INCREASED OXIDATIVE DAMAGE AND PREMATURE PLACENTAL AGING CONTRIBUTE TO THE AETIOLOGY OF STILLBIRTH

by

Zakia Sultana

B.Pharm (Honours), M.Pharm

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy in Reproductive Medicine

School of Medicine and Public Health

Faculty of Health and Medicine

The University of Newcastle, Australia

August, 2018

Statement of Originality

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide for loan and photocopying when deposited in the University's Digital Repository, subject to the provisions of the Copyright ACT 1968.

.....

Zakia Sultana

August, 2018

Declaration of Collaboration

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers. I have included as part of the thesis a statement clearly outlining the extent of collaboration, with whom and under what auspices.

.....
Zakia Sultana

Statement of Contribution to Joint Publications

I hereby certify that the work embodied in this thesis contains published papers/scholarly work of which I am a joint author. I have included as part of the thesis a written declaration, endorsed by my supervisor, attesting to my contribution to the joint publications/scholarly work.

Candidate: Date: 01/08/18

By signing below, I confirm that Zakia Sultana has made a primary and original contribution to the publications, and manuscripts submitted and in preparation for publication, included in this thesis as detailed below.

Supervisor: Date: 3/8/2018

Chapter	Title	Status	Contribution
2	Oxidative stress, placental ageing-related pathologies and adverse pregnancy outcomes	Published	Conception and designing of the review, research to find the publications referred to, manuscript writing, designing and preparation of the figures.

3	Is there a role for placental senescence in the genesis of obstetrical complications and fetal growth restriction?	Published	Conception and designing of the review, research to find the publications referred to, manuscript writing and designing and preparation of the figures.
5	Evidence that fetal death is associated with placental aging	Published	Designing and executing in vitro experiments, data analysis, result interpretation, preparation of the figures and manuscript writing.
6	Effect of serum-starvation on lipid peroxidation and expression of sirtuins in human placental explants: implication for aldehyde oxidase 1 and G-protein coupled estrogen receptor 1 in placental oxidative damage and aging	Prepared for publication	Designing and performing experiments, data analysis, result interpretation, preparation of the figures and manuscript writing.
7	Growth factor depletion in placental trophoblast cells increases lipid peroxidation, reduces mTORC1 activity and alters mitochondrial function via aldehyde oxidase and GPER1 mediated pathways	Prepared for publication	Designing and executing experiments, data analysis, result interpretation, preparation of the figures and manuscript writing.

Acknowledgements

First of all I wish to express sincere gratitude to my PhD supervisors **Laureate Professor Roger Smith** and **Dr Kaushik Maiti**. Roger, thank you for sharing your brilliance, enthusiasm for the unknown and critical thinking skills. Thank you for imparting your knowledge and skills on me and for encouraging me to do my very best. I am really grateful for your compliment, encouragement, thoughtful guidance, and support. Kaushik, you have continuously demonstrated exceptional generosity with investments of your intellect, time and energy. Thanks for all the encouragement, guidance and support. You are a fantastic young supervisor and I am very proud to be your first PhD student. To both, thank you for your trust and confidence and for allowing me to grow as a research scientist. I look forward to our ongoing research endeavours.

I must thank all of my colleagues at the Mothers and Babies Research Centre for welcoming me into their group and helping me to grow as a scientist. In particular, I would like to thank Dr Cheng Chan, Dr John Fitter, Dr Jonathan Paul, Dr Giavanna Angeli, Ms Maria Bowman, Rachael Taylor, Binod Sharma, for intellectually challenging discussions, input and many enjoyable moments. I have enjoyed every moment working with you. This PhD wouldn't be the same without you and I am sincerely grateful for all of your help. A special thanks to Dr Prema Monogar (Prof Phil Hansbro's Lab) for her kind help with the Seahorse assay. A warmest thanks also to Ms Lee Dedman (Natural history illustration) for her time in illustrating those wonderful images for me. I also would like to thank Mrs Anne Wright (Research Midwife) for her help in collecting placentas. Thanks to the women who volunteered to participate in the study, as well as the staff at the delivery suite. Thank you to

all the staff and researchers at the Hunter Medical Research Institute for providing me with the type of research environment that stimulates and develops creativity and knowledge into medical research.

