenteric artery responses to methacholine (MCh) in male offspring

Vascular responses to MCh were assessed at 7 (**A & B**) and 13 (**C & D**) months of age. **B & D**: summary data of vascular sensitivity (pEC_{50}) to MCh. At 7 months of age, responses to MCh were unaffected by either maternal hypoxia or nMitoQ treatment. At 13 months of age, nMitoQ increased sensitivity to MCh in both Norm and pHyp offspring. Data are presented as mean ± SEM. * $P < 0.05$ group effect of maternal treatment, n=4-8/group.

In female offspring at 7 months of age, there was no effect of maternal hypoxia on mesenteric artery responses to MCh; however, nMitoQ reduced sensitivity to MCh in the maternal normoxia group (Figure 4.10A & B). Conversely, at 13 months of age maternal hypoxia reduced offspring sensitivity to MCh and this was increased by maternal nMitoQ treatment in both groups (Figure 4.10C & D). The contribution of NO to MCh-induced vasorelaxation was unaltered by hypoxia or nMitoQ treatment in 7- or 13-month-old female offspring [(delta AUC of MCh ± L-NAME (a.u.): NormS: 95 ± 18, NormQ: 158 ± 36, pHypS: 97 ± 34, pHypQ: 76 ± 29) and (delta AUC of MCh ± L-NAME (a.u.): NormS: 154 ± 35, NormQ: 143 ± 47, pHypS: 142 ± 37, pHypQ: 141 ± 32) respectively].

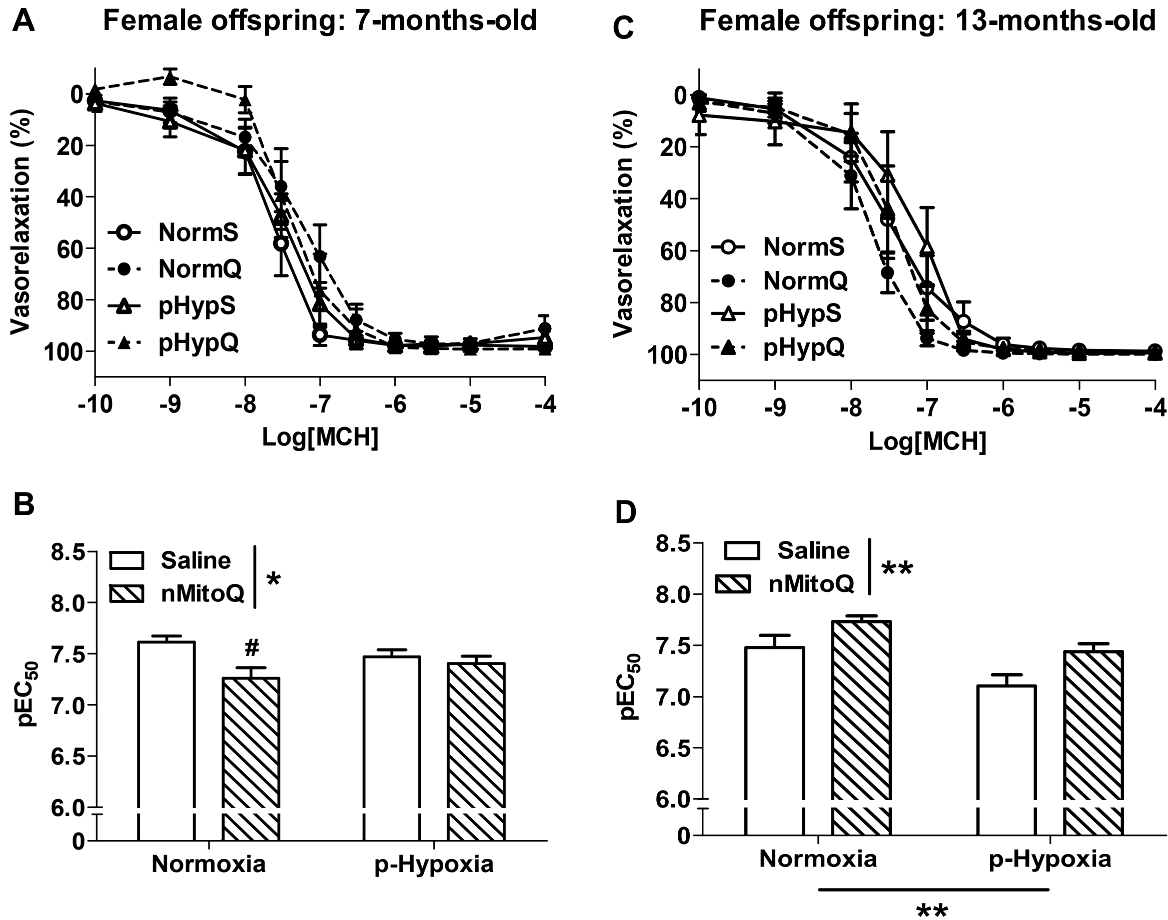


Figure 4.10 Mesenteric artery responses to methacholine (MCh) in female offspring

Vascular responses to MCh were assessed at 7 (**A & B**) and 13 (**C & D**) months of age. **B & D**: summary data of vascular sensitivity (pEC_{50}) to MCh. At 7 months of age, there was no effect of exposure to maternal hypoxia but nMitoQ treatment decreased sensitivity to MCh, particularly in the Norm group. At 13 months of age, maternal hypoxia reduced sensitivity to MCh while nMitoQ treatment increased sensitivity in both maternal environment groups. Data are presented as mean ± SEM. * $P < 0.05$, ** $P < 0.01$ group effect of maternal environment or maternal treatment, # $P < 0.05$ Tukeys post-hoc effect vs. NormS group, n=3-7/group.

4.4 DISCUSSION

The current study was undertaken in order to examine the potential benefit of maternal treatment with a placentally-targeted antioxidant for preventing the developmental programming of adult onset cardiovascular dysfunction in a rat model of maternal hypoxia. Our studies were performed in both young adults and aged adults time point in order to use aging as a secondary insult that could amplify a phenotype and allow subsequent investigation of treatment effects. We found in Chapter 3 that maternal hypoxia led to oxidative stress in placentas of male and female fetuses and nMitoQ treatment prevented oxidative stress in placentas of both sexes. The present study showed that in later life, male offspring exposed to hypoxia *in utero* developed a sex-specific phenotype that included indices of cardiac diastolic dysfunction and reduced NO modulation of vascular constriction in young adult males (7 months). Interestingly, these effects were entirely absent in young adult female offspring. With aging to 13 months, male offspring of hypoxic pregnancies developed further signs of cardiovascular dysfunction including cardiac wall thinning and increased mesenteric artery vasoconstriction compared to normoxic counterparts, in addition to persistent diastolic dysfunction, similar to previous studies from our lab (289). The effect of aging in females from hypoxic pregnancies was less severe; females developed only minor indices of diastolic dysfunction and reduced endothelium-dependent vasorelaxation compared to aged offspring of normoxic pregnancies. The relative protection of females from the detrimental outcomes of a compromised prenatally development has been observed in previous studies both within our own laboratory, as well as by other investigators (68).

