Treating the Placenta with a Nanoparticle-Encapsulated Mitochondrial Antioxidant Improves Placental Function and Fetal Cardiomyocyte Development in a Rat Model of Prenatal Hypoxia

By

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Abstract

Introduction: Pregnancy complications associated with prenatal hypoxia have been linked to the development of cardiovascular disease in adult offspring. Prenatal hypoxia (due to maternal or placental hypoxia) has been shown to increase placental oxidative stress and impair placental function in a sex-specific manner, thereby affecting fetal development. Studies suggest that prenatal hypoxia can reduce mitochondrial respiratory capacity, which in turn, could lead to placental dysfunction and impaired fetal organ development. Recently, we, together with our collaborators, have demonstrated that prenatal hypoxia-induced placental oxidative stress can impair development of key fetal organ system (such as the brain) through the release of placenta-derived factors into the fetal circulation. However, the effects of placenta-derived factors on fetal cardiomyocyte development are yet to be determined. Given that oxidative stress is central to placental dysfunction and altered fetal organ development, placental function may be improved by the use of an antioxidant. However, drug use in pregnancy is restricted due to potential adverse effects on the fetus should transplacental passage occur. Thus, the main objective of my PhD studies was to improve placental function without direct drug exposure to the fetus in order to avoid off-target effects during development. I assessed a placenta-targeted innovative treatment strategy using a mitochondrial-targeted antioxidant (MitoQ) encapsulated into biodegradable nanoparticles (nMitoQ) as a delivery system, which diffuses into the placental syncytium to release the antioxidant treatment within the placenta without crossing the placental basal membrane to reach the fetus. I tested whether this placenta-targeted nMitoQ treatment targeted in hypoxic dams improved placental oxidative stress and placental morphology and function. In addition, I assessed whether nMitoQ treatment in hypoxic

dams prevents fetal programming of cardiac diseases via the release of placenta-derived factors.

Methods: Pregnant Sprague-Dawley rats were intravenously injected with saline or nMitoQ (100µl of 125 µM) on gestational day (GD) 15 and exposed to either normoxia (21% O_2) or hypoxia (11% O_2) from GD15-21. On GD21, placentae from both sexes were collected for detection of oxygenation, superoxide, nitrotyrosine, nitric oxide, CD31 (endothelial cell marker), and maternal and fetal blood spaces, *Vegfa* and *Igf2* expression in the placental labyrinth zone. In a second set of experiments, on GD21, male and female placental labyrinth zones were collected for mitochondrial functional assessments. To assess the effects of placenta-derived factors on fetal cardiomyocyte development *in vitro*, on GD21, male and female placentae were harvested, placed in culture, and conditioned media (containing placental-derived factors) was collected after 24h. Male and female cardiomyocytes from control dam fetuses were incubated with same sex placental conditioned media.

Results: nMitoQ treatment reduced the prenatal hypoxia-induced increase in placental superoxide levels in both male and female placentae but improved oxygenation in only female placentae. Without altering nitric oxide levels, nMitoQ treatment reduced nitrotyrosine levels in hypoxic female placentae. Prenatal hypoxia reduced placental *Vegfa* and *Igf2* expression along with reduced maternal and fetal blood spaces and fetal capillaries area in the labyrinth zone from both sexes, while nMitoQ increased *Vegfa* and *Igf2* expression only in hypoxic female placentae. Prenatal hypoxia reduced mitochondrial complex IV activity in male placentae, which was improved by nMitoQ treatment. Interestingly, in females, nMitoQ had no effect on placental mitochondrial function, but prenatal hypoxia increased contribution of the N-pathway (through complex I) and decreased contribution of the S-pathway (through complex II). nMitoQ treatment led to increased oxygenation in hearts and livers of hypoxic female fetuses, whereas oxygenation was improved in hearts but not livers of nMitoQ treated prenatally hypoxic male fetuses. Furthermore, factors derived

from hypoxic placentae of dams treated with nMitoQ prevented increases in the percentage of binucleated cardiomyocytes (marker of terminal differentiation) and the size of mononucleated and binucleated cardiomyocytes (sign of hypertrophy).

Conclusion: In summary, my studies suggests that treatment strategies targeted against placental oxidative stress can improve placental function in complicated pregnancies and may prevent fetal programming of cardiac disease. However, the mechanisms (i.e. changes in placental morphological and mitochondrial functional capacity) were distinct between males and females. Thus, sex differences need to be taken into account when developing placenta-targeted therapeutic strategies to improve fetal development in complicated pregnancies.

Preface

This thesis is an original research work by Esha Ganguly. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Pregnancy Complications", and AUP # 242.

Some of the research conducted for this thesis forms part of an international research collaboration, led by Dr. Patrick C. Case at the University of Bristol, with Dr. Sandra T. Davidge being the lead collaborator at the University of Alberta. The University of Bristol has submitted a patent application for the nanoparticle formulation referred to in chapters 2, 3 and 4 in this thesis. The mitochondrial function experiment was designed with the assistance of Dr. Helene Lemieux. The literature review in chapter 1 and data presented in chapters 2, 3 and 4 are my original work.

Part of Chapter 1 of this thesis have been published as "***Ganguly E**, Hula N, Spaans F, Cooke CLM and Davidge ST. Placenta-Targeted Treatment Strategies: An Opportunity to Impact Fetal Development and Improve Offspring Health in Later Life. Pharmacol Res 157, 104836. 2020."* Ganguly E and Hula N was responsible for the manuscript composition. Spaans F assisted with manuscript figure preparation and manuscript edits. Cooke CLM assisted with manuscript composition and edits. Davidge ST was the supervisory author and was involved in concept formation and manuscript formation.

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Dedication

This thesis is dedicated to my mom and dad.

Acknowledgement

It is hard to express my gratitude that I have for all the people who have helped me complete this journey. I will start by thanking my mentor, Dr. Sandra T. Davidge. Sandy, you have been a perfect teacher, a supportive mentor, and a great role model. Thank you for all your patience, enthusiasm, kindness, and more importantly for your trust in me. I not only learned great science from you, but you have also taught me to be a humble scientist. It has been a privilege to be part of the 'The Davidge Lab' and, I will be forever grateful.

Sandy always says, "It takes a village to raise a child", and my PhD would not have been possible without support from 'The Davidge Lab'. I have had the chance to work with Jude, Floor, Raven, Anita, Natasha, Mais, Tamara, Mazhar, Roberto, and Amy. Thank you all for being such wonderful colleagues. I am thankful for helping me think through my project, for sharing your knowledge, for the encouragement, support, and friendship. PhD is a long and challenging process, and my PhD is certainly a combined effort of everyone that I have had a chance to work with over all these years.

I would like to thank my supervisory committee members, Dr. Zamaneh Kassiri and Dr. Denise Hemmings for their thoughtful guidance throughout my degree and invaluable feedback on my research project. Thank you for all your support. I would also like to acknowledge, Dr. Helene Lemieux, with whom I have had the opportunity to collaborate and learn about the mitochondria.

I would also like to acknowledge the support of the following funding agencies that have supported my PhD work: Canadian Institute of Health Research (CIHR), the Women and Children's Health Research Institute (CIHR), the Faculty of Medicine and Dentistry, Department of Physiology and Department of Obstetrics and Gynecology.

Most importantly, I would like to thank my family and friends for their unconditional love and

support with all my decisions. This journey would not have been possible without my parents supporting my decision of moving continents and starting all over again in a new place. Thank you for believing in me, more than what I believed in my own self. I would like to thank my Uncle (Dr. Paul Ganguly), Riya, and Saty who have been my family and a constant source of support and inspiration in Canada. Finally, I would like to thank Gaurav for his unending support and motivation in getting my thesis completed. You have taught me to keep my cool and think through before taking any decisions. Thank you for all that you do. I hope that I can make everyone who has been a part of my PhD journey proud someday.

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List of abbreviations

The following abbreviations have been used in the thesis.

Abbreviation	Meaning
ADP	Adenine dinucleotide phosphate
ANOVA	Analysis of variance
AR	Adrenergic receptor
ATF4	Activating transcription factor 4
ATP	Adenosine triphosphate
a.u.	Arbitrary unit
Bcl-2	B-cell lymphoma 2
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
C19MC	Chromosome 19 miRNA cluster
CAT	Catalase
CAMKII	Calcium/calmodulin-dependent protein kinas II
Chek1	Checkpoint kinase 1
$COMT^{-/-}$	Catechol-O-methyl transferase
DAF-FM	Diamino fluorescein-FM
DAPI	4′, 6-diamidino-2-phenylindole
DHE	Dihydroethidium

DNA	Deoxyribonucleic acid
DNMT3b	DNA methyltransferase 3 beta
DMR2	Differentially methylated region 2
DOHaD	Developmental origins of health and disease
Drp1	Dynamin-related protein 1
eIF2α	Eukaryotic initiation factor 2 alpha
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ER	Estrogen receptor
EVT	Extravillous trophoblast
$FADH_2$	Flavin adenine dinucleotide
FGR	Fetal growth restriction
g	Gram
GD	Gestational day
GLUTs	Glucose transporters
GRP75	75-kDa glucose-regulated protein
H&E	Hematoxylin and Eosin
HBSS	Hank's Balanced Salt Solution
H_2O_2	Hydrogen peroxide
HIF-1α	Hypoxia-inducible factor 1α
HSP60	Heat shock protein 60
HSP70	Heat shock protein 70

HSD11B2	Hydroxysteroid 11-beta dehydrogenase 2
HUVEC	Human umbilical vein endothelial cell
ICR	Imprinting control region
IGF-1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor 1 receptor
IGF-2	Insulin-like growth factor 2
IGF2R	Insulin-like growth factor 2 receptor
ILs	Interleukins
ILR2B	Interleukin 2 receptor subunit beta
IMM	Inner mitochondrial membrane
iNOS	Inducible nitric oxide synthase
I/R	Ischemia / reperfusion
INSR	Insulin receptor
JAK1	Janus kinase 1
L-PAE	L-pheylalanine ethyl ester
μM	Micromolar
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MFN1	Mitofusin 1
MFN 2	Mitofusin 2
miRNAs	MicroRNAs
MMP2	Matrix metallopeptidase 2

MMP9	Matrix metallopeptidase 9
mtDNA	Mitochondrial DNA
MYH6/7	Anti-heavy chain cardiac myosin
N-Pathway	NADH-Pathway
n DNA	Nuclear DNA
NADPH	Nicotinamide adenine dinucleotide hydrate
NR3C1	Nuclear receptor subfamily 3 group C member 1
nMitoQ	MitoQ encapsulated into nanoparticles
NO	Nitric oxide
OCT	Optimal cutting temperature
$\cdot OH$	Hydroxyl radical
OPA1	Optic atrophy 1
O_2	Oxygen
OXPHOS	Oxidative phosphorylation
peIF2α	Phosphorylated eukaryotic initiation factor 2 alpha
PBS	Phosphate buffered solution
PGF	Placental growth factor
PI3K	Phosphoinositide 3-kinase
PKCε	Protein kinase C epsilon
PND	Postnatal day
RNS	Reactive nitrogen species
SLC2A1	Solute carrier family 2 member 1

SLC2A3	Solute carrier family 2 member 3
SLC38A1	Solute carrier family 38 member 1
SLC38A2	Solute carrier family 38 member 1
S-Pathway	Succinate-Pathway
SEM	Standard error of mean
sFlt-1	Soluble fms-like tyrosine kinase 1
SIRT1	Sirtuin 1
SOD	Superoxide dismutase
TIMP3	Tissue inhibitor of metalloproteinase 3
TIMP4	Tissue inhibitor of metalloproteinase 4
TNFα	Tumor necrosis factor alpha
t RNA	Transfer RNA
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
VEGFR-1	Vascular endothelial growth factor receptor-1
VEGFR-2	Vascular endothelial growth factor receptor -2
XO	Xanthine oxidase

CHAPTER 1:

General Introduction

Part of this chapter has been published:

***Ganguly E**, Hula N, Spaans F, Cooke CLM and Davidge ST. Placenta-Targeted Treatment Strategies: An Opportunity to Impact Fetal Development and Improve Offspring Health in Later Life. Pharmacol Res 157, 104836. 2020.*

Author contributions: EG and **NH;** draft preparation and critical revision of manuscript, **FS;** figure preparation; **EG, NH, FS, CLC** and **STD**; critical revision of manuscript.

1.1 Fetal Programming of Chronic Adult Diseases

The developmental origin of health and disease (DOHaD) theory is based on epidemiological evidence that a compromised prenatal and early postnatal environment leads to increased risk of long-term development of adult disease [reviewed in (1-4)]. Since the publication of a landmark study by Dr. David Barker demonstrating a correlation between low birthweight and subsequent death from cardiovascular disease later in life (5), several epidemiological studies and experimental animal studies have led to the emergence of an ever-expanding body of literature in support of the DOHaD hypothesis. A large body of evidence now available regarding the role of fetal programming of long-term health suggests that an impoverished *in utero* environment leads to an increased risk of cardiovascular disease later in life [reviewed in (6)]. Importantly, cardiovascular diseases are the leading cause of mortality worldwide and claim over 17 million lives per year (7, 8). Cardiovascular diseases produce immense health and economic burdens globally and despite extensive public policy efforts in implementing behavioral, lifestyle and pharmacologic interventions, cardiovascular diseases continue to be one of the most resilient health problems in modern society. Considering the major socio-economic burden of cardiovascular disease, it is clear that prenatal programming of chronic diseases has the potential to greatly impact the health of our (future) population.

The underlying etiology of fetal programming of chronic diseases is multi-factorial and involves complex mechanisms, including (but not limited to) prenatal hypoxia, malnutrition, environmental exposures, and inflammation [reviewed in (3, 9, 10)]. Of all these factors, hypoxia in pregnancy is associated with placental dysfunction (such as fetal growth restriction; FGR), which can itself, as a single factor, lead to fetal programming of chronic diseases (such as adult cardiovascular disease) (2). Moreover, chronic fetal hypoxia associated with ‘placental

insufficiency' (i.e. inadequate placental supply of nutrient and oxygen to the fetus) is one of the most common consequences of pregnancy complications; known to be associated with alterations in fetal cardiac development and cardiovascular dysfunction in adult life (4, 11).

1.2 Hypoxia in Pregnancy

Hypoxia in pregnancy is associated with a variety of maternal, placental and fetal conditions that may have different pregnancy outcomes (12). According to Kingdom and Kaufmann, hypoxia in pregnancy can be classified into three subtypes based on its origin (13): 1) *preplacental hypoxia:* Both the mother and her fetus is hypoxic. Preplacental hypoxia usually results from maternal exposure to low oxygen levels and/or pre-existing maternal respiratory and cardiovascular diseases. Maternal causes of hypoxia include environmental factors (such as high-altitude and smoking) and/or cardiovascular factors (such as chronic maternal hypertension, heart failure and cyanotic congenital heart disease) (12); 2) *uteroplacental hypoxia:* maternal oxygenation is normal but uteroplacental and fetoplacental circulation is hypoxic due to abnormal (impaired) placentation (such as in preeclampsia and FGR). Placental hypoxia may result from inadequate oxygen delivery, abnormal trophoblast invasion, impaired uterine artery remodeling, and/or placental insufficiency (14); and 3) *postplacental hypoxia:* only the fetus is hypoxic. Regardless of the cause, the consequences of placental hypoxia are impaired placental morphology and function and, ultimately chronic fetal hypoxia; which have been linked to impaired fetal organ development and increased risk of developing cardiovascular and metabolic diseases in adult offspring born from complicated pregnancies.

Given that placental hypoxia is central to the pathophysiology of pregnancy complications

leading to placental insufficiency and fetal hypoxia, research in this thesis focuses on placental hypoxia. Since the fetus has limited reserves *in utero* to compensate for a reduced oxygen supply (11), prenatal hypoxia therefore is considered as one of the most common insults in pregnancy complications that can impact fetal development. Studies have extensively utilized pregnant animals (such as sheep, rodents or guinea pigs) subjected to acute and/or chronic hypoxic conditions using gas-regulated chambers (wherein the partial fraction of oxygen inspired by the dam is controlled), as a tool to elucidate the effects of prenatal hypoxia on fetal development and long-term offspring health (15-17). Although animal models of prenatal hypoxia do not completely represent the clinical pathophysiology of pregnancy complications associated with placental insufficiency, advantages include that the level of oxygen, duration and timing of the hypoxic insult can be manipulated (15, 16, 18).

Overall, placental hypoxia is one of the most common factors in pregnancy complications associated with placental dysfunction and poor fetal and offspring health outcomes (19). Therefore, to better understand the effects of placental hypoxia on fetal development, it is essential to understand normal development and function of the placenta.

1.3 Development of the Placenta

Pregnancy is a complex physiological condition that involves a number of systemic adaptive changes in the mother to ensure an adequate supply of nutrients and oxygen essential for optimal fetal growth. Normal pregnancy is characterized by an increase in total maternal blood volume by about 40% with concomitant increases in maternal cardiac output by 30-35% at late gestation (20,

21). In humans, and in experimental animals such as sheep and rats, total uteroplacental blood flow increases almost 17-fold above the non-pregnant level, with over 90% of blood flow being directed to the placenta by late gestation (21, 22). The maternal uterine arteries undergo extensive uteroplacental vasculature remodeling and enhanced vasodilation (i.e. functional changes in uterine artery reactivity), which facilitates increased maternal blood flow to the placenta, and transplacental transfer of nutrients and oxygen to the developing fetus (20). The placenta serves as the primary interface between the maternal and fetal circulations, and provides the developing fetus with sufficient nutrients and oxygen (23). Furthermore, the placenta secretes hormones and growth factors that modulate maternal physiology and metabolism in order to support fetal growth (24).

The placenta is a composite organ of two distinct cell lineages that arise from the fertilized embryo early during development (25, 26). One of the first two cell lineages within the blastocyst (pre-implantation embryo) is the trophoectoderm (outer cellular layer of the blastocyst) (27). The trophoectoderm differentiates into trophoblast cells, which, in turn, forms the epithelial covering of the placenta (27). The other earliest lineage is the inner cell mass, which forms the embryo proper (28). The vascular network of the placenta is derived from the extra-embryonic allantoic mesoderm (formed from inner cell mass lineage) (29). Apart from the establishment of embryo-derived trophoblast cell lineage that forms the placenta, a key feature of placental development is the transformation of maternal endometrial tissue into decidual tissue, which forms the maternal portion of the mature placenta. The differentiation of endometrial stromal cells into decidual cells is initiated upon implantation of the blastocyst into the maternal endometrium. This process in commonly referred to as decidualization and is essential to support normal placentation and embryonic growth.

1.3.1 The chorioallantoic placenta

The chorioallantoic placenta is found in all eutherian mammals (30). It is formed from the maternal endometrium, the trophoectoderm and the extra-embryonic mesoderm (31). This type of placentation is generally classified into three main placenta types, based on the "invasiveness of fetal tissues and their access to maternal blood flow" as proposed by Grosser et al (Figure 1.1) (32). These include: (i) Epitheliochorial placentation is the least invasive form lacking significant invasion of the uterine tissues. In this form of placentation, the fetal tissues are separated from the maternal blood by the maternal uterine epithelium and maternal vascular endothelium (Figure 1.1A); (ii) Endotheliochorial placentation is partially invasive, wherein the fetal tissues are separated from the maternal blood by only the endothelial wall of the maternal blood vessels (Figure 1.1B); and (iii) Haemochorial placentation is the most invasive form and found in many mammalian species, such as humans, nonhuman primates and rodents (e.g. rats and mice). In this form of placentation, fetal tissues are in direct contact with the maternal blood (Figure 1.1C) (32). The next section focuses on the development and structure of the haemochorial placenta found in rats, in comparison to that in humans.

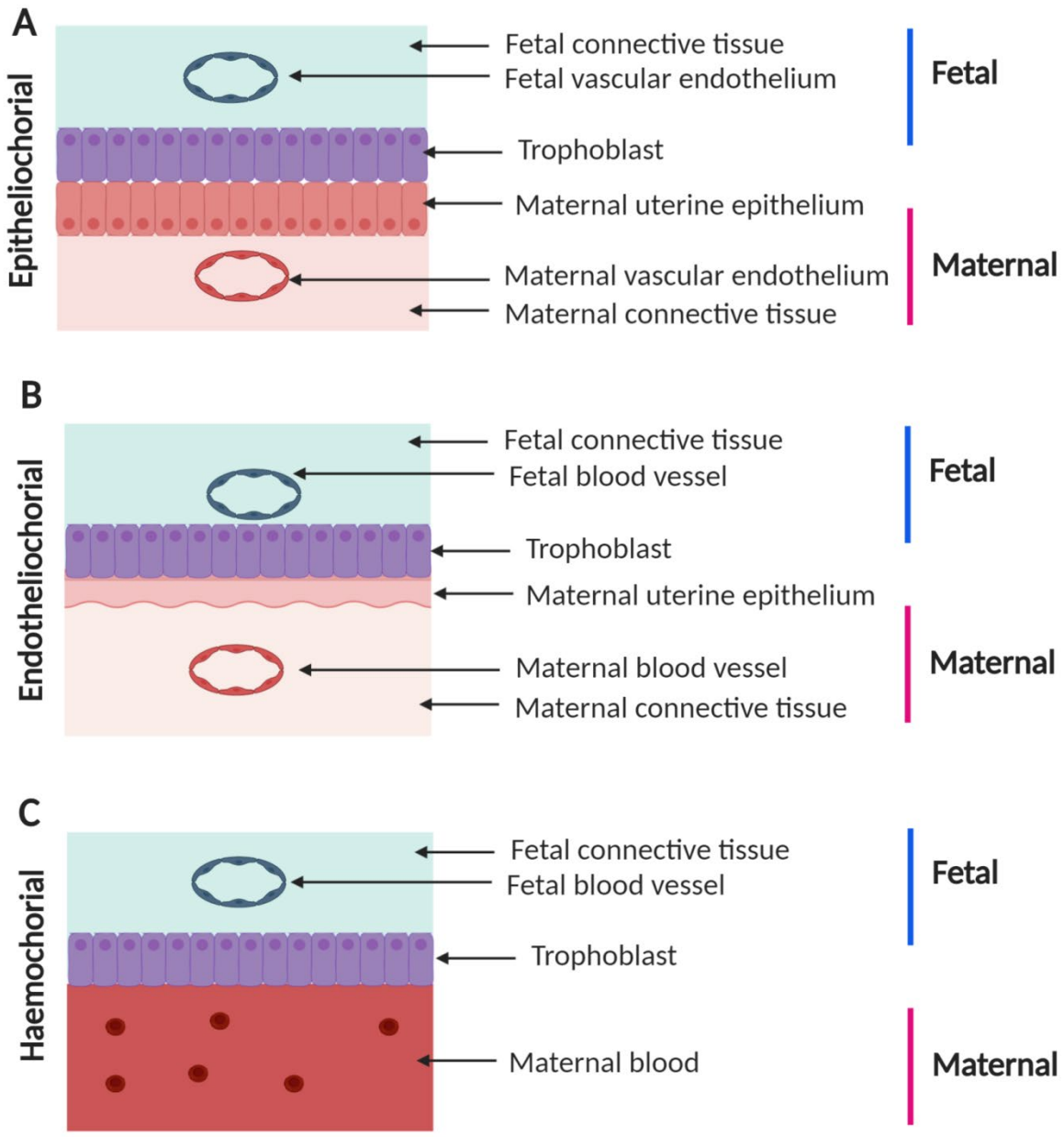

Figure 1.1 Types of placentation

A: Epitheliochorial placentation is the least invasive having three layers of maternal tissue separating the fetus from the maternal blood. **B:** Endotheliochorial placentation is partially invasive and only the maternal endothelium separates the fetus from the maternal blood. **C:** Haemochorial placentation is the most invasive type in which fetal tissues are in direct contact with maternal blood. Designed by Esha Ganguly. Created with BioRender.com

1.3.2 The haemochorial rat placenta

Around the time of implantation (gestational day [GD] 4.5), the trophoectoderm cells proliferate and give rise to the extraembryonic ectoderm of the early post implantation conceptus (Figure 1.2) (33). The haemochorial rat placenta develops from the trophoblast cells arising from the trophoectoderm and is established approximately at GD 12 (term=22 days) (33, 34). As placental development proceeds, the trophoectoderm expands to form the ectoplacental cone and the chorionic trophoblasts, which subsequently forms the junctional zone and labyrinth zone, respectively (Figure 1.2) (33, 35).

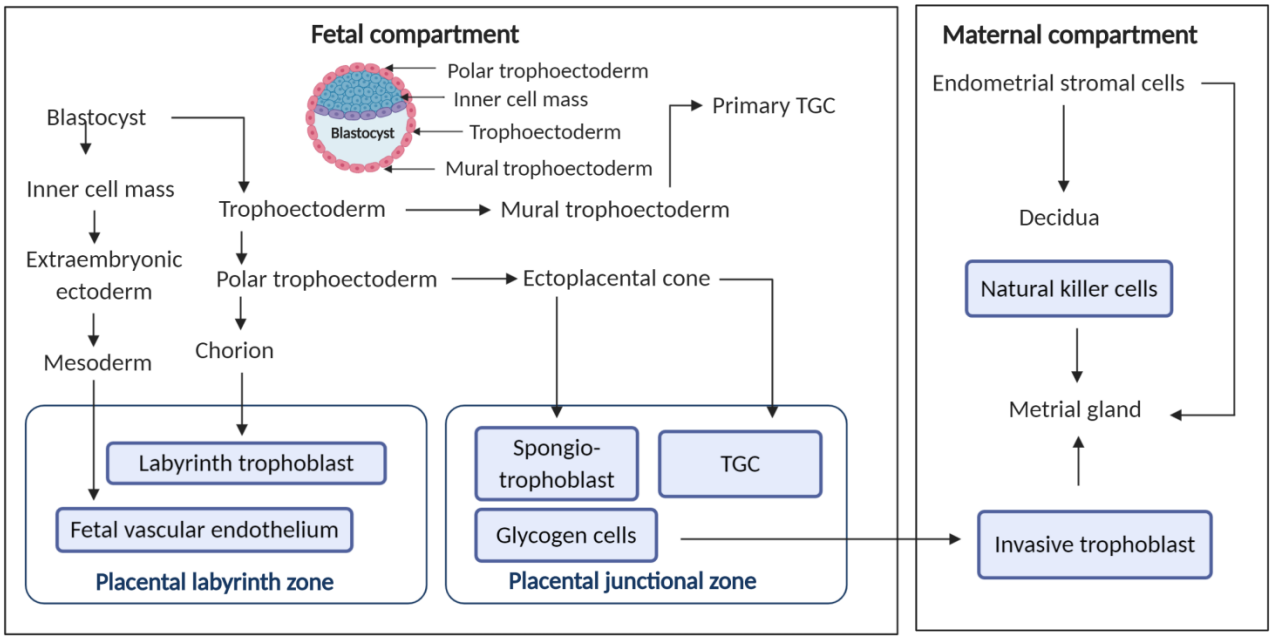

Figure 1.2 Structural components of the haemotrichorial rat placenta
The trophoectoderm cells within the blastocyst differentiate into trophoblast cells, which form the epithelial covering of the placenta. The vascular endothelium of the placenta is derived from the extra-embryonic mesoderm. The ectoplacental cone is the source of trophoblast cells comprising the junctional zone. The junctional zone is composed of spongiotrophoblast, trophoblast giant cells (TGCs), glycogen cells, and invasive trophoblast cells. The invasive trophoblast cells penetrate into the rat mesometrial uterus and maternal decidua. The chorionic trophoblasts along with the fetal vascular endothelium form the labyrinth zone. The labyrinth zone forms the interhaemal membrane or exchange surface of the rat placenta. Designed by Esha Ganguly. Created with BioRender.com

1.3.2.1 The junctional zone

The ectoplacental cone is the source of trophoblast cells comprising the junctional zone (33). The junctional zone separates the labyrinth zone from the maternal decidua and is comprised of four differentiated cell types, including a layer of trophoblast giant cells, spongiotrophoblast cells, glycogen cells, and invasive trophoblast cells (Figure 1.3) (33). The trophoblast giant cells are located at the maternal-placental interface and border the mesometrial decidua. The trophoblast giant cells are one of the major endocrine cells of the placenta. They synthesize and secrete steroid and peptide hormones that regulate flow of maternal blood to the implantation site, ovarian progesterone synthesis and lactogenesis. The trophoblast giant cells also produce luteotropic and lactogenic hormones during pregnancy. The spongiotrophoblast cells are located beneath the trophoblast giant cell layer and act as the endocrine compartment that maintains secretion of progesterone from the corpus luteum (36). The glycogen cells are essential for normal function of the placenta and are believed to provide nutrients to the placenta and/or the fetus because of their glycogen content (36). Being the main endocrine compartment of the placenta, the junctional zone produces several hormones, growth factors and cytokines that play a key role in regulating maternal physiology for maintenance of normal pregnancy (Figure 1.3) (33).

The junctional zone of the rodent placenta is considered “operationally analogous” to the extravillous trophoblast column of the human placenta (Figure 1.4) (26, 37). The human placenta can be divided into two major functionally distinct compartments: (i) villous; and (ii) extravillous. The progenitor cytotrophoblast cells, in humans, give rise to the invasive trophoblast lineage (37). Extravillous trophoblast cells are primarily the invasive cell types which are responsible for the invasion and remodeling of the maternal spiral arteries (37). Similarly, in rats, invasive trophoblast cells arise from the junctional zone (38). The invasive trophoblast cells penetrate into the rat

mesometrial uterus through the decidua using two routes that include: i) interstitial invasion (trophoblasts migrate between spiral arteries); and ii) endovascular invasion (trophoblasts migrate within the spiral arteries, replacing the endothelium) (39, 40). Both rat and human placentae exhibit deep trophoblast invasion into the decidua. On the contrary, in mice, trophoblastic invasion is very limited and confined to the decidua (34).

1.3.2.2 The labyrinth zone

The labyrinth zone is involved in the exchange of gases, nutrients and waste products between maternal and fetal circulations and plays a critical role to sustain normal fetal growth and development (41). The labyrinth zone is composed of trophoblast giant cells and two syncytial trophoblast cell layers formed via fusion of the trophoblast progenitor cells (Figure 1.3) (29). The "interhaemal membrane" or exchange surface of the rat placenta, from the maternal to the fetal side, is composed of three continuous cell layers. These include one cytotrophoblast layer in contact with the maternal blood, and two syncytial cell layers in contact with fetal vascular endothelium (Figure 1.3) (41). The labyrinth zone is involved in the transport of gases, nutrients and waste products between maternal-fetal circulations and plays a critical role to sustain normal fetal growth and development (41). The analogous structure to the rat labyrinth layer is the villous compartment of the human placenta (Figure 1.4). The chorionic villi in the human placenta are formed of connective tissue that contains fetal mesenchymal cells and blood vessels, and are surrounded by an underlying layer of cytotrophoblast and the outer syncytiotrophoblast (41). The syncytiotrophoblast is a multinucleate cell that forms the surface of the villous placenta, and is in direct contact with the maternal blood. Thus, the "interhaemal membrane" of the human placenta comprises of a syncytiotrophoblast layer, an underlying cytotrophoblast layer and the fetal vascular endothelium (41). However, the number of layers of trophoblast cells between the maternal blood and the fetal

capillaries in the rodent placenta is greater than in the human placenta.

The placenta performs various functions, such as transfer of nutrient and oxygen to the fetus while eliminating waste products, secretion of hormones which are important for fetal growth, and also acts as a selective barrier (42). As pregnancy complications commonly associated with placental dysfunction lead to poor pregnancy outcomes, the main functions performed by the placenta, especially those functions that have been shown to affect fetal and offspring health outcomes will be discussed in the section below.

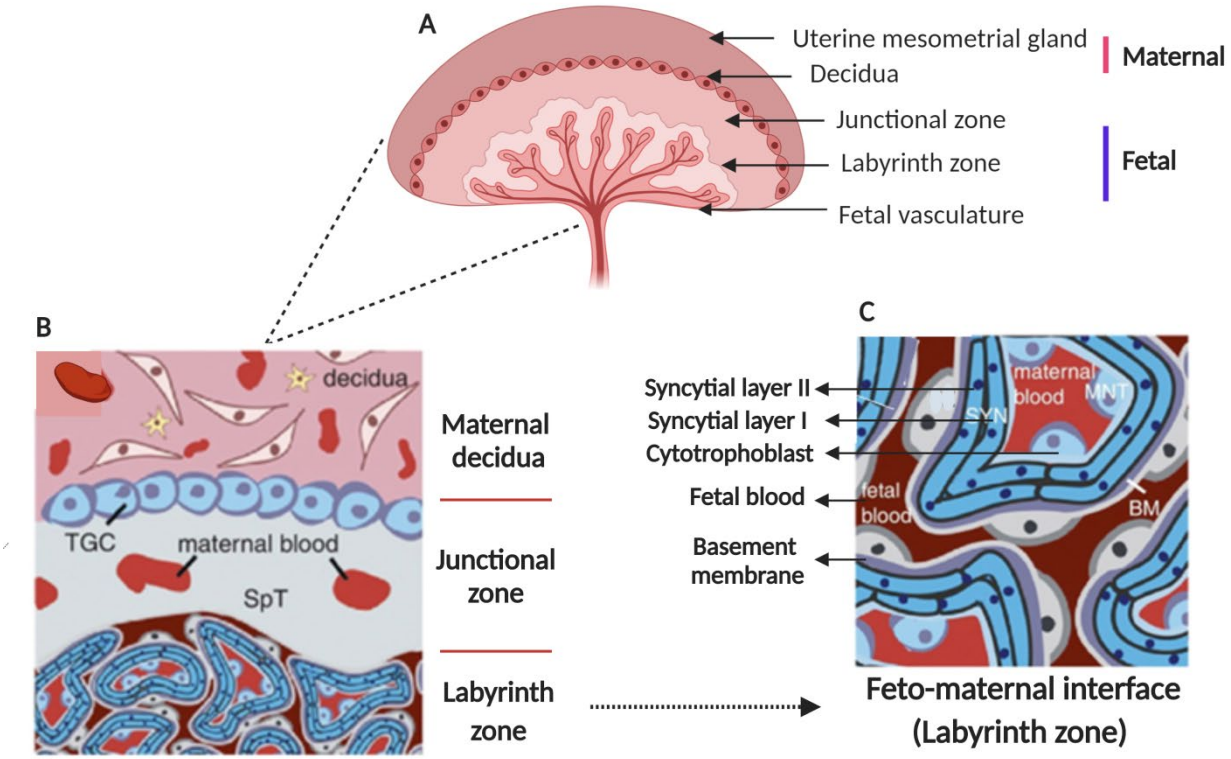

Figure 1.3: Structure of the rat placenta

A: An illustration of the ultrastructure of the rat placenta. **B:** Schematic diagram of the mature chorioallantoic placenta**.** The junctional zone separates the maternal decidua from the labyrinth zone and is comprised of trophoblast giant cells (TGC), spongiotrophoblast (SpT) and the glycogen cells. The labyrinth zone is composed of one cytotrophoblast layer in contact with the maternal blood, and two syncytial layers in contact with fetal vascular endothelium. **C.** Schematic diagram of the rat haemotrichorial labyrinth zone also known as the maternal-fetal exchange surface of the rat placenta. Adapted from Robbins *et al*. (43).

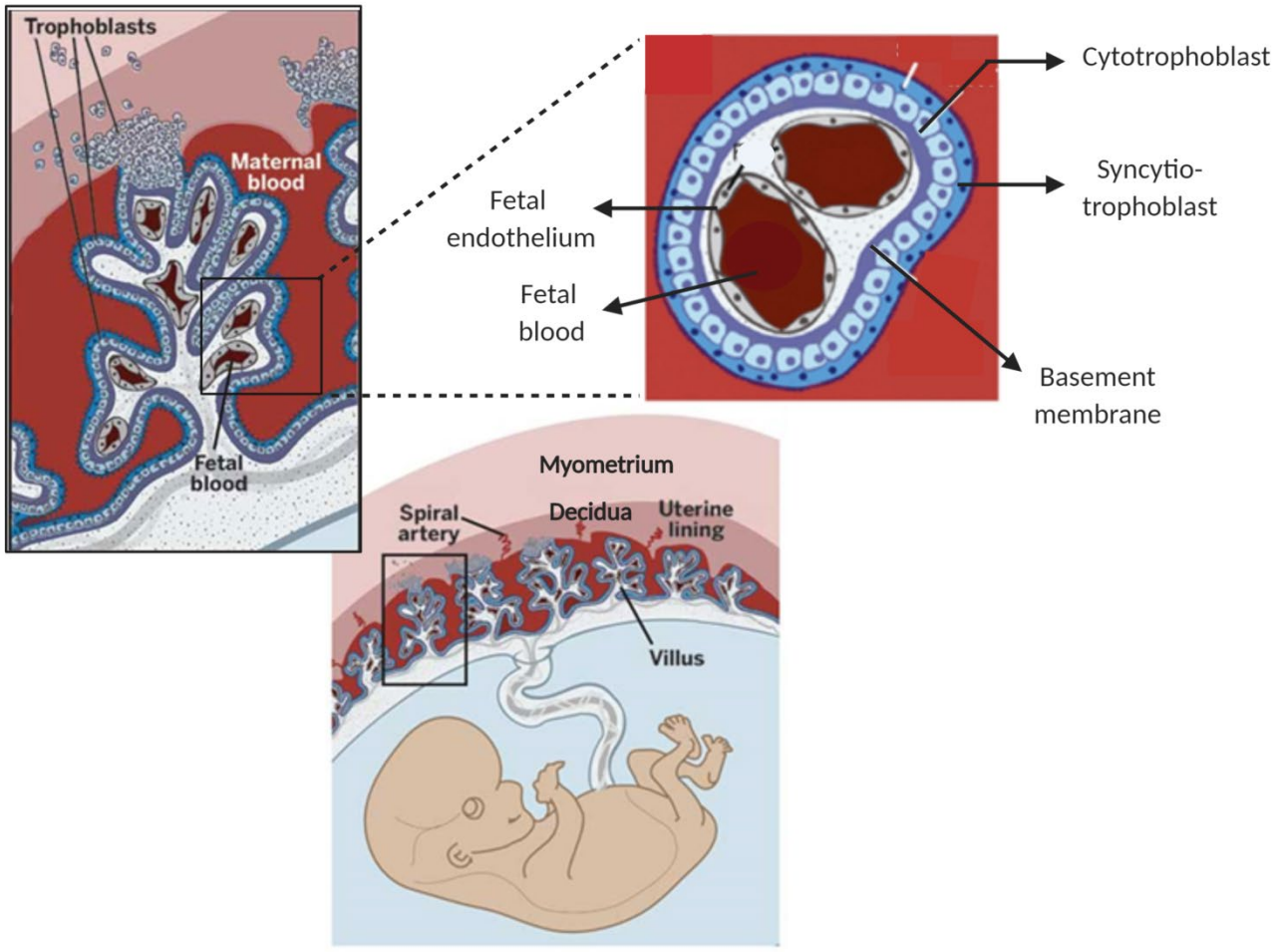

Figure 1.4: Structure of the human placenta
A: Schematic diagram of the chorionic villi in the human placenta that contains fetal mesenchymal cells and blood vessels, and are surrounded by an underlying layer of cytotrophoblast and the outer syncytiotrophoblast **B.** Schematic diagram of the human haemomonochorial maternal-fetal exchange surface of the human placenta comprising of a syncytiotrophoblast layer, an underlying cytotrophoblast layer and the fetal vascular endothelium. Adapted from Robbins *et al*. (43).

1.4 Placental Function

1.4.1 Placental transport of oxygen

Oxygen transfer across the placenta takes place via simple diffusion. Simple diffusion is defined as the passage of small uncharged molecules through lipid bilayers of the cell membrane (42). This is a passive process and is not dependent on adenosine triphosphate (ATP) production (42). Oxygen transfer to the fetus mainly depends on the oxygen partial pressure gradients between maternal and fetal blood circulations (42, 44). Hence, exchange of oxygen is considered to be "flow-limited", and changes in the uteroplacental and fetoplacental blood flow can directly influence the net amount of oxygen molecules transferred (45). Oxygen transfer to the fetus is also proportional to the maternal-fetal surface area for exchange and inversely proportional to the placental membrane thickness (42). Therefore, exchange of these molecules is "membrane- or diffusion-limited", and placental morphology (such as barrier thickness and surface area) plays a major role in the transfer of oxygen.

1.4.2 Placental transport of glucose and amino acids

Glucose is a primary energy source for the fetus and is transferred from the maternal blood to the fetoplacental circulation because of low fetal gluconeogenesis *in utero* (46). In order to meet fetal demands, transplacental transport of glucose takes place via facilitated diffusion through glucose transporters (GLUTs) (47, 48). In the rat placenta, both GLUT1 and GLUT3 are localized on the apical surface of syncytial layer I facing the maternal circulation, whereas only GLUT1 was detected in the basal membrane of syncytial layer II facing the fetal side, suggesting that these two layers function as a unit to transport glucose (46, 49). Net glucose transfer to the fetus depends on its concentration gradient between maternal-fetal circulations, the placenta's

own metabolic requirements, and expression and/or activity of GLUT transporters.

The placenta mediates net transfer of amino acids essential for protein synthesis and metabolic processes by the fetus. The active transport of amino acid across the placenta to the fetus via a range of amino acid transporters requires energy to mediate the transfer (50-52). Overall, the activity of amino acid transporters is determined by factors that regulate amino acid concentrations in both the maternal-fetal circulations and within the placenta.

1.4.3 Endocrine functions of the placenta

The placenta secretes a wide range of hormones that play a key role in maternal adaptations in order to support fetal growth. The chorionic gonadotropin secreted from the placental trophoblasts, maintains the corpus luteum and continued secretion of ovarian progesterone and estrogens (24). Chorionic gonadotropin also acts in an autocrine and paracrine manner at the maternal-fetal interface. For instance, chorionic gonadotropin promotes angiogenic vascular growth factor (VEGF) secretion by trophoblasts (53) and can suppress the maternal immune system from "mounting a response against the allogeneic fetus" (54).

In rodents, the corpus luteum is the primary source of circulating steroid hormones, such as estrogens and progesterone, while in humans the placenta serves as the primary source of steroid hormones (55). Placental steroid hormones, in women and rodents, help to reduce maternal insulin sensitivity during pregnancy (56). The later stage of pregnancy is marked by a state of insulin resistance in the peripheral maternal tissues, which is mediated in part by the placental growth hormone (57). The placental growth hormone further stimulates the secretion of insulin-like growth factor-1 (IGF-1) by the maternal liver [reviewed in (58)]. Maternal IGF-1 in turn stimulates placental cell proliferation and increases placental supply of glucose and amino acids to the fetus

(59). Other placental hormones include progestins (supports the endometrium and prevents contractility of uterine smooth muscles) and relaxins (works with progesterone to maintain pregnancy) [reviewed in (24)].

A number of factors are known to affect placental structure and function. As the placenta lacks autonomic and cholinergic innervation, placental morphology and function is dependent on locally derived factors (60). Therefore, a few of many other important factors implicated to impair placental structure and functions in pregnancy complications are discussed below.

1.5 Factors Affecting Placental Morphology and Function in Normal Pregnancy

1.5.1 Oxygen

Oxygen levels are an important factor that regulates placental development and function throughout pregnancy. The development of the embryo begins in a relatively low-oxygen environment (i.e. partial pressure of oxygen [PO_2] < 20 mm Hg, which is equivalent to oxygen concentration of around 1-3%) (31). The 'physiological hypoxia' during the first trimester in humans is essential for organogenesis in the embryo. At this stage, the fetus is highly sensitive to reactive oxygen species (ROS) because it possesses low antioxidant capacity, and therefore hypoxia during first trimester protects the developing fetus against the teratogenic effects of ROS (61). The low oxygen levels also have beneficial effects on early placental development. The hypoxic environment leads to increased cytotrophoblast proliferation (62). Caniggia *et al.*, demonstrated that first trimester human placental explants maintained at 3% oxygen *in vitro* increased extravillous cytotrophoblast proliferation, but not invasion, compared to controls (63). The low oxygen

environment further promotes placental vasculogenesis and angiogenesis through the expression of growth factors, such as VEGF, placental growth factor (PGF) and endothelial nitric oxide synthase (eNOS), all of which are activated by hypoxia-inducible factor-1α (HIF-1α; a transcription factor and master regulator of cellular responses to low oxygen levels) (62, 64). In addition to HIF-1α, another HIFα subunit, including hypoxia-inducible factor-2α (HIF-2α), have been shown to positively regulate the hypoxic response (65). Placentae from HIF-2α knockout embryos exhibit impaired placental vascularization, poor decidual invasion, and defective labyrinth zone formation (65). However, chronic activation of HIF-2α expression in hypoxic placentae from FGR and preeclampsia was associated with hypoxia-induced increased production of soluble Fms-like tyrosine kinase-1 (sFlt-1) in trophoblasts, which could reduce bioavailability of the pro-angiogenic factor, VEGF (66). This suggests that although the HIFα subunits are essential for placental development, sustained expression of HIFα subunits in hypoxic placentae have been associated with complications of pregnancy.

