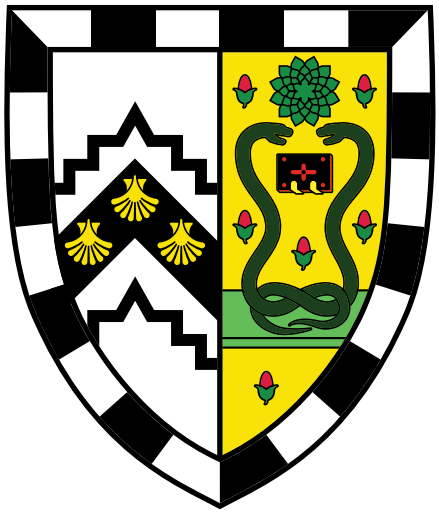
Direct Effects of Glucocorticoids on the Developing Cardiovascular System:

Studies in the Chicken Embryo



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This thesis is submitted for the degree of Doctor of Philosophy

September 2019

SUMMARY:

Glucocorticoid therapy for threatened preterm birth and in preterm infants has become common practice in the last 40 years. This treatment is based on the pioneering work of Liggins who discovered that development of fetal tissues was dependent upon the pre-partum surge in fetal cortisol. Therefore, exposure to synthetic glucocorticoids in premature offspring could accelerate pulmonary maturation, reducing risk of respiratory complications. Ante- and post-natal glucocorticoid therapy has since been demonstrated to significantly reduce morbidity and mortality in the preterm infant. However, several aspects of this therapy are not optimised, including drug choice or drug formulation. There is increasing animal and human data that suggest exposure to synthetic glucocorticoids may have a detrimental effect on the developing cardiovascular system. Therefore, there is an urgent need to understand possible adverse direct effects of current clinical therapy. The chicken embryo model was used to isolate the direct effects of glucocorticoids used in human clinical practise on the developing cardiovascular system, without confounding influences on the maternal or placental physiology. Specifically, this work compared the effects between Dexamethasone phosphate (Dex) Betamethasone phosphate (Phos), acetate (Ace) or combined (Beta). Treatment with 0.1mgkg^{-1} glucocorticoid at 60-70% of incubation resulted in significant growth restriction at term, which was more severe following Beta treatment due to the acetate formulation. Cardiac function was impaired by all treatments, again with Beta having a more severe effect. Dex treatment resulted in enhanced peripheral vasoconstrictor reactivity, cardiomyocyte hypertrophy and induced oxidative stress, caspase 3-mediated apoptosis, with p38-mediated reduced proliferation. In contrast, Beta treatments impaired peripheral vasodilator reactivity, reduced total cardiomyocyte number, promoted excessive GR activation due to loss of negative feedback, and led to p53-mediated apoptosis with reduced cardiomyocyte proliferation. Beta acetate shared the loss of GR negative feedback and enhanced p53 expression, whereas Beta phosphate did not. Therefore, combined, the data in this thesis support direct and divergent adverse effects of glucocorticoids used in human clinical practise on the developing cardiovascular system. The work offers insight into mechanisms underlying detrimental effects, providing a platform to modify current clinical antenatal glucocorticoid therapy and make it safer for the treatment of the preterm baby.

DECLARATION

This dissertation is the result of my own work and includes nothing, which is the outcome of work done in collaboration, except where specifically indicated in the text and acknowledgements.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other university or similar institution. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other university or similar institution.

This thesis does not exceed the word limit set by the Biology Degree Committee (60,000 words).

Tessa A.C. Garrud

Date

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'We are at the very beginning of time for the human race. It is not unreasonable that we grapple with problems. But there are tens of thousands of years in the future. Our responsibility is to do what we can, learn what we can, improve the solutions, and pass them on. It is our responsibility to leave the people of the future a free hand...

…It is our responsibility as scientists, knowing the great progress which comes from a satisfactory philosophy of ignorance, the great progress which is the fruit of freedom of thought, to proclaim the value of this freedom; to teach how doubt is not to be feared but welcomed and discussed; and to demand this freedom as our duty to all coming generations.'

Richard Feynman, 1988

ACKNOWLEDGEMENTS:

I will be forever grateful for the help of my supervisor **Prof. Dino Giussani** in completing this thesis and the three years of research that led up to its writing. The hard work he has dedicated both to the project, and to my own development as a scientist cannot be understated. I certainly wouldn't have been able to achieve the things I have without his support; from international conference presentations, writing and publishing a review paper, collaborating on numerous exciting projects, to gaining my official ninja training certificate in the remote hills of Japan.

Also essential to the success of this project are the members, past and present, of 'team chicken'. **Dr Nozomi Itani, Dr Katie Skeffington, & Dr Christian Beck**, not only established the chicken laboratory and many of the techniques used extensively in this thesis, but also introduced me to the fun of working with chicken embryos. **Dr Youguo Niu, Mr Sage Ford, Dr Kim Botting, Dr Qiang Lyu, and Ms Wen Tong**, have also contributed invaluably to all the effort that goes into ordering, incubating, dosing, and hatching eggs, including many weekend treks to the Barcroft.

I also owe many thanks to various colleagues from our lab and others, for their contribution to experimental techniques used in this project. **Dr Youguo Niu** for his expertise in Langendorff preparations, and **Miss Fiona Conlon** for her contribution to the Langendorff experiments. **Dr Kim Botting** for her advice and practical help in establishing a chicken cardiomyocyte dissociation protocol. **Ms Wen Tong** for her ability to run western blots at all hours of the night. **Dr Noor Teulings** for working to establish this project and contributing to Q-PCR experiments. I also wish to thank **Prof. Sue Ozanne** for her advice on various aspects

of this project, and for her lab members for helping me run molecular experiments, especially **Dr Lisa Nicholas**. **Prof. Mike Murphy** generously provided us with MitoB probe, and mass spec analysis was conducted by **Dr Sabine Arnst, & Dr Angela Logan** in his lab. Also, thanks to **Dr Bernardo Krause** for expanding my myography horizons, and singing Pink Floyd with me after too many glasses of wine.

I am also extremely grateful to the **Wellcome Trust** for funding the PhD programme which made this work possible. Also, to the **Society for Reproductive Investigation**, **The Physiological Society**, & **Gonville and Caius college** for their contributions towards travel costs such that work from this thesis could be presented at international conferences. Additional thanks go to the **Centre for Trophoblast Research** for allowing us to use their microscope equipment.

Finally, thanks to all the people who have had to put up with me for the past three years; especially my family and friends. My sister **Em** for her support and willingness to have two-hour long conversations about true crime documentaries. My close friends **Ailish, Alex & Mairead** who have stuck with me throughout this whole process. I don't think there is any way I could ever repay them or express how much they have done for me. Thanks to my parents, **Paul & Joy**, for their help and support, and to **Jet, Mel & Sue** for their companionship. My grandpa **Tom Garrud** passed away during the completion of this project. He was endlessly curious about the world, and happy to have a discussion on any subject. Whilst he will not get to see this thesis, I know how proud he would be and that if he were here, he not only would love to read a copy but also to discuss with me his many thoughtful questions.

ABBREVIATIONS:

ACh	Acetylcholine
AGT	Antenatal glucocorticoid therapy
ANOVA	Analysis of variance
BPD	Broncho-pulmonary Dysplasia
CFR	Coronary flow rate
CRL	Crown rump length
CO_2	Carbon dioxide
CVD	Cardiovascular disease
DoHAD	Developmental origins of health and disease
DNA	Deoxyribonucleic acid
eNOS	Endothelial Nitric Oxide Synthase
ET-1	Endothelin-1
g	Gram
H_2O_2	Hydrogen peroxide
IVH	Intra-ventricular haemorrhage
K^+	Potassium
kg	Kilogram
L-NAME	
LVDP	Left ventricular developed pressure
LVEDP	Left ventricular end diastolic pressure
m	Metre
M	Molar
mm	Millimetre
mM	Millimolar
mmHg	Millimetres of mercury
NEC	Necrotising Enterocolitis
NHS	National Health Service
ng	Nanogram
nL	Nanolitre
NO	Nitric Oxide
O_2	Oxygen
PE	Phenylephrine
ROS	Reactive oxygen species
SNP	Sodium Nitroprusside
SOD	Supra-oxide dismutase
UK	United Kingdom
USA	United States of America
%	Percent
°C	Degrees Celsius
µg	Microgram
µL	Microlitre
µM	Micromolar
5-HT	Serotonin

CHAPTER 1: GENERAL INTRODUCTION

1.1 PATHO-PHYSIOLOGY OF PRETERM BIRTH

Healthy human pregnancy lasts an average of 40 weeks, with labour typically occurring between 37- and 42-weeks of gestation. If a neonate is born prior to 37 weeks of gestational age, then the birth is considered preterm (Purisch, 2017). A preterm birth can be the result of iatrogenic intervention due to maternal or fetal pathology such as pre-eclampsia, or other types of abnormal placentation. However the majority of preterm births are idiopathic and occur spontaneously (Goldenberg et al., 2008). Preterm birth is the greatest cause of neonatal morbidity and mortality in the UK (NICE, 2015), with preterm infants carrying a greater risk of immediate health complications to their respiratory, immunological, nervous, gastrointestinal and other systems as well as long-term negative health implications (Harrison & Goldenberg, 2016). The associated psychological and economic burden to the families of preterm infants is significant, and more widely the economic burden on societies is huge (Behrman, 2007). There are currently limited preventative or even predictive strategies to address preterm birth (Purisch, 2017). As such, advances in understanding the aetiology, outcomes and treatment of premature birth are essential to improve the health of children of all nations.

The Office of National Statistics in the UK lists 53,887 births occurring at <37 weeks gestational age in England and Wales in 2017, accounting for 8% of live births for which gestational age was recorded. This rate has remained relatively unchanged over the past decade, despite improvements in obstetric care (Fig.1.1). A similar percentage is recorded in the United States at 9.93% in 2017, again with no observable decrease in recent years. There is substantial

heterogeneity in preterm birth rate between different countries; for example, within Europe the rate varies from as low as 5% up to 11% (Fig.1.1).

Current understanding for the epidemiological variance in preterm birth rate and predisposing factors continues to be poor. Groups at higher risk include black, African-American and Afro-Caribbean mothers, those with low educational or socioeconomic status, women with a short cervix, and very young or old mothers (Goldenberg et al., 2008). Multiple pregnancies also carry a much greater risk than singleton pregnancies. Certain environmental factors have been associated with preterm birth risk, such as lead exposure (Andrews et al., 1994), air pollution (Stieb et al., 2012), and tobacco smoke (Behrman, 2007). Environmental or psychological stressors may also have an impact; instances of violence and discrimination have a positive correlation with pre-term birth rates (Hill et al., 2016; Mendez et al., 2014), as well as maternal depressive illness (Orr et al., 2002).

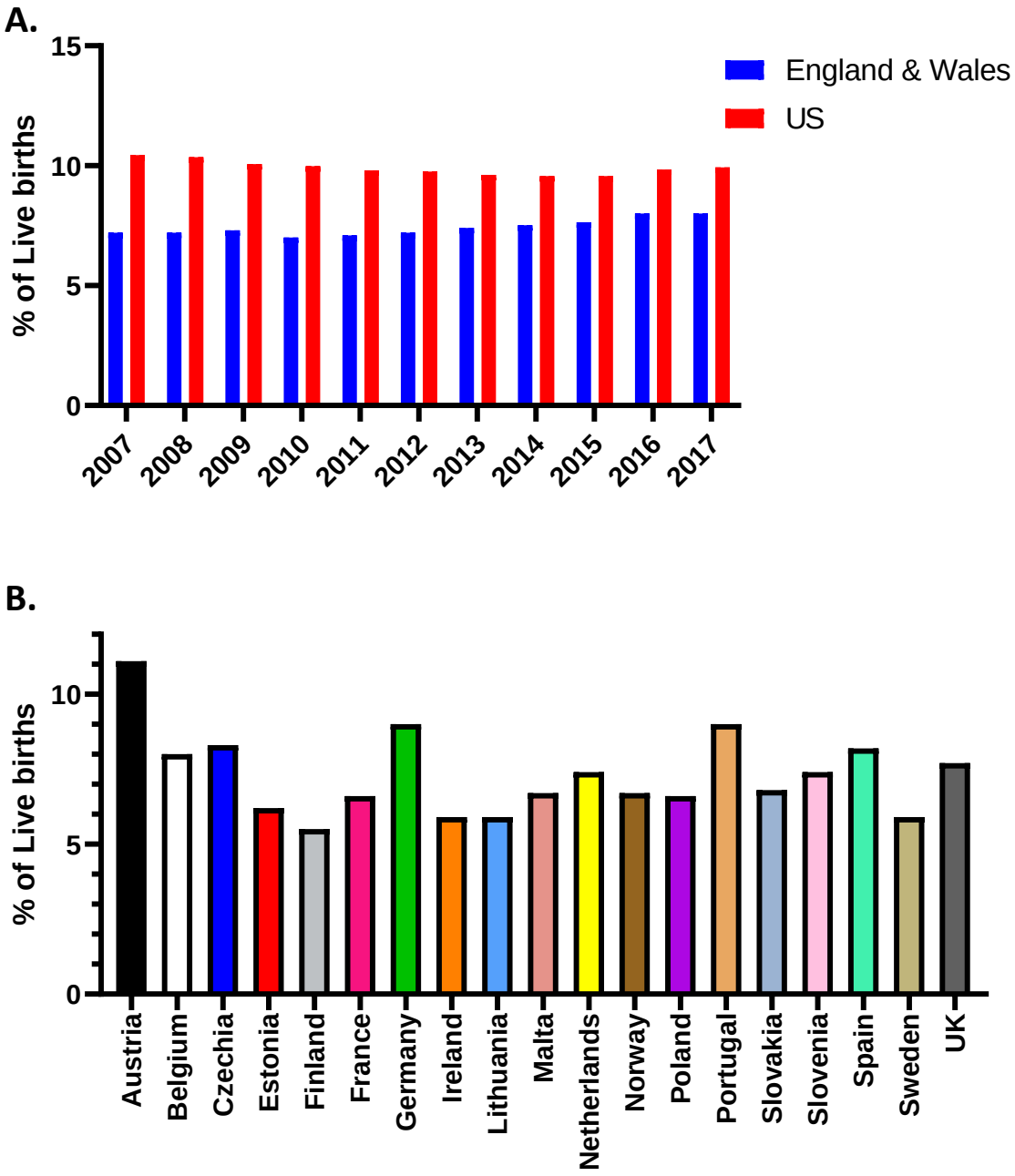

Figure 1.1 Preterm birth rates. A., US and England/Wales preterm birth rates from 2007-2017, B. preterm birth rates reported in 2008 for various European countries. Data from ONS (UK), CDC (US), and Zeitlin et al. (2013).

Understanding the physiological aetiology of preterm parturition and how the myriad of associated factors increasing its likelihood is key to identifying at risk women and developing preventative treatments. An overarching thesis explaining preterm birth is that several

different physiological changes activate a common central pathway resulting in increased uterine contractility, premature rupture of membranes and accelerated cervical maturation, resulting in preterm labour (Di Renzo et al., 2018). The premature induction of myometrial contractility is associated with altered inflammatory signalling in the decidua, resulting in expression of specific interleukins (IL-1, -6, -8), and contraction related proteins, including connexin-43 and prostaglandin receptors (Wadhwa et al., 2001). Further changes in proteases, and breakdown of fibronectin lead to separation of the decidua and chorioamniotic membranes, and eventually membrane rupture, again secondary to changes in immunological signalling. Cervical dilatation is induced by alterations in extracellular membrane proteins such as collagen, which decreases cervical tensile strength (Di Renzo et al., 2018). Our overall understanding of the mechanisms of parturition suggests immune signalling in the decidua acts as an essential switch towards activation of the uterus and cervix. Indeed, intra-amniotic infection has been reported in up to 50% of spontaneous preterm births, suggesting that dysregulated immune signalling can affect the decidual clock (Gonçalves et al., 2002). Therefore, infection or any other factor that may prime the maternal immune system, leading to an exaggerated immunological response to a subsequent insult may prematurely initiate the inflammatory changes associated with labour (Norwitz et al., 2015). Similarly, in women with vaginal bleeding or placental lesions, there is a shift towards a pro-inflammatory response and an increased likelihood of preterm labour (Di Renzo et al., 2018).

Current treatment options available for preventing or stalling preterm labour largely focus on the management of risk factors; identifying at risk women, treating infections, and monitoring

fetal wellbeing (Harrison & Goldenberg, 2016). Lifestyle interventions, such as advising women to quit smoking prior to pregnancy can also help to eliminate certain risk factors (McCowan et al., 2009). Women who present with a short cervix during pregnancy may be offered several treatments, including cervical cerclage (use of sutures to strengthen and close the cervix) or progesterone administration as a pessary (a silicone device) placed at the cervical opening, or vaginally (Koullali et al., 2016). These interventions have had mixed results in different populations varying from ineffective use of cerclage (Berghella et al., 2010) to a significant reduction in preterm birth when vaginal progesterone was administered. A meta-analysis demonstrated a relative risk of preterm birth <35 weeks gestation as 0.69 compared to placebo treatment (Romero et al., 2012). However, even when preventative treatment may be of benefit, detailed obstetric history as well as evaluation of pregnancy-related symptoms is vital, which may not be available in all healthcare settings.

1.2 Offspring Outcomes Following Preterm Birth

In terms of health outcomes for the preterm infant, the most immediate and significant consideration is of course neonatal survival. Despite the consistency in preterm birth rate in recent years, the percentage of survival of the preterm infant has increased over the past ten years, due to improvements in neonatal care (Patel, 2016). For instance, survival rate in Taiwan between 2007 and 2012 improved from 72% to 78% (Su et al., 2015), whilst in the US survival improved from 85.7% in 2000 to 87.5% in 2009 (Horbar et al., 2012). There is a negative correlation between survival rate and gestational age at birth, for example those born at 22 weeks have <40% survival rate (Patel, 2016). The majority of deaths of preterm neonates are listed as being due to prematurity or complications associated with preterm birth, making it difficult to dissect the exact pathological mechanisms involved. For deaths in which a specific cause is listed, the condition described as respiratory distress syndrome (RDS), immaturity of the neonatal lungs and therefore inability to ventilate, is the greatest contributor, but this has seen significant reductions as neonatal care has improved. In the UK respiratory causes of neonatal death have reduced from contributing to 64% of deaths between 1988-1994 to 49% between 2002-2008 (Berrington et al., 2012). Over the same time period deaths due to necrotising enterocolitis (NEC) have increased from 4% to 11%, making this the second largest cause of infant death.

The most common short-term sequalae of preterm birth are associated with respiratory dysfunction. RDS can be immediately treated via administration of surfactant and providing continuous positive airway pressure, thus allow ventilation of the lungs. Estimates of incidence of Respiratory Distress Syndrome (RDS) vary by country but have been estimated

at around 1.72% of all live births, and as high as 57.14% for those born at 32 weeks of gestation (Ghafoor et al., 2003). Following RDS, further respiratory issues may develop. Babies born pre-term are at a high risk of developing Broncho-Pulmonary Dysplasia (BPD) – a chronic lung disease affecting both the airways and parenchyma, that is defined by the need for oxygen supplementation for babies >36 weeks post-menstrual age (Ryan, 2006). BPD was initially defined by Northway in 1967, following investigation into the potential sequalae following respiratory distress syndrome (Northway et al., 1967). High levels of inflammation are seen in the lungs of neonates suffering from BPD, and it may lead to scarring and cellular abnormalities. BPD is also very predictive of future neurological impairment, and long-term respiratory dysfunction (Kobaly et al., 2008; Gough et al., 2014). Patent ductus arteriosus is also very common in preterm neonates and is associated itself with risk of RDS (Schena et al., 2015). Respiratory difficulties lead to inability to fully oxygenate the blood, which can lead to wide ranging damage in the body if oxygen delivery is compromised.

Several other serious short-term outcomes are secondary to immaturity of the neonatal circulatory system. Haemorrhage in the new-born brain is a common and serious finding in premature neonates. Intraventricular haemorrhage (IVH) occurs at a very high rate in extremely premature infants (45% of neonates) and occurrence rates remain relatively unchanged (Wilson-Costello et al., 2005; Jain et al., 2009). IVH is secondary to a number of factors, including immaturity of the germinal matrix vasculature in the brain, alterations in cerebral perfusion such as hypo- and hypertension, and alterations to the blood coagulation system (Ballabh, 2010). Hypotension is itself a common issue in premature neonates, who struggle to regulate and maintain arterial blood pressure (Fanaroff & Fanaroff, 2006). IVH is

associated with increased risk of several adverse neurological outcomes (Payne et al., 2013). Another outcome associated with an immature circulatory system is retinopathy of prematurity (ROP) leading to blindness due to reduced retinal vascular growth. Excessive supplementation with oxygen can worsen ROP by further affecting vascular endothelial growth factor (VEGF) levels in the retina (Chen & Smith, 2007).

The main metabolic morbidity present in preterm neonates is NEC in which portions of the bowel die leading to feeding intolerance, which may progress to intestinal perforation and peritonitis. NEC affects up to 10% of preterm infants, and a large proportion of very low birth weight neonates with NEC will die (20-30%; Patel & Denning, 2015). Again, the pathophysiology is multifactorial and complex, but it is thought that prematurity of the gastro-intestinal system may predispose to NEC due to insufficient immune defences and a propensity towards inflammatory responses (Nanthakumar et al., 2000), as well as decreased intestinal barrier function (Sharma et al., 2007). It is also thought that a lack of the normal commensal microbiota in the intestines may contribute (Patel & Denning, 2015).

Neurodevelopmental outcomes can also be seriously affected by preterm birth, with a spectrum of dysfunction listed in the literature. Cerebral Palsy represents a permanent movement disorder secondary to a brain injury that occurs at high rates in preterm infants (Patel, 2016). The gross motor function classification system (GMFCS) is typically used to describe cerebral palsy ranging from level 1 where speed, balance, and coordination are affected but children can walk and run, to level 5 where children are permanently wheelchair bound and have a limited ability to control head and trunk movement (Graham et al., 2016).

As with many other outcomes of preterm birth, the risk of cerebral palsy increases with decreasing gestational age. The pathogenesis of the brain injury that results in cerebral palsy is thought to be secondary to hypoxia or ischaemia, which are more common when respiratory difficulties are present (Graham et al., 2016).

As more infants are surviving preterm birth and the associated immediate jeopardies, the number of children with long-term health implications is increasing. A prospective study in the East Midlands estimated the difference in societal cost of healthcare between a single term born infant and a single preterm infant over the first 24 months of life to be £4657 (Khan et al., 2015). Clinical follow up of preterm born children have demonstrated increased risk of various adverse neurological outcomes. Rates of both deafness and blindness are higher in survivors of extreme prematurity (Doyle & Anderson, 2010). Developmental delay is also more common in survivors of preterm birth as are rates of major neurological disability diagnosed in early childhood (Msall & Tremont, 2002). Cognitive disability in the first 3 years of life has been reported in various studies following preterm birth, with rates as high as 42% of preterm children affected (Hack et al., 2000). At 6 years of age, cognitive impairment was reported in 21% of children born extremely preterm in the UK (Marlow et al., 2005). A meta-analysis of preterm born children assessed after 5 years of age also demonstrated increased risk of lower cognitive test scores in children born preterm, with more severe effects on those born at a lower gestational age (Bhutta et al., 2002). Follow up studies at adolescence have demonstrated increased instances of grade-repetition and special education measures in preterm born teenagers in Ontario (Saigal et al., 2000). Clearly, a spectrum of neurodevelopmental disorders is associated with preterm birth, even the least severe of

which can cause substantial negative effects on individuals. This is also not accounting for those with serious disability, such as cerebral palsy due to neonatal brain damage, which are diagnosed much earlier in life.

Long-term respiratory illness is also common in individuals born preterm. Asthma rates have been shown to be increased in some but not all studies (Doyle & Anderson, 2010). In terms of pulmonary function, there is evidence for decreased forced expiratory volume (FEV) and increased bronchial hyper-responsiveness in adolescents born extremely preterm (Halvorsen et al., 2004). In the same study there was also an increased likelihood of pneumonia or asthma reported during childhood. At 21 years of age, survivors of preterm birth are more likely to self-report occurrence of respiratory symptoms including asthma, coughing or wheezing (Narang et al., 2008). At adulthood, reduced total alveoli numbers have been demonstrated, as well as bronchiole hyper-reactivity, both of which can impair lung function by reducing the surface area for gas exchange (O'Reilly et al., 2013). Further exposure to environmental factors, such as tobacco smoke or air pollution may exasperate these conditions leading to outright respiratory disease. As those populations at higher risk of preterm birth are also often at higher risk of being exposed to negative environmental factors such as air pollution, this is concerning (Stieb et al., 2012).

Preterm birth is also highly associated with later cardio-metabolic dysregulation, which in many instances is associated with adult-onset hypertension and diabetes – two of the main components of the metabolic syndrome. At 30 years of age, individuals born preterm have a significantly increased systolic blood pressure (3.5mmHg greater than control group), and

insulin resistance (Dalziel et al., 2007). A similar increase in blood pressure has been demonstrated in other cohorts of young adults born preterm (Hovi et al., 2016). Whilst these blood pressure increases are not particularly large, a small increase in early adulthood is highly associated with increased risk of cardiovascular disease in later life (Chen & Wang, 2008). Cardiac structure is also known to be altered in preterm individuals, specifically an increase in left ventricular mass and reductions in functional cardiac parameters (Lewandowski *et al.*, 2013a). Increased left ventricular mass is a risk factor for heart failure, and there is some evidence linking preterm birth with increased risk of heart failure (Carr et al., 2017). As well as insulin resistance, an increase in plasma low-density lipoprotein (LDL) level has been reported in ex-preterm adults (Parkinson *et al.*, 2013), which is itself associated with atherosclerosis. Higher fasting insulin levels, and increased instance of gestational diabetes and type-2 diabetes have also been demonstrated in survivors of prematurity (Luu et al., 2017). In a world in which many consider us to be amidst an epidemic of non-communicable diseases including diabetes and heart disease, large numbers of children being born with a predisposition to develop these conditions is again concerning (Murray et al., 2012). A summary of the reported short- and long-term effects of preterm birth on an individual's health is shown in Table 1.1.

When the World Health Organisation (WHO) released their 'Global Action Report on Preterm Birth' in 2012, they estimated that around 1 million children globally every year die due to complications of prematurity. In 2015 this number remained unchanged despite advances in neonatal care (Liu et al., 2016). Even with reductions in preterm associated mortality in economically developed countries, such as in England and Wales, the estimated cost to the

public sector remains huge, with an estimated cost of £2.946 billion per year (Mangham et al., 2009). Clearly, it continues to be vital to increase our understanding of the pathophysiology leading to preterm birth as well as to improve its treatment to further reduce both mortality and morbidity associated with prematurity.

Physiological System	Short-term Outcomes	Long-term Outcomes
Respiratory	• Broncho-pulmonary Dysplasia • Patent Ductus Arteriosus	• Asthma • Airway Hyperreactivity • Reduced Alveoli Number
Circulatory	• Intra-ventricular haemorrhage • Peri-ventricular haemorrhage • Hypotension • Retinopathy of Prematurity	• Increased Systolic Blood Pressure • Increased Diastolic Blood Pressure • Increased Left Ventricular Mass • Increased Risk of Heart Failure
Neurological	• Cerebral Palsy & Motor Impairment	• Major Neurological Disability • Reduced IQ Score • Deafness • Blindness • Special Education Measures
Metabolic	• Necrotizing Enterocolitis	• Increased Fasting Insulin • Increased Insulin Resistance • Increased Plasma LDL levels • Increased Risk of Gestational and Type-2 Diabetes

Table 1.1 Overview of main short- and long-term outcomes on offspring of preterm birth.

1.3 Developmental Effects of Glucocorticoids in the Perinatal Period

It has been established for many years, that in almost all animal species studied, there is a prepartum surge in fetal plasma levels of glucocorticoids (Silver, 1988; Fig.1.1). This pre-partum surge is intricately linked with maturation of various physiological systems such that the neonatal transition can occur (Fowden & Forhead, 2015). It is also characterised by a switch away from tissue accretion, and towards distinct cellular maturational changes (Silver, 1988). As such, endogenous glucocorticoids in many ways act as the indispensable orchestrator of fetal maturation, either directly inducing tissue maturation or acting via other endocrine systems.

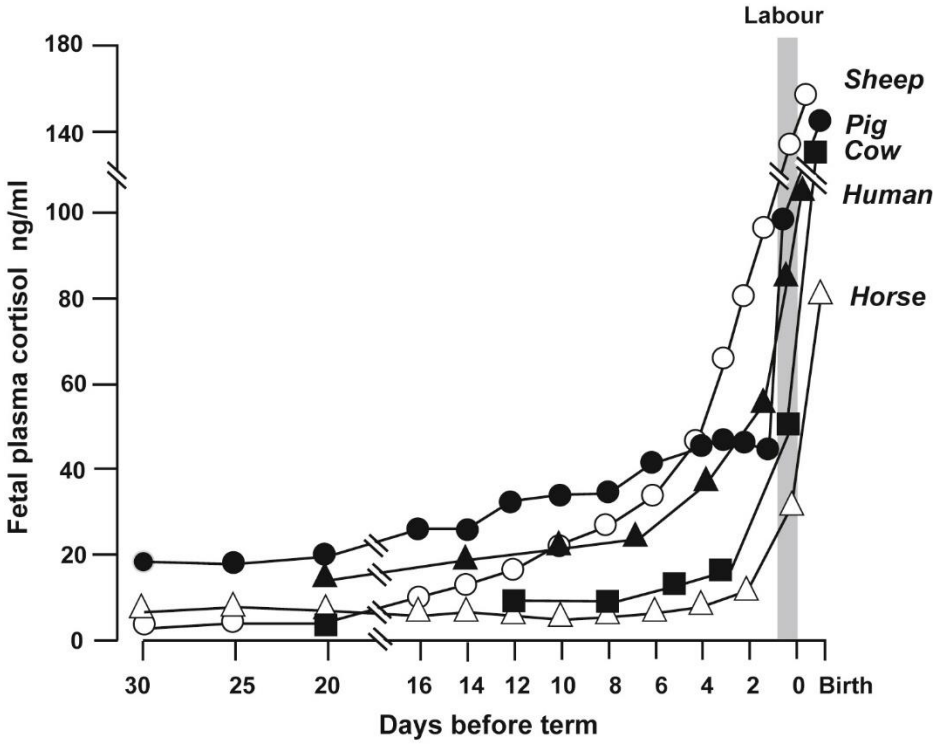

Figure 1.2 Ontological rise of fetal plasma cortisol, in the 30 days prior to term, in various species. Reproduced from Fowden & Forehead, 2015. Length of pregnancy: pig 115 d (filled circles), sheep 145 d (open circles), humans 280 d (filled triangle), cow 280 d (filled squares), and horses (pony) 335 d (open triangles).

During pregnancy in the human, the majority of cortisol present in the fetal circulation is of maternal origin as it can pass directly into fetal blood via the placenta (Fowden et al., 2016). The placenta contains relatively high levels of inactivating enzyme 11-βHSD2, which converts active cortisol into comparatively bio-inactive cortisone, however around 20% of maternal cortisol passes through without becoming inactivated (Mastorakos & Ilias, 2003). Maternal cortisol production rises during pregnancy as the maternal adrenal glands become hypertrophic. However, in late gestation the fetal hypothalamo-pituitary-adrenal (HPA) axis is also active (Fig.1.2). In sheep, it has been shown that the pre-partum cortisol surge is predominantly fetal in origin (Mastorakos & Ilias, 2006). There is also some evidence that in primates, CRH may be of placental origin with trophic effects on the fetal pituitary-adrenal axis (MacLean et al., 1995). Parturition in humans is thought to be triggered primarily by fetal androgens (DHEAS predominantly), arising from the fetal adrenal gland (Nathanielz et al., 1998).

Glucocorticoids may act directly to alter gene expression via the MR (Mineralocorticoid receptor) or the GR (Glucocorticoid receptor), which once activated can bind to Glucocorticoid Response Elements (GREs) in the promoters of specific genes with or without other transcription factors to alter transcription (Oakley & Cidlowski, 2013; Fig.1.3.). This is thought to reflect the main pathway for glucocorticoids to directly alter cellular maturation. The transcriptional effects are clearly dependent on tissue receptor expression but also on local glucocorticoid availability which may be altered by inactivating enzymes such as 11β-HSD2 which is prominently expressed for example in the developing brain and the placenta (Wyrwoll et al., 2011; Fowden et al., 2016). The response will also be tissue specific,

depending on the developmental trajectory and thus epigenetic state of the cells receiving the glucocorticoid signal. Hence, the timing of exposure is also critical.

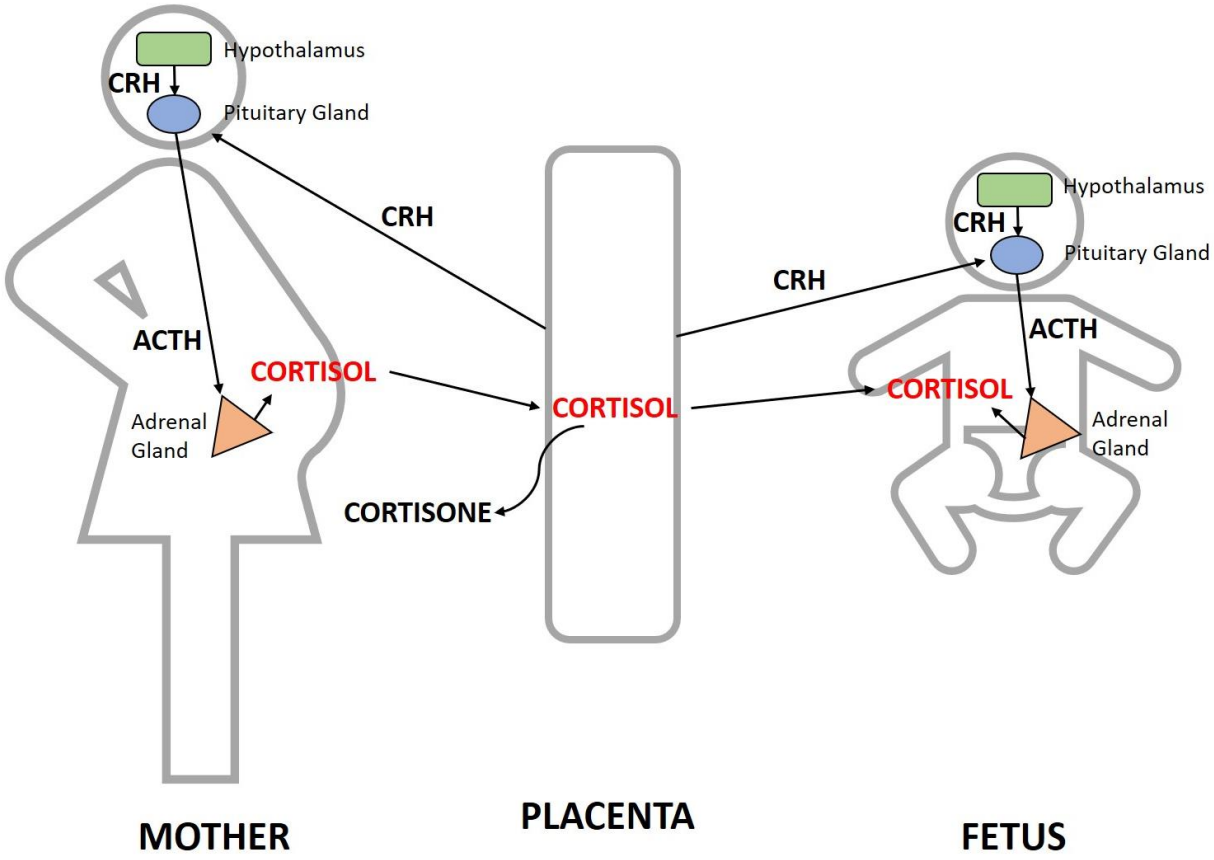

Figure 1.3 Sources of fetal plasma cortisol. Maternal hypothalamus produces CRH, stimulating ACTH production from the anterior pituitary gland, which induces cortisol release from the adrenal. Maternal cortisol may cross the placenta into the fetal circulation or become inactivated by placental 11β-HSD2. Fetal cortisol may originate from the fetal HPA axis, or from the maternal circulation. Both the maternal and fetal HPA axes can be stimulated by placental origin CRH.