Birth weight is a common index of a compromised *in utero* environment that has been used extensively in a clinical setting. Our previous study showed that nMitoQ treatment rescued neonatal body weight after maternal exposure to hypoxia in rats (249). In Chapter 3 we expanded

on this finding by assessing fetal body weights in male and female fetuses separately, which showed a sex-specific response to nMitoQ treatment; IUGR was prevented in female but not male fetuses. I in the current chapter, by investigating neonatal body weight, the hypoxia-induced phenotype of IUGR was still evident in both male and female neonates. However, the effect of nMitoQ treatment was subtle; while neonatal body weight seemed to improve after maternal nMitoQ treatment in male and female offspring of dams exposed to hypoxia, it was not normalized to control neonatal weights. The difference in the body weight results between fetal and neonatal life may be due to confounding variables. For example, neonatal body weight may be affected by suckling of the offspring (thus affecting body weight). Because this variability is avoided with fetal weight measurements, this may represent a more accurate assessment.

In the current study, maternal exposure to hypoxia had no effect on cardiac morphology in either male or female offspring at a young adult age. However, developmental programming effects on cardiac morphology may vary depending on age and sex of the offspring, and on the utilized animal model. For example, in a rat model of low protein diet, female offspring did not demonstrate altered cardiac morphology at 3 months of age; males were not assessed in that study (384). Another study using a low protein rat model, male IUGR offspring exhibited cardiac hypertrophy at 8 months of age, illustrated by a thicker posterior wall compared to the male offspring from normal pregnancy (175). Our study found that in male offspring aged to 13 months, maternal hypoxia led to decreased ventricular wall thickness; which may ultimately result in a dilated cardiomyopathy. Assessment of cardiac function showed that maternal hypoxia induced left ventricular diastolic dysfunction (decreased diastolic filling as assessed by a decreased A wave and increased E/A ratio) and pulmonary artery dysfunction (decreased peak velocity) in young adult (7 month) male offspring that persisted with aging; while females were unaffected as young

adults. In our previous studies we did not observe cardiac diastolic dysfunction at the younger (4 month) age point in male or female rat offspring exposed to hypoxia in fetal life compared to normoxic controls, without imposing the additional hit of a postnatal high-fat diet (289, 309). Aging to 12 months, however, uncovered a hypoxia-induced diastolic dysfunction phenotype that, similar to the current study, encompassed changes in more parameters in male than female offspring (289).

Left ventricular systolic function [as measured by parameters such as ejection fraction (EF), shortening fraction (FS) and cardiac output (CO)] appears to be more resistant to the effects of developmental programming. In both the current and previous studies from our laboratory, systolic function was not altered by hypoxia or age in male or female offspring (289). In a maternal protein restriction model, Menendez-Castro *et al* demonstrated a reduced contractility (ejection fraction) and more distensible myocardium at day 70 of life in IUGR rat male offspring compared to controls normal pregnancy (204). Furthermore, maternal undernutrition in rats, led to a lower ejection fraction and larger left ventricular mass in 22-month-old male and female offspring compared to the sex-matched controls (276). These data, combined with the results from the current study, suggest that cardiac dysfunction is evident in IUGR adult offspring, although the cardiac parameters affected may be dependent on the type of maternal insult.

The effects of maternal treatment with the antioxidant nMitoQ on cardiovascular function in offspring are complex. We have previously shown that applying a postnatal antioxidant intervention (resveratrol; supplemented in a high-fat diet starting from the weaning date for a period of 9 weeks) in the maternal hypoxia animal model prevented susceptibility to ischemia/ reperfusion injury, but was unable to prevent diastolic dysfunction in adult male offspring (309). In the current study, treatment with nMitoQ also had no effect on diastolic function. However,

nMitoQ treatment reduced the pulmonary artery peak velocity back to control levels. With the additional impact of aging, the beneficial effects of nMitoQ treatment on pulmonary artery function were lost. nMitoQ treatment was also unable to prevent the development of cardiac wall thinning in male offspring, following exposure to hypoxia *in utero.*

In contrast to male offspring, female offspring were unaffected by hypoxia *in utero* as young adults and developed only very mild indices of altered diastolic function (decreased A wave). In response to maternal nMitoQ treatment, young adult female offspring of dams exposed to normoxia or hypoxia showed significantly reduced E/A ratios. However, female offspring exposed to hypoxia *in utero* demonstrated a tendency towards an increased E/A ratio, possibly suggesting impaired diastolic ventricular filling. Thus, nMitoQ treatment may be beneficial in limiting the development of diastolic dysfunction in young adult females of dams exposed to hypoxia in pregnancy. The remainder of the cardiopulmonary effects of maternal treatment with nMitoQ on female offspring demonstrated several interaction effects; however, none of these interactions were reflected as statistically significant effects of either the *in utero* environment or maternal treatment with nMitoQ and thus the physiological significance of these outcomes remains uncertain.

Our own and others' studies have demonstrated vascular dysfunction following exposure to a suboptimal *in utero* environment; namely, an increased vasoconstrictor phenotype (increased adrenergic, angiotensin and endothelin reactivity) and decreased vasorelaxant phenotype (decreased nitric oxide and endothelium-dependent hyperpolarization), and increased oxidative stress [reviewed in (216)]. In the current study we observed that a hypoxic *in utero* environment, in decreased basal NO activation in young adult male offspring and increased vasoconstriction to adrenergic agonists in aged male offspring compared to their normoxic counterparts. Aging itself

can reduce vascular sensitivity to vasoconstrictors; for instance, contractile responses of human subcutaneous resistance arteries to noradrenaline and phenylephrine were reduced with aging (227). Furthermore, maximal vasoconstriction to phenylephrine and norepinephrine was diminished in adipose resistance arteries from aged compared to the control young rats (257). Similarly, we observed a reduction in mesenteric artery sensitivity to phenylephrine with aging from 7 to 13 months in male rats from a normal *in utero* environment. Exposure to hypoxia *in utero* had no effect on vasorelaxation in either young or aged males. In young adult female offspring, the vasoconstrictor and vasorelaxant pathways investigated in the current study were unaltered by exposure to hypoxia *in utero*. However, with the additional impact of aging, there was a significant reduction in sensitivity to methacholine in aged female hypoxic offspring compared to normoxic controls; suggesting that aging has a greater impact on endothelial function in female offspring exposed to hypoxia *in utero* than in their male counterparts. Previous studies in rats have demonstrated a reduction in NO function or bioavailability with aging that occurred earlier in females than males (50, 131, 217, 218). This effect of aging on hypoxic female offspring may leave them more vulnerable to cardiovascular insults.

Interestingly, treatment with nMitoQ differentially affected vasoconstriction of male offspring mesenteric arteries to phenylephrine in the young and aged groups. In young adult males, maternal treatment with nMitoQ reduced sensitivity to phenylephrine independent of maternal environment, and reduced NO-mediation of vasorelaxation in only offspring exposed to hypoxia *in utero*. In aged male offspring, conversely, nMitoQ treatment increased sensitivity to phenylephrine, offsetting the decrease observed with aging, and increased sensitivity to vasorelaxation independent of the *in utero* environment. Interestingly, maternal nMitoQ treatment increased sensitivity to MCh in both male and female, normoxic and hypoxic offspring at 13

months of age; demonstrating a beneficial effect of antioxidant treatment on improving vascular endothelial function.