At the end of first trimester, the concentration of intraplacental oxygen increases approximately 3-fold (i.e. oxygen concentration of around 6-8%), which, in turn, has been associated with increased generation of placental ROS (62). During normal pregnancy, the placenta adapts to these physiological ROS levels through increased expression and/or activity of several key antioxidant enzymes (such as superoxide dismutase, SOD) (67). Physiological levels of ROS serve as a signaling molecule that drives trophoblast differentiation and invasion *in vivo*, and are considered beneficial for normal placental development, and ultimately optimal fetal growth (68). The increased placental oxygen concentrations in the second and third trimesters in humans are essential for placental protein synthesis and active transport of nutrients required to support the rapid growth of the fetus during late gestation (69). Given that oxygen levels are central to cellular

metabolism and regulate placental development and functions, chronic hypoxia during late gestation leading to placental oxidative stress has been implicated to play a key role in the etiology of pregnancy complications, such as FGR (70, 71).

1.5.2 Nitric oxide

During early pregnancy, nitric oxide (NO), a potent vasodilator plays an important role in regulating embryo development and implantation, and promoting trophoblast invasion (72-74). During later stages of pregnancy, NO not only maintains placental vascular resistance but also mediates placental vascular development and morphology, and ultimately placental function (64). During the last half of gestation exponential increase in fetal growth is primarily dependent on transplacental exchange of oxygen and nutrients between maternal-fetal circulations; therefore, establishment of a functional fetoplacental circulation is essential for healthy pregnancy outcomes (75). The rate of transplacental exchange of nutrients depends primarily on uterine and umbilical blood flow velocities (64). The blood flow velocities, in turn, are dependent on enhanced vasodilation of the maternal uterine and uteroplacental vessels, and placental vascular growth and morphology (75).

Placental vasculogenesis (formation of first blood vessels) and subsequent angiogenesis (development of new vessels from already existing vessels) are of pivotal importance in maintenance of placental morphology and function, and it is imperative that they are appropriately regulated (76). NO has also been implicated to play a key role in the development of the feto-placental vascular network via increased expression of the pro-angiogenic, VEGFA (77, 78). The feto-placental circulation lacks both cholinergic and autonomic innervation; therefore, placental vascular development and function are dependent on angiogenesis which is mediated in part by

locally derived vasoactive factors such as NO and VEGFA (60, 79, 80). During normal pregnancy, NO derived from the eNOS in the endothelium of the fetoplacental vasculature is crucial for maintenance of low fetoplacental vascular resistance by promoting vasculogenesis and angiogenesis (81). Indeed, in human placental cotyledons and placental arteries, NO contributes to maintenance of low vascular resistance in the placental circulation and promotes angiogenesis (82, 83). In contrast, decreased NO (via eNOS inhibition and/or deletion) has been associated with impaired angiogenesis and increased feto-placental vascular resistance (84, 85). In human placental tissues, the eNOS isoform is predominantly expressed in syncytiotrophoblast and endothelial cells [reviewed in (86)], while inducible NOS (iNOS) isoform is expressed in the placental smooth muscle cells, syncytiotrophoblasts, extravillous trophoblasts, and hofbauer cells of the villous stroma [reviewed in (80)]. Studies suggest that pregnancy complications associated with chronic fetal hypoxia are marked by increased placental vascular resistance and reduced uterine artery blood flow, which in turn, was shown to be associated with decreased NO (via eNOS inhibition) and inadequate feto-placental vascularization (87, 88). Additionally, increased iNOS expression in the placental endothelium from FGR pregnancies shows a positive correlation with increased umbilical pulsatility index and vascular dysfunction determined *in vivo* (88). Furthermore, human umbilical vein endothelial cells (HUVEC) exposed to oxidative stress down-regulate eNOS and up-regulate iNOS expression, which may lead to nitrosative stress and cell apoptosis (89). Thus, increased NO levels are not necessarily associated with improved placental endothelial function.

1.5.3 Growth factors

As mentioned above, placental morphology and function is dependent on locally-derived growth factors. Two growth factors that play a key role in placental angiogenesis and

morphogenesis are 1) pro-angiogenic factor, VEGF; and 2) growth factor, insulin-like growth factor-2 (IGF-2).

The VEGF family consists of VEGFA, VEGFB, VEGFC, VEGFD and PGF. Of these, VEGFA (also referred to as VEGF) is a potent pro-angiogenic factor that is normally expressed at high levels in the human placenta and regulates angiogenesis by binding to two tyrosine kinase receptors, VEGFR-1/Flt-1 and VEGFR-2/KDR, present on endothelial cells (76). The *Vegf* gene (in humans and rodents) is encoded from eight exons with alternate splicing of the sixth and seventh exons, resulting in five different isoforms of VEGF (such as $VEGF_{120}$, $VEGF_{144}$, $VEGF_{164}$, $VEGF_{188}$, $VEGF_{205}$ in rodents), each with distinct number of amino acids (90). $VEGF_{120}$ (known as $VEGF_{121}$ in humans) is a freely secreted isoform and acts as an endothelial cell mitogen (90). Other isoforms, such as $VEGF_{164}$ (known as $VEGF_{165}$ in humans) and $VEGF_{188}$ (known as $VEGF_{189}$ in humans), have limited bioavailability due to their sequestration within the extracellular matrix (90).

Expression of VEGF is correlated to increased number of fetoplacental capillaries at the placental interface in humans, increased placental vascularization in ewes, and an increase in vascularization of the placental labyrinth zone in rodents, suggesting that VEGF-VEGFR system is involved in placental angiogenesis that may promote transplacental transfer of nutrients essential for optimal fetal development (76). Studies suggest that VEGF-induced angiogenesis requires NO, suggesting that various local factors interact with each other and can affect transplacental oxygen diffusion in the placenta (91, 92). Additionally, NO has been shown to regulate expression of pro-angiogenic VEGF in the rat placenta (93). Placental dysfunction in pregnancies complicated by placental hypoxia has been linked with reduced expression of VEGFA and impaired fetoplacental vascularization (80, 94). In rodents, deletion and/or decreased expression of *Vegf* is associated with decreased labyrinth region vascularization, causing FGR due to placental insufficiency.

Insulin-like growth factors (particularly IGF-2) play a key role in maternal-fetal resource allocation via regulation of placental morphology. IGF-2 is expressed at high levels by the placenta in all species studied till date (such as rodents, guinea pigs and humans) (95). IGF-2 can bind to a variety of receptors, such as insulin-like receptors (IGF1R and IGF2R), insulin receptor (INSR), and a hybrid IFG1R-INSR, but with varying affinity (95). The main actions of IGF-2 are mediated via the IGF1R, which activates the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways (95). Genetic manipulations of *Igf2*, *Igf2r*, and the downstream signaling pathways are known to affect placental development and its capacity to modulate supply of nutrients to the fetus (95). For example, *Igf2* overexpression causes placental and fetal overgrowth, while genetic deletion of total *Igf2* in the placenta and reduced fetal and placental weights (96, 97). The reductions in fetal and placental weights in response to reduced total *Igf2* relate to alterations in placental labyrinth zone formation (such as surface area, labyrinth zone thickness/volume, and vascularization, all of which can regulate transplacental oxygen and/or nutrient delivery to the fetus). In addition, passive permeability of the placenta to hydrophilic nutrients and transplacental efficiency is reduced in complete *Igf2* knockout mice. For example, the *Igf2* null placenta has been shown to transport less neutral amino acids via the System A transporters (96, 98). In addition to total *Igf2*, placenta-specific transcript of IGF2, *Igf2P0*, has been shown to play an important role in placental morphological adaptation (95). For instance, in mice lacking the *Igf2P0* transcript, the volume of the exchange labyrinth region was reduced in proportion to the junctional zone (99). Furthermore, deletion of *Igf2P0* transcript in mice led to reduce fetal and placental growth (98). Thus, by regulating the placental morphological phenotype IGF-2 plays an important role in regulation of placental supply of nutrients to the fetus. Moreover, IGF-2 has been shown to be altered in response to adverse intrauterine environments (such as hypoxia) and decreased placental

IGF-2 is associated with FGR (95). Studies suggest that alterations to IGF-2 are in line with changes in placental morphology and function, thus linking maternal intrauterine environments to placental supply of substrates (nutrients and oxygen) and fetal development (100, 101). Maternal exposure to 10-12% O_2 during late gestation in mice led to decreased *Igf2* expression, which was associated with reductions in placental labyrinth zone volume, reduced maternal blood space area and exchange surface area, all of which could limit supply of oxygen and nutrients (glucose and amino acids) to the fetus, and ultimately result in FGR (100, 101).

Taken together, all the above factors that play a key role in placental morphology and function have been shown to be altered in response to hypoxia in pregnancy. In addition to all the above-mentioned factors, the placenta also requires energy for its own growth, metabolism and transport functions. Mitochondria are the main site for ATP and concomitant physiological levels of ROS generation in the placenta (70). Therefore, mitochondrial function under normal physiological conditions and factors controlling mitochondrial respiratory capacity will be discussed in the section below.

1.5.4 The Mitochondria

Mitochondria are double-membrane bound organelles that are essential for numerous intracellular processes such as regulation of cellular metabolism, including amino acids, steroid and cholesterol biosynthesis, iron-sulphur cluster biogenesis, and, most importantly, the production of ATP (an energy-carrying molecule used by cells and tissues for performing biological functions) (102). The mitochondria consume oxygen supplied by cellular respiration to generate ATP across a proton gradient via oxidative phosphorylation (OXPHOS) (103). Mitochondria possess their own genome (known as mitochondrial DNA, mtDNA) that encodes 13 key protein subunits of the

OXPHOS system, while the remaining OXPHOS subunits and mitochondrial proteins are encoded by the nuclear DNA (nDNA). The mtDNA thus works in concert with the nDNA to maintain mitochondrial structural and functional integrity. Mitochondria act as a "signaling system" and regulate cell fate, cellular differentiation and apoptosis through the control of cellular redox homeostasis and ROS production. Mitochondria also serve as "oxygen sensors". ROS production by mitochondrial OXPHOS may vary in response to changes in oxygen availability (such as ROS levels are increased in response to low oxygen levels) [reviewed in (104, 105)].

1.5.4.1 Generation of ATP and ROS by mitochondrial OXPHOS under physiological conditions

Mitochondrial ATP generation results from oxido-reduction reactions performed by a series of multi-enzymatic respiratory complexes, which form the functional unit of the OXPHOS system, and are embedded across the inner mitochondrial membrane [reviewed in (106, 107)]. These reactions involving high energy electron carrier molecules reduced by mitochondrial respiratory complexes are coupled to a proton gradient that is used by ATP synthase to produce ATP from adenosine diphosphate (ADP) and inorganic phosphate [reviewed in (107)].

The mitochondrial OXPHOS system is composed of four multi-enzymatic respiratory complexes (complexes I-IV) and ATP synthase (complex V) [reviewed in (106-108)] (Figure 1.5). In addition to the OXPHOS system, the Krebs cycle (also known as the citric acid cycle) is essential for mitochondrial ATP generation. The Krebs cycle occurs in the mitochondrial matrix and produces nicotinamide adenine dinucleotide (NADH) and intermediate amino acids (such as Succinate), which are further oxidized by complex I (NADH:ubiquinone oxidoreductase) and complex II (Succinate: ubiquinone oxidoreductase) respectively (Figure 1.5). Following which

electrons are transferred to complex III (ubiquinol: cytochrome *c* oxidoreductase), which reduces cytochrome *c* (a soluble electron carrier located in the intermembrane space) (Figure 1.5). Finally, electrons are transferred from cytochrome *c* to complex IV (cytochrome *c* oxidase). Complex IV transfers electrons to the terminal electron acceptor (i.e. molecular oxygen). The mitochondrial respiratory chain continuously reduces oxygen into water in the mitochondrial matrix. The flow of electrons through the OXPHOS system is linked to simultaneous efflux of protons into the intermembrane space, thus generating a proton-motive force. The proton-motive force consists of an electro-chemical trans-membrane potential across the inner mitochondrial membrane that is essential not only for ATP synthesis but also helps to sustain other mitochondrial functions (such as ion exchange and import of mitochondrial precursor proteins from the cytosol). (109). In the final step of OXPHOS, the resulting proton-motive force is used to drive ATP production by complex V (109).

Under physiological conditions, ROS are produced as a normal by-product of mitochondrial OXPHOS. For example, the one-electron reduction of oxygen during OXPHOS leads to production of superoxide (110). It has been estimated that less than 0.1% of the electrons flowing through the mitochondrial respiratory chain leak onto oxygen to form superoxide under normal conditions [reviewed in (111)] . The ROS levels within the "normal" or "physiological" levels are maintained by the intramitochondrial antioxidant defense systems [such as MnSOD; glutathione peroxidases; and catalase] [reviewed in (112)].

Mitochondrial ROS production is not only dependent on expression and/or activity of the respiratory complexes, but also depends on mitochondrial morphology. Mitochondrial morphology is regulated via continuous cycles of fusion/fission (known as mitochondrial dynamics) and mitochondrial biogenesis (113). From a physiological perspective, through dynamic changes in

their morphology, mitochondria can influence the cell's bioenergetic needs and maintain cellular redox homeostasis in responses to stress signals. Mitochondrial morphology not only affects mitochondrial function, but also controls other cellular processes, such as apoptosis. Because the dynamic morphological changes in the mitochondria are essential for the maintenance of mitochondrial function, it is important to discuss the processes of mitochondrial fusion and fission, and biogenesis.

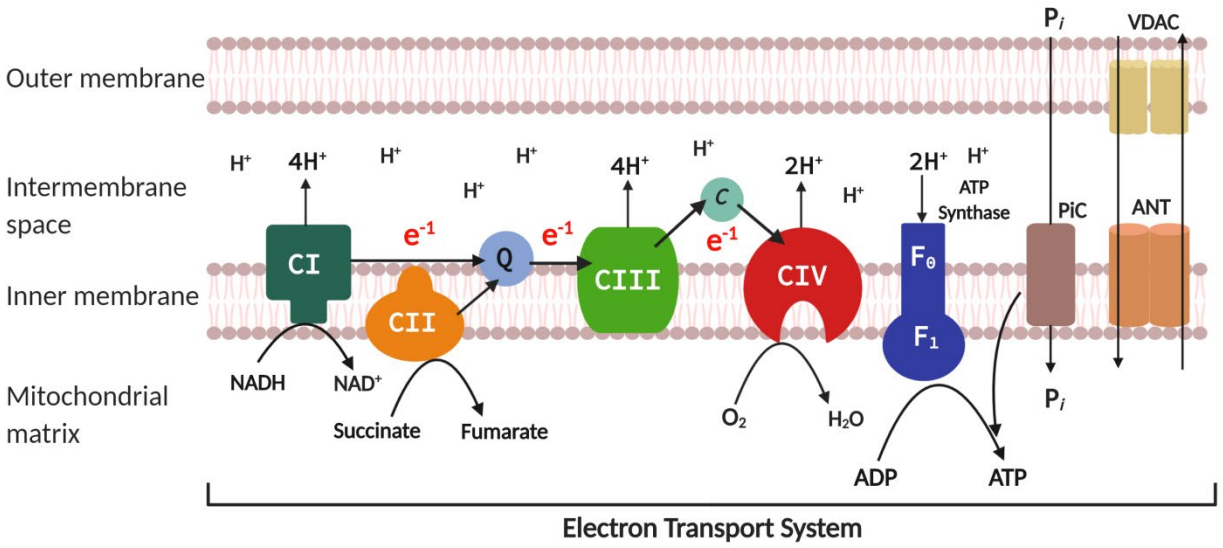

Figure 1.5 Scheme of oxidative phosphorylation in mitochondria
The electron transport system (ETS) located in the mitochondrial inner membrane includes complexes I (CI), II (CII), III (CIII), IV (CIV). The electrons enter into the ETS via CI, CII, and electron transferring flavoprotein, and glycerophosphate dehydrogenase (later two not shown). Substrates for CI provide electrons from NADH via the citric acid cycle. Succinate provides $FADH_2$ feeding electrons into CII. Electrons (e^{-1}s) from CI and CII converge at the Q-cycle before being transferred to CIII and CIV. When e^{-1}s are transferred into the ETS, protons are pumped into the intermembrane space at CI, CIII and CIV. The proton gradient is used by the phosphorylation system to produce adenine triphosphate (ATP). The phosphorylation system is composed of ATP synthase, the adenine nucleotide translocase (ANT), and the phosphate carrier (PiC). Adenine diphosphate (ADP) enters the mitochondrial outer membrane via the voltage dependent anion channel (VDAC) and the mitochondrial inner membrane through the ANT. The phosphate freely passes through the outer membrane and is transported through the inner membrane via the PiC. The ATP synthase uses the phosphate and the ADP to make ATP. Adapted from Lemieux *et al.* (114). Created with BioRender.com

1.5.4.2 Mitochondrial dynamics and biogenesis regulate mitochondrial OXPHOS and ROS production

Mitochondrial dynamics (fusion and fission) are not only required to ensure mitochondrial morphology but also play a critical role in maintaining mitochondrial function when the cells experience metabolic and environmental stresses (115). Mitochondrial morphology can range from "giant spherical" form to "hyperfused reticular" networks and primarily depends on the balance between fusion and fission (116, 117). These processes are carried out by groups of dynamin-related guanosine triphosphatase (GTPase) enzymes (such as Optic Atrophy 1 (OPA1), Mitofusin-1 (MFN1), and Mitofusin-2 (MFN2) for mitochondrial fusion and Dynamin-related protein 1 (Drp1) for mitochondrial fission) (117). Mitochondrial fusion results in the generation of hyperfused and interconnected mitochondrial network, which helps to mitigate stress-related effects and promote cell survival by mixing/exchanging mitochondrial components (such as mtDNA, metabolites and proteins) of partially damaged mitochondria with other healthy mitochondria. Conversely, mitochondrial fission favors the generation of fragmented and isolated mitochondria but is also essential for elimination of damaged mitochondrial fragments through mitophagy (autophagy of mitochondria). Therefore, the highly dynamic fusion and fission is proposed to balance two competing processes: "compensation of damage by fusion and elimination of damage by fission" (Figure 1.6). Moreover, from a functional standpoint, a pro-fusion environment (inducing mitochondrial elongation and enhanced respiratory capacity) has been associated with reduced mitochondrial ROS generation, whereas a pro-fission environment (leading to mitochondrial fragmentation and accumulation of damaged mtDNA) increased mitochondrial ROS generation (116).

The production of new mitochondria, a process known as mitochondrial biogenesis, depends

on tight co-ordination between mitochondrial fusion/fission and mitophagy (a mitochondrial quality control process that removes damaged/dysfunctional mitochondria segregated by mitochondrial fission) (118, 119). Mitochondrial biogenesis is regulated by a nuclear encoded protein family, the peroxisome proliferator-activated receptor gamma coactivator (PGC) such as PGC-1α (Figure 1.5) (120). PGC-1α acts as a co-transcriptional regulation factor that induces mitochondrial biogenesis by activating nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) (121). NRF-1 and -2 can induce the expression of mitochondrial transcription factor A (Tfam), which drives transcription and replication of mtDNA (120). Moreover, mitochondrial biogenesis can be increased and/or reduced in response to adverse stress conditions (119). Taken together, mitochondrial dynamics (fusion and fission) and biogenesis play a critical role in cellular adaptations during physiological and pathophysiological conditions because of their role in energy production and the capacity to generate signals that may promote cellular adaptations.

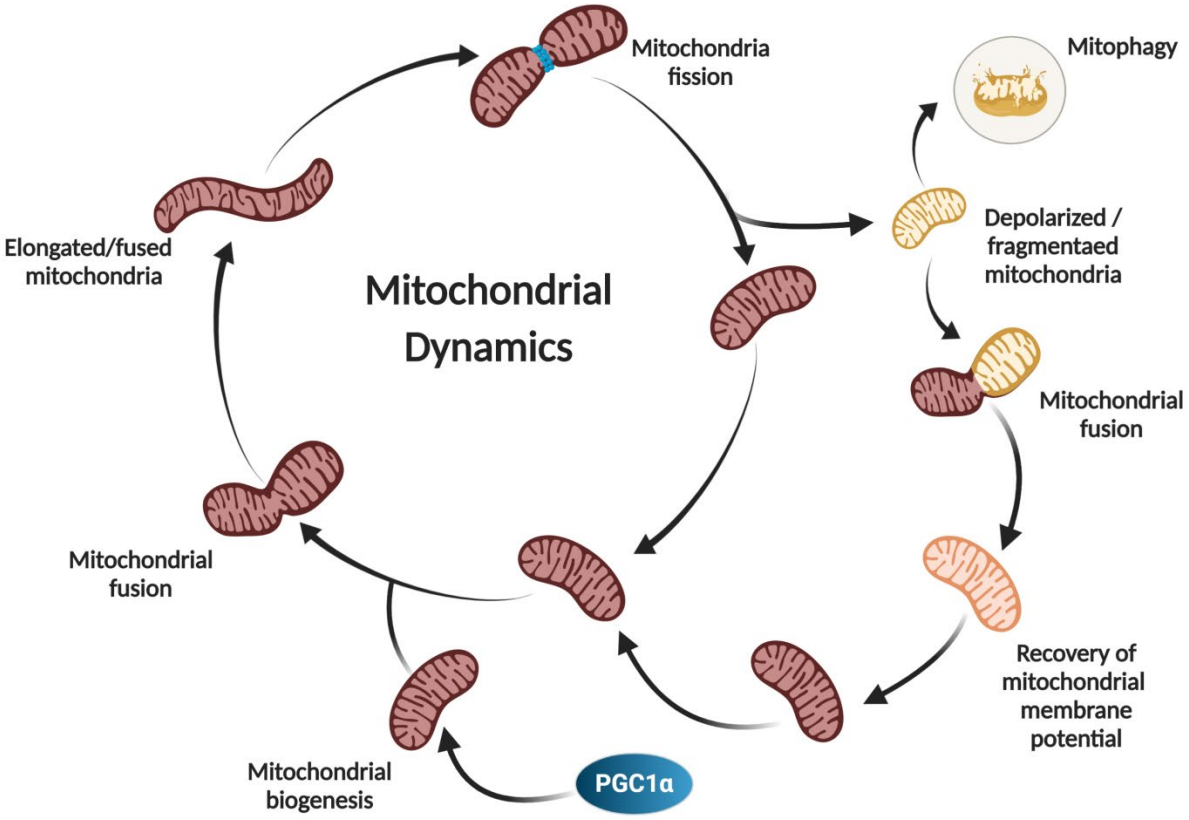

Figure 1.6: mitochondrial fusion, fission, and biogenesis
Mitochondria are highly dynamic organelles undergoing continuous cycles of fusion and fission, with a balance between mitochondrial biogenesis and elimination of damaged mitochondria (mitophagy). Mitochondrial biogenesis through PGC1α is required to compensate from decreased mitochondrial mass resulting from increased mitochondrial degradation (mitophagy). Mitochondrial fusion results in elongated and fused network of mitochondria. Mitochondrial fission leads to isolation of depolarized and fragmented mitochondria for removal by mitophagy. The recovery of mitochondrial membrane potential after fusion allows the mitochondria to enter the life cycle. An ongoing mitochondrial fusion-fission cycle allows mitochondrial functional and genetic complementation and the proper distribution of newly synthesized mitochondria during cell cycle. Therefore, an imbalance between mitochondrial fusion, fission, biogenesis and mitophagy could lead to changes in mitochondrial mass, morphology and functions. Designed by Esha Ganguly. Created with BioRender.com

1.5.4.3 Role of mitochondrial function in placentae of normal pregnancy

Placental mitochondrial function and morphology adapt throughout gestation in response to changes in the metabolic demands of the placenta in order to support fetal growth [reviewed in (122)]. The mitochondrial sub-populations present in various placental cell types are morphologically distinct [reviewed in (122)]. For example, mitochondria from the placental syncytiotrophoblast in humans are smaller, fragmented and irregular in shape, and have modified atypical cristae morphology (123). Conversely, mitochondria from the underlying cytotrophoblast layer are larger, less dense, and contain abundant cristae (122, 123). In addition to morphological differences, placental mitochondrial respiratory capacity adapts developmentally throughout gestation. During early pregnancy, placental oxygen levels are low as the maternal blood supply to the placenta is incomplete (31). In the first trimester human placenta, in order to efficiently utilize the limited oxygen supply, mitochondrial respiration remains low with no change in mitochondrial content at around 11 weeks of gestation (124). Mitochondrial respiration increases sharply between 12 to 13 weeks of gestation in humans when maternal blood flow to the placenta is established (124). In term human placentae, mitochondrial respiration, and mitochondrial content was higher compared to first trimester placenta, suggesting that greater blood supply to the placenta leads to higher mitochondrial content, which is usually associated with higher respiration (124).

A recent study demonstrated gestational age-related (early versus late gestation) and zone-related (junctional versus labyrinth) differences in mitochondrial respiration in placentae from mice (70). The labyrinth zone had lower rates of mitochondrial oxygen consumption and pyruvate-supported respiration rates at earlier gestational stages compared to labyrinth zones from term placentae (70). A possible explanation for lower rates of pyruvate-supported mitochondrial respiration and oxygen consumption in the labyrinth zone during late gestation could be an adaptive

strategy to spare oxygen and glucose for transfer to the fetus during its rapid period of growth. These observations are in line with previous studies showing that in human term placentae the syncytiotrophoblast had lower mitochondrial respiration compared to the highly metabolically active cytotrophoblast layer (125). Overall, placental mitochondrial morphology and function adapts to support fetal growth.

1.6 The Effects of Sex on Placental Development and Function

As discussed in the previous section(s), normal development and function of the placenta is critical for optimal fetal growth and survival. The placenta from male and female fetuses, however, develop differently, suggesting that sex differences in fetal growth trajectories could be mediated in part by sex-specific differences in placental growth and morphology (126). Current research suggests that morphological and functional adaptations in placentae from male and female fetuses are sexually dimorphic in response to suboptimal *in utero* environments. Moreover, ample evidence supports the idea that sexually dimorphic responses of male and female fetuses to adverse prenatal environments are likely to be mediated by sex-specific differences in placental adaptations to prenatal insults. Therefore, discussing sexual dimorphism in normal placental development and functions in the absence of any prenatal insult is of considerable importance as sex differences are an important determinant of fetal development and is the focus of our current study.

The placenta was once considered an asexual organ and therefore many studies focusing on the placenta have not taken the sex of the fetus into account (127). But given its extraembryonic origin, the placenta is now considered to have the same sex as that of the embryo, and studies suggest that sex differences in the placenta can originate early in development (127, 128). Ishikawa *et al.* showed that male (XY) placentae were significantly larger than female (XX) placentae and that such differences are independent of the effects of circulating androgens (129). Thus, sex of the embryo may affect size of both fetal and placental growth. Furthermore, the trajectory for fetal growth in response to adverse prenatal environments is determined by the fetal sex (130, 131). For example, male fetuses adopt a “minimalist approach” in response to adverse *in utero* environments where they continue to grow normally (127). This response strategy

places male fetuses at a greater risk if an adverse event occurs during gestation. In contrast, female fetuses adapt to suboptimal intrauterine environments by reducing their growth (127). This allows the female fetuses to adapt better in suboptimal *in utero* environments. Thus, males and females are different in how they adapt and respond to adverse prenatal environments. The specific mechanism for differential capacity of the male and female fetuses to adapt during adverse prenatal conditions are yet to be understood, but it is often speculated that this could be due to the sex-specific differences in the normal expression and regulation of genes, proteins and steroid pathways in placentae from male and female fetuses. This may explain the differential responses of male and female placentae under adverse *in utero* conditions.

There is evidence that some molecular pathways that regulate placental development and morphology are differentially regulated in males and females, for instance in the expression and activity of immune pathways. Sood *et al.* found higher expression of genes related to immune pathways, such as *JAK1*, *IL2RB*, *CXCL1*, *IL1RL1* and *TNF* receptor, in female placentae from normal human pregnancies (132). Expression of 5α-reductase (the enzyme that converts testosterone to its bioactive form of dihydro-testosterone) was found to be greater in female term placentae from humans (133). Taken together, these studies suggest that sex-specific differences in expression of immune and inflammatory pathways in the placenta could result in account for male and female fetuses exposed to varying concentrations of cytokines and testosterone, which may ultimately impact fetal development.

Furthermore, sex-specific differences in placental gene expression may account for sex differences found in placental morphology. Kalisch-Smith *et al* found that in mice, at mid-gestation, placentae from female fetuses had reduced labyrinth volume, fetal and maternal blood spaces, and fetal blood space surface area along with decreased expression of IGF2 compared to male placentae

(126). However, by GD 20, these sex-specific differences in placental morphology were no longer present, but female fetuses had lower body weights compared to male fetuses (126). This suggests that differences in placental growth factor expression and morphology between males and females likely contributes to the lower fetal weights in females compared to males at GD 20.

A more recent study by Saoi *et al* found sex-specific metabolic differences in the mouse placenta during normal pregnancy (134). The female placenta exhibited higher antioxidant capacity along with greater mitochondrial respiration compared to male placentae (134). The higher levels of metabolites, such as acylcarnitines, suggest that fatty acid β-oxidation may be a more preferred substrate for energy generation in the female placenta during normal gestation. Taken together, all these studies suggest that sexual dimorphism observed in fetal development in response to adverse prenatal environments (such as prenatal hypoxia) is due at least in part, to differences in placental morphological adaptations and bioenergetic capacity. In the next section we will focus on how hypoxia in pregnancy affects placental morphology and function and the potential mechanisms leading to placental dysfunction.

1.7 Effects of Hypoxia in Pregnancy on Placental Morphology and Function

1.7.1 Prenatal hypoxia impairs placental morphology and function

As discussed in Section 1.2, placental hypoxia may result from inadequate oxygen delivery, placental insufficiency, and/or abnormal spiral artery remodeling. Regardless of the cause, the consequence of placental hypoxia is impaired placental morphology/function, which could potentially contribute to impaired fetal organ development in offspring born from complicated pregnancies. Experimental exposure of pregnant rodents and guinea pigs to acute and/or chronic hypoxic environments has been extensively used to study the effects of reduced oxygen availability on placental morphology/functions, fetal outcomes, and long-term offspring health (Table 1.1). Studies have demonstrated that the severities of the hypoxic insults on placental and fetal outcomes are dependent on the duration, magnitude and timing of the gestational hypoxia (101). Our laboratory and others have shown that maternal exposure to hypoxia in the last third of gestation (11% O_2, GD 15-21) in rats led to placental hypoxia and significant reductions in placental and fetal weights (in both males and females) (15, 100, 135). Further studies showed that in this model, there is evidence for increased placental oxidative stress as well as morphological effects such as reduced in labyrinth and junctional zone volumes, and reduced fetal capillary volume (101). The adverse morphological changes were associated with reduced placental efficiency and transport functions (such as a reduction in transplacental amino acid transport) (101). Chronic maternal hypoxia in rats and guinea pigs also led to impaired trophoblast invasion and uterine artery remodeling, and ultimately FGR (136, 137). However, maternal exposure to hypoxia early during gestation (13% O_2, GD 6-20) in rats led to increased placental weights with no changes in fetal weights (138). Furthermore, hypoxia early in gestation led to morphological adaptations in the placenta, such as

increased absolute junctional and labyrinth zone volume, increased fetal capillary surface area (135). This suggests that the impact of hypoxia on placental and fetal development is dependent on the timing of the hypoxic insult.

The effects of maternal exposure to hypoxia on placental morphology depended on the severity of the hypoxic insult. For example, in mice, maternal exposure to 12-13% O_2 (GD 14-19) enhanced the labyrinth zone volume, which could be a strategy to optimize maternal-fetal substrate delivery (101). On the contrary, maternal exposure to 10% O_2 in mice (GD 14-19) led to decreased labyrinth zone volume with concomitant expansion of the junctional zone (101). Therefore, depending on the timing and severity of the prenatal hypoxic insult, placental morphological changes could be associated with impaired trophoblast invasion and maternal spiral artery remodeling, increased indices of oxidative stress, and altered expression of genes and proteins involved in trophoblast proliferation, differentiation and apoptosis, and fetoplacental vascularization (summarized in Table 1.1) [reviewed in (139)].

Table 1.1 Effects of prenatal hypoxia on placental and fetal outcomes

Degree of hypoxia	Duration of exposure	Species	Outcomes				Sex	Refs
			Placental			Fetal		
			Weight	Morphology and stress markers	Function			
9.5% O_2	GD 14.5-17.5	Rats	↓placental weight (21%)	↓ trophoblast invasion ↑ trophoblast apoptosis (caspase 3)	ND	↓ fetal weight (24%)	ND	(140)
10% O_2	GD 6-19	Rats		↑ collagen fibres and reticular fibres fragmented ↓ inner diameter of chorionic plate arteries and intraplacental arteries ↓ chorionic plate arteries	ND	ND	ND	(136)
10.5% O_2	GD 6-21	Rats	↔ placental weight	↓ trophoblast invasion ↓ uteroplacental vascular remodeling	ND	↓ fetal weight	ND	(141)
11% O_2	GD 6.5-13.5	Rats	↑ total cross-sectional area	↑ uterine mesometrial blood vessels ↑ EVT invasion ↑ EVT-guided maternal uterine artery remodeling ↑ junctional zone and labyrinth zone cross-sectional areas (greater expansion of junctional zone vs. labyrinth zone)	ND	ND	ND	(142)

11% O_2	GD 15-21	Rats	↓placental weight ↓placental weight	ND	ND	↓ fetal weight ↓ fetal weight	Male Female	(15)
13% O_2	GD 6-20	Rats	↑placental weight (5%)	↔ junctional zone, labyrinth zone volume ↑ oxidative stress	ND	↔ fetal weight	ND	(138)
13% O_2	GD 6-20	Rats	ND	↑ absolute placental volume (junctional zone, labyrinth zone and decidua ↑ fetal capillary surface area) ↔ maternal surface area and blood space volume ↔ interhaemal membrane thickness ↑ ER UPR (GRP78 and ATF4) ↑ mitochondrial UPR (GRP75 and TID1)	ND	↓ fetal weight	ND	(135)
10% O_2	GD 14-19	Mice	↔ placental weight	↓ labyrinth zone fetal capillaries (percentage volume) ↓ interhaemal membrane (placental barrier) thickness	↓ placental efficiency ↔ glucose acid Transport ↓ labyrinth zone amino acid transport ↔ labyrinth zone amino acid transporters (*Slc38a1*, *Slc38a2* and *Slc38a3*)	↓ fetal weight (21%)	ND	(101)

12% O_2	GD 14.5-18.5	Mice	↔ placental weight	↔ placental length, width and depth ↔ maternal blood spaces ↔ *Vegfa* mRNA ↔ *Igf2* mRNA	↔ amino acid transporter (*Slc38a1* and *Slc38a2*) ↔ glucose transporter (*Slc2a1* and *Slc2a3*)	↓ fetal weight ↓ fetal liver weight ↔ fetal brain weight	Male	(100)
12% O_2	GD 14.5-18.5	Mice	↔ placental weight	↔ placental length, width and depth ↓ maternal blood spaces ↔ *Vegfa* mRNA ↓ *Igf2* mRNA	↑amino acid transporter (*Slc38a1*) ↔ amino acid transporter (*Slc38a2*) ↓ glucose transporter (*Slc2a1*) ↔ glucose transporter (*Slc2a3*)	↓ fetal weight ↔ fetal liver weight ↔ fetal brain weight	Female	(100)
13% O_2	GD 0-18.5	Mice	↑placental weight (10%)	↔ oxidative stress (4-HNE formation, marker for lipid peroxidation) ↑ ER stress markers (p-eIF2α/IF2α and ↑ XBP-1) ↑ mitochondrial stress (HSP-60) ↑ maternal blood spaces (arterial and venous blood spaces) ↔ absolute volume blood of junctional zone, labyrinth zone and decidua	ND	↓ fetal weight (11.7%)	ND	(143)

13% O_2	GD 14-19	Mice	↔ placental weight	↓ interhaemal membrane (placental barrier) thickness ↔JZ volume (absolute and percentage volume)	↓ placental efficiency ↑ glucose transport ↔ amino acid transport ↑System A amino acid transporter (*Slc38a3*)	↓ fetal weight (~5%)	ND	(101)
13% O_2	GD 11-16	Mice	↔ placental weight	↑ maternal blood spaces ↑ labyrinth zone surface area and absolute volume	↔ placental efficiency ↔ glucose transport ↔ amino acid transport	↔ fetal weight	ND	(143)
16% O_2	GD 0.5-18.5	Mice	↔ placental weight	ND	ND	↔fetal weight	ND	(143)
10.5% O_2	GD 20-40	Guinea pigs	ND	↑ trophoblast proliferation ↓ trophoblast migration and invasion into maternal spiral arteries	ND	ND	ND	(137)
10.5% O_2	GD 20-64	Guinea pigs	↑absolute placental weight (10.1%) ↑relative placental weight (31.8%)	ND	ND	↓ fetal weight (16.1%)	ND	(137)

Table legend: eNOS, endothelial nitric oxide synthase; EVT, extravillous trophoblast; SLC2A1, solute carrier family 2 member 1; SLC2A3, solute carrier family 2 member 3; SLC38A1, solute carrier family 38 member 1; SLC38A2, solute carrier family 38 member 2; SLC38A2, solute carrier family 38 member 3; HNE, hydroxynonenal; ER, endoplasmic reticulum; eIF2α, eukaryotic initiation factor 2 alpha; peIF2α, phosphorylated eukaryotic initiation factor 2 alpha; XBP1, x-box binding protein 1; HSP60, heat shock protein 60; VEGFA, vascular endothelial growth factor A; IGF2, insulin-like growth factor 2; HSD11B2, hydroxysteroid 11-beta dehydrogenase 2; NR3C1,

nuclear receptor subfamily 3 group C member 1; NR3C2, nuclear receptor subfamily 3 group C member 2; UPR, unfolded protein response; GRP78, 78-kDa glucose-regulated protein; ATF4, activating transcription factor 4; GRP75, 75-kDa glucose-regulated protein; GD, gestational day; ND, not determined.

1.7.2 Mechanisms by which hypoxia in pregnancy may lead to placental dysfunction and impaired fetal organ development

There are multiple mechanisms by which hypoxia in pregnancy can contribute to placental dysfunction and impaired fetal organ development. Therefore, I will address the main potential mechanisms by which prenatal hypoxia may lead to impaired placental function.

1.7.2.1 Placental mitochondrial dysfunction and excessive ROS production

During normal pregnancy, mitochondrial respiration adapts to oxygen fluctuations over the course of gestation to support fetal development (124). However, studies suggest that placental dysfunction and impaired fetal development found in pregnancies complicated with prenatal hypoxia could be associated, in part, with impaired mitochondrial respiratory capacity (70, 144-146). For instance, as described in Table 1.1, maternal exposure to hypoxia in mice (13% O_2, GD 0.5-18.5) led to increased mitochondrial stress along with reduced expression of the mitochondrial respiratory complexes, and ultimately FGR (143). Furthermore, hypoxic human placental villous tissues from severe preeclamptic pregnancies were shown have impaired mitochondrial function (i.e. decreased expression of complex I and reduced complex III activity) and increased ROS levels (94). Human placental samples from high-altitude pregnancies showed evidence of reduced complexes I and IV expression compared to sea-level placentae, suggesting impaired mitochondrial function in placentae from high-altitude pregnancies (145). A more recent study by Sferruzzi-Perri *et al*. showed that maternal exposure to hypoxia during last third of gestation in mice (10.5% O_2) led to decreased rates of mitochondrial oxygen consumption and increased protein carbonylation (marker of oxidative stress) in the placental labyrinth zone (70). However, mitochondrial respiration and oxidative stress in the placental junctional zone remained

unaffected by maternal hypoxia on GD19 (70). This suggests that the mitochondrial respiration in the placental junctional and labyrinth zones adapt differently in response to prenatal hypoxia in order to support fetal growth and development. Maternal exposure to hypoxia in guinea pigs during late gestation (10.5% O_2 GD 15-19) led to decreased placental oxygenation and reduced complexes I and IV activity via increased nitration of both complexes by peroxynitrite in placentae from male but not female offspring (144). This suggests that prenatal hypoxic conditions can lead to placental mitochondrial dysfunction and increased placental ROS production that, in turn, can impair placental function. However, the sex-specific differences in mitochondrial OXPHOS capacity in response to prenatal hypoxia remain to be investigated.

1.7.2.2 Causes and consequences of placental oxidative and nitrosative stress

Prenatal hypoxia was shown to cause placental oxidative stress (increased production of ROS) and fetal programming of cardiovascular disease (2). Maternal exposure to hypoxia in rats leads to increased markers of oxidative stress in the placenta, and ultimately FGR (2, 138). Placental oxidative stress arises when the production of ROS exceeds the capacity of cell's antioxidant defense mechanisms. All the major antioxidant systems, both enzymatic (Copper/Zinc SOD [Cu/MnSOD], MnSOD and catalase) and non-enzymatic (glutathione, glutathione peroxidase, glutathione S-transferase, peroxiredoxin, thioredoxin) systems are present in the placenta (147). As described in previous section(s), mitochondria are considered to be the major source of ROS under conditions of low oxygen in the placenta. During hypoxic conditions, a reduction in molecular oxygen in the mitochondria, result in increased leakage of electrons and generation of superoxide, thus leading to oxidative stress. The polarized superoxide anion remains within the mitochondrial matrix where it is enzymatically converted to hydrogen peroxide (H_2O_2) by mitochondrial MnSOD

(148). Further, H_2O_2 can either react with ferrous iron to produce hydroxyl ($^{\bullet}OH$) radicals (known as Fenton reaction) (149) or reduced to water by catalase or glutathione peroxidase (150).

Oxidative stress targets plasma and organelle membranes because phospholipids are easily oxidized by superoxide anion. ROS can directly interact and oxidize amino acids of proteins and nucleic acids (such as DNA), which can disrupt normal cellular structure and functions (151). Moreover, ROS can directly interact with mitochondrial DNA, proteins and membranes, which may compromise assembly of the OXPHOS subunits into functional complexes, thereby limiting ATP production and increasing the risk of further mitochondrial ROS production (152).

Under conditions of oxidative stress, increased superoxide reacts with NO to produce peroxynitrite (153). Both peroxynitrite and peroxynitrite-derived radicals act as powerful oxidants that can lead to oxidation and/or nitration of tyrosine and cysteine residues in proteins, thiol, lipids and DNA (68, 151). From a functional perspective, increased formation of the oxidant peroxynitrite can attenuate placental vascular reactivity possibly through depletion of NO and increased nitration of proteins in the fetal vasculature (154). Increased formation of the pro-oxidant peroxynitrite can lead to FGR, as demonstrated previously in the hypoxic catechol-*O*-methyl transferase-deficient ($COMT^{-/-}$) mice (155). Taken together, evidence suggests that hypoxia in pregnancy leads to placental oxidative and nitrosative stress, which could be a potential linking mechanism to impaired fetal development.

Overall, prenatal hypoxia can directly lead to (i) placental oxidative and nitrosative stress; and (ii) impaired placental mitochondrial function which, in turn, can lead to placental dysfunction and poor fetal and offspring health outcomes. However, the precise mechanisms by which placental hypoxia may negatively impact fetal organ development and health remain

elusive. In addition to reduced oxygen and nutrient delivery to the developing fetus due to placental dysfunction, recent research suggests that there may also be factors released into the fetal circulation affecting organ development (156, 157). These factors could be derived from the mother and cross the placental barrier, or derived from the placenta, to eventually impair or affect fetal development. Moreover, placental factors released into the maternal circulation have been extensively studied and shown to impair maternal vascular function and reduce uteroplacental blood flow, thereby perpetuating placental dysfunction even further (as reported in pregnancies complicated by preeclampsia) (reviewed in (158)). Prior to the work described in this thesis, we, together with our collaborators, have demonstrated that an oxidatively stressed placenta can lead to adverse fetal brain development through the release of circulating factors into the fetal circulation in a rat model of prenatal hypoxia (156); however, the effects of placenta-derived factors on fetal cardiac development have not yet been determined. The potential placenta-derived factors released into the fetal circulation that, I theorize, maybe altered by placental oxidative stress are discussed below.

1.7.2.3 Potential placenta-derived factors that may affect fetal organ development

The placenta releases several circulating factors such as growth factors VEGF, PGF, IGF2, cytokines (e.g., tumor necrosis factor [TNFα] and interleukins [ILs]), and microRNAs (miRNAs), which we theorize, may affect development of key fetal organ systems (159-161). The balance between pro- and anti-angiogenic factors and their receptors in pregnancy is a complex and dynamic process. Pro-angiogenic factors, such as VEGFA, can be secreted by the placental syncytiotrophoblast into the maternal circulation. In response to oxidative stress,

placentae from preeclamptic pregnancies contribute to increased secretion of anti-angiogenic factors, such as soluble isoform of fms-like tyrosine kinase, sFLT-1, into the maternal circulation with concomitant decreases in the levels of pro-angiogenic VEGFA. However, it is not well understood to date if circulating VEGFA from the maternal circulation can cross the placental barrier to the fetal circulation via active transport and protein transporters. Proteins above the threshold size of 5 kilodalton (kDa) were shown to not cross the placental barrier from the maternal circulation (162); therefore, it can be theorized that VEGFA (molecular weight of ~ 40 kDa) may be produced and secreted directly from the syncytiotrophoblast to the fetal circulation.