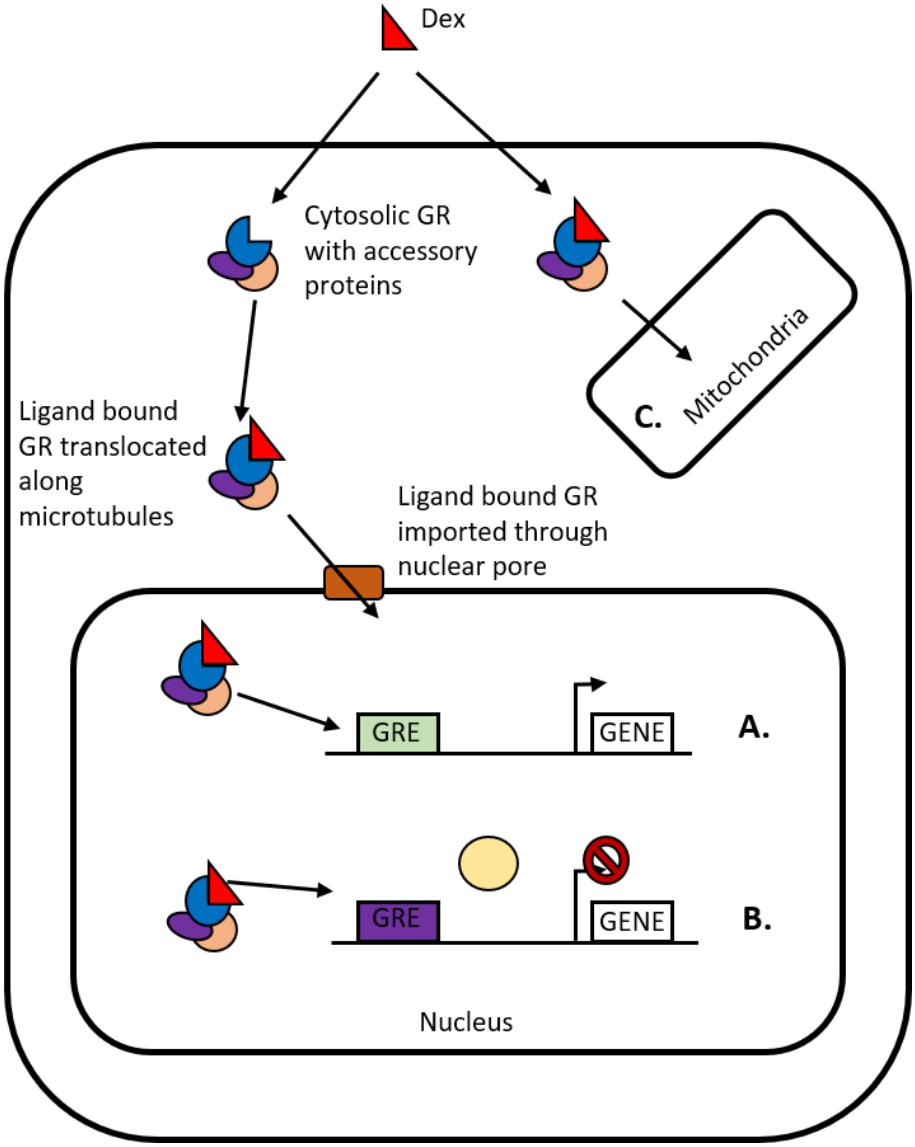

Figure 1.4 Simplified Glucocorticoid Receptor (GR) pathway. Canonical GR signalling to induce genomic effects occurs via binding of ligand to cytosolic GR (with accessory proteins), leading to translocation of the ligand-receptor complex into the nucleus. A. positive transcriptional regulation can occur via; direct binding of GR dimer to a glucocorticoid response element (GRE), concerted binding of GR with a secondary transcription factor (TF), binding of GR onto a TF either directly or indirectly. B. Negative regulation of gene expression can occur via; GR dimer binding to a negative-regulator GRE (nGRE), competitive binding with an overlapping TF site, binding of GR onto a TF, TF sequestration. C. ligand bound GR can also induce genomic effects in mitochondria when bound to mtDNA. Image adapted from (Scheschowitsch, Leite and Assreuy, 2017).

Glucocorticoids are known to have additional rapid cellular effects (termed the non-canonical pathway), mediated by cytoplasmic receptors. The nature and functional consequences of this mode of signalling are less well understood, particularly in relation to fetal maturation, but various cellular kinases have been implicated (Oakley & Cidlowski, 2013). It is also known that the GR may activate a non-canonical signalling pathway which is cytoplasmic and may induce rapid changes, although the exact role of this pathway is unknown (Stahn & Buttgereit, 2008). Fetal maturation is also known to be secondary to changes in the functionality and set points of other hormonal systems including the thyroid hormones (Forhead et al., 2006), the insulin-like growth factors (IGFs; Fowden, 2003), leptin (De Blasio et al., 2015), and insulin signalling (Jellyman et al., 2012). Cortisol is also known to mature the adrenal gland, leading to increased sensitivity and magnitude of catecholamine release in response to stress, which is vital during parturition and neonatal life (Liggins, 1994; Fletcher et al., 2006). The effects of a plethora of hormonal changes are diverse and distinct changes are seen throughout the fetal body, both directly and indirectly in response to the pre-partum cortisol surge (Fig.1.5).

Figure 1

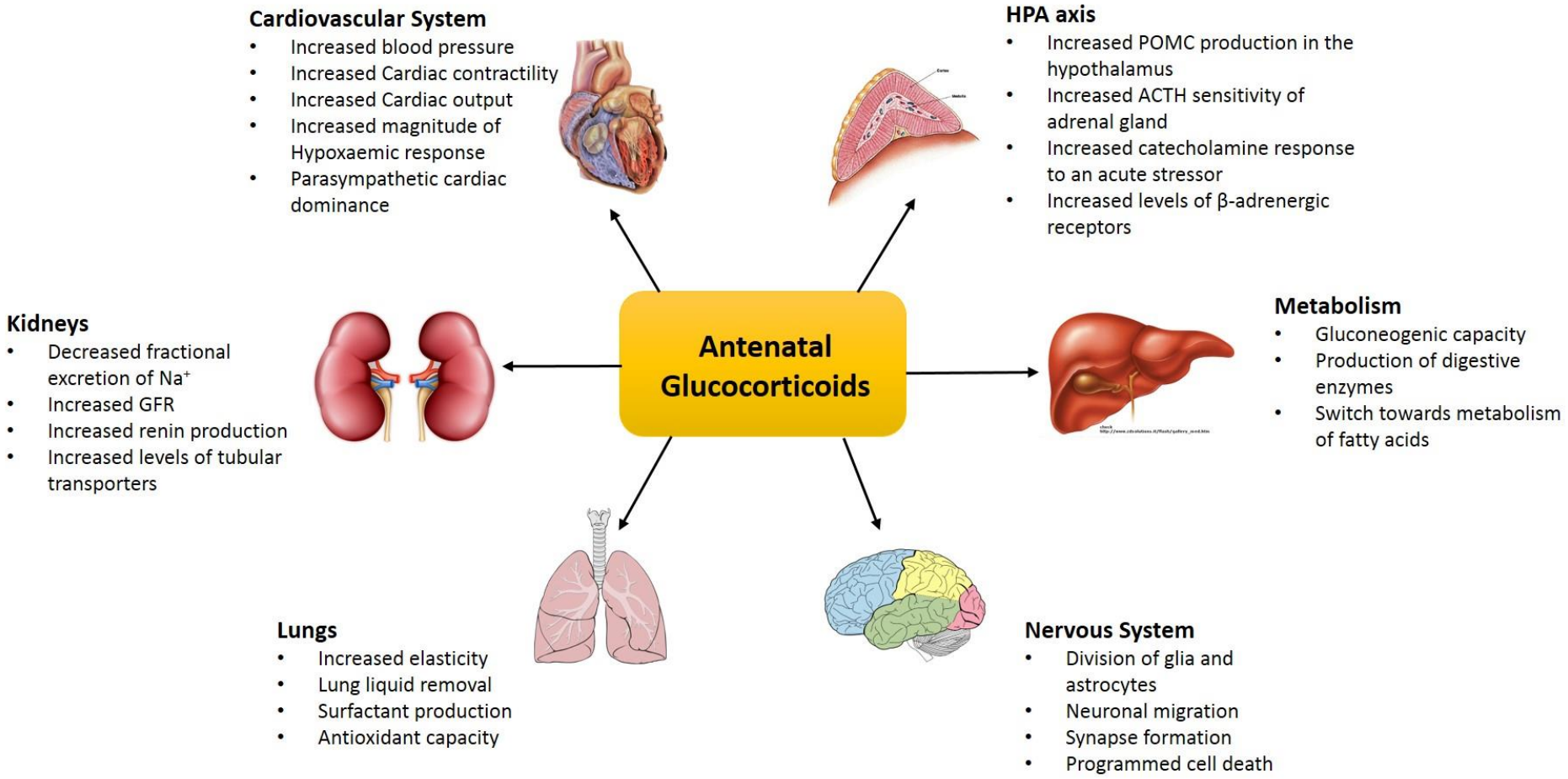

Figure 1.5 Maturational effects of antenatal glucocorticoids on; the fetal cardiovascular system, renal system, lungs, nervous system, metabolism and HPA axis functioning.

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The Maturational effects of glucocorticoids have perhaps been most studied in the fetal lung. The fetal lungs are fluid filled, and relatively inelastic (Harding & Hooper, 1996). However, the neonate must be able to inflate the lungs to begin respiration immediately after birth. As development progresses to term, levels of surfactant proteins increase and histologically surfactant granules can be seen in type II pneumocytes (DiFiore & Wilson, 1994). Lung liquid also begins to be removed (Jain & Eaton, 2006), and elastin levels increase (Liggins, 1994). These changes have been shown *in vivo* and *in vitro*, to rely on the surge in circulating levels of glucocorticoids (Gonzales et al., 1986; Liley et al., 1987; Banks et al., 1999; Bird et al., 2015). Antioxidant defences in the lung are also up-regulated prior to term, including pulmonary superoxide dismutase (SOD) and paraoxanase-3, again these changes are thought to be secondary to the increased plasma glucocorticoid levels (Walther et al., 1991; Belteki et al., 2010; Erenberg et al., 1982).

Similarly, glucocorticoid dependent changes are seen in other systems essential for neonatal survival. Fetal metabolism switches towards a gluconeogenic state, and production of digestive enzymes is initiated (Fowden et al., 1998; Fowden et al., 2006). Pancreatic development is also stimulated (Fowden et al., 2005). The fetal circulation likewise changes substantially towards term. Fetal arterial blood pressure increases with increasing gestational age (Forhead et al., 2000; Shinozuka et al., 2000; Giussani et al., 2005). This change can be experimentally stimulated by exogenous administration of synthetic glucocorticoids (Fletcher et al., 2002). The fetal baroreflex has different responsiveness depending on gestational age (O'Connor et al., 2006; Blanco et al., 1988). In sheep, the set-point increases towards term to allow a greater resting arterial blood pressure, again in a manner which can be replicated by

administering exogenous glucocorticoids (Fletcher et al., 2002). There are also significant maturational effects of glucocorticoids on the fetal heart. They stimulate cardiomyocyte maturation; specifically hypertrophy and bi-nucleation, and induce a switch away from hyperplasia (Rog-Zielinska et al., 2014a). The electrical conduction system in the heart is matured in preparation for the increased cardiac workload at birth (Davies et al., 1996). Left ventricular developed pressure also increases towards term, and a switch towards parasympathetic cardiac dominance occurs, although it is not clear what role glucocorticoids play in this maturation (Fletcher et al., 2005). During acute stress, for example a period of fetal hypoxia, the fetal cardiovascular system typically redistributes blood flow to vital circulations, such as the brain – the so called fetal brain sparing effect (Giussani, 2016). The extent of this response increases with advancing gestational age (Fletcher et al. 2006) and this maturation can be accelerated in the preterm fetus by maternal (Jellyman et al., 2005) or fetal (Fletcher et al. 2003) treatment with Dexamethasone. The fetal HPA axis is vital for acute stress responses by means of a rapid rise in blood cortisol levels (Newby et al., 2015). The sensitivity of the HPA axis to an acute stressor increases with gestational age, secondary to changes in production of POMC hormones in the pituitary (Yang et al., 1991), and increased sensitivity of the adrenal itself to ACTH (Fraser et al., 2001). There is also evidence to support that exposure of the preterm fetus to synthetic glucocorticoids accelerates the maturation of the fetal adrenal gland, for instance by increasing adrenocortical sensitivity to ACTH during a period of acute hypoxic stress (Fletcher et al., 2004).

During fetal life the kidneys do not act as the main osmoregulatory organ, that role is distributed to the placenta, however they do produce large volumes of relatively hypo-

osmotic urine which enters the amniotic fluid (Wlodek et al., 1992). The fetal kidneys are poor at retaining sodium and consequently have a large fractional excretion of sodium (FENa). This allows for the excretion of the large amounts of fluid that cross the placenta and must be lost from the fetal circulation (Holtbäck & Aperia, 2003). As gestational age increases, FENa is reduced as transporters increase in number and activity in the nephron (Holtbäck & Aperia, 2003), this can be induced experimentally by glucocorticoids (Aperia et al., 1981). This prepares the kidneys for sodium retention upon birth to maintain blood volume. For example, in the sheep fetus FENa decreases from around 15% at 0.75 of gestation to 5% at term (Segar et al., 1992), and continues to decrease during early neonatal life (Smith & Lumbers, 1989). In human babies, FENa also decreases during neonatal life (Benson, 2008). Glomerular filtration rate (GFR) in the fetal kidneys is also low. Again, this increases towards term, in part due to an increased cardiac output and renal blood flow (Chang et al., 2003) which increases from around 3% to 25% in the neonate (Holtbäck & Aperia, 2003). Maturation of the kidney is also essential for several endocrine systems. The renin-angiotensin system (RAS) is vital for sodium homeostasis and long-term control of blood pressure. Renin production increases with gestational age (Robillard & Nakamura, 1988), concomitant with changes in expression of AT_{-1} and AT_{-2} receptors in various target organs (Shanmugam & Sandberg, 1996). Aldosterone production has also been shown to increases before birth (Robillard et al., 1992), and whilst the fetal kidneys are relatively insensitive (Martinerie et al., 2009), the neonate has an active responsiveness. The period of nephrogenesis occurs in sheep (Moritz et al., 2003) and humans (Hinchliffe et al., 1992) predominantly during late gestation (in humans no nephrogenesis occurs after 36 weeks). The pre-term glucocorticoid surge is involved in a decrease in nephrogenesis in exchange for cellular changes within the tubules themselves (Moritz et al., 2003).

The need for the neonate to have various neurological functions at birth is clear – homeostatic regulatory mechanisms must become active to ensure survival. The relationship between the pre-partum cortisol surge and neurological development is complex, and different cell populations and brain regions are affected differently. Neurogenesis occurs predominantly during early gestation, and by late gestation the principal events occurring are: multiplication of glia and astrocytes and their subsequent migration, programmed cell death, neuronal migration, synapse formation, pruning, and myelination (Knuesel et al., 2014). Glucocorticoids are thought to be highly involved in regulating these processes. Specifically, activation of the canonical GR pathway is thought to be vital in stabilising vascular endothelial cells in the brain (thus reducing the risk of haemorrhage), and in regulating proliferation of neural stem/progenitor cells (NSPCs; Vinukonda et al., 2010; Peffer et al., 2014). Administration of synthetic glucocorticoids has been demonstrated to alter cell number and size within specific cellular populations, and to cause long-term alterations in synaptic function (Kreider et al., 2006). Glucocorticoids have been implicated in balancing apoptosis in the developing brain (Almeida et al., 2000). Neuronal maturation of several endocrine axes has also been related to glucocorticoids, e.g. the HPA axis (Davis et al., 2006), and the renin-angiotensin system (RAS; Dodic et al., 2002).

A brief look at the myriad of effects of the prepartum glucocorticoid surge highlight the potential difficulties if a neonate is born prior to exposure to this surge, and hence is not sufficiently matured for survival in the extra-uterine environment.

1.4 Clinical Benefits of Glucocorticoids in Perinatal Life

The first steps towards clinical use of glucocorticoids to treat pre-term birth occurred with Liggins' serendipitous observation that synthetic glucocorticoid injection into the pregnant ewe triggered fetal lung maturation (Liggins, 1969). He correctly inferred that glucocorticoids must be critical for fetal maturation, and immediately saw the clinical potential. Together with obstetrician Howie an initial clinical trial using AGT when women presented at hospital with threatened pre-term labour was completed in 1972, with clear positive results – incidence of respiratory distress syndrome (RDS) in the neonate was reduced from 25.8% in the control group to 9% in the glucocorticoid treated group, whilst perinatal death was reduced from 18% to 6.4% (Liggins & Howie, 1972). Several other clinical trials then followed (Roberts & Dalziel, 2006), demonstrating a decrease in various neonatal morbidities following AGT, with the positive effects comparable for older and more recent studies (in which other advances in neonatal care were also available). Initially, the obstetrics community was reluctant to adopt AGT as standard. However, in 1994 the NIH released a consensus statement (NIH, 1994) which purported that:

> *''Antenatal corticosteroid therapy is indicated for women at risk of premature delivery with few exceptions and will result in a substantial decrease in neonatal morbidity and mortality, as well as substantial savings in health care costs."*

In the years since then AGT has become almost universally implemented for threatened pre-term birth. Four Cochrane systematic reviews have been conducted on AGT, concluding that there is an associated decrease in several neonatal morbidities including respiratory distress syndrome (RDS), and an associated decreased risk of perinatal death (Sotiriadis et al., 2009; Brownfoot et al., 2013; Crowther et al., 2015; Roberts et al., 2017) with the latest update cementing the drastic benefits of AGT (Roberts et al., 2017). Consequently, AGT is now

considered to be an indispensable treatment by most health organisations. The World Health Organisation lists antenatal glucocorticoids for preterm labour as an essential medicine (Lawn & Althabe, 2013) and AGT is recommended by the Royal College of Obstetricians and Gynaecologists for all pregnancies at risk of preterm birth between 26-34 weeks (Royal College of Obstetricians & Gynaecologists, 2010).

The positive effects of AGT on neonatal respiratory outcomes are mainly due to direct effects on the fetal lungs leading to accelerated maturation (Ballard, 2000). AGT has been shown in animal models to induce structural changes leading to greater alveolar volume (Willet et al., 1999), drastically increased lung volume (Jobe et al., 1993), and increased surfactant production (Ballard, 2000). These changes lead to improvements in lung compliance and ventilation efficiency (Ikegami et al., 1997). In clinical trials, AGT results in a significant reduction in RDS, moderate/severe RDS, and the need for mechanical ventilation, whilst not having an effect on instance of chronic lung disease (Roberts & Dalziel, 2006). These beneficial effects on the lungs are also independent of post-natal surfactant therapy (Martin & Fanaroff, 2013).

As well as improved respiratory outcomes for preterm babies, AGT significantly reduces other associated morbidities. Rates of IVH are significantly reduced in AGT treated neonates (Roberts et al., 2017). This may be due to maturation of the cardiovascular system, for example these neonates have a higher mean blood pressure (and are less likely to suffer from hypotension; Dempsey, 2017), and the brain microvasculature itself is likely directly affected (Garton et al., 2017). There is also a significant decrease in NEC following AGT treatment. This

may be secondary to decreased bacterial translocation across the intestinal barrier, decreased colonisation with aerobic bacteria, and/or increased activity of enzymes including lactase, sucrase, and the Na^+/K^+ ATPase in intestinal epithelial cells (Lin et al., 2014).

AGT coverage of women at risk of preterm birth, between 24 and 34 weeks of gestation, has a target of 85% in the UK (the other 15% being comprised of women with contraindications; Royal College of Paediatrics and Child Health, 2015), however global coverage does in fact vary drastically. Those countries with the most developed health care systems typically have coverage at or approaching the 85% mark. However, developing countries often have far poorer coverage. For example, Afghanistan and the Democratic Republic of Congo had reported coverage between 2010 and 2011 of only 16% in pregnancies 26-34 weeks (Vogel et al., 2015). Few of the clinical trials have been conducted in low-income countries and so efficacy in these settings is yet to be made entirely clear. Exposure to AGT is also not limited to these groups, in fact many women who are exposed to AGT will go on to deliver after 35 weeks, or up to a week after drug administration (Makhija et al., 2016; Razaz et al., 2015), especially in those developed countries with high coverage. Clearly, with the number of fetuses at risk of preterm birth and the expected coverage with AGT, vast numbers of individuals have been and will go on to be exposed to this treatment

As well as AGT, it is also relevant that synthetic glucocorticoids may be administered to the new-born infant in the immediate post-natal period, predominantly to treat BPD, provide blood pressure support and ameliorate sepsis. Steroids such as Dexamethasone administered in this period have also been shown to successfully decrease inflammation and allow

extubation of the infant (Farstad et al., 2011). The use of steroids for this purpose rose during the 1990's, with predominantly Dexamethasone being utilised (Kobaly et al., 2008; Mammel et al., 1983). However, it was soon observed that those children who had received post-natal glucocorticoids demonstrated both severe immediate and long-term adverse side effects from this treatment, including gastrointestinal bleeding, hypertension, and neurological impairment. In 2002, the American Academy of Pediatrics released a consensus statement advising against the use of steroids for treatment of BPD (American Academy of Paediatrics, 2010). The result was a rapid decrease in use of this therapy, except in the most severe cases of BPD, despite lack of large controlled trial data (DeMauro et al., 2014). Since then, the DART study indicated no strong association with long term consequences when low-dose Dexamethasone treatment was trialled (Doyle et al., 2007). The rates of BPD in very low birth weight, and very low gestational age neonates has not changed since the early 2000s (remaining at just over 50%). In Finland, of babies born preterm between 22-25 weeks, the incidence of BPD was 67.3%, and between 26-30 weeks this decreased to 36.6% (Farstad et al., 2011). Whilst the rate of neonatal steroid use has decreased since the early 2000s, it still affects around 8% of neonates in this category. What's more, the rate of antenatal steroid use in this group is high. A retrospective analysis in North America estimated antenatal steroid coverage of this group of new born children at 61-75% (Walsh et al., 2006), meaning that many preterm individuals are exposed to both antenatal and postnatal steroid therapy.

1.5 Adverse Effects of Glucocorticoids in the Perinatal Period

Despite the clear life-saving benefits of AGT, there is mounting concern about adverse effects of exposure to high levels of synthetic glucocorticoids on organ systems other than the lungs. Adverse changes to physiological systems in the fetal and neonatal period may result in permanent dysfunction than increases risk of disease in the adult; what is termed developmental programming of adult disease (DoHaD; Barker, 2007). Our understanding of these detrimental effects of synthetic steroids is compounded by the use of multiple animal models, timings of glucocorticoid exposure, glucocorticoid dose, and timing of outcome measurements throughout the literature. The human data are also difficult to interpret as the majority of children exposed to AGT experienced a sub-optimal pregnancy and hence it is not possible to disentangle the effects between multiple factors.

Despite these difficulties, there is significant evidence that exposure to glucocorticoids in early life impairs growth. In animal studies both single and repeat doses of antenatal glucocorticoids lead to growth restriction (Newnham et al., 1999; Jobe et al., 1998). Antenatal glucocorticoids are also associated with decreased placental weight (Newnham & Moss, 2001), alterations in placental blood flow (Jellyman et al., 2004), and amino acid transport (Audette et al., 2014) – all factors associated with impaired fetal growth. Studies in non-human primates also observe a growth restriction following one or multiple antenatal doses (Doyle et al., 2014). However in humans, in the Cochrane reviews of single AGT, there is no significant difference in birth weight between treatment and placebo groups, suggesting that in humans a single course may not be sufficient for serious growth impairment (Roberts et al., 2017).

Exposure to excess glucocorticoids during early life can also have several effects on the fetal and adult cardiovascular system. There are a number of rapid alterations to fetal haemodynamics and movement (Koenen et al., 2002; Mulder et al., 2009; Fletcher et al., 2002), primarily a transient hypertension and a decrease in fetal movements. Exposure to low levels leads to a shift in set-point of the fetal baroreflex and hypertension up to 48-hours after the initial injection (Fletcher et al., 2002). In the neonate and adult, the most commonly reported long term effect is dysregulation of blood pressure (Lindsay et al., 1996). Sheep exposed in utero have been reported to have elevated blood pressure at 6 months of age, and an attenuated baroreflex (Shaltout et al., 2012; Davis et al., 2006). In human clinical follow-up studies, both systolic and diastolic blood pressure in exposed individuals at 14 years of age is increased (Cheong et al., 2014). In both humans and animal models, there are several reported changes directly to the vasculature, such as endothelial dysfunction (a well-known harbinger of future cardiovascular disease), changes in aortic arch stiffness, and blood flow pulsatility (Docherty et al., 2001; Pulgar & Figueroa, 2006; Kelly et al., 2012). Direct cardiac effects have been shown including transient hypertrophy, altered autonomic responses, impaired Starling mechanism, systolic dysfunction, and increased end diastolic volume and pressure (Yunis et al., 1999; Dodic et al., 2001; Niu et al., 2013). Many of these changes are seen in cardiovascular disease and heart failure.

The renal system is also of great importance when discussing cardiovascular and metabolic disease. The kidney, as a key regulator of blood pressure, is a central thespian in hypertension. Chronic kidney disease (CKD) is also highly associated with diabetes, and levels are rising at a

serious rate (Webster et al., 2016). Given the costs and detriment to wellbeing of treatments for CKD, this rise is worrying. Several studies have demonstrated that antenatal glucocorticoid exposure can reduce nephron number (Figueroa et al., 2005; Ortiz et al., 2001; Moritz et al., 2011) and can lead to reduced glomerular filtration rate (GFR) in the adult (Finken et al., 2008). This has been proposed to result in adult hypertension via a positive feedback mechanism of glomerular damage. This is summarise by the Brenner hypothesis that states that increased load of filtrate per nephron leads to an increased glomerular pressure, this causes glomerular damage leading to further loss of GFR and hypertension, hypertension causes more glomerular damage (Brenner et al., 1988). However, the hypertension observed after antenatal glucocorticoid exposure is not proportional to nephron number, hence other factors are also contributing (Zhang et al., 2010). A reduction in nephron number may also impair the response to a later renal insult. In male sheep treated antenatally with betamethasone, exposure to Angiotensin II significantly increased markers of oxidative stress and increased protein excretion (Bi et al., 2014). Glucocorticoids may also alter the developing renal RAS (Connors et al., 2010; Contag et al., 2010; Forhead et al., 2015). Alterations in the set-point of this system again may contribute towards hypertension.

There are several known links between antenatal glucocorticoids and future metabolic disease. In the fetus, exposure to glucocorticoids can alter pancreatic development leading to a decrease in beta cell endowment in the islets. Several studies have observed dysfunction in insulin sensitivity (Blanco et al., 2014), and glucose tolerance at adulthood (Nyirenda et al., 1998). Exposure to antenatal glucocorticoids may also increase risk for obesity and abnormal fat deposition (Drake et al., 2010; Finken et al., 2011). Alterations in the leptin axis have also

been reported (Sugden et al., 2001). These parameters are risk factors for type-II diabetes, which itself increases risk of both CKD and cardiovascular disease. Being exposed to an altered maternal metabolic profile in utero (e.g. pregnancy with maternal diabetes or obesity) may itself programme future metabolic dysfunction in the offspring (Fernandez-Twinn & Ozanne, 2006).

Several animal studies have also suggested a reduction in brain weight following antenatal or postnatal glucocorticoid exposure. Specifically, this appears to be associated with a decrease in volumes of cortical and deep grey matter, and hippocampal neuronal soma volume (Uno et al., 1990; Tijsseling et al., 2013; Camm et al., 2011). This decrease in brain volumes may be transient or persistent (Noorlander et al., 2014). Cellular alterations include delayed myelination (Antonow-Schlorke et al., 2009), alterations in gap junction proteins (Sadowska & Stonestreet, 2014), and specific changes in gene expression (Labaune et al., 2002; Pascual et al., 2014). Functionally, it seems that these changes may lead to impaired performance in various neurological tests and altered behaviour in the adult (van der Voorn et al., 2015). There are also reports of altered stress and anxiety responses (Oliveira et al., 2006, 2012; Alexander et al., 2012). This may be secondary to alterations in the HPA axis (Shaltout et al., 2011; Li et al., 2013). Follow-up studies of school children exposed to post-natal glucocorticoids suggest a decrease in IQ (Hitzert et al., 2014). Adolescents at age 14-17 who have been exposed to postnatal Dexamethasone have adverse motor function, impaired neuropsychological test scores, and the females were more likely to need special education measures (ter Wolbeek et al., 2013). This group also displays decreased brain volumes, specifically of the white matter, thalami, and basal ganglia, with a potential dose-dependent

relationship (Cheong et al., 2014), and altered HPA axis function (Ter Wolbeek et al., 2015). There is human evidence of altered stress responses following antenatal glucocorticoid exposure, for example increased cortisol response to stress tests in adolescents (Moisiadis & Matthews, 2014a). In primates there is also an increase in HPA activity in juveniles, and alterations in hippocampal mRNA129 have been observed in sheep models. The implications of neurological effects of antenatal glucocorticoids for clinical treatment are great. Clearly, there is a scale of neurological impairment, and whilst follow-up studies may focus on manifest neurological diseases, such as cerebral palsy, even modest changes in neural function can significantly impact future health and wellbeing.

1.6 Mechanisms of Dysfunction Induced by Glucocorticoids

The missing piece in preventing the lifelong unwanted implications of AGT, and more broadly of fetal and/or neonatal exposure to elevated levels of glucocorticoids, is a lack of clear understanding of the mechanisms underlying these adverse effects. Because of the pleiotropic nature of glucocorticoids in different tissues and at different gestational ages, it is very difficult to isolate specific causes. However, the changes observed in different studies throughout the literature can be summarised into several categories of potential mechanistic changes in the aetiology of the dysfunctional adult phenotype (Fig.1.6.).

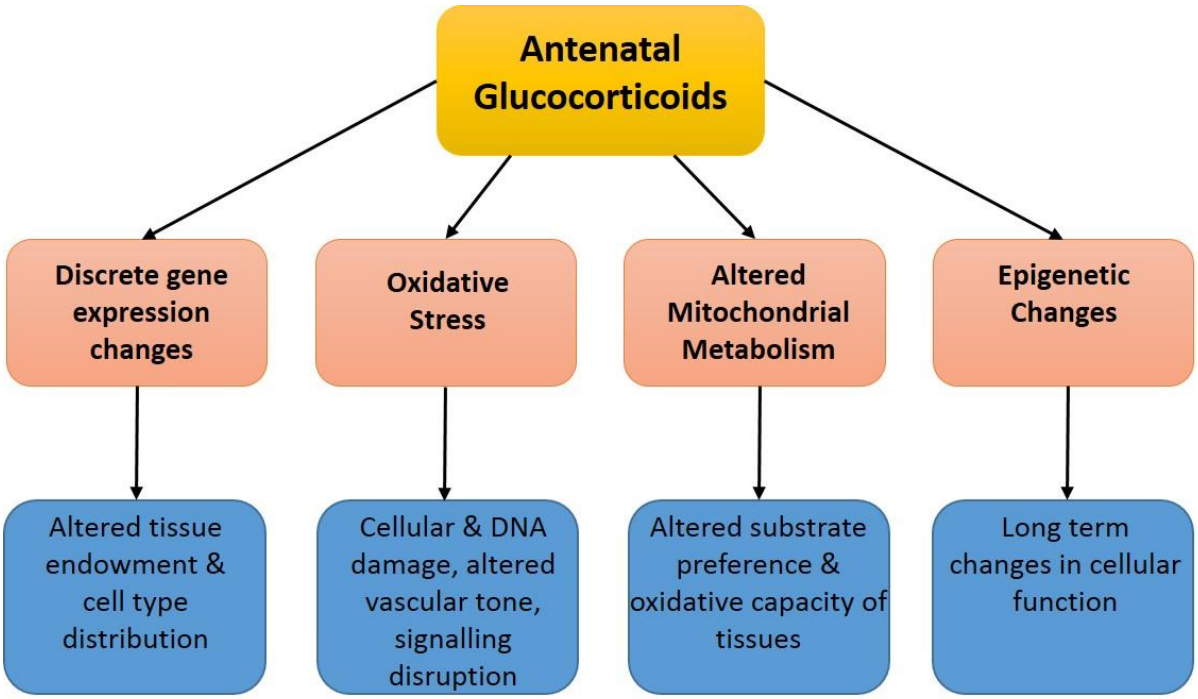


Figure 1.6 Mechanisms of antenatal glucocorticoid induced dysfunction.

One of the main effects of glucocorticoids in the fetus is a switch away from tissue accretion towards maturation (Newnham & Moss, 2001; Fowden et al., 2016). Prematurely exposing the fetus to high levels of synthetic glucocorticoids may therefore induce this switch earlier, resulting in a decrease in growth. Decreased body growth, or of specific organs has been demonstrated in many animal models. For certain organs in which the endowment of particular cell types is set during prenatal development, this premature switch could lead to a life-long impairment. For example, in many animals, cardiomyocyte number is established in late gestation, and the pre-partum cortisol surge is known to cause a shift from cell division to cellular hypertrophy (Thornburg et al., 2011; Gay et al., 2015). Therefore, antenatal exposure to glucocorticoids at levels inappropriate for the stage of gestation can prematurely reduce cardiomyocyte number, potentially leading to impaired contractility, excessive hypertrophy, and cardiac dysfunction in adulthood (Gay et al., 2015). Similarly, as we have discussed, a decrease in nephron number may pre-dispose someone to hypertension and kidney disease in adulthood (Brenner et al., 1988). Equally, early exposure to glucocorticoids may alter the ratio of different cell types in a particular tissue. Exposure to Dexamethasone has been shown to reduce the proportion of beta cells in the pancreas (Dumortier et al., 2011). This could in turn lead to insufficiency of insulin production, with potential downstream changes in the sensitivity of insulin target organs, such as adipose tissues, leading eventually to an increased risk of insulin resistance (Dumortier et al., 2011). Brain weight can be reduced by antenatal glucocorticoid exposure, and loss of cell number is specific brain areas may be in part responsible for the neurological deficits described (Tijsseling et al., 2013).

In adults, there is a clear link between glucocorticoids and oxidative stress, and further this oxidative stress is associated with cardiovascular and metabolic dysfunction (Wu et al., 2014; Aboelwafa and Yousef, 2015; Takeshita et al., 2015). Indeed, *in vitro*, it seems that glucocorticoid administration in high doses can increase cellular oxidative stress, and a meta-analysis of the link between glucocorticoids and oxidative stress in adult animals suggests that there is a significant effect, which is larger in long term studies (Costantini et al., 2011; Tang et al., 2013). In the fetus, this relationship is less clear, with some organs appearing to have a reduction in oxidative stress following antenatal exposure (Walther et al., 1998). However, it is probable that in some tissues an induction of cellular oxidative stress may be responsible for some of the observed dysfunction. Endogenous cortisol matures fetal mitochondria, leading to an increase in oxidative capacity by up-regulation of specific electron transport chain components (Lee et al., 2013). A mismatch between substrate availability and mitochondrial activity could lead to an increase in generation of oxidative stress. Free radicals, most notably reactive oxygen species (ROS), may subsequently damage cellular components, affect signalling pathways, and alter oxidant tone of the vasculature by decreasing NO availability (Keaney & Chen, 2004). There is evidence of oxidative stress in the heart and brain in animal models of AGT (Wallwork et al., 2003; Zhang et al., 2004, 2005; Adler et al., 2010; Camm et al., 2011; Tijsseling et al., 2013). A study in which HUVECs (Human Umbilical Vein Endothelial Cells) were treated with Dexamethasone also demonstrated an increase in hydrogen peroxide production and decreases in cellular NO, secondary to ROS production via NAD(P)H Oxidase, Xanthine Oxidase, and the Mitochondrial electron transport chain (Iuchi et al., 2003). In humans, there is evidence that Betamethasone may cause an acute repression of Glutathione peroxidase 3 (GPx3; Verhaeghe et al., 2009).

Glucocorticoids alter mitochondrial biogenesis, oxidative phosphorylation, and substrate preference, either directly or via changes in nuclear genes (Lee et al., 2013). In late gestation, there is a switch towards increased numbers of mitochondria and towards metabolism of fatty acids in energy demanding tissues such as the heart (Rog-Zielinska *et al.*, 2014a). A premature transition to this state may lead to ROS build-up within the mitochondria, potentially damaging mitochondrial proteins and DNA. Excessive glucocorticoid levels may thus result in mitochondrial dysfunction, which may persist into adulthood, as mitochondria are not generated de novo, but by replication of the existing organelles. Mitochondrial dysfunction is a hallmark of many diseases including diabetes, atherosclerosis and heart failure (Ma et al., 2012; Yu & Bennett, 2014; Goldenthal, 2016). For example, it is thought that mitochondrial dysfunction may lead to a build-up of intermediates of lipid metabolism, which may cause lipotoxicity and contribute towards development of insulin resistance (Bakar et al., 2015). There is relatively little research into the role of mitochondria in programming by antenatal glucocorticoids, but it is known to exist in other models of developmental programming, e.g. in maternal under-nutrition programming of diabetes (Reusens & Remacle, 2006).