I would like to acknowledge my incredibly supportive and generous family. The first people I want to thank in my family are my parents. Words cannot express how grateful I am to my mother and father for all their unwavering support, love, care and sacrifices for educating and preparing me for my future. Thank you for always believing in me and giving me all the good advice. Thank you for always being there for me and allowing me to go my own way when needed. I thank to my parents-in-law who supported me unconditionally during this journey. Thanks to my brother, sister, uncles, aunts, my neighbours, and friends for encouraging me through all my years of schooling and university, and their endless support and love. You have all contributed to helping me reach this incredible goal. I am extremely lucky to have you all in my life.

Thanks to the one who made the biggest sacrifice during my PhD, my precious little boy Tawseef. I couldn't have done this PhD possible without your sacrifice. I am sorry for not being there for you when you needed me most. You are a wonderful little boy and I love you more than anything in the entire world.

Lastly but certainly not least, I have to thank my biggest supporter during this time, my loving husband, Dr Md Mominul Haque. You have been the most supportive husband I could have ever hoped for. You always take care of me, let me unload all my stresses and make sure I

feel good enough to make everything, including this PhD possible. Thank you for standing by me this whole time and always pushing me forward.

I gratefully acknowledge the assistance of a scholarship from the University of Newcastle during this research work.

I dedicate this thesis to my beloved parents, my husband and my son for their constant support and love.

Publication List

Journal Publications (full text)

The work presented in this thesis has directly resulted in the following publications:

1. **Sultana Z**, Maiti M, Dedman L, Smith R. Is there a role for placental senescence in the genesis of obstetrical complications and fetal growth restriction? American Journal of Obstetrics and Gynecology, 2018; 218: S762–S773.
2. Maiti K, **Sultana Z**, Aitken RJ, Morris J, Park F, Andrew B, Riley SC, Smith R. Evidence that fetal death is associated with placental aging. American Journal of Obstetrics and Gynecology, 2017;217: 441.e1-14.
3. **Sultana Z**, Maiti K, Aitken J, Morris J, Dedman L, Smith R. Oxidative stress, placental ageing related pathologies, and adverse pregnancy outcomes. American Journal of Reproductive Immunology, 2017;77:e12653.

Abstract Publications

The work presented in this thesis has directly resulted in the following abstract publications as part of conference presentations:

1. **Sultana Z**, Maiti K, Smith R. Use of an in vitro human placental explant culture model to interrogate the effects of starvation on placental function. Placenta, 2015;36:A49.
2. Maiti K, **Sultana Z**, Aitken J, Smith R. The human placenta at 41 weeks of gestation shows evidence of aging with shortened telomeres, DNA oxidation and changes in IGFR2, autophagy and mTOR. Placenta, 2015;36:A49.

Conference Presentations

The work presented in this thesis has directly resulted in the following conference presentations:

1. **Sultana Z**, Maiti K, Smith R, Hansbro PM, Nair PM. Growth Factor Depletion in Placental Cells Increases Lipid Peroxidation and Reduces Mitochondrial Function and mTOR Activity via Aldehyde Oxidase Mediated Pathways. Reproductive Sciences, 2018;25;229A. Poster presentation delivered at the 65th Annual Meeting of the Society for Reproductive Investigation, March 6-10, San Diego, CA.
2. Maiti K, **Sultana Z**, Aitken J, Smith R. Unexplained Antepartum Stillbirth Is Associated with Biochemical Evidence of Placental Aging. Reproductive Sciences, 2017;24:216A. Poster presentation delivered at the 64th Annual Scientific Meeting, Society for Reproductive Investigation, March 15-18, 2017, Orlando, Florida.
3. Maiti K, **Sultana Z**, Aitken J, Smith R. Evidence That Stillbirth Is Linked to Placental Aging. Reproductive Sciences, 2016;23:76A. Poster presentation delivered at the 63rd Annual Scientific Meeting, Society for Reproductive Investigation, March 16-19, 2016, Montreal, Canada.
4. **Sultana Z**, Maiti K, Smith R. Use of an in vitro human placental explant culture model to interrogate the effects of starvation on placental function. Placenta, 2015;36:A49. Poster presentation delivered at the International Federation of Placenta Association (IFPA), September 8-11, 2015, Brisbane, Australia.
5. Maiti K, **Sultana Z**, Aitken J, Smith R, The human placenta at 41 weeks of gestation shows evidence of aging with shortened telomeres, DNA oxidation and changes in IGFR2, autophagy and mTOR. Placenta, 2015;36:A49. Poster presentation delivered

at the International Federation of Placenta Association (IFPA), September 8-11, 2015, Brisbane, Australia.