In female offspring, vasoconstrictor function was unaltered by either exposure to hypoxia or treatment with nMitoQ at either age studied. While vasorelaxation was unaffected by the *in utero* environment at 7 months of age, maternal treatment with nMitoQ reduced mesenteric sensitivity to methacholine in normoxic offspring only. This reduction in vascular sensitivity to vasorelaxation in female offspring of control dams that did not suffer from pregnancy complications is considered as unwanted side effect of nMitoQ treatment in pregnancy. With aging, however, nMitoQ treatment improved sensitivity to methacholine in offspring of dams styed in normoxia or exposed to hypoxia in pregnancy. Both of these actions of nMitoQ appeared to be independent of NO production given that the contributions of both basal and methacholine-induced NO were similar among the groups.

It is well known that the placentas of male and female fetuses develop and respond to a suboptimal *in utero* environment via different mechanisms [reviewed in (59)]. Furthermore, cellular production of oxidative stress differs in male and female fetuses in normal pregnancy as well as in response to maternal stress (51, 75, 350). Therefore, we speculate that our results demonstrated dichotomous sex-specific and exposure-specific effects on offspring cardiovascular parameters. Future studies, specifically designed to directly address the complex pathophysiological responses in males versus females, are warranted.

4.5 PERSPECTIVES

In summary, our study demonstrated that maternal hypoxia has some sex- and age-dependent effects on offspring cardiac and vascular function later in life. Furthermore, the current

study highlights that using nanoparticles to target an antioxidant treatment (MitoQ) to the placenta could have potential benefits in offsetting the cardiovascular pathologies manifested in adulthood following gestational hypoxia. Indeed, maternal treatment with nMitoQ increased sensitivity to vasorelaxation in aged male and female offspring, prevented pulmonary artery dysfunction in young male offspring of dams exposed to hypoxia in pregnancy and led to improved systolic function in aged female offspring of dams exposed to hypoxia in pregnancy compared to the saline treated controls. This illustrates a possibly compliment role of the placenta in developmental programming and that targeting interventions to the placenta may become a valuable strategy to improve cardiovascular function in offspring born from compromised pregnancies.

CHAPTER 5: GENERAL DISCUSSION AND FUTURE DIRECTIONS

5.1 SUMMARY OF THE MOST SIGNIFICANT FINDINGS

The main focus of this PhD thesis was to assess the effect of hypoxia in pregnancy on maternal vascular function, fetal development and offspring cardiovascular function later in life. The current PhD project as well aimed to propose an intervention to improve offspring cardiovascular health in IUGR pregnancies. As placental oxidative stress is important in the pathophysiology of many pregnancy complications including IUGR and developmental programming, the mitochondrial antioxidant MitoQ loaded onto nanoparticles was proposed as an intervention to prevent placental oxidative stress and optimally prevent IUGR and fetal programming of cardiovascular disease. Nanoparticles were used to prevent nMitoQ treatment from crossing the placental barrier to the fetus. This aim was set to avoid risking direct fetal exposure to the antioxidant treatment (249).

Using an established animal model, we found that hypoxia in pregnancy can increase maternal blood pressure and cause vascular bed-dependent alterations in maternal vascular function. Furthermore, maternal hypoxia can alter uteroplacental and fetoplacental blood flow velocities, which caused a reduction in uterine artery RI and an increase in umbilical vein RI. Using a similar animal model but with a shorter period of maternal hypoxic exposure, we found that maternal hypoxia did not affect placental weight but increased oxidative stress in placentas taken from male and female fetuses. Maternal hypoxia as well led to IUGR, a reduction in placental fetal and maternal blood space area, and an increase in the relative heart weight to body weight (cardiac hypertrophy) in both male and female fetuses. However, hypoxia led to an increased cardiomyocyte size in only male but not female fetuses. Additionally, in young (7 months) and aged (13 months) male and female offspring, maternal hypoxia led to sex-dependent cardiovascular dysfunction. This included indices of cardiac diastolic dysfunction and reduced NO

modulation of vascular constriction in young adult males (7 months), cardiac wall thinning and increased mesenteric artery vasoconstriction compared to normoxic counterparts, in addition to persistent diastolic dysfunction in aged adult males (13 months). Furthermore, aged females (13 months) of dams exposed to hypoxia developed diastolic dysfunction and reduced endothelium-dependent vasorelaxation compared to aged offspring of dams stayed in normoxia all over the pregnancy.

nMitoQ treatment in fetal life was used to assess the effect of preventing placental oxidative stress on placental and fetal development and on offspring cardiovascular function after maternal exposure to hypoxia. Our results showed that nMitoQ treatment prevented placental oxidative stress in both male and female fetuses. nMitoQ did not affect placental weight or fetal and maternal blood space area in male fetuses but led to an increase in placental weight of female fetuses, which was associated with a reduction in placental fetal and maternal blood space area in normoxic placentas and an increase in fetal and maternal blood space area in hypoxic placentas. Additionally, nMitoQ treatment prevented IUGR and the increase in the relative heart weight in only female fetuses. However, nMitoQ rescued cardiomyocyte growth which was increased due to hypoxia in male fetuses. nMitoQ treatment also, partly improved cardiovascular function in offspring of both sexes. For example, nMitoQ improved systolic function in aged female offspring of dams exposed to hypoxia compared to the nMitoQ untreated controls increased sensitivity to vasorelaxation in aged male and female offspring.

Maternal nMitoQ treatment, however, had some unwanted side effects on the offspring. These unwanted side effects were seen more in female than male offspring and more in female offspring of normoxic dams than hypoxic dams. For example, maternal nMitoQ treatment increased fetal body weight in female offspring of normoxic dams which can increase the

vulnerability to transgenerational obesity in the offspring (103). This increase in fetal body weight of female offspring was also associated with a reduction in fetal and maternal blood space area per field of view in the placenta which could have a long-term impact on the vulnerability of these females to develop cardiovascular disease later in life. This possibility has been investigated in this thesis. For example, maternal nMitoQ led to interaction effects in adult life in female offspring whereby in most cases maternal nMitoQ tended to improve cardiovascular outcome in female offspring of hypoxic dams, nMitoQ tended to enhance cardiovascular dysfunction in female offspring of normoxic dams. For example, in young female offspring of normoxic dams, maternal nMitoQ treatment tended to increase pulmonary valve peak velocity which could reflect diastolic dysfunction in the right ventricle. However, in young female offspring of hypoxic dams, maternal nMitoQ treatment tended to normalize pulmonary valve peak velocity to a level closer to the level seen in offspring of normoxic saline-treated dams. Similarly, systolic function was also altered differentially by nMitoQ treatment at both 7 months (stroke volume was decreased in normoxic and increased in hypoxic offspring) and 13 months (ejection fraction and fractional shortening were both decreased in normoxic and increased in hypoxic while the left ventricular volume in systole was increased in normoxic and decreased in hypoxic offspring) of age. Furthermore, nMitoQ differentially altered cardiac morphology at 13 months of age in females; the left ventricular internal diameter in systole was increased in normoxic and decreased in hypoxic offspring. Given that none of these interaction effects were reflected in significant effects of either the *in utero* environment or maternal treatment with nMitoQ, the physiological significance of these outcomes remains uncertain and requires further investigation.