As well as discrete changes in gene expression, which may alter organ and tissue growth, glucocorticoids can also act via epigenetic mechanisms resulting in persistent long-term changes in gene expression. Epigenetics refers to trans acting mechanisms that alter gene expression without altering the underlying genetic code. The most common mechanisms of this type of control include alterations in DNA methylation, histone modifications, and changes induced via miRNAs (Moisiadis & Matthews, 2014b; Sales et al., 2017). Cytosine

residues in DNA can be reversibly methylated, leading to recruitment or prevention of binding of other regulatory factors. Methylation of gene promoter or enhancer regions may lead to either up or down-regulation of expression. Histone modifications, including acetylation and methylation, can alter histone-DNA interactions leading to compaction or loosening of chromatin, or can also recruit further effector proteins. miRNAs act via a post-transcriptional mechanism to reduce gene expression – complementary miRNA regions target specific gene transcripts for degradation via the DICER complex. Glucocorticoids may act either directly via the GRE, or secondarily by changing levels of epigenetic regulators or other hormones to alter these systems. Overall, this leads to an incredibly complex network of gene regulatory mechanisms that can be either transient or persist throughout the life course. Persistent modifications in gene expression could lead to fundamental alterations in parameters of homeostatic systems, especially in the fetus where the epigenome is relatively plastic (Jang & Serra, 2014). It has been shown that antenatal dexamethasone treatment leads to changes in DNA methylation in the guinea pig hippocampus, in a gene specific manner (Crudo et al., 2012), and alters mRNA levels of imprinted genes in the rat potentially secondary to methylation changes (Drake et al., 2011). Gay et al. (2015) demonstrated that the reduction in cardiomyocyte proliferation following dexamethasone treatment in rat pups was secondary to changes in DNA methylation and down-regulation of cyclin D272. This may be most pertinent in systems that are heavily controlled by epigenetic mechanisms, for example key HPA axis regulatory genes NR3C1, NR3C2, CRH, POMC, and 11HSD2 (Moisiadis & Matthews, 2014b). In other models of developmental programming there is also increasing evidence for the contribution of epigenetic changes (Pham et al., 2003; Bogdarina et al., 2007; Aagaard-Tillery et al., 2008; Dudley et al., 2011; Lie et al., 2014). A further implication of

epigenetic mechanisms of programming is that changes may persist for multiple generations, if the changes are resistant to embryonic epigenetic reprogramming (Yao et al., 2014).

1.7 A WAY FORWARD? UNKNOWNS AND CONSIDERATIONS

The lifesaving benefits of AGT mean this treatment is unlikely to become out of favour with obstetricians, despite the growing evidence for negative effects on the developing heart, brain and other organs and systems. However, it is clear that more basic research is needed to improve understanding of the effects of AGT, and to tailor current clinical practise to minimise the detrimental effects whilst maintaining the clinical benefits. Current estimates suggest that the number needed to treat with AGT to prevent one neonatal death is 798 at 34 weeks gestational age (Travers et al., 2017), hence there is still much room for improvement.

The current dose regimen used in clinical practise is based largely on the original clinical trials and is not adjusted for any maternal or fetal factors (e.g. maternal weight, age, twinning; Ballard, 1995). Further, if a mother threatened with pre-term labour does not deliver within 7 days of receiving AGT, a repeat course may be given (FIGO committee report, 2019). There is no clear consensus on how many repeat courses should be given to maintain beneficial effects of AGT (Bonanno et al., 2007), and as early as 1996, 58% of responders to a US based survey answered they would give 6 or more courses if pre-term labour had still not occurred (Planer et al., 1996). RDS instance is slightly reduced by multiple courses (Crowther et al., 2015), and a rescue course has been shown to reduce neonatal morbidity (Garite et al., 2009). However, there is growing concern about the more severe negative effects of repeat doses (Romejko-Wolniewicz et al., 2014). So much so, that in 2003, a second NIH Consensus panel advised against the use of repeated courses of AGT (Crane et al., 2003). There is also evidence from animal studies that significantly lower equivalent doses than those used in clinical

practise can achieve similar levels of lung maturation (Jobe et al., 2009). However, some clinical evidence of incomplete course of AGT have shown smaller benefits in preventing RDS when compared to neonates who completed a full course, although the partial course was still effective in reducing IVH and neonatal death (Chien et al., 2002). Further research is therefore clearly needed into the optimal dose, whether it should be adjusted for maternal factors, and how many courses could be given before the potential future detrimental effects outweigh the benefits.

There are also unanswered questions surrounding the correct method of drug administration. Administering glucocorticoids to the mother rather than directly to the fetus has been reported to cause a more significant growth retardation (Jobe et al., 1998), likely as a result of additional detrimental effects of AGT on placental blood flow and function (Jellyman et al., 2004). However, fetal administration with synthetic glucocorticoids produced smaller improvements in postnatal lung function than with maternal administration despite higher fetal plasma glucocorticoid levels (Jobe & Soll, 2004). Intra-amniotic administration has been shown to be successful in maturing fetal lungs in the sheep and the monkey (Gilbert et al., 2001), but has a high associated morbidity making it potentially unsuitable for clinical use (Moss et al., 2003). Any potential route of fetal administration needs to be investigated thoroughly prior to introduction to clinical practise. It may also be unfeasible in low-income populations, or where obstetric care is less available. Another possibility for route of drug administration is to give AGT orally to the mother. The only clinical trial to use oral dexamethasone as an alternative to standard intra-muscular treatment was ceased after IVH

levels were found to be significantly increased in the oral compared to the intra-muscular administration group (Egerman et al., 1998).

Another impending unanswered question as to the optimum clinical treatment is the choice of glucocorticoid for AGT. The synthetic glucocorticoids that are mostly used are Dexamethasone and Betamethasone (Table.1.2; Jobe & Soll, 2004). Both drugs are synthetic analogues of endogenous cortisol, which can readily cross the placenta, with a substantially increased affinity for the GR. They also will not interact with the MR, or with inactivating enzyme 11βHSD2 (Buttgereit et al., 1999). Betamethasone was initially used by Liggins and Howie for the first clinical trials because the acetate formulation of the drug was thought to have a long plasma half-life (Liggins & Howie, 1972). Since then many trials have been conducted using either Dexamethasone or Betamethasone, with few directly comparing the two steroids. A comparison of meta-analyses by Jobe suggests that Betamethasone may be more effective in reducing IVH and death than Dexamethasone (Jobe & Soll, 2004), however this is only based on a small number of studies. Animal studies have also demonstrated that the acetate formulation of Betamethasone may be as effective at stimulating lung maturation as the combined phosphate and acetate formulation used in clinical practise (Jobe et al., 2009; Schmidt et al., 2019). There clearly needs to be more research into the drug of choice, and formulation of that drug. The WHO currently only lists Dexamethasone as an essential medicine, largely due to it being both cheaper and more widely available than Betamethasone (WHO, 2015). However, the WHO highlighted this as an area in need of further research in their 2015 report on preterm birth outcomes, stating:

> *'There is no conclusive evidence on the comparative efficacy of dexamethasone and betamethasone that would support a recommendation of one over the other.'*

Drug	Dexamethasone	Betamethasone
Formulation	Phosphate	50:50 Phosphate/Acetate
Route of Administration	Maternal intra-muscular injection	Maternal intra-muscular injection
Clinical Administration	4x6mg doses, 12hrs apart	2x12mg doses, 24hrs apart
Total Dose	24mg	24mg
MSP		

Table 1.2 Comparison of Dexamethasone and Betamethasone dosing regimens for AGT (WHO, 2012)**.**

The lack of a clear consensus surrounding so many aspects of the clinical use of AGT has been reported by several public health bodies. The World Health Organisation (WHO) in 2015 (WHO, 2015) identified the following questions as priority research directions:

- What are the long-term outcomes of all infants exposed to antenatal corticosteroids (including term infants)?
- What are the effects of antenatal corticosteroid at different gestational ages at birth?
- What is the minimum effective dose of corticosteroids to achieve fetal lung maturation and other improved outcomes?
- What is the minimum dose required for repeat courses of antenatal corticosteroids?
- What are the most effective regimen and dose for antenatal corticosteroids?

1.8 Isolating Effects of Glucocorticoids in the Perinatal Period

Part of the difficulty in improving our understanding of how AGT can affect fetal physiological systems differentially, is the impossibility in isolating effects of AGT on the mother, the placenta and the fetus in human clinical studies or in basic science studies using mammalian animal models. In this regard, studies in avian species circumvent these problems, allowing us to research the direct effects of a clinically relevant treatment of glucocorticoid on the fetus, without additional confounding influences on the mother and the placenta, as the avian embryo develops in isolation within the egg. In addition, in the chicken, the temporal developmental milestones for cardiac maturation are much more similar to the human in terms of the % of development at which specific events occur, in contrast with rats and mice, which are born highly immature and maturation of the cardiovascular system extends well into the postnatal period (Sissman, 1970; Marcela et al., 2012; Itani et al., 2018). Due to these similarities, an insult applied at a similar time of development to the chick embryo can be expected to have similar effects on cardiovascular development as an insult applied at that time to a human fetus (Itani et al., 2018)(Fig.1.7).

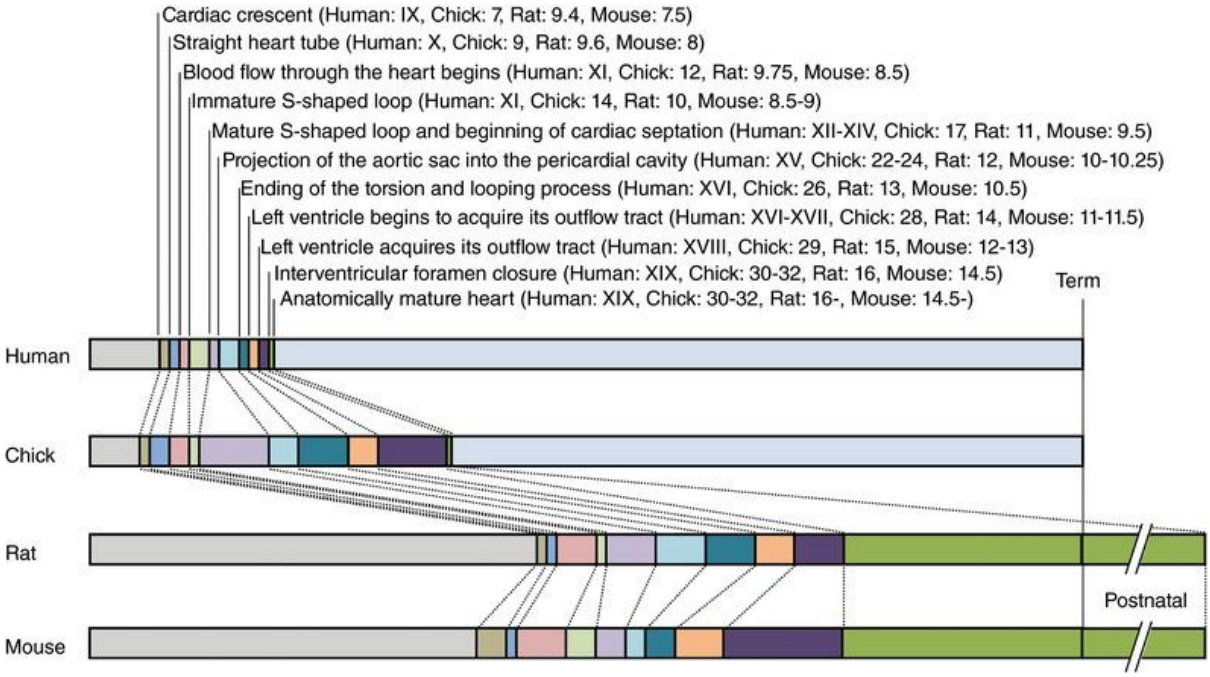


Figure 1.7 Cardiac developmental milestones in various species. (Taken from Itani et al., 2018).

In addition to a similar course of cardiovascular development, it is vital to find a model in which a comparable role and timing of the glucocorticoid surge is seen. In the chicken embryo it has been demonstrated that the HPA axis is active from as early as 5 days of incubation (total incubation time in the chicken is 21 days; Siegel & Gould, 1976). There are two distinct periods in which an increase in plasma glucocorticoid levels are observed; between embryonic days 14-16 there are increases in both circulating corticosterone and cortisol (Kalliecharan & Hall, 1974), whilst immediately prior to hatching there is a drastic rise in circulating corticosterone (Scott et al., 1981; Tanabe et al., 1986; Fig.1.8) – the latter being the primary endogenous glucocorticoid in most birds. It is thought that this pre-hatching rise is secondary to increased sensitivity of adrenal cells to ACTH, as well as increased CRH and ACTH production in the hypothalamus and pituitary (Carsia et al., 1987). This secondary increase in corticosterone is qualitatively similar to the pre-partum cortisol rise in the human

fetus (Jenkins & Porter, 2004). Other favoured animal models do not necessarily have an analogous glucocorticoid profile. Rodents are well known to have a delayed glucocorticoid surge that happens in the post-natal period, with peak corticosterone levels in the rat occurring approximately at post-natal day 20 (Pignatelli et al., 2006).

More importantly, this glucocorticoid rise in birds has been demonstrated to have a similar maturational role as human cortisol. In the chicken embryo lung administration of glucocorticoids results in increased surfactant production (Sullivan & Orgeig, 2001). Glucocorticoids have also been demonstrated to alter neuronal division and maturation in a manner similar to that seen in the mammalian brain (Austdal et al., 2016; Aden et al., 2011).

Studies in our laboratory have previously demonstrated that it is possible to dose the chicken embryo directly via application of a solution onto the chorioallantoic membrane, and that drugs applied in this manner are readily taken up into the fetal circulation and tissues (Itani et al., 2015).

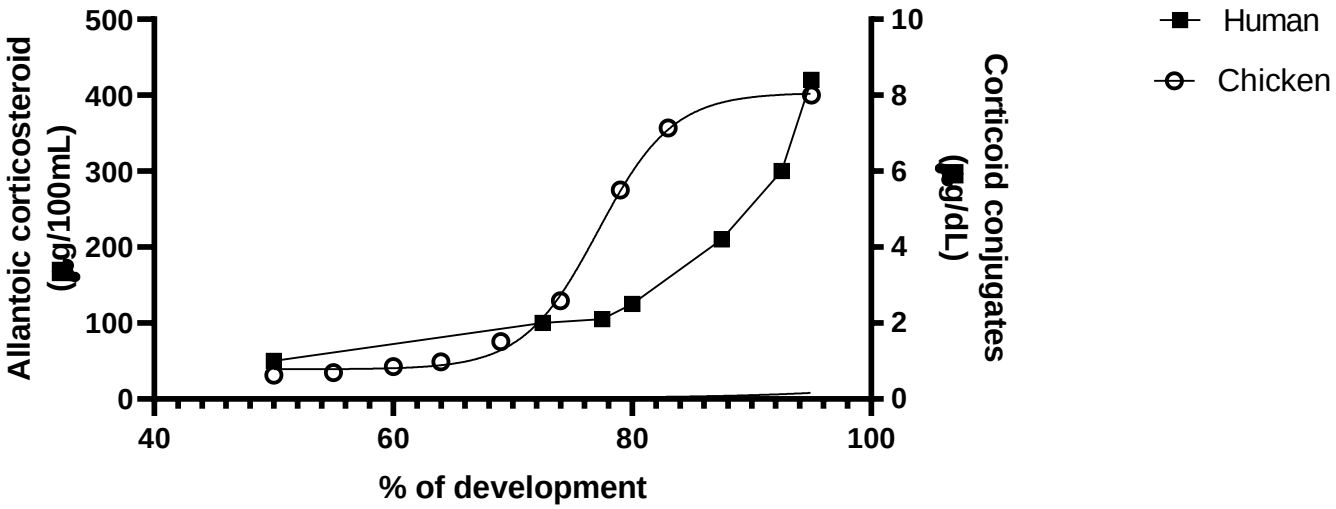


Figure 1.8 Ante-natal glucocorticoid surge, in chicken egg allantoic cavity and human fetal plasma. Data compiled from (Murphy 1978; Tona et al. 2003; Woods et al. 1970).

The chicken embryo has the final advantage of being reasonably large, having an average weight at day 19 is ca.25g (Itani et al., 2015), compared with the term rat fetus, which weighs ca.4g (Camm et al., 2011). Hence, the chicken embryo is amenable to cardiovascular functional tests, including assessment of cardiac function via the Langendorff preparation, and of peripheral vascular function via small vessel wire myography (Itani et al., 2018) in addition to studies at the cellular and molecular levels.

1.9 AIMS AND OBJECTIVES OF THIS THESIS

Therefore, using the chicken embryo model system, the main aims of this thesis were:

A. To determine the direct effect on the developing cardiovascular system of a human clinically relevant dose of Dexamethasone;
B. To compare the direct effects on the developing cardiovascular system of human clinically relevant doses of Dexamethasone versus Betamethasone; and
C. To isolate the direct effects on the developing cardiovascular system of human clinically relevant doses of Betamethasone using different drug formulations.

Within each aim, there were 5 specific objectives:

1. To determine direct effects on embryonic survival and growth;
2. To determine direct effects on the cardiac structure and function;
3. To determine direct effects of vascular function;
4. To determine molecular pathways mediating direct effects;
5. To determine direct effects on lung maturation.

CHAPTER 2: GENERAL METHODS

All procedures were performed under the UK Animals (Scientific Procedures) Act 1986 and approved by the University of Cambridge Ethical Review Board.

2.1 EGG INCUBATION AND DRUG ADMINISTRATION

Fertilised Bovans Brown eggs (Medeggs, Henry Stewart & Co., UK) were delivered weekly and further stored at 14°C in a cooler (Bosch KTW18V80GB, Robert Bosch Limited, UK) to arrest development, as standard (Fasenko, 2007). Eggs were also manually rotated daily during storage to prevent fusion of the embryo and the chorioallantoic membranes (Tazawa, 1980). After one to five days of storage, the eggs were weighed and placed in an incubator under normoxic (21 % O_2) optimal conditions (37.9 °C, 45 % humidity, 12:12 hour light: dark cycle, automatic rotation every hour, incubator Mod.75-A and Mod.M-240, Masalles, Spain; Fig. 1.1.). This was termed as day 1 and the start of the incubation period. Eggs were candled daily from day 13 onwards and any displaying no evidence of fetal development, indicated by a clear air cell at one end of the egg, were discarded.

A dose of 0.1mg.kg^{-1} was administered on day 14 of the 21-day incubation period. This dose was selected initially as it is lower than the clinical dose experienced by a human fetus, yet lead to significant growth effects in preliminary studies. Glucocorticoid in the chicken embryo was administered at 66% of the incubation period, as this is equivalent to ca. 26/27 weeks of human gestation, which reflects an early time point at which antenatal glucocorticoid therapy

(AGT) is administered clinically, and at which this treatment has been shown to be effective in improving neonatal outcomes (Roberts et al., 2017). The dosing regimen and experimental timeline is shown in Fig.1.2. Outcome measurements were taken at day 19 of incubation, which is equivalent to term in a human pregnancy. This time point was chosen as throughout days 21-23 of incubation chicken embryos will begin to 'pip' through the chorioallantoic membrane and switch to air breathing. Thus to characterise effects on the embryo prior to any neonatal transition occurring, day 19 was chosen. In preliminary studies we noted a significant impairment in hatching rate of glucocorticoid treated embryos, making it practically unfeasible to measure outcomes in the newly hatched chick which would be more relatable to the human neonate.

To dose the eggs, candling reveals the air space, which can then be accessed via drilling of a small hole through the shell. Drug administration can then be made through this hole topically onto the chorioallantoic membrane with substances being absorbed by the chorioallantoic circulation. Previous studies in this laboratory have established this as an effective and non-invasive method of egg dosing (Itani et al., 2015).

In this thesis 4 cohorts of chicken embryos were utilised to achieve an *n* number of 8-10 for each outcome described below, for control and treatment groups. Cohort 1 was used for biometry and snap freezing of tissues. Cohort 2 was used for Langendorff and small vessel wire myography. Cohort 3 was used for perfusion fixation. Cohort 4 was used for molecular digestion of the embryonic heart to isolate individual cardiomyocytes.

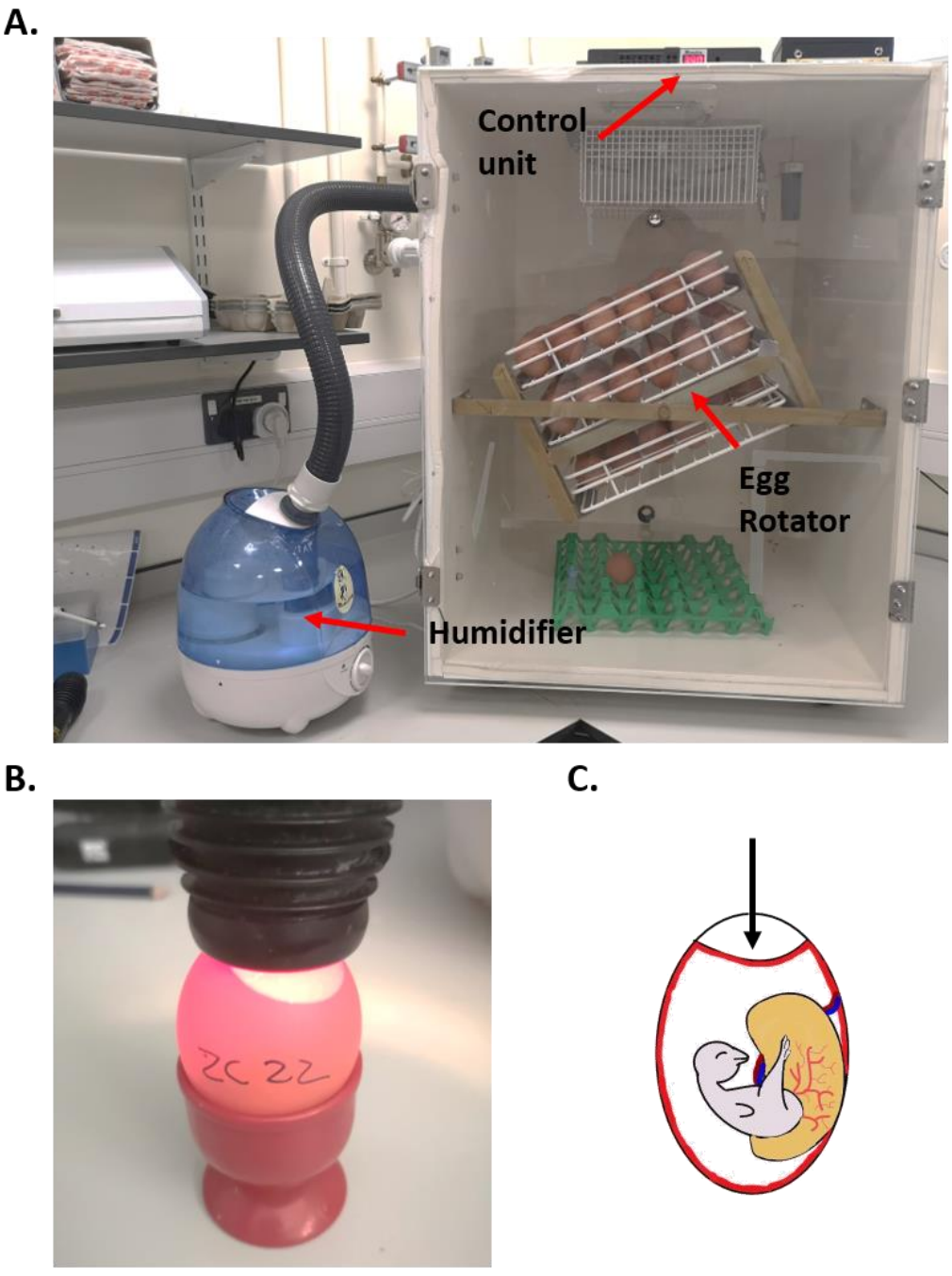


Figure 2.1 Egg incubation and dosing procedures. A. Eggs were incubated in an incubator with automatic rotation, controlled humidity of 45% and temperature of 37.9°C, B. Eggs were candled to locate air cell, as this both provides evidence for embryonic growth and demonstrates location of the chorioallantoic membrane for

drug administration, C. Drugs were administered via a small hole drilled through the shell, directly onto the chorioallantoic membrane (shown in red), where they are readily taken up by the chorioallantoic vasculature.

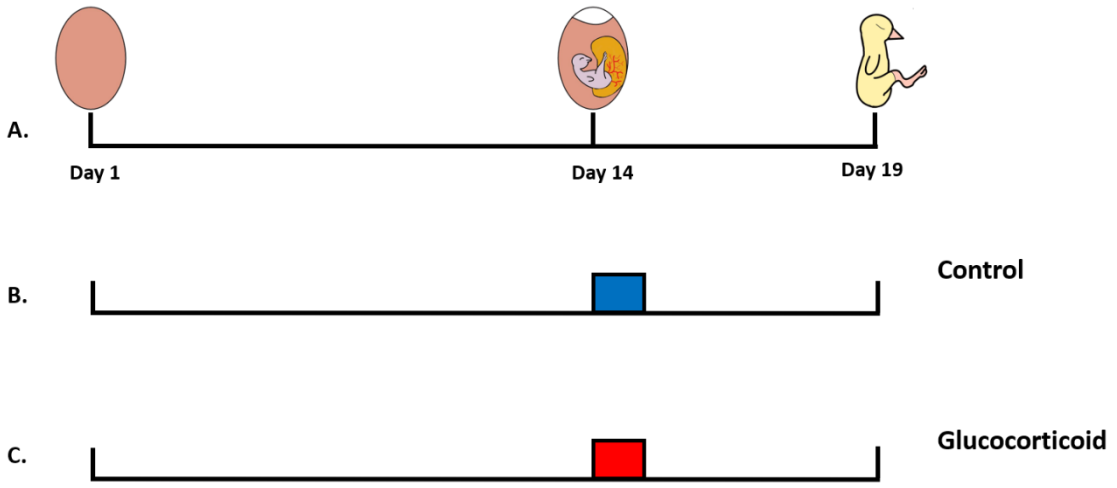
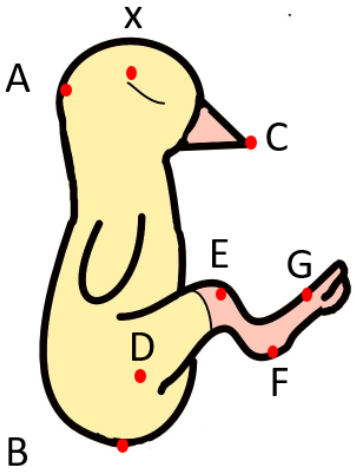


Figure 2.2 Dosing scheme for both control and glucocorticoid treatments. Eggs are dosed with 100µL of either sterile water or aqueous glucocorticoid (to give a total dose of 0.1mgkg^{-1}) on day 14 of incubation.

2.2 Assessment of Embryonic Growth

On Day 19 of incubation, embryos were removed from their eggshells and euthanized by spinal cord transection. Embryo mass (g) and mass of the eggshell, yolk, non-embryonic tissues, and any remaining fluid was measured and recorded. Measurements of fetal size were made using an electronic calliper (Mitutoyo 500-181- 20, Japan). Crown Rump Length (CRL) was taken as length between the crown of the skull, and base of the back. Head diameter (HD) was measured just behind the eyes (Fig. 1.3.). Animals were further dissected such that heart and brain mass could also be recorded.



- CRL = A to B
- Head length = A to C
- Femur length = D to E
- Tibia length = E to F
- Metatarsal length = F to G
- Head diameter measured transverse across skull at point x

Figure 2.3. Biometry on the day 19 chicken embryo.

2.3 Assessment of Ex Vivo Cardiac Function

On day 19, embryos were removed from their eggshells and euthanized by spinal cord transection. The hearts were then rapidly excised and placed into ice-cold Krebs Buffer solution (NaCl 120mM, KCl 4.7mM, $MgSO_2.7H_2O$ 1.2mM, KH_2PO_4 1.2mM, $NaHCO_3$ 25mM, glucose 10mM, $CaCl_2.2H_2O$ 1.3mM, all reagents from Sigma, UK). The glucose concentration utilised was based on evidence of embryonic chicken plasma glucose levels (Latour et al., 1996; Sunny & Bequette, 2014; Shi et al., 2014). Excess tissue was removed, and upon identification, the aorta was cannulated onto a 19-gauge blunt needle thereby mounting the heart onto the Langendorff apparatus (Fig.1.4). In this set up, the heart is perfused via the coronary arteries by oxygenated Krebs Buffer (95% O_2, 5% CO_2) at 40°C, at a pressure of

40cmH_2O (systolic pressure at day 19 of incubation; Tazawa, 2004). The buffer was continuously re-circulated around the apparatus and cleaned with a nitro-cellulose filter (8.0µm SCWP, Merck Millipore, USA). The heart was then allowed to stabilise for several minutes, before an incision was made into the top of the left atrium. A small non-flexible balloon, made of cling film and attached to a cannula and a pressure transducer (Argon Medical Devices, UK), was then inserted to the base of the left ventricle. The balloon was filled using a Hamilton syringe by incrementally introducing 5µL of water into the cannula, until a final volume of ca. 30µL and a stable optimal pressure reading of between 5-10mHg LVEDP were reached (Giussani et al., 2012). The final filling volume of the syringe was established during preliminary experiments and use of the same volume throughout ensured any changes seen were due to changes in ventricular pressure. The heart was further allowed to stabilise for a minimum of 10 minutes. Data were acquired from the pressure transducer via the M-PAQ data acquisition system and IDEEQ software (Maastricht Programable AcQuisition System, Maastricht, NL).

Analysis of the Langendorff experiments was conducted on the LabChart software (Version 7, ADInstruments), following export of the IDEEQ files to a text document. Baseline readings of several variables were taken (left ventricle end diastolic pressure LVEDP; left ventricle developed pressure, LVDP; maximal rate of contraction, dP/dt max; Cycle Duration; Diastolic and Systolic Cycle Duration; Tau, τ; Contractility Index; Fig.1.5). In addition, coronary Flow Rate (CFR) was measured gravimetrically by timing collection of 2mL of perfusate. In vitro responses to the sympathetic β_1-adrenergic receptor agonist Isoprenaline (Sigma, UK, in the range 10^{-9} – 10^{-7}M) and the parasympathetic cholinergic agonist Carbachol (Sigma, UK, in the range 10^{-8} – 10^{-6}M) were then assessed by diluting these drugs into Krebs Buffer, warming

them, and administering them in increasing doses to the heart via the perfusing cannula, with care taken to match perfusion rate to coronary flow rate. To assess drug responses a representative baseline and response section of the trace were highlighted and used to derive measurements of heart rate (HR) and LVDP, which were then calculated as percentage changes from baseline. The heart was given time to return to baseline measurements between each incremental dose.

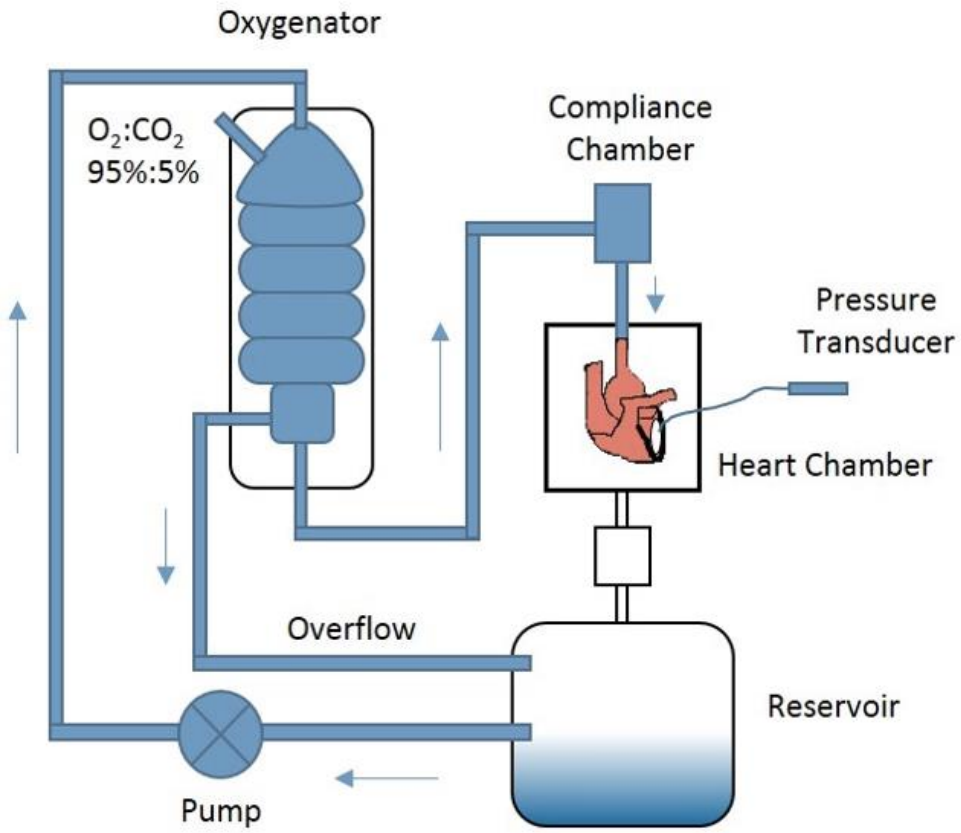


Figure 2.4 Langendorff apparatus. Blue arrows indicate direction of flow. Krebs solution is pumped from the reservoir through an oxygenator and then to the heart via the compliance chamber. Perfusion of the heart occurs via the coronary arteries, and drainage via the pulmonary artery. A non-distensible balloon is inserted into the left ventricle and connected to a pressure transducer. All glassware is heated to maintain the heart at ca. 38°C (chicken body temperature).

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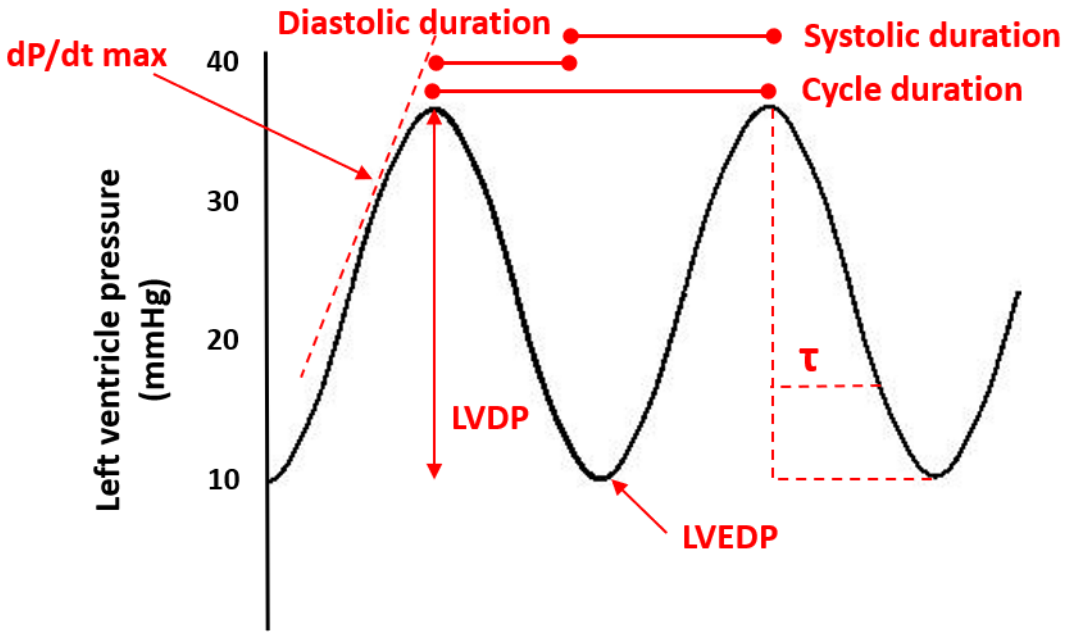


Figure 2.5. Calculation of cardiac parameters. LVDP is calculated as amplitude of contraction, LVEDP is the lowest pressure reached during diastole, cycle duration is calculated as time taken between the peak of each contraction, systolic duration is the time from the lowest pressure to the peak of the contraction curve, diastolic duration is time taken from the peak to reach the lowest pressure, τ is the time taken for the ventricle to relax by (1-1/e)%, contractility index is calculated as dP/dT max divided by maximum pressure generated.