Besides growth factors, cytokines are produced by the placental trophoblasts and also by macrophages and stromal cells of the placenta [as reviewed in (163)]. During normal pregnancy, the placenta secretes anti-inflammatory cytokines such as interleukin- 4 (IL-4) and interleukin- 10 (IL-10) that play beneficial roles in normal physiological processes, such as trophoblast invasion and proliferation (163). Hypoxic and oxidatively stressed placental tissues are associated with increased levels of pro-inflammatory cytokines (164, 165) such as tumor necrosis factor-alpha (TNF-α) (166), interleukin-6 (IL-6) (167) and interleukin-2 (IL-2) (160), and decreased placental production of anti-inflammatory cytokines such as IL-10 (168). Moreover, cytokines can cross the placental barrier to the fetus. Dahlgreen *et al.* showed that IL-6 administered into pregnant rats during mid-gestation (GD11-13) and late-gestation (GD17-19) was able to cross the placental basal membrane in the fetal circulation (169). Some cytokines (such as TNF-α and IL-6) have also been shown to cross the human placenta *ex vivo* (170). On the contrary, other studies showed that cytokines cannot be transported across the placenta from maternal to fetal circulation in both humans (171) and rodents (172). In pregnant rats, maternal administration of TNF-α does not

cross the placenta to the fetal circulation (172). Therefore, it can be speculated that the placenta can directly produce and secrete cytokine in the maternal as well as fetal circulation and/or maternal circulating cytokines could also enter the fetal circulation via the placenta. Additionally, cytokines in the fetal circulation can also be synthesized within the fetus (173).

The placenta-also secretes specific miRNAs into the maternal and fetal circulations (174). miRNAs are small highly conserved single stranded non-coding RNAs (around 18-22 nucleotides in length) and bind to the 3′-untranslated regions (3′-UTRs) of target mRNA for subsequent mRNA degradation (175). Chang *et al*, found that in pregnant transgenic mice, which exclusively express the primate specific chromosome 19 miRNA cluster (C19MC) in the placenta, these placenta-specific C19MC miRNAs were detected in the fetal compartment (176). A recent study further confirmed that circulating miRNAs in diabetic mice can cross the placenta to enter the fetal circulation, eventually leading to fetal cardiac dysfunction (177). This suggests that placenta-derived miRNAs can be directly trafficked into the fetal compartments and detected in fetal organs. The expression profile of miRNAs is altered in placental tissues from FGR pregnancies (178). Furthermore, expression profiles of miRNAs and associated gene expression are altered by oxidative stress in the human villous first trimester trophoblasts (178). Overall, this suggests that miRNAs derived from oxidatively stressed placentae from complicated pregnancies can impair fetal organ development. Indeed, together with our collaborators we found that oxidative stress in the placenta altered placenta-derived miRNA secretion which could be related to abnormal gene expression in the fetal brain, eventually leading to impaired neuronal development (156). However, it is remains to be determined whether placenta-derived factors released into the fetal circulation may be one of the mechanisms by which placental oxidative

stress can impair fetal heart development.

1.8 Effects of Hypoxia in Pregnancy on Fetal Cardiac Development

As reviewed by Alexander *et al*., research has demonstrated an association between hypoxia in pregnancy and abnormal cardiac function in both fetal and adult life (179). However, to understand how prenatal hypoxia may impair fetal cardiac development and lead to increased risk of later life cardiac disease, it is first important to understand the growth and maturation of fetal heart in normal pregnancy.

1.8.1 Cardiac development in fetuses from normal pregnancies

Cardiogenesis is the process in which the heart develops from the embryonic mesoderm which occurs in early gestation (i.e. middle of the 3rd week of gestation in humans and around GD 7.5 in rodents). During early gestation, after cardiogenesis, fetal hearts increase in mass by hyperplastic growth of mononucleated cardiomyocytes (180). This is followed by a rapid decline in the proportion of mononucleated cardiomyocytes and a corresponding increase in the proportion of terminally differentiated binucleated phenotype (180). Binucleation occurs when mononucleated cardiomyocytes undergo karyokinesis (DNA synthesis) and nuclear mitosis but fail to complete cytokinesis (181). These binucleated cardiomyocytes lack the capacity to proliferate and therefore subsequent increases in cardiac mass occur via hypertrophy of binucleated cardiomyocytes (182). This process begins before birth in some species, such as humans and sheep. For example, in fetal sheep cardiomyocyte terminal differentiation begins at about GD 110 and continues until birth (term= GD 145) (180). Therefore, at the time of birth around 60-70% of ovine cardiomyocytes are binucleated and can no longer divide (180). However, this growth pattern differs in rodent (rat and mouse) hearts, where they are born with an immature cardiovascular system and cardiomyocyte

maturation occurs after birth. In rodents, this process continues until postnatal day (PND) 14, or even later (183). During the fetal period, cardiac growth occurs through the process of hyperplasia (i.e. an increased number of cardiomyocytes, which are the functional units of the heart). During the early postnatal period (PND 1-3), in rodents, growth and maturation of cardiomyocytes at first still occurs by hyperplasia, where most of the cardiomyocytes are mononucleated (184). This is followed by a rapid transitional phase (PND 4-14) where mononucleated cardiomyocytes transform into a binucleated (terminal differentiated) phenotype (184, 185). On PND 14 and later, cardiac growth occurs by hypertrophy (increase in cell size) of pre-existing cardiomyocytes (184, 185). Cardiomyocyte endowment at birth is thus determined by a balance between cellular proliferation and terminal differentiation (180).

1.8.2 Prenatal hypoxia impairs fetal cardiomyocyte development

As discussed in section 1.1, hypoxia in pregnancy can itself lead to fetal programming of chronic diseases (2). The direct effect of prenatal hypoxia on fetal cardiac development, morphology and function has been extensively studied in different animal models of hypoxia (186). In rats, maternal exposure to hypoxia led to increased fetal cardiac oxidative stress and increased cardiomyocyte apoptosis that eventually resulted in reduced number of cardiomyocytes in fetal hearts (16). Furthermore, prenatal hypoxia reduced fetal cardiomyocyte proliferation, reduced markers of cell division (such as p21), and increased the percentage of binucleated cardiomyocytes (16). The subsequent increases in relative heart weight through increased cardiomyocyte size could be a compensatory response for reduced cardiomyocyte number at birth; all of which may increase susceptibility to cardiac dysfunction in fetal and adult life (187). Studies also showed that maternal exposure to hypoxia in rats caused ventricle septal defects, produces myocardium thinning of

ventricles, and epicardium detachment from myocardium in the hypoxic fetal hearts (188). Moreover, ovine fetuses exposed to high altitude hypoxia during pregnancy demonstrated increased expression of hypoxic biomarkers (such as HIF1α) along with decreased expression of the cardioprotective PKCε, increased expression of markers for autophagy in hypoxic hearts and reduced post-ischemic cardiac recovery (189). In guinea pigs exposed to late gestation hypoxia (10.5% O_2, GD45-60) increased relative heart weights in hypoxic fetuses were associated with increased cardiac expression of pro-inflammatory cytokines (such as TNFα and IL6), increased matrix metalloproteinase-2 and -9 expression, and increased expression of inflammation-induced iNOS expression (190-192).

The effects of prenatal insults on cardiomyocyte endowment at birth are dependent on the timing and duration of the insult, which in turn, can have different impacts on cardiac morphology. For example, in rats, maternal exposure to hypoxia earlier in pregnancy (13% O_2, GD6-20) resulted in unchanged relative heart weights (18), whereas we have observed increased relative heart weight in offspring from rats exposed to hypoxia during late gestation (11% O_2, GD15-21) (15). However, despite the differential effects on heart weights, hypoxia in pregnancy led to increased indices of oxidative stress, decreased cardiomyocyte proliferation, and increased apoptosis (summarized in Table 1.2); all of which could lead to cardiac dysfunction later in life. Likewise, in sheep, long term high-altitude exposure to hypoxia impaired cardiac function in fetal hearts as evidenced by decreased cardiac contractility and output, and reduced post-ischemic recovery (189). Taken together, hypoxia in pregnancy can lead to altered fetal cardiac development and function (summarized in Table 1.2), eventually resulting in increased risk of cardiac dysfunction later in adult life.

As described in the above section, studies have looked into the direct effects of prenatal hypoxia on fetal cardiac development and function. Given that placental hypoxia and associated oxidative stress is central to the pathophysiology of pregnancy complications leading to placental insufficiency and impaired fetal growth, this thesis focuses on placental hypoxia and/or placental oxidative stress affecting fetal development. The effects of placental hypoxia and/or placental oxidative stress on fetal organ development could be independent of the fetus being hypoxic. The next section describes the use of antioxidant treatment strategies that can target placental oxidative stress, and ultimately lead to improved pregnancy outcomes.

TABLE 1.2 Effects of prenatal exposure to hypoxia on fetal cardiac development							
Degree of hypoxia	**Duration of exposure**	**Species**	**Fetal cardiac outcomes**			**Sex**	**Refs**
			Weight	**Cellular and morphological**	**Function**		
10.5% O_2	GD 15-21	Rats	↑ heart-to-body weight ratio	↓ Ki-67 and BrdU (proliferation marker) ↓ cyclin D2 (cell division marker) ↑ p27 (cell division inhibitor) ↑ TIMP-3 and TIMP-4 (inhibits proliferation)	ND	ND	(193)
			↓ absolute heart weight ↑ heart-to-body weight ratio	↑ % binucleation of cardiomyocytes ↑ size of binucleated cardiomyocyte ↑ cell death (apoptosis) ↑ active caspase 3 (pro-apoptotic) ↑ Fas protein levels (apoptotic receptor) ↓ Bcl-2 protein levels (anti-apoptotic) ↓ HSP70 protein levels ↑ beta 1-AR levels	ND	ND	(16)
			↑ heart-to-body weight ratio	↑ LV myocardium wall thickness ↓ cardiomyocyte proliferation	ND	ND	(193, 194)
				↑ ROS levels	ND	ND	(195)
				↓ PKCε mRN ↑ ER α and β isoforms	ND ND	Male Female	(196)

10.5% O_2	GD 19-21	Rats	↑ heart-to-body weight ratio	↑ cytochrome c levels (marker of apoptosis)	ND	ND	(197)
13% O_2	GD 6-20	Rats	↔ heart-to-body weight ratio	↑ aortic thickening ↑ nitrotyrosine ↑ HSP70 levels	ND	ND	(138)
8% O_2	GD 11.5-12.5	Mice	↓ ventricular mass	↓ ventricular wall thickness ↑ ventricular myocardium thinning ↑ epicardium detachment from myocardium ↓ proliferation ↑ cell death (apoptosis) ↓ DNA synthesis	ND	ND	(188)
10.5% O_2	GD 45-60	Guinea pigs	↓ absolute heart weights ↑ heart-to-body weight ratio	↑ eNOS mRNA and protein in coronary artery segments ↓ eNOS protein in cardiac tissues	ND	ND	(190-192, 198)
				↑ TNF-α, IL-6, MMP2 and MMP9 mRNA ↑ MMP2 activity ↑ MMP9 protein levels	ND	ND	
				↑ iNOS mRNA and protein levels ↑ nitrite/nitrate levels	ND	ND	

Long-term high altitude hypoxia	GD 30-140	Sheep	↓ heart weights	↑ RV capillary diameter than LV ↑ lactate dehydrogenase and citrate synthase activity ↓ cardiomyocyte glycogen content and mitochondrial content	↓ cardiac contractility ↓ RV and LV cardiac outputs ↓ RV and LV function	ND	(199-203)
				↑ HIF-1α protein levels ↑ DNMT3b protein levels ↓ PKCε protein levels ↑ miR-210 levels ↑ autophagy markers (LC3B-II)	↓ post-ischemic recovery	Male and Female	189)

Table legend: ER, Estrogen receptors; Bcl-2, B-cell lymphoma 2; HSP70, heat shock protein 70; AR, adrenergic receptor; BrdU, Bromodeoxyuridine; TIMP3, tissue inhibitor of metalloproteinase 3; TIMP4, tissue inhibitor or metalloproteinase 4; eNOS, endothelial nitric oxide synthase; TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; MMP2, matrix metallopeptidase 2; MMP2, matrix metallopeptidase 2; MMP9, matrix metallopeptidase 9; miR, microRNA; iNOS, inducible nitric oxide synthase; RV, right ventricle; LV, left ventricle; HIF-1α, hypoxia-inducible factor 1-alpha; DNMT3b, DNA methyltransferase 3 beta; PKCε, protein kinase C epsilon; GD, gestational day; ND, not determined.

1.9 Antioxidant Treatment Strategies in Pregnancy

1.9.1 Maternal systemic antioxidant treatment

Given the contribution of oxidative stress in the underlying pathophysiology of *in utero* origins of chronic disease, many studies using animal models of DOHaD have attempted to reverse or prevent later life onset of chronic disease in the offspring with treatments and/or interventions in the mother. In addition to the benefit of systemic maternal treatments (such as antioxidant treatment) in complicated pregnancies to improve outcomes in the adult offspring, studies have shown that many maternal systemic treatments are able to cross the placenta and affect fetal development and long term offspring health, sometimes to a detrimental extent [as reviewed in (204)]. For example, in a randomized, placebo-controlled trial Poston *et al.* reported that supplementation with vitamin C and vitamin E from the second trimester of pregnancy until delivery does not prevent preeclampsia in women at risk and increases the rate of low birthweight babies (205). Moreover, antioxidant treatments (such as tempol) have been shown to cross the placental barrier to the fetus and may even have detrimental (off-target) effects on normal fetal development (205, 206). For instance, Stanley *et al.* showed that in an eNOS knockout mice model of FGR, administration of the anti-oxidant tempol in drinking water not only increased body weight of $eNOS^{-/-}$ mice but also increased fetal weight of control mice, which may have detrimental effects (207). Therefore, due to the potential for off-target effects of maternal systemic interventions on the developing fetus, new studies have focused on placenta-targeted treatments (Figure 1.7). A recent comprehensive review by Sibley has highlighted the importance of selectively targeting therapeutics to the oxidatively stressed and dysfunctional placentae in order to reduce any adverse off-target effects on fetal development (208).

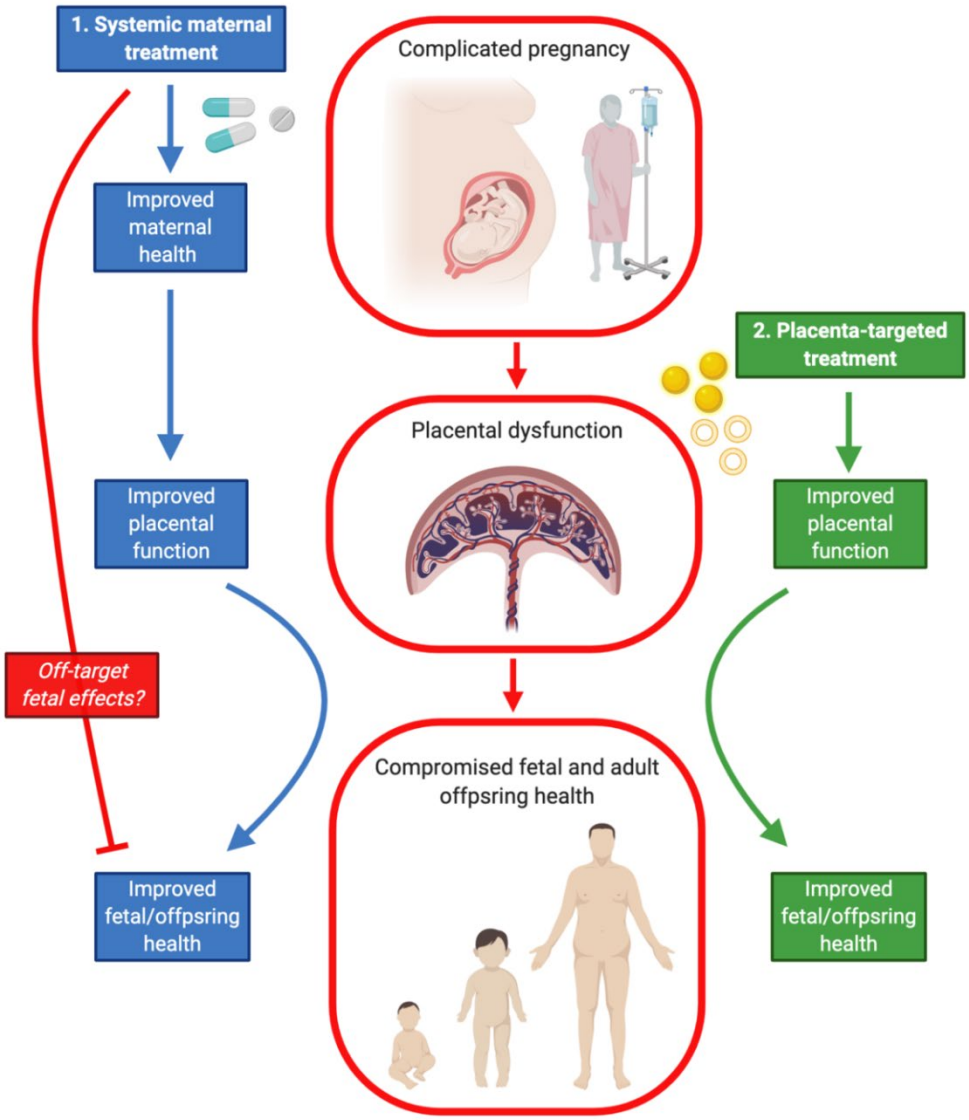

Figure 1.7. An illustration showing the effects of maternal systemic interventions and placenta-targeted treatment strategies on fetal and adult offspring health. Systemic maternal treatments (such as antioxidant treatment) in complicated pregnancies improve outcomes in the adult offspring, but are able to cross the placenta and affect fetal development and long term offspring health, sometimes to a detrimental extent. Placenta-targeted treatment strategies can be used to improve placental function and improve pregnancy outcomes with minimal direct drug exposure to the fetus and avoid any potential off-target effects during fetal development. Ganguly *et al.* (209). Created with BioRender.com

1.9.2 Benefits of placenta-targeted treatment strategies

Targeted drug delivery to specific cells and/or organs offer several advantages, including (but not limited to) sustained release of therapeutic drugs *via* encapsulation, which prevents degradation and promotes extension of the half-life of the drugs. In addition, targeted drug delivery may also lower the required therapeutic dosage by increasing local drug bioavailability (reviewed in (210)). Nanoparticle delivery strategies can also be optimized to target a specific tissue and/or cell type via alterations in nanoparticle size and surface composition (which determines the charge/zeta potential and hydrophilic nature of the nanoparticle), which influences nanoparticle uptake and interaction with the plasma membrane (211). For instance, liposomes coated with tumor-homing peptides (CGKRK and iRGD) accumulated within the outermost syncytium without crossing into the underlying cytotrophoblast layer in first-trimester human placental explants and accumulated within the placental labyrinth region (fetal vascular endothelium and trophoblast cells) in mice, with no evidence of transfer to fetal tissues (212). Notably, placenta-targeted delivery of drugs, such as growth factors, was more effective in improving fetal outcomes than maternal systemic administration (212). For example, intravenous administration of iRGD-coated liposomes containing IGF-2 in a well-characterized model of FGR (i.e. placenta-specific IGF-2 knockout mice) in mice was more effective in improving fetal growth than systemically administered IGF-2 (212). The particle size and surface charge of these drug carriers can be altered to allow for controlled drug release targeted to specific organs (e.g. placenta) over an extended period of time in order to achieve greater therapeutic potential. Importantly, the improved efficacy of targeted drug-delivery systems may permit the use of lower therapeutic dosages and/or reduced frequency of drug administration (e.g. number of intravenous injections required) over the course of treatment.

1.9.3 Nanoparticle delivery to target antioxidant treatment to the placenta

Because the use of a nanocarriers to target therapeutics offer several advantages, our recent studies have utilized polymeric nanoparticles composed of a poly (γ-glutamic acid) (γ-PGA) hydrophilic outer shell and L-phenylalanine ethylester (L-PAE) hydrophobic inner core to specifically target the placenta (156, 213). These nanoparticles are specifically taken up by the outer syncytial membrane due to 1) their relatively large size (~180 nm), 2) their negative charge (zeta potential of -20 mV which reduces phagocytic elimination of nanoparticles), 3) their hydrophilic outer surface composition (reduces particle aggregation and facilitates modest cellular uptake depending on the plasma membrane properties), and 4) their biodegradable properties (undergoes delayed hydrolysis and/or rapid enzymatic degradation by γ- glutamyl transpeptidase enzyme) (156, 213-215).

Given that mitochondria are a major source of placental oxidative stress in pregnancy complications associated with placental dysfunction (see section 1.5.4), I used a mitochondrial-targeted antioxidant to prevent oxidative damage. MitoQ is a mitochondria-specific antioxidant because of its ability to selectively accumulate within the inner mitochondrial membrane *in vivo.* MitoQ consist of a ubiquinone moiety covalently attached to a lipophilic triphenylphosphonium (TPP^+) cation through a ten carbon alkyl chain. The positively charged TPP cation enables MitoQ to rapidly permeate through the phospholipid bilayers of the plasma membrane and accumulate several thousand-fold within the inner mitochondrial matrix in response to the large negative mitochondrial membrane potential (-150 mV to -170 mV). Once inside the mitochondrial matrix, the ubiquinone in MitoQ is reduced to the antioxidant ubiquinol by complex II of the electron transport system. In acting as an antioxidant against oxidative damage, the ubiquinol is oxidized to ubiquinone by reactive oxygen species such as superoxide, which is then reduced to ubiquinol by

complex II ((216), reviewed in (217, 218)). A recent study by Nuzzo et al. showed that MitoQ (without nanoparticles) fed daily to hypoxic pregnant dams (at a dose of 500 μmol/L) in the drinking water from GD 6-20 reduced markers of mitochondrial stress, enhanced placental efficiency to control levels, and increased fetal capillary surface area and maternal blood spaces in the placental labyrinth region compared to placentae from normoxic pregnancies (135). Although maternal treatment with MitoQ improved placental morphological adaptations in hypoxic pregnancies, the safety and the efficacy may be increased by selectively targeting MitoQ to the placenta. Therefore, together with our collaborators, our laboratory have been investigating the antioxidant benefits of MitoQ encapsulated into polymeric γ-PGA nanoparticles (nMitoQ) to target the placenta in order to minimize direct drug exposure to the fetus and avoid any potential off-target effects during feral development (156, 219).

There is a possibility that nMitoQ delivered to the placenta via intravenous injection could be taken up in other maternal organs. However, due to the large sized polymeric nanoparticles, accumulation in maternal organs was shown to be limited (156). Our lab, together with our collaborators, showed that the nanoparticles do not cross the placenta to the fetus (156). However, since the hydrophobically-modified γ-PGA nanoparticles are charged polar molecules, they may enter the placental trophoblast cells by endocytosis. Indeed, Mukai *et al.* showed that cellular entry of γ-PGA nanoparticles is mediated via phagocytosis and/or macropinocytosis and that upon entry into the cell γ-PGA nanoparticles were trapped inside endosomal vesicles (220). Furthermore, polymeric γ-PGA nanoparticles were shown to only possess membrane disruptive properties at endosomal pH ranges and are able to bypass the endosomal degradation process after cellular uptake (214). Thus, I theorize that in our studies, the nanoparticles enter the placental syncytiotrophoblast, deliver their antioxidant content (MitoQ) inside the cytosol by endosomal

escape and are then degraded by γ-glutamyl transpeptidase. It is possible that once MitoQ is released inside the cytosol; it rapidly accumulates in the mitochondria of the syncytiotrophoblast. However, this remains to be confirmed.

Previous studies from our laboratory showed that maternal nMitoQ treatment improved neuronal development in fetuses exposed to prenatal hypoxia. Moreover, in a sex-specific manner, nMitoQ treatment prevented development of cardiac diastolic dysfunction in adult female offspring and improved pulmonary artery function in adult male offspring (219). However, the effects of nMitoQ treatment on placental morphology and function, and fetal cardiac development have not yet been studied.

1.10 Hypotheses and Aims

Overarching hypothesis

A placental-targeted nMitoQ treatment in a rat model of prenatal hypoxia will improve placental function by targeting placental oxidative stress, and prevent the release of factors derived from a hypoxic and oxidatively stressed placenta, thus leading to improved fetal cardiomyocyte development.

Hypotheses

- Prenatal hypoxia will lead to mitochondrial dysfunction (i.e. decreased mitochondrial respiratory capacity), increased placental oxidative and nitrosative stress, and ultimately impaired placental morphology via decreased expression of growth factor, IGF2 and pro-angiogenic factor, VEGFA; all of which can contribute to placental dysfunction and poor fetal

outcomes. Placenta-targeted nMitoQ treatment will improve mitochondrial function, decrease prenatal hypoxia-induced placental oxidative and nitrosative stress along with increasing IGF2 and VEGFA expression, improving placental morphology, and ultimately result in improved fetal outcomes (Chapter 2 and 3).

- Factors released from prenatally hypoxic placentae will impair fetal cardiomyocyte maturation (induce terminal differentiation) and growth (increased cell size). Furthermore, nMitoQ treatment will prevent the release of factors derived from an oxidatively stressed placenta, thereby improving cardiac development (Chapter 4).
- Moreover, since placentae from male and female fetuses were previously shown to respond to prenatal stresses differently, I hypothesize that there is a sex-specific divergence in the placental response to prenatal hypoxia-induced oxidative/nitrosative stress and the effectiveness of nMitoQ treatment (Chapter 2, 3 and 4).

Aims

To test my hypotheses I used a rat model of prenatal hypoxia, wherein pregnant rats were exposed to 11% O_2 during last third of gestation (i.e. GD 15-21; term= 22 days) (15, 16, 219). Previous studies from our laboratory and others have shown that maternal exposure to hypoxia during late-gestation can lead to placental oxidative stress (156, 219), FGR (15, 219), abnormal cardiomyocyte development (16), and cardiovascular dysfunction in adult offspring (15). Therefore, using this established model of prenatal hypoxia, I aimed to:

Aim 1: Assess the effects of prenatal hypoxia and nMitoQ treatment on oxidative stress, nitrosative stress, oxygenation, and on placental morphology (such as area of the fetal capillaries and maternal and fetal blood space area) and measuring expression of pro-angiogenic factor *Igf2* and growth factor *Vegfa* in placentae from male or female fetuses.

Aim 2: Assess the effects of prenatal hypoxia and nMitoQ treatment on mitochondrial function, and expression of markers of mitochondrial fusion (OPA1) and fission (Drp1) in placentae from male or female fetuses.

Aim 3: Assess the effects of factors secreted from placentae of dams exposed to prenatal hypoxia on development (maturation and growth) of cardiomyocytes from males or females *in vitro*, and if this can be prevented by use of placenta-targeted nMitoQ treatment.

CHAPTER 2:

Sex-Specific Effects of Nanoparticle-Encapsulated MitoQ (nMitoQ) Delivery on Placental Function in a Rat Model of Fetal Hypoxia

A version of this chapter has been published:

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2.1 Introduction

As previously discussed in Chapter 1, placental dysfunction in pregnancies complicated by chronic fetal hypoxia is often associated with placental oxidative and nitrosative stress (i.e. an imbalance in the formation of ROS/RNS). An imbalance in ROS/RNS levels, caused by excessive generation of ROS such as superoxide anions and/or a fall in endogenous antioxidants such as SOD, can lead to impaired placental development and altered normal placental function and potentially reduce oxygen and nutrient delivery, thus impairing fetal development (reviewed in (221, 222)). One impact of excessive superoxide anions is the resultant scavenging of the vasodilator NO to produce RNS, such as peroxynitrite (147); therefore, increased superoxide levels could reduce NO bioavailability and impair the important contribution of NO in fetoplacental angiogenesis (154). As the placenta lacks both autonomic and cholinergic innervation, placental morphology and function are dependent on locally derived growth factors such as VEGFA and IGF2 (64, 223). Expression of the pro-angiogenic factor VEGFA is regulated by the oxygen sensing family of transcription factors such as HIF-1α (100), and reduced VEGFA expression can impair fetoplacental vascular development (60). Decreased IGF2 expression, associated with FGR, is also critical to placental morphology and nutrient transfer to the fetus (98). Therefore, in the current study, we assessed the effects of hypoxia during pregnancy on placental oxidative/nitrosative stress and placental morphology using an established model of prenatal hypoxia.

Interestingly, the placental response to oxidative stress appears to be different in placentae from males compared with females (reviewed in (224)). Human studies have shown that the placental oxidative stress response to adverse maternal environments in general appear to be more pronounced in male versus female placentae (146, 225-227). In rodents, similar results were found,

where adverse maternal environments altered placental morphology, placental gene expression and enzymes for epigenetic modifications (e.g. DNA methylation) in a sexually dimorphic manner (131, 228-230). Hence, we examined sex-specific differences in the placental responses to prenatal hypoxia.

Given that oxidative stress is a central mediator of placental dysfunction in fetal hypoxia, and mitochondria are a major source of cellular ROS in the placenta (as reviewed in (122)), our lab and others have been studying the potential use of the mitochondrial-specific antioxidant MitoQ to target placental oxidative stress in complicated pregnancies (135, 156, 219). Indeed, in a rat model of prenatal hypoxia, MitoQ treatment prevented the increase in mitochondrial stress markers in the placental labyrinth zone (135). Recently we have shown that nMitoQ is a delivery approach to access the placental syncytium without crossing the placental basal membrane to reach the fetus (2, 156). With this treatment strategy, previous study from our laboratory showed that maternal treatment with nMitoQ in a rat model of prenatal hypoxia increased fetal weight in female fetuses, improved neuronal development and had sex-dependent beneficial effects on *in vivo* cardiovascular function in prenatally hypoxic adult offspring (156, 219). Furthermore, nMitoQ treatment of human preeclamptic placental explants improved neuronal development *in vitro* (231). However, the effect of nMitoQ treatment on placental morphology, the mechanisms via which reduced placental oxidative stress might improve placental function and fetal growth, as well as any potential sex differences, remain to be investigated.

Therefore, in the current study, I aimed to further identify the effect of nMitoQ treatment on placental function and oxidative stress, and the sex-specific effects of the treatment, in a rat model of fetal hypoxia. We hypothesized that nMitoQ treatment would decrease prenatal hypoxia-induced oxidative/nitrosative stress along with increasing VEGFA and IGF2 expression, improving

placental morphology, and ultimately resulting in improved fetal growth. Moreover, since it has been shown that placentae from male and female fetuses respond to prenatal stress differently, we hypothesized that there is a sex-specific divergence in the placental response to prenatal hypoxia-induced oxidative/nitrosative stress and the effectiveness of nMitoQ treatment.

2.2 Materials and Methods

All procedures described were approved by the University of Alberta Animal Policy and Welfare Committee, and were in accordance with the guidelines of the Canadian Council on Animal Care (AUP #242).

2.2.1 Preparation of nanoparticle encapsulated MitoQ (nMitoQ)

MitoQ loaded nanoparticles were synthesized as previously described (156, 219). Briefly, an amphiphilic copolymer of poly (γ-glutamic acid) and L-phenylalanine ethylester (γ-PGA-Phe) was synthesized as described previously (213). γ-PGA-Phe (10 mg/mL) dissolved in dimethyl sulfoxide (DMSO) was added to an equivalent volume of sodium chloride (NaCl; 0.15M), dialyzed against distilled water using a dialysis membrane, freeze-dried and resuspended in phosphate-buffered Saline (PBS; 10 mg/mL). Nanoparticles were then measured by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK) as diameter (180 nm), zeta potential (-20 mV) and polydispersity index (0.12). γ-PGA-Phe nanoparticles (10 mg/mL) were mixed with MitoQ (2 mg/mL) at equivalent volume in NaCl (0.2M). Nanoparticles were isolated by centrifugation, washed and resuspended in PBS (10 mg/mL). The amount of MitoQ (278 nM) which was adsorbed to nanoparticles was evaluated by UV absorption measurement, as previously described (156).

2.2.2 Prenatal hypoxia rat model

Female Sprague-Dawley rats, 3 months of age (weighing 250-275 g), were obtained from Charles River (Quebec, Canada) and housed in a temperature and light controlled room (10:14h light/dark cycle) with *ad libitum* access to food and water. Females were housed with Sprague–Dawley males overnight and pregnancy was confirmed the following morning by the presence of sperm in a vaginal smear, which was defined as gestational day (GD) 0 of pregnancy. On GD 15 pregnant dams were randomly assigned into two groups that were exposed to either prenatal hypoxia (11% O_2) by placing them in a Plexiglas hypoxic chamber from GD 15-21, or were housed at atmospheric oxygen (21% O_2) as controls. Pregnant dams received an intravenous injection via the tail vein on GD 15 with either saline or nMitoQ (100 µl of 125 µM nMitoQ). As MitoQ is recycled and lasts up to one week *in vivo*, the nMitoQ treatment protocol consisted only of a single injection (156). The dose of nMitoQ was based on our previous studies (156, 219). As our study is focused on nMitoQ, as a single entity, we have a saline control rather than a nanoparticle along group as the properties may be different without the MitoQ and, ultimately, nanoparticles alone would never be used in practice. Previous studies have demonstrated that these nanoparticles are inert (156, 231). At the end of gestation, on GD 21, rats were euthanized prior to parturition and fetuses with their respective placentae were removed. Fetal sex was determined using the anogenital distance and fetal parameters, including body weight, crown-to-rump length and abdominal circumference, and placental weights, were measured and averaged per litter. Whole placentae (labyrinth and junctional zone) from male and female fetuses (two/sex/litter) were processed and embedded in either paraffin or optimal cutting temperature (OCT) compound for immunofluorescent and other staining procedures, as listed below. In other placentae (two/sex/litter) the placental labyrinth zones was isolated and snap frozen in liquid nitrogen for RNA analysis.

Placental labyrinth zone was used for both immunofluorescent staining and RNA analysis. The labyrinth zone acts as the nutrient and gas exchange layer between maternal-fetal circulations. Therefore, I specifically chose the placental labyrinth zone due to its high-energy demand for active transport of nutrients to the fetus during late gestation, and hence more susceptible to oxidative stress.

2.2.3 Dihydroethidium staining for superoxide production and diaminofluorescein-FM (DAF-FM) for NO levels

Placental cryostat sections (10 μm) were thawed, washed three times with Hank's balanced salt solution (HBSS) and incubated with dihydroethidium (DHE) to measure superoxide levels (200 μM, Biotium, Burlington, Canada) or DAF-FM to measure nitric oxide levels (10 μM, Thermo Fisher Scientific, Eugene, OR, USA) in HBSS at 37ºC for 30 min. Sections were washed with HBSS (3 x 2 min) and covered with a drop of 4′, 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Sections were protected from light and pictures were immediately taken to prevent photobleaching.

2.2.4 Immunofluorescent nitrotyrosine staining for placental peroxynitrite levels and CD31 staining to assess placental labyrinth feto-placental vascular capillaries

Nitrotyrosine residues are the molecular footprint of peroxynitrite generation and can be used as a marker of peroxynitrite (154). CD31 was used as an endothelial marker (232) to assess placental vascularization. Placental cryostat sections (8 μm) were thawed, fixed in ice-cold acetone for 10 min, washed thrice in PBS and non-specific binding was blocked using 2% bovine serum albumin (BSA) in PBS for 60 min. Sections were incubated overnight at 4ºC with a primary

antibody for nitrotyrosine (1:10; mouse-anti-tyrosine, NOVUS Biologicals, Oakville, ON, Canada) or CD31 (1:200 mouse-anti-CD31/PECAM-1, NOVUS Biologicals) in 2% BSA/PBS. The next day, sections were washed thrice with PBS and incubated with secondary antibody (1:250 in 2% BSA/PBS; donkey anti-mouse IgG (H+L), AF488, Alexa, Invitrogen) for 1 hour at room temperature. Sections were washed with PBS thrice, mounting medium containing DAPI was added (Vector Laboratories; Burlingame, CA, U.S.A) and slides were covered, protected from light and left to dry overnight. Images were taken the next day.

2.2.5 Immunofluorescent staining for placental HIF-1α expression

Placental levels of the transcription factor hypoxia-inducible factor-1α (HIF-1α) were measured as a marker of hypoxia. Placental PFA-fixed sections (8 μm) were dewaxed in xylene and rehydrated in ethanol (100%, 95% and 80%). Endogenous peroxidase activity was blocked using 10% H_2O_2 in distilled water for 10 min, and incubated in sodium citrate buffer supplemented with 0.05% Tween 20 at 90ºC for 20 min for antigen retrieval. Nonspecific staining was blocked with 2% BSA/PBS for 60 min at room temperature, and incubated overnight at 4ºC with a primary antibody against HIF-1α (1:250; rabbit-anti-HIF1 alpha, NOVUS Biologicals, Oakville, ON, Canada) in 2% BSA/PBS. The next day, sections were washed twice with Tris buffered saline containing 0.05% Tween 20 (PBS-T) and incubated with secondary antibody (1:250; goat-anti-rabbit IgG (H+L)-AF546, Invitrogen, Carlsbad, CA, USA) in PBS-T for 60 min. at room temperature. After incubation with secondary antibody, sections were washed twice in PBS-T, once in distilled water and mounted using mounting medium containing DAPI (Vector Laboratories). Slides were protected from light and left to dry overnight. Images were taken the next day.

2.2.6 Immunofluorescent staining for placental and fetal tissue oxygenation

In a separate group of dams, tissue oxygenation levels were assessed by intraperitoneal (i.p.)

injection of either pimonidazole hydrochloride (60 mg/kg) (Hypoxyprobe™ -1, Burlington, USA) or an equivalent volume of vehicle (saline) as a control on GD20. Six hours post injection, dams were euthanized and placentae, fetal hearts and fetal liver were collected and snap frozen. Pimonidazole levels in placental tissues and fetal cardiac and hepatic tissues from both sexes were assessed by immunostaining. Pimonidazole hydrochloride (also known as 2-nitroimidazoles) distributes to all tissues but is activated by reduction in cells exposed to oxygen concentration less than 14 micromolar, which is equivalent to a partial pressure pO_2 = 10 mmHg at 37ºC. The activated intermediate forms stable adducts with proteins containing thiol groups (i.e. reduced pimonidazole, the staining product). Placental cryosections (8 μm) were fixed in acetone (10 min.), washed in PBS thrice and non-specific staining was blocked using 2% BSA/PBS for 1 hour. Sections were incubated overnight at 4ºC with monoclonal anti-pimonidazole antibody (1:200; Hypoxyprobe™ Kit) in 2% BSA/PBS. The next day, sections were washed thrice in PBS and incubated with secondary antibody (1:250; donkey anti-mouse IgG (H+L), AF488, Alexa, Invitrogen) in 2% BSA/PBS for 60 min. Sections were washed with PBS three times and mounted using mounting medium containing DAPI (Vector Laboratories). Slides were protected from light and left to dry overnight. Images were taken the next day.

2.2.7 Morphological analysis of placenta

Using an established Haematoxylin and Eosin (H&E) staining protocol, placental PFA-fixed sections (8 μm) 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, covered and left to dry overnight.

2.2.8 Image analysis

All images were taken on an Olympus IX81 fluorescence microscope with CellSens

Dimensions software (Olympus, Japan) with TRITC at 532 nm (for DHE, HIF-1α staining) or FITC at 488 nm (for nitrotyrosine, DAF-FM and CD31 staining) wavelength, respectively. Three representative pictures of the placental labyrinth zone were randomly taken from each of the tissue section at 20X magnification. All pictures were corrected to the blank controls (i.e. samples without DHE, or samples incubated only with secondary antibodies) to remove background staining. Fluorescent images were analyzed using ImageJ 1.48 software (National Institutes of Health, Bethesda, MD, USA) to determine mean fluorescence intensity (MFI). MFI values from the DHE, nitrotyrosine and pimonidazole staining were normalized to the nuclei counts per image. The average MFI of the three representative images per sample per experimental group was taken.

For placental morphological analysis, images were taken with a digital camera mounted on a bright field microscope (EVOS XL Core Imaging System, Thermo Fisher Scientific, Canada) at a 2X magnification. For the other assessments, three randomly selected representative fields from each placenta were obtained at a magnification of 40X. Then, using Image J software, maternal and fetal blood space area in each field of view was converted into black and remaining placental tissue into white for quantification. Total area of maternal and fetal blood spaces per field of view were calculated using Image J software and the values were averaged per experimental group. Briefly, images were opened in Image J and converted into 16 bit binary images. Following which a threshold was set automatically by the program, which converted the maternal and fetal blood space area into black and remaining placental tissue into white. Particle count of maternal and fetal blood space was analyzed on ImageJ using the option of “analyze particles” in the software which resulted in a surface area value for the black space, i.e. the placental blood space.

2.2.9 Real-time RT-PCR for placental gene expression of IGF and VEGFA

Total RNA was isolated from the placental labyrinth using RNeasy plus Mini Kit (QIAGEN

Inc., Ontario, Canada). Total RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (AB Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Using gene specific primers for *Igf2, Igf2P0, Igf1r, Igf2r, Vegfa* quantitative real time RT-PCR (qPCR) was performed using iQ™ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) (See Table 1 for primers). Briefly, thermal cycling was initiated by a 5 min. denaturation at 95ºC, followed by 40 cycles of 95ºC for 30s, annealing at 60ºC for 15s, and 72ºC for 30s. Samples without reverse transcriptase (RT) using the same PCR primers were done as a control for presence of genomic DNA. The gene expression levels in each sample (absolute quantification) were calculated from the standard curve (for each primer set) and normalized to rat Cyclophilin A (PPIA) expression.

2.2.10 Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.04 software (GraphPad Software, U.S.A.). All data are expressed as mean ± S.E.M. All data were analyzed using a two-way ANOVA, with hypoxia and nMitoQ treatment as the two independent factors, followed by Sidak's multiple comparison post hoc tests. A value of $p<0.05$ was considered significant.

Gene	Forward primer (5’ – 3’)	Reverse primer (5’ – 3’)
Igf2	TGT CTA CCT CTC AGG CCG TAC TT	TCC AGG TGT CGA ATT TGA AGA A
Igf2P0	GAT CAT CGT CCA GGC AAT TT	GTT GCG TAG TTC CCG AAG TT
Igf1r	AAG GAT GGC GTC TTC ACC A	GAG TGG CGA TCT CCC AGA G
Igf2r	CTG CAG GCG GGA AAG	TTC CAC TCT TAT CCA CAG CAC
Vegfa	GTG CAC TGG ACC CTG GCT TT	TTC ACC ACT TCA TGG GCT TTC TG
Ppia	AGC ATA CAG GTC CTG GCA TC	TTC ACC TTC CCA AAG ACC AC

Table 2.1: Quantitative real-time PCR primers.

2.3 RESULTS

2.3.1 Offspring and placental characteristics

In male fetuses, prenatal hypoxia decreased fetal weight and abdominal girth without affecting placental weight (Table 2.2). nMitoQ treatment increased male abdominal girth in the prenatal hypoxia exposed group, while no effects of nMitoQ treatment were observed on fetal or placental weights in males (Table 2.2). Prenatal hypoxia reduced placental efficiency, expressed as ratio of fetal weight/placental weight in male fetuses, which was not significantly improved by nMitoQ treatment, however, placental efficiency in the hypoxia/nMitoQ group was no longer significantly different than normoxia/saline controls (Table 2.2), suggesting an effect of nMitoQ treatment. Crown-to-rump length was similar between all experimental groups in male fetuses (Table 2.2).

Variables	Normoxia		p-Hypoxia		Main Effect		
	Saline	nMitoQ	Saline	nMitoQ	p-Hypoxia	nMitoQ	Interaction
Fetal weight (g)	5.81±0.24	5.6±0.15	5.21±0.19	5.13±0.26	*	-	-
Placental weight (g)	0.61±0.04	0.58±0.03	0.61±0.02	0.62±0.03	-	-	-
Fetal weight / Placental weight	9.05±0.39	9.76±0.50	7.96±0.78	8.69±0.56	-	-	-
Abdominal girth (cm)	3.87±0.08	3.72±0.05	3.32±0.13	3.82±0.13†	*	-	**
Crown-rump length (cm)	4.78±0.09	4.92±0.12	4.47±0.18	4.82±0.13	-	-	-

Table 2.2: **Fetal and placental characteristics of male offspring.** Body weight, placental weight, placental efficiency (body weight/placental weight), abdominal girth, crown-rump length from male fetuses collected on GD21. Data are represented as mean ± SEM. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test. n= 6 dams/group. *$p<0.05$ overall group effect of prenatal hypoxia. †$p<0.05$ versus corresponding hypoxia-saline group.

In female fetuses, prenatal hypoxia had no effect on fetal weight or abdominal girth but increased placental weight, which was significantly reduced by nMitoQ treatment (Table 2.3). nMitoQ treatment had no effect on female fetal weight or abdominal girth (Table 2.3). Prenatal hypoxia reduced placental efficiency, expressed as ratio of fetal weight/placental weight in female fetuses, which was not significantly improved by nMitoQ treatment, however, placental efficiency in the hypoxia/nMitoQ group was no longer significantly different normoxia/saline controls (Table 2.3). Crown-to-rump length was similar between all experimental groups in female fetuses (Table 2.3).