Alongside these interaction effects which were seen using echocardiography in female offspring, nMitoQ treatment had differential unwanted side effects in both male and female

offspring of dams exposed to normoxia or hypoxia in pregnancy. For example, maternal treatment with nMitoQ reduced mesenteric artery sensitivity to methacholine in female offspring of normoxic dams at 7 months of age. Maternal nMitoQ as well, increased mesenteric artery sensitivity to PE in male offspring of normoxic and hypoxic dams at 13 months of age. Thus, more assessment of nMitoQ safety and an overall evaluation of benefits versus detrimental side effects of the treatment are still needed.

5.2 EFFECT OF HYPOXIA ON MATERNAL VASCULAR FUNCTION

Abnormal maternal vascular function can affect fetal growth and development and contribute to the pathophysiology of IUGR [reviewed in (310)]. Therefore, understanding how a suboptimal environment (such as maternal hypoxia) can affect maternal vascular function is important for developing an intervention to prevent IUGR. It has been shown previously in rats that exposing pregnant rats to hypoxia can lead to an increase in maternal blood pressure via an increase in endothelin-1 signaling (383). However, the effect of maternal hypoxia on maternal vascular responses to vasodilators and vasoconstrictors, and on placental blood flow velocities were not investigated (383). Because of that, we conducted a study to assess the mechanisms of maternal vascular responses to hypoxia in rats. One of the most significant findings was showing that maternal vascular beds (mesenteric and uterine) respond to hypoxia in different ways. For example, hypoxia led to a lower nitric oxide contribution to vasodilation in uterine arteries, but it increased the in nitric oxide contribution to vasodilation in mesenteric arteries. Different vascular bed responses of to stimulants or inhibitors in hypoxia were shown previously. In pregnant guinea pigs at high altitude, for example, uterine arteries showed a diminished (but not reversed) vasorelaxation response to acetylcholine after adding a nitric oxide inhibitor. In the same dams, however, the vasorelaxation response of thoracic arteries to bradykinin was completely reversed

after adding a nitric oxide inhibitor showing different vascular bed responses to nitric oxide inhibition (361). These results illustrate that understanding the pathophysiology of pregnancy complications should take into consideration the different responses of vascular beds.

In our maternal study, one of our initial hypotheses was that maternal hypoxia will increase the RI of the uterine artery lead to a reduction in blood flow to the placenta and IUGR. Furthermore, if uterine artery RI was abnormally high in hypoxic pregnancy, then using an intervention which prevented an increase in uterine artery RI might attenuate IUGR and rescue fetal development. However, contrary to our expectations we found that uterine artery RI was reduced in hypoxic pregnant rats compared to the normoxic control. This reduction in uterine artery RI was associated with preserved placental weight but not attenuated fetal growth restriction. These findings are supported by a Cahill *et al*. who assessed placental physiological responses to hypoxia (45). They found that compared to normoxic pregnant mice, placentas of those who were exposed to hypoxia experienced capillary expansion, thinning of the interhaemal membrane (facilitates maternal-fetal exchange) and an increase in the diameter of the radial artery, resulting in a 2.6-fold drop in the total utero-placental vascular resistance. These changes in placental physiology due to hypoxia were accompanied by a preserved placental weight but a drop in fetal weight. Consequently, we concluded that our finding utilized that improving uterine artery RI preserved placental weight, but it is not enough to rescue fetal growth.

5.3 EFFECT OF HYPOXIA ON PLACENTAL AND FETAL DEVELOPMENT

As the reduction of uterine artery RI was not enough to rescue fetal growth, we looked for a new target to attenuate fetal growth restriction and improve offspring well-being in hypoxic/complicated pregnancy. Through our collaboration with the University of Bristol and using an established rat model of hypoxia in our laboratory, we found that maternal nMitoQ can

prevent placental oxidative stress, IUGR and abnormal fetal neural development, effects which have been linked to fetal programming of neurological disorders (249). Furthermore, we found that the effect of maternal hypoxia on fetal neural development was mediated via placental oxidative stress and placental secreted factors (249). As maternal hypoxia can affect fetal cardiomyocyte development as well (21), and maternal hypoxia is related to developmental programming of cardiovascular disease (113, 289), we aimed to implement the same animal model of hypoxia being used by our collaborators from Bristol to assess the effect of nMitoQ treatment on fetal growth and cardiomyocyte development. Moreover, we were interested in sex differences. It was established previously that male and female fetuses respond to the suboptimal intrauterine environment in a different way; and the difference in the effects of maternal hypoxia on male and female offspring continues in offspring young and aged life [reviewed in (59, 216)]. Therefore, the effect of maternal hypoxia and nMitoQ treatment was assessed in male and female fetuses separately.

Our results showed sex-dependent effects of hypoxia and nMitoQ treatment on fetal development. As the placenta is the hallmark in the pathophysiology of many pregnancy complications including IUGR and developmental programming; and because the antioxidant treatment was targeted to the placenta but not to the fetus, we speculate that the sex-difference in response to hypoxia and nMitoQ treatment was at least in part mediated by the placenta in sex dependent ways (229, 280). By assessing placental weight and structure we found that hypoxia did not alter placental weight in male or female fetuses but reduced placental fetal and maternal blood space area in both sexes. nMitoQ treatment did not prevent the reduction in placental fetal and maternal blood space area in male fetuses but did so in female fetuses of hypoxic dams and was associated with an increase in the body weight. A previous study showed a reduction in absolute

fetoplacental arterial vascular volume and in total vessel segments in murine placenta after maternal exposure to hypoxia which was associated with IUGR (45). We, therefore, suggest that the reduction in placental fetal and maternal blood space area contributed to the reduction in the body weight of hypoxic male and female fetuses. After nMitoQ treatment, the increase in placental fetal and maternal blood space area in female fetuses of hypoxic dams could explain the increase in female body weight in that group. In normoxic female fetuses, however, the reduction in placental fetal and maternal blood space area was not associated with a reduction in the body weight in female fetuses of normoxic dams. We anticipate that the increase in the body weight of normoxia/nMitoQ female fetuses might come at the expense of their developing organs (because the placenta was not efficient, and the fetus adapted to this). Indeed, this was shown in adult life in the normoxia/nMitoQ female offspring, as female offspring from dams exposed to hypoxia/nMitoQ had a better cardiovascular function than their control hypoxia/saline female offspring as shown by a closer to normal (control) cardiac morphology, systolic function, and sensitivity to the vasorelaxant MCh. However, normoxic female offspring of dams exposed to nMitoQ had cardiovascular dysfunction compared to their control normoxia/saline female offspring shown by an increase in the left ventricular internal diameter at diastole, systolic dysfunction, and reduction in the sensitivity to the vasorelaxant MCh. These findings suggest a possible link between abnormal placental development and offspring cardiovascular dysfunction.