2.4 Assessment of Ex Vivo Peripheral Vascular Function

At day 19 of incubation, embryos were removed from their eggshells and euthanized by spinal cord transection. A 2nd order branch of the femoral artery (diameter ca. 300µm) was dissected into cold Krebs buffer (118.5mM NaCl, 25mM $NaHCO_3$, 4.7mM KCl, 1.2mM $MgSO_4.7H_2O$, 1.2mM KH_2PO_4, 2.5mM $CaCl_2$, 2.8mM D-glucose; all reagents Sigma, UK). Two platinum wires of 40µm diameter were fed through a vessel section of ca. 2mm in length and used for mounting onto the jaws of each channel of a small vessel wire myograph (Multi-wire Myograph System 610M and 620M, DMT, Fig.1.6). Vessels were warmed to 37°C and bubbled

with 95%O_2/5%CO_2. After warming, vessels were normalized to the internal circumference needed to generate a pressure of k x 2.66kPa (mean blood pressure of the chicken embryo at day 19; Altimiras & Crossley, 2000), where k=0.9. Preliminary results from our laboratory suggest that the k=0.9 normalisation factor used gives the maximum difference between passive and active tension in these vessels, and is a widely used normalisation value (Ping et al., 2014). Vessels then underwent a 'wake-up' procedure in which they were exposed to 120mM KCl for 3 minutes, then responses to Norepinephrine (NE) 10^{-4} and Acetylcholine (ACh) 10^{-4} were assessed to check for endothelial and vascular smooth muscle integrity. To assess vasoconstrictor responsiveness, vessels were exposed to increasing doses of NE, Serotonin (5-HT), Phenylephrine (PE), and Endothelin-1 (ET-1; all from Sigma, UK) at 2min intervals (Fig.1.5). To assess vasodilator function vessels were pre-constricted with 64mM KCl to induce a sub-maximal constriction, then exposed to increasing doses of ACh to determine endothelium-dependent relaxation, or Sodium-nitroprusside (SNP) to determine vascular smooth muscle-dependent relaxation (Fig 1.6). To further assess the nitric oxide (NO) dependent component of the Ach response, vessels were treated with 5µm of the NOS inhibitor N(G)-Nitro-L-arginine methyl ester (L-NAME; Sigma, UK). Vessels were allowed to re-establish baseline during 20 minutes between each dosing curve and washed repeatedly with Krebs solution after each dose-response curve to remove any residual drug from the bathing solution.

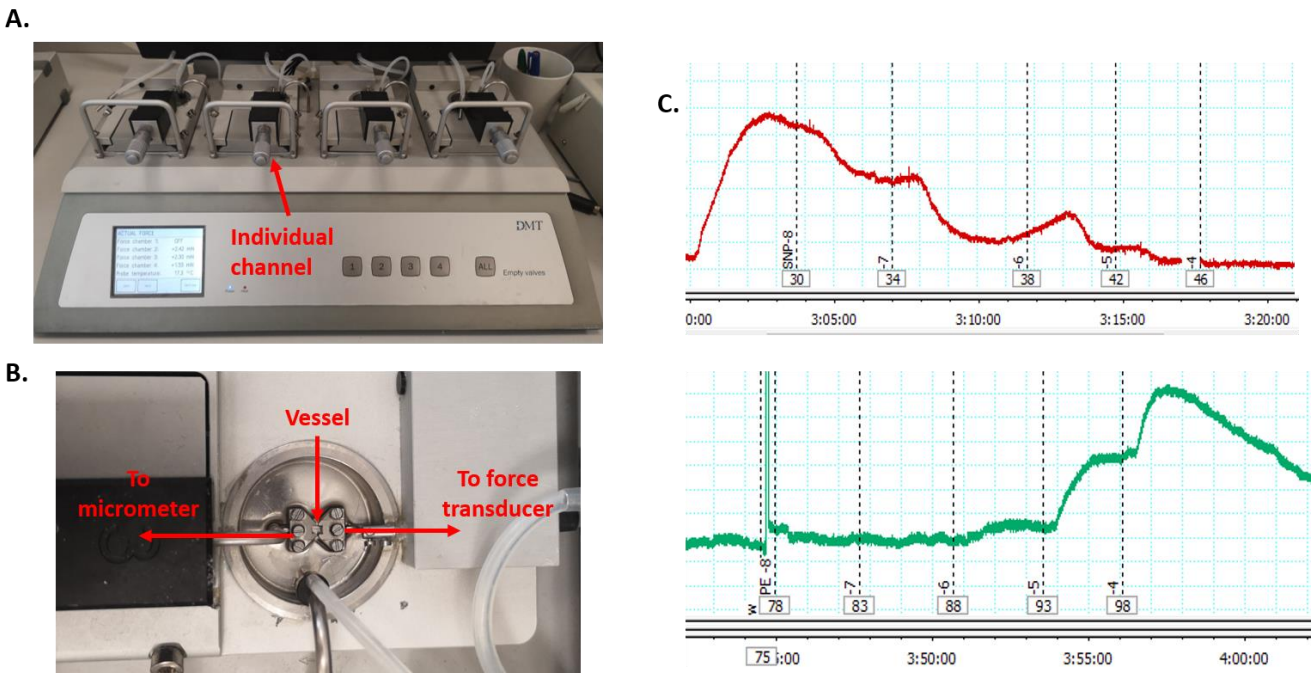


Figure 2.6 Small vessel wire myography apparatus. A. DMT 620M wire mygoraph with four separate channels for vessels, B. Individual channel; 2mm segment of vessel is attached via two 40µm platinum wires to the jaws, one side leads to a micrometer so diameter between the jaws can be adjusted to stretch/relax the vessel, the other side leads to a force transducer which records tension generated by the vessel in mN, C. Example of dose response curves. Vessels are preconstricted with 64mM KCl then exposed to increasing doses of SNP and the %relaxation is calculated, similarly, vessels are exposed to increasing doses of PE and the total constriction at each dose is measured.

To analyse vascular responses, a baseline was set at the start of each dosing curve, and then the ΔmN between baseline and the maximal response at each dose was measured. Dose-response curves were plotted on GraphPad Prism 7. A best-fit curve was then fitted to the data, and the GraphPad program was used to generate a maximal response value, and area under or above the curve. The area above the curve for the Ach response curves for each vessel both before and after L-NAME treatment were calculated. The area above the post L-NAME curve was taken as the NO-independent component of the Ach response, whilst the

area above the pre-L-NAME curve minus the area above the post L-NAME curve was taken as the NO-dependant component.

2.5 Assessment of Embryonic Cardiac Stereology

Embryos were removed from their eggshells and anaesthetised with sodium pentobarbital (intraperitoneal 0.3mL of Pentoject, Animalcare Ltd., UK). The heart was exposed via the chest cavity and the right atrium cut. A 27 gauge needle was inserted through the left ventricle and heparinized saline (Sigma, UK) followed by 4% paraformaldehyde (PFA; Sigma, UK) were flushed through this system via spontaneous contraction of the heart for approximately 15 minutes at 40cmH_2O (Tazawa, 2004). The hearts were dissected into PFA, and after 24 hours placed in Phosphate Buffered Saline (PBS, Sigma, UK), in which they were stored in at 4°C until assessment.

Fixed hearts were sectioned into 1mm slices (Acrylic mouse heart slicer matrix with coronal section intervals; Zivic Instruments, UK). The slices were arranged in ascending order (apex side down) and photographed (Nikon camera). Images were analysed using the ImageJ software (version 1.46, National Institute of Health, USA). Gridlines of 0.5mm intervals were imposed on the images, and a mid-cardiac section was then chosen to analyse. Width measurements were made at points where the outer edges crossed the gridlines. To measure areas, a cross grid of 1mm^2 was superimposed onto the images and crosses within each compartment (left ventricle, LV; left lumen, LL; right ventricle, RV; right lumen RL) were scored if the centre of the cross was on the compartment (Fig.1.7). Compartment areas were

assessed in all heart sections. Areas were then calculated according to the Cavalieri principle (Gundersen et al., 1988), whereby:

$$\text{Area } (\text{mm}^2) = \text{A}(\rho) * \sum \text{P}$$

$$\text{Volume } (\text{mm}^3) = \text{ area} * \text{section thickness} * \text{no. of sections}$$

Where A(ρ) is the area associated with each point, and ΣP is the number of points in each compartment.

The initial 5mm of the descending aorta was also dissected from the top of each fixed heart and processed in paraffin using a tissue processing machine (TP1020, Leica Biosystems, UK). The section of the aorta was then embedded into a paraffin block and sectioned at 5µm (RM2235 Vibratome, Leica Microsystems, UK). Consecutive sections (10) for each aorta segment were arranged onto a microscope slide, deparaffinised with xylene (Sigma, UK), and then stained with Gill's Haemotoxylin (Sigma, UK). Slides were subsequently dehydrated by processing through 70%, 80%, and 100% ethanol (Sigma, UK). Aortic slices were quantified using NewCast software 2.0 (Computer Assisted Stereological Toolbox, Integrator software version 4.6.3.857, Visiopharm, UK) attached to a microscope (Leica DMI 6000B, UK). Gridlines of 100µm were imposed on the images and measurements were made at gridline intersections with the outside of the blood vessels. To quantify area, a grid of points was placed over the image and values calculated using the Cavalieri principle, as described for the heart chambers above.

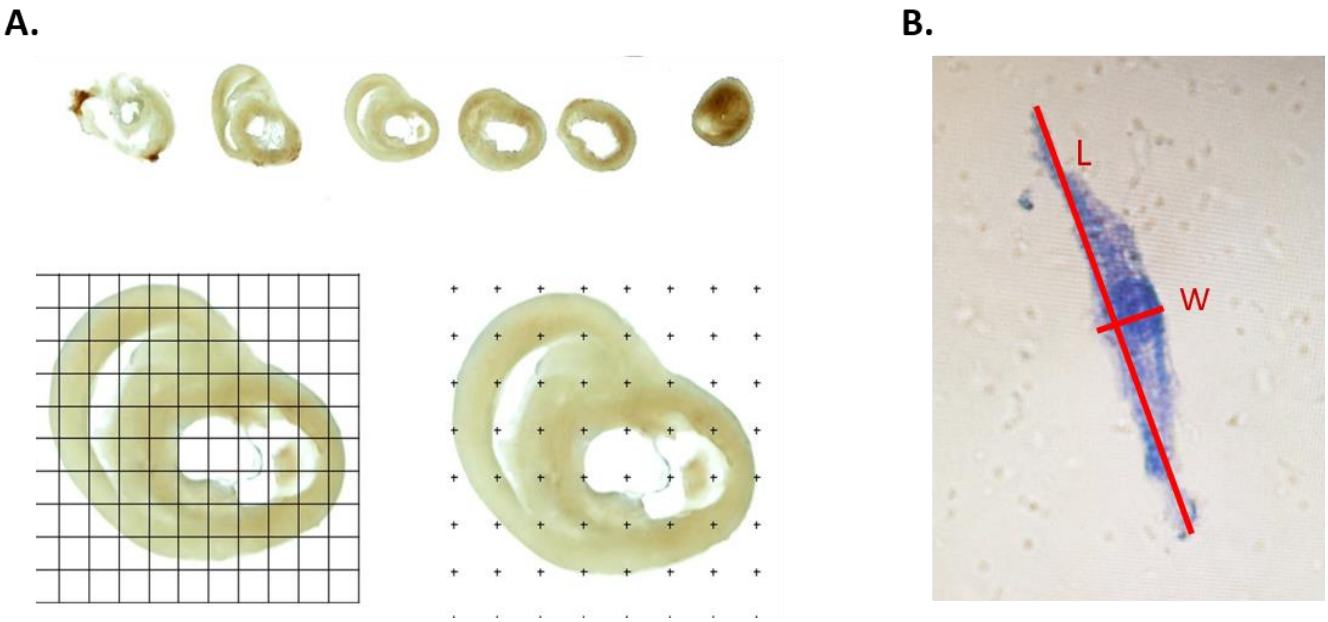


Figure 2.7 Cardiac Stereology. A. Perfusion fixed hearts were sliced into 1mm sections, and imaged. Width measurements were made where a superimposed grid line met the outer edge of either ventricle; points falling on tissue were assigned a count of $1mm^2$, B. Isolated cardiomyocyte stained with Methyl Blue, width is measure across the nucleus, length is from furthest apart edges.

To quantify cardiomyocyte nuclear density, two mid cardiac sections were selected from each fixed heart and dissected into ca. $1mm^3$ pieces. These tissues were transferred to histological cassettes and soaked in 100% ethanol overnight. The following day the tissues were exposed to 2 x 1-hour incubations in 100% ethanol, then soaked overnight in 100% butanol. On the third day the tissues were soaked in Technovit 7100 resin (Hereus Kulzer, DL), for 4 hours and then overnight. On the fourth day, the tissues were transferred to moulds and embedded in Technovit 7100 resin plus hardener and left at 40°C to set for several days. Tissues were then sectioned using a glass blade microtome (2050 Microtome, Reichart-Jung, DL) at a thickness of 30µm, then stained with Haematoxylin (Sigma, UK) for 15 minutes, agitated with water for 30 minutes, stained with 0.15% basic fuchsine (Sigma, UK) for one minute, and dipped three times in tap water.

NewCast software 2.0 (Computer Assisted Stereological Toolbox, Visiopharm Integrator, software version 4.6.3.857) attached to a Leica DMI 6000B microscope, was again used to assess nuclear density. On 10x magnification, each tissue piece was traced to define a region of interest. The software was then used to set up a meander sampling of approximately 40 counting frames of 400µm^2. For this sampling, the central 10µm of each 30µm thick section was assessed. Using the counting frame, nuclei within the frame or touching the top or right lines were counted, whilst those outside the frame or touching the bottom or left lines were excluded. The top right and bottom left corners were used to count tissue area. If the corner fell on cardiac tissue, then it was assigned a count of 200 µm^2 (half of the counting frame). If it did not fall on tissue, then no count was assigned. This process was repeated until at least 100 nuclei were counted per heart. These results could then be used to determine numerical density of cardiomyocyte nuclei for each heart using the following equation:

$$\text{nuclear density } (\mu\text{m}^{-3}) = \frac{\sum \text{nuclei}}{10 * 200 * \sum \text{P}}$$

Where nuclei is the nuclei counted in that heart, and P is the ventricular tissue points assigned in the heart (10 µm is the height of the optical dissector, whilst 200 µm^2 is the area associated with each point of ventricular tissue). These results can then further be used to estimate total number of nuclei in the left ventricle by multiplying nuclear density (µm^{-3}) by total left ventricular volume (mm^3).

2.6 ASSESSMENT OF ISOLATED CARDIOMYOCYTES

On day 19 of incubation embryos were euthanised by spinal cord transection, and the heart was subsequently exposed in the thoracic cavity. Heparinised saline (0.4mL; Sigma, UK), followed by saturated KCl solution (0.2mL; Sigma, UK) were injected into the heart via a 24-gauge needle into the aorta to flush any remaining blood clots and ensure the cardiomyocytes are all in diastole. Hearts were then excised and mounted onto a modified Langendorff preparation. In this set up the heart was also cannulated via the aorta, and initially perfused with Tyrode's buffer (140mM NaCl, 5nM KCl, 1mM MgCl2, 10mM Glucose, 10mM HEPES, pH adjusted to 7.35, all reagents Sigma, UK) until bleaching of the ventricles occurred, demonstrating that the majority of blood had been flushed from the heart. After 10 minutes of perfusing the heart with Tyrode's, the solution was switched to Tyrode's containing 160Uml^{-1} of type-II collagenase (Gibco, UK) and 0.78Uml^{-1} of type-XIV protease (Sigma, UK). This enzymatic solution was allowed to perfuse the heart for 12-15 minutes, until digestion of the heart was apparent, but it still had enough integrity to remain attached to the perfusing cannula. The heart was then perfused further with Krebs buffer (as per Section 1.3) for 10 minutes to wash out any remaining active enzymes. Once this had been completed, the heart was removed from the cannula and placed into a Falcon tube containing 5mL of Krebs buffer and gently inverted several times to dislodge the dissociated cardiomyocytes. PFA was then added to make the solution up to a final concentration of 4% PFA, then stored at room temperature.

To assess both nuclearity and cardiomyocyte length or width of, solutions were stained with 50% Methyl Blue stain (Sigma, UK) and visualised under a light microscope (Leica DMI

6000B, UK). The NewCast software (Computer Assisted Stereological Toolbox, Visiopharm Integrator system version 4.6.3.857) was used to randomly search each 15µL solution sample to measure 50 randomly chosen cardiomyocytes per heart. Cardiomyocytes were assessed for nuclearity and both thickness (as defined as the central point through the mononucleated nucleus, or directly between the binucleated nuclei), and length (Fig.1.7.B). Estimated cardiomyocyte volumes were then calculated as per Österman et al.(2015) using the mathematical approximation of two cones with cone base width equal to cardiomyocyte width, and cone length equal to half of the cardiomyocyte length:

$$\text{volume} = \frac{2}{3}\pi\frac{\text{lw}^2}{4}$$

2.7 Assessment of Cardio-Pulmonary Molecular Pathways

2.7.1 Protein Extraction

To freeze tissues for molecular analysis, organs were dissected immediately following euthanasia of the animal via spinal cord transection, then weighed and snap frozen in liquid nitrogen. The samples were then stored at -80°C. For samples of frozen cardiac tissue, only the ventricles were frozen. For lung, liver, and brain samples the entire organ was frozen.

Prior to molecular extraction, frozen tissue was sectioned on dry ice using a razor blade. The tissue was then aliquoted into roughly 50mg portions in Eppendorf tubes and again stored at -80°C. Extraction of protein was conducted using cell lysis buffer (10x solution, Cell Signalling, UK) with addition of a protease inhibitor cocktail (cOmplete Mini, EDTA-free, Roche Diagnostics, Dl). One aliquot from each animal was transferred to a lysing matrix tube (Lysing

Matrix A, MP Biomedicals, UK) and 500µL of lysis buffer was added. These samples were then homogenised using a tissue homogeniser (MagNA Lyser, Roche Diagnostics, Dl) for 20 seconds, they were then placed in ice for 2 minutes before being homogenised for a further 20 seconds. Samples were then centrifuged at 4°C, 13000rpm, for 5 minutes (Thermo Scientific, Dual rotor, UK). The supernatants were collected into new Eppendorf tubes and assayed for protein content.

A BCA assay was conducted to assess protein levels in extractions. Neat protein samples were diluted to a concentration of 1 in 25, and then 25µL of each sample was loaded in duplicate into a 96 well plate, along with a series of Bovine Serum Albumin (BSA; Sigma, UK) standards. Assay solution (200µL of) was then added to each well. Assay solution was prepared by mixing Bichorionic acid (Sigma, UK) and Copper Sulphate (Sigma, UK) in a 50:1 ratio. The plate was allowed to incubate at room temperature for 10 minutes before being read at 562nm on an automatic plate reader (ELx800, BioTek, UK). The standard curve was then used to extrapolate concentrations in the diluted, and thus in the neat samples.

2.7.2 Western Blots

Western blotting was used to assess levels of proteins of interest in these samples. The reagents used to dilute the protein samples, make the acrylamide gels, running and transfer buffers are all listed in Table 1.1 (all reagents from Sigma, UK). Gels were set between two glass plates, with a 21-space plastic comb being inserted into the stacking gel prior to setting. Once set, the gels were placed into an electrophoresis chamber (ATTO AE6500, JP), which was

subsequently filled with running buffer. Protein samples in SDS buffer (Table 1.1) were boiled at 90°C for 10 minutes then allowed to cool. Samples were then loaded into the gel wells at a total protein amount of 15µg per well, and a molecular marker ladder (Page-Ruler Plus, 10-250KDa, Thermo-Scientific, UK) was added to the first and final wells. Gels were run at 110V, 12mA for 4 hours.

Samples were then transferred from the agarose gel to a membrane (Polyvinyl difluoride Immobilin-P membrane, Millipore, USA), using a semi-dry transfer apparatus (Bio-Rad semi-dry transfer pack, Bio-Rad Laboratories, UK). Three pieces of filter paper soaked in transfer buffer (Table 1.1) were placed onto the transfer apparatus. The membrane was activated in methanol for 1 minute, then placed on top of the filter paper stack. The gel was removed from the glass plates and placed on top of the transfer membrane, followed by three further transfer buffer-soaked pieces of filter paper. A roller was used to ensure no air bubbles were left between the membrane and the gel. The transfer apparatus was run for 90 minutes at a current of 200mA. Once this was completed the membrane was removed, stained with Ponceau S solution (Sigma, UK) for 5 minutes, and an image was taken. Subsequently, the membrane was washed in TBST (Table 1.1), then soaked for 1 hour in blocking buffer (Table 1.1) at room temperature prior to antibody staining. Primary antibodies were diluted in either 5% BSA, or 5% milk powder (Marvel Original, Premier Foods, UK) and the membrane was incubated overnight at 4°C. Secondary antibody incubation was performed at room temperature for 1 hour. TBST was used to wash the membranes between blocking, primary and the secondary incubation steps. Antibodies were detected by using West Pico chemiluminescent substrate (Thermo Scientific, UK), then exposing the membranes to a

photo-sensitive film (GE Healthcare Amersham Hyperfilm ECL, Fisher Scientific, UK). The intensity of the bands was assessed using ImageJ software (version 1.46, National Institute of Health, USA), and normalised to the total protein expression determined by the Ponceau stain. Antibodies used are listed in Table 1.2.

Buffer/Gel	Components
SDS Gel Loading Buffer	200mM Tris-Base (pH adjusted with HCl to pH 6.8) 400mM Dithiothreitol 8% Sodium dodecyl sulphate (SDS) 40% Glycerol a few crystals of Bromophenol blue.
Stacking Gel	1.5M Tris-Base (pH 6.8) 12.5mM 30% Acrylamide/Bis (19:1 ratio) 5% 10% SDS 1% 10% Tetramethylethylemediamine (TEMED) 0.005% 10% Ammonion Persulfate (APS) 0.05%
Running Gel	1.5M Tris-Base (pH 8.8) 37.5mM 30% Acrylamide/Bis (19:1 ratio) 10.05% 10% SDS 1% 10% TEMED 0.005% 10% APS 0.05%
Running Buffer	25mM Tris-Base 250mM Glycine 0.5% SDS
Transfer Buffer	39mM Glycine 48mM Tris-Base 0.037% SDS 20% Methanol
TBS-T Wash Buffer	20mM Tris-Base 0.15M Sodium Chloride pH adjusted with HCl 0.1% Tween-20

Table 2.1 Western blot buffer and gel reagents.

Target	Dilution	Antibody
ERK1/2	1:1000	Cell Signalling Technology 9102
Phosphorylated ERK1/2	1:1000	Cell Signalling Technology 9101S
SAPK/JNK	1:1000	Cell Signalling Technology 2402S
Phosphorylated SAPK/JNK	1:1000	Cell Signalling Technology 99251S
Hsp27	1:1000	Cell Signalling Technology 2402S
Hsp70	1:2000	Cell Signalling Technology 4872
PDI	1:1000	Abcam ab3672
Hsp60	1:2000	Abcam ab46798
Cleaved caspase-3	1:1000	BD Biosciences 610539
Caspase-3	1:1000	Abcam ab90437
DNPH	1:200	Chemicon Oxyblot
GR	1:1000	Santa Cruz Biotechnology, Inc. sc-393232

Table 2.2 Antibodies used for western blot.

2.7.3 RNA Extraction

A second aliquot of frozen cardiac ventricular tissue was used to extract RNA for gene expression analysis. Extraction was completed using Qiagen RNeasy Mini Kit (Qiagen, UK). Tissue was disrupted using an electrical homogeniser (Polytron PT1200, Kinematica, UK) and RLT Buffer from the Qiagen kit (600µL buffer to 30mg tissue). The lysate was centrifuged for 3 minutes at 12,000g and the supernatant was moved to fresh Eppendorf tubes. Ethanol (70%) was added to the supernatant and mixed by pipetting, and then 700µL of the sample

was transferred to a RNeasy Mini spin column in a collection tube. Tubes were centrifuged for 15 seconds at 8,000g and the flow through was discarded. Then, 700µL RW1 was added to the spin column and the centrifuge step was repeated, again with flow-through discarded. This was further repeated with 500µL Buffer RPE, and then a further 500µL Buffer RPE. The spin column was then transferred to a new collection tube, 50µL of RNase-free water was added directly to the spin column membrane, and then the tubes were centrifuged at 1 minute for 8000g to elute the RNA. This final step was repeated due to large amounts of tissue being used. Once extracted, the RNA samples were kept on ice and for RNA concentration, and quality of extracted RNA using a NanoDrop spectrophotometer (Thermo Fisher Scientific, UK).

The synthesis of cDNA for real-time quantitative PCR (RT-QPCR) was achieved using a First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, UK). RNA samples were diluted to give a total amount of 20ng, 1µL random primer, 4µL of 5x Reaction Buffer, 1µL of RiboLock RNase Inhibitor, 2µL 10mM dNTP Mix, and 1µL RevertAid M-MulVRT were added and then total volume was adjusted to give a 20µL reaction volume in a PCR tube. Tubes were then gently mixed. The synthesis reaction was carried out in a thermocycler (S1000, BioRad, UK). Samples were heated to 25°C for 5 minutes then 42°C for 60 minutes, and finally heated to 70°C for 5 minutes to terminate the reaction. cDNA was stored at -20°C.

2.7.4 Real-time Quantitative PCR

Q-PCR primers were designed using Primer3 software (National Institutes of Health, USA), based on the *Gallus gallus* reference genome, then ordered from the Sigma Simple Primer ordering system. Primers were diluted to 100µM combined forwards and reverse pair stock solutions. The RNA primers used are listed in Table 1.3. Prior to running with experimental samples, primers were tested on control cDNA to ensure there was an amplification efficiency of 90-110%, and that a single amplification product was present (Fig. 1.8). SYBR Green PCR master mix (Thermo Fisher Scientific, UK) was added to template cDNA, 30µL RNA primers (forward and reverse pair), and made up to a 6µL total of reaction volume. All samples were run in duplicate, and a minimum of two house-keeper genes were run for each sample. A QuantStudio 7 Flex (Applied Biosystems, UK) Q-PCR machine was used to run the reaction, completing a hold stage of 2 minutes at 50°C then 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C then 1 minute at 60°C. Finally, a melt curve was produced from the reaction products by heating to 95°C for 15 seconds, allowing to cool to 60°C for 1 minute, then a further 15 seconds at 95°C. Threshold levels for Ct value determination were set manually following the Q-PCR reaction, and automatic analysis was carried out by the Q-PCR machine. Samples were discarded from the analysis for a gene if the Ct replicate values had a greater than 0.5 standard deviation difference. Other criteria for exclusion from analysis included no amplification, multiple peaks in melt curve, or other machine-based errors that led to no Ct value being produced. To analyse expression levels the ΔΔCt method was employed (Livak & Schmittgen, 2001). Briefly, the difference between Ct values of the gene of interest in a control vs. experimental condition was calculated, and then compared to the difference between Ct values in a housekeeper gene between the control and experimental

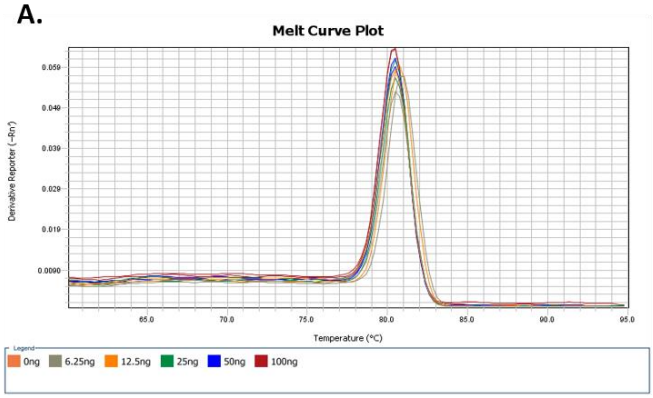
condition to give a ΔΔCt value. A value for expression fold change vs. control can be calculated using the following equation:

$$\text{fold change}(\text{vs. control}) = 2^{(-\Delta\Delta Ct)}$$

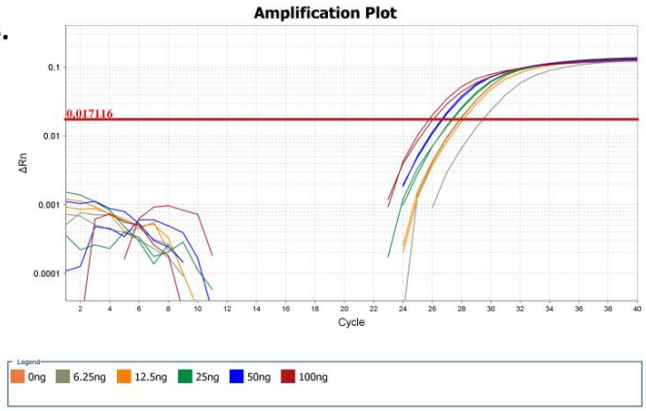
Target Gene	Forward Primer	Reverse Primer
sdha (housekeeper)	TCTGTCCATGGTGCTAATCG	TGGTTTAATGGAGGGGACTG
β-actin (housekeeper)	TGCTGTGTTCCCATCTATCG	TTGGTGACAATACCGTGTTCA
gapDH (housekeeper)	CCTCTCTGGCAAAGTCCAAG	CAACATCAAATGGGCAGATG
catalase	ACTGGTGCTGGCAACCC	ACGTGGCCCAACTGTCAT
GPx	TTGTAAACATCAGGGGCAAA	TGGGCCAAGATCTTTCTGTAA
eNOS	GGGCTCTGCGGTTCTCTG	CACAGAAGGTCTCGCAGGC
P38	CGAAATGACCGGCTACGTGG	CACTTCATCGTAGGTCAGGC
SOD2	CTTCCTGACCTGCCTTAC	CGTCCCTGCTCCTTATT
MKK3	CTACTTGGTGGACTCGGTGG	CGACTTCACGTTGTAGCCCT
P53	GTGGGCTCTGACTGTACCAC	GCCCTCCAGTGTAAGGATG
P16	TCCTCCTCCTACCAGAGATG	TGTACCTGAGGCTCCTTGTC

Table 2.3 RNA Primers for RT-QPCR.

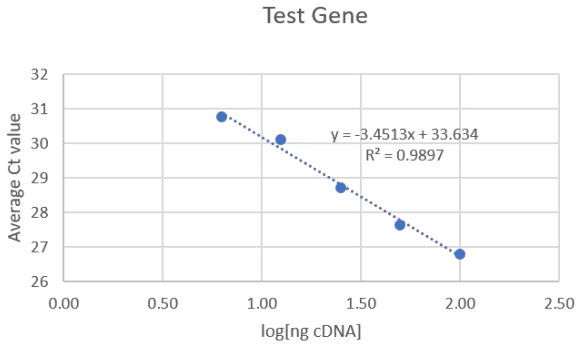
A.



B.



C.



D.

$$Efficiency\ (\%) = \left(10^{\frac{-1}{slope}} - 1\right) * 100$$

Figure 2.8 PCR primer optimisation. A. Primers were tested using control cDNA to ensure that only one product was present in the melt curve. B. Primer efficiency was tested by using various amounts of control cDNA and measuring amplification, a threshold was manually set in the linear region of the amplification curve (in red), C. Average Ct values from the amplification plot in B. are plotted against log[cDNA] to determine the amplification efficiency, D. Primer efficiency can then be calculated from the slope of the Ct vs log[cDNA] graph. An efficiency of 90-110% is required.

2.8 Assessment of Embryonic Lung Maturation

Following perfusion fixation of the embryo (Section 1.5), upper thoracic embryonic lungs were also dissected into 4% PFA, then changed to PBS after 24 hours. Lung tissues were transferred to histological cassettes, then soaked in 100% ethanol overnight. The following day, samples were transferred to fresh 100% ethanol for 1 hour, then further 100% ethanol for another hour, before being soaked in 100% butanol overnight. On the third day, samples were soaked

in molten paraffin for 3 hours using a wax-processing machine (Section 1.6). Samples were then embedded in paraffin with the posterior side of the lungs at the base of the embedding mould. Tissues were then sectioned using an ultramicrotome at a thickness of 8µm, floated on warm water (40°C) and then allowed to dry on glass slides. Sectioning began at the posterior end of the embedded lungs, 10 tissue sections were taken, with the 7th and 8th chosen for analysis.

Once dry, slides were stained and imaged using the same computer software (Section 1.6). Using a protocol adapted from Bjørnstad et al. (2014), for each animal, the outlines of the left and right lung lobes were traced and the software then produced a random sampling within that area. A total of 20 measurements were made for: parabronchial atrial diameter, atrial septum thickness, air capillary diameter, and the distance between the air capillary and the nearest blood vessel – the diffusion distance (Fig.1.9). In addition, 5 measurements were made of thickness of the gas exchange zone (Fig 1.9).

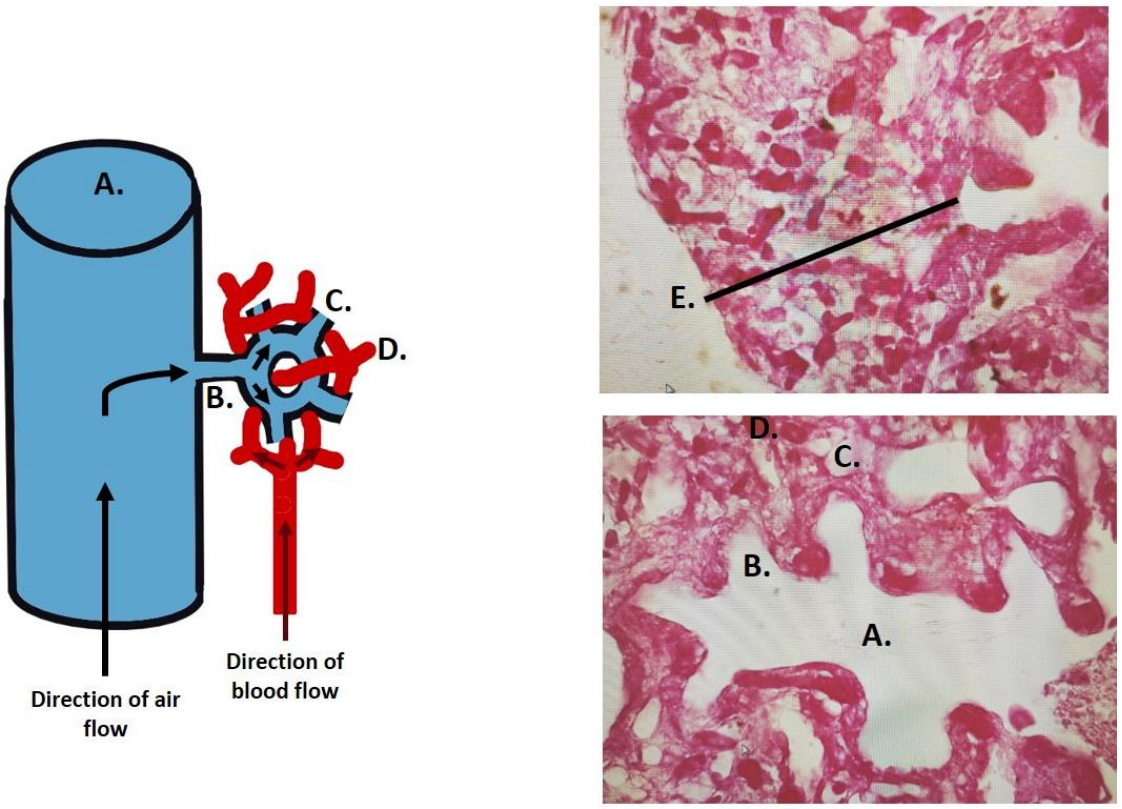


Figure 2.9 Lung Histology Measurements. Diagrammatic representation of gas exchange unit in the avian lung next to histological images of stained lung sections; A – bronchiole, B - atrium into air capillaries, C - branching air capillaries, D – closely associated vasculature, E – thickness of gas exchange area wherein air capillaries and vasculature are in close association.

2.9 Power Calculations & Statistics

Appropriate power calculations have been established to determine the minimum sample size required to achieve statistical significance from previously published data in the chicken embryo using similar outcomes and in a similar number of experimental groups. The variable which has been found to have the smallest mean value difference between the experimental groups is; heart rate for isolated organ work, and cardiac wall:lumen area ratio

for cellular techniques. Power calculations were conducted using G*Power software (Heinrich Heine Universität, Dusseldorf, DL)(Faul et al., 2007). Calculations comparing these variables between 4 animal groups are shown in Table 2.4 below:

	Heart Rate (isolated organ)	**Wall:Lumen ratio (stereology)**
Difference between means	3.3	2.9
Standard Deviation	1.5	1.5
Power (95% confidence when α=0.05)	>0.8	>0.8
Minimum Required Sample Size	8	8

Table 2.4 Power Calculations

Two additional animals were generated where possible per group to compensate for experimental failure, such as increased mortality, cardiac arrhythmia for the isolated organ work, or provision of sufficient material to run experiments in duplicate for the cellular and molecular studies.

The resulting experimental groups and there use in this thesis are show below in Fig.2.10. There were 40 eggs in each treatment group, of which 10 each were used for the molecular, stereology, biometry & isolated organ, and cardiomyocyte isolation studies. New control groups were used on each occasion (Chapters 3, 4 & 5) and new groups for all the other experimental treatments except dexamethasone, where results from the original group of

40 eggs were used in the later studies (see Fig.2.10). For some experimental groups and techniques, an n-number of 8 was not possible due to practical restrictions, for example the number of available wells on western blot gel. All n-numbers are listed in figure legends. For some experimental groups and techniques, an n-number of 8 was not possible due to practical restrictions, for example the number of available wells on western blot gel. All n-numbers are listed in figure legends.