Variables	Normoxia		p-Hypoxia		Main Effect		
	Saline	nMitoQ	Saline	nMitoQ	p-Hypoxia	nMitoQ	Interaction
Fetal weight (g)	5.16±0.29	5.81±0.14	5.17±0.16	5.26±0.09	-	-	-
Placental weight (g)	0.56±0.01	0.59±0.01	0.62±0.02	0.56±0.01†	-	-	*
Fetal weight / Placental weight	9.06±0.54	9.7±0.31	7.7±0.62	8.72±0.44	-	-	-
Abdominal girth (cm)	3.67±0.11	3.77±0.13	3.38±0.13	3.7±0.12	-	-	-
Crown-rump length (cm)	4.7±0.15	4.8±0.10	4.56±0.14	4.63±0.07	-	-	-

Table 2.3: Fetal and placental characteristics of female offspring. Body weight, placental weight, placental efficiency (body weight/placental weight), abdominal girth, crown-rump length from female fetuses collected on GD21. Data are represented as mean ± SEM. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test. n= 6 dams/group. *$p<0.05$ overall group effect of prenatal hypoxia. †$p<0.05$ versus corresponding hypoxia-saline group.

2.3.2 nMitoQ treatment improved placental oxidative/nitrosative stress in female offspring

Superoxide and peroxynitrite levels were assessed as markers of oxidative/nitrosative stress. In male offspring, superoxide levels were significantly increased in offspring exposed to prenatal hypoxia compared to normoxic control offspring (Figure 2.2A+B). nMitoQ treatment significantly decreased placental superoxide generation in prenatally hypoxic male offspring and had no effect in the control group (Figure 2.2A+B). Nitrotyrosine levels (Figure 2.2C+D) or nitric oxide levels (Figure 2.2E+F) were not affected by prenatal hypoxia or nMitoQ treatment in placentae from male offspring.

In female offspring, superoxide levels significantly increased in offspring exposed to prenatal hypoxia was significantly decreased by nMitoQ treatment in prenatally hypoxic offspring and had no effect in the control group (Figure 2.3A+B). Nitrotyrosine levels tended to be increased in the placentae of prenatally hypoxic female offspring (Figure 2.3C+D). Moreover, there was a significant interaction between nMitoQ treatment and prenatal hypoxia exposure in which nMitoQ treatment decreased nitrotyrosine levels in placentae of only female hypoxic offspring (Figure 2.3C+D). However, hypoxia significantly increased nitric oxide levels in placentae from female offspring but there was no effect of nMitoQ treatment (Figure 2.3E+F).

Male Placentae

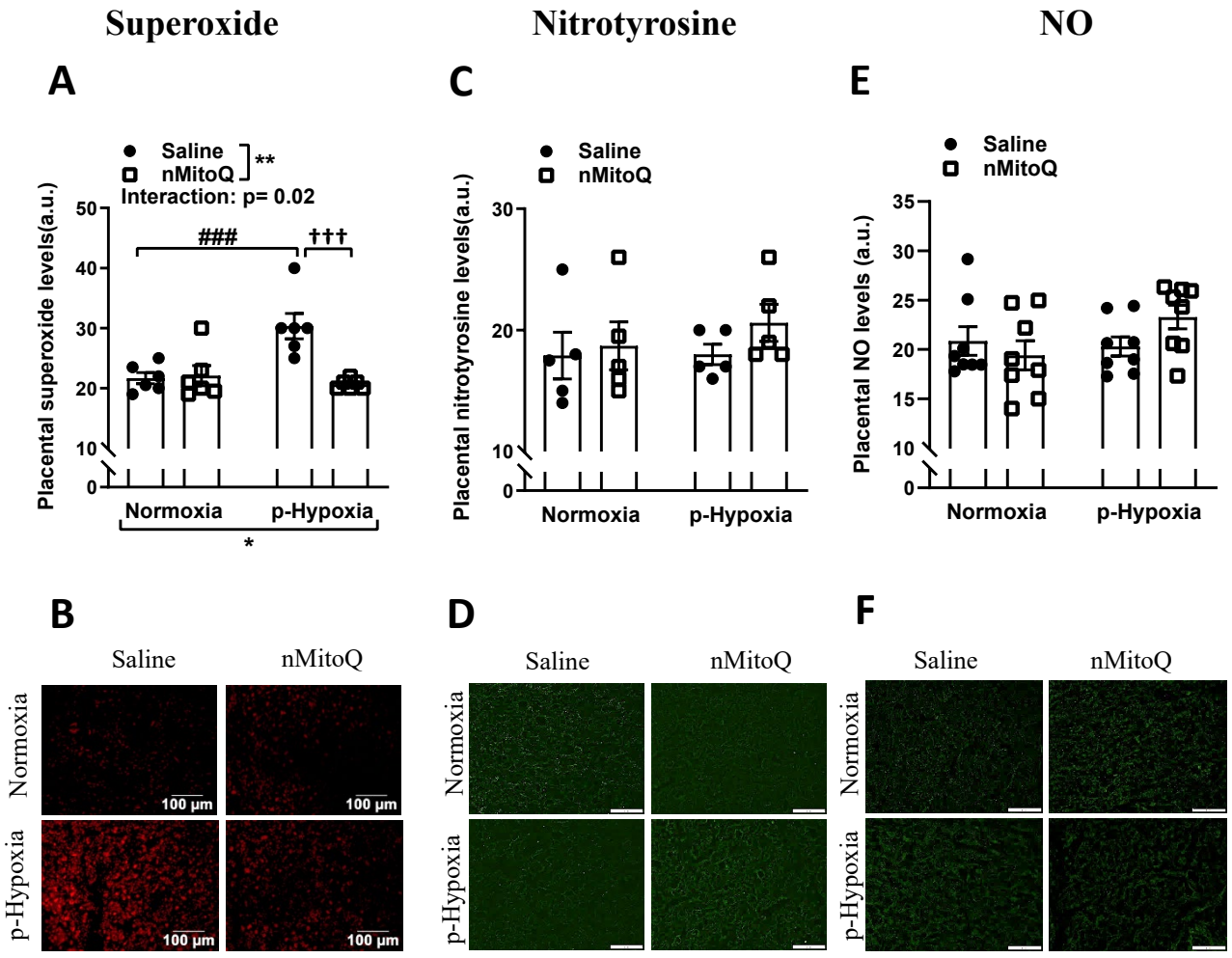

Figure 2.1. Effects of nMitoQ treatment on placental superoxide, peroxynitrite and NO levels in normoxic and hypoxic placenta of male offspring. Normoxic and hypoxic dams were treated with nMitoQ or saline and superoxide levels were assessed by DHE staining in male placentae (**A+B**), peroxynitrite levels were detected by staining for nitrotyrosine (the footprint of peroxynitrite production) in male placentae (**C+D**) and NO levels were assessed by DAF-FM staining in male placentae (**E+F**) on GD21. Data are represented as mean ± SEM. a.u.: arbitrary units. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test (n= 6-7/group). *p<0.05, **p<0.01 group effect of prenatal hypoxia or nMitoQ treatment, $^{###}$p<0.001 compared to normoxia-saline, †††p<0.001 compared to hypoxia-saline group.

Female Placentae

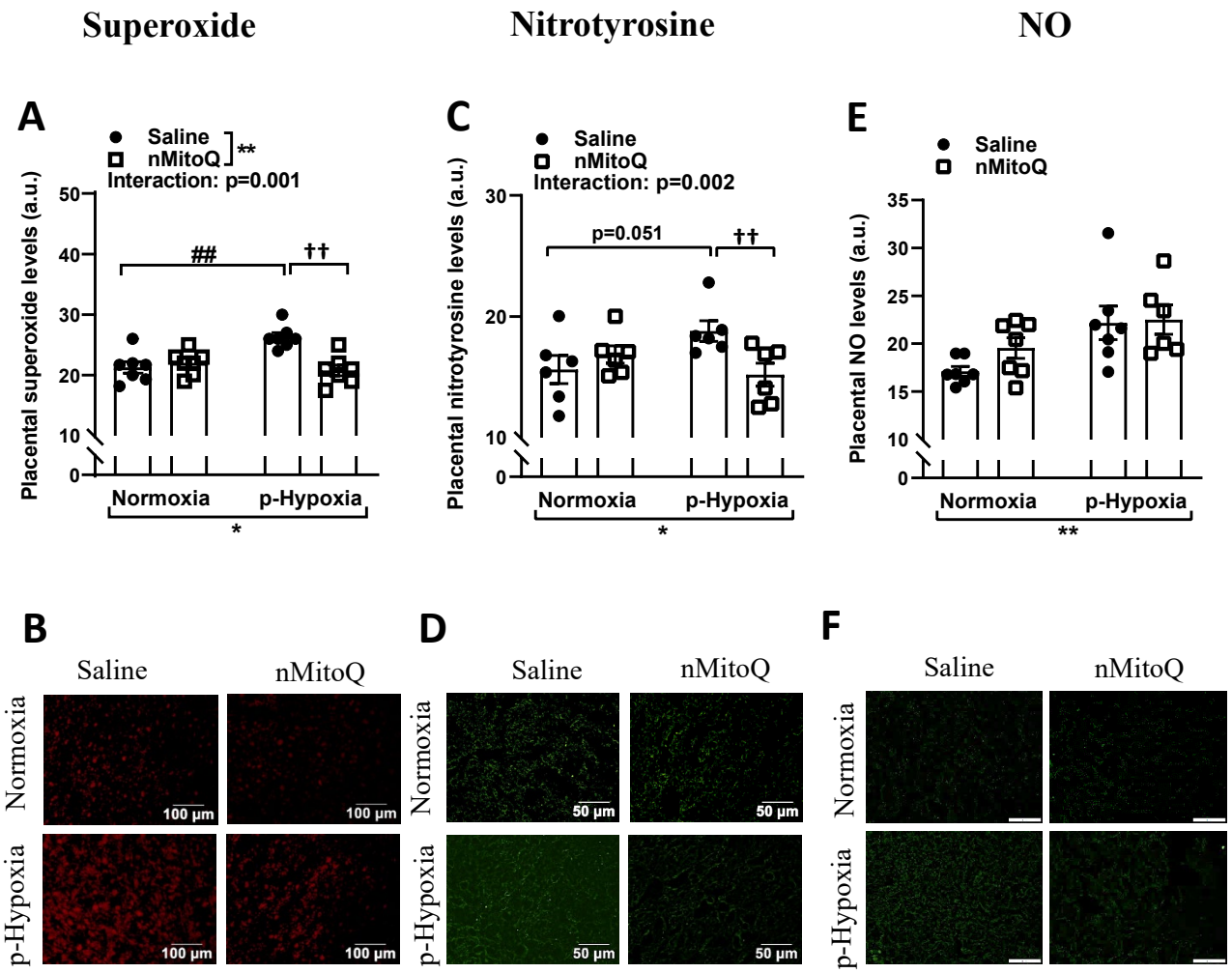

Figure 2.2. Effects of nMitoQ treatment on placental superoxide, peroxynitrite and NO levels in normoxic and hypoxic placenta of female offspring. Normoxic and hypoxic dams were treated with nMitoQ or saline and superoxide levels were assessed by DHE staining in male placentae (**A+B**), peroxynitrite levels were detected by staining for nitrotyrosine (the footprint of peroxynitrite production) in male placentae (**C+D**) and NO levels were assessed by DAF-FM staining in male placentae (**E+F**) on GD21. Data are represented as mean ± SEM. a.u.: arbitrary units. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test (n= 6-7 dams/group). *p<0.05, **p<0.01 group effect of prenatal hypoxia or nMitoQ treatment, $^{\#\#}$p<0.01 compared to normoxia-saline, ††p<0.01 compared to hypoxia-saline group.

2.3.3 nMitoQ treatment decreased markers of placental and fetal hypoxia in female offspring

We next assessed HIF1-α protein levels as a marker for tissue hypoxia and confirmed placental oxygenation levels by pimonidazole staining. HIF1-α expression was significantly increased in prenatal hypoxia-exposed placentas from male offspring, which was not reduced by nMitoQ treatment (Figure 2.4A+B). There was a significant interaction between nMitoQ treatment and prenatal hypoxia in male placentae, whereby nMitoQ increased HIF1-α expression in placentae from normoxic male offspring (Figure 2.4A+B). Placental oxygenation was decreased (as shown by increased pimonidazole staining) in placentae of male offspring which was unaffected by nMitoQ treatment (Figure 2.4C+D). Prenatal hypoxia decreased oxygenation in fetal hearts of only male offspring, while nMitoQ increased oxygenation in only prenatally hypoxic hearts of male offspring (Figure 2.4E+F). Hepatic oxygenation was reduced in livers of male offspring but nMitoQ treatment had no effect (Figure 2.4G+H).

Prenatal hypoxia significantly increased placental HIF1-α expression in female offspring (Figure 2.5A+B). nMitoQ treatment decreased HIF1-α expression in placentae from female offspring exposed to prenatal hypoxia (Figure 2.5A+B). There was a significant interaction between nMitoQ treatment and prenatal hypoxia in female placentae, whereby nMitoQ increased HIF1-α expression in placentae from normoxic female offspring (Figure 2.5A+B). Placental oxygenation was also decreased (as shown by increased pimonidazole staining) in placentae of female offspring exposed to prenatal hypoxia (Fig 2.5C+D). nMitoQ treatment significantly increased oxygenation in placenta of only female offspring exposed to prenatal hypoxia (Figure 2.5C+D). There was no effect of prenatal hypoxia on oxygenation in fetal hearts of female offspring while nMitoQ increased oxygenation in hearts of female offspring (Figure 2.5E+F). Hepatic oxygenation was

reduced in livers of prenatally hypoxic female offspring, and nMitoQ treatment increased oxygenation in livers of only female offspring exposed to prenatal hypoxia (Figure 2.5G+H).

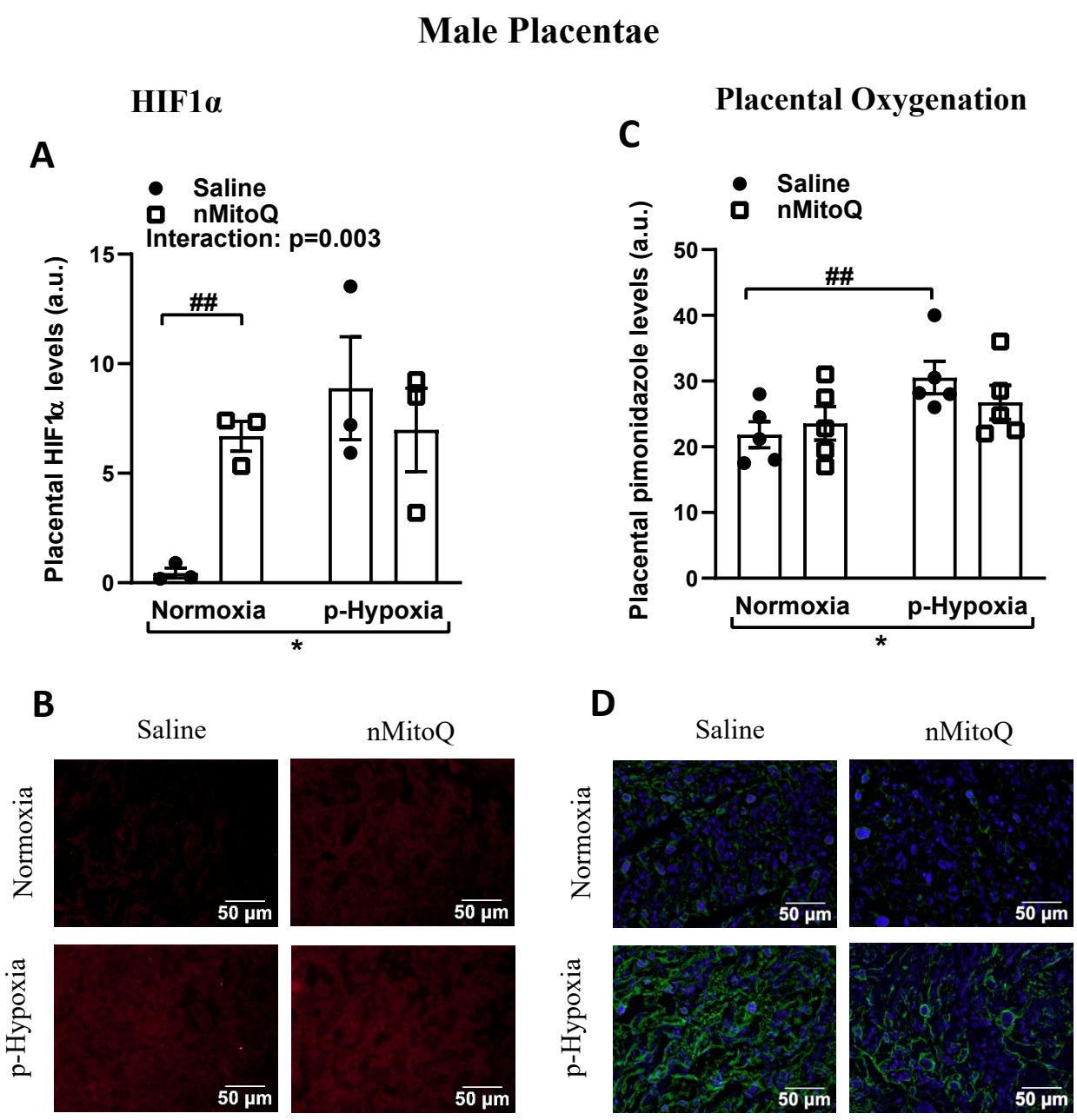

Figure 2.3. Effects of nMitoQ treatment on marker of hypoxia and oxygenation levels in normoxic and hypoxic placentae of male offspring. Expression of HIF1-α protein, a marker for tissue hypoxia (**A+B**) and oxygenation levels as assessed by pimonidazole staining (**C+D)** in placentae obtained from male fetuses on GD21. Data are represented as mean ± SEM. a.u.: arbitrary units. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test (n= 5-6 dams/group). *p<0.05 group effect of prenatal hypoxia and nMitoQ treatment, $^{\#\#}$p<0.01 compared to normoxia-saline.

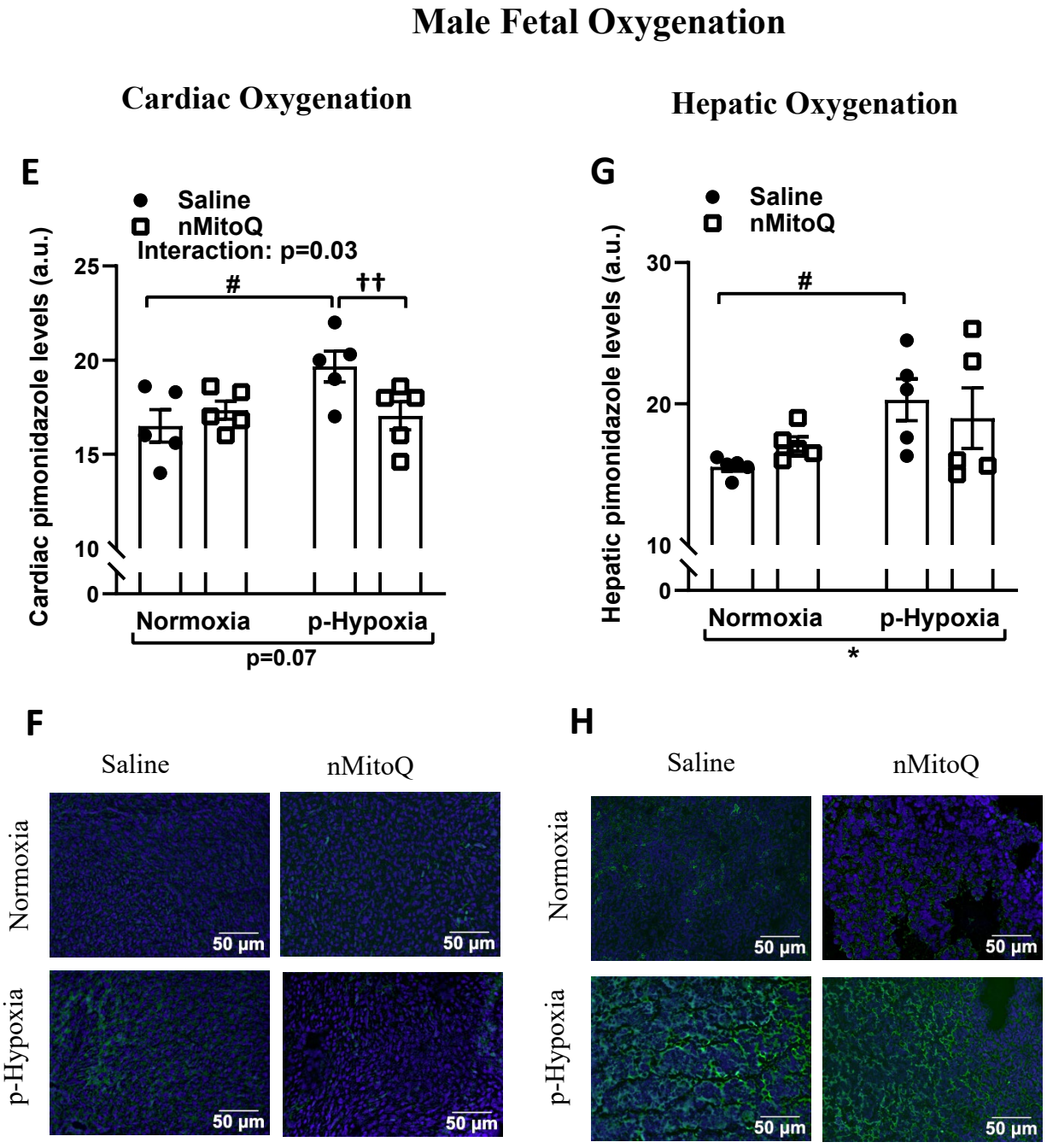

Figure 2.3. Effects of nMitoQ treatment on cardiac and hepatic oxygenation levels in normoxic and hypoxic male fetuses. Oxygenation levels as assessed by pimonidazole staining in cardiac tissues **(E+F)** and hepatic tissues of **(G+H)** male fetuses on GD21. Data are represented as mean ± SEM. a.u.: arbitrary units. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test (n= 5-6 dams/group). *$p<0.05$ effect of prenatal hypoxia and nMitoQ treatment, $^{\#}p<0.05$ compared to normoxia-saline, $^{\dagger\dagger}p<0.01$ compared to hypoxia-saline group.

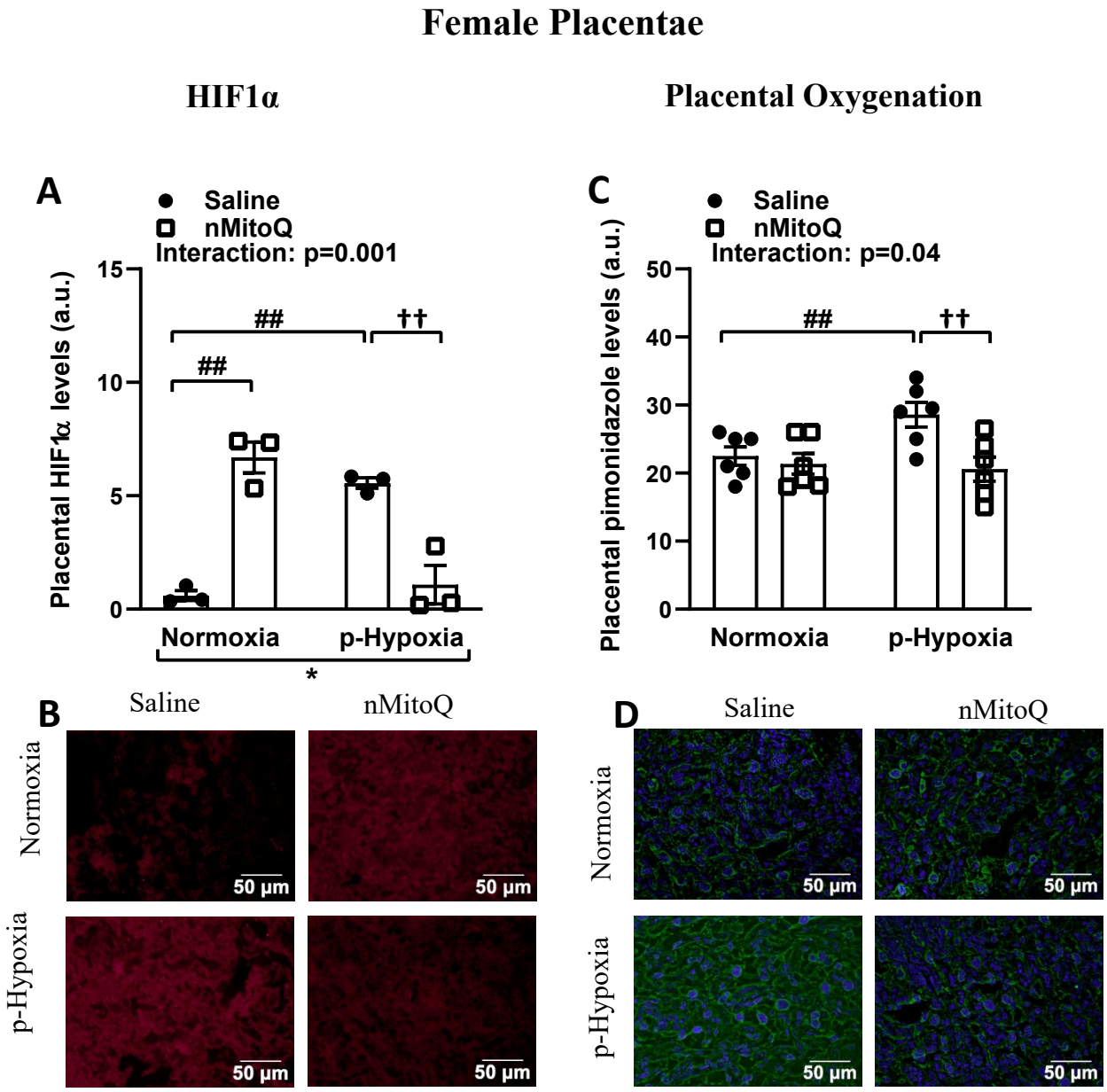

Figure 2.4. Effects of nMitoQ treatment on marker of hypoxia and oxygenation levels in placentae of normoxic and hypoxic female offspring. Expression of HIF1-α protein, a marker for tissue hypoxia (**A+B**) and oxygenation levels as assessed by pimonidazole staining (**C+D)** in placentae obtained from male fetuses on GD21. Data are represented as mean ± SEM. a.u.: arbitrary units. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test (n= 5-6 dams/group). *$p<0.05$ group effect of prenatal hypoxia and nMitoQ treatment, $^{\#}p<0.01$ compared to normoxia-saline, $^{\dagger\dagger}p<0.01$ compared to hypoxia-saline group.

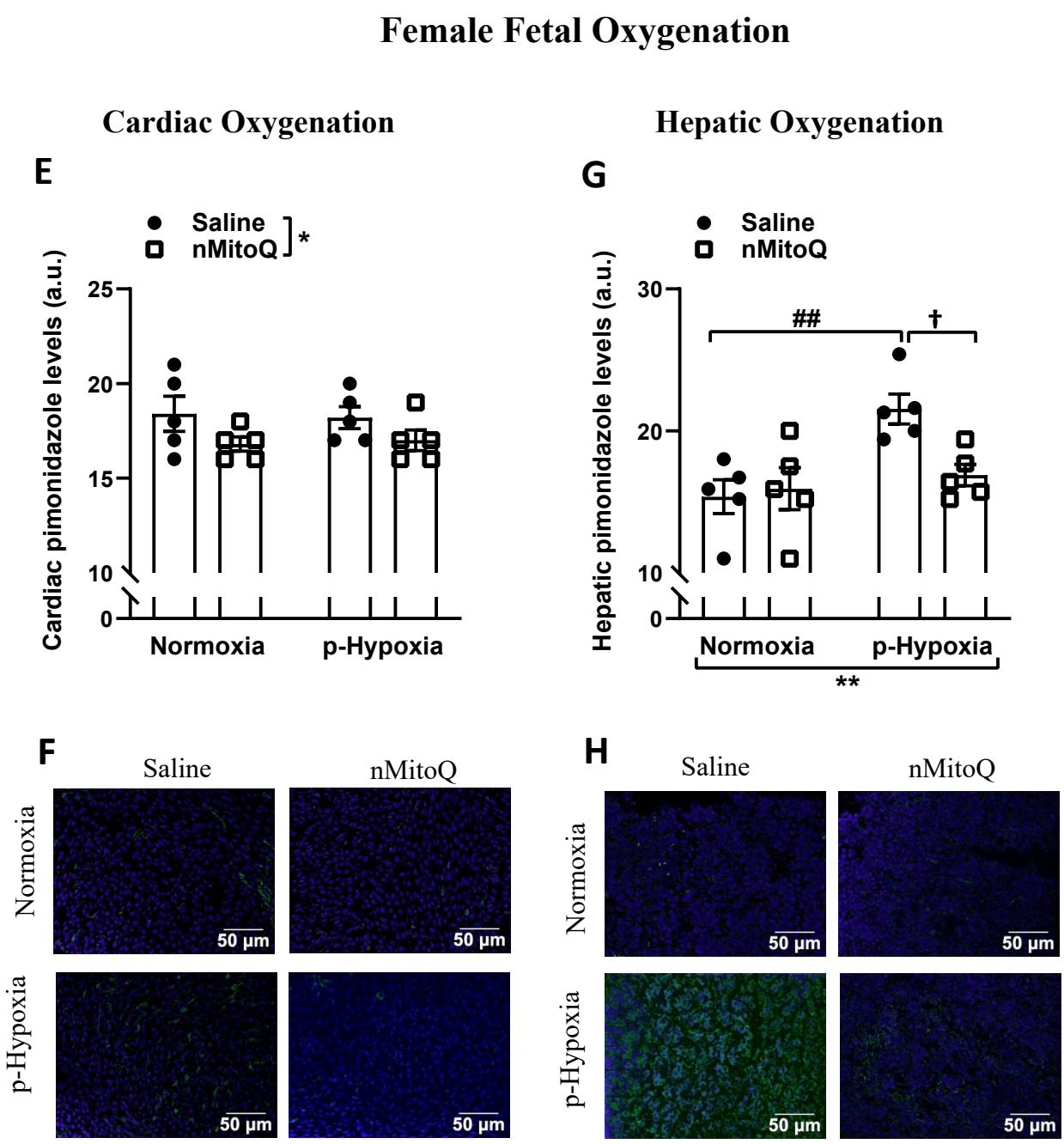

Figure 2.4. Effects of nMitoQ treatment on cardiac and hepatic oxygenation levels in normoxic and hypoxic female fetuses. Oxygenation levels as assessed by pimonidazole staining in cardiac tissues (**E+F)** and hepatic tissues (**G**+**H)** of female fetuses on GD21. Data are represented as mean ± SEM. a.u.: arbitrary units. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test (n= 5-6 dams/group). *p<0.05, **p<0.01 group effect of prenatal hypoxia and nMitoQ treatment, $^{\#\#}$p<0.01 compared to normoxia-saline, †p<0.05 compared to hypoxia-saline group.

2.3.4 nMitoQ treatment increased angiogenesis and vascularization in placentae of female offspring

Placental hypoxia is commonly associated with altered expression of the placental pro-angiogenic peptide vascular endothelial growth factor (VEGF) (233, 234). In male offspring, prenatal hypoxia decreased placental *Vegfa* mRNA expression, which was not altered by nMitoQ treatment (Figure 2.6A). Prenatal hypoxia also reduced CD31-positive area of staining (i.e. the fetal capillaries) (Figure 2.6B). There was a significant interaction between nMitoQ treatment and prenatal hypoxia in male placentae, whereby nMitoQ reduced both *Vegfa* and CD31 staining in placentae from normoxic male offspring (Figure 2.6A+B).

In female offspring, prenatal hypoxia decreased placental *Vegfa* mRNA expression, which was increased by nMitoQ treatment in placentae from prenatally hypoxic female offspring (Figure 2.6C). CD31-positive area of staining (i.e. the fetal capillaries) was reduced by prenatal hypoxia in placentae from female offspring (Figure 2.6D). Similar to the *Vegfa* expression pattern, there was a significant interaction between nMitoQ treatment and prenatal hypoxia in female placentae, whereby nMitoQ increased CD31 staining only in the placentae from prenatal hypoxic female offspring (Figure 2.6C+D).

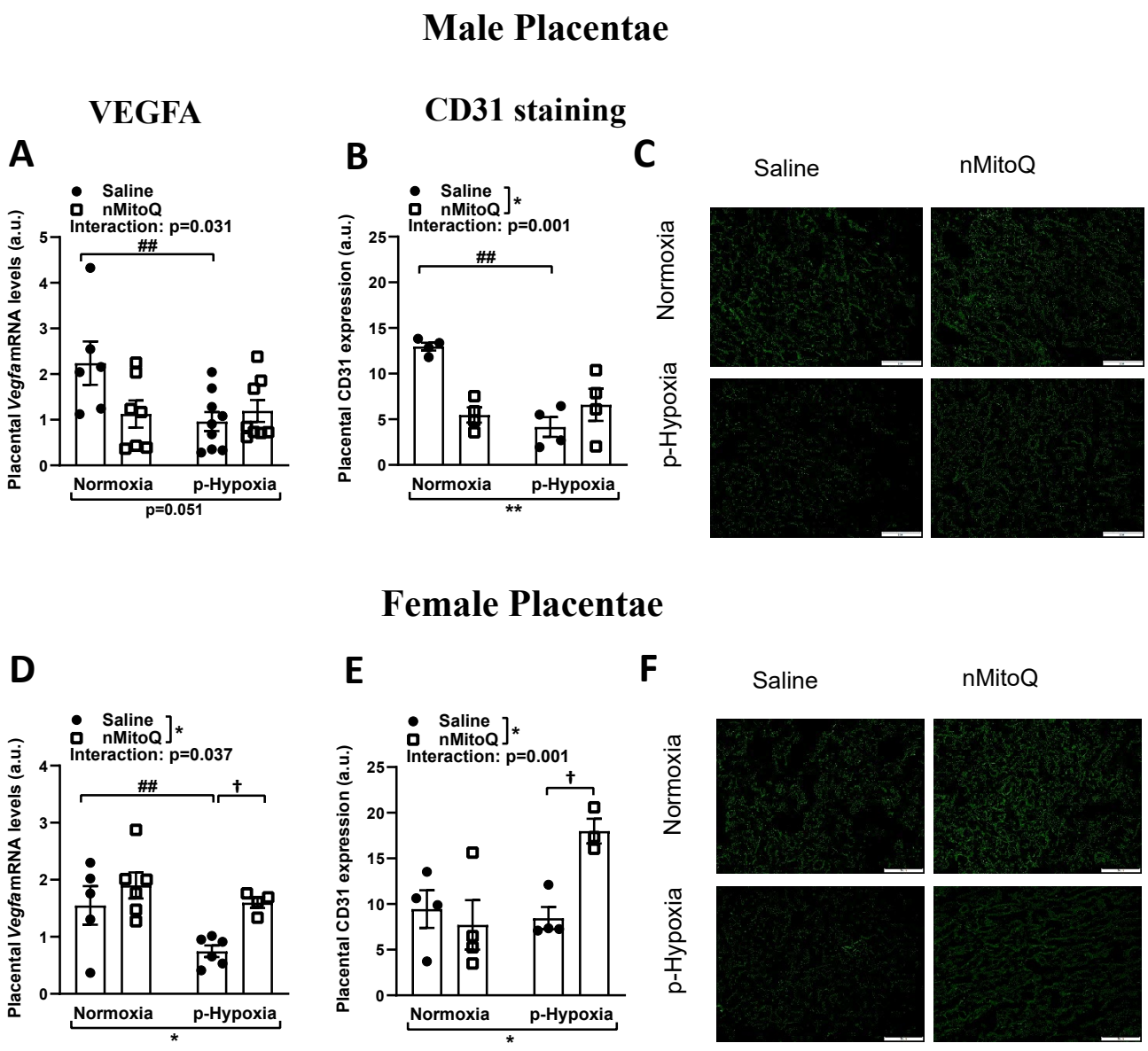

Figure 2.5. Effects of nMitoQ treatment on markers of angiogenesis and vascularization in normoxic and hypoxic placentae of male and female offspring. Proangiogenic factor *Vegfa* mRNA levels were assessed by qPCR in placental tissue obtained from male (**A**) and female (**C**) fetuses on GD21. Feto-placental vascular capillaries as assessed by CD31 staining in placentae obtained from male (**B**) and female (**D**) fetuses. Representative images of CD31 stained placental labyrinth sections in placentae of male (**C**) and female offspring (**F**). Data are represented as mean ± SEM. a.u.: arbitrary units. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test (n= 5-9 dams/group). p=0.051, *p<0.05, **p<0.01 group effect of prenatal hypoxia and nMitoQ treatment, $^{\#\#}$p<0.01 compared to normoxia-saline, †p<0.05 compared to hypoxia-saline group.

Sufficient maternal and fetal blood space in the placental labyrinth zone is essential for oxygen and nutrient exchange between the maternal and fetal circulations (31). We found that prenatal hypoxia reduced maternal and fetal blood space area in placentae from only male offspring (Figure 2.7A+B). nMitoQ did not change maternal and fetal blood space area in placentae from males (Figure 2.7A); however, in female offspring, nMitoQ treatment increased fetal blood space area in placentas exposed to prenatal hypoxia (Figure 2.7C+D).

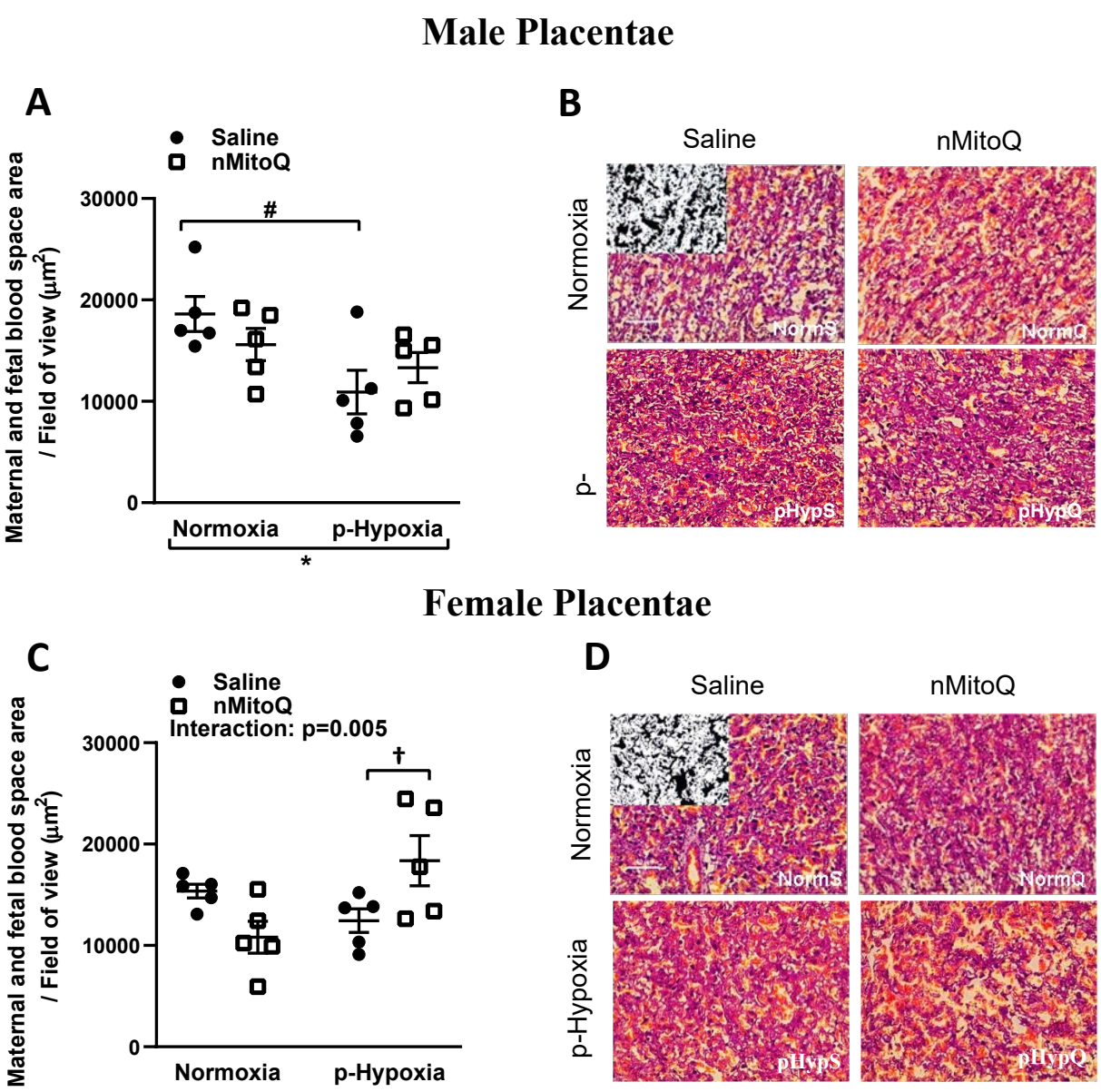

Figure 2.6. Effects of nMitoQ treatment on maternal and fetal blood space area in normoxic and hypoxic placentae of both male and female offspring. Maternal and fetal blood space area per field of view in labyrinth zones of placenta obtained from male (**A**) and female (**C**) fetuses on GD21. Representative images of H&E stained placental labyrinth sections in placentae of males (**B**) and females (**D**). Inset: Image J was used to convert maternal and fetal blood space area into black and remaining placental tissue white for quantification. Total area of maternal and fetal blood spaces per field of view were calculated using Image J software and the values were averaged per experimental group. Data are represented as mean ± SEM. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test (n= 5/group). *$p<0.05$ group effect of prenatal hypoxia, $^{\#}p<0.05$ compared to normoxia-saline, $^{\dagger}p<0.05$ compared to hypoxia-saline group. **Data acquisition and data analysis: Mais Aljunaidy**

2.3.5 nMitoQ treatment increased placental Igf2 in female offspring

IGF2 plays an important role in placental development (235), and total placental *Igf2,* and *Igf2* expressed only in the placental labyrinth region (i.e. *Igf2P0*), were shown to regulate the nutrient exchange characteristics of the placenta (97). In male offspring, prenatal hypoxia decreased total *Igf2* and placental-specific *Igf2* expression, which was not affected by nMitoQ treatment (Figure 2.8A+B). In placentae from female offspring, total *Igf2* and placental-specific *Igf2* expression was significantly decreased by prenatal hypoxia (Figure 2.8C+D). Moreover, nMitoQ treatment increased *Igf2* expression in the prenatal hypoxia-exposed female placentae only (Fig 2.8C). Expression levels of the IGF2 receptors, *Igf1r* and *Igf2r*, were decreased in placentae of both prenatally hypoxic male and female offspring, but were not affected by nMitoQ treatment (Figure 2.9A-D).

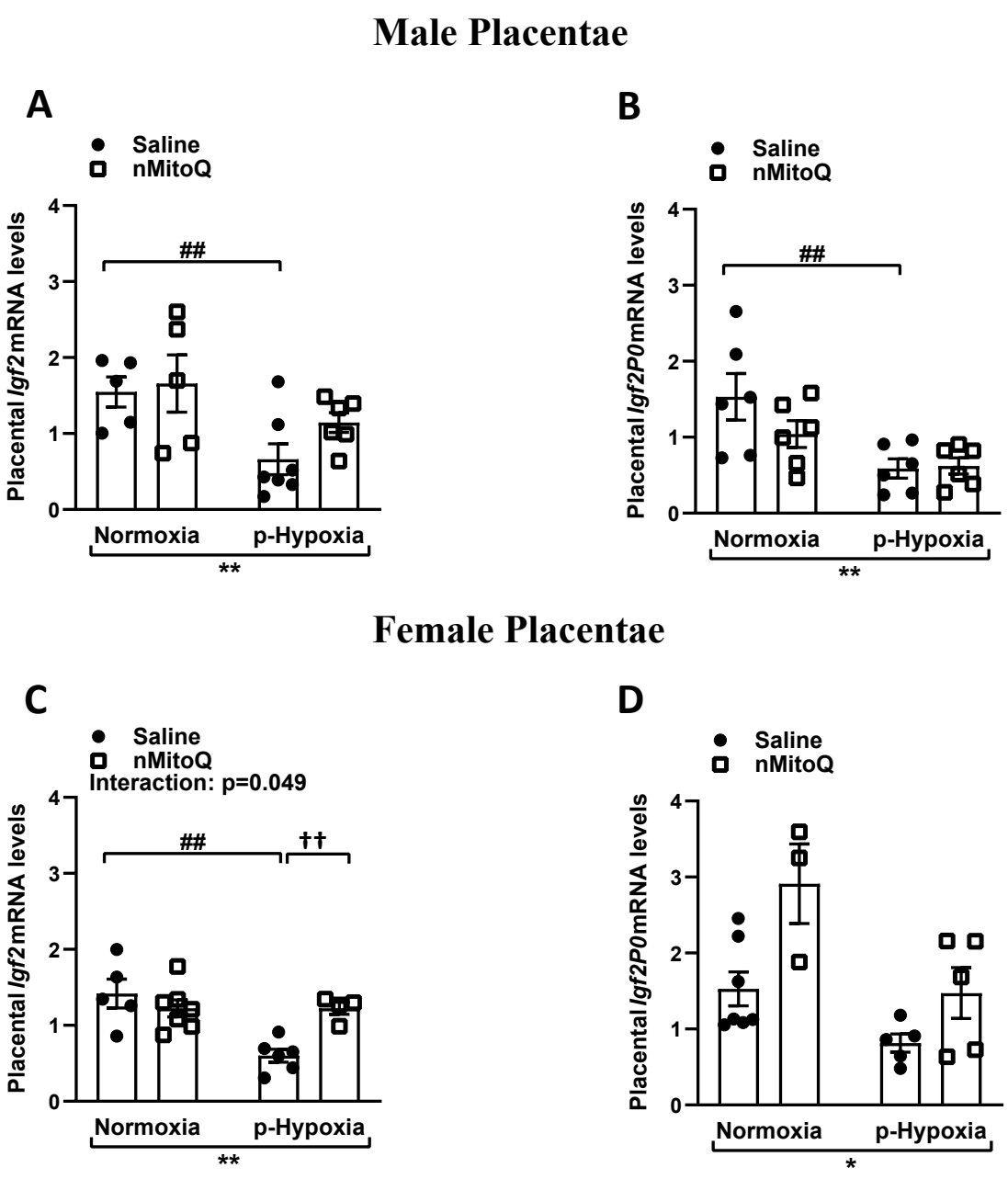

Figure 2.7. Effects of nMitoQ on placental *Igf2* mRNA expression in normoxic and hypoxic placentae of both male and female offspring. Growth factor *Igf2* (**A**+**C**) and *Igf2p0* (**B**+**D**) mRNA levels as assessed by qPCR in placentae obtained from male (**A**+**B**) and female (**C**+**D**) fetuses on GD21. Data are represented as mean ± SEM. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test (n=5-9 dams/group). *p<0.05, **p<0.01 group effect of prenatal hypoxia, $^{\#\#}$p<0.01 compared to normoxia-saline, ††p<0.01 compared to hypoxia-saline group.