5.4 EFFECT OF MATERNAL HYPOXIA ON OFFSPRING CARDIOVASCULAR FUNCTION

It has been established that hypoxia in pregnancy can cause cardiovascular dysfunction in adult offspring and that these adverse effects of hypoxia on the offspring are linked to placental dysfunction [reviewed in (11, 216)]. We, therefore, asked whether an intervention that targets

placental oxidative stress can prevent developmental programming of cardiovascular disease later in life. One of the strengths of the current thesis was the assessment of the effect of maternal hypoxia and nMitoQ treatment on male and female offspring separately. The difference in the offspring susceptibility to cardiovascular disease in both sexes has been shown previously in our own laboratory and by other investigators (10, 289). The mechanisms were usually explained by hormonal and genetic differences in males and females that make male offspring more vulnerable to cardiovascular disease (223, 336). Consequently, understanding the differences in male and female susceptibility to cardiovascular disease is important for developing sex specific interventions.

Another strength of this thesis was the assessment of the effect of maternal hypoxia and nMitoQ treatment on male and female offspring at both young and ages. We found that young male offspring (7 months) of dams exposed to hypoxia in pregnancy had a sex-specific phenotype that included cardiac diastolic dysfunction, pulmonary artery dysfunction and reduced vascular basal nitric oxide modulation of vasoconstriction. Interestingly, in young female offspring, these effects were completely absent. At a more advanced age (13 months), male offspring of dams exposed to hypoxia in pregnancy developed additional signs of cardiovascular dysfunction including a reduction in the thickness of the cardiac wall and an increase in vascular contractility. These signs were associated with a persistent diastolic and pulmonary artery dysfunction, which agrees with our previous study (289). With aging, female offspring developed few signs of cardiovascular dysfunction including signs of diastolic dysfunction and a reduction in endothelium-dependent vasorelaxation compared to the control offspring from normoxic dams. This difference in adult male and female offspring susceptibility to develop cardiovascular disease cannot be linked to hormonal changes (as it is usually justified in studies on human beings) because

female rats do not experience menopause when they age. Instead, they become infertile by ovarian senescence and their estrogen levels remain constant compared to the physiological reduction in estrogen levels seen in humans after menopause (65). Therefore, aging by itself can be a unique risk factor to develop cardiovascular disease and should be considered when deciding an intervention.

The effect of maternal nMitoQ treatment on cardiovascular morphology and function was assessed as well in adult male and female offspring at different ages. Compared to the offspring of dams that did not receive nMitoQ treatment, our results showed that nMitoQ had beneficial effects including a reduction in the sensitivity to the vasoconstrictor PE in young male offspring, and closer to normal cardiac morphology and systolic function in aged hypoxic female offspring. However, alongside having beneficial effects, nMitoQ treatment had detrimental effects as well as illustrated by diastolic dysfunction in young female and aged male offspring and by an increase in the left ventricular internal diameter at diastole and systolic dysfunction in aged normoxic offspring. This means that the safety of maternal nMitoQ treatment needs more investigation.

5.5 PROJECT LIMITATIONS

In this PhD project, the first established animal model of IUGR (pregnant rats were exposed to hypoxia between day 6 and 20 of pregnancy) was chosen to enhance maternal vascular resistance. The choice of this animal model was based on a previous study, which showed that exposing pregnant rats to hypoxia between day 6 and 21 of pregnancy increases maternal blood pressure (383). We anticipated that the increase in maternal peripheral vascular resistance might be associated with an increase in uterine artery RI as well, which could be possibly targeted to prevent IUGR. As the project progressed, the results showed that hypoxia led to an increase in maternal blood pressure but a reduction in the uterine artery RI which was associated with IUGR.

Therefore, as the reduction in the uterine artery RI was still accompanied by IUGR, there was a need for a new target to prevent IUGR and improve pregnancy outcome. Placental dysfunction is the hallmark of many pregnancy complications (such as IUGR). Thus, targeting an intervention to the placenta was chosen to improve fetal development and offspring well-being in complicated pregnancy. For that purpose, a second animal model of IUGR for my PhD project was chosen (pregnant rats were exposed to hypoxia between day 15 and 21 of pregnancy). The reason for choosing the second animal model is that our laboratory has previously assessed the phenotype of placental and fetal development, and offspring cardiovascular function using this time period (34, 217, 218, 289, 309), which made me feel more confident of proceeding to use it in my PhD project.

One of the weaknesses of the animal models being used here is that they do not completely mimic the clinical pathophysiology of IUGR in human beings. In the general clinical settings, placental hypoxia is not the result of entire maternal body exposure to hypoxia. Whereas, in these animal models, IUGR was created by placing pregnant dams in hypoxia. Maternal exposure to hypoxia (in animal models or in women living at high altitude) can lead to maternal adaptions to compensate for the low oxygen level, such as an increase in maternal heart rate and alveolar ventilation or alterations in normal maternal vascular responses [reviewed in (211)].

Another weakness of the study is that rats which were exposed to maternal hypoxia could have experienced stress that could impact on fetal development and/or maternal behavior. Future studies to assess maternal corticosterone levels (the main glucocorticoid involved in regulation of stress response in rodents) as a biological indicator of stress would be advised. In Chapter 3, I presented data showing that maternal hypoxia did not alter corticosterone level in the plasma of male fetuses but reduced it in the plasma of female fetuses. Thus, fetal corticosterone levels were not elevated but maternal corticosterone was not assessed. Moreover, although, maternal behavior

did not appear to be perturbed (e.g. they prepared their nests and fed their offspring), cross feeding pups could be performed in the future studies to overcome any doubts about this matter.

There are other animal models of IUGR due to reduced oxygen supply that could be implemented here in studying developmental programming. For example, the reduced uterine perfusion pressure (RUPP) animal model, which is usually created in pregnant rats or mice by ligating the uterine artery to enhance placental ischemia, leading to increased placental oxidative stress (303), low birth weight, high maternal blood pressure (102) and abnormal maternal vascular function (35). However, creating the RUPP model involves a mechanical disruption of uterine blood flow, thus testing intervention strategies is limited. In addition, the model is associated with an increased incidence of miscarriage, premature delivery and high fetal resorption rate compared to sham operated control (no ligation) (303). Sheep models of placental reduction in uterine blood flow are also considered animal models of placental hypoxia and can be created by placental embolization (38) or caruncle removal (215). This causes a reduction in fetal body weight and abnormal fetal cardiomyocyte development (38, 215). Studies from research groups globally using different species are necessary for further translation to human pregnancies.

In the current project nMitoQ treatment was begun before maternal exposure to hypoxia and nMitoQ was still effective in preventing placental oxidative stress at the day of tissue collection which was after two weeks of nMitoQ application. However, the timing for nMitoQ intervention still needs to be defined in the clinical settings as pregnancy in humans lasts for months and multiple doses might be needed. Also, in humans choosing the best time of intervention is challenging and has yet to be determined because early screening methods of pregnancy complications (and therefore the ideal time to apply antioxidant treatment) are still not available.