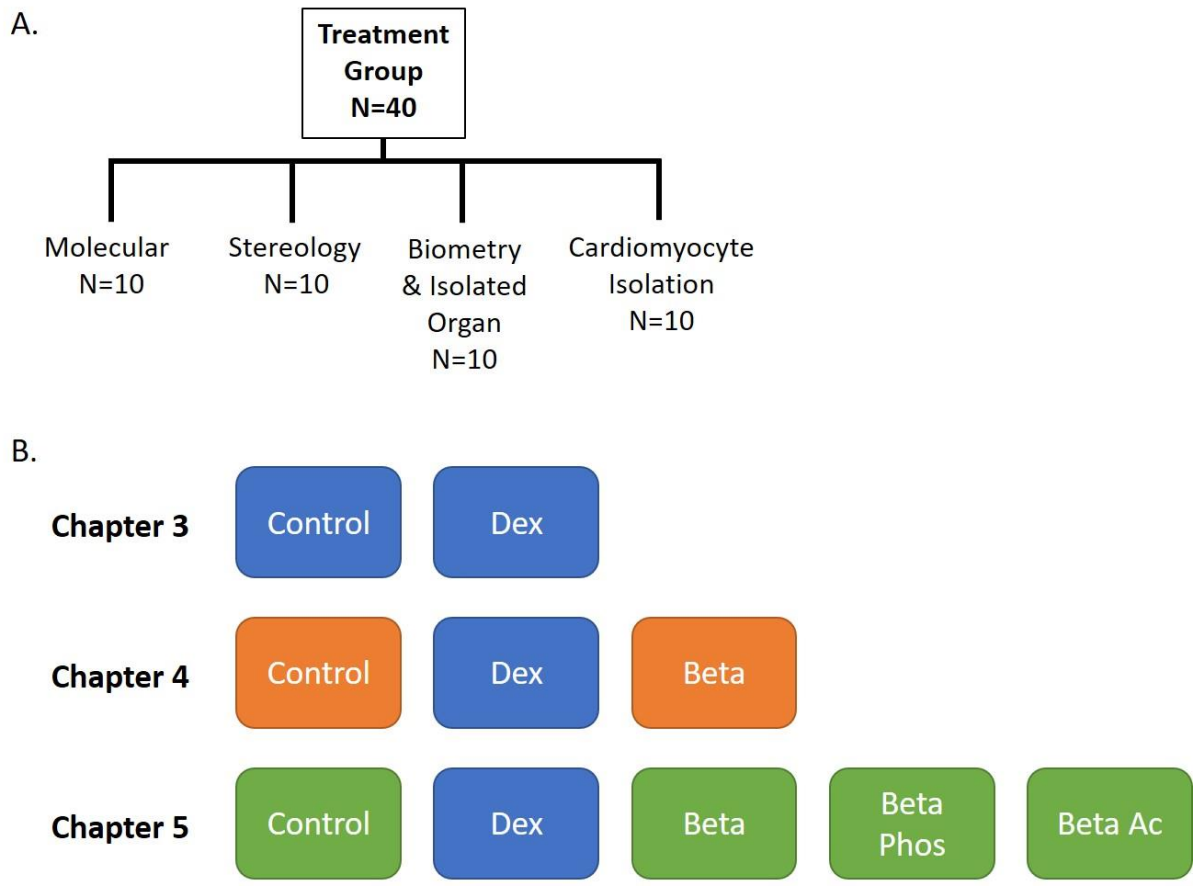


Figure 2.10 Use of animal groups. A. Each experimental group was generated to give 40 embryos to establish all outcomes, B. Use of experimental groups in each chapter, where different colours represent different egg cohorts.

Due to limitations of incubation equipment and cost, it was not possible to repeat the Dex group for chapters 4 & 5. However new control and Beta eggs were generated for chapters 4 & 5, to ensure no seasonal or temporal effects on embryo growth. As eggs were purchased from a research egg supplier, it is not known whether eggs are from the same or different chickens throughout the experiments. Throughout the various chicken embryo projects conducted in our laboratory we have seen no significant changes over time on embryo growth, or isolated organ parameters.

In chapter 3, statistical significance was calculated via use of a parametric t-test, whereby significance was set at P=0.05 and denoted by: * P<0.05, ** P<0.01, *** P<0.001, ****P<0.0001. For isolated organ drug response curves a two-way ANOVA with Tukey post-hoc test was used, again with P=0.05. If a significant interaction was reported (between dose and treatment group) then multiple comparisons were examined and denoted by * P<0.05, ** P<0.01, *** P<0.001, ****P<0.0001, directly above the corresponding dose.

In chapters 4 & 5, statistical significance was calculated via a one-way ANOVA with Tukey post-hoc test with significance set at P=0.05. If a significant interaction was reported (between dose and treatment group) then multiple comparisons were examined and denoted using letters to represent statistically different groups (i.e. if a letter is shared between any 2 experimental groups, then those groups are not statistically different from each other). For isolated organ drug response curves a two-way ANOVA with Tukey post-hoc test was used, again with P=0.05. If a significant interaction was reported (between dose and treatment group) then multiple comparisons were examined and denoted by * P<0.05 if

significantly different to the control value, and † P<0.05 if significantly different to the Dex value.

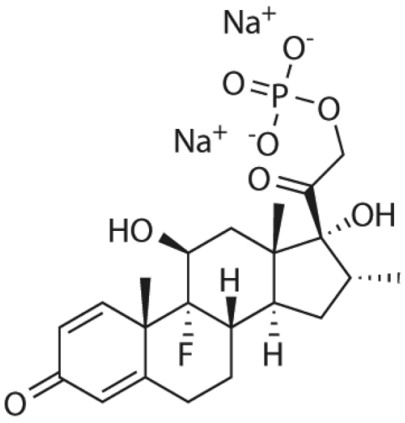
All statistical tests were conducted using GraphPad Prism 7 software (Graphpad Software, CA, USA).

CHAPTER 3: DEXAMETHASONE IN THE CHICKEN EMBRYO

3.1 INTRODUCTION

Since initial clinical trials in 1972, antenatal glucocorticoid therapy (AGT) has become a standard treatment for obstetricians, and has significantly reduced neonatal mortality and morbidity (Liggins, 1969)(Roberts & Dalziel, 2006). Numerous clinical studies have been conducted on AGT, concluding that there is a significant associated decrease in several neonatal morbidities including respiratory distress syndrome (RDS), intraventricular haemorrhage (IVH) and necrotising peritonitis (NEC) (Sotiriadis et al., 2009; Brownfoot et al., 2013; Crowther et al., 2015; Roberts et al., 2017).

Dexamethasone (Dex) is one of the most popular glucocorticoids of choice for AGT in the majority of countries, primarily due to its wide availability for other medical treatments and its low cost per course (Lawn et al., 2012). Dex is a synthetically produced analogue of endogenous glucocorticoid cortisol (Fig.3.1). It has an added Fluoride atom at the 9 C position, which is thought to enhance its biological activity. Insertion of an additional 1,2 carbon-carbon double bond selectively augments glucocorticoid over mineralocorticoid activity, decreasing the rate of metabolic clearance and increasing the biological half-life of in plasma (Buttgereit et al., 1999). Mineralocorticoid activity is further elisminated by methylation at the 16 C position (He et al., 2014). Thus, Dex has a negligible mineralocorticoid activity but a glucocorticoid potency approximately 25-fold that of cortisol (Parker & Schimmer, 1996) and the half-life for clearance from the plasma of fetal sheep is extended by approximately 6-8 h compared to cortisol (Jellyman et al., 2009a).



Dexamethasone Phosphate	
Dose	6mg intramuscularly
No. of doses (per AGT course)	4, 12hrs apart
Total dose (per AGT course)	24mg in 48hrs
Adjustments	Not adjusted for any maternal factors
Rescue courses	If delivery has not occurred within 48hrs rescue course may be given, up to 6 courses reported.

Figure 3.1 Structure and dosing regimen of Dexamethasone phosphate for use in AGT.

In addition to AGT, synthetic glucocorticoids, particularly Dex, may be administered to the neonate in the immediate post-natal period to treat Broncho-pulmonary dysplasia (BPD; Davidson et al., 2017). There is no consensus of dosing regimen for post-natal Dex administration. Initially very high doses were used, however these were shown to have negative effects and hence were advised against (Mammel et al., 1983; Kobaly et al., 2008; American Academy of Pediatrics, 2010). Since then, the DART study indicated no strong association with long term consequences when low-dose Dexamethasone treatment was trialled delivering 0.89mgkg^{-1} over a 10-day course (Doyle et al., 2007). This lower dose is the most commonly used clinically in the new-born period.

Despite the clear benefits of AGT, there is mounting concern about potential damaging effects on the fetal developing cardiovascular system and cardiovascular function in adulthood. In

the neonate and adult, the most commonly reported long term cardiovascular effect is dysregulation of blood pressure (Lindsay et al., 1996). Sheep exposed *in utero* have been reported to have elevated blood pressure at 6 months of age, and an attenuated baroreflex (Shaltout et al., 2012; Davis et al., 2006). In human clinical follow-up studies, both systolic and diastolic blood pressure are increased in youths at 14 years of age (Cheong et al., 2014). In both animal models and in human clinical studies there are several reported changes directly to the vasculature such as endothelial dysfunction (a well-known harbinger of future cardiovascular disease), changes in aortic arch stiffness, and blood pulsatility (Docherty et al., 2001; Pulgar & Figueroa, 2006; Kelly et al., 2012). Direct cardiac effects have been shown including transient hypertrophy, altered autonomic responses, impaired Starling mechanism, systolic dysfunction, and increased end diastolic volume and pressure (Yunis et al., 1999; Dodic et al., 2001; Niu et al., 2013).

In order to assess the effects of AGT on the fetus, and to isolate mechanistic changes leading to altered cardiovascular function, it was vital to find a suitable model system. The chicken embryo model is unique in allowing us to research the direct effects of a clinically relevant treatment of Dex on the fetus, without the confounding influence of the mother and placenta, as the embryo develops in isolation within the egg. This is important as there is evidence for maternal cardiovascular changes in the acute period following dexamethasone administration (Elwany et al., 2018), and to changes in placental function (Audette et al., 2014). Of added value, cardiac maturation is similar in the chicken embryo to that of the human fetus (Sissman, 1970; Marcela et al., 2012). Studies in our laboratory have previously demonstrated that it is possible to dose the chicken embryo directly via application of

aqueous solution to the chorioallantoic membrane, and that drugs applied in this manner are readily taken up into the fetal circulation and tissues (Itani et al., 2015). The chicken embryo has the final advantage of being reasonably large (average weight is *ca.* 25g (Itani et al., 2015)), and so amenable to various ex vivo cardiovascular tests, including investigation of cardiac function via an isolated Langendorff preparation, and assessment of peripheral vascular function via small vessel wire myography.

Therefore, in this chapter we have addressed the hypothesis that Dex administration at day 14 of incubation would result in cardiac and peripheral vascular dysfunction by the end of incubation (day 19). To address this hypothesis, there were six objectives:

1. To develop a clinically relevant model of antenatal Dex treatment in the chicken embryo;
2. To investigate the effects of Dex treatment on embryonic survival and growth of the chicken embryo;
3. To investigate the effects of d Dex treatment on the cardiac function and structure of the chicken embryo;
4. To investigate the effects of Dex treatment on peripheral vascular function in the chicken embryo;
5. To investigate the molecular pathways induced by Dex treatment in the chicken embryo;
6. To investigate the effects of Dex on lung maturation in the chicken embryo.

3.2 METHODS

3.2.1 Experimental Protocol

Details of the methodology are presented in Section 2. In brief, all procedures were performed under the U.K. Animals (Scientific Procedures) Act 1986 and were approved by the Ethical Review Board of the University of Cambridge. Fertilised Bovans Brown chicken eggs were weighed, irradiated with UV light, and placed into a normoxic incubator at 38°C and 45% humidity for 19 days (term is 21 days; see Section 2.1.). Eggs were dosed with dexamethasone phosphate in aqueous solution, or water on day 14 of development via injection through a small hole in the shell (situated above the air sac) onto the chorioallantoic membrane, as previously described (Itani et al., 2015).

The Dexamethasone dose used in this study was 0.1mgkg^{-1}. AGT was administered at two thirds of gestation, which is equivalent to 24 weeks in human pregnancy; a relevant, early period at which glucocorticoids can realistically be administered in the clinical setting (Ballard & Ballard, 1995; Roberts et al., 2017).

3.2.2 Assessment of Embryonic Growth

On Day 19 of incubation, embryonic growth was assessed using a high precision calliper (Mitutoyo 500-181-20, U.K.) to determine embryonic lengths. Embryo mass and mass of the eggshell, yolk, extra tissues (chorioallantoic membrane and connected blood vessels), and any remaining liquid was measured and recorded. The ratio of partitioning of resources was

calculated as embryonic tissue mass/extra-embryonic tissue mass. Crown-rump length, head diameter were recorded (Section 2.2). Individual organs were also dissected and weighed.

3.2.3 Assessment of Cardiac Structure & Function

At day 19 of the incubation period, 10 embryos per group were anaesthetised with sodium pentobarbitate (0.3mL of Pentoject (Animalcare Ltd., York, UK; intraperitoneal), then perfusion fixed with paraformaldehyde (Section 2.5) at 2.66kPa (embryonic day 19 blood pressure (Altimiras and Crossley, 2000)) inserted into the left ventricle via a cannula. Fixed hearts were sectioned into 1mm slices and photographed. Areas of cardiac compartments in each section were counted, and a mid cardiac section was chosen to measure widths. Volumes were then calculated as per section 2.5. The initial 5mm of the descending aorta was dissected from the top of each fixed heart and processed in paraffin. Consecutive sections (n=10) for each aorta were arranged onto a microscope slide, de-paraffinised, and then stained with Gill's Haemotoxylin. Slides were subsequently dehydrated and imaged (Section 2.5). Thicknesses and areas were then counted.

Cardiomyocytes were also isolated from hearts of a different cohort of day 19 embryos (Section 2.6). The coronary vasculature was perfused with collagenase and protease solution. After 10 minutes, the hearts were perfused with Kreb's buffer to remove enzymes, and then suspended in buffer for 40 minutes. Cells were stored at 4% PFA. Cardiomyocyte suspensions were mixed with 10% methylene blue in a 1/1 ratio and visualised under a light microscope (Section 2.6). Cell nuclearity of 300 random cardiomyocytes per heart was assessed, and

measurements of length and width were taken from 50 random cardiomyocytes. Cell volumes were then calculated.

To assess *ex vivo* cardiac function, hearts from a different cohort were rapidly excised and placed into icy Krebs' Buffer solution. The aorta was cannulated onto a 19-gauge blunt needle and mounted onto the Langendorff apparatus. Pressure recordings were subsequently made (Section 2.3). In brief, following a period of normalisation, a small non-distensible balloon made from saran wrap and connected to a pressure transducer, was introduced through the left atrium into the left ventricle. The balloon was then filled with water in 5µL increments until a smooth baseline recording was established. Baseline recordings were made and Coronary Flow Rate (CFR) was assessed. *In vitro* responses to the sympathetic agonist Isoprenaline (Sigma-Aldritch, in the range 10^{-9} – 10^{-7}) and the parasympathetic agonist Carbachol (Sigma-Aldrich, in the range 10^{-8} – 10^{-6}) were then assessed by administering via the perfusing cannula. Analysis of the Langendorff experiments was conducted on the LabChart software (Version 7, ADInstruments).

3.2.4 Assessment of Peripheral Vascular Function

A 2nd order branch of the femoral artery was dissected into cold Krebs solutions and then 2mm sections were mounted onto a wire myograph (Section 2.4). Vessels were maintained at 38°C in Krebs solution, and were aerated with 95% O_2/5% CO_2 gas. To assess vasoconstrictor responses vessels were exposed to increasing doses of NE, 5-HT, PE, and ET-1 at 2-minute intervals. To assess vasodilator function vessels were pre-constricted with K+,

then exposed to increasing doses of Ach, or SNP. To assess the NO dependent component of the Ach response, vessels were treated with 5µM L-NAME.

3.2.5 Assessment of Molecular Pathways in the Heart

Frozen heart tissue was powdered on dry ice, and separated into 50mg aliquots, then stored at -80°C. Extraction of proteins was conducted in TK lysis buffer (Section 2.7.1). For Western blot analysis, protein extracts were separated on a 10% agarose gel, then stained with primary antibody and secondary antibodies (Table 2.2). Gel bands were then quantified as described in section 2.7.2.

A further set of frozen tissue aliquots were used to conduct RNA extraction using a Quiagen RNEasy mini kit (Section 2.7.3). RNA samples were then converted into cDNA libraries using Quiagen Reverse Transcriptase kit. DNA primers to various mRNAs of interest were designed and tested. Only primers that gave a single peak, with a clear dose-dependent relationship were used further (table 2.3). mRNA expression analysis was then conducted as per section 2.7.4.

3.2.6 Assessment of In Vivo Oxidative Stress

In this chapter, further experiments were undertaken to assess in vivo production of mitochondria-derived reactive oxygen species using the molecular probe MitoB, kindly supplied to us by Professor Michael Murphy of the Mitochondrial Biology Unit, Addenbrookes

Hospital, Cambridge. The probe, MitoB, comprises a triphenyl-phosphonium (TPP) cation driving its accumulation within mitochondria, conjugated to an arylboronic acid that reacts with H_2O_2 to form a phenol, MitoP. Quantifying the MitoP/MitoB ratio by liquid chromatography tandem mass spectrometry (LC-MS/MS) enables measurement of a weighted average of mitochondrial H_2O_2 that predominantly reports retrospectively on tissue mitochondria of the living chicken embryo (Cochemé *et al.*, 2012). Therefore, on day 19 of incubation, a subset of embryos were dosed with 20nmol MitoB in aqueous solution. Precisely one hour after treatment, embryos were killed and heart, brain, liver, and skeletal muscle were snap frozen in liquid nitrogen. To extract the molecules for analysis by mass spectrometry; 50mg of tissue was homogenised in pH 7 Tris buffer (Bullet Blender, Next Advance, New York, USA), then 600µL of acetonitrile (ACN, Romil, Cambridge, UK) mixed with 0.1% formic acid (Sigma, Gillingham, UK). An internal standard was added, then samples were centrifuged at 16,000g for 10 minutes and the supernatant collected into fresh Eppendorf tubes. A further 500µL of 95%ACN/0.1% formic acid was added to the remaining pellet then the previous step repeated, and the two resulting supernatants combined. The tubes were left overnight to evaporate in a SpeedVac, and the following day 170µL of 20%ACN/0.1% formic acid was added and the centrifugation step repeated. The supernatant was then filtered (0.22µm PVDF filters, Millipore, Watford, UK), into mass spectrometry autosampler vials (Thermo Fisher Scientific, Loughborough, UK). Simultaneously to the experimental sample extraction, standard curves for both MitoB and MitoP were generated by spiking control tissue with known amounts of MitoB (0, 10, 25, 100, 250, 1000pmol) or MitoP (0, 1, 5, 10, 100pmol). The MitoB and MitoP contents of the samples were then analysed on an I-class Acquity UPLE attached to a Xevo TQ-S triple quadruple mass spec (Waters, Milford, USA).

The mass spectrometry and analysis were conducted by Dr. Angela Logan (Mitochondrial Biology Unit, Addenbrookes Hospital, Cambridge, UK).

3.2.7 Assessment of embryonic lung maturation

Perfusion fixed lungs were embedded in paraffin, then sectioned into 8µm sections with an ultramicrotome (Section 2.8). Slides were stained with haemotoxylin and eosin (Section 2.6), then imaged. In tissue isolated from each embryo, 20 measurements of parabronchial atrial diameter, atrial septum thickness, air capillary diameter, and distance between air capillary and nearest blood vessel (diffusion distance), and 5 measurements of thickness of gas exchange zone were taken.

3.2.8 Statistics

All results except for dose-response curves were compared for statistical significance using the Student's *t* test for unpaired data. Dose-response curves were assessed using a mixed-model two-way ANOVA with the Tukey post hoc test. Statistical tests were conducted on GraphPad Prism 7 (Graphpad Software, CA, USA) software and significance was accepted when $P<0.05$. All significant differences are indicated by: * $P<0.05$, ** $P<0.01$, *** $P<0.001$, ****$P<0.0001$.

3.3 Effects on Embryonic Growth

Following assignment to treatment groups on day 14 the survival rate for control embryos was 100%, whilst for dexamethasone treated embryos it was reduced to 66.7% ($P<0.05$). At day 19, dexamethasone treated embryos had a significantly lower total haemoglobin level, and a higher blood glucose concentration (Fig.3.2).

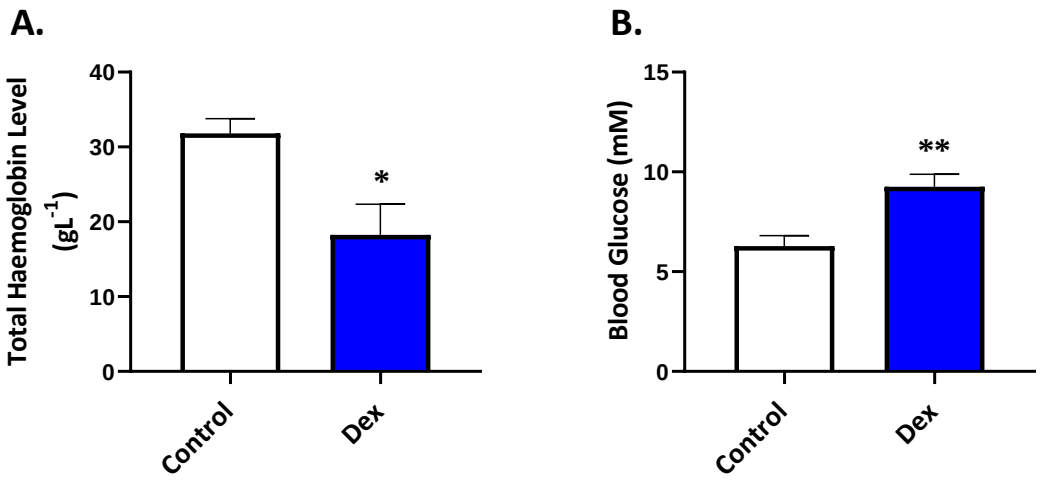


Figure 3.2 Day 19 blood parameters. A. total blood haemoglobin levels (g/L), B. blood glucose concentration (mM) Control n=12, Dexamethasone n=12. Parametric t-test, * P<0.05, ** P<0.01, *** P<0.001, ****P<0.0001.

Embryos treated with Dexamethasone had a significant growth restriction at day 19 of incubation (Fig.3.3), both in absolute terms, and relative to the day 1 and day 19 egg weights. The ratio of partitioning of resources between embryonic and extra-embryonic tissues was significantly reduced in dexamethasone treated embryos, and they had a significantly higher mass of liquid in the day 19 egg. Relative head diameter and brain weight were increased comparative to control embryos, as was relative heart weight, whilst crown-rump length (CRL) was significantly reduced.

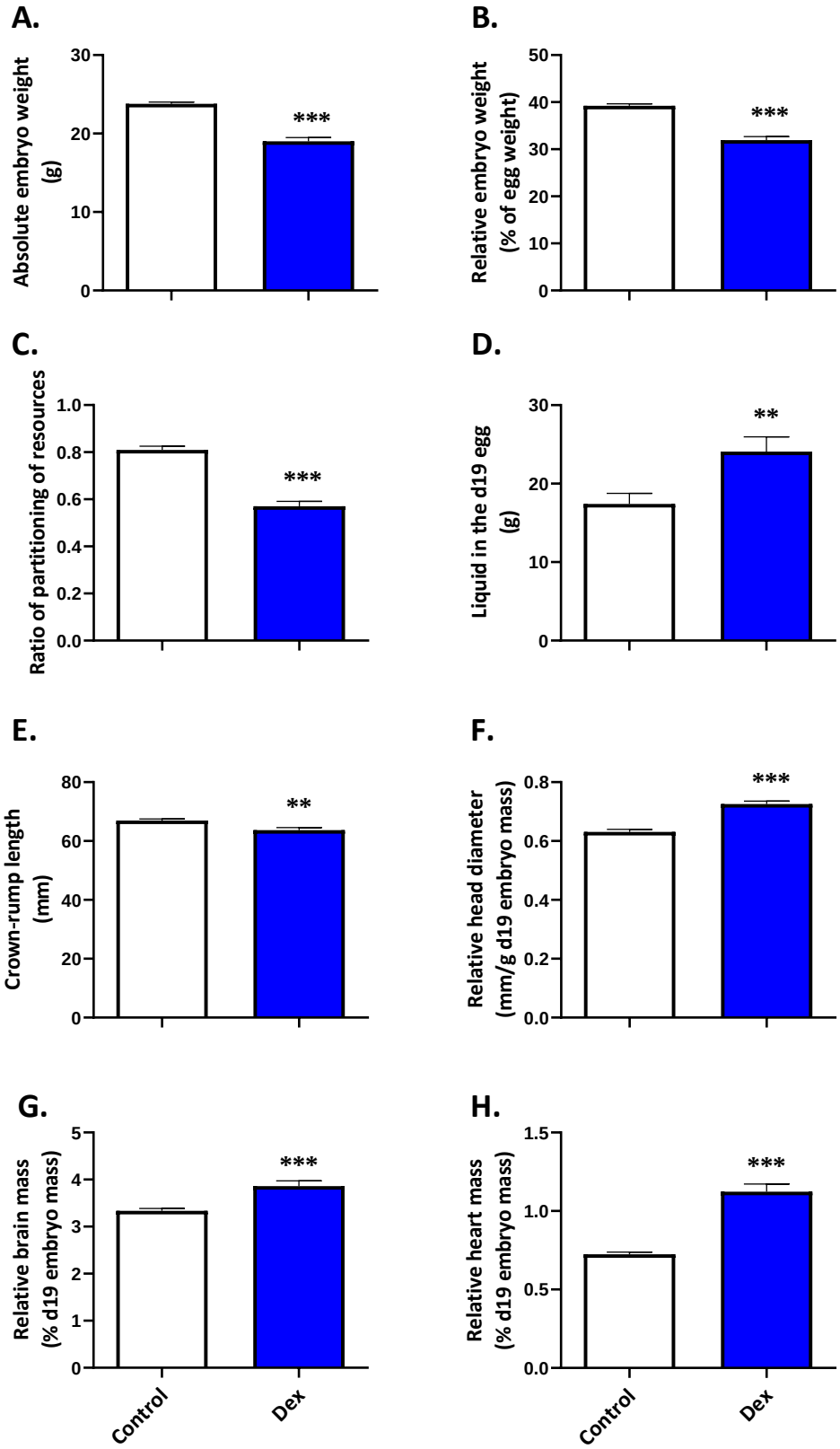
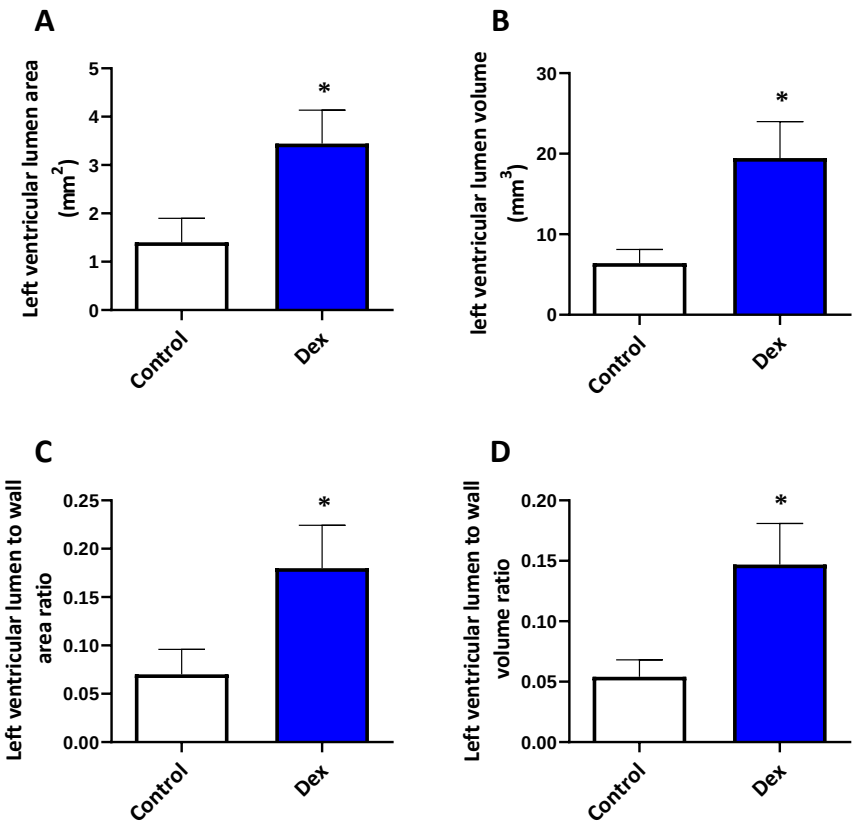


Figure 3.3 Day 19 biometry. A. absolute embryo mass (g), B. relative embryo mass (% d19 egg mass), C. ratio of embryonic to extra-embryonic mass, D. liquid mass (g), E. crown-rump length (mm), F. head diameter relative to embryo mass (mm/g), G. relative brain mass (% embryo mass), H. relative heart mass (% embryo mass) Control n=12, Dexamethasone n=12. Parametric t-test, * P<0.05, ** P<0.01, *** P<0.001, ****P<0.0001.

3.4 Effects on Cardiac Structure & Function

The area of the left ventricle wall and septum was unaltered in dexamethasone treated embryos relative to controls, whilst the area of the left ventricle lumen was significantly increased (Fig.3.4). The same trend was observed when muscle and chamber volumes were assessed, with the left ventricle wall having an unchanged volume and the left ventricle lumen a significantly increased volume. The ratio of left ventricle lumen to wall was increased in terms of both area and volume, suggesting left ventricular dilatation. The right ventricular wall volume and right ventricular lumen volume were increased differentially in dexamethasone treated embryos, resulting in a significant increase in the right lumen to wall volume ratio, also suggesting right ventricular dilatation.



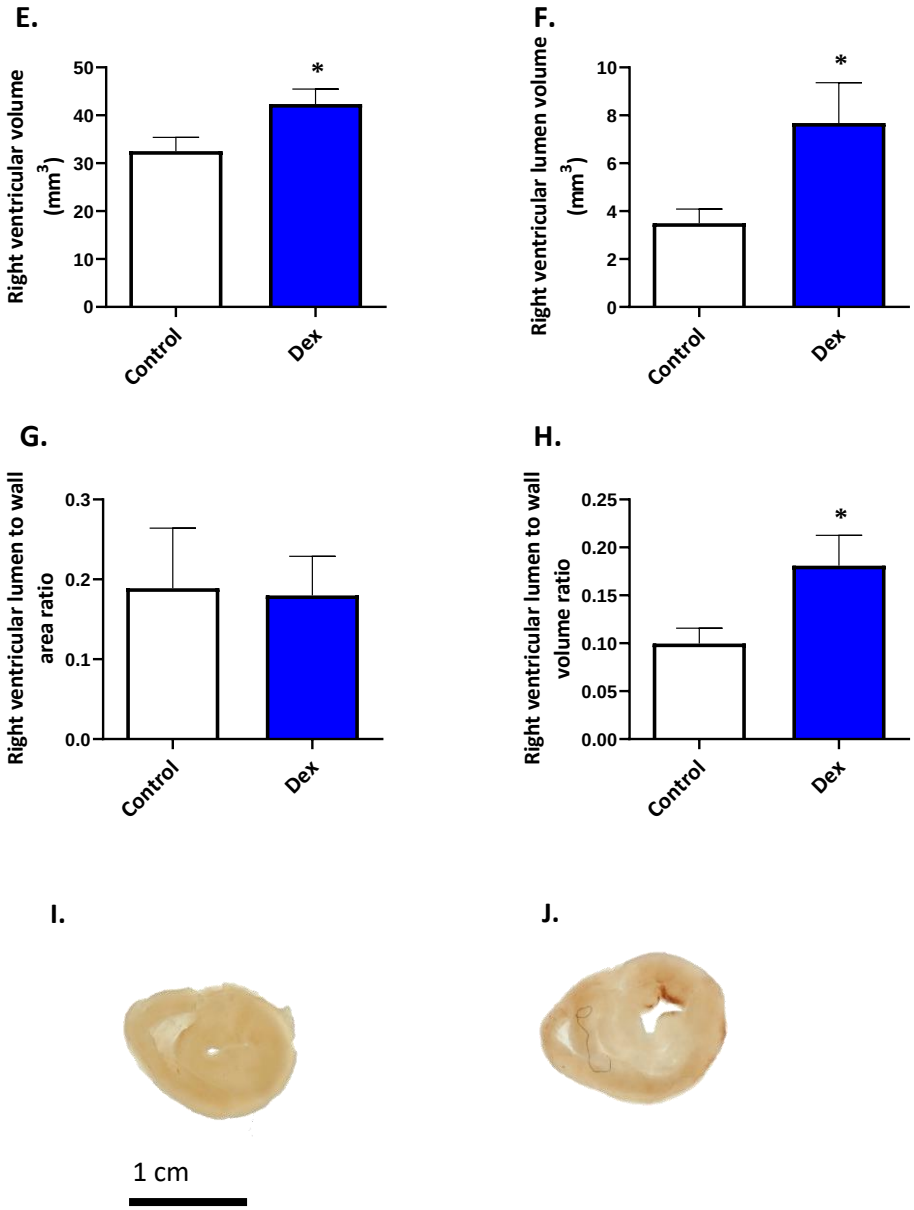


Figure 3.4 Ventricle areas, volumes, and ratios. A. left ventricular lumen area, B. left ventricular lumen volume, C. Ratio of left ventricular lumen to wall area, D. ratio of left ventricular lumen to wall volume left ventricle wall volume, E. right ventricular volume, F. right ventricular lumen volume, G. ratio of right ventricular lumen to wall area, H. ratio of right ventricular lumen to wall volume, I. representative mid-section of control embryo heart, J. representative mid-section of Dex treated heart, in control (n=10) and dexamethasone treated (n=10) embryos, at day 19 of incubation. Parametric t-test, * P<0.05, ** P<0.01, *** P<0.001, ****P<0.0001.

Aortic wall thickness was significantly reduced in dexamethasone treated embryos (Fig.3.5), whilst aortic lumen diameter was unaltered.

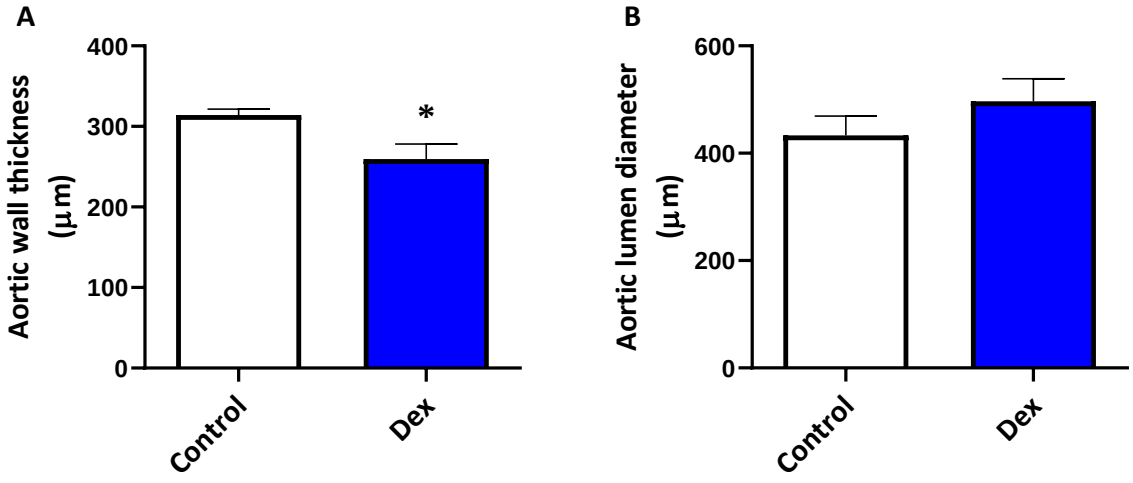


Figure 3.5 Aortic thickness. A. Aortic wall thickness, B. aortic lumen diameter, in control (n=7) and dexamethasone treated (n=10) embryos at day 19 of incubation. Parametric t-test, * P<0.05, ** P<0.01, *** P<0.001, ****P<0.0001.

Isolated cardiomyocytes were quantified to assess cellular size and nuclearity. Dexamethasone treated animals had a significantly larger cardiomyocyte width, and calculated cell volume. There was also a significant reduction in the % of cardiomyocytes that were mononucleated (Fig.3.6). Left ventricular cardiomyocyte nuclear density was also significantly reduced in dexamethasone treated embryos.