Manuscripts Prepared for Publication

1. **Sultana Z** et al. Effect of serum-starvation on lipid peroxidation and expression of sirtuins in human placental explants: implication for aldehyde oxidase 1 and G-protein coupled estrogen receptor 1 in placental oxidative damage and aging.
2. **Sultana Z** et al. Growth factor depletion in placental trophoblast cells increases lipid peroxidation, reduces mTORC1 activity and alters mitochondrial function via aldehyde oxidase and GPER1 mediated pathways.

Table of Contents

CHAPTER 4

CHAPTER 7

Growth Factor Depletion in Placental Trophoblast Cells Increases Lipid Peroxidation, Reduces mTORC1 Activity and Alters Mitochondrial Function via Aldehyde Oxidase 1 and GPER1 Mediated Pathways 213

List of Tables

List of Figures

List of Abbreviations

General

•OH	hydroxyl radicals
4E-BPs	eukaryotic translation initiation factor 4E-binding proteins
4HNE	4-hydroxynonenal
8OHdG	8-hydroxydeoxy-guanosine
AChE	acetylcholinesterase
ADP	5'–adenosine di-phosphate
AMP	5'–adenosine mono-phosphate
AMPK	5' AMP–activated protein kinase
AOX1	aldehyde oxidase 1
ATP	5'–adenosine tri-phosphate
AUC	area under the curve
BCA	bicinchoninic acid assay
BMI	body-mass index
BSA	bovine serum albumin
Ca^{2+}	Calcium ion
cAMP	cyclic 3',5'–adenosine monophosphate
CAT	catalase
CDK	cyclin-dependent kinases
cDNA	complimentary deoxyribonucleic acid

Ct	cycle threshold
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco’s modified eagle’s medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside 5′-triphosphates
ECAR	extra-cellular acidification rate
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
ETC	electron transport chain
FADH	flavin adenine dinucleotide
FBS	fetal bovine serum
FCCP	carbonyl cyanide-4 (tri-fluoromethoxy) phenylhydrazone
FGR	fetal growth restriction
FOXO	forkhead box class O
GPER1	G-protein coupled estrogen receptor 1
GSH-Ps	glutathione peroxidases
GSH-T	glutathione s-transferase
H_2O_2	hydrogen peroxide
HRP	horseradish peroxidase
ICD	international classification of disease
ICM	inner cell mass
IGF	insulin-like growth factor

IgG	immunoglobulin G
IHC	immunohistochemistry
IL	interleukin
IUGR	intrauterine growth restriction
LAMP2	lysosomal activated membrane protein 2
LC3B	microtubule-associated proteins 1A/1B light chain 3 B
LDS	lithium dodecyl sulphate
MAPK	mitogen activated protein kinase
MDA	malondialdehyde
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
mTOR	mammalian/mechanistic target of rapamycin
mTORC1/mTORC2	mTOR complex 1/complex 2
$NADH/NAD^+$	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
O_2^-	superoxide anion radical
OCR	oxygen consumption rate
OD	optical density
OS	oxidative stress
p70S6K	phosphoprotein 70 ribosomal protein S6 Kinase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PI3K	phosphoinositide 3-kinase
pPROM	preterm premature rupture of membranes
pRB	retinoblastoma tumor suppressor protein
PVDF	polyvinylidene fluoride
RAS	renin-angiotensin system
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	reverse transcription
RT	room temperature
RT-qPCR	real-time quantitative polymerase chain reaction
SAHF	senescence-associated heterochromatin foci
SASP	senescence-associated secretory phenotype
SA-β-gal	senescence-associated β-galactosidase
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
Ser	serine
SGA	small for gestational age
SIDS	sudden infant death syndrome
Sir2	silent information regulator 2
SIRT	sirtuins
SOD	superoxide dismutase
SQSTM1	sequestosome 1
STBMs	syncytiotrophoblast microparticles