Another matter that could be questioned in my PhD project concerns that possible effects of nanoparticles alone (without MitoQ) on placental development, fetal development, and offspring cardiovascular function. In my PhD project, nMitoQ treatment was looked at as a modification of the MitoQ treatment alone. The aim was to assess the effect on nMitoQ treatment as a compound but not as a separate entity (MitoQ and nanoparticles separately). Therefore, assessing the effect of nanoparticles alone is not useful in the current project as applying MitoQ alone was not intended. However, we have shown previously that γ-PGA-graft-L-PAE nanoparticles (alone without MitoQ) do not possess a toxic or an antioxidant effect on placental and fetal neural cells, and do not have a role in the placental-mediated effect on fetal neural development (249). Therefore, we speculate that the effects of nMitoQ treatment on placental oxidative stress and fetal development herein might mainly be mediated by MitoQ but not by the accompanied nanoparticles. However, confirming this is encouraged in future studies.

Another aspect that could be questioned in this project is about combining placental conditioned medium from male and female fetuses, and applying it on male and female cardiomyocytes separately. The combined placental media did not show an effect on cardiomyocyte growth and maturation. However, placentas of male and female fetuses might develop, function and respond to pregnancy complications in different ways [reviewed in (59)]. Thus, using separate male and female placental conditioned medium could have revealed an effect of placental secreted factors on fetal cardiomyocyte development. Furthermore, no positive control was used in this study when assessing placental inflammatory factors. Additionally, when placental conditioned medium was collected, the first 24 hours placental conditioned medium was discarded, and the next 24 hours placental conditioned medium was collected and used for the experiments. Discarding the first 24-48 hours placental conditioned medium was done previously

to allow the placenta to acclimatize after exposure to trauma (removal from the animal body) (82, 124, 249). However, assessment of the level of placental secreted factors in the first 24 hours placental conditioned medium and their impact on cardiomyocyte development was not performed. Furthermore, by collecting the placental cultured medium we can not decipher factors that are released towards the fetus from those released into the mother [i.e. apical vs. basement membrane of syncytiotrophoblast cells have different expression of transporters, reviewed in (354)]. Using fetal plasma (contains placental secreted factors) instead of placental cultured medium could be another option to assess how placental secreted factors can affect fetal cardiomyocyte development. However, using fetal plasma has some limitations as well because it does not contain only factors secreted by the placenta, but also other factors produced and secreted by the fetus itself or passed the placental barrier from maternal circulation to the fetus such as hormones and proteins. All these matters discussed about placental secreted factors will be taken in consideration in the future in our laboratory, as expanding on the effect of placental secreted factors on fetal cardiomyocyte development will be continued in the next few years.

5.6 FUTURE DIRECTIONS

One of the biggest questions to be answered is why male and female offspring responded differently to hypoxia and nMitoQ treatment, and what are the mechanisms or pathways that mediate these differences. Although hormonal and genetic factors were suggested (223, 336), detailed studies (such as assessment of epigenetics, hormones and growth factor pathways) are still needed to fully understand how these factors influence placental and fetal growth and then offspring susceptibility to cardiovascular disease later in life.

Furthermore, if MitoQ is released by nanoparticles in the cytoplasm, the rat placental barrier could be more protected from MitoQ than a human placental barrier. This is because in rats

there are three trophoblast layers in the placenta that may help to prevent MitoQ transfer to the fetus. However, in humans, there are only two trophoblast layers in the placenta which could make it easier for MitoQ to pass to the fetus. This is an interesting aspect to look at in the future to understand the difference in MitoQ transportation among different species. However, localization of nanoparticles in the trophoblast cells should be performed first.

Other aspects that should be assessed in the future are the optimal dosage and timing of antioxidant treatment to achieve the best outcome in hypoxic pregnancies. This includes an assessment of how long nMitoQ can stay in the body and be effective in humans and in different animal species especially those animals that have a longer gestational period than rodents. This also includes the best timing of intervention in humans that will be tightly linked to the availability of early screening methods of pregnancy complications. Additionally, additional assessment of nMitoQ on maternal vascular function and, importantly, the safety and overall evaluation of benefits versus detrimental side effects of the treatment should be looked at in the future.

This PhD project focused on the big physiological picture of how maternal hypoxia and nMitoQ treatment can affect fetal development and offspring well-being. However, more detailed exploration at a molecular level is still needed. For example, still needed is assessment of the effect of maternal hypoxia and nMitoQ treatment on metabolic and mitochondrial function in the placenta and in fetal and offspring cardiac cells (129, 226), placental cellular stress markers [such as heat shock proteins; (27)], and placental endoplasmic reticulum stress markers [such as phospho-eukaryotic initiation factor 2; (379)]. Also, a full assessment of the effect of maternal hypoxia and nMitoQ treatment on placental histology in both sexes is needed. Additionally, an assessment of the level of placental hypoxia (i.e. by using real-time photoacoustic imaging) should be carried out to determine if nMitoQ treatment can have any effect on it. Also, the effect of

maternal hypoxia and placental secreted factors was assessed on cardiomyocyte growth and maturation, but other aspects of cardiomyocyte development such as metabolic function, apoptosis and proliferation still need determination.

In an offspring adult life, assessment of nMitoQ effect in preventing offspring cardiovascular disease could be done after exposing offspring of hypoxia-exposed dams to a second hit (such as high-fat diet) that could create a stronger phenotype (289). In adult offspring as well, using wire myography does not reflect the vascular function in an intact cardiovascular system. Thus, other cardiovascular assessment methods can be used in the future to assess vascular function *in vivo* and to expand on the cardiac function assessment which has been performed here using echocardiography. For example, some methods were done previously in mice and can be implemented in cardiovascular assessment in rats such as, two-photon imaging which was used to assess Ca^{2+} signaling in arteriole smooth muscle cells of anesthetized mice (198), and non-invasive ultrasonic methods, which were used to assess blood flow velocity of peripheral vessels, including carotid and coronary artery in anesthetized mice as well (121, 122). Furthermore, non-invasive electrocardiogram (ECG) recording can be used in conscious rats to assess cardiac disease such as abnormal cardiac autonomic control (246). Myocardial metabolism assessment in fully conscious and mobile rats can be performed in the future as well by using positron emission tomography (368). Ultimately, future studies on adult cardiac function are needed to determine with nMitoQ intervention in offspring exposed to prenatal hypoxia reduces their susceptibility to cardiovascular disease.

5.7 SIGNIFICANCE OF THE THESIS RESULTS

This thesis has elucidated factors that play an important role in the pathophysiology of IUGR and developmental programming of cardiovascular disease. This thesis as well showed that

targeting antioxidant treatment to the placenta can impact fetal body weight, fetal cardiac development and offspring cardiovascular function in adult life, which means that interventions do not necessarily need to target the fetus to improve pregnancy outcomes. Furthermore, this PhD project showed sex-dependent differences in the response to maternal hypoxia and antioxidant treatment. This is very important as different responses to suboptimal environment and interventions in male and female offspring might impact the choice of the intervention in each sex.

The current PhD thesis helped set the stage for future studies about employing nanoparticles to deliver interventions to specific targets in pregnancy in order to improve offspring cardiovascular function in complicated pregnancy.