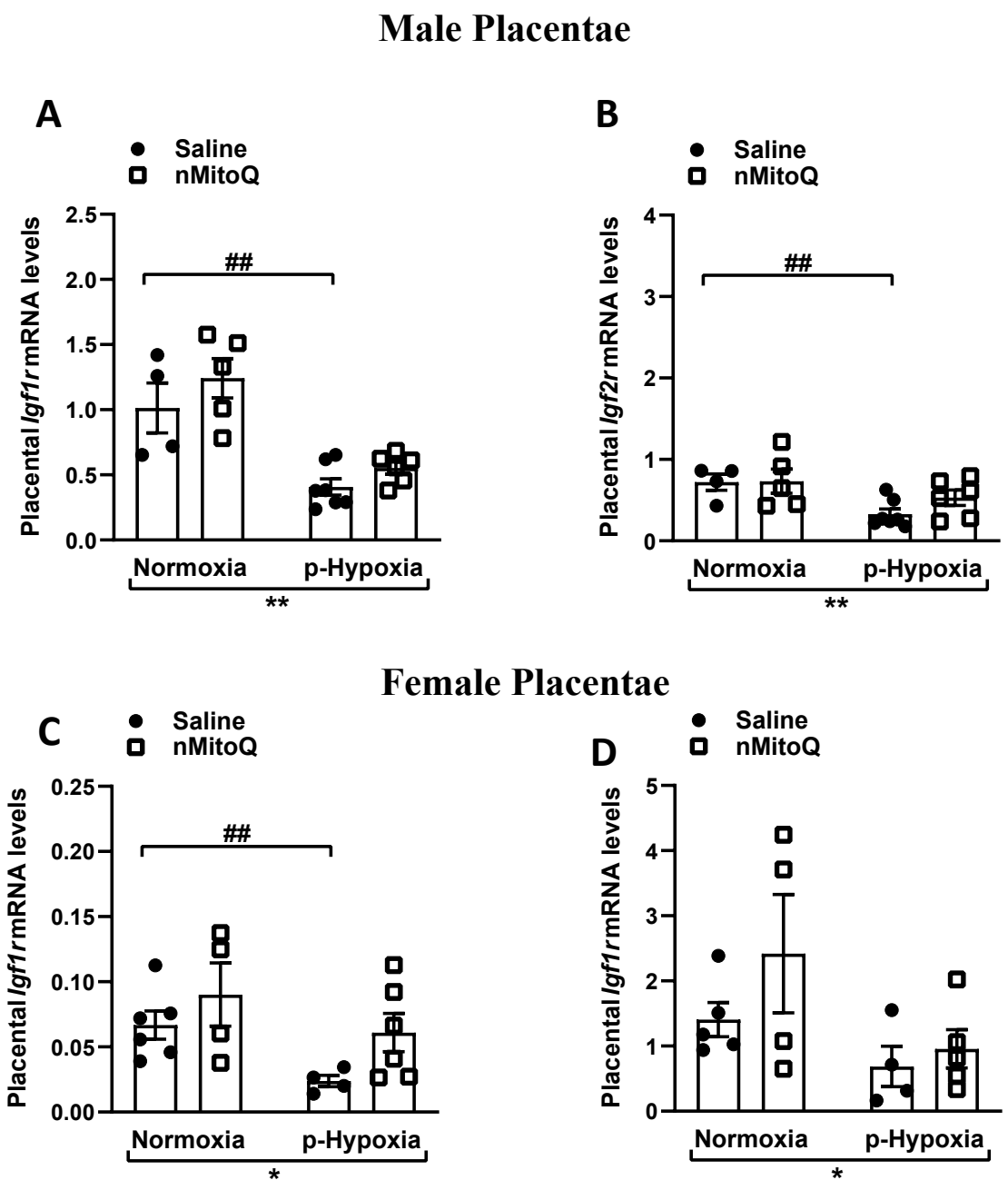

Figure 2.8. Effects of nMitoQ treatment on *Igf1r* and *Igf2r* mRNA expression in normoxic and hypoxic placentae of both male and female offspring. Receptors for IGF2, *Igf1r* (A+C) and *Igf2r* (B+D) mRNA levels were assessed by qPCR in the labyrinth zones of placentae taken from male (A+B) and female (C+D) fetuses on GD21. Data are represented as mean ± SEM. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test (n=4-5 dams/group). *p<0.05, **p<0.01 group effect of prenatal hypoxia or nMitoQ treatment, ##p<0.01 compared to normoxia-saline.

2.4 Discussion

We showed that a reduction in placental ROS/RNS by nMitoQ treatment improved oxygenation, increased expression of VEGFA (known to affect placental morphology) and increased expression of *Igf2* in placentae of female offspring. Moreover, nMitoQ treatment was more effective in placentae of female offspring compared with males. Our data demonstrated that the placental response to prenatal hypoxia is different in males versus females, and that the response of the placentae to nMitoQ treatment is sexually dimorphic, resulting in sex differences in the effect of nMitoQ treatment on placental oxidative stress, vascularization and placental and fetal growth.

Given that oxidative stress is a central mediator of placental dysfunction, we and others have studied the use of an antioxidant to protect the placenta against oxidative stress (135, 156). Recently, the antioxidant benefits of MitoQ treatment on placental adaptations from hypoxic pregnancies was extensively studied in male offspring (135). Nuzzo *et al.* showed that MitoQ (not encapsulated within nanoparticles) given daily to hypoxic pregnant dams in drinking water from GD 6 to GD 20 restored placental efficiency to control levels, increased absolute placental volume, fetal capillary surface area in placental labyrinth zone and maternal blood spaces relative to placentae from normoxic pregnancies (135). The placental morphological adaptations (i.e. increased placental weight, increased fetal capillary surface area) to early-onset hypoxia (GD 6 – GD 20) demonstrated in males were contrary to the placental morphological phenotype (i.e. unchanged placental weight and reduced fetal capillary area) observed in our model of late-onset hypoxia (GD 15 – GD 21) in male offspring. Thus, diverse prenatal hypoxia insults can affect pregnancies differently, which needs to be taken into consideration for clinical translation of findings. In our study, using MitoQ encapsulated in nanoparticles (nMitoQ) to target the placenta in order to limit direct drug exposure to the fetus and avoid potential off-target effects during fetal development, we

also observed beneficial effects; however, nMitoQ treatment was more effective in placentae of female offspring compared with males.

In our model of prenatal hypoxia, placental efficiency (fetal weight/placental weight) was reduced in both male and female fetuses, which was partially improved by nMitoQ treatment in both males and females. Interestingly, fetal weight was reduced by prenatal hypoxia only in males but not females, indicating that males may be more severely affected by prenatal hypoxia than females, as has been suggested in other studies (146, 225, 226). This reduction in fetal weight suggests insufficient oxygen and nutrient delivery to the fetus in the hypoxia exposed dams. Interestingly, in females, prenatal hypoxia increased placental weight, which was prevented by nMitoQ treatment. This may suggest that females, in compensation for the hypoxic environment, increased placental growth in order to increase oxygen and nutrient supply to the fetus and prevent growth restriction; which was indeed successful, as fetal weights in the females were not affected by prenatal hypoxia.

We showed that prenatal hypoxia increased oxidative stress (superoxide generation in male and female placentae), as demonstrated previously (135, 138, 219), and nitrosative stress (peroxynitrite formation in only female placentae). Oxidative and nitrosative stress can inhibit normal placental development in various ways (e.g. inhibit mitochondrial electron transport and oxidation of DNA) and potentially reduce oxygen and nutrient delivery, thus impairing fetal development (68, 151). The nMitoQ treatment was therefore specifically designed to prevent oxidative stress in the placenta, and indeed nMitoQ treatment significantly decreased placental superoxide and peroxynitrite levels. This supports our hypothesis that using MitoQ loaded nanoparticles as a targeted delivery system to the placenta is an effective treatment against the generation of reactive oxygen/nitrogen species in the placenta in compromised pregnancies.

Interestingly, in female offspring, we observed that the increased superoxide/peroxynitrite levels by prenatal hypoxia coincided with increased NO levels. Under normal pregnancy conditions, NO bioavailability promotes feto-placental vasodilation and induces angiogenesis (236, 237). The placental circulation lacks autonomic and cholinergic innervation, thus feto-placental angiogenesis and placental vascular resistance and function is dependent on locally derived vasoactive factors (e.g. NO) and growth factors (e.g. VEGF and IGF2) (60, 79, 80). In the human placenta, all NOS isoforms are expressed, but differentially within the tissues. eNOS isoform is predominantly expressed in syncytiotrophoblasts and endothelial cells (reviewed by (86), while neuronal NOS (nNOS) and inducible NOS (iNOS) isoform is expressed in the placental smooth muscle cells, syncytiotrophoblasts, extravillous trophoblasts and hofbauer cells of the villous stroma (reviewed by (80). Moreover, studies have correlated increased placental vascular resistance and placental dysfunction to decreased eNOS and increased iNOS isoform expression, leading to increased nitrosative stress (87, 88). Interestingly, FGR has been associated with higher levels of placental NO, together with increased nitrosative stress and inadequate feto-placental vascularization (153, 238, 239). Therefore, the increased NO levels in female placentae may be an immediate adaptive mechanism to prenatal hypoxia, which may be associated with placental vascular dysfunction. Although I have not assessed NOS isoforms and the contribution of various cell types to the increased placental NO levels in females, the above studies may suggest that increased iNOS expression and/or activity could be a potential source for increased placental NO observed in our model of prenatal hypoxia.

We found that prenatal hypoxia decreased placental and fetal oxygenation (in males and females), which coincided with an increase in HIF-1α levels in both sexes. Systemic and placental responses to hypoxia are orchestrated by hypoxia-inducible factors (such as HIF-1α) and, being a

marker of hypoxia, this finding is in accordance with the reduced placental oxygenation we observed. Notably, in only the female prenatally hypoxic placentae, nMitoQ treatment effectively improved placental oxygenation. Moreover, the effect of prenatal hypoxia on oxygenation was more pronounced in the female fetal liver compared to the fetal heart, as previously reported by our laboratory but only in male offspring (15). Interestingly, nMitoQ treatment improved oxygenation in the hearts of both male and female fetuses, but in the liver, only female fetuses showed improved oxygenation with nMitoQ. Moreover, placental HIF-1α was only decreased by nMitoQ in the female placentae. This may explain why the female fetuses did not show any signs of significant growth restriction, as nMitoQ effectively decreased oxidative stress, HIF-1α and reduced placental weight in the female placentae. The underlying mechanisms for reduced effectiveness of nMitoQ treatment in males are not fully understood and remain to be further studied. A possible explanation might be that males show greater growth rate *in utero* (240), therefore the higher oxygen and nutrient demand by the male fetus may predispose them to a greater risk of adverse developmental outcomes following oxygen deprivation (241).

Oxidative stress is known to contribute to abnormal placental growth, function and angiogenesis (242). As mentioned above, the placenta lacks autonomic innervation, and locally derived NO, growth factors such as the proangiogenic factor VEGF, play essential roles in placental vascular development and function (60). HIF-1α (in hypoxic conditions) has been shown to increase VEGFA expression, while a reduction in VEGF has been observed in placentae from complicated pregnancies such as preeclampsia (93, 243). Therefore, placental angiogenesis is dependent on locally derived VEGFA. Our study showed that prenatal hypoxia decreased *Vegfa* mRNA expression and the area of fetal blood capillaries (measured by endothelial cell marker CD31) in placentae of both male and female offspring, suggesting decreased angiogenesis and

vascularization. The decreased *Vegfa* mRNA expression could be due to decreased binding of HIF-1α to the hypoxia responsive element (HRE) on the *Vegfa* gene, which was previously shown by Myatt et al. to decrease *Vegfa* expression in preeclamptic placentae with increased ROS (94). Interestingly, nMitoQ treatment only improved *Vegfa* mRNA expression, the area of fetal blood capillaries and the placental blood space in the female placentae but not in the male placentae. The specific mechanisms remain to be investigated but it may be speculated that this could be mediated in part by increased placental NO levels present in the prenatally hypoxic placentae, which were not affected by nMitoQ treatment and could increase VEGFA. However, the effects of NO on VEGFA expression vary for different tissues and cell types (93) therefore, NO mediated VEGFA expression within the placenta may be highly dependent on the different cell types. Our data are in accordance with previous studies, where MitoQ (not bound to nanoparticles) was shown to increase maternal blood space surface area in the placenta in males only (135). Taken together, our data could suggest that reduction of superoxide by nMitoQ could lead to enhanced angiogenesis via increased *Vegfa* expression and fetal capillary area and blood space in placentae of female offspring.

Another important growth factor for placental morphogenesis is IGF2 (95). In humans, decreased placental IGF2 has been associated with FGR (244). In mice, genetic knockouts of *Igf2* showed impaired placental development and fetal growth with reduced placental transfer of nutrients to the fetus (96, 98). We observed that prenatal hypoxia decreased expression of *Igf2* and *Igf2P0* in the labyrinth zone of both male and female offspring. This is in line with previous studies showing that maternal exposure to hypoxia has direct effects on total *Igf2* expression in the placenta: maternal exposure to 10-12% oxygen during late gestation decreased *Igf2* expression which affected the labyrinth layer morphology and decreased maternal blood space (100, 101). Hence the reduction in placental *Igf2* expression could also explain the decreased fetal blood spaces

by prenatal hypoxia that we observed. The expression of *Igf2* is regulated by methylation of the *Igf2* gene at the imprinting control region (ICR) and the differentially methylated region 2 (DMR2). Recent studies suggest that a suboptimal intrauterine environment leads to epigenetic changes in the *Igf2* gene and associated *Igf2* expression in growth restricted offspring of rats exposed to bilateral uterine artery ligation (245). Interestingly, hypoxic stress responses in general appear to be driven by epigenetic changes (246). For example, prenatal hypoxia increased placental ROS and DNA methylation enzymes (247). Therefore, we speculate that the methylation status at ICR and DMR2 may differ in a sex-specific manner, and changes in DNA methylation could account for the altered *Igf2* expression, which will be the focus for our future investigations.

It is well known that the placenta functions and adapts to an adverse intrauterine environment in a sex-specific manner (reviewed by (127)). Furthermore, generation of oxidative stress differs in both male and female fetuses and placentae under conditions of adverse prenatal stress (248). Our current study further demonstrates a dichotomous sex-specific and nMitoQ-specific effect on the placenta, and ultimately fetal development. Overall, our data suggested that nMitoQ treatment decreased oxidative/nitrosative stress, improved oxygenation, was effective in bringing *Vegfa* and *Igf2* expression back to control levels, and increased fetal blood space only in female placentae; thereby showing that nMitoQ treatment may protect offspring from the detrimental effects of a hypoxic insult in a sexually dimorphic manner with increased effectiveness in females. Of note, our data showed that nMitoQ treatment also affected the placenta in the control groups. For example, in normoxic controls, nMitoQ treatment increased HIF-1α expression in both male and female placentae, and reduced CD31 area in the male offspring. Previous studies have demonstrated that maternal antioxidant supplementation with ascorbic acid in normal pregnancies was associated with vascular dysfunction and weight gain (138). Therefore, continuing these studies

we need to keep in mind that this treatment is specifically designed to treat pregnancies complicated by fetal hypoxia and be aware that there might be detrimental effects of maternal antioxidant intervention during normal pregnancy.

To conclude, we demonstrated that nMitoQ treatment reduced placental nitrosative stress, improved oxygenation and placental morphology via increased VEGFA and IGF2 expression in a sex-specific manner; showing more effectiveness in placentae from female offspring. Moreover, our study shows that male fetuses appear to be more susceptible to an adverse *in utero* environment. Although the exact mechanism(s) remain to be further investigated, a higher pro-oxidant state with reduced antioxidant capacity in the male placentae may explain the increased susceptibility of the male offspring under adverse conditions. In addition, our current study illustrates that the placenta is a contributing factor in the sexual dimorphism that has been observed in fetal programming. Thus, sex differences will need to be taken into account when developing placental-targeted therapeutic interventions to prevent fetal hypoxia and ultimately optimize fetal development in complicated pregnancies.

CHAPTER 3:

Nanoparticle-Encapsulated Antioxidant Improves Placental Mitochondrial Function in a Sexually Dimorphic Manner in a Rat Model of Prenatal Hypoxia

A version of this chapter has been published:

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Author contributions: EG, **RK**, **FS**, **CLC**, **HL**, and **STD**; designed the research, **EG**, **RK**, and **CDH**; performed the research, **TP**,**CPC**, **MPM**; contributed to study reagents, **EG, FS, CDH, HL,** and **STD**; analyzed the data, **EG, FS**, and **STD**; drafted the manuscript, **EG, RK, FS, CDH, TP**, **CPC, CLC, MPM, HL, STD**; revised the manuscript.

3.1 Introduction

As discussed previously in Chapter 1, the placenta, being a highly metabolic organ meets its high energy demand by using oxygen to generate ATP via OXPHOS in the mitochondria (69, 249). ROS are a normal by-product of OXPHOS, making the mitochondria a major site of ROS generation in the placenta (122).

During normal pregnancy, the placental mitochondria produce ROS at physiological levels, which are essential for placental vascular responses and functions (70). However, placentae from pregnancies complicated by preeclampsia and fetal growth restriction showed evidence of increased placental oxidative stress (250, 251). Moreover, excessive generation of ROS and increased levels of hypoxia-inducible factor-1α (HIF-1α) in human placental tissues from pregnancies complicated by preeclampsia were associated with mitochondrial dysfunction (94). However, in pregnancies complicated by adverse maternal environments, such as prenatal hypoxia, excessive generation of ROS can negatively affect mitochondrial respiratory capacity (68, 252). A recent study demonstrated that prenatal hypoxia during late gestation in mice led to increased oxidative stress and decreased rates of mitochondrial oxygen consumption in the placental labyrinth zone (70). Placental tissues from hypoxic pregnancies in guinea pigs were shown to have increased nitrosative stress along with reduced expression and/or activity of the mitochondrial respiratory complexes I and IV, which was more pronounced in male versus female placentae (144). Together, these changes can impair normal placental function and potentially reduce nutrient and oxygen delivery to the fetus, thus affecting fetal organ development.

We have shown in the previous chapter that prenatal hypoxia can lead to increased oxidative and nitrosative stress, decreased placental and fetal oxygenation, and impaired placental

morphology via decreased expression of VEGFA and IGF2 along with reduced area of fetal capillaries in a sex-specific manner; all of which can affect normal placental function and potentially reduce oxygen and nutrient delivery, thus impairing fetal development. Since there is a sex-specific divergence in the placental response to prenatal hypoxia, in the current chapter I will investigate any potential sex-specific differences in the placental mitochondrial function in response to prenatal hypoxia.

Furthermore, mitochondrial fission and fusion also have a critical role in maintaining functional mitochondria when cells experience metabolic or environmental stresses (253). Previous studies have shown that oxidative stress in the preeclamptic placentae lead to increased mitochondrial fragmentation, which was associated with decreased expression of the pro-fusion fusion proteins OPA1 and MFN1, and increased expression of the fission protein Drp1. This suggests that increased oxidative stress in the placentae could potentially lead to a pro-fission environment and impaired mitochondrial function (122, 254) (71, 255). Therefore, in this chapter, we will further assess any potential changes in the expression of placental mitochondrial fusion and fission proteins in male and female placentae from dams exposed to prenatal hypoxia.

In the previous chapter, we showed that maternal treatment with placenta-targeted nMitoQ prevented oxidative stress and improved oxygenation and morphology in the placenta of a rat model of fetal hypoxia. Moreover, in our study, nMitoQ treatment was more effective in reducing the hypoxia-induced effects on placental oxidative stress in the female placentae. However, in this model, the possible mechanisms by which nMitoQ treatment improves placental morphology and function in a sexually dimorphic manner are not known. Therefore, the effect of nMitoQ treatment on placental mitochondrial function was investigated herein. We hypothesized that prenatal hypoxia leads to mitochondrial dysfunction, and that nMitoQ treatment improves placental mitochondrial

function and promotes a pro-fusion environment, thereby leading to improved fetal outcomes. Moreover, since placentae from male and female fetuses were previously shown to respond to prenatal stress differently, we hypothesized that there is a sex-specific divergence in the prenatal hypoxia-induced placental mitochondrial dysfunction and the effect of nMitoQ treatment.

3.2 Materials and Methods

3.2.1 Rat model of prenatal hypoxia

Three-month-old female Sprague-Dawley rats (weighing 250-275 g) were housed with male Sprague–Dawley rats overnight and pregnancy was confirmed the following morning by the presence of sperm in a vaginal smear (defined as gestational day (GD) 0 of pregnancy). Following which, pregnant dams were treated as described in Section 2.2.2. At the end of gestation, on GD21 (term: GD22), dams were euthanized prior to parturition and fetuses with their respective placentae were removed. Fetal sex was determined using the anogenital distance and fetal parameters, including body weight, crown-to-rump length and abdominal circumference, and placental weights, were measured and averaged per litter. Placental labyrinth zones (three/sex/litter) were isolated and used for mitochondrial function experiments immediately without freezing. In addition, placental labyrinth zones (two/sex/litter) were isolated and snap frozen in liquid nitrogen for Western blot analysis. All frozen tissues were stored at -80ºC until further processing. Being the nutrient and gas exchange layer between maternal-fetal circulations, the labyrinth zone, has a high-energy demand for active transport of nutrients to the fetus during late gestation. Therefore, I chose the labyrinth zone as it is more susceptible to oxidative stress.

3.2.2 High-resolution respirometry

To assess placental mitochondrial function, respirometry measurements were performed on freshly isolated labyrinth zones from male and female placentae using High-Resolution Respirometry (Oxygraph-O2k, OROBOROS Instruments, Innsbruck, Austria). The oxygraph was calibrated at 37 °C as per manufacturer's instructions and each chamber was filled with 2 ml of mitochondrial respiration medium 05 (MiR05; 110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 1 g/L fatty-acid free bovine serum albumin [BSA], 3 mM $MgCl_2$-$6H_2O$, 20 mM taurine, 10 mM KH_2PO_4, and 20 mM K-HEPES; pH 7.1, osmolarity 330 mOsm). Datlab software (OROBOROS Instruments, Innsbruck, Austria) was used for data acquisition and analysis.

Approximately 15 mg of wet weight placental labyrinth tissues were immediately transferred to 2 mL of ice-cold MiR05, and gently homogenized on ice with a Potter-Elvehjem tissue homogenizer attached to an overhead stirrer (Wheaton Instruments, Millville, NJ, USA). After 10 passes at intensity level 2, 200 µl of homogenate was added to each oxygraph chamber containing 1.8 ml of MiR05. The remaining tissue homogenate was flash frozen at -80ºC for citrate synthase (CS) activity assay (see mitochondrial content section below). The titration protocol used to evaluate mitochondrial function measured respiration at two different states: LEAK and OXPHOS. The LEAK respiration represents the nonphosphorylated state, before the addition of ADP, and measures the oxygen flux compensating for proton leak and slip, cation cycling and electron leak across the mitochondrial inner membrane. The OXPHOS state represents oxygen consumption coupled to phosphorylation of ADP to ATP in the presence of saturating ADP (256).

The titration protocol used includes the measurement of LEAK respiration in the presence of 5 mM pyruvate and 5 mM malate, OXPHOS capacity for the NADH pathway (N-pathway; flux through complex I in the presence of pyruvate, malate and 2.5 mM ADP), for the NADH & Succinate pathway (NS-pathway; convergent electron flux through complex I and II in the presence

of pyruvate, malate, 10 mM succinate, and ADP), and for the Succinate pathway (S-pathway, electron flux through complex II after inhibition of complex I with 1 μM rotenone), residual oxygen consumption (ROX, after inhibition of complex III with 5 uM Antimycin A), as well as complex IV activity (in the presence of 2 mM ascorbate and 0.5 mM tetramethylphenylenediamine (TMPD feeding electrons into complex IV), and chemical background (with 100 mM sodium azide, subtracted from complex IV activity).

In the placental tissues, uncoupling with dinitrophenol (DNP) after addition of succinate did not affect mitochondrial respiration (data not shown), which confirms that there was no limitation of the OXPHOS capacity by the phosphorylation system, as previously demonstrated in other tissues of rodents (257-259). The OXPHOS capacities were measured after the addition of exogenous cytochrome *c* (10 μM) to avoid bias resulting from damage to the outer mitochondrial membrane during tissue preparation. An increase of respiration of more than 15% following cytochrome c addition was used as an exclusion criterion and occurred in less than 10% of total experiments.

Data of mitochondrial function are presented either as oxygen flux per mass and as Flux Control Ratios (FCR), normalized for maximal OXPHOS capacity in the presence of substrates feeding electrons simultaneously into the N- (through complex I) and S- (through complex II) pathways. Mitochondrial respiratory capacity for each individual male and female placenta within a dam (3 placentae/sex/dam) was treated as a single data point in all experimental groups. This was based on a previous study showing that in permeabilized cardiac fibers of human patients the variability in mitochondrial respiratory capacity expressed as FCRs within the same cardiac tissue was similar to the variability observed between samples from different cardiac tissues in the same group (260).

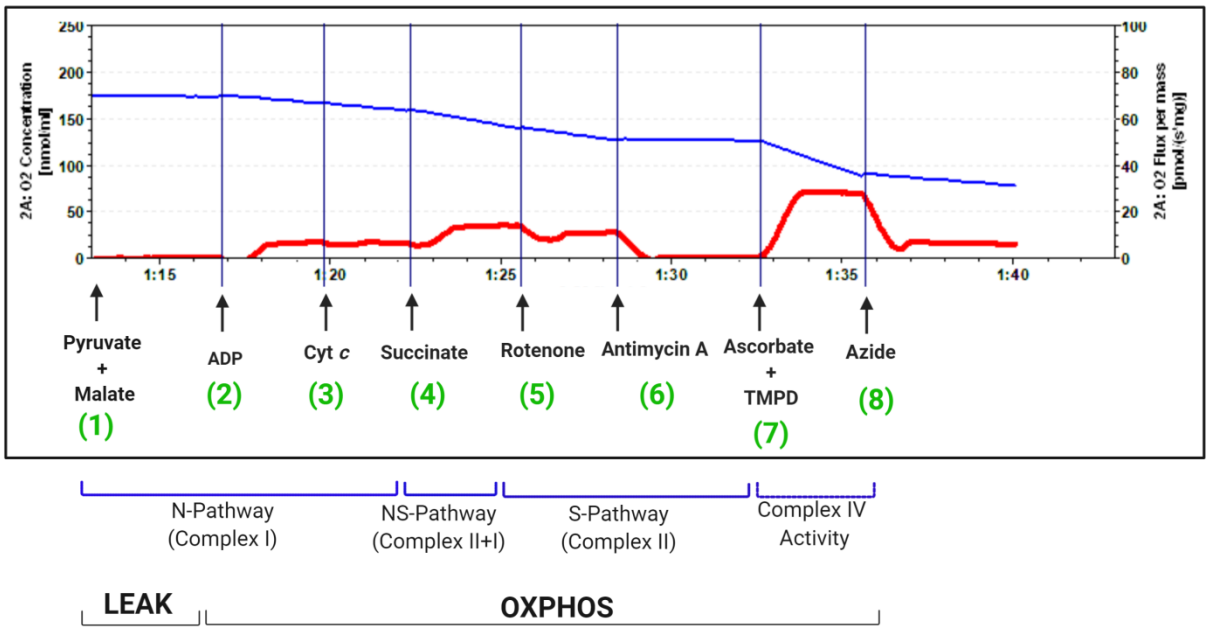

Figure 3.1. Representative tracing for the evaluation of mitochondrial respiratory capacities in placental tissues (labyrinth zone) in placentae of male and female fetuses on GD21 with a multiple substrate-inhibitors titration protocol. The trace represents the oxygen consumption as a function of time. The measurement was performed at 37ºC in the placental tissues. Mitochondrial coupling states are distinguished as LEAK (without ADP) and OXPHOS (saturating ADP). The multiple titration protocol comprised the following steps: (1) LEAK respiration in the presence of N-pathway (complex I) substrates pyruvate and malate; (2) OXPHOS respiration in the presence of N-pathway (complex I) substrate; (3) addition of cytochrome *c* (Cyt *c*) to test for integrity of the outer mitochondrial membrane; (4) addition of succinate to measure respiration in the presence of the substrates of NS—pathway (complexes I and II) such as pyruvate and malate and succinate; (5) S-pathway (succinate-supported) respiration after inhibition of N-pathway (complex I) with rotenone; (6) residual oxygen consumption after inhibition of complex III with antimycin A; (7) complex IV respiration in the presence of ascorbate and TMPD**;** and (8) inhibition of complex IV with azide. Arrows indicate steps and times of titrations of the substrates and inhibitors.

3.2.3 Mitochondrial content

CS activity was measured as previously described (261) at 37ºC using a UV/Vis spectrophotometer (Ultrospec 2100 Pro; Cambridge, MA, USA) equipped with a thermostated cell holder and a circulating water bath. Placental tissue homogenates were thawed on ice and underwent two additional cycles of homogenization with a conical glass homogenizer for 15 sec to ensure complete homogeneity of the samples. The absorbance was measured at 412 nm during 5 min, following the reduction of 0.1 mM/L 5,5'-dithiobis-2-nitrobenzoic acid (ε: 13.6 $ml.cm^{-1}.\mu mol^{-1}$) in the presence of 0.25% Triton X-100, 0.5 mM oxaloacetatic acid, 0.31 mM acetyl coenzyme A, 100 mM Tris-HCl, and triethanolamine-HCl buffer (pH 8.0).

3.2.4 Western blotting for expression of mitochondrial biogenesis, fusion and fission proteins

Placental tissue homogenates were prepared in lysis buffer (composition: 20 mM Tris [pH 7.4], 5 mM EDTA, 10 mM sodium pyrophosphate tetrabasic, 100 mM sodium and 9 mM fluoride with 1% Nonidet P-40) containing a protease inhibitor cocktail (1x Halt protease inhibitor, Thermo Scientific) 1 mM PMSF (Fluka Biochemika), and phosphatase inhibitor (2 mM sodium orthovanadate, Sigma). Total protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL). A total of 100 µg of protein was loaded, and separated on 7.5% SDS-polyacrylamide gels by electrophoresis. Proteins were transferred to a nitrocellulose membrane (0.2 µm, Bio-Rad) for 1 hour at 4ºC. Total protein in each lane was stained using the REVERT™ Total Protein Stain (LI-COR Biosciences, Lincoln, NE, USA). Three separate membranes were incubated with 50% blocking buffer for fluorescent Western blotting (Rockland Immunochemical) for 2 hours (room temperature), and incubated overnight at 4ºC with primary antibodies for the following: (1)

Peroxisome proliferator-activated receptor gamma, coactivator 1 (PGC1α) (1:250; Abcam, Catalogue # ab54481, Cambridge, United Kingdom); (2) Nuclear respiratory factor 1 (NRF1) (1:1000; Proteintech, Catalogue # 12482-1-AP, Rosemont, IL, USA); (3) OPA1 (1:3500; Cell Signaling Technology (CST), Catalogue # 80471, Danvers, MA, USA); (4) Mitofusin 1 (MFN1) (1:500; Proteintech, Catalogue # 13798-1-AP, Rosemont, IL, USA); (5) Drp1 (1:150; Santa Cruz Biotechnology, Catalogue # sc-271583, Dallas, TX, USA), and Phospho-Drp1 (Ser616) (1:4500; Invitrogen, Catalogue # PA5-64821, Waltham, MA, USA). The next day, membranes were incubated with the corresponding secondary antibodies: IRDye 800 anti-rabbit or IRDye 680 anti-mouse (1:10,000; LI-COR Biosciences). Blots were washed thrice with Phosphate Buffered Saline with Tween-20 (PBS-T), and then visualized with a LI-COR Odyssey Bioimager and quantified by densitometry with Odyssey V3.0 software (LI-COR Biosciences). Protein band quantifications were normalized to the total amount of protein in each lane. The ratio of the long form of OPA1 (L-OPA1) to the short form of OPA1 (S-OPA1) is expressed as L-/S-OPA1. Drp1 phosphorylation levels were expressed as the ratio between phosphorylated and total Drp1 (i.e. pDrp1/total Drp1). All other data are expressed as percent increases/decreases over the corresponding control (placental tissue from normoxia/saline group for each sex).

3.2.5 Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.02 software (GraphPad Software, U.S.A.). All data are expressed as mean ± S.E.M, and were analyzed using a two-way ANOVA for the main effects of two independent factors (prenatal hypoxia and nMitoQ treatment), followed by Sidak's multiple comparison *post hoc* tests. A value of $p<0.05$ was considered significant. Significant outliers were removed from final analyses (using Grubb's test).

3.3 RESULTS

3.3.1 Placental and fetal outcomes

In male fetuses, prenatal hypoxia decreased fetal weight and crown-to-rump length without affecting placental weight, while nMitoQ treatment increased fetal weight in the prenatal hypoxia exposed group (Table 3.1). Prenatal hypoxia reduced fetal/placental weight ratios in male fetuses (Table 1). There was no overall effect of nMitoQ treatment; however, there was a significant interaction between prenatal hypoxia and nMitoQ treatment, in the fetal/placental weight ratios (Table 1). Abdominal girth was similar between all experimental groups in male fetuses (Table 3.1).

In female fetuses, prenatal hypoxia reduced fetal weight but had no effect on placental weight, fetal/placental weight ratios, crown-to-rump length or abdominal girth (Table 3.2). nMitoQ treatment had no effect on female fetal or placental weights, fetal/placental weight ratios, crown-to-rump length or abdominal girth (Table 3.2).

Variables	Normoxia		p-Hypoxia		Main Effect		
	Saline	nMitoQ	Saline	nMitoQ	p-Hypoxia	nMitoQ	Interaction
Fetal weight (g)	5.92±0.22	5.38±0.30	**4.75±0.11**	**5.58±0.16†**	*	*	**
Placental weight (g)	0.59±0.02	0.55±0.02	0.58±0.03	0.60±0.02	-	-	-
Fetal weight / Placental weight	10.16±0.43	9.72±0.52	**8.11±0.27**	9.35±0.48	*	-	*
Abdominal girth (cm)	4.05±0.14	3.79±0.12	3.77±0.05	3.8±0.11	-	-	-
Crown-rump length (cm)	5.01±0.08	4.82±0.11	4.71±0.05	4.78±0.07	*	-	-
Litter size (# of males)	5.6±0.51	6.4±0.40	6±0.075	6.5±0.67	-	-	-

Table 3.1: Fetal and placental characteristics of male offspring. Body weight, placental weight, placental efficiency (body weight/placental weight), abdominal girth, crown-rump length, and litter size from male fetuses collected on GD21. Data are represented as mean ± SEM. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test. n= 6 dams/group. *$p<0.05$, **$p<0.01$ overall group effect of prenatal hypoxia. $^{\dagger}p<0.05$ versus corresponding hypoxia-saline group.

Variables	Normoxia		p-Hypoxia		Main Effect		
	Saline	nMitoQ	Saline	nMitoQ	p-Hypoxia	nMitoQ	Interaction
Fetal weight (g)	5.70±0.17	5.24±0.36	**4.46±0.09**	4.83±0.23	**	-	-
Placental weight (g)	0.58±0.04	0.55±0.03	0.54±0.02	0.55±0.04	-	-	-
Fetal weight / Placental weight	9.95±0.67	9.57±0.52	8.71±0.27	9.15±0.63	-	-	-
Abdominal girth (cm)	3.94±0.07	3.83±0.09	3.84±0.08	3.69±0.16	-	-	-
Crown-rump length (cm)	4.83±0.06	4.84±0.19	4.78±0.08	4.70±0.02	-	-	-
Litter size (# of females)	5.4±0.93	7.2±0.73	5.87±0.64	5.2±0.60	-	-	-

Table 3.2: Fetal and placental characteristics of female offspring. Body weight, placental weight, placental efficiency (body weight/placental weight), abdominal girth, crown-rump length, and litter size from female fetuses collected on GD21. Data are represented as mean ± SEM. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test. n= 6 dams/group. **$p<0.01$ overall group effect of prenatal hypoxia.

3.3.2 Mitochondrial function

Flux per mass

Mitochondrial respiration in placental tissues was first expressed as oxygen flux per tissue mass. Prenatal hypoxia and nMitoQ treatment had no effect on the N- pathway (through complex I) and S-pathway (through complex II) mass-specific capacity in the placentae from male offspring (Figure 3.2A, C). However, the data showed a decrease in maximal respiratory capacity through the combined NS-pathway (through complex I and II) (Figure 3.2B), as well as a decrease in complex IV activity in prenatal hypoxia-exposed placentae from male offspring (Figure 3.2D). nMitoQ treatment significantly increased complex IV activity in placentae from the prenatal hypoxia group compared to the normoxia group (Figure 3.2D).

In placentae from female offspring, there was no effect of either prenatal hypoxia or nMitoQ treatment on oxygen flux per unit mass through N-pathway (complex I), NS-pathway (complex I and II), S-pathway (complex II), and Complex IV respiration (Figure 3.3A-D).

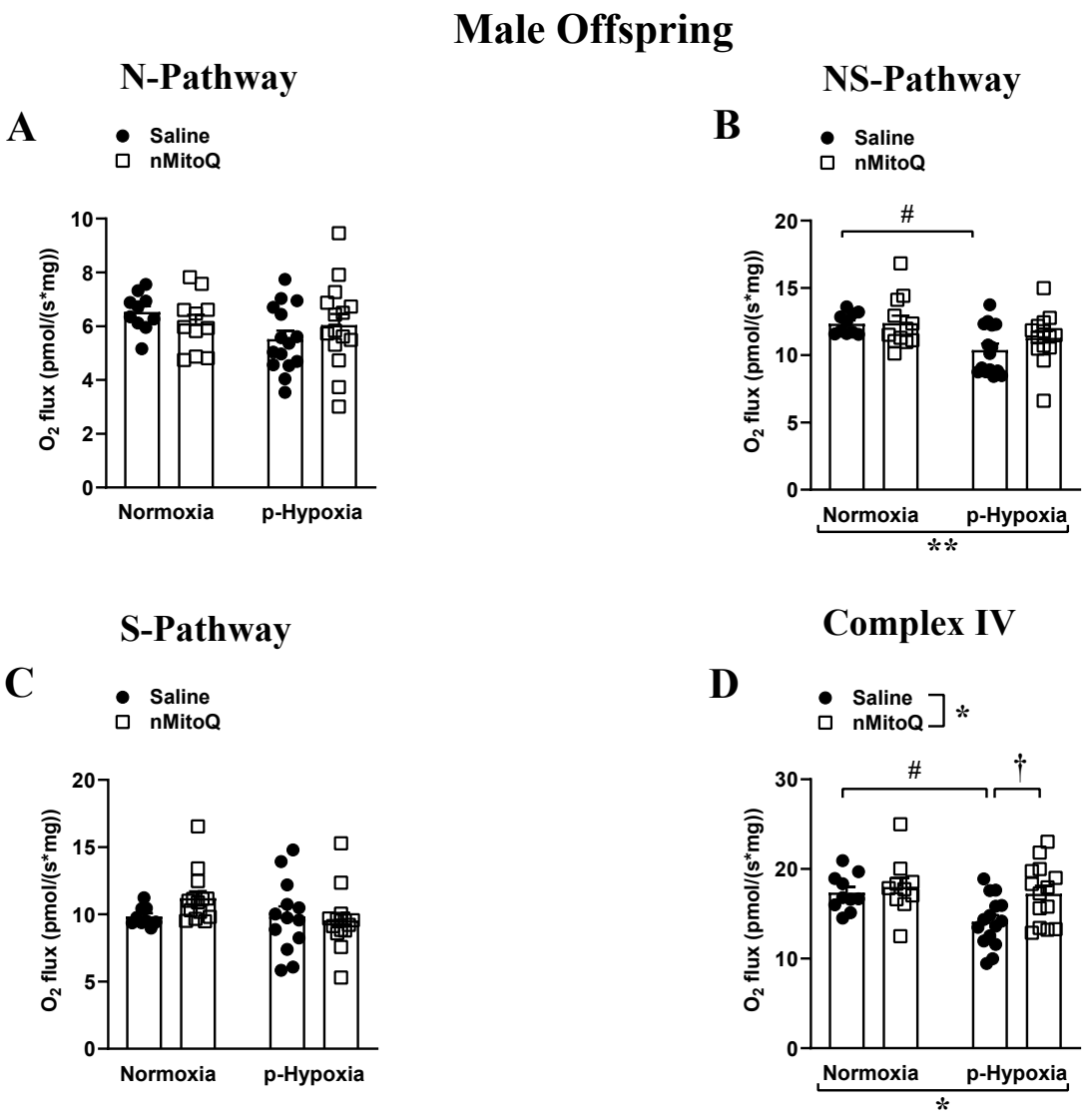

Figure 3.2. Effects of prenatal hypoxia and nMitoQ treatment on mitochondrial respiration (expressed as oxygen flux per unit mass of tissue) in placentae of male fetuses on GD21. Oxidative phosphorylation (OXPHOS; coupled to ADP) capacity for the NADH pathway (N-pathway; NADH-linked substrates pyruvate and malate entering via complex I), NADH & Succinate pathway (NS-pathway; convergent electron flux through complex I and II), Succinate pathway (S-pathway; succinate entering via complex II after complex I inhibition with rotenone), and complex IV activity (presence of ascorbate and tetramethylphenylenediamine (TMPD) feeding electrons into complex IV) was measured in placentae from male (**A-D**) fetuses. Data are presented as mean ± SEM. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test. A single data point represents an individual placenta with n=3 placentae/dam in all groups, number of dams used= 5-6 per group. *p<0.05, **p<0.01 group effect of prenatal hypoxia and nMitoQ treatment, $^{\#}$p<0.05 compared to normoxia-saline, †p<0.05 compared to hypoxia-saline group.