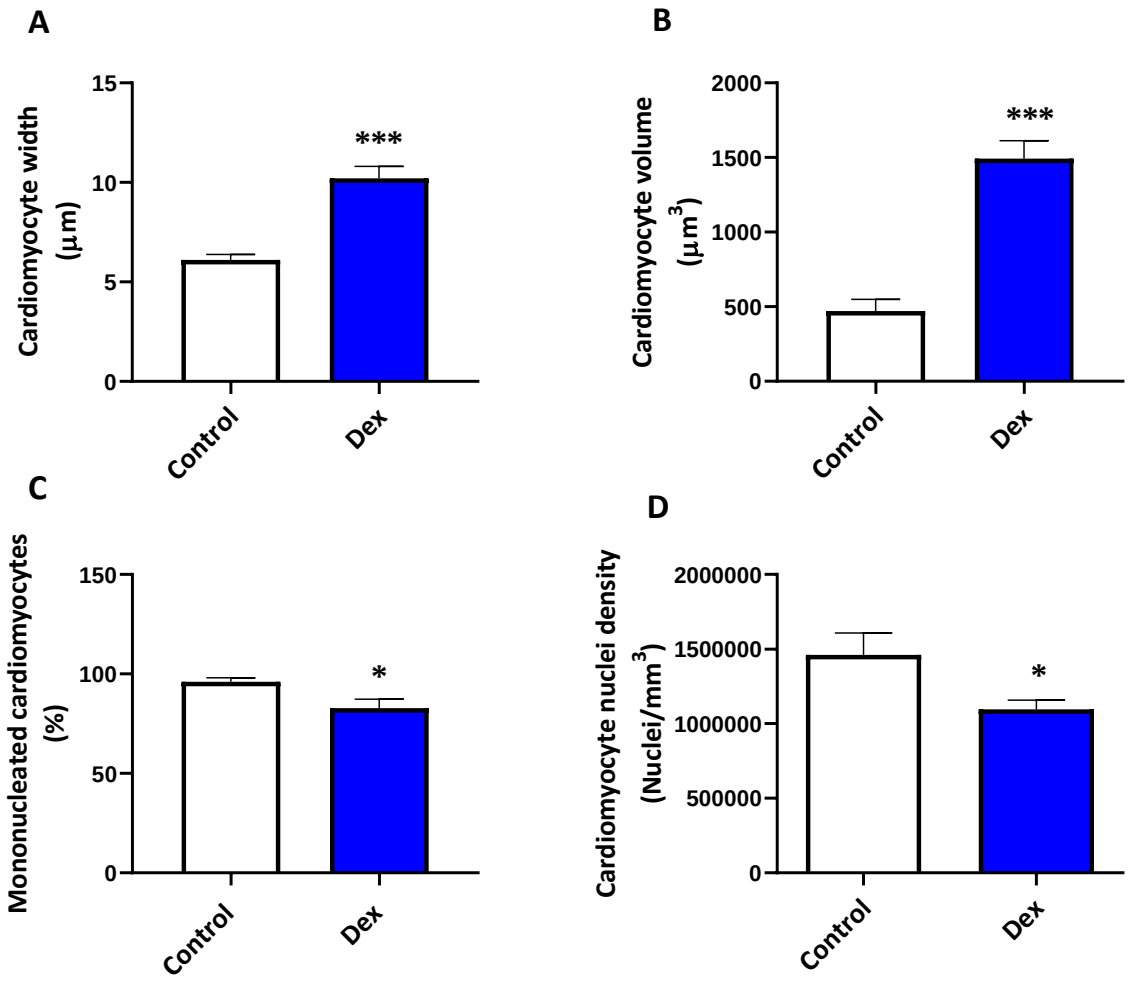


Figure 3.6 Cardiomyocyte analysis. A. cardiomyocyte width in um, B. calculated cardiomyocyte volume in mm3, C. percentage of all cardiomyocytes counted that were mononucleated, D. number of cardiomyocyte nuclei per mm3 of left ventricular tissue, in control (n=6) and dexamethasone treated (n=6) embryos at day 19 of incubation. Parametric t-test, * P<0.05, ** P<0.01, *** P<0.001, ****P<0.0001

In terms of basal cardiac function, dexamethasone treated animals had a significantly increased LVEDP, suggestive of diastolic dysfunction (Fig.3.7). This was accompanied by a significant decrease in Tau, which is an index of the speed of left ventricle relaxation. Dexamethasone treated animals also displayed systolic dysfunction, with a significantly impaired LVDP and contractility index (Fig.3.7).

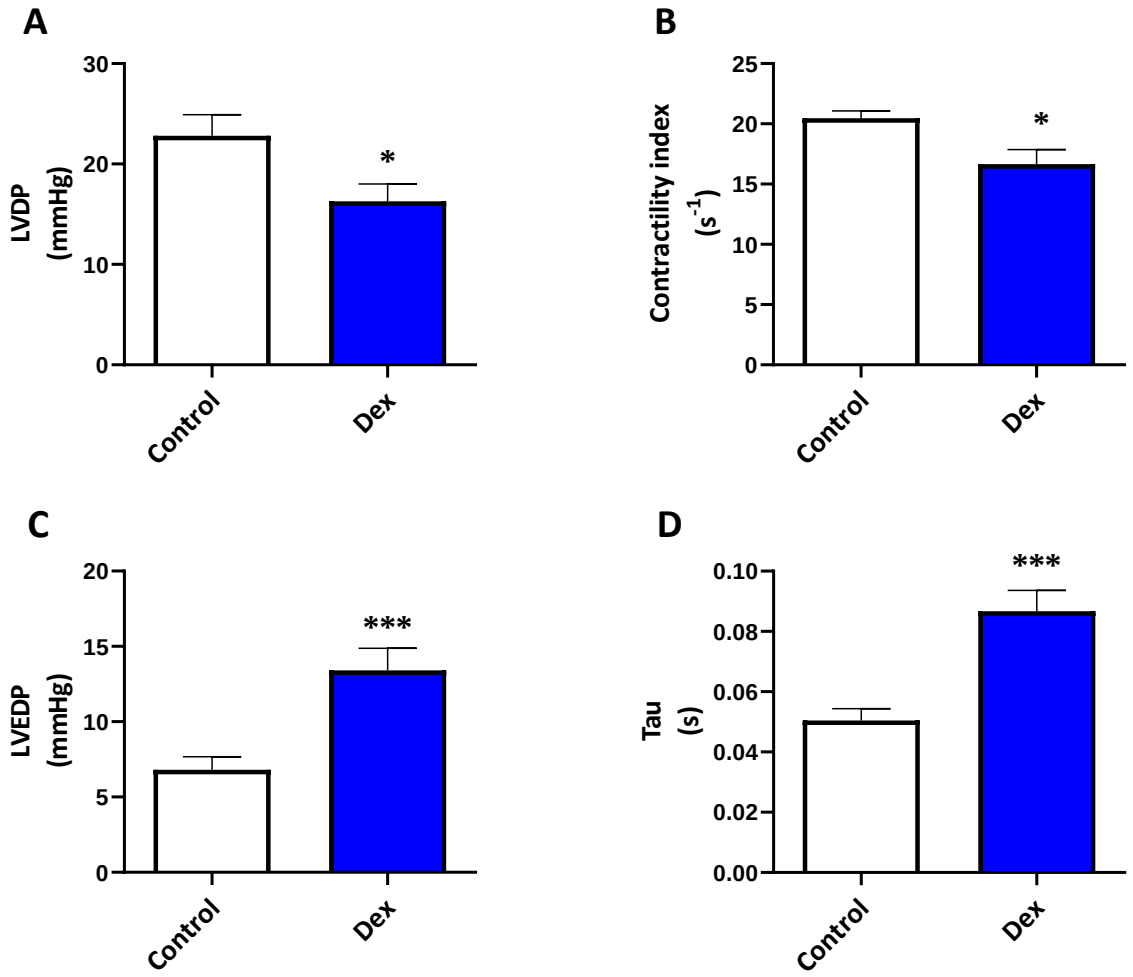


Figure 3.7 Diastolic and systolic function. A. Left ventricular developed pressure, B. contractility Index, C. Left ventricular end diastolic pressure, D. Tau, in control (n=10) and dexamethasone treated (n=10) day 19 embryos. Parametric t-test, * P<0.05, ** P<0.01, *** P<0.001, ****P<0.0001.

Coronary flow rate was slightly reduced in dexamethasone treated animals, although this did not reach significance. Systolic duration was significantly reduced in dexamethasone treated animals, whilst diastolic duration was unaltered (Fig.3.8).

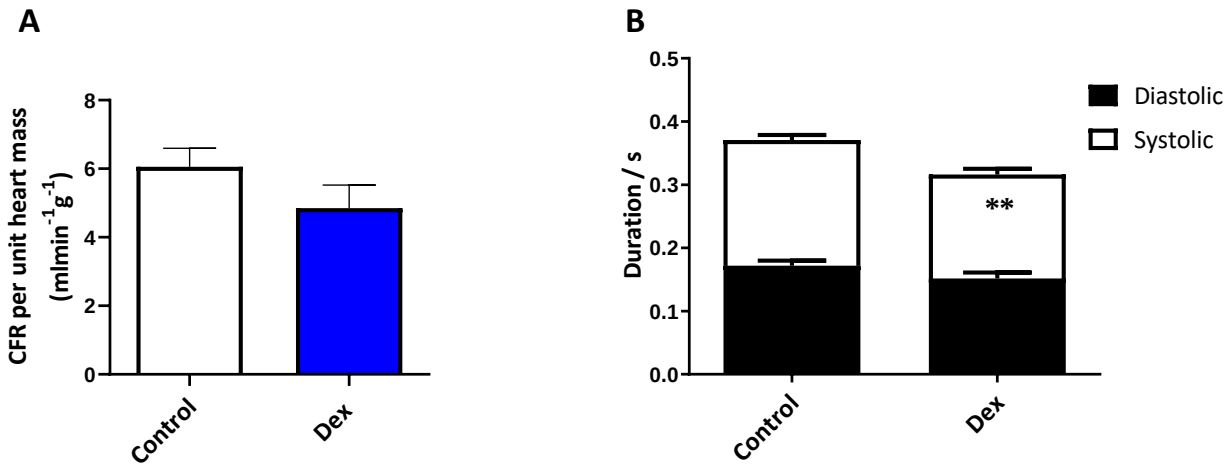


Figure 3.8 Coronary flow rate and heart cycle duration. A. Coronary flow rate relative to heart weight, B. Systolic and diastolic cycle duration, in control (n=10) and dexamethasone (n=10) treated embryos at day 19. Parametric t-test, * P<0.05, ** P<0.01, *** P<0.001, ****P<0.0001.

In terms of stimulated cardiac function, the developed pressure response of dexamethasone treated embryos to the muscarinic agonist carbachol was significantly impaired relative to control embryos (Fig.3.9), however the heart rate response, and responses to the adrenergic agonist isoprenaline were not altered significantly.

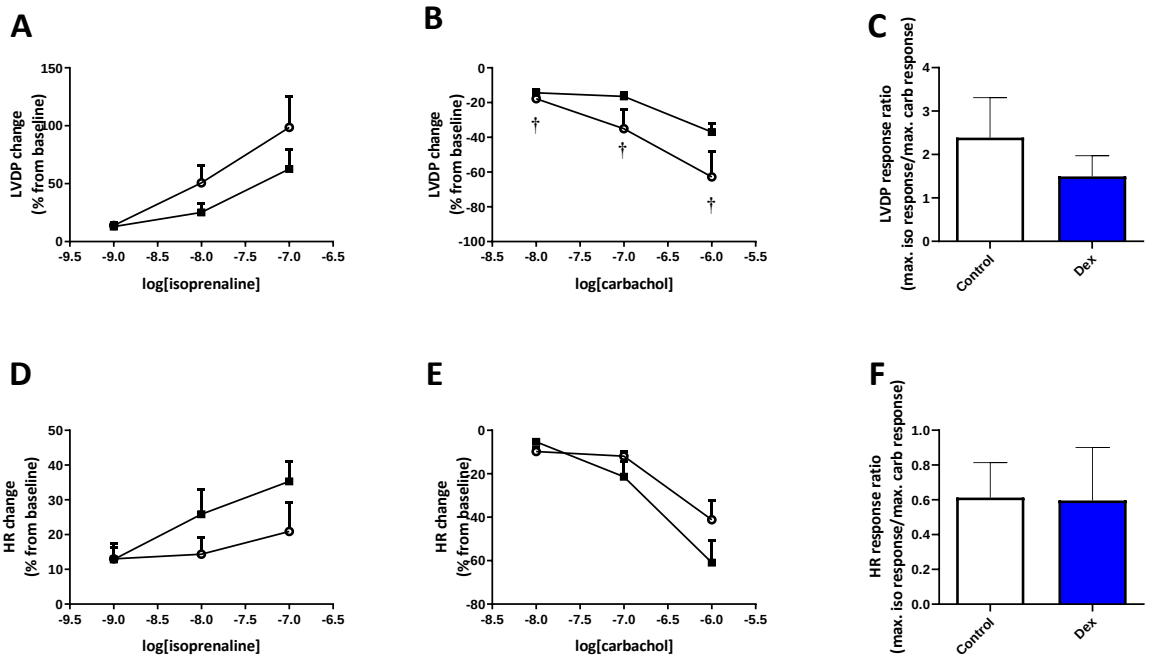


Figure 3.9 Cardiac responses to autonomic agonists. A. left ventricular developed pressure response to isoprenaline, B. left ventricular developed pressure response to carbachol, C. ratio of the maximum developed pressure responses to isoprenaline and carbachol, D. heart rate response to isoprenaline, E. heart rate response to carbachol, F. ratio of heart rate response to isoprenaline and carbachol, in control (O n=10) and dexamethasone treated (•n=10) embryos at day 19 of incubation. † P<0.05, control vs. Dex, two-way ANOVA.

3.5 Effects on Peripheral Vascular Function

The femoral arteries response to K^+-induced constriction was significantly increased in the dexamethasone group (Fig.3.10). There was no significant difference in sensitivity of the dose-dependent response to vasoconstrictor PE between treatment groups (as assessed by pD2), and the magnitude of the response, relative to each vessel's maximum K+-induced response, was unchanged (Fig.3.10). The dose-dependent vasoconstriction response to endothelin-1 (ET-1) was also significantly enhanced in the dexamethasone treated embryos (Fig.3.10). The response to serotonin (5-HT) however, was impaired.

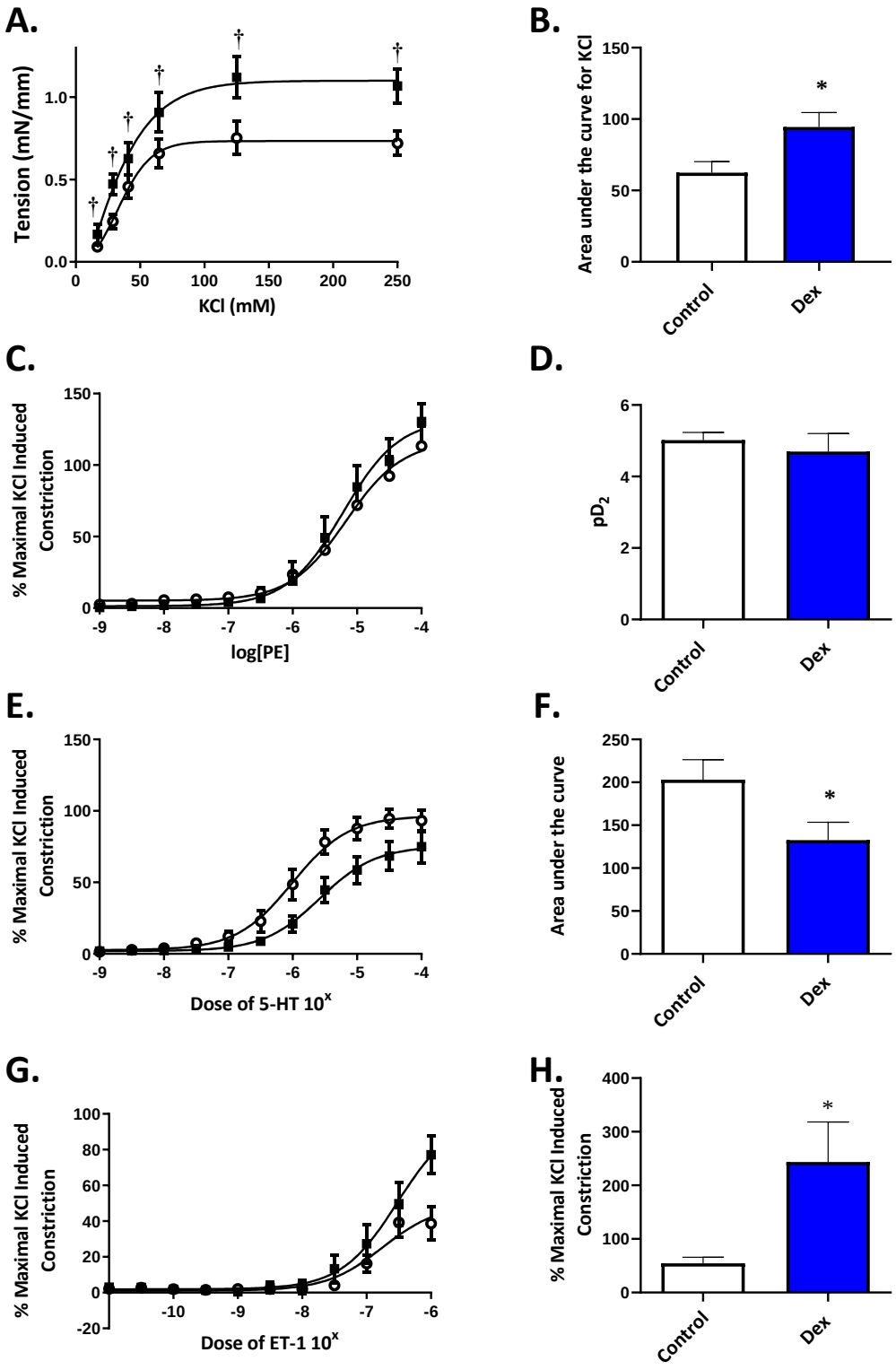


Figure 3.10 Vasoconstrictor responses. A. tension generated in response to KCl (mM), B. Area under the curve of the KCL response (arbitrary units), C. response to PE as a % of maximal KCl response, D. pD2 of PE response, E. response to 5-HT as a % of maximal KCl response, F. area under the curve of 5-HT response (arbitrary units), H. response to ET-1 as a % of maximal KCl response, H. maximal ET-1 response as a % of maximal KCl response, in control (O n=10) and Dex (•n=10) treat embryos, two-way ANOVA with Tukey post-hoc test, A parametric t-test * P<0.05, ** P<0.01, *** P<0.001, ****P<0.0001, † P<0.001, where † represents a main effect, two-way ANOVA.

There was no significant difference observed between control or dexamethasone treated animals in response to the vasodilator Ach (Fig.3.11). The proportion of the Ach-induced relaxation attributable to NO-dependant or NO-independent mechanisms was also similar between the two groups (Fig.3.11). The response to NO donor SNP was also unchanged in the dexamethasone treated embryos relative to the control embryos, suggesting no impaired vasorelaxation is present (Fig.3.11).

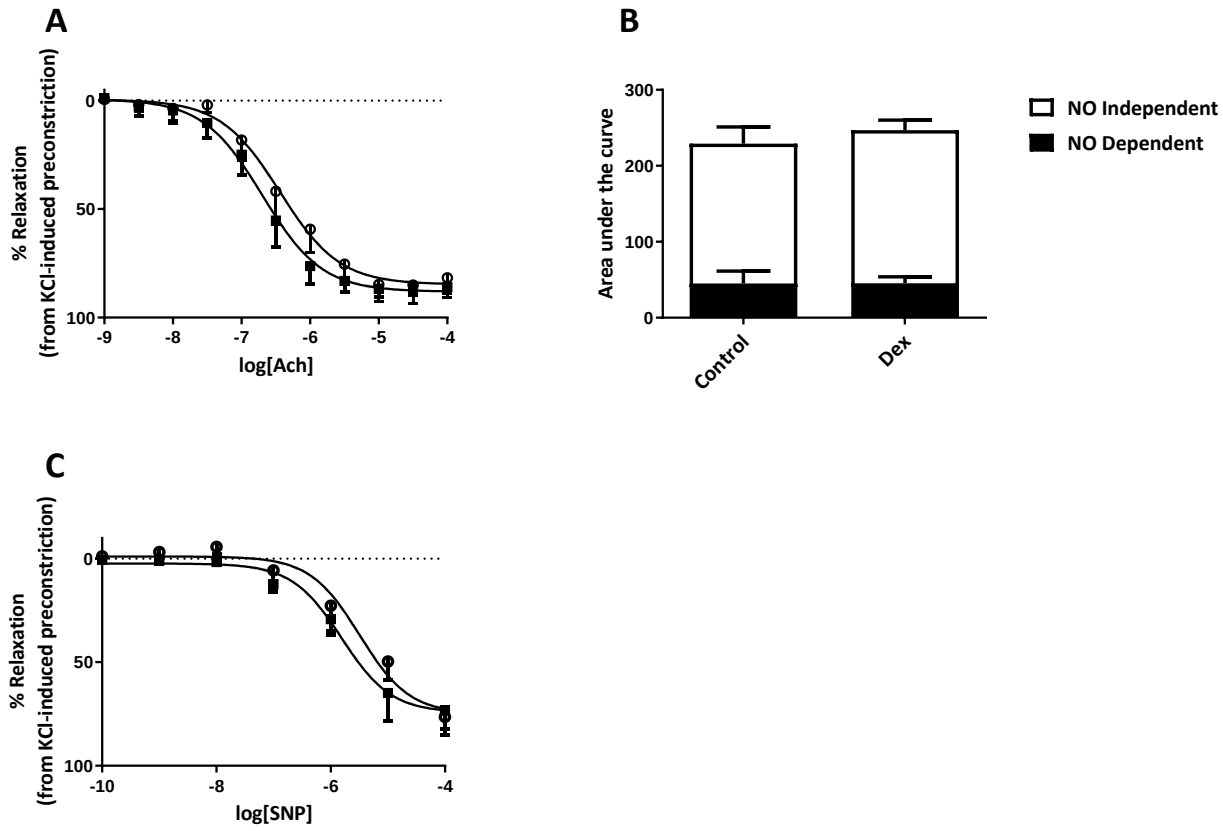


Figure 3.11 Vascular vasodilator responses. A. vascular relaxation from KCl-induced pre-constriction in response to increasing doses of Ach, B. breakdown of the vasodilator response to Ach, by treating vessels with the eNOS inhibitor L-NAME, and assessing the area under the Ach dose-response curve, before and after inhibition, the area under the curve dependent on eNOS and hence NO can be determined, C. vascular relaxation from KCl-induced pre-constriction in response to NO donor SNP, in control (O n=10) and dexamethasone treated (•n=10) embryos.

3.6 Effects on Molecular Pathways in the Heart

Endogenous in vivo H_2O_2 production, assessed via the molecular probe MitoB, revealed that at 5 days post dexamethasone exposure, there was increased H_2O_2 production in the fetal liver (Fig.3.12) Further evidence for oxidative stress was given by a decrease in mRNA expression of antioxidants Catalase, and Super-oxide Dismutase (Mn-dependent), and an increase in the mRNA expression of the pro-oxidant cp450 2A1. There was also a significant increase in protein carbonylation levels the chicken embryo, which is evidence of oxidative protein damage.

Dex treated embryonic hearts had a significant up-regulation of cell stress kinases at day 19 of incubation, specifically the pERK and pSAPk/JNK pathways (Fig.3.13). There was also demonstrated to be a significant up-regulation of protein chaperones that are known to be up-regulated in the unfolded protein response (UPR). Hsp27 is associated with endoplasmic-reticulum (ER) unfolded protein stress, whilst hsp70 is associated with cytoplasmic unfolded protein stress, and TID-1 and hsp60 are mitochondrial chaperones associated with unfolded protein stress. As well as evidence for cellular stress in Dex treated embryos, there was also evidence for an increase in cellular apoptosis in the day 19 embryonic heart. There was a significant increase in total caspase-3 levels, cleaved caspase-3 levels, and no change in levels of the anti-apoptotic protein BCL (Fig.3.13). This suggests a significant increase in levels of apoptosis in the day 19 heart. Whilst there was no change in levels of key cell cycle regulator p53 mRNA in Dex treated embryos, there was a significant increase in mRNA expression of MKK3, p38, and p16 (Fig.3.13). These are all kinases associated with cell dynamics and known

to be up-regulated in response to various stresses and induce cellular senescence or apoptosis.

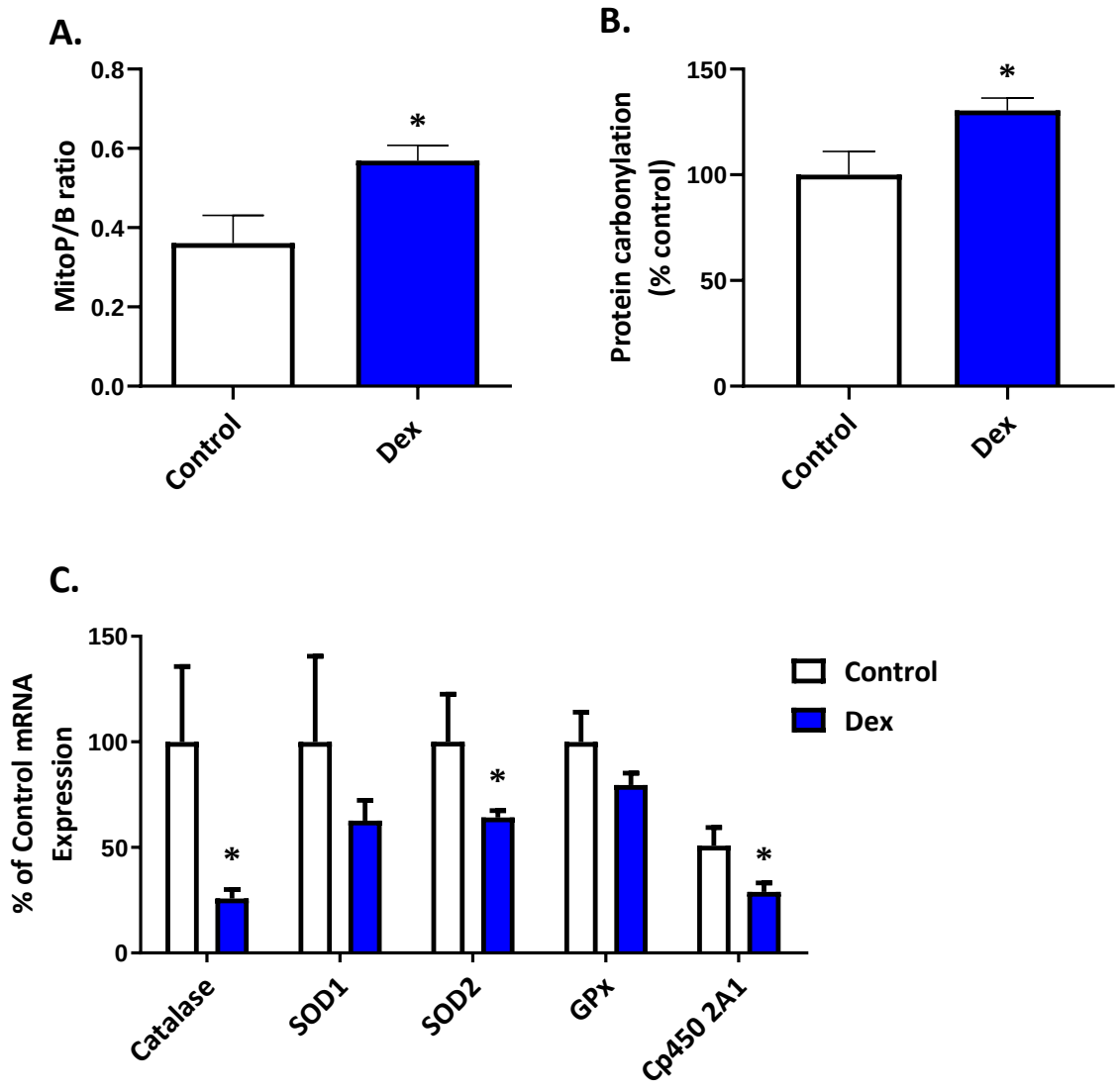


Figure 3.12 Oxidative stress following Dex exposure. A. MitoP/B ratio in the fetal liver 6 days post Dex administration, B. Levels of protein carbonylation detected by western blot, C. mRNA expression of Catalase, SOD1 (Zn/Cu), SOD2 (Mn), Glutathione peroxidase (GPx) and cp450 2A1, in control (n=10) and dexamethasone (n=10) treated embryos. Each graph assessed by parametric t-test, * P<0.05.

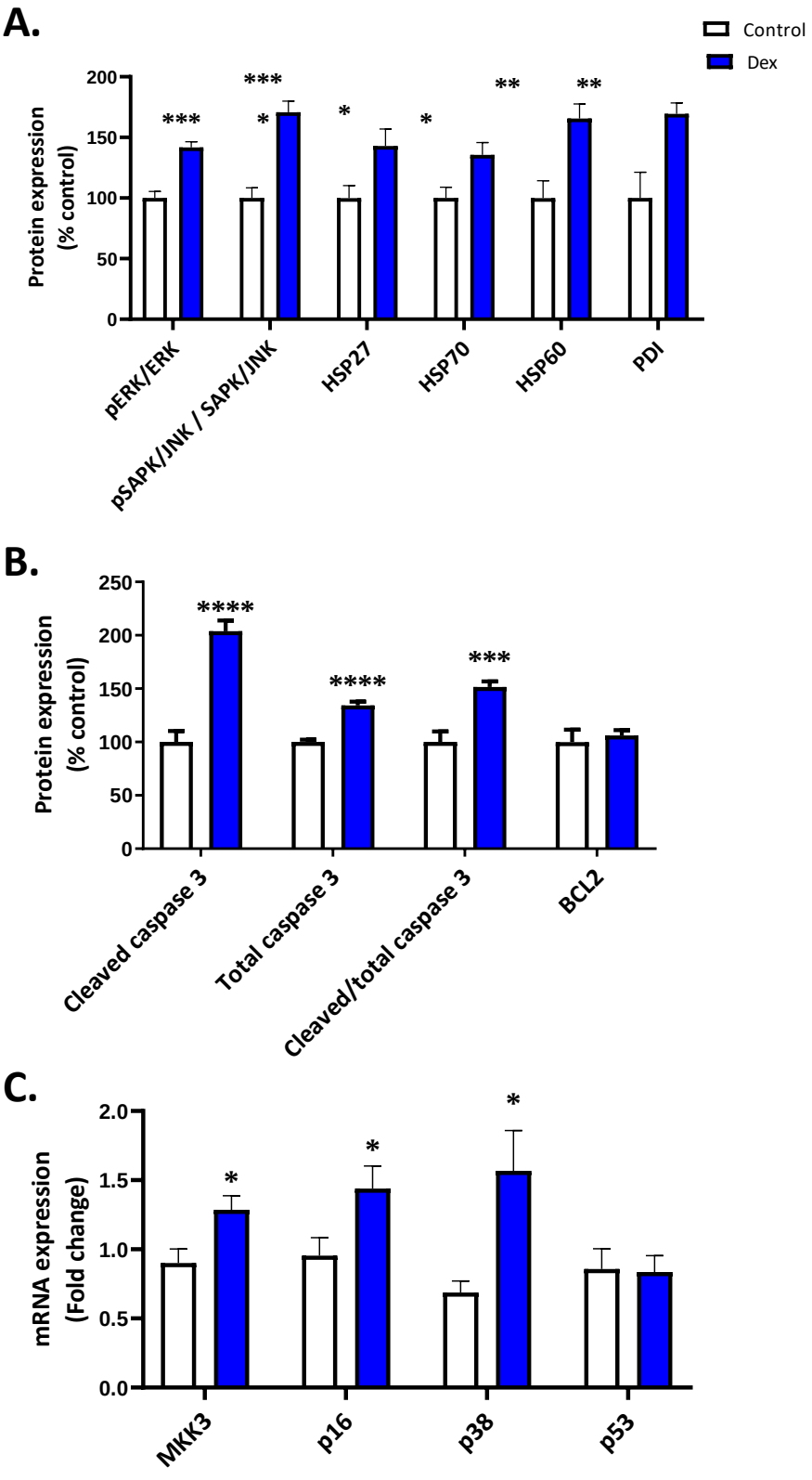


Figure 3.13 Cellular stress, apoptosis, and cell cycle markers. A. phosphorylated ERK protein/non-phosphorylated levels, phosphorylated SAPK/JNk / non-phosphorylated levels, protein expression of chaperones HSP27, HSP70, HSP60, and PDI B.. protein levels of caspase-3 cleaved caspase-3, ratio of cleaved caspase-3 to total caspase 3, BCL, C. mRNA levels relative to control of MKK3, p16, p38, p53, in control (n=6) and dexamethasone (n=6) treated embryos. Parametric t-test, * P<0.05, ** P<0.01, *** P<0.001, ****P<0.0001.

3.7 Effects on Lung maturation

In the day 19 embryonic lung, there was a significant increase in protein expression of the key surfactant protein SP-B (Fig.3.15). There was a trend towards an increase in caveolin-1, which is a cellular marker of lung maturation, although this increase did not reach significance.

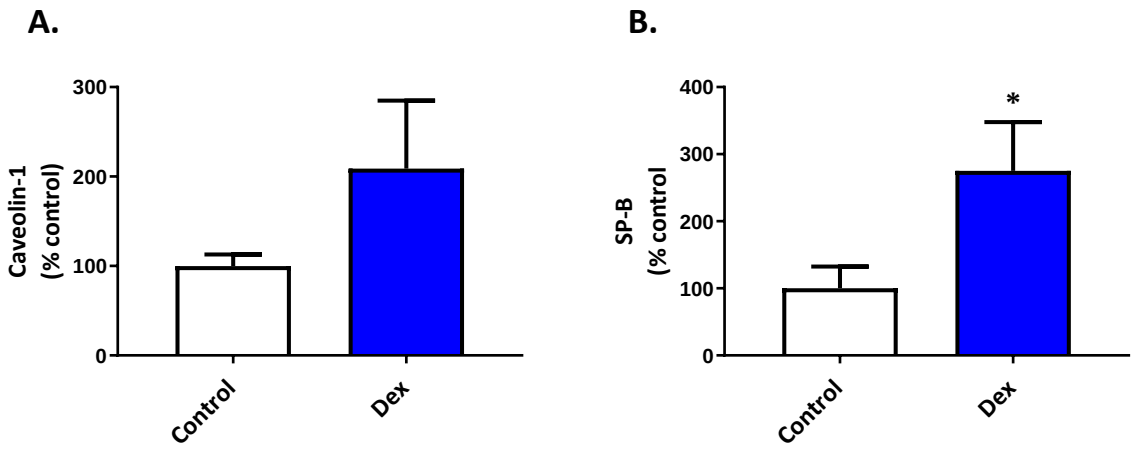


Figure 3.14 Protein markers of lung maturation. A. protein levels of caveolin 1, B. protein levels of SP-B, in control (n=6) and dexamethasone (n=6) treated embryos. Parametric t-test, * P<0.05.

In terms of morphological maturation of the embryonic lungs, there was a significant increase in parabronchial atrial diameter, air capillary diameter, and total thickness of the gas exchange region adjacent to the parabronchi (Fig.3.16). There was also a significant decrease in diffusion distance between air capillaries and the closest blood capillary, for oxygen exchange.