TAE	tris-acetate EDTA
TBS	tris-buffered saline
TBST	tris-buffered saline with 0.1% tween-20
TCA	tri-carboxylic acid
TERC	telomerase RNA component
TERT	telomerase reverse transcriptase
Thr	threonine
Tris	trisaminomethane (2-amino-2-hydroxymethyl-propane-1,3-diol)
TSC1/2	tuberous sclerosis complex 1/2
ULK1	Unc-51 like autophagy activating kinase 1
WHO	world health organization
XF	extracellular flux
XO	xanthine oxidase

Units

%	percent
°C	degrees Celsius
g	grams
hrs	hours
kDa	kilo Dalton
L	litre
M	molar
mg	milligrams

min	minutes
mL	millilitres
mm	millimitres
mM	millimolar
nM	nanomolar
sec	seconds
V	volts
U	unit
µg	micrograms
µm	micromitres
µM	micromolar
g	gravity

Prefixes

m	milli (10^{-3})
µ	micro (10^{-6})
n	nano (10^{-9})
p	pico (10^{-12})

Symbol

α	alpha
β	beta
Δ	delta

Abstract

Stillbirth is a neglected public health problem affecting more than two million women and families globally each year with devastating and long-lasting psychosocial and financial impact. Rates of stillbirth, even in high-income countries with access to optimal obstetric care, have remained static in the past two decades. The causes of, or associations with, stillbirth that have been identified clinically include fetal factors such as genetic/structural abnormalities and growth restriction, maternal factors such as preeclampsia and infections and placental factors such as abruption and placenta previa. However, no specific cause has been established for the majority of stillbirths at term, and the rate of this category of death rises drammatically as gestation progresses beyond 38 weeks. Taking into account the functional definition of aging that is an increase in the risk of death with time, and the existence of placental pathologies in the unexplained stillbirth pregnancies resembling aging in other organs, we hypothesise that premature placental aging may be the primary factor in the aetiology of unexplained stillbirth. Premature aging may occur when cells experience increased oxidative stress that causes damage to cellular macromolecules, including DNA, RNA and lipids, and alters protein expression patterns, especially those that are crucial for cellular survival and function.

Therefore, the primary aim of this thesis was to investigate evidence that the placenta from late-gestation shows biochemical signs of oxidative damage and aging that would also be present in placentas associated with stillbirths. A further aim was to investigate the pathways that mediate the oxidative damage and aging in the placenta in pathologic pregnancies. We have shown that placentas from both late-term and stillbirth pregnancies show biochemical

signs of aging in the form of increased DNA and lipid oxidation. Also, the expression of aldehyde oxidase 1 (AOX1), which is known to be involved in reactive oxygen species (ROS) generation and oxidative stress, is increased in placental tissues obtained from both late-gestation and stillbirth pregnancies. We tested the association of AOX1 in stillbirth pregnancy as an RNA sequencing study performed in our laboratory identified a significant increase in AOX1 mRNA in late-term placentas compared to term healthy placentas (unpublished). The demonstration of G-protein coupled estrogen receptor 1 (GPER1), a cell surface estrogen receptor, localisation on the apical surface of the normal placental syncytiotrophoblast and its role in the reduction of ROS generation and oxidative damage indicate that this receptor may be a critical step in the pathway of placental ROS induced oxidative damage.

Using a placental explant and a cell line culture model, we then tested the pathways that regulate placental oxidative damage and aging. Results presented in this thesis revealed that growth factor removal resulted in placental oxidative damage, with impaired mitochondrial function, decreased expression of sirtuins (proteins that control aging), alteration of nutrient sensing mammalianTORC1, and energy sensing AMP activated protein kinase pathways, all the changes are known to be associated with oxidative damage and aging in other tissues. Inhibition of AOX1 or stimulation of estrogen activation at GPER1 resulted in the blocking of all the changes observed after removal of growth factors. Together, these findings support the hypothesis that placental oxidation is regulated by estrogen activation at the GPER1 and inhibition of AOX1 leading to the inhibition of ROS generation and oxidative stress. Our study identifies potential biomarkers of oxidative damage and aging in stillbirth placentas that raise the possibility that these biomarkers of placental oxidative damage and aging may

be released into the maternal blood where they may have diagnostic value in predicting the fetus at risk for stillbirth. Treatment targeting AOX1 and/or GPER1 may arrest the oxidative damage in the placenta in pregnancies identified at risk and may lead to novel therapeutic strategies for delaying placental aging, as well as preventing stillbirth and other age-related adverse pregnancy outcomes.