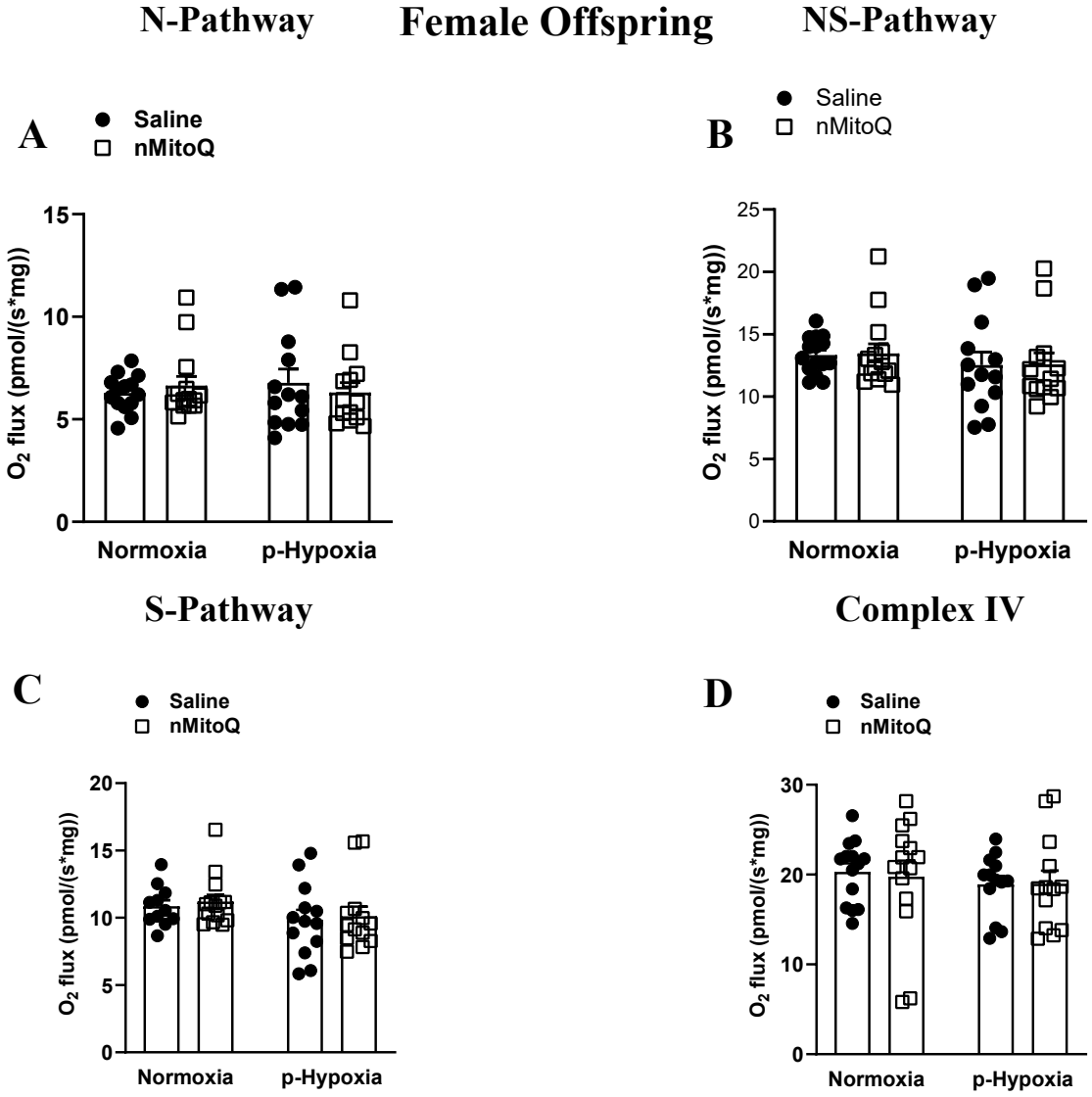

Figure 3.3. Effects of prenatal hypoxia and nMitoQ treatment on mitochondrial respiration (expressed as oxygen flux per unit mass of tissue) in placentae of female fetuses on GD21. Oxidative phosphorylation (OXPHOS; coupled to ADP) capacity for the NADH pathway (N-pathway; NADH-linked substrates pyruvate and malate entering via complex I), NADH & Succinate pathway (NS-pathway; convergent electron flux through complex I and II), Succinate pathway (S-pathway; succinate entering via complex II after complex I inhibition with rotenone), and complex IV activity (presence of ascorbate and tetramethylphenylenediamine (TMPD) feeding electrons into complex IV) was measured in placentae from female (**A-D**) fetuses. Data are presented as mean ± SEM. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test. A single data point represents an individual placenta with n=3 placentae/dam in all groups, number of dams used= 5-6 per group.

Flux control ratio

Mitochondrial respiration was then expressed as flux control ratio (FCR). The FCRs represent the proportional contribution of specific respiratory pathways to the maximal OXPHOS capacity, and therefore are dictated by the mitochondrial qualitative properties rather than the mitochondrial content (256). In placentae from male offspring, neither prenatal hypoxia nor nMitoQ treatment affected FCR for the N-pathway (through complex I), S-pathway (through complex II) or complex IV activity (Figure 3.4A-C). However, nMitoQ treatment increased the FCR for the S-pathway (through complex II) in both normoxic and prenatally hypoxic male placentae (overall nMitoQ effect) in the males (Figure 3.4B).

In the female placenta, prenatal hypoxia caused a shift in pathway utilization for energy production with an increase in contribution of the N-pathway (through complex I) (Figure 3.5A), and a decrease in contribution of the S-pathway (through complex II) (Figure 3.5B). There was no effect of nMitoQ treatment on the FCR for the S-pathway contribution (through complex II) and for the N-pathway (through complex I) (Figure 3.5A, B). There were no effects of prenatal hypoxia or nMitoQ treatment on the FCR for complex IV activity in female placentae (Figure 3.5C).

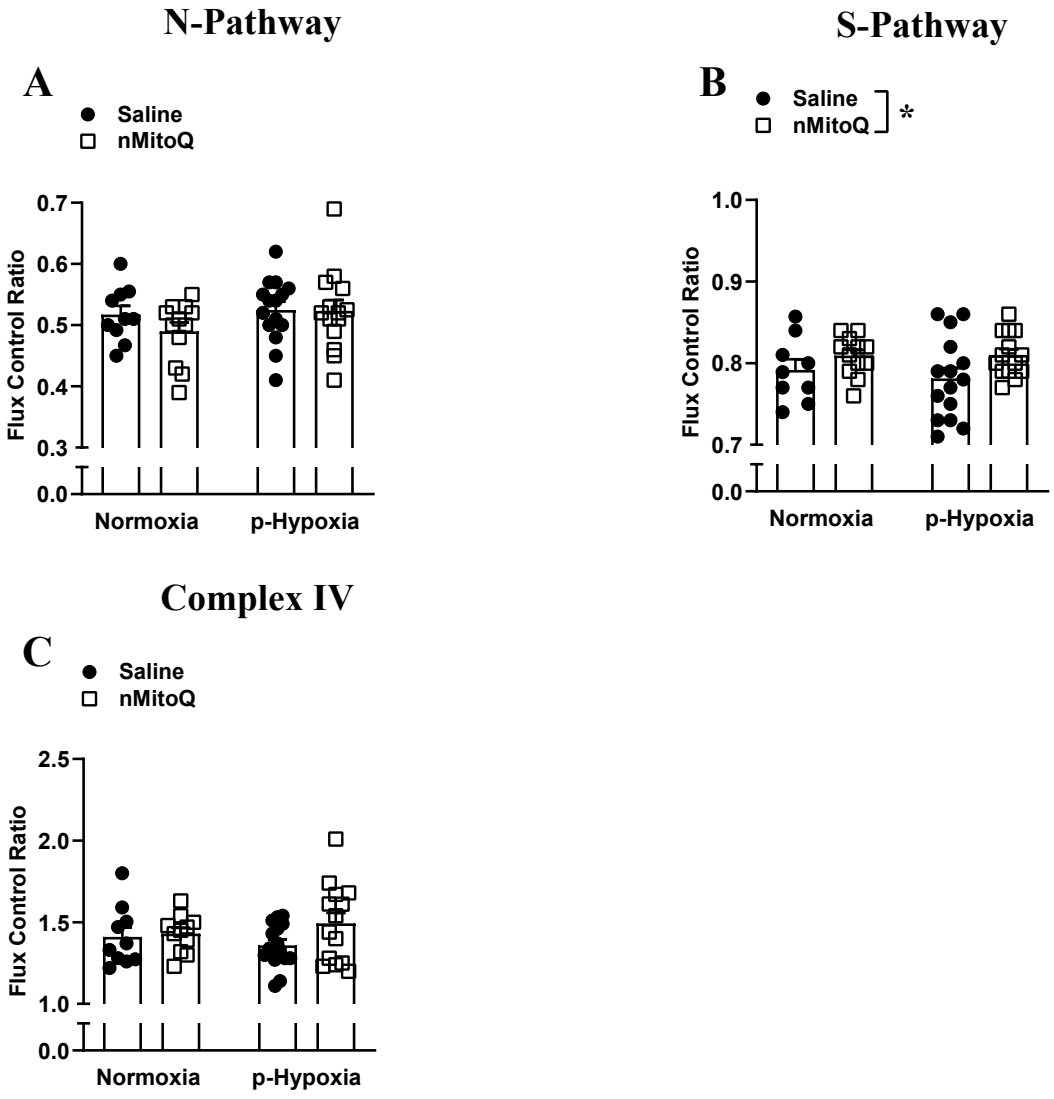

Figure 3.4. Effects of prenatal hypoxia and nMitoQ treatment on mitochondrial respiration (expressed as flux control ratio; FCR) in placentae of male fetuses on GD21. Oxidative phosphorylation (OXPHOS; coupled to ADP) capacity for NADH pathway (N-pathway; NADH-linked substrates pyruvate and malate entering via complex I), Succinate pathway (S-pathway; succinate entering via complex II after complex I inhibition with rotenone), and complex IV activity (presence of ascorbate and TMPD feeding electrons into complex IV) was measured in placentae from male (**A-C**) fetuses. Data are presented as mean ± SEM. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test. A single data point represents an individual placenta with n=3 placentae/dam in all groups, number of dams used= 5-6 per group. *$p<0.05$ group effect of prenatal hypoxia or nMitoQ treatment.

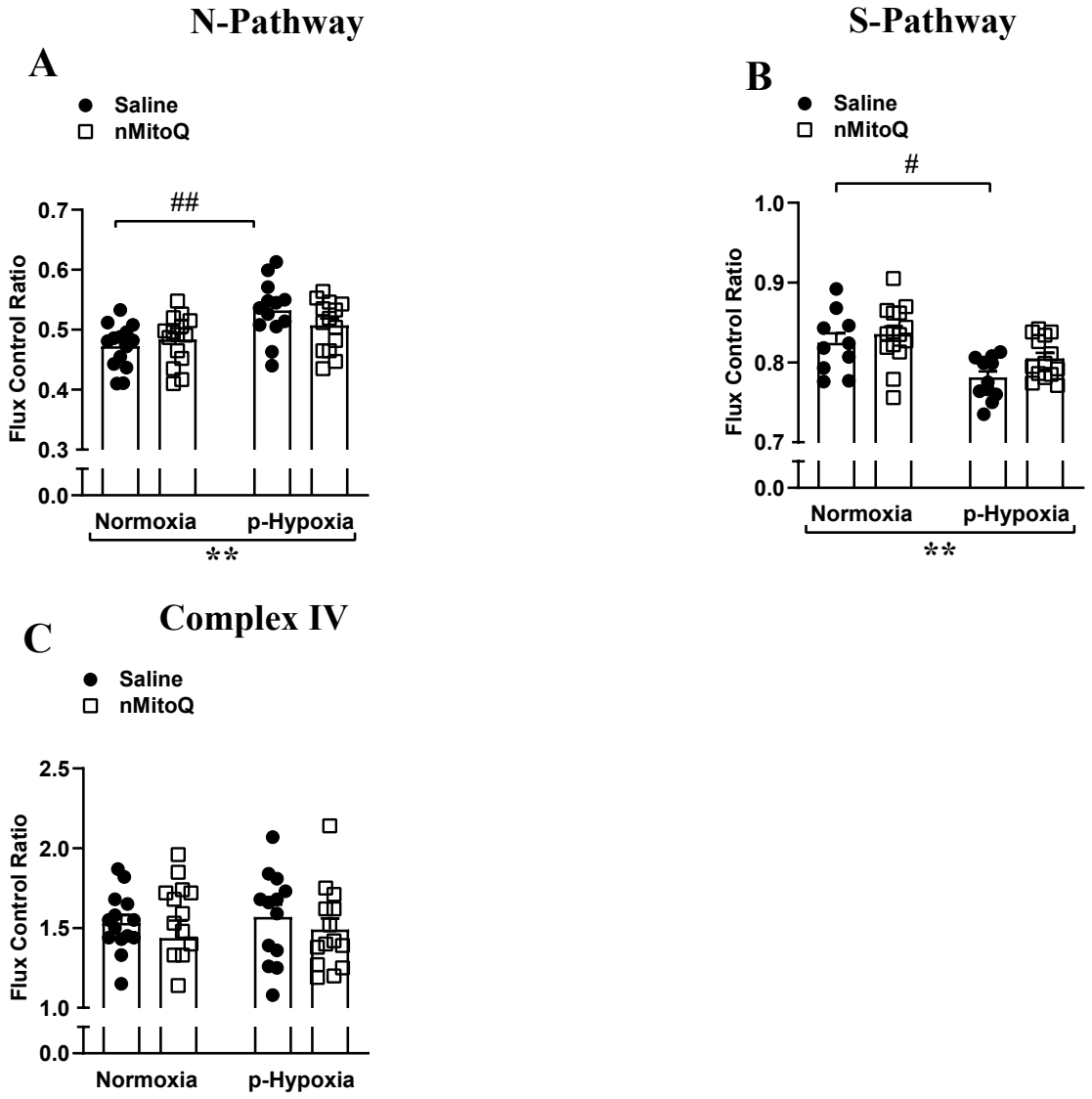

Figure 3.5. Effects of prenatal hypoxia and nMitoQ treatment on mitochondrial respiration (expressed as flux control ratio; FCR) in placentae of both female fetuses on GD21. Oxidative phosphorylation (OXPHOS; coupled to ADP) capacity for NADH pathway (N-pathway; NADH-linked substrates pyruvate and malate entering via complex I), Succinate pathway (S-pathway; succinate entering via complex II after complex I inhibition with rotenone), and complex IV activity (presence of ascorbate and TMPD feeding electrons into complex IV) was measured in placentae from female (**A-C**) fetuses. Data are presented as mean ± SEM. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test. A single data point represents an individual placenta with n=3 placentae/dam in all groups, number of dams used= 5-6 per group. *p<0.05, **p<0.01 group effect of prenatal hypoxia or nMitoQ treatment, $^{\#}$p<0.05, $^{\#\#}$p<0.01 compared to normoxia-saline.

3.3.3 Mitochondrial coupling (LEAK)

The FCR for LEAK in the presence of NADH-linked substrates was used to as an indicator of mitochondrial coupling independent of the mitochondrial content and OXPHOS capacity (262). A minimum value of 0.0 indicates a fully coupled system, while a value of 1.0 indicates a fully non-coupled system. All our values were below 0.1 and indicated good mitochondrial coupling in both the male and female placentas (Figure 3.6A, B). In male offspring, the FCR for LEAK was increased in prenatally hypoxic placentae compared to placentae from normoxic controls, indicative of less coupled mitochondria (Figure 3.6A). Moreover, there was a significant interaction between nMitoQ treatment and prenatal hypoxia in male placentae, whereby nMitoQ increased the FCR for LEAK in placentae from normoxic male offspring, but had no effect in the hypoxic placentae (Figure 3.6A). In placentae from female offspring, there was no effect of prenatal hypoxia on the FCR for LEAK (Figure 3.6B). However, nMitoQ increased LEAK respiration in placentae from normoxic female offspring, but had no effect in placentae from female fetuses exposed to prenatal hypoxia (significant interaction between nMitoQ treatment and prenatal hypoxia exposure; Figure 3.6B).

The addition of exogenous cytochrome *c* after pyruvate, malate and ADP was used as an indicator of outer membrane damage. We observed similar fractional increases in respiration between groups in male placentae (Normoxia-Saline: 0.06±0.01; Normoxia-nMitoQ: 0.06±0.01; p-Hypoxia-Saline: 0.04±0.01; p-Hypoxia-nMitoQ: 0.06±0.01) and female placentae (Normoxia-Saline: 0.06±0.01; Normoxia-nMitoQ: 0.05±0.01; p-Hypoxia-Saline: 0.05±0.01; p-Hypoxia-nMitoQ: 0.08±0.01), indicating integrity of the outer mitochondrial membrane was similar in placental tissues among treatment groups, and well preserved compared to other tissues and mitochondrial preparation (259, 261, 262).

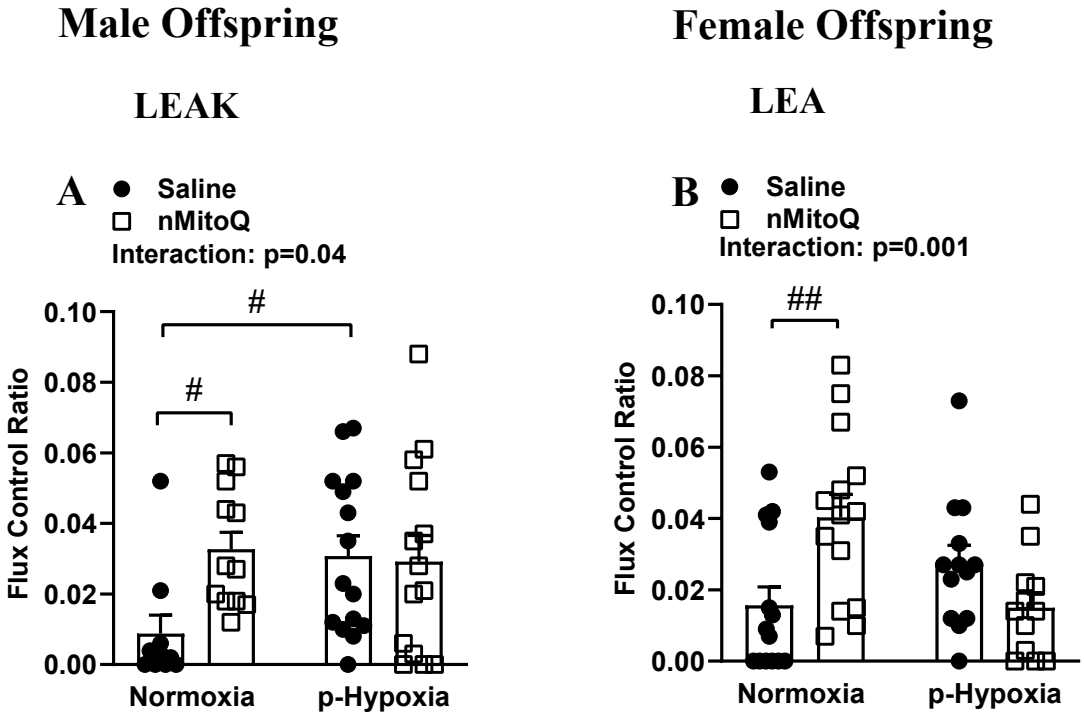

Figure 3.6. Effects of prenatal hypoxia and nMitoQ treatment on mitochondrial LEAK respiration (expressed as flux control ratio; FCR) in placentae of both male and female fetuses on GD21. Normoxic and hypoxic dams were treated with nMitoQ or saline and mitochondrial LEAK (respiration in the absence of ADP) was measured in placentae from male (**A**) and female (**B**) fetuses. Data are presented as mean ± SEM. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test. A single data point represents an individual placenta with n=3 placentae/dam in all groups, number of dams used= 5-6 per group. $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to normoxia-saline.

3.3.4 Mitochondrial content and biogenesis

CS activity was used as a recognized marker of mitochondrial content (263). In placentae from male and female offspring, there were no alterations in CS activity by either prenatal hypoxia or nMitoQ treatment (Figure 3.7A, B). PGC-1α and NRF1 are critical proteins involved in mitochondrial biogenesis (119). In placentae from both male and female offspring, PGC-1α protein expression was not affected by either prenatal hypoxia exposure or nMitoQ treatment (Figure 3.8A, B). However, NRF1 levels were not different in placentae from male offspring, while prenatal hypoxia increased NRF1 levels in placentae from female offspring, without effect of nMitoQ treatment ($p=0.04$; Figure 3.8C and D).

Figure 3.7. Effects of prenatal hypoxia and nMitoQ treatment on markers of mitochondrial content in placentae of both male and female fetuses on GD21. Mitochondrial content was measured by citrate synthase activity in placental homogenates obtained from male (**A**) and female (**B)** fetuses on GD21. Data are presented as mean ± SEM. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test. n= 6 dams/group.

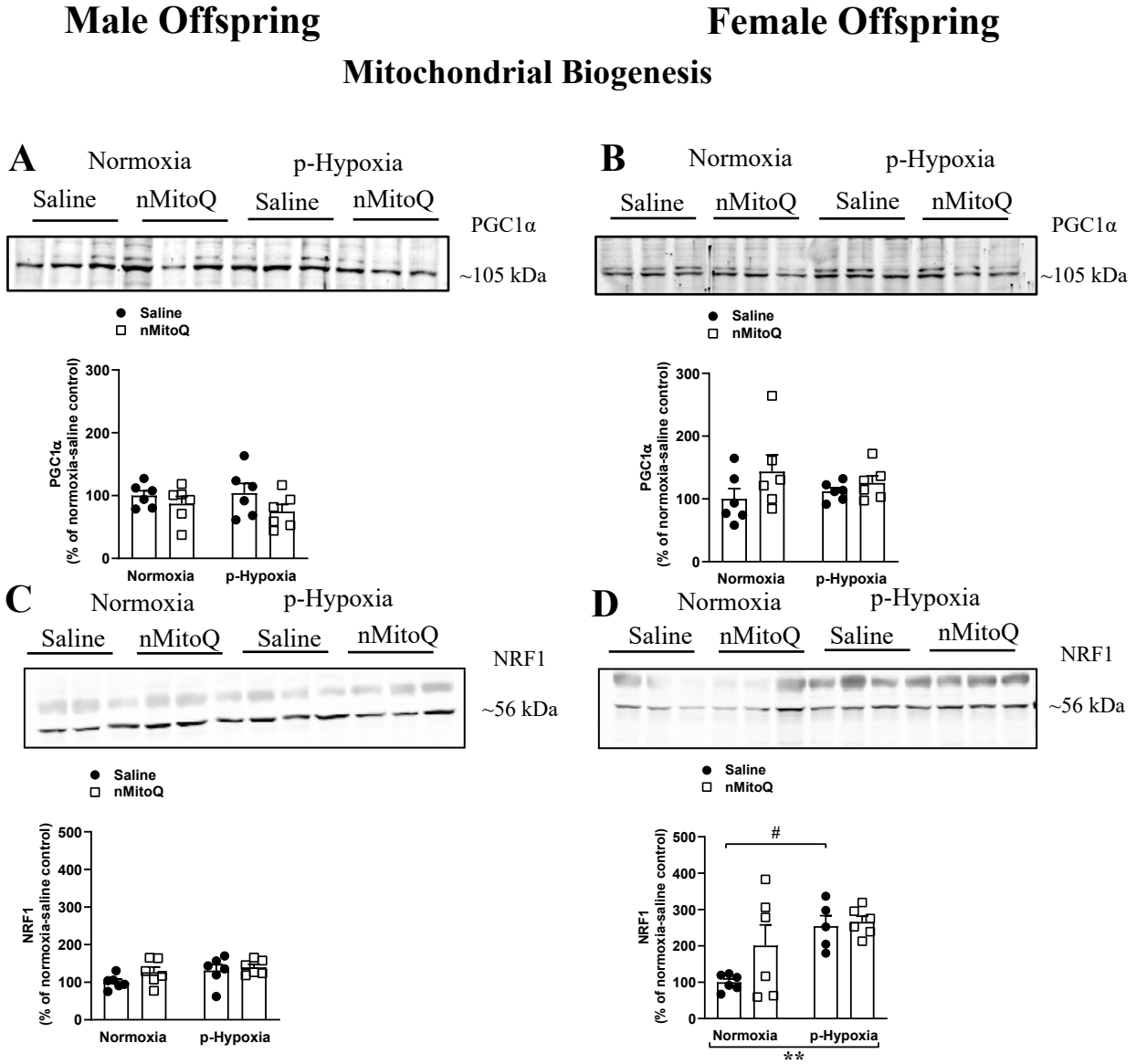

Figure 3.8. Effects of prenatal hypoxia and nMitoQ treatment on markers of mitochondrial biogenesis in placentae of both male and female fetuses on GD21. Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) protein expression was measured in placentae obtained from male (**A**) and female (**B**) fetuses. Nuclear respiratory factor 1 (NRF1) protein expression was measured in placentae from male (**C**) and female (**D**) fetuses. All data are presented as a ratio of the protein of interest to total amount of protein in each lane. PGC-1α and NRF1 data were calculated as the percent change compared to the mean of the normoxia-saline control group (set as 100%). Representative images of Western blot membranes probed for PGC-1α and NRF1 are shown above the graphs. Proteins of interest were normalized to the total amount of protein in each lane used as loading control. Data are presented as mean ± SEM. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test. n= 6 dams/group.

3.3.5 Mitochondrial fusion and fission protein expression

OPA1 has been identified as one of the key proteins that regulate mitochondrial fusion. Uncleaved (long) form of OPA1 (L-OPA1) promotes mitochondrial fusion, and a cleaved (short) form of OPA1 (S-OPA1) that is generated by proteolysis of L-OPA1 facilitates mitochondrial fission (264). In placentae from male offspring, L-OPA1 protein levels were significantly decreased by prenatal hypoxia; but were not affected by nMitoQ treatment (Figure 3.9A and B). The L-OPA1/S-OPA1 ratio was reduced in placentae exposed to prenatal hypoxia (Figure 3.9C) but was not altered by nMitoQ treatment (Figure 3.9C).

In placentae from female offspring, L-OPA1 and S-OPA1 expression was not significantly affected by either prenatal hypoxia or nMitoQ treatment (Figure 3.10A and B). However, even though prenatal hypoxia did not alter the L-OPA1/S-OPA1 ratio, it was increased after nMitoQ treatment in placentae of both normoxic and prenatally hypoxic offspring (Figure 3.10C).

Mitofusin 1 (MFN1) is required in inner mitochondrial membrane fusion mediated by OPA1 (265). In placentae from both male and female offspring, MFN1 expression was not different after either prenatal hypoxia or nMitoQ treatment compared to normoxia controls (Figure 3.9D and 3.10D).

Phosphorylation of Drp1 (a cytosolic GTPase involved in mitochondrial fission (266, 267) at serine 616 promotes Drp1-mediated mitochondrial fission and is a commonly used marker of mitochondrial fission (268, 269). In placentae from both male and female offspring, neither prenatal hypoxia nor nMitoQ treatment affected Drp1 phosphorylation (pDRP1/DRP1 ratio; Figure 3.11 A and B).

Male Offspring

Mitochondrial Fusion Protein

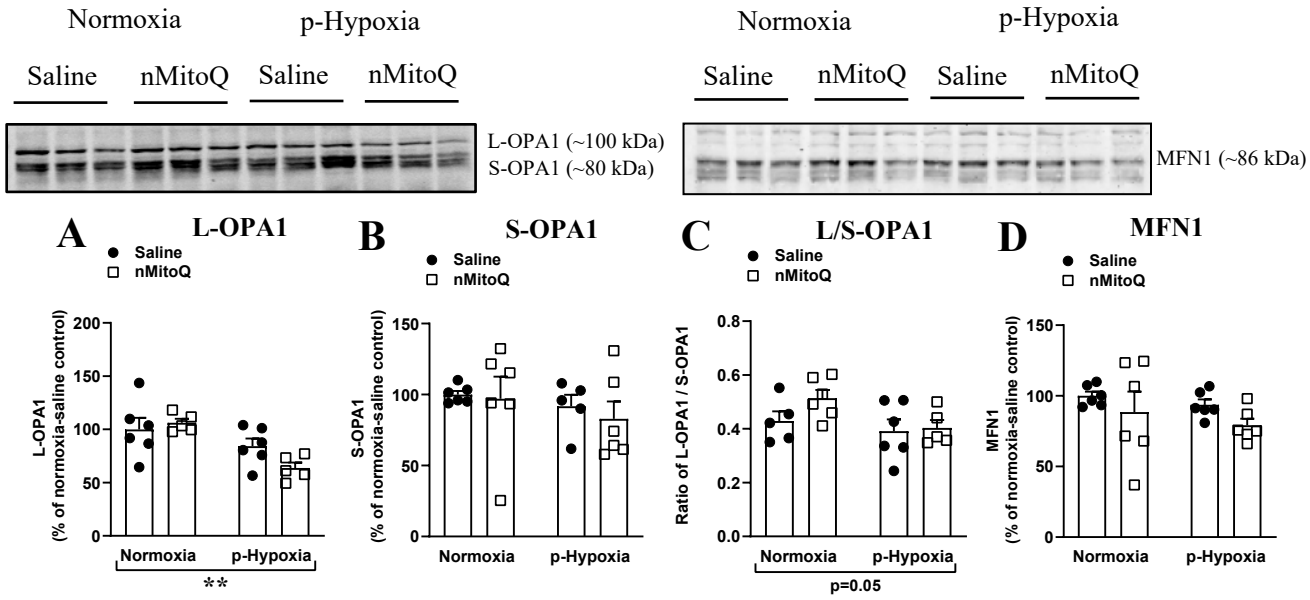

Figure 3.9. Effects of prenatal hypoxia and nMitoQ treatment on mitochondrial fusion protein (OPA1 and MFN1) expression in placentae of male fetuses on GD21. Mitochondrial inner membrane fusion protein Optic atrophy protein 1 (OPA1) expression was measured in placentae obtained from male (**A-C**) fetuses. Data for long (L-) OPA1 and short (S-) OPA1 are presented as a ratio of the protein of interest to total amount of protein in each lane. Data for L- OPA1/S- OPA1 were presented as ratio of the long (L-) OPA1 to short (S-) OPA1 for all experimental groups. Mitochondrial outer membrane fusion protein Mitofusin 1 (MFN1) expression was measured in placentae from male (**D**) fetuses. The normoxia-saline group was normalized to 100% and then the percent change for all other experimental groups was assessed. Representative images of Western blot membranes probed for OPA1 and MFN1 are shown above the graphs. Data are presented as mean ± SEM. Proteins of interest were normalized to the total amount of protein in each lane used as loading control. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test. n= 6 dams/group. *p<0.05, **p<0.01 group effect of prenatal hypoxia or nMitoQ treatment.

Female Offspring

Mitochondrial Fusion Protein

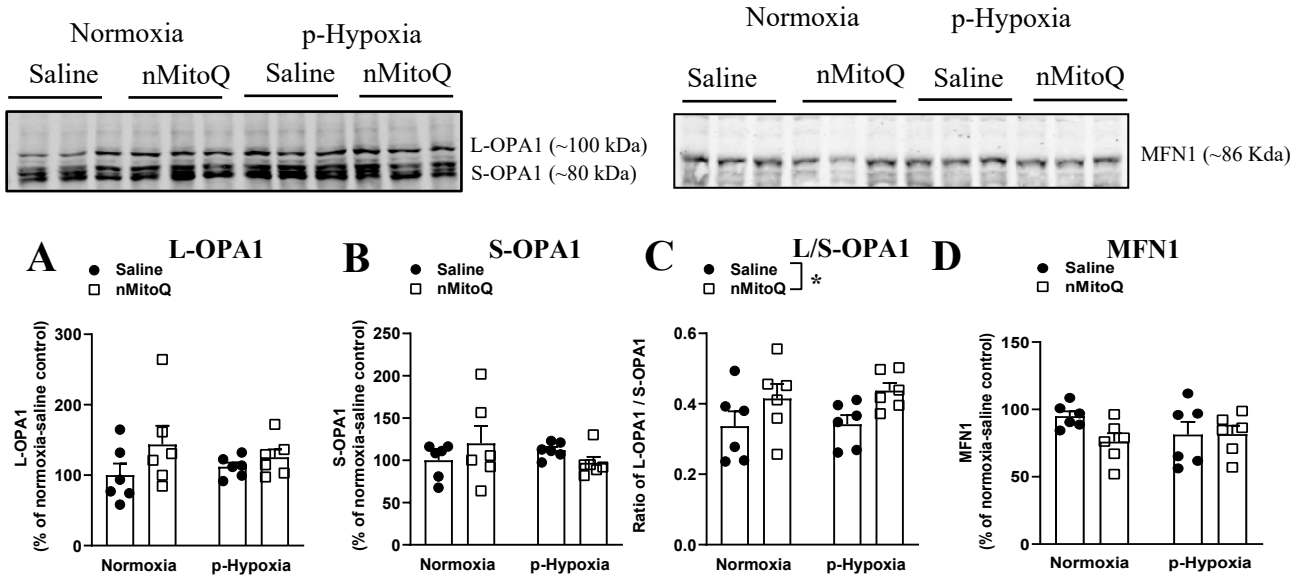

Figure 3.10. Effects of prenatal hypoxia and nMitoQ treatment on mitochondrial fusion protein (OPA1 and MFN1) expression in placentae of female fetuses on GD21. Mitochondrial fusion protein Optic atrophy protein 1 (OPA1) protein expression was measured in placentae obtained from female (**A-D**) fetuses. Data for long (L-) OPA1 and short (S-) OPA1 are presented as a ratio of the protein of interest to total amount of protein in each lane. The normoxia-saline group was normalized to 100% and then the percent change for all other experimental groups was assessed. Data for L- OPA1/S- OPA1 were presented as ratio of the long (L-) OPA1 to short (S-) OPA1 for all experimental groups. Representative images of Western blot membranes probed for OPA1 are shown. Data are presented as mean ± SEM. Proteins of interest were normalized to the total amount of protein in each lane used as loading control. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test. n= 6 dams/group. *$p<0.05$ group effect of nMitoQ treatment.

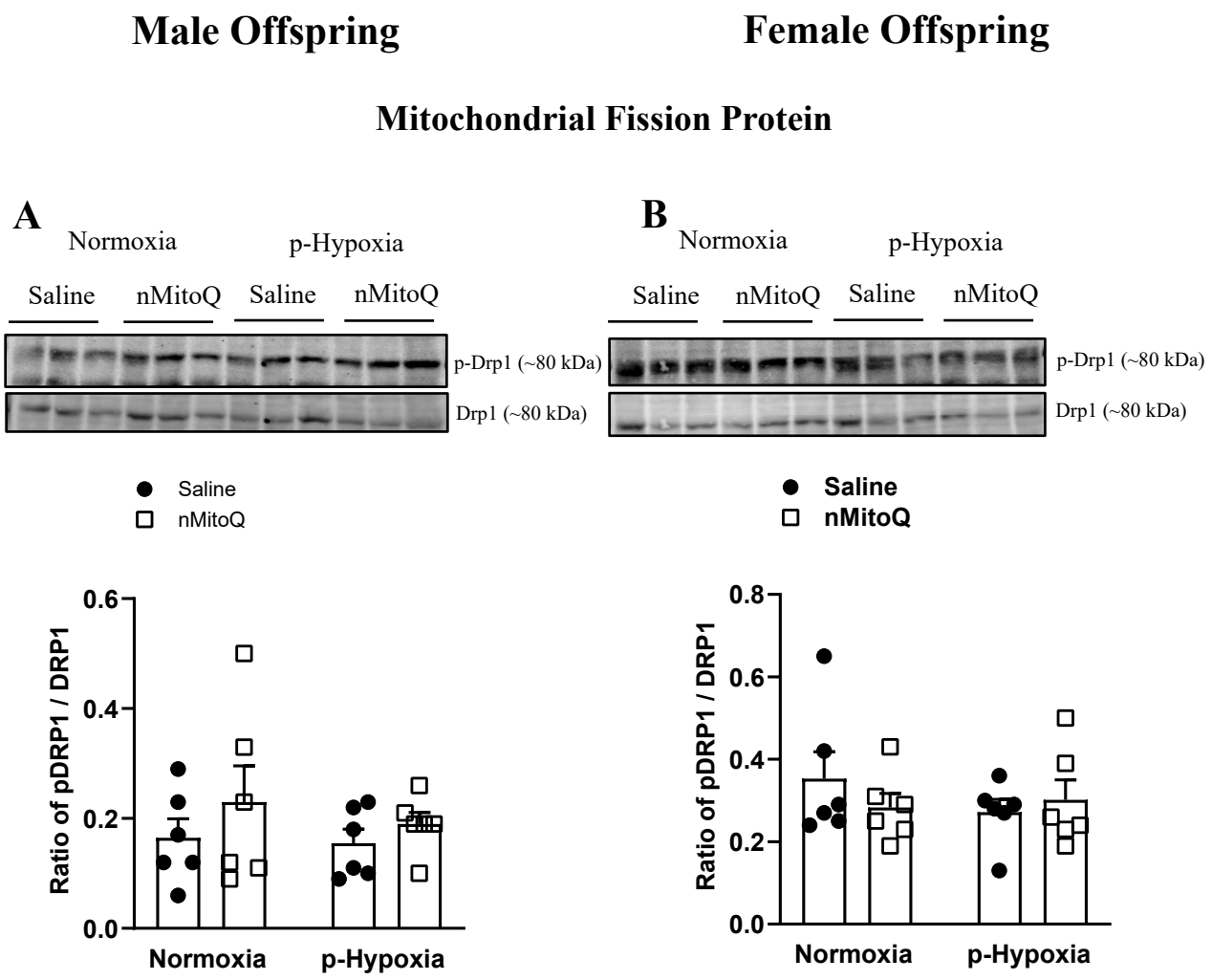

Figure 3.11. Effects of prenatal hypoxia and nMitoQ treatment on mitochondrial fission protein (Drp1) expression in placentae of both male and female fetuses on GD21. Mitochondrial fission protein Dynamin-related protein 1 (Drp1; total Drp1 and pDrp1) protein expression was measured in placentae obtained from male (**A**) and female (**B**) fetuses. All data are presented as a ratio of phosphorylated Drp1 to total Drp1 protein. Representative images of western blot membranes probed for total Drp1 and pDRP1 in placentae of males (**A**) and females (**B**). Proteins of interest were normalized to the total amount of protein in each lane as loading control. Data are presented as mean ± SEM. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test. n=6 dams/group.

3.4 Discussion

The current study was designed to examine the effects of prenatal hypoxia and nMitoQ treatment on placental mitochondrial function. We showed that prenatal hypoxia reduced complex IV activity in flux per mass in placentae from only male fetuses, and the decrease was prevented by nMitoQ treatment. Furthermore, prenatal hypoxia decreased expression of pro-fusion protein, L-OPA1 isoform, in male placentae. However, in placentae from female fetuses, prenatal hypoxia increased contribution of the N-pathway (through complex I) and decreased contribution of the S-pathway (through complex II). nMitoQ treatment had no effect on mitochondrial function but led to increased expression of the profusion protein, L-OPA1, in placentae from hypoxic female offspring. Overall, my data suggests that placental mitochondria adapt and/or respond to prenatal hypoxia in a sex-specific manner via changes in OXPHOS capacities of different respiratory pathways and steps, and reduced mitochondrial fusion (Figure 3.12A and B). In addition, my data also suggests that the effect of nMitoQ treatment on placental mitochondrial OXPHOS capacities were sexually dimorphic (Figure 3.12A and B).

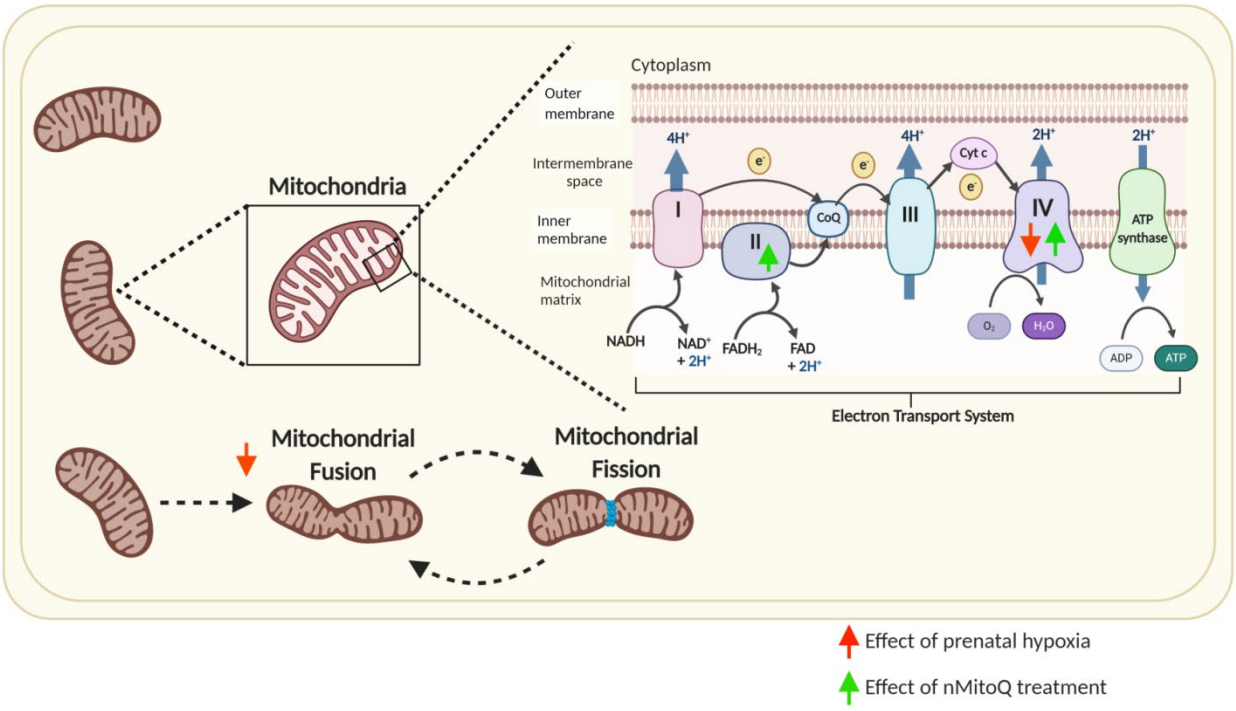

Figure 3.12. Schematic summary of the effects of prenatal hypoxia and nMitoQ treatment on the mitochondria in placentae from male fetuses. In male placentae, prenatal hypoxia decreased complex IV activity and mitochondrial fusion, while nMitoQ treatment increased complex IV activity and mitochondrial respiration through complex II **(A)**. Arrows indicate effect of prenatal hypoxia (red) and nMitoQ treatment (green). Created with BioRender.com

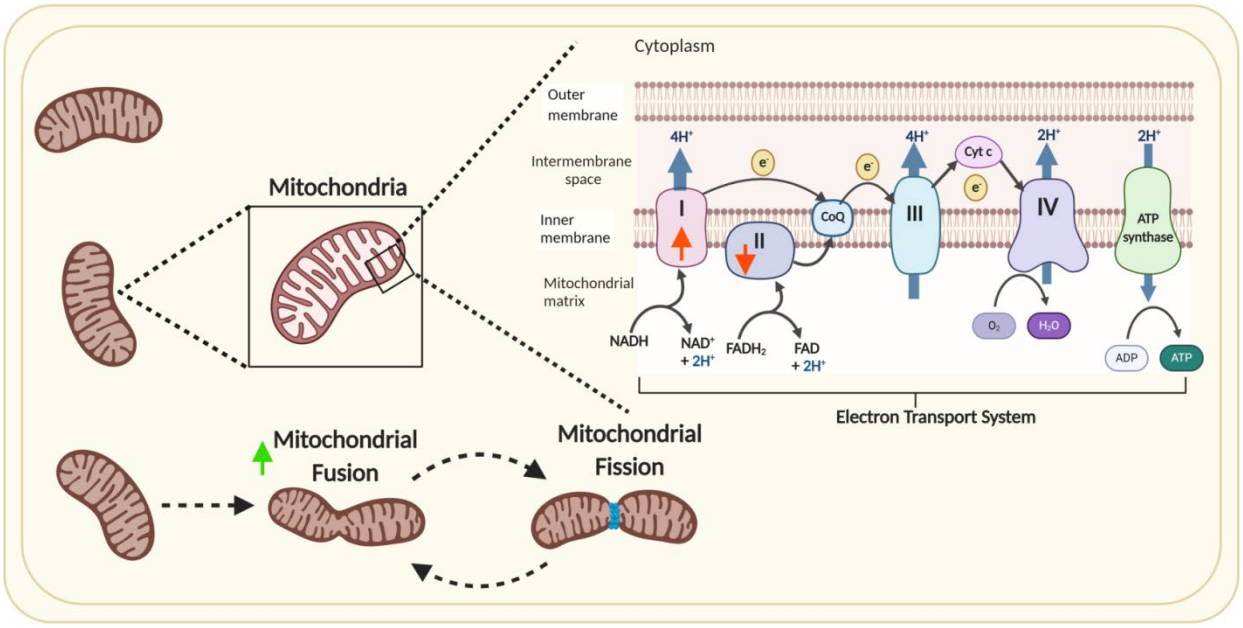

Figure 3.12. Schematic summary of the effects of prenatal hypoxia and nMitoQ treatment on the mitochondria in placentae from female fetuses. In female placentae, prenatal hypoxia increased mitochondrial respiration through complex I, while nMitoQ treatment increased mitochondrial fusion in hypoxic female placentae **(B)**. Arrows indicate effect of prenatal hypoxia (red) and nMitoQ treatment (green). Created with BioRender.com

In the current study, male and female fetal weights were reduced by prenatal hypoxia while nMitoQ treatment improved fetal weight only in hypoxic male fetuses. In Chapter 2, nMitoQ prevented reduced fetal weight in females but not males. However, in Chapter 2, the fetal weight phenotype was not as robust and the overall percent reduction in fetal weights was only 10% in comparison to the 20% reduction seen in Chapter 3. Thus, we speculate that the fetal phenotype in the current chapter was not severe enough for nMitoQ to observe a statistically significant effect on fetal weights. The exact reasons for the less severe phenotype in Chapter 2 are not known; however, the fetal phenotype of the prenatal hypoxia model observed in the current chapter is similar to previous studies from our group (219, 270, 271). Despite the differential effects of placenta-targeted nMitoQ treatment on fetal weights, we previously showed that nMitoQ treatment reduced placental oxidative stress, enhanced placental morphology and function, prevented pulmonary artery dysfunction in prenatally hypoxic adult male offspring and led to improved vascular reactivity in aged male and female offspring (156, 219, 272). This suggests that a treatment targeted towards placental oxidative stress, irrespective of its effects on fetal weights, can lead to improved long-term health outcomes. Our findings lay emphasis on the fact that although studies have correlated fetal weight (as estimation of size) as an indicator of fetal organ development and long-term health outcomes, birth weight does not necessarily equate with fetal development and later life health outcomes in the offspring (273) In addition, we found that fetal weight/placental weight ratio, used as a surrogate indicator of placental efficiency, was reduced by prenatal hypoxia only in males but not in females. Moreover, nMitoQ treatment in males improved placental efficiency. In contrast, the reduction in the female fetal weight by prenatal hypoxia was not accompanied by changes in placental efficiency.