APPENDICES

Fetal characteristic data form

Pup No.	Body weight	Length	Abdominal girth	Heart weight	Kidney weight	Brain weight	Placental weight		Placental diameter
							Wet	Dry	
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									
16									
Average									

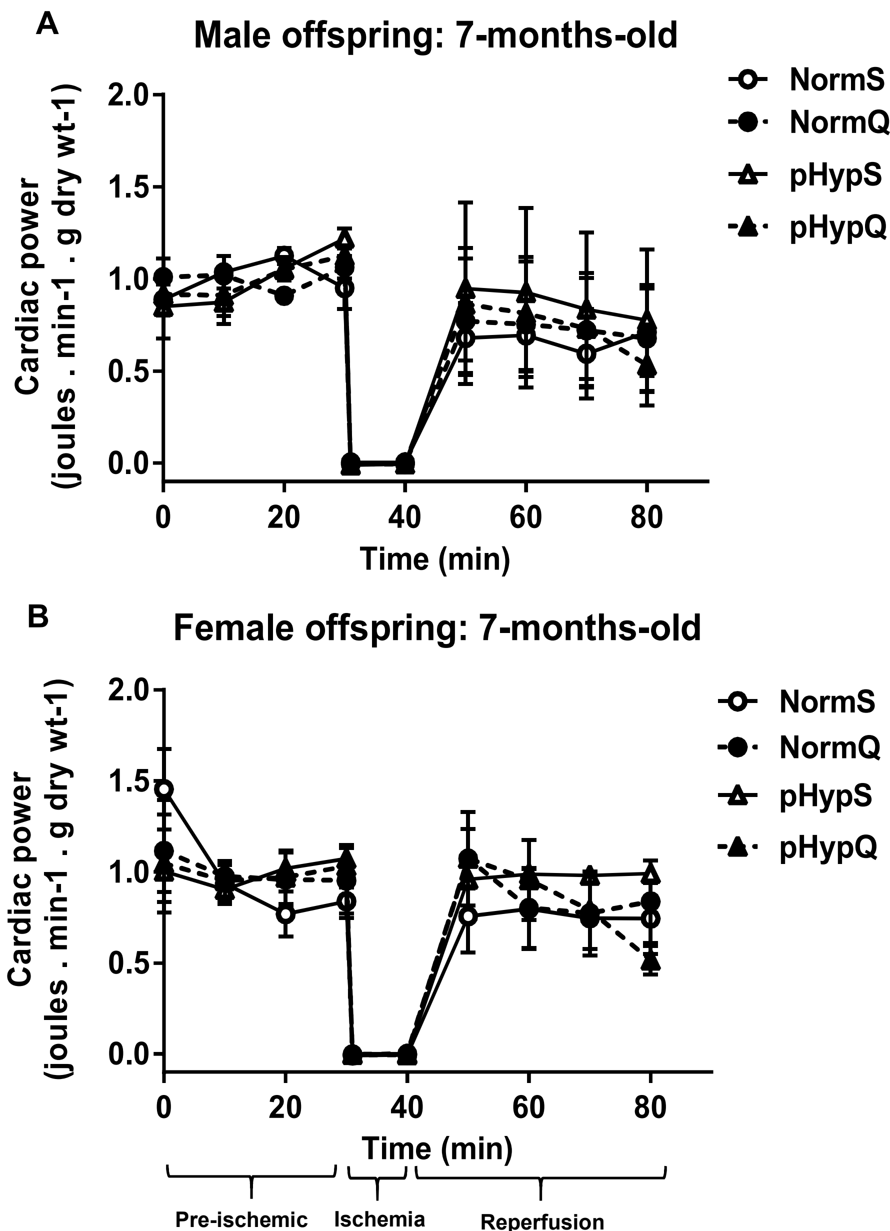


Figure 6.1 Effect of maternal hypoxia and nMitoQ treatment on myocardial susceptibility to ischemia/reperfusion injury in male and female offspring at 7 months of age

Average cardiac power was developed over time during *ex vivo* cardiac aerobic perfusion (pre-ischemia) and after 10 minutes (reperfusion) of stopping the flow (ischemia). This pilot study was done as part of the adult offspring cardiovascular assessment. Data showed no difference in cardiac power between the groups in male and female offspring at 7 months of age. Glucose was used as a source of cardiac energy in the perfused krebs–henseleit solution. Future assessments of myocardial susceptibility to ischemia/reperfusion injury will be done in our laboratory using fatty acids as an ideal physiological source of energy in the heart. Data are presented as mean ± SEM. Male: n=3-5/group. Female: n=2-6/group. Data acquisition and analysis: Dr. Jude Morton.