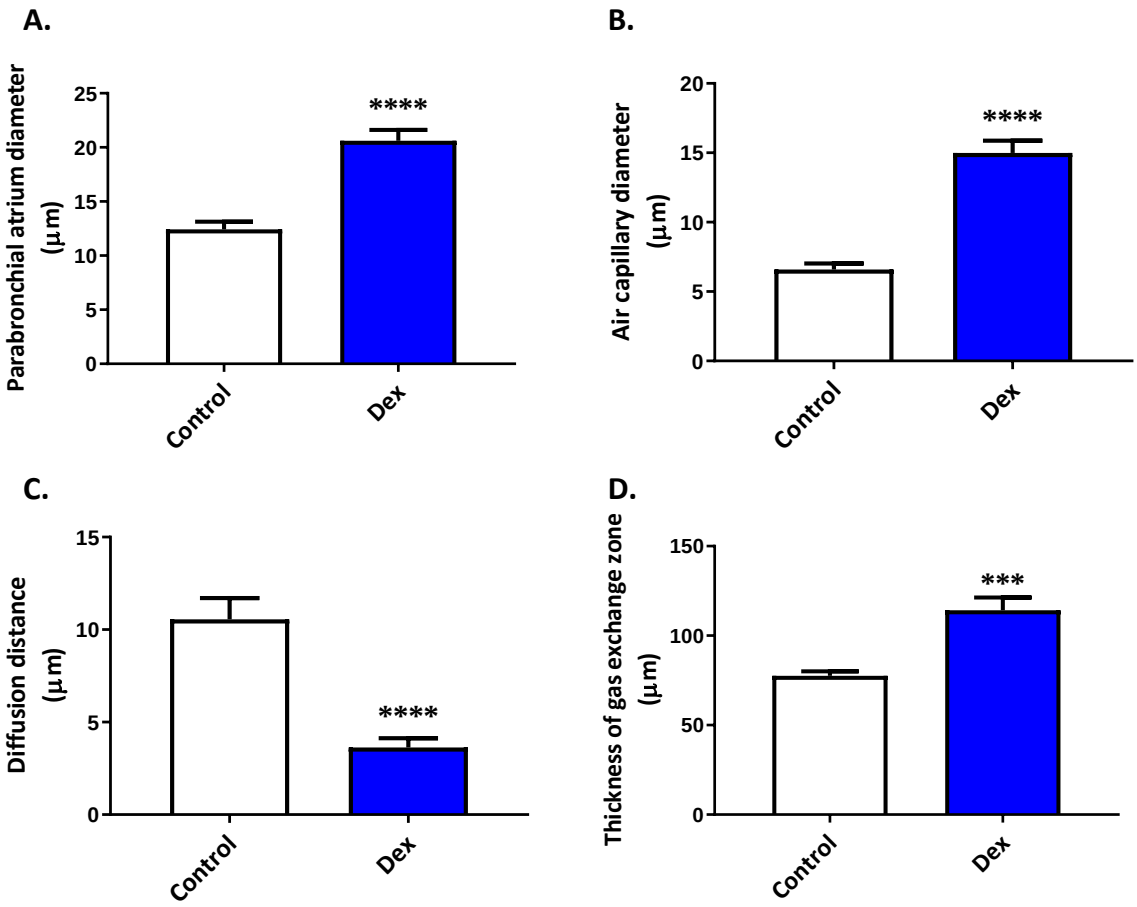


Figure 3.15 Morphological markers of lung maturation. A. parabronchial atrial diameter (µm), B. air capillary diameter (µm), C. diffusion distance (µm), D. thickness of gas exchange region adjacent to parabronchi (µm), in control (n=10) and dexamethasone (n=10) treated embryos. Parametric t-test, *** P<0.001.

3.8 DISCUSSION

3.8.1 Summary

Dexamethasone treatment did not alter egg mass at any stage of incubation, however it resulted in a decrease in embryonic survival from day 14 of incubation. Dexamethasone treatment resulted in significant asymmetric growth restriction in the day 19 embryo in terms of absolute mass, mass relative to egg mass and head diameter to body mass ratio. Embryonic cardiac function was significantly impaired following dexamethasone treatment. Coronary flow rate remained unchanged, however systolic duration in the cardiac cycle was significantly reduced. Dexamethasone treated embryos had a significant impairment in left ventricular developed pressure (LVDP) and in contractility index, as well as increased left ventricular end diastolic pressure (LVEDP) and Tau (time taken to reach 67% ventricular relaxation). In terms of cardiac structure, there was a significant left ventricular and right ventricular dilatation observed in dexamethasone treated embryos. Peripheral vascular constriction to KCl was increased and there was an enhanced vascular response to ET-1 and 5-HT, but no effect on the dilator responses to either SNP, or Ach.

Hearts displayed various indices of oxidative stress, including an increase in protein carbonylation, and changes in antioxidant/oxidant related genes, including catalase, Mn-SOD, and cp450 2A1. Dex treated embryos demonstrated an activation of cell stress kinases pERK and pSAPK/JNK, and upregulation in associated cellular proteins known to be important in the UPR response (hsp27, hsp70, hsp60, and PDI). Up-regulation of total and cleaved caspase 3 suggest an apoptotic phenotype. This was associated with an up-regulation of p53 mRNA

expression. Furthermore, both MKK3 and p38 kinases were significantly up-regulated at the transcriptional level, as were cell cycle mediators p16 and p38. Dex treated embryos also demonstrated a negative feedback effect on the glucocorticoid receptor (GR) in the heart, as protein expression levels were decreased relative to controls.

Various markers of lung maturation were up-regulated in the day 19 embryonic lungs from Dex treated embryos relative to controls. SP-B expression was up-regulated, and there were morphological markers consistent with pulmonary maturation, including parabronchial atrial diameter and air capillary diameter.

In this chapter we addressed the hypothesis that Dex exposure in late cardiovascular development will result in cardiac and peripheral vascular dysfunction. Therefore, the data presented here support this hypothesis.

3.8.2 Effects on Embryonic Growth & Survival

We have demonstrated in this model of embryonic glucocorticoid administration a profound growth restriction by the end of development. This is an effect of fetal over-exposure to glucocorticoids that has been reported in various animal models including rodents, sheep, and non-human primates (Jobe et al., 1998; Newnham & Moss, 2001; Doyle et al., 2014; Fowden & Forhead, 2009). Conversely, glucocorticoid deficiency during pregnancy, e.g. following adrenalectomy, results in increased fetal mass relative to control animals (Fowden and Forhead, 2009a). In systematic reviews of the human data however, there is no clear

difference in growth between treatment and placebo groups, although this is compounded by difficulties in disentangling the effects of glucocorticoids with those of complicated pregnancy (Roberts et al., 2017). The likely cause of this overall growth restriction is the glucocorticoid mediated induction of the late development switch away from tissue accretion towards maturation (Fowden & Forhead, 2015). The majority of organs display a decrease in mass following early glucocorticoid exposure, as well as changes in cell number and function (Fowden and Forhead, 2009b). Mechanistically, glucocorticoids have been demonstrated to act via the GR pathways to alter; cellular receptors, ion channels and transporters, enzyme production, cytoskeletal proteins, and intracellular growth pathways (Fowden and Forhead, 2009a).

Interestingly in this study we have demonstrated that the growth restriction induced by Dex exposure is asymmetric, with the embryonic brain and heart being spared relative to the overall body mass. This could be an effect secondary to differential expression of GR in different tissues. It may also be secondary to reduced Dex levels in certain tissues due to exclusion by drug efflux transporter P-glycoprotein, which is known to be highly expressed in the brain (Karssen et al., 2002). This may lead to overall lower activation of the GR pathway in the fetal brain. GR however is known to be highly expressed in the fetal heart and so it is unlikely that a lower level of receptor is the reason for the sparing of heart growth (Rog-Zielinska *et al.*, 2014b). Another possibility is that certain organs may be spared due to differential effects of Dex on the vasculature. Whilst in the periphery (e.g. the femoral and mesenteric circulations) glucocorticoids induce vasoconstriction, thereby reducing delivery of oxygen and nutrients (Pulgar & Figueroa, 2006; Lee et al., 2014), this occurs to a lesser extent

in vital circulations, such as the heart and brain (Jellyman *et al.*, 2009b). This is a key feature of the fetal stress response, which generally acts to protect the fetal brain at the expense of other tissues, and is known to be at least partly due to glucocorticoid mediated signalling (Giussani, 2016).

In this chapter we also report a significant decrease in embryonic survival at day 19 of incubation in Dex treated embryos relative to controls (66.7% vs. 100%). This is clearly not the case in the human situation in which survival of the fetus following AGT is not impaired (Roberts et al., 2017). This difference could be due to the relative growth trajectory of the chicken embryo relative to the human fetus. Between day 14 and day 19 of incubation the chicken embryo mass increases from an average of 5.7g to 19g (preliminary studies), an increase of 233%. Comparatively the human fetus increases from an average of 1031g at 27 weeks to 2624 at 36 weeks, an increase of 154% (Salomon et al., 2007). Hence restriction of growth during this period may have a greater effect on the chicken embryo, leading to more severe growth restriction and decreased survival. The chicken embryo also lacks any maternal responses to AGT which may blunt the negative effects on survival.

3.8.3 Effects on Cardiac Structure and Function

Functionally, hearts displayed profound systolic and diastolic dysfunction, showing an inability to generate appropriate left ventricular pressure and a slower than normal ventricular relaxation. This was associated with a biventricular dilator phenotype. This is significant as these are all indices of a failing heart (Tanai & Frantz, 2015).

In dexamethasone-treated embryos, there was also a reduced total number of left ventricular cardiomyocytes and the myocytes were significantly larger than in control hearts. Typically, in late gestation, both systolic and diastolic cardiac function improve as the ventricular walls thicken due to hyperplastic growth of cardiomyocytes (Harada et al., 1999; Corrigan et al., 2010; Oparil et al., 1984; Christoffels et al., 2000). There is also growth of the t-tubular and sarcoplasmic reticulum systems, and alterations in ion channel density and identity (Davies et al., 1996; Siedner et al., 2003; Fahmi et al., 2004). Finally, there is a profound change in cardiac metabolism as the number of mitochondria increases and the fetal heart switches towards primarily fatty acid metabolism and increased ATP production (Bartelds et al., 2000; Mizuno et al., 2010). Endogenous glucocorticoids are known to be either directly or indirectly involved in many of these key maturational processes, and loss of glucocorticoid signalling during cardiac development has been shown to lead to a dilated phenotype, with poorly functioning cardiomyocytes (Rog-Zielinska et al., 2013; Oakley & Cidlowski, 2015). In our model, it appears as though the cardiomyocytes have prematurely switched away from a proliferative phenotype towards binucleation and hypertrophic growth, which is normally associated with the post-natal heart (Porrello et al., 2008; Pohjoismäki et al., 2013). This has led to both hypertrophied cardiomyocytes and an overall decrease in cardiomyocyte number. The resulting systolic and diastolic dysfunction could therefore arise from the reduced number of cardiomyocytes, impairing the ability of the left ventricle to develop significant pressure, and premature maturation of the cardiomyocytes imparting electrical conductance and contractility alterations leading to the dysfunctional cycle duration and diastolic relaxation. A dilated phenotype in the ventricles is in the adult individual is typically associated with acute decompensated heart failure, when neuro-hormonal compensatory changes eventually fail

to maintain systolic function, resulting in a volume overload in the left ventricle and a failure to pump (Japp et al., 2016; Kurmani & Squire, 2017). Whilst we have no direct measurement of blood pressure in these animals, it is plausible that a combination of dexamethasone-induced reductions in cardiomyocyte number and increased afterload have resulted in an inability of the heart to pump sufficiently, leading to a pressure overload and dilated cardiomyopathy.

In most animals cardiomyocyte endowment is dependent on myocyte proliferation during early development. Chicken cardiomyocytes proliferate throughout incubation with the greatest rate occurring in early development (days 3 and 4), however this proliferation also continues into post-hatching life (up to 6 weeks of age) (Li et al., 1997; Sedmera & Thompson, 2011). Therefore, any insult which decreases proliferation during incubation, either by inhibiting progression of the cell cycle or stimulating cell cycle exit and terminal differentiation, can affect endowment. However, the ability of chicken cardiomyocytes to proliferate after hatching means that the decreased numbers of cardiomyocytes shown at day 19 may not persist to adulthood. In comparison in mammals there is little to no post-natal cardiomyocyte proliferation, meaning endowment is largely established during gestation (Thornburg et al., 2011). Glucocorticoids are known to play a role in the switch towards binucleation and terminal differentiation, either directly or indirectly (for example via stimulation of the thyroid hormone axis) (Porrello et al., 2008; Thornburg et al., 2011).

3.8.4 Effects on Peripheral Vascular Function

When peripheral femoral vascular function was assessed ex vivo, no change was seen to the vasodilator responses to ACh or SNP, however a significantly increased response to K+, ET-1 and 5-HT was measured in the Dex treated embryos. The elevated response to vascular smooth muscle cell (vSMC) depolarisation, suggests either vSMC hyperplasia or hypertrophy resulting in more contractile units in the vascular wall. Glucocorticoids have been shown in other studies to induce proliferation and increase contractility of vSMCs directly, *in vivo* and *in vitro* (Volk et al., 2010; McLellan et al., 1992). This increased contractile capacity of the peripheral circulation is likely associated with increased resistance and decreased flow in these circulations. This would fit with the apparent restriction in growth in the embryo leg length, as a reduction in delivery of oxygen and nutrients will enhance the reduction in tissue accretion. Whether this femoral hypercontractile phenotype results in hypertension in the fetal circulation is unclear from this experiment but this effect may contribute in the asymmetric growth restriction effects of dexamethasone and increased cardiac afterload in this model. In other models of antenatal Dex exposure, a subsequent hypertension has been reported, often associated with an increase in femoral vascular resistance (Figueroa et al., 2005; Docherty et al., 2001; Celsi et al., 1998). As blood pressure increases towards term, this could be simply a premature switch towards the increased afterload seen with advancing gestation in preparation for neotnatal life (Giussani et al., 2005; Forhead et al., 2000). It could also be a stress response, as endogenous glucocorticoids are known to mediated responses to hypoxia and undernutrition by redistributing blood flow towards vital organs (Anwar et al., 2016).

The significantly increased vascular response to ET-1 in Dex exposed is mirrored in other studies (Pulgar & Figueroa, 2006; Kutzler et al., 2003). Dex has been shown *in vitro* to increase both ET-1 production and release from vSMCs, and also to potentiate the contractile effects of ET-1 (Kanse et al., 1991; Sato et al., 1992; Rouhert et al., 1993). ET-1 acts on the vasculature via way of two separate receptors (Fig.3.17), the $ET\text{-}_A$ and $ET\text{-}_B$ receptor. The $ET\text{-}_A$ receptor is present on the vSMCs and induces an intense vasoconstriction, whilst the $ET\text{-}_B$ receptor is present on the vascular endothelium and is known to induce NO production via nitric oxide synthase (NOS; Davenport et al., 2016). Thus the balance of $ET\text{-}_A$ to $ET\text{-}_B$ receptors in a given circulation is important in determining the overall vasomotor response to ET-1. There is evidence that antenatal glucocorticoid exposure causes an up-regulation of $ET\text{-}_A$, thus shifting towards a vasoconstrictor phenotype (Kutzler et al., 2003). As the responses reported here are due to direct administration of ET-1 to the vasculature, it is likely the changes seen are due to alterations in vascular receptors, or the downstream contractile pathways. However, there is no altered response to nor-adrenaline or phenylephrine in this model, which both act via α_1-adrenergic receptors to induce IP_3 production and thus show a convergence with the $ET\text{-}_A$ receptor pathway. This suggests that the downstream pathway is unchanged and rather alteration at the level of the ET-1 receptors is favoured. However, other groups have demonstrated alterations to downstream vasoconstrictor pathways, such as the cd38/cADPR pathway (Lee et al., 2014). Abnormality of the endothelin signalling in the present model is of biological and clinical significance, as endothelin dysregulation has been associated with various adult cardio-pathologies, and has been shown to be present in pregnancy complicated by gestational diabetes and pre-eclampsia (Dieber-Rotheneder et al., 2006, 2012).

We also observed an impaired response to 5-HT in the Dex treated embryos, becoming more apparent at higher concentrations. 5-HT is known to act via a multitude of receptors and to have divergent effects in different vascular system (Watts, 2016). The dose-dependent vasoconstriction is thought to mainly be due to action of the 5-HT-$_{2A}$ receptor (Watts, 2005), and 5-HT has long been associated with hypertension in the adult (Huzoor-Akbar et al., 1989; Watts & Thompson, 2004; Nichols, 2009). Here, we also report a dose-dependent vasoconstriction in isolated femoral arteries. The reduced response in Dex exposed embryos could be secondary to a variety of factors including distribution and identity of 5-HT receptors in the femoral artery. Other studies have demonstrated that glucocorticoids can attenuate 5-HT mediated vasoconstriction (Angeles et al., 2006; Selli & Tosun, 2016). A further study of 5-HT receptors sub-types would help elucidate the mechanism behind the altered 5-HT vascular constriction reported here.

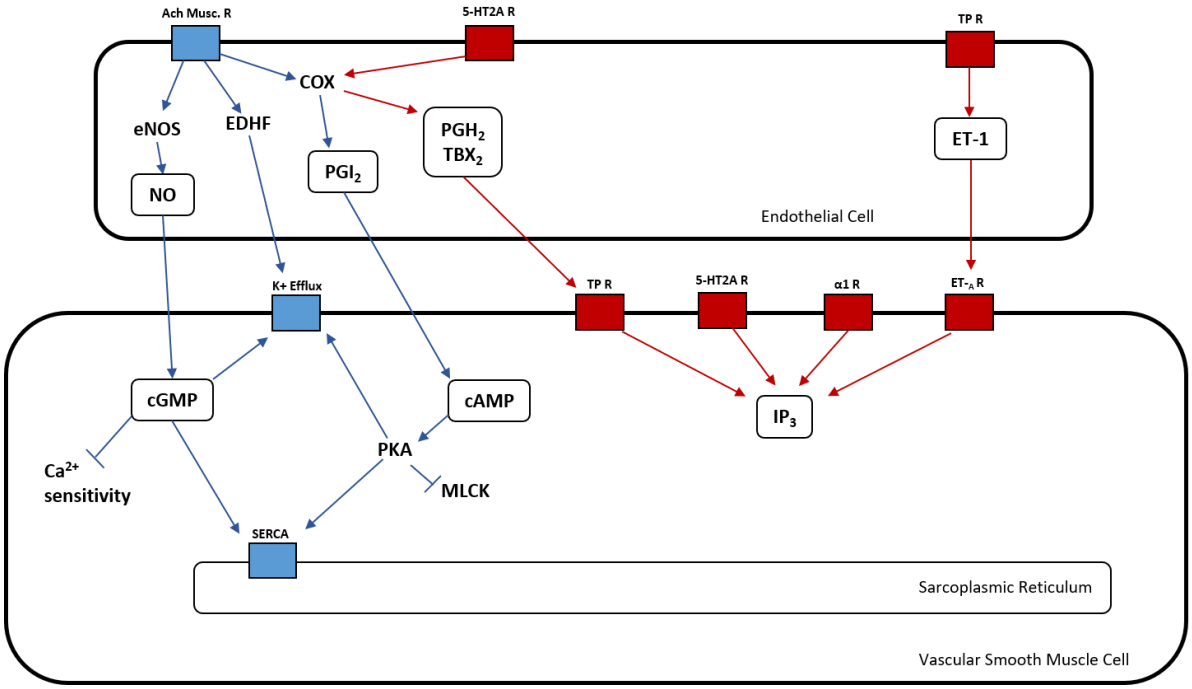


Figure 3.16 Vascular interactions. Interactions of vasodilators Ach which activates nitric-oxide synthase (eNOS), cyclo-oxygenase (COX) and endothelium derived hyperpolarising factors (EDHF). Production of NO and prostacyclins (PGI_2) leads to production of secondary messengers cAMP and cGMP in the vSMCs, which activate K^+ efflux channels, protein kinase A (PKA), SERCA, and inhibit myosin light-chain kinase (MLCK) and reduced Ca^{2+} sensitivity of sarcomeres. Vasoconstrictors acting on 5-HT_{2A} receptors or thromboxane receptors (TP Rs) on the endothelial cells stimulate production of ET-1, thromboxane (TBX_2) and PGH_2. Activation of 5-HT_{2A}, TP, ET-$_A$, or α_1-adrenergic receptors leads to production of secondary messenger IP3 and a subsequent increase in cytosolic Ca^{2+}.

3.8.5 Effects on Molecular Pathways in the Heart

There is significant evidence for activation of cellular stress pathways in the Dex exposed embryonic heart, secondary to generation of oxidative stress. Both direct measurements of *in vivo* mitochondria-derived oxidative stress (MitoP/B measurements) and of oxidative protein damage (protein carbonylation) show elevated levels in the Dex exposed embryos. There was also a significantly down-regulation of mRNA levels of antioxidants catalase and (Mn)-SOD. In adult tissues it is well known that Dex can induce oxidative stress (Lv et al., 2018; Yazıcı et al., 2018; Costantini et al., 2011). This is likely a combination of down-regulation of

antioxidant defences, up-regulation of pre-oxidant pathways, and direct actions on the mitochondria (Goodman et al., 1996; Takahashi et al., 2002; Du et al., 2009; Mutsaers & Tofighi, 2012). Oxidative stress has also been demonstrated in other models of AGT, and in additional organs such as the brain (Camm et al., 2011), whilst antioxidant treatment has been shown to protect against some of the glucocorticoid induced dysfunction (Zhang et al., 2004; Herrera et al., 2010; Wu et al., 2014). The effects of an increase in cellular oxidative stress include direct protein and DNA damage, disturbances to Ca^{2+} homeostasis, membrane potential, and mitochondrial function (Tönnies & Trushina, 2017).

There is evidence for a significant up-regulation of cellular stress pathways, likely secondary to the increase in oxidative stress. This includes the pERK, pSAPK, p38, and MKK3 pathways. There was also significant up-regulation of downstream markers of the unfolded protein reponse (UPR) stress, including hsp27, hsp70, hsp60 and PDI, and of cell cycle regulators p53 and p16. Likely consequences of activation of these pathways include a decrease in protein synthesis in an attempt to reduce protein stress in the ER; if this stress cannot be alleviated then the UPR response can lead to induction of apoptosis (Scull & Tabas, 2011) and an inflammatory response (Hotamisligil & Davis, 2016). There is evidence for this in our model as both total and cleaved caspase-3 protein levels were significantly increased, and there was a significant reduction in total nuclei in the left ventricle. Therefore, a combination of reduced cardiomyocyte number with impaired contractility and relaxability due to cellular stress in the cardiomyocytes is likely to be in part responsible for the systolic and diastolic dysfunction shown in this model. The molecular pathways leading to observed cardiac dysfunction are shown in Fig.3.18

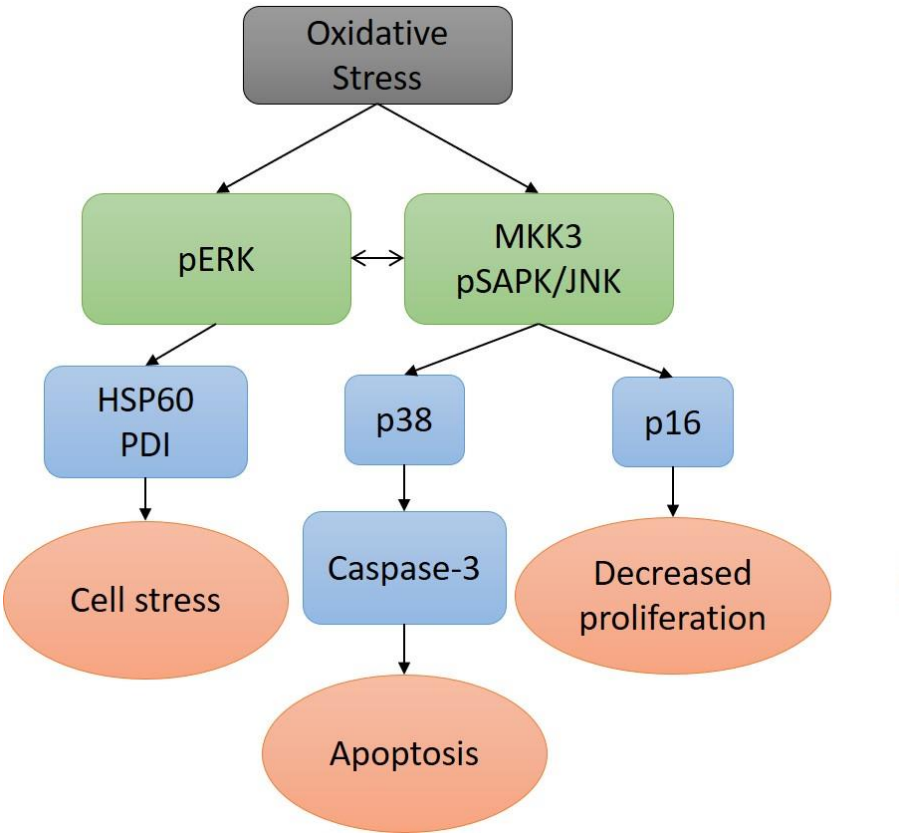


Figure 3.17 Summary of molecular pathways activated in the embryonic heart following Dex administration.

3.8.6 Effects on Lung Maturation

As one of the greatest beneficial effects of AGT in the pre-term neonate is accelerated lung maturation and a reduction in respiratory distress (McKinlay et al., 2015), it is pertinent to assess the effects of Dex administration in the lungs of our chicken embryo model. However, the avian lung is different to the mammalian lung in several key aspects. Mammalian lungs consist of highly compliant lobular gas exchange regions containing millions of alveoli, attached to a tubular conducting pathway which allows for bi-directional flow of gas into and

out of the lungs (Schittny, 2017; Weibel, 2017). This acts as the equivalent of a 'pool' exchange system whereby the inhaled alveolar gas equilibrates with the pulmonary circulation, resulting in carbon dioxide efflux and oxygen influx (Weibel, 2017). Avian lungs conversely are comprised of a series of relatively rigid gas exchange tubes (air capillaries) through which air flows unidirectionally (Harvey & Ben-Tal, 2016; Cieri & Farmer, 2016). These tubes are ventilated by sets of compliant 'air sacs'; the caudal abdominal air sacs, and the cranial air sacs. On inspiration the air sacs expand resulting in air flowing into the lungs into the caudal air sacs, and air in the gas exchange region to flow into the cranial air sacs. Upon expiration the air sacs are compressed, causing air in the caudal sacs to enter the gas exchange region whilst air in the cranial sacs can exit the lungs via the bronchus (Harvey & Ben-Tal, 2016). Functionally this results in a higher efficiency of gas exchange owing to the counter-current and cross-current air and blood flows established and has led to the long-held theory of this type of respiration being necessary for the evolution of the metabolic demands of flight (Cieri & Farmer, 2016; Maina, 2015).

Structurally, the gas exchange unit in the avian lung, the air capillary, arises from tubular parabronchi which are themselves a tertiary division of the main bronchus (Cieri & Farmer, 2016). Several air capillaries arise from the parabronchial 'atria' along their length. Air capillaries are histologically like alveoli in that they consist of a very thin epithelium and basement membrane, in close proximity to the endothelium of the pulmonary circulation (Maina, 2015). Previous work has demonstrated that the gas exchange regions of chicken lungs mature in the late developmental period in a manner similar to humans (Hylka & Doneen, 1982; Bjørnstad et al., 2014; Sullivan & Orgeig, 2001). Specifically, there is a late-

developmental switch towards production of type-II pneumocytes, and concomitantly increases in surfactant production (Bjørnstad et al., 2014). Increases in both surfactant lipids and proteins (such as SP-A and SP-B) have been shown during late embryonic development, peaking at around day 21, when chick pipping occurs (Hylka & Doneen, 1982; Bjørnstad et al., 2014). In cell culture, it has been shown that these changes may be induced by glucocorticoid exposure (Sullivan and Orgeig, 2001). Structurally the air capillaries mature in late development, with increased parabronchial atrial diameter, increased air capillary diameter, and decreased distance between the lumen of air capillaries and endothelium (referred to here as gas exchange distance) (Bjørnstad et al., 2014; Maina, 2015).

Here, we showed an increase in Dex embryos of protein levels of both caveolin-1 and SP-B which are known to increase towards term (Bjørnstad et al., 2014). Surfactant components are vital for reducing lung compliance such that the neonate can ventilate the lungs (Ballard et al., 1995), hence this observation is important in demonstrating the maintained beneficial effects of AGT on lung maturation in our chicken embryo model. We also report various histological indices of lung maturation following Dex exposure, including an increase in air capillary diameter, and in thickness of gas exchange region adjacent to the parabronchi. This further supports that Dex has accelerated lung maturation in these embryos.

3.8.7 Conclusions

The data in this chapter show that direct treatment of the chicken embryo with a human clinically relevant dose of dexamethasone at a developmental stage equivalent to the 27-week-old human fetus promotes asymmetric growth restriction and adversely affects

cardiovascular structure and function. Specifically, hearts isolated from dexamethasone treated embryos were heavier with significant biventricular luminal dilatation, and evidence of systolic and diastolic dysfunction. Mechanisms underlying the cardiovascular dysfunction in dexamethasone treated embryos included blunted inotropic responses to autonomic receptor agonists and cardiomyocyte hypertrophy with reduced proliferation. Enhanced *in vivo* generation of mitochondria-derived ROS, protein carbonylation and activation of ROS-induced cardiomyocyte senescence pathways provide a molecular link between dexamethasone treatment with adverse cardiovascular changes in the chicken embryo. Therefore, despite maturational beneficial effects on the lungs, the data provide evidence for direct adverse effects on the cardiovascular system of the developing offspring independent of effects on the mother and/or placenta of dexamethasone in doses relevant to those used in human clinical practise. It is of great importance that we further assess the mechanisms behind these negative effects such that clinical therapy can be adjusted to reduced negative effects on the cardiovascular system whilst maintaining maturational effects on the lungs.

CHAPTER 4: DEXAMETHASONE VS. BETAMETHASONE

4.1 INTRODUCTION

Antenatal glucocorticoid therapy (AGT) has been used extensively in the clinic for treatment of threatened pre-term birth since the 1990's (NIH, 1994). It has been shown that AGT prior to preterm birth dramatically reduces the risk of mortality and several severe morbidities in the preterm infant, such as Broncho-pulmonary dysplasia (BPD) and necrotising enterocolitis (NEC; Roberts et al., 2017). However, AGT has also been shown to cause potential significant adverse side effects on other organs and systems, such as the developing brain and cardiovascular system (Fowden et al., 2016). This has triggered serious questions as to how to optimise this therapy to maintain benefits but to minimise detrimental effects.

Notably, two different synthetic steroids Dexamethasone (Dex) and Betamethasone (Beta) are used interchangeably for this treatment, with no clear consensus on the optimal drug of choice (Schmidt et al., 2019). Beta and Dex are both fluorinated synthetic analogues of cortisol which is the primary endogenous fetal glucocorticoid (Fig.4.1). Fluorination at the 9-C position enhances the biological activity of the synthetic glucocorticoids whilst insertion of the 1,2 carbon-carbon double bond selectively augments glucocorticoid over mineralocorticoid activity (Buttgereit et al., 1999). This leads to reduced clearance from plasma and hence a prolonged biological half-life. Mineralocorticoid activity is further eliminated in synthetic glucocorticoids by methylation at the 16-C position (He et al., 2014). Beta and Dex are stereoisomers of each other, with the configuration of the 16-C methyl group varying between them (Buttgereit et al., 1999). Due to these additions Dex and Beta

have negligible mineralocorticoid activity, but their glucocorticoid potency is approximately 25-fold that of natural cortisol (Parker & Schimmer, 1996; Newnham & Moss, 2001). Further, the half-time for clearance from the plasma of fetal sheep of Dex and Beta is extended by approximately 6-8 h compared to cortisol (Jellyman et al., 2009; Gough et al., 2014).

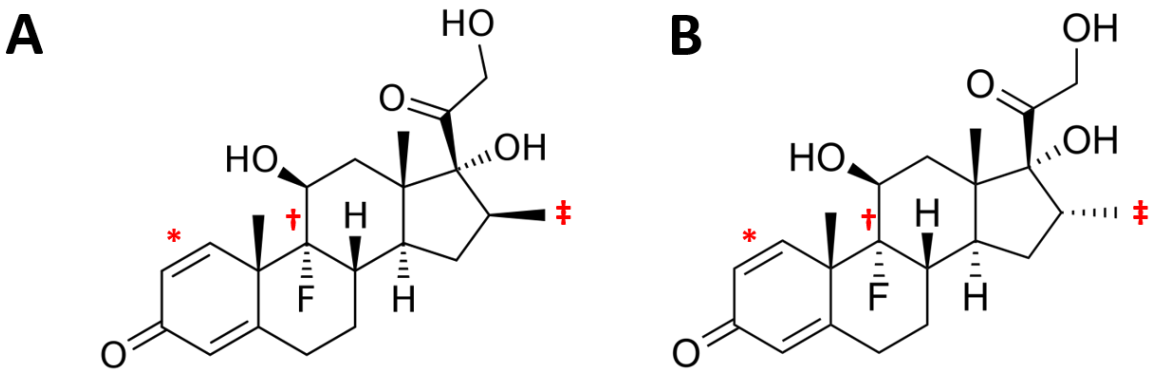


Figure 4.1 Chemical structures of the synthetic glucocorticoids. Betamethasone and Dexamethasone. A) Betamethasone, where * highlights the C1-C2 double bond, † highlights the fluorination of C9, and ‡ highlights the stereoisomeric methyl group. B) Dexamethasone, where * highlights the C1-C2 double bond, † highlights the fluorination of C9, and ‡ highlights the stereoisomeric methyl group. Taken from Garrud & Giussani (2019).

The dosing protocols for the different glucocorticoids are also historically different, with Dex being given as 4x12mg intramuscular doses, every 6 hours over a 48-hour period, whilst Beta is only given as 2x24mg doses 12 hours apart (Schmidt et al., 2019). This is due to the specific formulations of the drugs being utilised; Dex is given as a sodium ($Na^{+)}$ phosphate form of the drug, whilst Beta is given as a 50:50 mix of Na^{+}-phosphate and Na^{+}-acetate form. The acetate form of Beta has been shown to have a substantially longer half-life than the phosphate form, hence it is thought it will remain active in the fetal plasma for longer and hence fewer doses are required (Schmidt et al., 2018). The choice between which drug will be used by an obstetrician depends on several factors; some countries have historically used Beta over Dex

such as New Zealand, however Dex is often both cheaper, and more readily available (*WHO*, 2015).

There are few clinical studies that have directly compared the efficacy of Dex vs. Beta when treating threatened preterm birth. Individual studies have demonstrated that Beta has a greater efficacy in preventing neonatal death and RDS, whilst others have suggested that Dex is superior at preventing intra-ventricular haemorrhage (IVH) while maintaining positive neurological outcomes (Lee et al., 2006; Elimian et al., 2007; Lee et al., 2008). Other studies have reported no observable differences between the two drugs (Bar-Lev et al., 2004). A meta-analysis by Brownfoot et al., (2014), which assessed 10 trials (1161 infants), demonstrated no significant difference in most primary outcomes (neonatal death, RDS, BPD, IVH, mean birthweight), except for a decreased incidence of IVH when Dex was used compared to Beta. As well as different efficacies in preventing morbidities associated with preterm birth, there is also evidence in animal studies for differences in adverse outcomes on other systems in the newborn. Beta may have a more pronounced acute effect on fetal heart rate and fetal body movements (Rotmensch et al., 1999). The relatively small numbers of clinical or basic science studies directly comparing Beta and Dex make it difficult to assess what the optimum drug treatment for AGT should be. Indeed, both the UN committee on maternal and neonatal health, and a report by the WHO on preventing preterm birth, conclude that there is not adequate evidence to recommend one over the other, and that more research is sorely needed.

One of the well-known mechanisms leading to gluccocorticoid induced hypertension following adult or fetal Dex exposure, is generation of oxidative stress (Safaeian & Zabolian, 2014; Tain et al., 2014). Dex is known to be a potent inducer of oxidative stress in cell culture, and treatment of dex-induced hypertensive animals with antioxidants can restore normal physiological function (Zhang et al., 2004; Tai et al., 2016; Lin et al., 2018). Beta has also been associated with increases in oxidative stress in fetal models (Verhaeghe et al., 2009; Stojanovska et al., 2018). Therefore, it is possible that molecular changes involved in AGT induced cardiovascular dysfunction relate to induction of oxidative stress and the associated cellular responses which may include activation of stress kinase pathways, cell cycle changes, and apoptosis (Kim et al., 2016). As the outcomes associated with Dex and Beta treatment have been shown to differ in some studies, it is therefore also possible that any underlying molecular mechanisms differ.

Therefore, the main aim of this chapter was to compare the effects of the same clinically relevant dose of Dex and Beta on the developing cardiovascular system in the purest form possible, minimising any potential confounding influences of either treatment. The chicken embryo model is unique and appropriate for this, as the embryo develops in isolation within the egg. Therefore, the model allows comparison of the direct effects on the developing individual of any therapy, isolated from additional effects on the mother and/or placenta. Studies in our laboratory have previously reported that it is possible to dose the chicken embryo directly via the air cell with topical application of an aqueous solution onto the chorio-allantoic membrane (Itani et al., 2015). In this chapter we addressed the hypothesis that Dex and Beta exposure in late incubation will result in cardiac and peripheral vascular dysfunction

in the chicken embryo and that the severity and nature of this dysfunction will differ depending on which steroid is administered. To test this hypothesis, there were six objectives:

1. To develop clinically relevant models of AGT in the chicken embryo;
2. To investigate the effects of Dex and Beta treatment on embryonic survival and growth of the chicken embryo;
3. To investigate the effects of Dex and Beta treatment on the cardiac function and structure of the chicken embryo;
4. To investigate the effects of Dex and Beta treatment on peripheral vascular function in the chicken embryo;
5. To investigate the molecular pathways induced by Dex and Beta treatment in the chicken embryo;
6. To investigate the effects of Dex and Beta on lung maturation in the chicken embryo.