My data showed that prenatal hypoxia leads to a decrease in oxygen flux per unit of mass

for both the maximal OXPHOS capacity through the NS-pathway (complexes I and II simultaneously) and complex IV activity. This occurred only in male, but not female, placentae. Without considering sex differences, a recent study in mice also showed that maternal exposure to hypoxia during late-gestation reduced placental labyrinth zone mitochondrial OXPHOS respiration through complex I and complexes I and II simultaneously (70). However, in guinea pigs, complex I activity was decreased by hypoxia during late-gestation (in only male placentae) (144). In addition, hypoxic human term placental tissues (sex not specified) from high-altitude and preeclamptic pregnancies were reported to have decreased expression of complexes I and IV, which may potentially lead to mitochondrial dysfunction and increased ROS production in hypoxic placentae (94, 145). Our data, when presented as FCR, clearly indicate that in the female placentae only, prenatal hypoxia increased respiration through the N-pathway (complex I) whereas respiration through the S-pathway (complex II) was decreased. An increase in electrons entering into the N-pathway (through complex I) versus the S-pathway (through complex II) is known to provide a higher phosphate/oxygen (P/O) ratio, i.e. ATP produced per oxygen atom reduced by the electron transport system (274). When NADH (N-pathway; complex I-linked substrate) is oxidized by the ETS, about 2.5 ATP are formed, in contrast to 1.5 ATP formed with Succinate (S-pathway; complex II-linked substrate), with both pathways utilizing same the amount of oxygen (274). This suggests that in prenatally hypoxic conditions, the female placentae may use oxygen more efficiently to produce greater amounts of ATP by increasing the flux through the N-pathway (complex I) compared to the S-pathway (complex II). However, whether such an adaptation leads to increased ATP generation in the hypoxic female placentae remains to be determined.

My data suggests that complex IV activity was reduced in male placentae from dams exposed to prenatal hypoxia. Complex IV acts as the terminal electron acceptor in the electron

transport system. Therefore, reduced complex IV activity leads to a higher leakage of electrons, and ultimately increased ROS production (275). These findings are in line with a recent study showing reduced complex IV activity in the prenatally hypoxic guinea-pig male placentae, but not in females.(144) Together with our own results, this indicates that prenatal hypoxia can directly impair complex IV respiration in a sex-specific manner (144). nMitoQ treatment effectively increased complex IV activity in placentae from hypoxic male fetuses, suggesting that this could be one of the mechanisms by which nMitoQ treatment can protect male placentae against placental ROS, as demonstrated previously in Chapter 2.

The flux control ratio for LEAK was used as an indicator of mitochondrial coupling independent of the mitochondrial content and OXPHOS capacity. In placentae from prenatally hypoxic male but not female fetuses, LEAK remained unaffected by nMitoQ treatment. However, we observed that nMitoQ treatment increased LEAK in both male and female normoxic placentae. MitoQ has been reported to act as a pro-oxidant in normal intact cells, whereas it serves as an antioxidant when ROS generation is increased under pathological conditions (276). In line with this, our data suggest that there might be detrimental effects of maternal antioxidant intervention during normal pregnancies, and that placenta-targeted antioxidant treatment strategies should thus be developed only to treat complicated pregnancies.

Studies have shown that hypoxic and nutritional stressors during pregnancy can alter placental mitochondrial function along with changes in mitochondrial biogenesis and content, all of which can affect normal placental function and reduce oxygen and nutrient delivery, thus impairing fetal development (70, 277). Mitochondrial biogenesis is regulated by PGC1α which acts as a co-transcriptional regulation factor that can bind to and activate the transcriptional activity of NRF1 (121). NRF1 can bind to the mitochondrial transcription factor A (*Tfam*) gene promoter and induce

expression of TFAM, which drives transcription and replication of the mitochondrial DNA (120). In our study, expression of mitochondrial biogenesis marker, PGC-1α, was not altered by prenatal hypoxia, while NRF1 expression was increased in prenatally hypoxic female placentae, without effect of nMitoQ treatment. However, despite increased NRF1 expression, mitochondrial content remained unaffected by both prenatal hypoxia and nMitoQ treatment. A possible explanation might be that increased NRF1 expression does not necessarily equate with increased transcriptional activity of NRF1. This suggests that alterations to the mitochondrial respiration observed in our study are predominantly through an intrinsic qualitative response rather than quantitative changes. It has been shown that the differences observed in mitochondrial biogenesis and content in response to adverse prenatal conditions (such as hypoxia) are dependent on severity and timing of the prenatal insult (122). For example, mitochondrial content is increased in placental tissues from early-onset but not late-onset preeclampsia, suggesting that in addition to the severity and timing of the hypoxic exposure, changes seen in mitochondrial content in response to pregnancy complications also depend on the length of the gestational insult (i.e. early- versus late-gestation) (71). Therefore, it could be speculated that in our model of prenatal hypoxia, changes in mitochondrial biogenesis and content may have been more evident when measured at an earlier gestational age and/or when exposed to prenatal hypoxia earlier in pregnancy for a longer period of time.

Additionally, mitochondrial function may be affected by altered mitochondrial fission and fusion. Of the core fusion and fission proteins, only Drp1 (a cytosolic GTPase that regulates mitochondrial fission) and OPA1 (mediates inner mitochondrial membrane fusion) expressions have been shown to be altered by ROS (278, 279). Drp1-mediated mitochondrial fission depends on phosphorylation of Drp1 at serine 616 residue that increases Drp1 activity and translocation of phosphorylated Drp1 to the outer mitochondrial membrane (268, 269). Mitochondrial

fragmentations via increased fission are linked to mitochondrial dysfunction and increased ROS generation (278, 280). There were no changes in Drp1 activity between the groups in whole tissue lysates from both male and female placentae, but there is a possibility that increased translocation of Drp1 to the mitochondrial outer membrane might enhance mitochondrial fragmentation, which remains to be further studied. Furthermore, despite no changes in MFN1 expression in prenatally hypoxic male and female placentae, we observed that prenatal hypoxia reduced the ratio of L- to S-OPA1 in the placentae from male, but not female, offspring. Furthermore, prenatal hypoxia reduced the ratio of L- to S- OPA1 in the placentae from male, but not female, offspring. Under normal conditions, OPA1 activity is dependent on its proteolytic cleavage by OMA1 proteases (constitutively cleaves L-OPA1 to S-OPA1) (264). However, in response to oxidative stress, enhanced activity of OMA1 results in loss of L-OPA1 (promotes fusion) and accumulation of S-OPA1 (prevents fusion of dysfunctional mitochondria). This suggests that oxidative stress-induced OPA1 processing inhibits mitochondrial fusion (281). Our male placental data is in line with previous studies showing that oxidative stress in placentae from preeclamptic pregnancies led to impaired mitochondrial respiratory capacity and decreased expression of L-OPA1 (282, 283). Moreover, this reduction in the pro-fusion environment may also explain the reduction in complex IV activity that we observed in hypoxic male placentae. In contrast, nMitoQ treatment increased the ratio of L- to S- OPA1 in only hypoxic female placentae. nMitoQ treatment had no effect on MFN1 expression in both male and female placentae but increased the ratio of L- to S- OPA1 in only hypoxic female placentae. The underlying mechanisms for a pro-fusion environment following nMitoQ treatment in females are not fully understood and remain to be further studied. A possible explanation might be that this could be mediated in part by the increased effectiveness of nMitoQ treatment in reducing oxidative/nitrosative stress and improving oxygenation in placentae from only

female offspring, as shown in chapter 2.

In summary, my data suggest sex-specific dichotomous effects of prenatal hypoxia and nMitoQ treatment on placental mitochondrial function. nMitoQ treatment increased complex IV activity in hypoxic male placentae, while promoting a pro-fusion environment in placentae from female offspring exposed to prenatal hypoxia. Thus, the sex-specific differences observed in the effectiveness of nMitoQ treatment in improving mitochondrial respiration could be due to changes in mitochondrial function in male and female placentae from dams exposed to prenatal hypoxia. Previous studies suggest that placental adaptations and responses to oxidative/nitrosative stress differ in male and female placentae under conditions of adverse intrauterine environments (248). Overall, my current study illustrates that placental mitochondrial function could be a contributing factor to the sexual dimorphism that has been observed in placental morphological and functional adaptations in response to prenatal hypoxia, and that placenta-targeted treatment strategies could possibly optimize fetal growth in complicated pregnancies.

CHAPTER 4:

Placenta-Targeted Treatment in Hypoxic Dams Improves Maturation and Growth of Fetal Cardiomyocytes in Vitro via the Release of Placental Factors

A version of this chapter has been published:

***Ganguly E**, Spaans F, Morton JS, Kirschenman R, Aljunaidy MM, Phillips TEJ, Case CP, Cooke CLM and Davidge ST. Placenta-Targeted Treatment in Hypoxic Dams Improves Maturation and Growth of Fetal Cardiomyocytes In Vitro Via The Release of Placental Factors. Experimental Physiology*. 2020; 105:1507-1514

Author contributions: **EG**; experimental design, data acquisition, data analysis, draft preparation and critical revision of manuscript, **FS**; experimental design, data analysis, draft preparation and critical revision of manuscript; **JSM**; experimental design and critical revision of manuscript; **RK**; data acquisition and critical revision of manuscript; **MMA**; experimental design, and critical revision of manuscript; **TP**; experimental design, provided essential study materials, critical revision of manuscript, **CPC**; experimental design, provided essential study materials, critical revision of manuscript, **CLC**; experimental design, data analysis and critical revision of manuscript, **STD**; experimental design, data analysis, draft preparation and revision of manuscript.

4.1 Introduction

As previously discussed in Chapter 1, recent research has demonstrated an association between a sub-optimal prenatal environment leading to chronic fetal hypoxia and abnormal cardiac function in both fetal and adult life [reviewed in (179)]. Adult rat offspring born from hypoxic pregnancies were shown to have increased susceptibility to ischemia and reperfusion (I/R) injury, suggesting that a sub-optimal prenatal environment may limit the potential of the heart to adapt to changes in cardiac load after birth (284). Moreover, prenatal hypoxia has also been shown to impair cardiac development in fetal life [reviewed in (4)]. For example, maternal hypoxia in rats leading to placental oxidative stress (138), increased percentage of fetal cardiomyocyte binucleation (a state in which cardiomyocytes become terminally differentiated and marker of premature exit from the cell cycle), and increased heart weight through an increase in cardiomyocyte size (i.e. hypertrophy) (16). However, the exact mechanisms by which prenatal hypoxia can affect fetal cardiomyocyte development still remains to be fully understood.

Together with our collaborators, we recently showed a potential mechanism linking placental oxidative stress to fetal organ development through the release of factors (oxidative stress- and hypoxia-related) from the placenta into the fetal circulation (156). In a rat model of maternal hypoxia, conditioned media containing factors secreted from a hypoxic placenta impaired fetal neuron development (156). However, it is not known whether factors derived from hypoxic placentae may also impair fetal cardiac development in a similar manner.

Using a model of fetal hypoxia, in our previous chapters, we showed that prenatal hypoxia leads to mitochondrial dysfunction, increased placental oxidative stress and impaired placental function; all of which may ultimately impair fetal organ development. Given that placental oxidative stress represents a potential link between hypoxia in pregnancy and impaired fetal organ

development [reviewed in (122)], we used our placenta-targeted nMitoQ treatment strategy to prevent placental oxidative stress and, thereby, impaired fetal organ development. In rats exposed to prenatal hypoxia, maternal treatment with nMitoQ improved mitochondrial function, prevented placental oxidative stress, and improved oxygenation and placental morphology in a sex-specific manner, as nMitoQ treatment was more effective in the female placentae (272). Furthermore, nMitoQ treatment improved fetal neuron development in hypoxic offspring (156), and conditioned media from nMitoQ-treated human preeclamptic placental explants improved neuronal development *in vitro* (231). However, whether nMitoQ treatment improves development of key fetal organ systems, such as the heart, and whether there are potential sex differences, are not known. We hypothesize that factors released from hypoxic placentae impair fetal cardiomyocyte development (i.e. terminal differentiation and hypertrophy), and that nMitoQ will prevent this effect, thereby improving fetal cardiac development.

4.2 Materials and Methods

4.2.1 Experimental model of prenatal hypoxia

Three-month-old female Sprague-Dawley rats (weighing 250-275 g) were housed with male Sprague–Dawley rats overnight and pregnancy was confirmed the following morning by the presence of sperm in a vaginal smear (defined as gestational day (GD) 0 of pregnancy). Following which, pregnant dams were treated as described in Section 2.2.2. On GD21 (term= GD22) dams were anaesthetized with isoflurane (by inhalation) and then killed by exsanguination via cardiac puncture. Following which fetuses were sexed and whole placentas (including labyrinth and junctional zone) from either male and female fetuses (three/sex/litter) were processed and cultured

for collection of placental conditioned media.

4.2.2 Placental culture and conditioned media collection

Placental conditioned media was collected using a previously established method (156). In short, placentas from three male and three female fetuses were collected from each of the dams per experimental group, cut into halves and placed into 12-well plates containing placental media (DMEM/F-12 [Dulbecco's Modified Eagle Medium/Ham's F-12] supplemented with 1% Gentamicin, 0.4% penicillin-streptomycin, 0.6% L-Glutamine and 10% Fetal Bovine Serum), separated by sex. Placentas were cultured [37ºC, 8% O_2 (normoxic conditions for placentas)] for 24h [as reviewed in (285)], during which the culture media was replaced with fresh placental media after 4h (to remove artefacts of a stressed placenta). The placental conditioned media was collected after 24h from three different male and female placentas from each of the dams per experimental group, which was pooled (separately based on the sex) and then frozen at -80ºC until further used for the cardiomyocyte treatments.

4.2.3 Cardiomyocyte isolation and culture

In a separate cohort of untreated normoxia dams (n=5), male and female fetuses were randomly selected from each litter. Ventricular cardiomyocytes were isolated from the fetal hearts and pooled based on the sex (n=5-6/dam/sex) using a modified protocol previously described for isolation of rat neonatal ventricular cardiomyocytes (286). Briefly, after euthanasia by decapitation, left ventricles were dissected in dissociation buffer (116 mM NaCl, 20 mM HEPES, 0.8 mM Na_2HPO_4, 5.6 mM glucose, 5.4 mM KCl, 0.8 mM $MgSO_4$, pH 7.35). Serial enzymatic digestions with an enzyme buffer (dissociation buffer containing collagenase type II (0.4 mg/ml) and pancreatin (0.6 mg/ml)) were performed at 37ºC. The remaining cell pellets were filtered through a 70 µm filter and spun once at 1300g. Following this, the resultant cell pellets were resuspended in

cardiomyocyte culture media (DMEM/F-12 media supplemented with 10% horse serum, 5% fetal bovine serum and 1% penicillin-streptomycin). Fibroblasts were separated from cardiomyocytes using differential adhesion. This pre-plating step removes fibroblasts which adhere to the uncoated cell-culture dish within a couple of hours. After incubation for 75 minutes, the media containing non-adherent cardiomyocytes was collected and number of viable cardiomyocytes was counted using the Trypan Blue dye exclusion test (Sigma-Aldrich, Oakville, ON, Canada). Cardiomyocytes were plated on 1% gelatin coated 24-well plates and grown [37ºC, 21% O_2] overnight in cardiomyocyte culture media.

4.2.4 Cardiomyocyte culture with placental conditioned media

After growing in 24-well plates for 24h, fetal cardiomyocytes from males and females were incubated with 500 µl per well of same sex placental conditioned media (supplemented with 20 mM cytosine b-D-arabinofuranoside, Sigma-Aldrich, Canada) to prevent the proliferation of fibroblasts. After 24h of incubation with placental conditioned media, cardiomyocytes were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton-X100 at room temperature for immunofluorescence analysis.

4.2.5 Immunofluorescence staining for MYH6/7

Cardiomyocytes were stained with MYH6/7 (anti-heavy chain cardiac myosin). Following which cardiomyocyte binucleation and cell size was determined using a previously described protocol (286). Briefly, after fixation, cardiomyocytes were incubated overnight with a primary antibody against MYH6/7 (1:150 in PBS; Abcam, Burlingame, CA, USA). The next day, cardiomyocytes were washed thrice in PBS and incubated with secondary antibody (1:200; goat-anti-mouse-AF488, Alexa, Invitrogen Canada, Burlington, ON, Canada) for 60 minutes in the dark. Cells were washed with PBS three times and incubated with DAPI (300 nM; Thermo scientific,

Waltham, MA, USA) for 20 minutes to stain the nuclei.

4.2.6 Imaging and assessment of cardiomyocyte cell size/binucleation

Images were taken immediately after staining using an Olympus IX81fluorescence microscope with CellSens Dimensions software (Olympus, Japan). For cardiomyocyte binucleation and cell size measurements, pictures (40X magnification) of ten random fields per well were taken for two replicate wells. Cell size (cross-sectional area) of mononucleated and binucleated cardiomyocytes were calculated using CellSens Dimension software (Olympus, Japan). The percentage of binucleation was calculated by counting the number of mononucleated and binucleated cardiomyocytes per microscopic field and expressed as number of binucleated cells / total number of cells per image.

4.2.7 Data analysis and statistics

Data and statistical analyses were performed using GraphPad Prism 8.02 software. All data were expressed as mean ± S.D and analyzed using a two-way ANOVA followed by Sidak's multiple comparison *post hoc* tests; $p<0.05$ was considered significant. Significant outliers (as assessed using Grubb's test) were removed from final analyses.

4.3 RESULTS

4.3.1 Percentages of proliferating (mononucleated) or terminally differentiated (binucleated) cardiomyocytes

There were no significant differences between our findings (such as percentage of mononucleated and binucleated cardiomyocytes, and area of mononucleated and binucleated cardiomyocytes) in male and female cardiomyocytes exposed to the same sex placental conditioned

media; therefore, data were combined with blue symbols indicating male cardiomyocytes and orange symbols indicating female cardiomyocytes. Conditioned media from prenatally hypoxic placentae reduced the percentage of mononucleated cardiomyocytes compared to conditioned media from normoxic placentae (Figure 4.1A). Moreover, placental conditioned media from hypoxic dams treated with nMitoQ increased the percentage of mononucleated cardiomyocytes to levels similar to normoxic conditions (Figure 4.1A). In line with the reduction in the percentage of mononucleated cardiomyocytes, conditioned media from prenatally hypoxic placentae increased the percentage of binucleated cardiomyocytes compared to conditioned media from normoxic placentae (Figure 4.1B). nMitoQ treatment prevented this increase in the percent binucleation only in cardiomyocytes exposed to conditioned media from prenatally hypoxic placentae (Figure 4.1B).

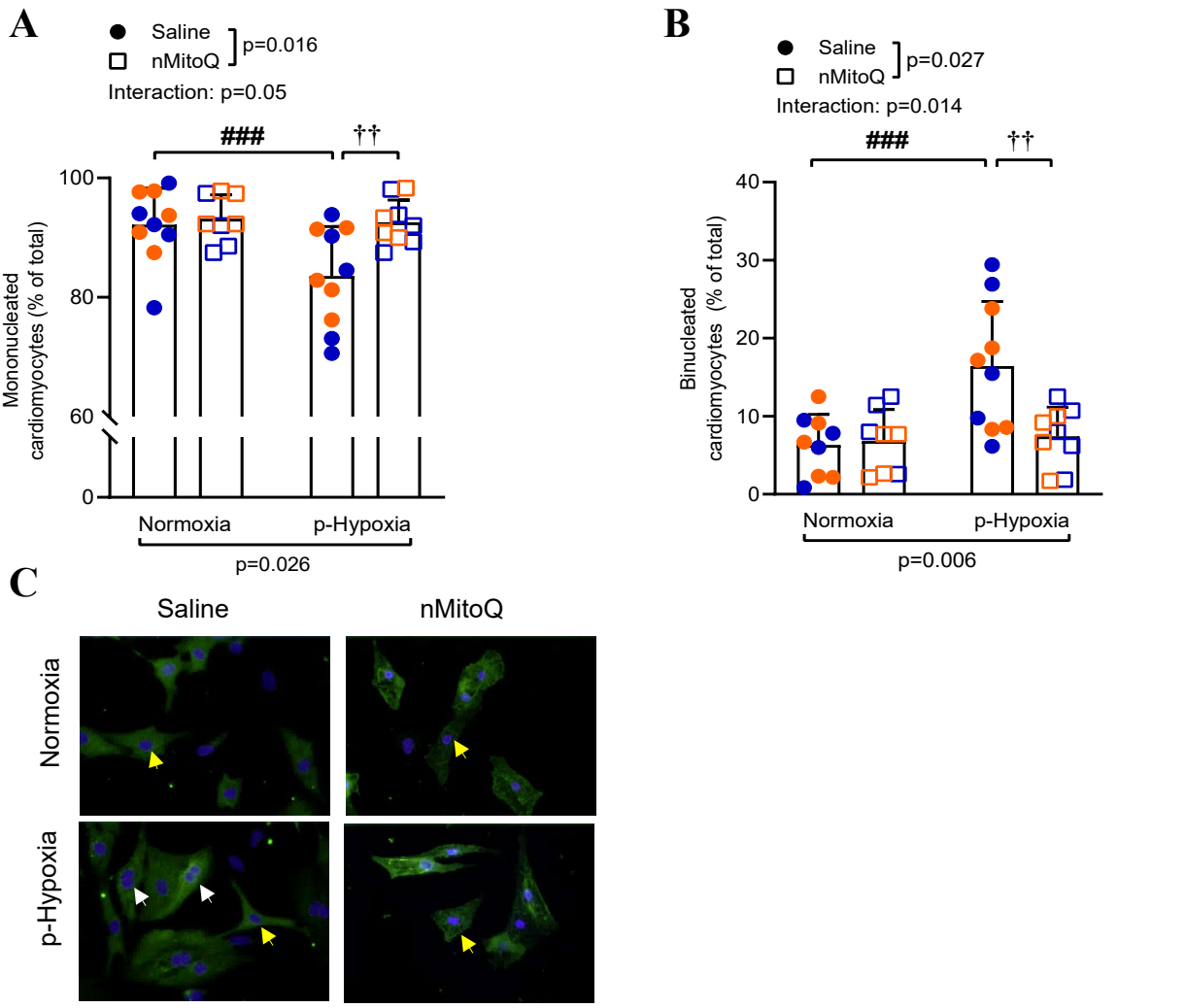

Figure 4.1. Effects of prenatal hypoxia-induced placental secreted factors and nMitoQ treatment on relative percentages of mononucleated and binucleated cardiomyocytes. Percentages of (**A**) mononucleated and (**B**) binucleated cardiomyocytes in isolated rat fetal cardiomyocytes after exposure to placental conditioned media from male (blue) and female (orange) placentas collected from normoxic and hypoxic dams that were treated with nMitoQ (open squares) or saline (solid circles). (**C**) Representative immunofluorescent images of mononucleated (yellow arrow) and binucleated (white arrow) cardiomyocytes stained with anti-cardiac heavy chain myosin (MYH6/7). Data are represented as mean ± SD. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test. $^{###}p<0.001$ compared to normoxia-saline, $^{††}p<0.01$, compared to hypoxia-saline group. n=5 dams with 5-6 pups (pooled)/sex/dam.

4.3.2 Mononucleated and binucleated cardiomyocyte size

The size of mononucleated and binucleated cardiomyocytes was increased after incubation with conditioned media from prenatally hypoxic placentae compared to conditioned media from normoxic placentae (Figure 4.2A+B). nMitoQ treatment decreased mononucleated and binucleated cell sizes only in cardiomyocytes exposed to placental conditioned media from prenatally hypoxic dams (Figure 4.2A+B).

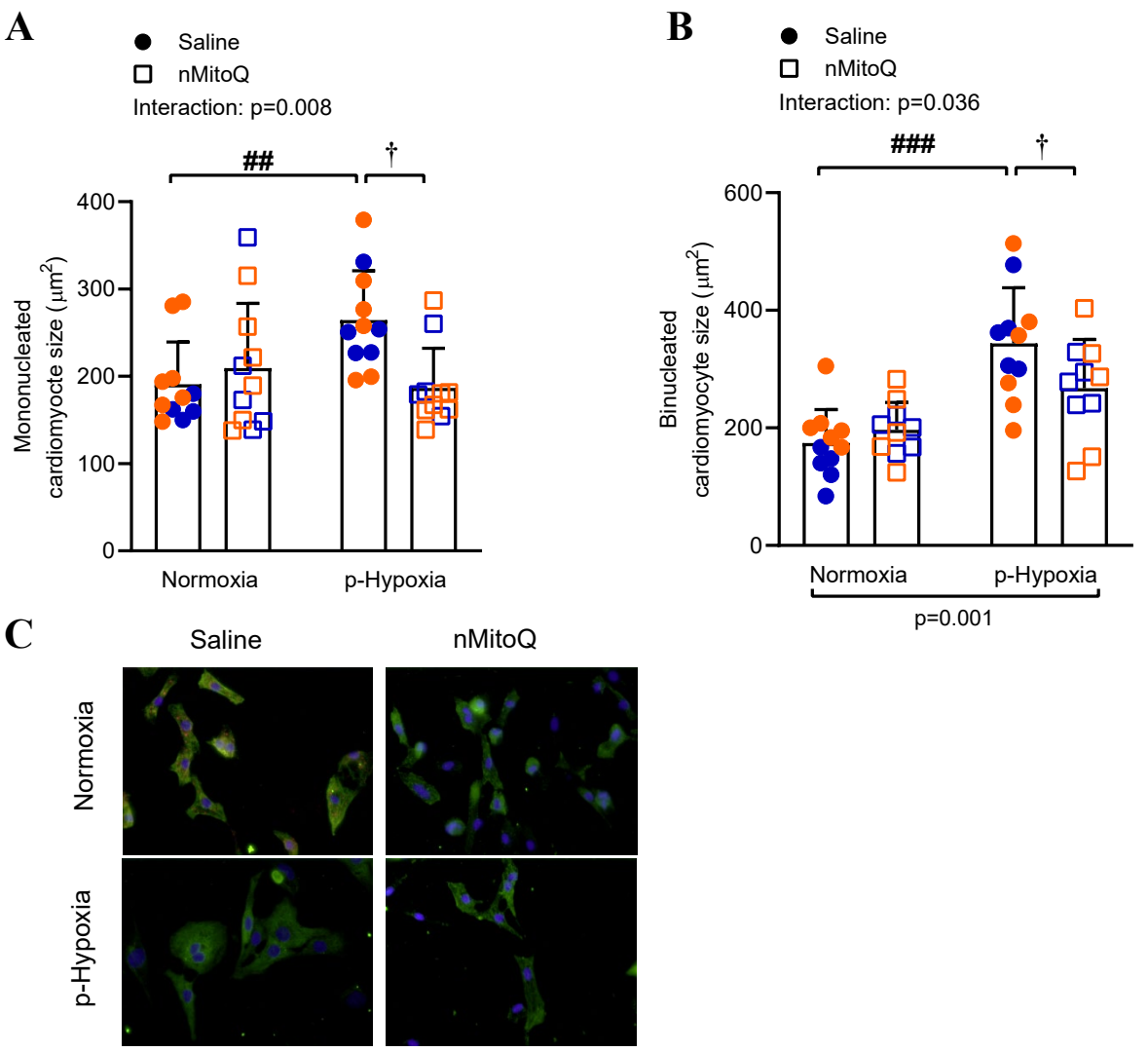

Figure 4.2. Effects of prenatal hypoxia-induced placental secreted factors and nMitoQ treatment on mononucleated and binucleated cardiomyocyte size. The size of (**A**) mononucleated and (**B**) binucleated cardiomyocytes was assessed after exposure to placental conditioned media from male (blue) and female (orange) placentas collected from normoxic and hypoxic dams that were treated with nMitoQ (open squares) or saline (solid circles). (**C**) Representative immunofluorescent images showing differences in cell sizes of mononucleated and binucleated cardiomyocytes stained with anti-cardiac heavy chain myosin (MYH6/7). Data are represented as mean ± SD. All groups were compared using a two-way ANOVA followed by Sidak's post hoc test. $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to normoxia-saline, $^{\dagger}p<0.05$ compared to hypoxia-saline group. n=5 dams, with 5-6 pups (pooled)/sex/dam.

4.4 Discussion

The current study was designed to examine the potential contribution of factors derived from placenta on fetal cardiomyocyte development in a rat model of prenatal hypoxia, with a specific focus on the effects of nMitoQ treatment on cardiomyocyte maturation and size. We demonstrated that factors derived from placentae previously exposed to prenatal hypoxia can impair cardiomyocyte development (i.e. induce terminal differentiation and increase cell size), which was not sex dependent. In addition, we showed that maternal treatment with nMitoQ resulted in release of factors derived from placentae that increased the proportion of mononucleated cardiomyocytes (suggested to have proliferative capacity) (184) and prevented increase in cardiomyocyte cell size.

The fetal and postnatal development of the heart is a complex biological process. Cardiomyocyte endowment (number of cells) at birth is thus determined by a balance between cellular proliferation and terminal differentiation (180). Moreover, cardiomyocyte endowment at birth is dependent on the timing and duration of prenatal insults (such as hypoxia), which have been shown to impact cardiac development differently. For example, unchanged relative heart weights has been observed in a model of early-onset hypoxia (13% O_2, GD6-20) (18) whereas we have observed increased relative heart weight in offspring exposed to late-onset hypoxia (11% O_2, GD15-21) (15). However, despite the differences in outcomes, these studies show that prenatal hypoxia can lead to cardiac changes and dysfunction later in adult life.

A reduction in the number of cardiomyocytes at birth could be compensated for by increases in cardiomyocyte size, all of which may increase susceptibility to cardiac dysfunction in fetal and adult life (187, 189). For example, ovine fetuses exposed to high altitude hypoxia during pregnancy had increased expression of hypoxic biomarkers (such as HIF1α) in the developing hearts and increased susceptibility to I/R injury (189). Moreover, adult offspring born from hypoxic

pregnancies had similar heart weights compared to normoxic controls but fewer and larger cardiomyocytes, which may be linked to the cardiac dysfunction that is observed in these adult offspring (287). In our study, we found that conditioned media from hypoxic placentae not only increased the percentage of binucleated cardiomyocytes but also increased sizes of both mononucleated and binucleated cardiomyocytes. These findings are in line with previous studies showing that a prenatal hypoxic insult in rats led to an *in vivo* increase in the proportion of binucleated cardiomyocytes which concomitantly resulted in a reduction of mononucleated cardiomyocytes (suggested to have proliferative capacity), and increased cardiomyocyte size (16, 288). Furthermore, even though cardiomyocyte development (in terms of the timing of cardiomyocyte maturation) is different between sheep and rats, growth-restricted hypoxaemic fetuses from sheep models of 'uteroplacental insufficiency' were also shown to have fewer but relatively larger cardiomyocytes at birth [reviewed in (186) (288)]. Therefore, together with our data, this shows that irrespective of the species, a prenatal hypoxic insult could directly lead to premature transition of cardiomyocytes to a terminally differentiated binucleated phenotype.

Our data demonstrated that factors released from a hypoxic and "distressed" placenta are able to promote premature transition of cardiomyocytes to a terminally differentiated binucleate phenotype lacking proliferative capacity and lead to increased cardiomyocyte cell size. Moreover, we showed that these effects of prenatal hypoxia (i.e. increased terminally differentiated cells and increased hypertrophy) were prevented with maternal nMitoQ treatment. As discussed in chapter 2, nMitoQ treatment was specifically designed to target placental oxidative stress, and was shown to decrease placental oxidative stress. Our data confirmed our hypothesis that treating the placenta alters the release of 'stress-related' factors from placentae previously exposed to prenatal hypoxia. Taken together, our data suggest that nMitoQ treatment strategy by preventing placental oxidative

stress can improve fetal cardiomyocyte development and has the potential to prevent fetal programming of cardiovascular diseases.

The placenta releases several circulating factors such as growth factors (e.g., PGF and VEGF, cytokines (e.g., TNFα and ILs), and miRNAs, which may affect development of key fetal organ systems (159-161). Interestingly, expression profiles of placental-derived miRNAs have been shown to be altered by oxidative stress in the placenta (289). Further, circulating miRNAs can cross the placenta to enter the fetal circulation. For example, in diabetic pregnant mice, circulating miRNAs were shown to enter the fetal circulation, eventually leading to fetal cardiac dysfunction (177). Interestingly, together with our collaborators we previously demonstrated that oxidative stress in the placenta altered placenta-derived miRNA secretion which could be related to abnormal gene expression in the fetal brain, eventually leading to impaired fetal organ development (156). miRNAs derived from an oxidatively stressed placenta may also be linked to abnormal fetal cardiomyocyte development. For example, an increase in certain miRNAs, such as increased miR-29a (which leads to decreased expression of cell cycle promoter, Cyclin D2 gene) reduced cardiomyocyte proliferation, (290), and increased miR-208a (which leads to upregulation of β myosin heavy chain (MYH7) gene expression associated with pathological hypertrophy) increased cardiomyocyte cell size (291). Previously we showed that both miR29a and miR208a were upregulated in placental conditioned media from prenatally hypoxic dams (156), which suggest that these placental-derived miRNAs could be one of the factors that are involved in the changes we observed in our study. Moreover, in a previous study from our laboratory together with our collaborators we found that in placental conditioned media from hypoxic dams treated with nMitoQ there was an upregulation of placental-derived miRNAs that could potentially increase cardiomyocyte proliferation (such as decreased miR-30, which suppresses Cyclin A2 gene

(promotes cell cycle activity)), reduce cardiomyocyte binucleation (such as increased miR-133a, which has been shown to reduce binucleation via reduced Sirtuin 1 expression), and reduce cardiomyocyte hypertrophy (such as decreased miR-208a, which targets MYH7 gene expression) (156). In addition, the altered expression profiles of miRNAs in the placental conditioned media from hypoxic dams after nMitoQ treatment were shown to improve fetal brain development (156). Taken together with our previous findings, it may be speculated that abnormal fetal cardiomyocyte development in response to factors released from a hypoxic and oxidatively stressed placenta could be mediated by placental-derived miRNAs; which will be a focus for our future studies.

Interestingly, in our current study there was no sex-specific divergence in the effects of factors released from prenatally hypoxic placentas on fetal cardiomyocyte development and the effectiveness of nMitoQ treatment. In summary, our data suggest a role for factors derived from the placenta in fetal cardiac development. Moreover, our study shows a dichotomous effect of prenatal hypoxia and nMitoQ treatment on fetal organ development. Overall, our data highlights the contribution of prenatal hypoxia and placental-derived factors in fetal cardiomyocyte development and shows that treatment targeted against placental oxidative stress could possibly prevent fetal programming of cardiac diseases.

CHAPTER 5: General Discussion

5.1 Summary of the Thesis

Oxidative stress has been described as a key mediator of placental dysfunction in complications of pregnancy commonly associated with fetal hypoxia and later-life cardiovascular diseases (292). We used a mitochondria-specific antioxidant, MitoQ, which was targeted against placental oxidative stress by encapsulation into biodegradable polymeric nanoparticles (nMitoQ). Moreover, recent evidence suggest that sex-specific fetal adaptations to adverse *in utero* environments are likely to be mediated by differential responses in placentae from males and female fetuses (224). Our studies therefore aimed to also assess the sex-specific effects of nMitoQ treatment. To our knowledge, the data presented in this thesis are the first to describe the sexually dimorphic effects of an antioxidant treatment strategy targeted to the placenta on its morphological and functional phenotype, and the contribution of factors derived from placentae of prenatally treated hypoxic dams to fetal cardiomyocyte development.

5.1.1 Male placenta: Effects of hypoxia and nMitoQ treatment

An overview of our findings in the male placenta is presented in Figure 5.1. In male placenta, we found that maternal exposure to hypoxia increased placental oxidative stress, which was prevented by nMitoQ treatment. Moreover, prenatal hypoxia decreased placental oxygenation and reduced mitochondrial complex IV activity. Without improving oxygenation (as shown by increased pimonidazole staining) in prenatally hypoxic placentae, nMitoQ treatment increased placental mitochondrial complex IV activity. Although placental weights remained unaffected, the prenatal hypoxic insult led to reduced fetal weights and fetal weight/placental weight (used as a surrogate of placental efficiency). Additionally, prenatal hypoxia decreased expression of growth

factors *Vegfa* and *Igf2* along with reduced maternal and fetal blood spaces, and fetal capillaries area in the labyrinth zone. nMitoQ treatment had no effect on placental weights and maternal and fetal blood spaces and vascularization, but improved placental efficiency to the normoxic control levels in the hypoxic placentae and led to increased fetal weights. Prenatal hypoxia led to decreased oxygenation in fetal hearts and livers and, nMitoQ treatment improved oxygenation in hearts but not livers of prenatally hypoxic male fetuses.

In summary, by targeting prenatal hypoxia-induced placental oxidative stress, nMitoQ treatment improved mitochondrial complex IV activity, enhanced placental efficiency, and improved oxygenation in hypoxic male fetal hearts as well as increased fetal weight in male fetuses.

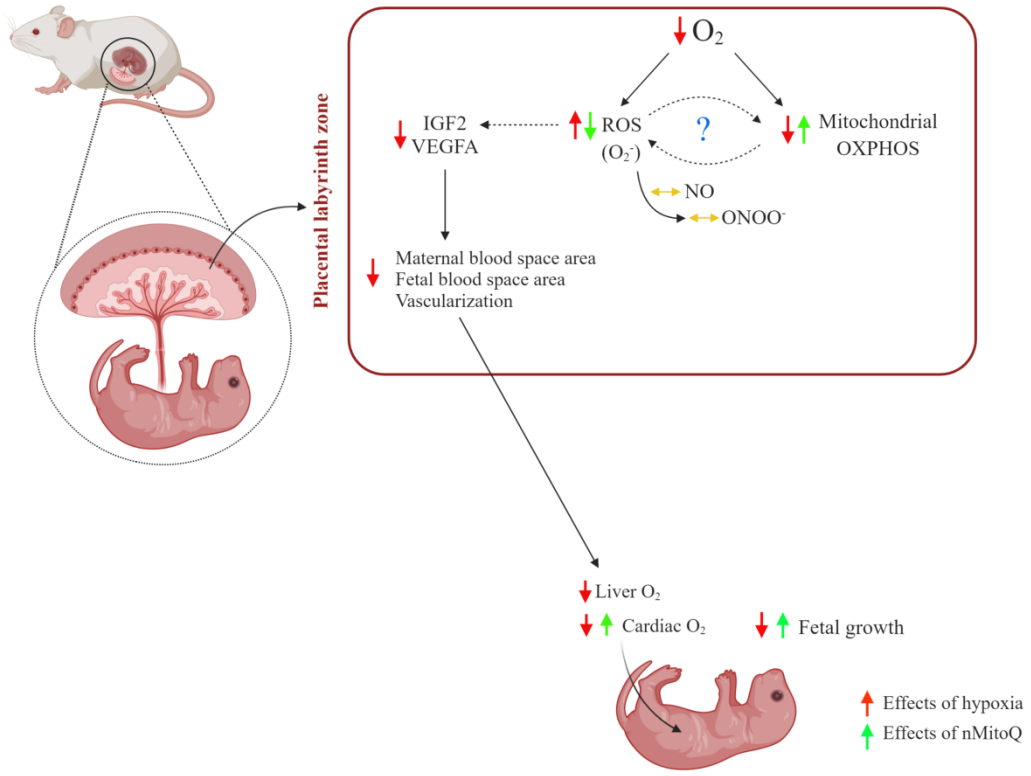

Figure 5.1. Schematic summary of the effects of prenatal hypoxia and nMitoQ treatment on placentae from male fetuses. In male placentae, prenatal hypoxia decreased placental oxygenation, increased ROS production, and decreased mitochondrial OXPHOS respiration (complex IV activity), while nMitoQ treatment without improving placental oxygenation led to decreased ROS production and increased mitochondrial OXPHOS respiration (complex IV activity). Prenatal hypoxia led to decreased maternal and fetal blood spaces and area of the fetal blood capillaries along with reduced *Vegfa* and *Igf2* expression. Fetal growth was reduced in prenatally hypoxic fetuses along with decreased cardiac and liver oxygenation. nMitoQ treatment led to improved oxygenation in fetal hearts compared to livers and increased fetal growth. Created with BioRender.com

5.1.2 Female placenta: Effects of hypoxia and nMitoQ treatment

An overview of the findings from the female placenta is presented in Figure 5.2. In female placenta, as with the males, we showed that maternal nMitoQ treatment prevented prenatal hypoxia-induced placental oxidative stress. Furthermore, our hypoxic insult not only reduced placental oxygenation, but also resulted in increased placental formation of the oxidant $ONOO^-$, which coincided with increased NO levels in the hypoxic placenta. nMitoQ treatment improved placental oxygenation and prevented prenatal hypoxia-induced $ONOO^-$ formation in the placenta without altering basal NO levels. Interestingly, nMitoQ treatment had no effect on placental mitochondrial function, but prenatal hypoxia increased contribution of the N-pathway (through complex I) and decreased contribution of the S-pathway (through complex II). This compensation can likely protect the female placentae from oxidative damage as the N-pathway (through complex I) will produce more ATP per oxygen atom reduced by the ETS in comparison to the S-pathway (through complex II). Indeed, there was no effect of either prenatal hypoxia or nMitoQ treatment on placental efficiency. Without having an effect on placental weight, prenatal hypoxia decreased placental expression of *Vegfa* and *Igf2* along with reductions in maternal and fetal blood spaces, and area of fetal blood capillaries in the placental labyrinth zone. nMitoQ treatment led to increased maternal and fetal blood spaces, and fetal capillary area which could be a result of increased *Vegfa* and *Igf2* expression as observed in the nMitoQ-treated hypoxic placenta. Prenatal hypoxia led to reduced fetal weights and decreased oxygenation in fetal hearts and livers. nMitoQ treatment without increasing fetal weights led to increased oxygenation as the fetal hearts and livers from nMitoQ treated dams were no longer hypoxic.

In summary, in hypoxic female fetuses, nMitoQ treatment reduced placental oxidative and nitrosative stress and improved placental morphology, which was further associated with improved

placental and fetal heart and liver oxygenation. Notably, placental mitochondrial function in hypoxic female fetuses could be an adaptation to support placental function in order to sustain fetal development under hypoxic conditions.

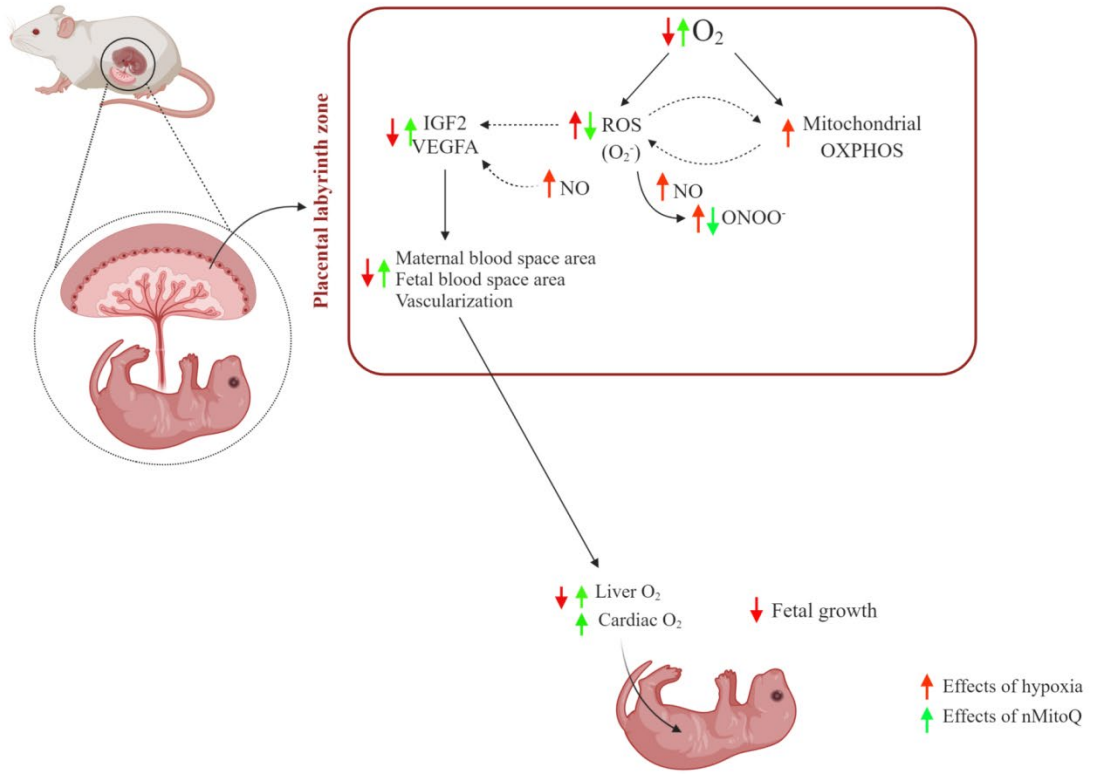

Figure 5.2. Schematic summary of the effects of prenatal hypoxia and nMitoQ treatment on placentae from female fetuses. In female placentae, prenatal hypoxia decreased placental oxygenation, increased ROS production, which was improved by nMitoQ treatment. Prenatal hypoxia increased mitochondrial OXPHOS respiration [(increased oxygen flux through the N-pathway (through complex I) and decreased S-pathway (through complex II)], which remained unaffected by nMitoQ treatment. Prenatal hypoxia led to decreased maternal and fetal blood spaces and area of the fetal blood capillaries along with reduced *Vegfa* and *Igf2* expression. nMitoQ treatment enhanced maternal and fetal blood spaces and area of the fetal blood capillaries and increased expression of *Vegfa* and *Igf2*. Fetal growth was reduced in prenatally hypoxic fetuses along with decreased cardiac and liver oxygenation. nMitoQ treatment led to improved oxygenation in fetal hearts and livers but did not affect fetal growth. Created with BioRender.com

5.1.3 Fetal cardiomyocyte development: Effects of placenta-derived factors and nMitoQ treatment

An overview of the findings from the effects of placenta-derived factors on fetal cardiomyocyte development is presented in Figure 5.3. In Chapter 4, we assessed the role of placenta-derived factors on fetal cardiac development. Factors derived from hypoxic placentae of dams treated with nMitoQ prevented increases in the percentage of binucleated cardiomyocytes (marker of terminal differentiation) and the size of mononucleated and binucleated cardiomyocytes (sign of hypertrophy). Despite the sex-specific effects of nMitoQ treatment on placental morphological and functional phenotype, we found that there were no sex-specific divergences in the effectiveness of our placenta-targeted nMitoQ treatment on fetal cardiomyocyte development. Taken together, our data suggest that a placenta-targeted prenatal treatment strategy that was used to prevent placental oxidative stress could possibly lead to improved fetal heart development.