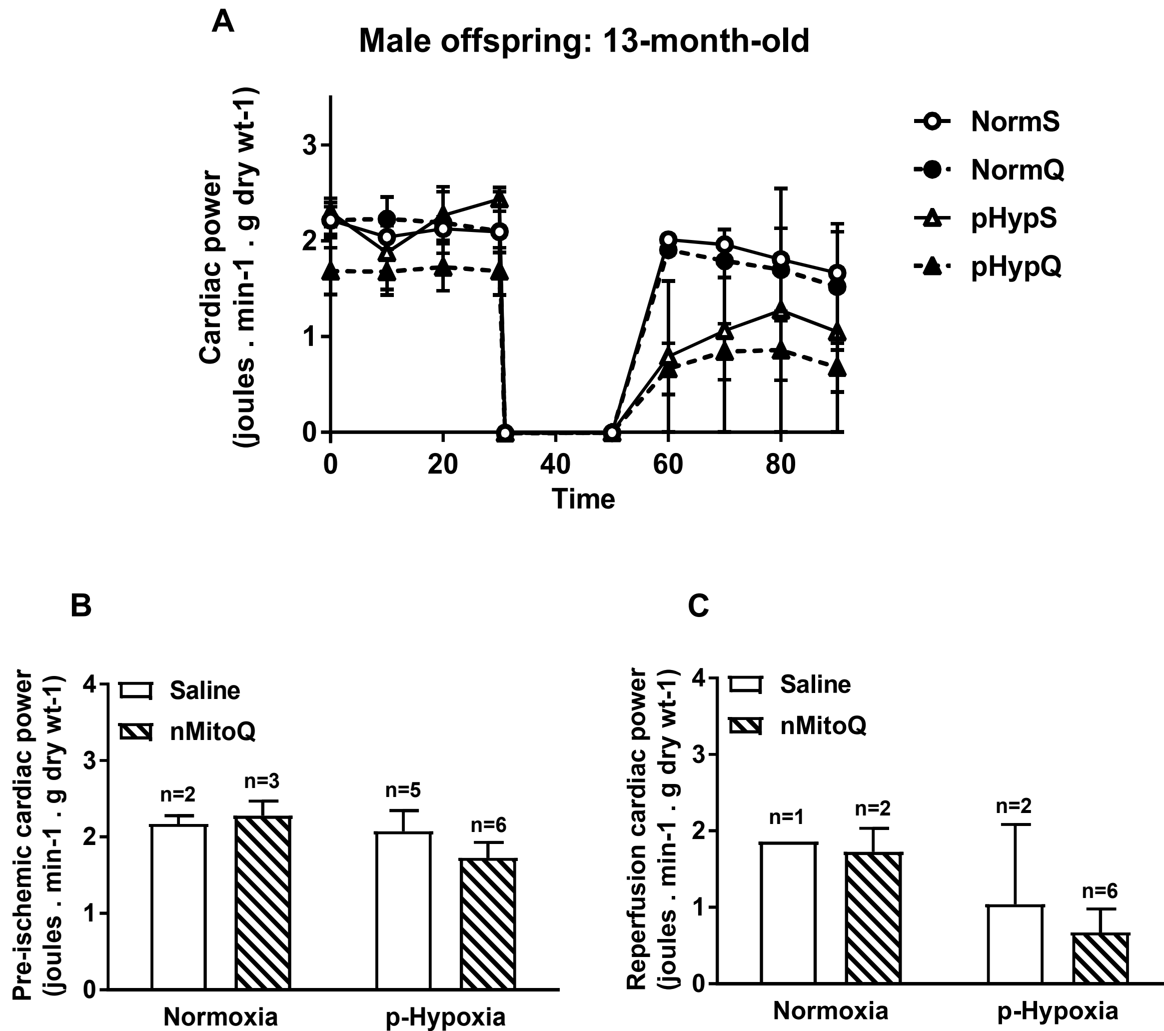


Figure 6.2 Effect of maternal hypoxia and nMitoQ treatment on myocardial susceptibility to ischemia/reperfusion injury in male offspring at 13 months of age

A: Average cardiac power was developed over time during *ex vivo* cardiac aerobic perfusion (pre-ischemia) and after 20 minutes (reperfusion) of stopping the flow (ischemia). **B**: Average maximal cardiac power developed during pre-ischemic period. **C**: Average maximal cardiac power developed during reperfusion. This pilot study was done as part of the adult offspring cardiovascular assessment. Data suggested a possible reduction in reperfusion cardiac power in offspring of dams exposed to hypoxia compared to offspring of dams stayed in normoxia. Maternal nMitoQ treatment did not prevent this possible reduction in reperfusion cardiac power. Glucose was used as a source of cardiac energy in the perfused krebs–henseleit solution. Future assessments of myocardial susceptibility to ischemia/reperfusion injury will be done in our laboratory using fatty acids as an ideal physiological source of energy in the heart. Data are presented as mean ± SEM. Data acquisition and analysis: Dr. Jude Morton.

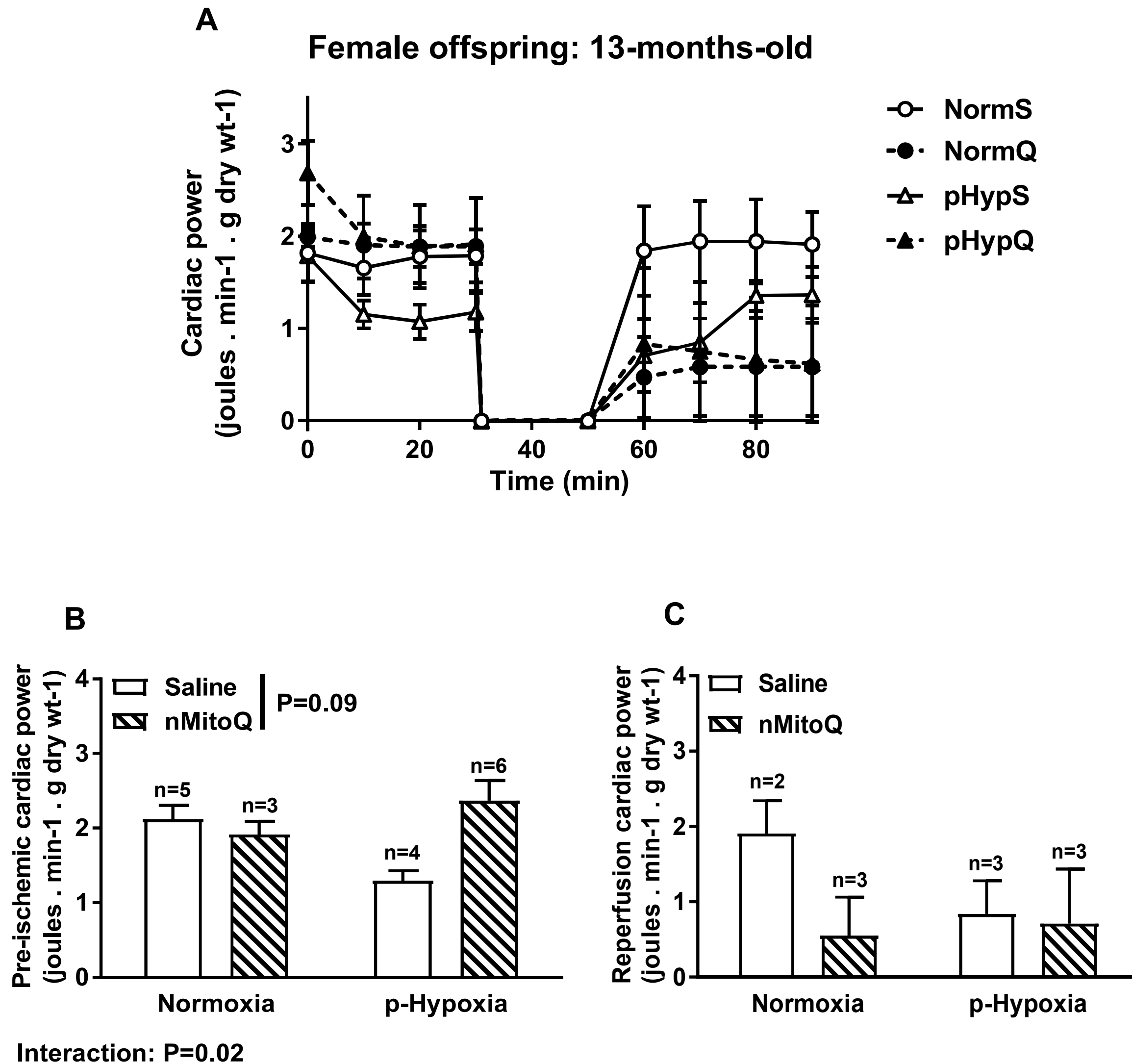


Figure 6.3 Effect of maternal hypoxia and nMitoQ treatment on myocardial susceptibility to ischemia/reperfusion injury in female offspring at 13 months of age

A: Average cardiac power was developed over time during *ex vivo* cardiac aerobic perfusion (pre-ischemia) and after 20 minutes (reperfusion) of stopping the flow (ischemia). **B**: Average maximal cardiac power developed during pre-ischemic period. **C**: Average maximal cardiac power developed during reperfusion. This pilot study was done as part of the adult offspring cardiovascular assessment. nMitoQ had a different effect in female offspring of dams stayed in normoxia or exposed to hypoxia in pregnancy whereby it did not alter baseline function in the females of dams stayed in normoxia but improved function in the females of dams exposed to hypoxia. Glucose was used as a source of cardiac energy in the perfused krebs–henseleit solution. Future assessments of myocardial susceptibility to ischemia/reperfusion injury will be done in our laboratory using fatty acids as an ideal physiological source of energy in the heart. Data are presented as mean ± SEM. Data acquisition and analysis: Dr. Jude Morton.

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