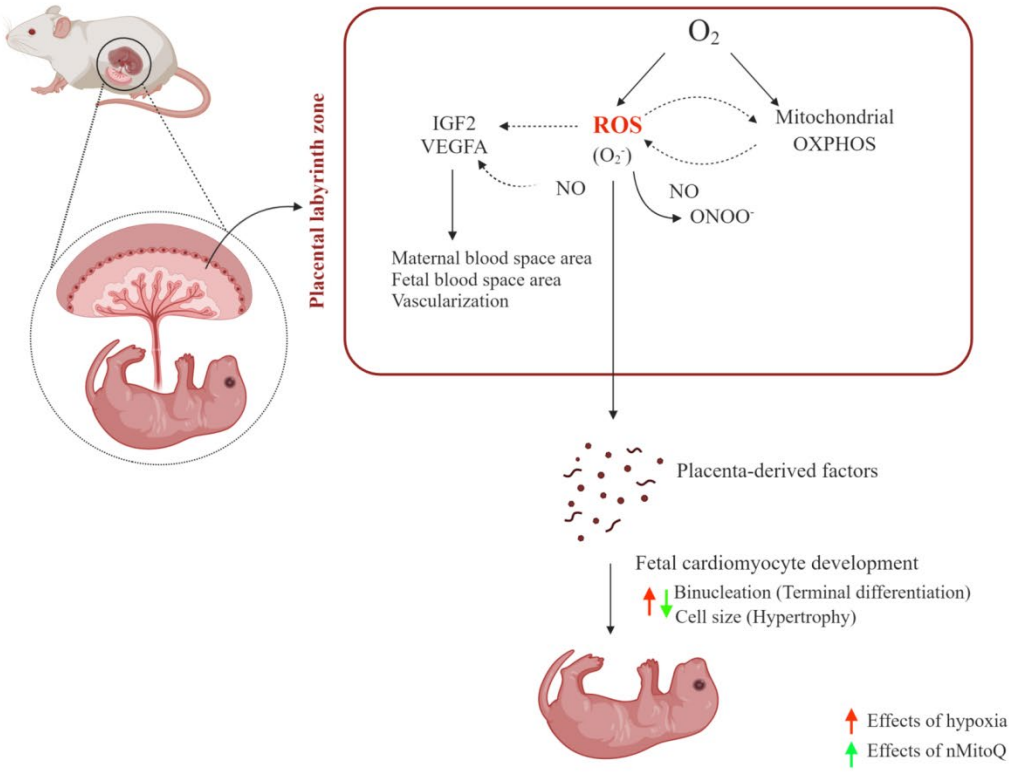

Figure 5.3. Schematic summary of the effects of placenta-derived factors from prenatally hypoxic and nMitoQ treated dams. In both male and female fetuses, placenta-derived factors from prenatally hypoxic and oxidatively stressed placentae led to increased cardiomyocyte binucleation (terminal differentiation) and cell size (hypertrophy), which was prevented by nMitoQ treatment. Arrows indicate effect of prenatal hypoxia (red) and nMitoQ treatment (green). Created with BioRender.com

5.2 Discussion

In accordance with our hypothesis, our data showed that maternal nMitoQ treatment affects placental morphology and functional capacity in a sex-dependent manner, and hence the potential mechanism(s) by which placenta-targeted nMitoQ treatment may impact fetal development are different in males and females.

5.2.1 Oxidative stress and placenta-targeted mitochondrial antioxidant (nMitoQ) treatment strategy

Mitochondrial dysfunction in the placenta results in excessive production of ROS leading to placental oxidative stress, which have been implicated to play a key role in the pathophysiology of several complications of pregnancy, such as FGR (68). Thus, mitochondrial dysfunction and ROS formation could be part of a vicious cycle and may represent a central mechanism of placental dysfunction in complications of pregnancy. Indeed, maternal exposure to hypoxia in mice (10.5% O_2, GD14-19) led to decreased mitochondrial OXPHOS respiration and increased oxidative stress in the placenta, which may account for defects in placental amino acid transport and, ultimately FGR, as previously demonstrated (70). Additionally, human placental tissues from preeclamptic pregnancies are commonly linked with a dysfunctional mitochondrial phenotype and increased ROS levels (293). Being a common feature of growth-restricted and preeclamptic pregnancies in humans, placental oxidative stress has been shown to reduce amino acid uptake in human trophoblasts *in vitro* (294, 295).

Given that placental oxidative stress is a central mediator of placental dysfunction in hypoxic pregnancies, we used the placenta-targeted mitochondrial antioxidant (nMitoQ) treatment strategy. The ubiquinone in MitoQ is rapidly activated to the antioxidant ubiquinol form by

complex II of the ETS (296). The antioxidant ubiquinol form of MitoQ donates a hydrogen atom from one of its hydroxyl groups to a lipid peroxyl radical, which prevents lipid peroxidation within the mitochondrial inner membrane, thus protecting against mitochondrial oxidative damage (217). As MitoQ selectively accumulates within the mitochondrial inner membrane *in vivo*, its effects in cells are thought to be largely due to prevention of mitochondrial oxidative damage (217). MitoQ has been shown to reduce oxidative stress, and there is also evidence that MitoQ decreases the release of ROS from mitochondria. Therefore, the antioxidant effects of placenta-targeted nMitoQ in our study could be due to a reduction in ROS production and protection against cellular and mitochondrial oxidative damage.

5.2.2 Effects of placenta-targeted nMitoQ treatment on prenatally hypoxic male placentae

Our results showed that nMitoQ treatment was unable to improve placental oxygenation, but reduced placental superoxide production (Chapter 2) in the hypoxic male placenta. A reduction in superoxide production could lead to improved mitochondrial complex IV activity (Chapter 3) as observed in the nMitoQ treated hypoxic male placenta. Complex IV acts as a cellular oxygen sensor and transfers electrons to oxygen (the terminal acceptor) (275). Under conditions of chronic hypoxia, a reduction in molecular oxygen can directly inhibit complex IV activity, which reduces electron flow through the ETS and higher leakage of electron, and ultimately increased superoxide production (275, 297). Chronic hypoxia-induced abnormal ROS levels, in turn, can lead to oxidative damage of mitochondrial ETS complexes, thereby perpetuating the effects of prenatal hypoxia on mitochondrial dysfunction even further (110, 144). Because nMitoQ treatment did not improve placental oxygenation (Chapter 2), we speculate that the effects of nMitoQ treatment on placental mitochondrial function are likely to be mediated by a reduction in ROS-induced oxidative

damage of mitochondrial complex IV activity.

Furthermore, the inability of nMitoQ treatment to improve placental oxygenation could be due to the fact nMitoQ treatment was unable to improve hypoxia-induced impaired placental vascularization (Chapter 2) and decreased maternal and fetal blood spaces (Chapter 2); all of which might lead to reduced placental perfusion and ultimately placental hypoxia. The placental vasculature is essential for providing oxygen to facilitate fetal growth, and is most affected in response to a suboptimal *in utero* environment (298). For instance, human placental tissues from FGR pregnancies often exhibit impaired placental vascularization and increased fetoplacental vascular resistance, which may ultimately lead to reduced placental oxygen delivery and abnormal ROS production (299). Placental ROS in turn have been shown to cause mitochondrial dysfunction, DNA damage, protein oxidation, lipid peroxidation and altered gene expression; which are known to affect normal placental function and potentially reduce oxygen and nutrient delivery, thus impairing fetal development (68, 151). Indeed, expression of placental amino acid and glucose transporters have been shown to be reduced in oxidatively stressed human placental explants (300). Therefore, it may be speculated that nMitoQ treatment may act to protect placental amino acid and glucose transporters against ROS-mediated oxidative damage.

nMitoQ treatment restored impaired placental efficiency as the male fetuses were no longer growth restricted (Chapter 3) and led to improved oxygenation in the hearts but not livers from male fetuses at term (Chapter 2). The fetus, in response to hypoxia, adapts to redistribute the cardiac output away from peripheral vascular beds and towards essential circulation to maintain perfusion of key organ systems such as the brain, heart and adrenal glands, a phenomenon known as “brain sparing” (301). Although placental oxygenation was not improved by nMitoQ treatment, increases in only fetal cardiac oxygenation compared to hepatic oxygenation suggest that the nMitoQ

treatment could result in increased blood flow as well as oxygen and glucose delivery away from the peripheral circulation towards the fetal heart and brain. In line with our data, a recent study by Botting *et al*. demonstrated that chronic maternal treatment with MitoQ in hypoxic sheep pregnancies (GD105-138) restored fetal growth while maintaining fetal brain sparing and improving NO-dependent dilatory responses in fetal femoral arteries (302). Therefore, it is possible that the increased oxygenation in male fetal hearts following nMitoQ treatment could be mediated by changes in vasodilatory responses in the fetal femoral arteries.

5.2.3 Effects of placenta-targeted nMitoQ treatment on prenatally hypoxic female placentae

In contrast, in placentae from hypoxic female fetuses, nMitoQ enhanced placental vascularity (i.e. increased fetal capillary surface area and enhanced maternal and fetal blood spaces) (Chapter 2). This may explain why the female placentae and fetuses were no longer hypoxic following nMitoQ treatment. The morphological adaptations in nMitoQ-treated hypoxic female placentae may be secondary to a reduction in oxidative stress and $ONOO^-$ formation, and an increase in placental NO bioavailability (Chapter 2). Studies indicate that NO is required for VEGF-mediated angiogenesis (303, 304), and I indeed found increased *Vegfa* expression only in nMitoQ-treated female placentae (Chapter 2), suggesting the increased NO bioavailability may be responsible for the increased *Vegfa* expression.

Although nMitoQ treatment had no effect on mitochondrial OXPHOS capacities in hypoxic female placentae, increased mitochondrial respiration through the N-pathway (complex I) in comparison to the S-pathway (complex II) could be an adaptation to produce more ATP per oxygen atom reduced by the ETS. When NADH (N-pathway; complex I-linked substrate) is oxidized by the ETS, about 2.5 ATP are formed, in contrast to 1.5 ATP formed with Succinate (S-pathway;

complex II-linked substrate), with both pathways utilizing same the amount of oxygen (274). The possibility of increased ATP production through the N-pathway (complex I) could lead to an increase in energy available for performing placental transport functions and may possibly explain the unaltered placental efficiency observed in hypoxic female fetuses. In the first animal cohort, there was no effect of prenatal hypoxia and nMitoQ treatment on female fetal weights (Chapter 2). However, in the second animal cohort, nMitoQ treatment did not alter the prenatal hypoxia-induced reductions in female fetal weights (Chapter 3). The reduction in fetal weights following the hypoxic exposure could be due to the slower growth trajectories of female fetuses that provide them with the flexibility to adapt to adverse *in utero* environments (127, 224).

Taken together, in placentae from hypoxic female fetuses, mitochondrial respiration adapts to produce more energy per oxygen atom reduced by the ETS. However, despite these adaptations in mitochondrial function, a prenatal hypoxic insult led to increased ROS in the female placenta. The increase in placental ROS generation could be due to a decrease in oxygen delivery to female placentae from dams exposed to prenatal hypoxia. The oxidatively stressed female placenta similar to the male placenta demonstrated reduced expression of growth factors, such as *Vegfa* and *Igf2*, which eventually led to decreased area of fetal blood capillaries, and maternal and fetal blood spaces in the hypoxic placenta, as observed in our study (Chapter 2). Although the mechanisms for decreased *Vegfa* and *Igf2* was not the focus of our study, we speculate that decreased *Vegfa* mRNA expression could be due to the reduced binding of the transcription factor, HIF1α, to the hypoxia responsive element (HRE) on the *Vegfa* gene. Myatt *et al.* previously demonstrated that increased HIF1α protein expression in the preeclamptic placenta did not lead to a corresponding increase in DNA-binding activity, thus resulting in reduced expression of HIF1α target genes (94). The changes in *Igf2* expression could be mediated by changes in the methylation status of the *Igf2* gene at the

imprinting control region (ICR) and differentially methylated region 2 (DMR2). The *Igf2* gene expression in a suboptimal intrauterine environment is regulated by epigenetic changes, which in general are driven by hypoxic and oxidative stress responses. Therefore, prenatal hypoxia-induced oxidative stress found in both male and female placentae may have contributed to changes in placental morphology and function via changes in expression of growth factors.

5.2.4 Potential causes for the sex-differences in placental response to prenatal hypoxia and nMitoQ treatment

Although nMitoQ treatment reduced ROS production in hypoxic placentae from both male and female fetuses, our data suggest that the effectiveness of nMitoQ treatment on male and female placentae was exerted via different pathways. nMitoQ improved mitochondrial function in placentae from only hypoxic male fetuses, while it enhanced placental morphology (area of fetal blood capillaries and maternal and fetal blood spaces) in only hypoxic female placentae. We found that by targeting placental oxidative stress, nMitoQ, might have a more pronounced effect on placental mitochondrial function in the male placenta, whereas in the female placenta it could lead to changes in placental morphology. The sex-specific differences in the placental response to nMitoQ could be explained by the differential effects of various factors, including (but not limited to) the antioxidant system, steroid hormones, cytokines, and growth factors (such as IGF2) on placentae from male and female fetuses.

Overall, the differential effects of nMitoQ treatment on the placenta could be due to the fact that female placentae are in general more protected against ROS-mediated oxidative damage than the male placentae. For instance, recent studies have shown that the female placentae exhibit a higher antioxidant capacity to maintain redox homeostasis both under conditions of normal (134) and complicated pregnancies (225). These sex-specific adaptations may protect the female fetuses

from developing adult diseases earlier in life, as previously demonstrated by our laboratory and others (15).

Studies have also linked sex-specific outcomes of pregnancy complications, such as preeclampsia and FGR, to the differential effects of steroid hormones on the placenta (305). For example, preeclamptic pregnancies with male fetuses have reported increased levels of maternal testosterone and a reduction in the placental expression of aromatase (a rate-limiting enzyme converting androgens to estrogen) (306). The importance of sex steroids in the regulation of a stressed environment have been extensively studied in other tissues with testosterone implicated to promote oxidative stress (307). The rat placenta is known to secrete testosterone mostly into the maternal circulation starting GD14 of pregnancy (308). Furthermore, prenatal exposure to testosterone in rats has been found to decrease placental oxygenation, which was associated with increased HIF1α expression and impaired vascularization in placentae from only male fetuses (309). This suggests that the rat placenta respond in a sex-specific manner to steroid hormones and thus alterations to placental and/or maternal testosterone levels in response to hypoxia could account for the sexually divergent responses observed in our study.

Moreover, a key factor in the regulation of cellular stress is O-linked-N-acetyl glucosamine transferase (OGT) (310). OGT is an important O-glycotransferase enzyme that plays a critical role in gene expression (311). This enzyme affects cellular functions through the post-translational modification, O-GlcNAc, on serine and threonine residues of intracellular proteins that can directly regulate chromatin configuration, and ultimately gene expression (312). Previous studies have shown that basal placental OGT is lower in males compared to females because it is encoded by an X-linked gene that escapes inactivation in the male placenta (312). Furthermore, OGT expression was reduced in response to prenatal stress in placentae from only male fetuses (312). Reduced

placental OGT was shown to cause decreased expression of gene sets involved in endocrine and anti-inflammatory signaling, suggesting that a reduction in OGT could account for functional changes in the placenta (312). Thus it is possible that differential expression of OGT in male and female placentae in our study could lead to sex-specific effects on placental transport and endocrine functions. However, the effects of prenatal hypoxia on placental OGT expression and associated gene expression remain to be studied.

Interestingly, human hypoxic placental tissues from male fetuses have reported elevated levels of cytokines such as TNFα, IL-6 and IL-8 compared to the female placentae (313). In addition to hypoxia, pro-inflammatory cytokines can also stabilize HIF1α in a number of normoxic tissues, including the placenta (314). It is known that HIF1α can activate the transcription factor, NFκB, which in turn, can initiate a cascade of inflammatory and immune response genes, such as those encoding pro-inflammatory cytokines, chemokines, and enzymes relevant for inflammation (315). Therefore, it is possible that in our study the differential expression pattern of cytokines in male and/or female placentae may lead to a pro-inflammatory environment under conditions of prenatal hypoxia and thus the effects exerted by the nMitoQ treatment could be sexually dimorphic. In addition to cytokines, male placentae from complicated pregnancies have been associated with increased expression of pro-apoptotic proteins, such as p53 and Bax compared to the female placentae, which contributes to increased trophoblast apoptosis in the male placentae (313).

5.2.5 Placenta-derived factors that may impair fetal cardiomyocyte development

In a recent study, Botting *et al*. showed that the protective effects of MitoQ on fetal growth in mammals could be due to effects of this antioxidant in the placenta (302). Although studies have commonly associated newborn weight as an indicator of impaired fetal organ development and poor long-term health outcomes, birth weight does not necessarily equate with later life health outcomes

in the offspring (273). Independent of fetal growth and birth weight, we, together with our collaborators, showed that factors derived from a hypoxic and oxidatively stressed placenta led to impaired fetal neuronal development *in vitro*, which was prevented by prenatal nMitoQ treatment (156). Furthermore, nMitoQ treatment of human preeclamptic placental explants prevented the release of "stress-related" placenta-derived factors, thereby improving neuronal development *in vitro* (231). This suggests that nMitoQ treatment by targeting placental oxidative stress could lead to alterations in factors derived from placentae of dams exposed to prenatal hypoxia and thus, prevent the effects of a dysfunctional and "distressed" placenta on impaired fetal organ development. In line with previous observations, in our current study, we found that placental factors derived from nMitoQ-treated hypoxic dams were able to prevent premature transition of fetal cardiomyocytes to a terminally differentiated mature (binucleated) phenotype and decreased cardiomyocyte cell size (hypertrophy) in both males and females, suggesting that by targeting placental oxidative stress nMitoQ treatment can mediate its protective effects on fetal cardiomyocyte development.

Although we showed that nMitoQ treatment could affect fetal organ development via placenta-derived factors, our studies were not aimed to identify the placenta-derived specific factors. As reported in Chapters 1 and 4, studies in our laboratory have shown altered miRNA expression profiles in response to prenatal hypoxia and nMitoQ treatment. Given that sex differences were not taken into account for the miRNAs measured in our previous study (156), in Chapter 4, we speculated on a few of the placenta-derived miRNAs that could lead to impaired fetal cardiomyocyte development. In our previous study, we found that in placenta-derived conditioned media from hypoxic dams treated with nMitoQ there was an upregulation of miRNAs that could potentially increase cardiomyocyte proliferation; reduce cardiomyocyte binucleation; and

cardiomyocyte hypertrophy. These include decreased miR-30, which suppresses expression of Cyclin A2 gene (promotes cell cycle activity), increased miR-133a, which has been shown to reduce binucleation via reduced Sirtuin 1 expression, and decreased miR-208a, which targets pro-hypertrophic MYH7 gene expression (156). However, in addition to miRNAs other factors can also play a role in fetal heart development. For instance, as discussed in Chapter 1, there is the possibility that pro-inflammatory placental and/or maternal cytokines (such as TNFα, IL-6 and IL-8) can cross the basal membrane into fetal circulation and may contribute to impaired fetal cardiomyocyte development. Previous studies have focused on the effects of placental cytokines on neuronal and brain development in complicated pregnancies (173). However, an increase in circulating pro-inflammatory cytokine levels (such as IL-6 and TNFα) has been shown to impair cardiac performance (such as left ventricle ejection time was reduced) in 6-day old neonatal mice (316). However, the role of placenta-derived cytokines as well as the contribution of cytokines present in fetal circulation on cardiomyocyte development remains to be studied.

Furthermore, we have shown that prenatal hypoxia led to increased placental oxidative stress, which could lead to increased apoptosis of trophoblast cells (305). Under conditions of cell injury particularly associated with apoptosis, the placentae can increase release of cargoes (containing miRNAs) by dead cells, as demonstrated previously in hypoxic human trophoblast cells (289). It is plausible that miRNAs released from apoptotic cells might lead to impaired cardiomyocyte maturation and growth. Altogether, our study illustrates that by acting against placental oxidative stress, nMitoQ treatment leads to improved fetal cardiomyocyte development via alterations in placenta-derived factors. The lack of sexual dimorphism in the effects of factors derived from nMitoQ treated hypoxic placentae is intriguing. Based on our findings from nMitoQ effects on hypoxic placentae, we speculate that placenta-derived factors in males and females could

be different. However, the specific placenta-derived factors yet remain to be confirmed.

5.2.6 Conclusion and clinical significance

In conclusion, our study provides compelling evidence of clinical importance for placenta-targeted prenatal therapeutics in pregnancies complicated by chronic fetal hypoxia. More importantly, our study suggests that sex-differences in placental function and treatment effects need to be taken into consideration, particularly when developing new therapeutic strategies to improve fetal development in pregnancies complicated by fetal hypoxia. In our experimental studies, nMitoQ treatment was administered at the time of the hypoxic insult (i.e. GD15), in clinical settings, however, treatment would be most likely administered subsequent to diagnosis and sometime after the 'insult' started (i.e. when the clinical signs appear). Therefore, using our pre-clinical rodent model, future studies need to be designed to test the nMitoQ treatment at different time points. Moreover, in humans, choosing the most relevant time point to administer and the dosages/frequency of the therapeutic intervention poses a challenge and has yet to be determined as the technology for early screening of pregnancy complications and assessing placental function and placental oxidative stress/oxygenation evolves. A recent study showed that spectral photoacoustic imaging methods can be used to longitudinally measure placental oxygenation using the Vevo 2100 ultrasound system *in vivo* in the reduced uterine perfusion pressure (RUPP) model of preeclampsia (317). This suggests that photoacoustic imaging of placental oxygenation could be used in the future to assess placental hypoxia in preclinical models of FGR and preeclampsia, which could help determine if prenatal treatments should be administered as well as the efficacy of potential therapeutic treatment. Although preclinical studies support the concept of applying photoacoustic imaging for the analysis of placental oxygenation in the clinic, translation of this technology in the

field of feto-maternal medicine is still in its early stages.

Of note, most studies focused on placenta-targeted treatment strategies have used pre-clinical rodent models as they possess a haemochorial placenta, which is similar to the human placenta. However, although pre-clinical rodent models provide an essential, useful and inevitable tool for pregnancy research, the rodent placenta is different from the human placenta in terms of the placental barrier, and thus species differences are a highly important consideration for successful translation of data from pre-clinical models to human clinical outcomes.

5.3 Project Limitations

To address our research questions in Chapters 2, 3 and 4, we used a rat model of prenatal hypoxia that was established in our laboratory by exposing pregnant dams to hypoxia during the last third of gestation. We chose this model system as previous studies from our laboratory have assessed the placental and fetal phenotype, and we have shown that prenatal hypoxia during late gestation eventually leads to later life cardiovascular dysfunction in the adult offspring (15, 219, 270). Therefore, this model system represents a consistent and reliable tool for our proof-of-principle study to test if placenta-targeted treatment strategy could prevent fetal programming of adult cardiovascular disease. Despite the advantages, some limitations exist with the regards to the animal model used in this thesis. One of the limitations of this rodent model is that the hypoxic insult affects both the dam and the fetus. Therefore, this model does not completely represent the clinical pathophysiology of pregnancy complications associated with placental insufficiency in humans. In a clinical setting, placental hypoxia is a consequence of failed uterine spiral artery remodeling in which the fetus, but not the mother, has restricted oxygen availability.

Another limitation of these studies is the timing of the placenta-targeted intervention. In our current study we administered nMitoQ at the time of exposure to hypoxia on GD 15. I chose this particular time point because of the technical difficulties involved in treating the dam once placed inside the hypoxic chamber. Despite the fact that a single dose of nMitoQ was effective in preventing placental oxidative stress until GD 21 (i.e. one week after nMitoQ application), future studies need to be designed to test the treatment at different time points as, in a clinical setting, treatment would be administered subsequent to diagnosis.

Furthermore, I used a placenta-targeted nMitoQ treatment strategy in Chapters 2, 3 and 4. As described previously in Chapter 1, in this treatment strategy, polymeric nanoparticle encapsulated mitochondrial antioxidant was specifically delivered to the placenta. The size and charge of the nanoparticle allows for its uptake by the placenta. Although the primary aim of my study was to treat the placenta and prevent off-target effects on the fetus, it would be of interest to assess any off-target effects of nMitoQ treatment strategy on the maternal immune system. This is important because when nanoparticles enter the body, interactions with the immune system are unavoidable. Depending on their physiochemical properties (size and charge), nanoparticles can interact with cells and proteins to stimulate or suppress the innate immune system, and similarly activate the complement system. The biodegradable nanoparticles used in my study have been previously characterized as an efficient and safe vehicle for drug delivery, but still it remains essential to understand in detail the effects of such nanoparticles on maternal immune cells (such as T-cells, B-cells, macrophages) during pregnancy.

Limitations exist for the work presented in Chapter 2 and 4, the first of which being that only mRNA levels were measured for growth factors (such as VEGFA and IGF2) in Chapter 2. The gene transcription level data can suggest whether or not the protein is present, but the changes in

gene expression are not always reflected at the protein levels. Gene expression provides useful information for identifying potential candidates for follow-up work at protein level. Therefore, to further understand the pathways regulating placental morphology, future experiments should address expression of growth factors at protein levels in the placenta. In Chapter 4, we cultured rat placental explants (from both normoxic and hypoxic groups) at 8% oxygen. The normoxic placenta is exposed to 8% oxygen *in vivo*, whereas the hypoxic placenta may experience 2-5% oxygen under *in vivo* conditions (285). We controlled for the experimental conditions by exposing placentae from all four experimental groups at 8% oxygen. We have also used appropriate positive control (cardiomyocytes treated with Angiotensin II) and conditioned media control (conditioned media without placenta) for our experiments. However, to eliminate the stress response in the hypoxic placentae due to variations between *in vivo* and *in vitro* oxygen levels, future experiments need to be performed by culturing placentae from normoxic and hypoxic groups at oxygen concentrations that they experience *in vivo*.

An additional limitation of the cardiomyocyte study presented in Chapter 4 is that the culture medium conditioned by rodent placentae and used for cardiomyocyte studies may consist of factors released by the placenta in both directions, i.e. to the fetal and maternal side. Therefore, there is a possibility that conditioned medium used in our study consists of placenta-derived factors released into the maternal and fetal circulation. In order to experimentally control for the directionality of placenta-derived factors, conditioned medium collected from a model of human placental barrier treated with nMitoQ under appropriate conditions *in vitro* may act as a more reliable tool to answer our research questions. For instance, our collaborators used conditioned media collected from human preeclamptic placental explants, which were treated with nMitoQ to study the effects of placenta-derived factors on fetal neuronal development *in vitro* (231). Overall,

these points are to be addressed in future studies, and need to be considered while developing future studies investigating the effects of placenta-derived factors on fetal organ development.

5.4 Future Directions

Studies conducted in this thesis illustrate that the placenta is a contributing factor in the sexual dimorphism that has been observed in fetal programming. While our data clearly denote sexual divergence in placental responses to prenatal hypoxia and nMitoQ treatment, further studies are still needed to delineate the underlying mechanisms that may contribute to sexual dimorphism in the placenta, which in turn, can help answer numerous questions regarding the increased susceptibility to develop later life cardiovascular disease in offspring born from hypoxic pregnancies.

5.4.1 Timing of the placenta-targeted intervention

As reported in Chapter 1, the haemochorial placentation in rats show marked invasion of the uterine wall by trophoblast. In rats, trophoblast invasion begins at around GD 15 and peaks around GD 18, followed by gradual regression of the invaded areas near term (39, 40). Our research question was focused on the effects of nMitoQ treatment on placental function and fetal development; therefore, we treated the rats at GD 15, which is at the trophoblast invasion and uterine spiral artery remodeling begins. As discussed in the previous sections, placental hypoxia is a consequence of failed uterine spiral artery remodeling in humans, and more importantly, in clinics, treatment would be most likely administered subsequent to diagnosis and sometime after manifestation of the clinical signs. Thus, an important future direction from these studies would be to investigate different time points for nMitoQ administration. The lack of technology for early

screening of pregnancy complications in humans makes it all the more essential to administer the intervention at different time points over the course of gestation to better understand the efficacy of the potential treatment strategy. It may be proposed to administer a single dose of nMitoQ and saline control on GD 6 or on GD 9, following which the rats would be subdivided and exposed to normoxia and/or hypoxia until term. The rat blastocyst implants at around GD 6, and GD 9 represents a pivotal developmental stage in rat placentation. During the interval between GD 8-9, the chorioallantoic placenta starts to form and cells are allocated to each specialized cell lineage of the chorioallantoic placenta, including the invasive trophoblast cell lineage (142). An earlier time point for the intervention would allow us to investigate spiral artery remodeling at around GD 18 in rats and/or assess the effects of the treatment on pregnancy outcomes at term.

5.4.2 Placental transport functions

Evidences suggest that transfer of substrates (such as glucose and amino acids) for fetal growth is largely determined by the use of substrates by the placenta for its own metabolism and energy requirements (300). Therefore, an important future direction would be the extension of the placental functional study. Transplacental transfers of amino acids but not glucose have been shown to be reduced in mice model of hypoxia (10.5% O_2, GD 14-19), ultimately leading to FGR (sex not specified) (101). Taking into account the sex-specific effects of nMitoQ treatment on the placenta, any possible sexual divergence in transport of nutrients need to be investigated. Most studies have widely studied the expression of the glucose and amino acid transporters without considering sex as a parameter and, thus the functional consequences of these nutrient transporter differences in male and female placentae remain to be determined.

5.4.3 Use of substrate for placental OXPHOS respiration

In relation to placental functional capacity, determining the preferred substrate used by

placental mitochondria to generate ATP may further help to understand the sexual dimorphism seen in the placenta. For instance, fatty acids have been shown to be a preferred substrate for energy production as reflected by higher concentrations of medium-chain acylcarnitines (play a key role in mitochondrial fatty acid beta-oxidation) in placentae from female compared to male fetuses during normal gestation (134). Fatty acids require 8-11% more O_2 to generate a given amount of ATP compared to glucose (70); therefore, it is plausible that under *in utero* hypoxic conditions, the substrate used for mitochondrial OXPHOS not only dictates transplacental nutrient transfer, but also placental mitochondrial OXPHOS capacity.

5.4.4 Cellular senescence and reduced telomere length

Hypoxia in pregnancy leading to mitochondrial dysfunction and abnormal levels of ROS generation can cause premature senescence and accelerated cellular aging of the placenta in complications of pregnancy, such as FGR (318). Premature aging, in general, is associated with reduced telomere length (318). Telomere shortening can cause increased DNA damage and activation of the apoptotic p53 pathway and promotes expression of senescence markers (such as p16 and p21) in placental trophoblasts (319). Cellular senescence leads to sustained activation of the transcription factor NF-κB and overexpression of pro-inflammatory cytokines such as TNFα, IL-6 (320). Human placental tissues from FGR pregnancies showed evidence of decreased telomere length (321, 322). Furthermore, studies have indicated an association between adverse *in utero* environment and telomere shortening in the offspring (323). Furthermore, reduced telomere length can lead to cardiomyocyte loss (324, 325), and increased susceptibility of other organs such as kidneys to I/R injury (326). Overall, adverse prenatal environments not only induce premature aging of the placenta, but could also lead to premature aging of essential organs (such as the heart) in young offspring from complicated pregnancies. Thus, an interesting future direction would be to

investigate whether placenta-targeted antioxidant treatment strategy affects placental and/or fetal organ aging and cellular senescence. Moreover, telomere length has been shown to be longer in female newborns compared to males (327). Therefore, it would be of interest to take into account the sex of the fetus and the offspring for all future studies.

5.4.5 Mitochondrial DNA and toll like receptors

Another exciting and interesting future direction would be to investigate if placental oxidative stress-induced telomere shortening and apoptosis could subsequently lead to release of placenta-derived cell free mitochondrial DNA (mtDNA) into the fetal circulation. mtDNA can lead to cardiomyocyte apoptosis and myocardial injury via its action on toll-like receptor (TLR) such as TLR9 and TLR4 (328). Upon ligand recognition, TLRs lead to activation of a kinase cascade, which triggers the activation of NFκB pathway and leads to changes in gene transcription and the production of pro-inflammatory cytokines (329). Furthermore, independent of mtDNA, a pro-inflammatory environment can also lead to activation of TLR signaling cascade. It has been hypothesized that placenta-derived mtDNA released into the maternal circulation could induce a systemic inflammatory response via activation of the TLRs, eventually leading to systemic maternal inflammation and vascular dysfunction in complications of pregnancy (330). Given the role of mtDNA and TLRs in vascular and cardiac dysfunction, it would be of interest to further explore if placenta-derived mtDNA could be released into the fetal circulation and whether it contributes to the development of vascular and cardiac dysfunction via TLR9 and/or TLR4 in offspring born from hypoxic pregnancies.

5.4.6 Placenta-derived factors and model systems

In the previous sections we speculated several factors derived from the hypoxic placenta that could lead to impaired fetal organ development. In line with that, an important next step would be

to assess placenta-derived factors in the conditioned media obtained from male and female fetuses. Previously, together with our collaborators, we have shown that the proteomic profiles in fetal blood and conditioned media were altered in response to prenatal hypoxia and nMitoQ treatment, but sex of the fetus was not taken into account. In addition to this it would be interesting to know if placenta-derived exosomes and/or extracellular vesicles are released into the fetal compartment in response to hypoxia and can nMitoQ prevent it.

As mentioned in section 5.3, a limitation of using the rodent placenta is that the directionality of released placental factors cannot be determined, thus we propose the use of placenta-on-a-chip model system to investigate transfer of placenta-derived factors into the fetal circulation to mimic the placenta *in vivo* (331). The *in vitro* placental barrier-on-a-chip microdevice would be an ideal candidate as it recapitulates the maternal and fetal interface during gestation by the co-culture of trophoblasts and endothelial cells on the 3D extracellular matrix, and has been used for drug transport and nanoparticle transfer study across the placental barrier (332). An alternative to the placenta-on-a-chip model would be a placental co-culture model with layers of trophoblasts and endothelial cells on a porous membrane, as described previously (333). The use of advanced model systems would provide insight into the molecules and factors transferred across the placental barrier into the fetal circulation.

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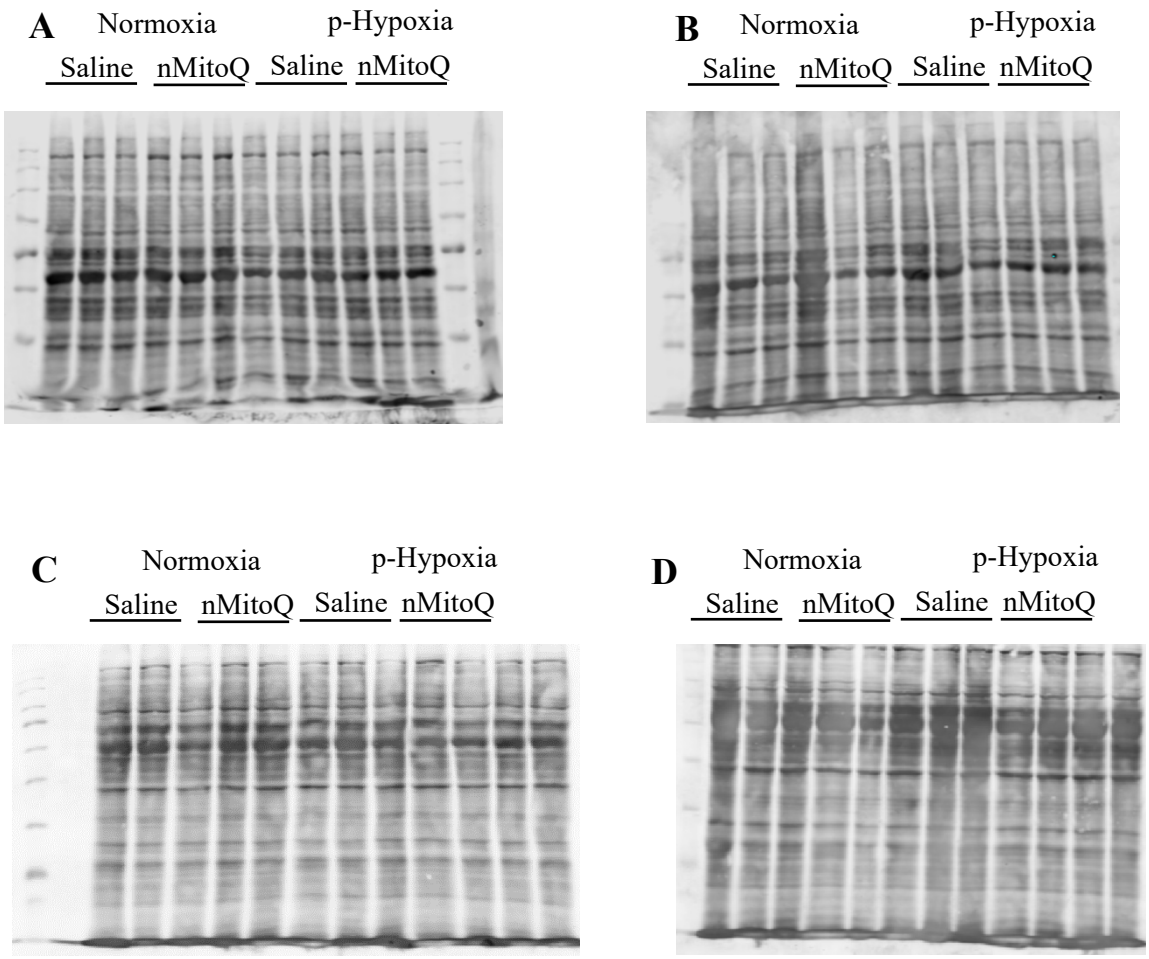
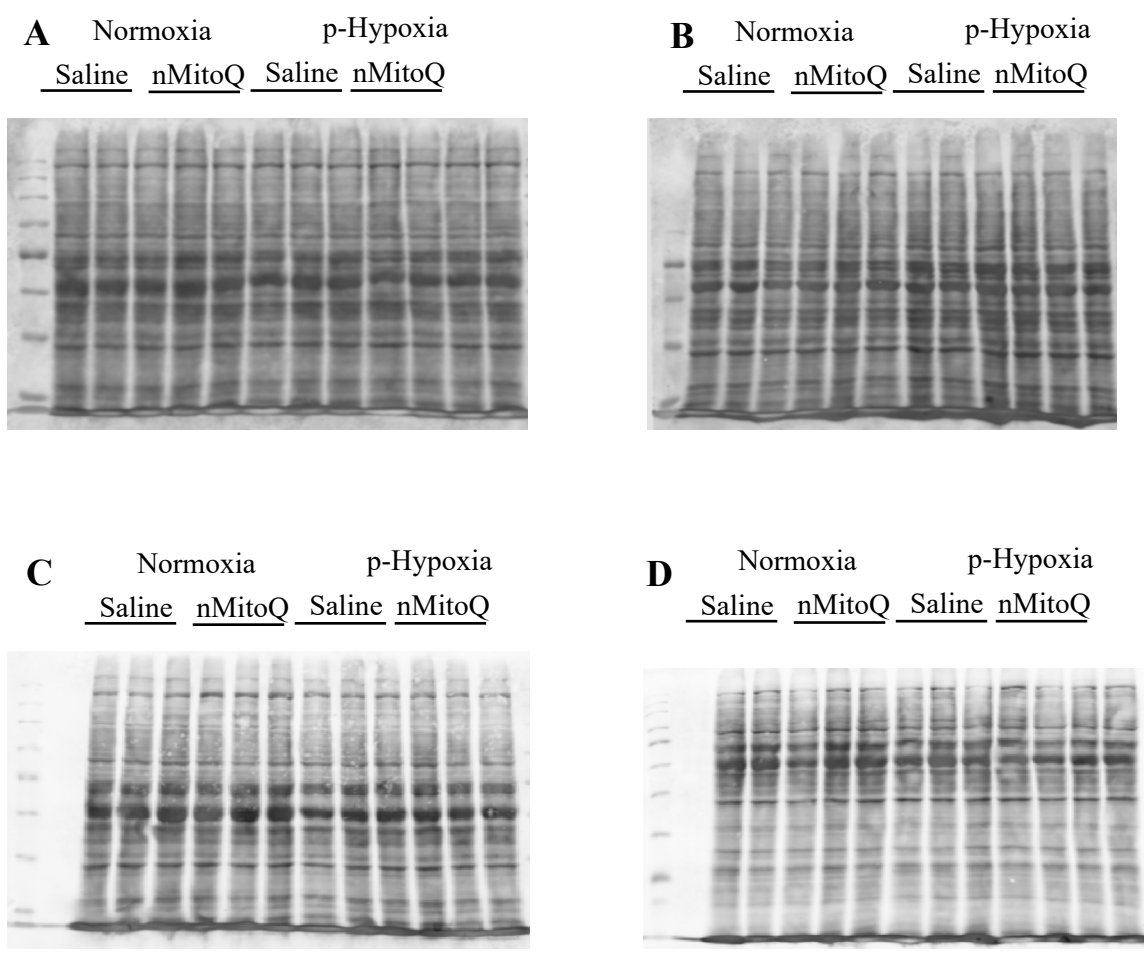
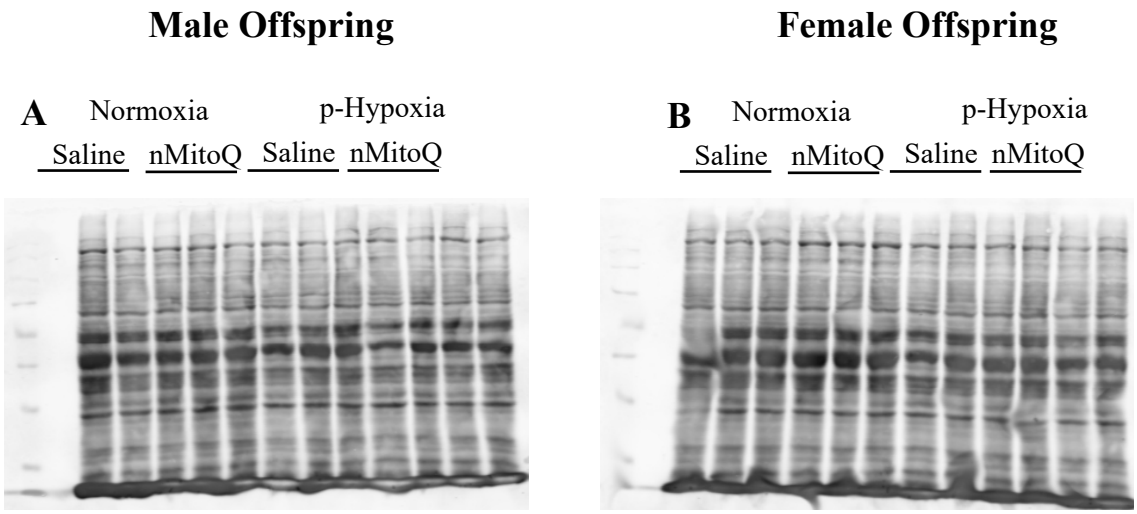
Appendices



Supplementary Figure S3.8. Representative total protein staining blots for mitochondrial biogenesis proteins peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α) and nuclear respiratory factor 1 (NRF1) in placentae of both male and female fetuses on GD21. Total amount of protein in each lane was used as a loading control to normalize PGC-1α and NRF1 protein expression in placentae obtained from male (**A+C**) and female (B+D) fetuses.



Supplementary Figure S3.9 and S3.10. Representative total protein staining blots for mitochondrial fusion proteins Optic Atrophy Type 1 (OPA1) and Mitofusin 1 (MFN1) in placentae of both male and female fetuses on GD21. Total amount of protein in each lane was used as a loading control to normalize OPA1 and MFN1 protein expression in placentae obtained from male (**A+C**) and female (B+D) fetuses.



Supplementary Figure S3.11. Representative total protein staining blots for mitochondrial fission protein Dynamin Related Protein 1 (Drp1) in placentae of both male and female fetuses on GD21. Total amount of protein in each lane was used as a loading control to normalize Drp1 protein expression in placentae obtained from male (**A**) and female (**B**) fetuses.