4.2 METHODS

4.2.1 Experimental Protocol

Detailed experimental protocols are explained in Section 2. In brief, all procedures were performed under the U.K. Animals (Scientific Procedures) Act 1986 and were approved by the Ethical Review Committee of the University of Cambridge. Fertilised Bovans Brown chicken eggs were weighed, irradiated with sterilising UV light, and placed into a normoxic incubator at 38°C and 45% humidity for 19 days (see Section 2.1.). Eggs were dosed with dexamethasone phosphate or betamethasone phosphate/acetate (Celestone, USA) in aqueous solution, or water on day 14 of development via injection through a small hole in the shell (situated above the air sac) onto the chorioallantoic membrane, as previously described (Itani et al., 2015).

The Dex/Beta dose used in this study was 0.1mgkg^{-1}. AGT was administered on day 14 of the 21 day incubation period, at two thirds of gestation, which is equivalent to 26/27 weeks in human pregnancy; a relevant, early period at which glucocorticoids can realistically be administered in the clinical setting (Roberts & Dalziel, 2006).

4.2.2 Assessment of Embryonic Growth

On Day 19 of incubation embryonic growth was assessed using a high precision calliper (Mitutoyo 500-181-20, U.K.) to determine embryonic lengths. Embryo mass and mass of the eggshell, yolk, extra tissues, and any remaininf liquid was measured and recorded. The ratio of partitioning of resources was calculated as embryonic tissue mass/extra-embryonic tissue mass. Crown-rump length, head diameter, head length were recorded (Section 2.2). Individual organs were also dissected and weighed.

4.2.3 Assessment of Cardiac Structure & Function

A cohort of 10 Embryos per group at day 19 of incubation were anaesthetised with sodium pentobarbitate (0.3mL of Pentoject; Animalcare Ltd., York, UK; intraperitoneal), then perfusion fixed using paraformaldehyde (Section 2.5) at 2.66kPa (embryonic day 19 blood pressure) infused into the left ventricle via a cannula. Fixed hearts were sectioned into 1mm slices and photographed. Areas of cardiac compartments in each section were counted, and a mid cardiac section was chosen to measure widths. Volumes were then calculated as per Section 2.5. The initial 5mm of the descending aorta was dissected from the level of the top of each fixed heart and processed in paraffin. Consecutive sections (n=10) for each aorta were arranged onto a microscope slide, deparaffinised, and then stained with Gill's Haemotoxylin. Slides were subsequently dehydrated and imaged (Section 2.5). Thicknesses and areas were then counted.

Cardiomyocytes were also isolated from hearts of day 19 embryos (Section 2.6)., by perfusing the coronary vasculature with collagenase and protease solution. After 10 minutes the hearts were perfused with Kreb's buffer to remove enzymes, and then suspended in buffer for 40 minutes. Cells were stored at 4% PFA. Cardiomyocyte suspensions were mixed with 10% methylene blue in a 1/1 ratio and visualised under a light microscope (Section 2.6). Cell nuclearity of 300 random cardiomyocytes per heart was assessed, and measurements of length and width were taken from 50 randomly chosen cardiomyocytes. Cell volumes were then calculated.

To assess *ex vivo* cardiac function, hearts were rapidly excised from another short of embryos and placed into icy Krebs' Buffer solution. The aorta was cannulated onto a 19-gauge blunt needle and mounted onto the Langendorff apparatus. Pressure recordings were subsequently made (Section 2.3). Following a period of normalisation, a small non-distensible balloon made from saran wrap and connected to a pressure transducer was introduced through the left atrium into the left ventricle. The balloon was then filled with water in 5µL increments until a smooth baseline recording was established. Baseline recordings were made and Coronary Flow Rate (CFR) was assessed. *In vitro* responses to the sympathetic agonist Isoprenaline (Sigma-Aldritch, in the range 10^{-9} – 10^{-7} M) and the parasympathetic agonist Carbachol (Sigma-Aldrich, in the range 10^{-8} – 10^{-6} M) were then assessed by administering via the perfusing cannula. Analysis of the Langendorff experiments was conducted on the LabChart software (Version 7, ADInstruments).

4.2.4 Assessment of Peripheral Vascular Function

A 2nd order branch of the femoral artery was dissected into cold Krebs solutions and then 2mm sections were mounted onto a wire myograph (Section 2.4). Vessels were maintained at 38°C in Krebs solution, and were aerated with 95% O_2/5% CO_2 gas. To assess vasoconstrictor responses vessels were exposed to increasing doses of NE, 5-HT, PE, and ET-1 at 2-minute intervals. To assess vasodilator function vessels were pre-constricted with K+, then exposed to increasing doses of Ach, or SNP. To determine the NO dependent component of the Ach response, vessels were treated with 5µm L-NAME.

4.2.5 Assessment of Molecular Pathways in the Heart

Frozen heart tissue was powdered on dry ice, and separated into 50mg aliquots, then stored at -80°C. Extraction of proteins was conducted in TK lysis buffer (Section 2.7.1). For Western blot analysis, protein extracts were separated on a 10% agarose gel, then stained with primary antibody and secondary antibodies (Table 2.2). Gel bands were then quantified as described in Secction 2.7.2.

A further set of frozen tissue aliquots were used to conduct RNA extraction using a Qiagen RNEasy mini kit (Section 2.7.3). RNA samples were then converted into cDNA libraries using Qiagen Reverse Transcriptase kit. DNA primers to various mRNAs of interest were designed and tested – only primers that gave a single peak, with a clear dose dependent relationship were used further (Table 2.3). mRNA expression analysis was then conducted as per section 2.7.4.

4.2.6 Assessment of Embryonic Lung Maturation

Perfusion fixed lungs were embedded in paraffin, then sectioned into 8µm sections with an ultramicrotome (section 2.8). Slides were stained with haemotoxylin and eosin (section 2.6) then imaged. Measurements (n=20) of parabronchial atrial diameter, atrial septum thickness, air capillary diameter, and distance between air capillary and nearest blood vessel (diffusion distance), and 5 measurements of thickness of gas exchange zone were taken per animal.

4.2.7 Statistics

All data except for dose-response curves were analysed using a one-way ANOVA with Tukey *post-hoc* test, conducted on GraphPad Prism 7 (Graphpad Software, CA, USA) software. Statistical differences ($p<0.05$) are demonstrated by different letters. Dose-response curves were assessed using a mixed-model two-way ANOVA. All significant differences to control values are indicated by *, whilst significant differences between Dex and Beta values are indicated by †. For all comparisons, significance was taken as $P<0.05$.

4.3 Effects on Embryonic Growth

Both Dex and Beta resulted in a significant reduction in embryo mass, relative embryo mass, partitioning of resources, and CRL (Fig.4.2), with the effects of Beta being more pronounced. Beta significantly increased relative head diameter, whilst Beta and Dex increased relative brain and heart mass, and liquid content of the egg with Beta having a greater effect**.**

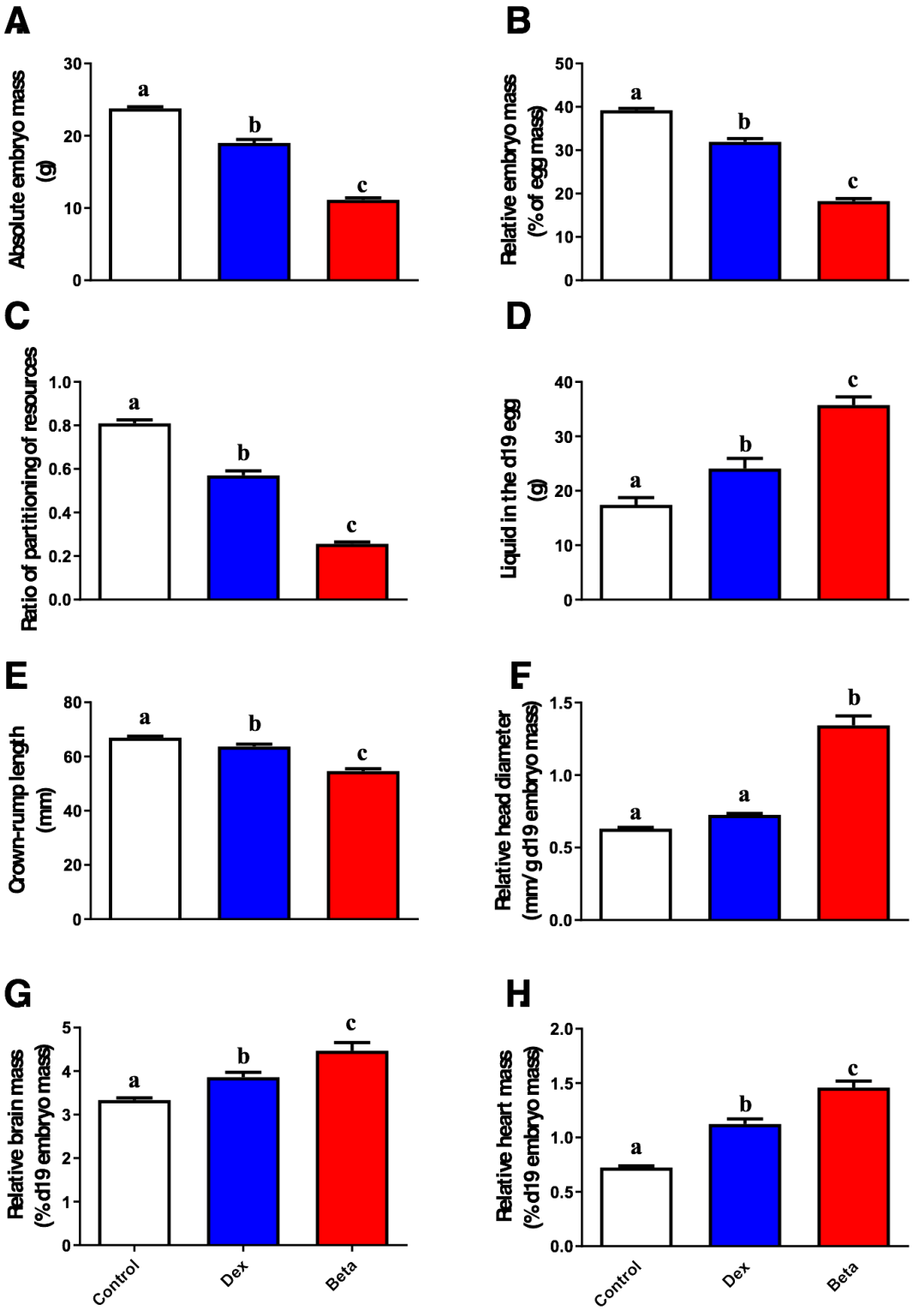
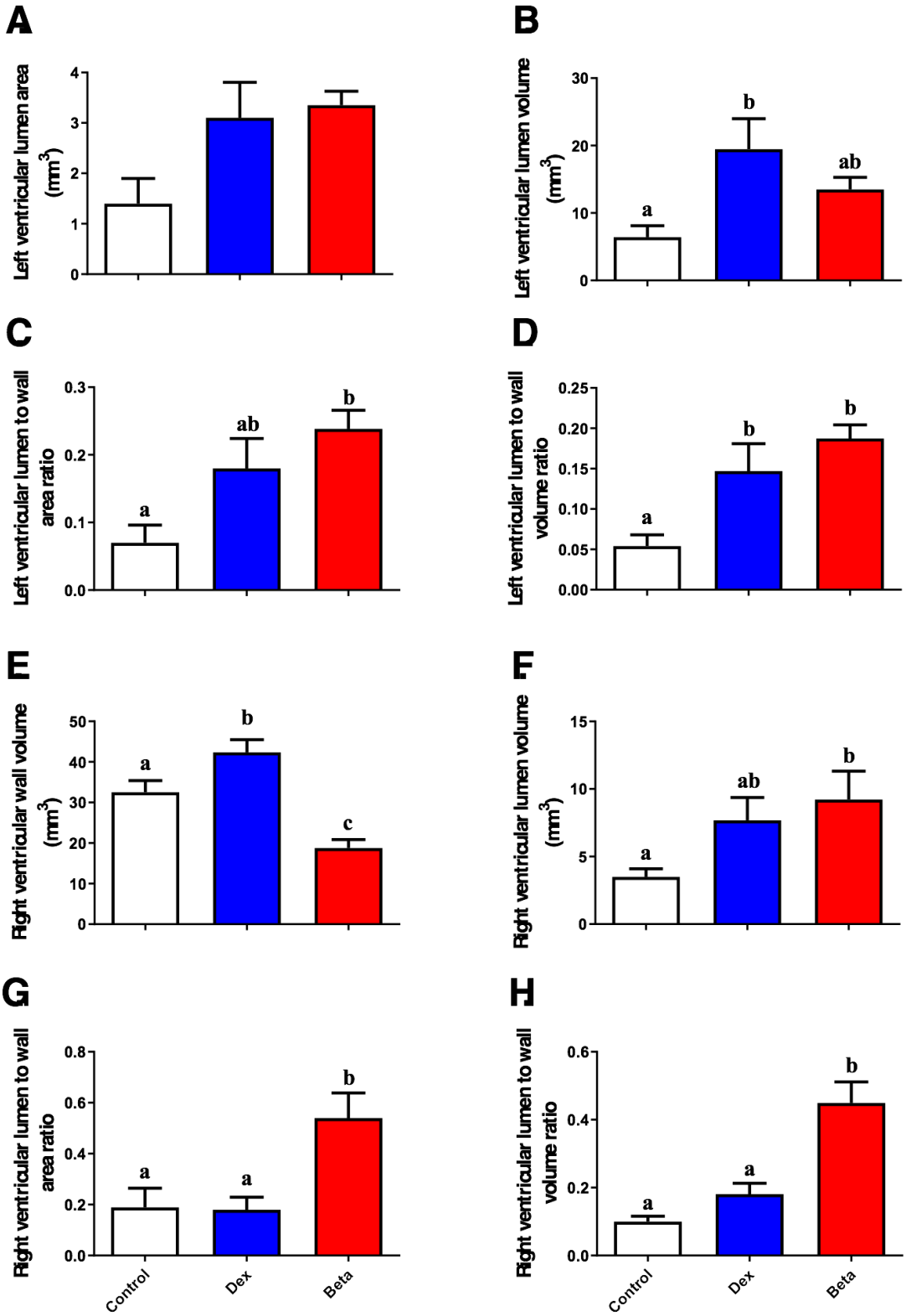


Figure 4.2 Day 19 biometry. A. Absolute embryo mass (g), B. Relative embryo mass as % of total d19 egg mass, C. Ratio of embryonic to extraembryonic tissues in the d19 egg, D.Liquid in the d19 egg (g), E. Crown-rump length (mm), F. Head diameter relative to d19 embryo mass (mmg^{-1}), G. Brain mass as % d19 egg mass, H. Heart mass as % d19 egg mass, in control (n=10), dexamethasone, and betamethasone treated (n=10) embryos, at day 19 of incubation. Ordinary one-way ANOVA with Tukey test, statistically significant differences denoted by different letters.

4.4 Effects on Cardiac Structure & Function

Both Dex and Beta induce left ventricular dilatation in terms of left lumen volume, ratio of left lumen to wall area and volume ratios (Fig.4.3). Beta also resulted in a significant dilatation of the right ventricle with a significantly increased lumen to wall area and volume ratio. This is also associated with a significant reduction in right ventricular wall volume. The aortic wall thickness was reduced following both Dex and Beta treatment, with Beta having a more severe effect (Fig.4.4). Dex resulted in an increase in aortic lumen diameter, whilst Beta resulted in a decrease.

In terms of cardiomyocyte structure, Dex significantly increased cardiomyocyte volume (Fig.4.5) and reduced % mono-nucleation, whilst Beta resulted in a significant reduction in cardiomyocyte nuclear density.


A
Left ventricular lumen area (mm3)
0
1
2
3
4
B
Left ventricular lumen volume (mm3)
0
10
20
30
a
b
ab
C
Left ventricular lumen to wall area ratio
0.0
0.1
0.2
0.3
a
ab
b
D
Left ventricular lumen to wall volume ratio
0.00
0.05
0.10
0.15
0.20
0.25
a
b
b
E
Right ventricular wall volume (mm3)
0
10
20
30
40
50
a
b
c
F
Right ventricular lumen volume (mm3)
0
5
10
15
a
ab
b
G
Right ventricular lumen to wall area ratio
0.0
0.2
0.4
0.6
0.8
a
a
b
Control
Dex
Beta
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Right ventricular lumen to wall volume ratio
0.0
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Beta

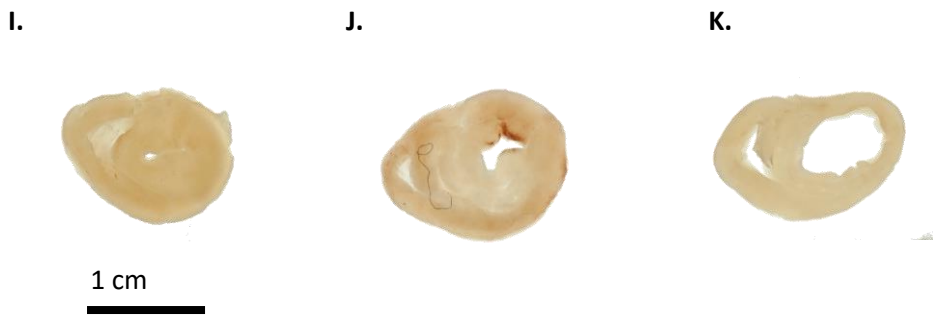


Figure 4.3 Cardiac stereology. A. left ventricular lumen area, B. left ventricular lumen volume, C. Ratio of left ventricular lumen to wall area, D. ratio of left ventricular lumen to wall volume left ventricle wall volume, E. right ventricular volume, F. right ventricular lumen volume, G. ratio of right ventricular lumen to wall area, H. ratio of right ventricular lumen to wall volume, I. representaive mid-section of control heart, J. representative mid section of Dex treated heart, K. representative mid section of Beta treated heart, in control (n=10), dexamethasone (n=10), and betamethasone treated (n=10) embryos, at day 19 of incubation. Ordinary one-way ANOVA with Tukey test, statistically significant differences denoted by different letters.

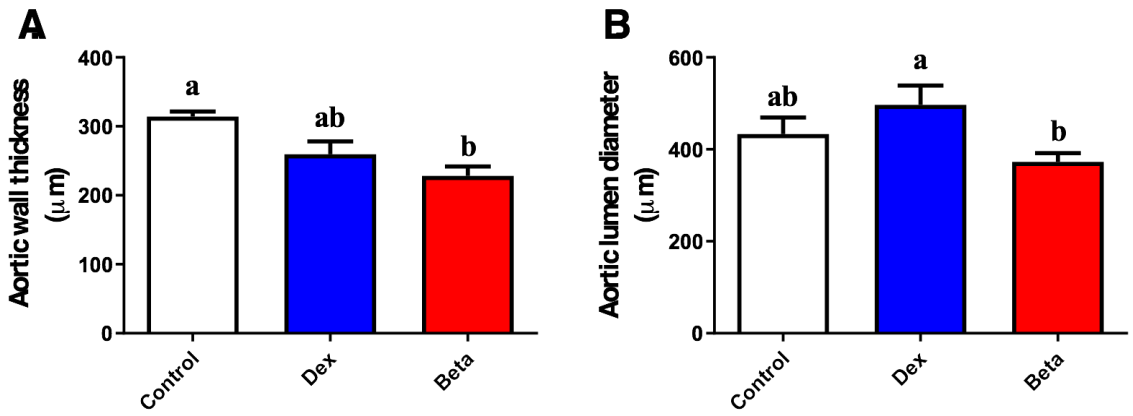


Figure 4.4 Aortic wall thickness and lumen diameter. A. Aortic wall thickness (µm), B. Aortic lumen diameter (µm) in control (n=10), dexamethasone, and betamethasone treated (n=10) embryos, at day 19 of incubation. Ordinary one-way ANOVA with Tukey test, statistically significant differences denoted by different letters.

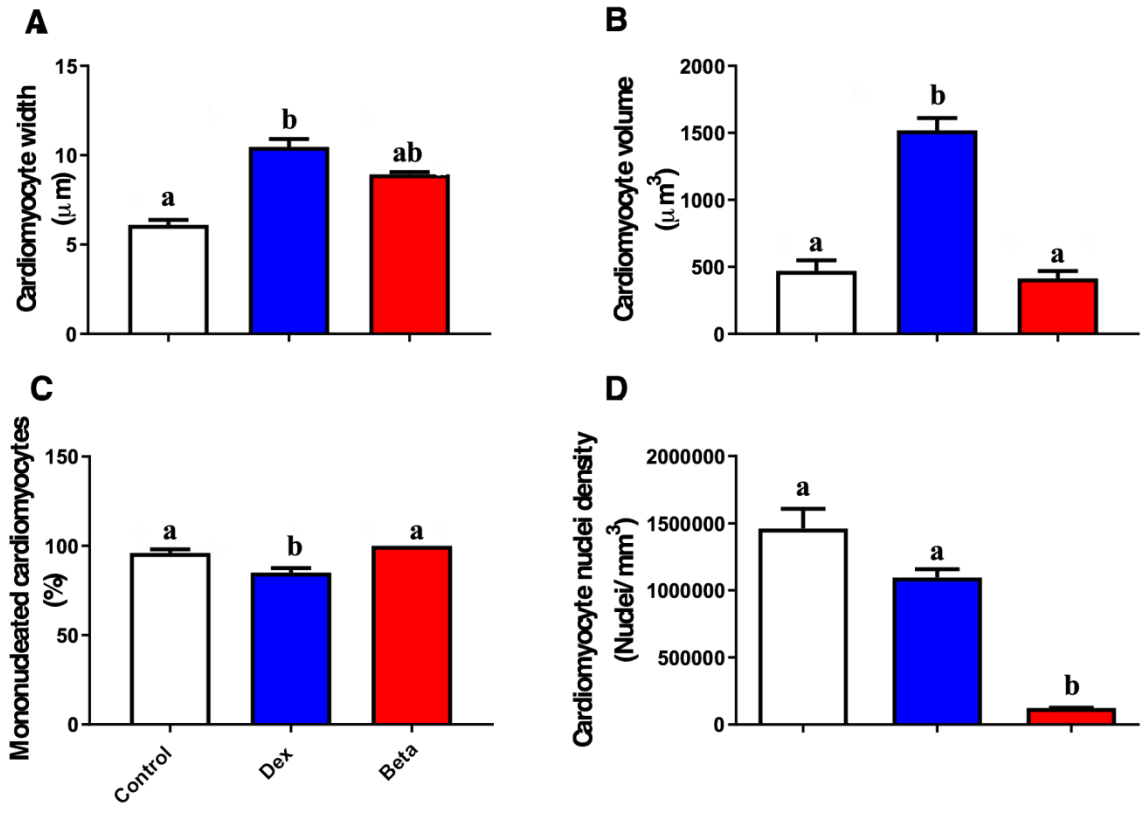


Figure 4.5 Cardiomyocyte analysis. A. cardiomyocyte width (µm), B. calculated cardiomyocyte volume (mm^3), C. percentage of all cardiomyocytes counted that were mononucleated, D. number of cardiomyocyte nuclei per mm^3 of left ventricular tissue in control (n=10), dexamethasone, and betamethasone treated (n=10) embryos, at day 19 of incubation. Ordinary one-way ANOVA with Tukey test, statistically significant differences denoted by different letters.

Beta significantly impaired systolic function in terms of both LVDP and contractility index (Fig.4.6). Both Dex and Beta significantly impaired diastolic function, with the effects of Beta being more pronounced.

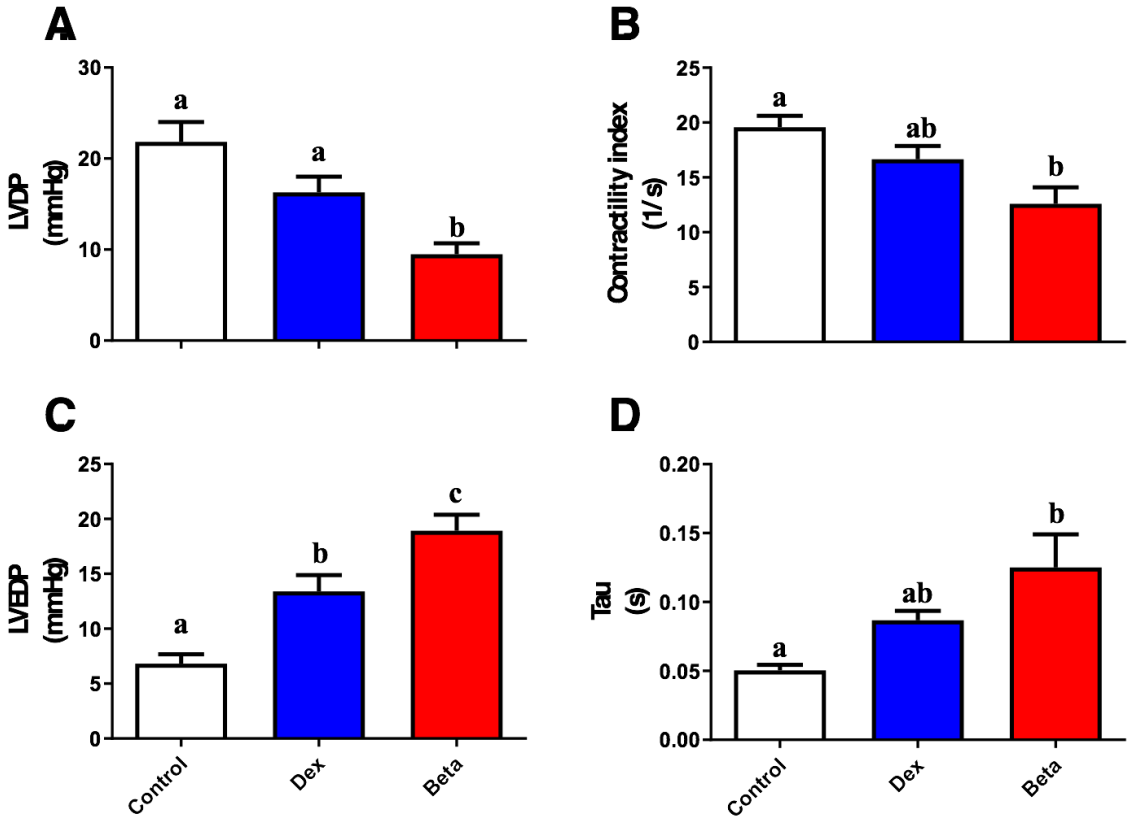


Figure 4.6 Diastolic and systolic function. A. Left ventricular developed pressure, B. contractility Index, C. Left ventricular end diastolic pressure, D. Tau, in control (n=10), dexamethasone, and betamethasone treated (n=10) embryos, at day 19 of incubation. Ordinary one-way ANOVA with Tukey test, statistically significant differences denoted by different letters.

Beta but not Dex significantly reduced CFR per unit heart mass, and cycle duration, with a significant reduction in systolic duration and no change in diastolic duration (Fig.4.7).

Both Dex and Beta significantly impaired the LVDP response to carbachol at the highest dose, and Beta also significantly impaired the HR response at this dose (Fig.4.8). There was no significant effect of either drug on isoprenaline responses or overall LVDP or HR response ratios.

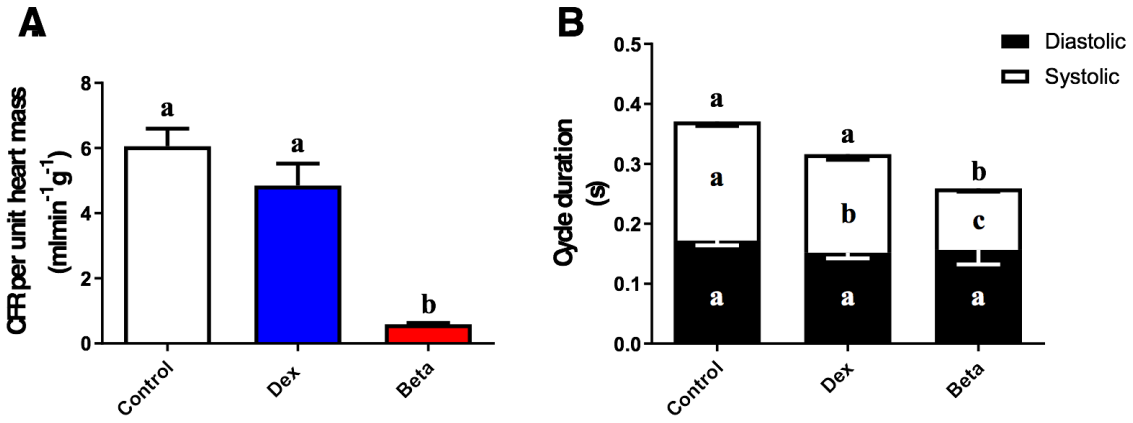


Figure 4.7 Coronary flow rate and heart cycle duration. A. Coronary flow rate relative to heart weight, B. Systolic and diastolic cycle duration in control (n=10), dexamethasone, and betamethasone treated (n=10) embryos, at day 19 of incubation. Ordinary one-way ANOVA with Tukey test, statistically significant differences denoted by different letters.

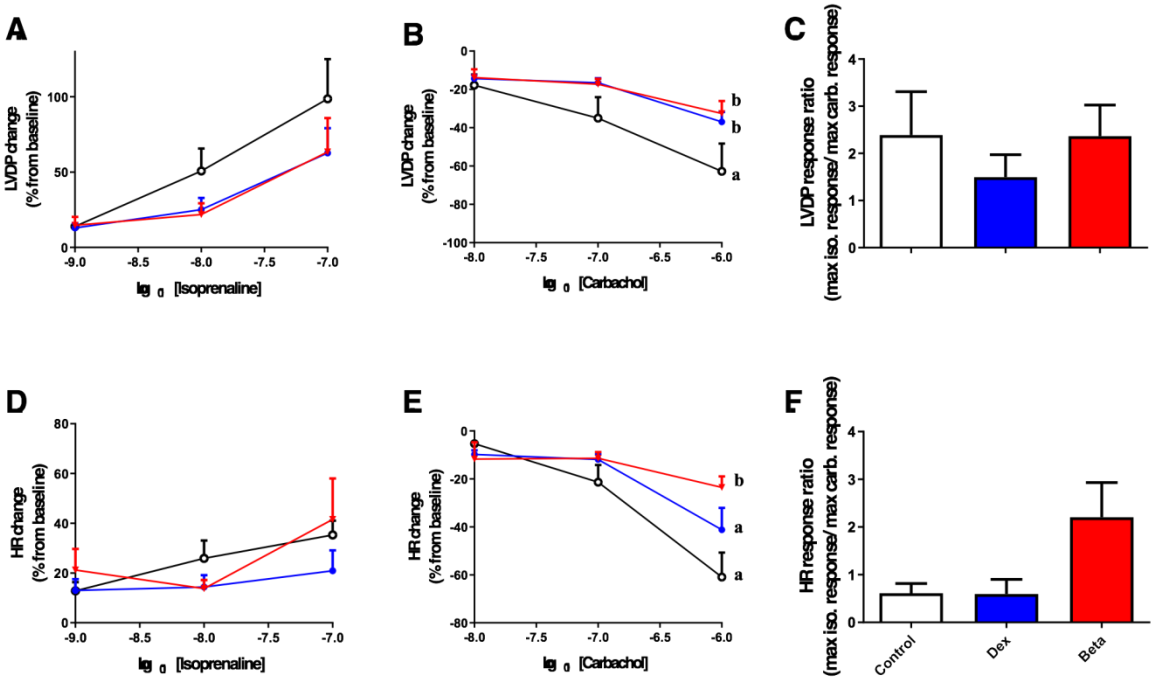


Figure 4.8 Cardiac responses to autonomic agonists. A. left ventricular developed pressure response to isoprenaline, B. left ventricular developed pressure response to carbachol, C. ratio of the maximum developed pressure responses to isoprenaline and carbachol, D. heart rate response to isoprenaline, E. heart rate response to carbachol, F. ratio of heart rate response to isoprenaline and carbachol, in control (n=10) Dex (n=10) and Beta

(n=10) treated embryos at day 19 of incubation. A,B,D,E two-way ANOVA with Tukey post-hoc test, C,F ordinary one-way ANOVA with Tukey test statistically significant differences denoted by different letters.

4.5 Effects on Peripheral Vascular Function

Dex significantly increased femoral vascular response to KCl, whilst Beta significantly reduced the area under the curve of the KCl response curve (Fig.4.9). Beta also significantly impaired the constriction to PE. There was no significant effect on 5-HT responses, however both Dex and Beta increased constriction in response to ET-1, with the effect of Dex being more pronounced.

Beta significantly reduced the relaxation response to Ach (Fig.4.10), which was associated with a significant impairment of NO-independent relaxation, and an increased in NO-dependent relaxation. Beta also significantly impaired SNP induced relaxation.

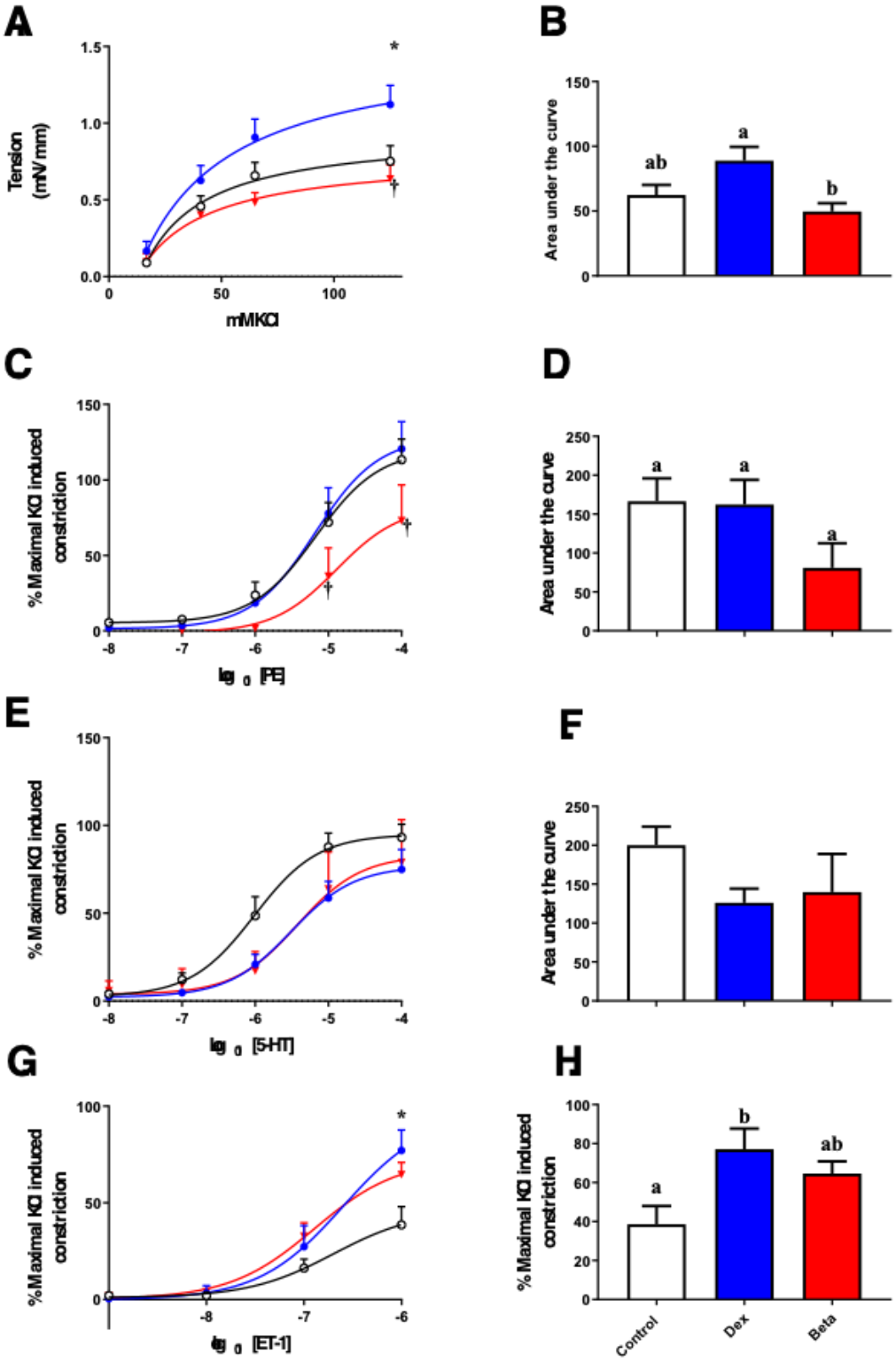


Figure 4.9 Vasoconstrictor responses. A. tension generated to KCl (mM), B. Area under the curve of KCL response (arbitrary units), C. response to PE as a % of maximal KCl response, D. pD2 of PE response, E. response to 5-HT as a % of maximal KCl response, F. area under the curve of 5-HT response (arbitrary units), H. response to ET-1 as a % of maximal KCl response, H. maximal ET-1 response as a % of maximal KCl response, in control (white, n=10) Dex (blue, n=10) and Beta (red, n=8) treated embryos, ACEG two-way ANOVA with Tukey post-hoc test where * represents statistically different to control, and † represents statistically different to Dex, BDFH ordinary one-way ANOVA with Tukey test. Statistically significant differences denoted by different letters.

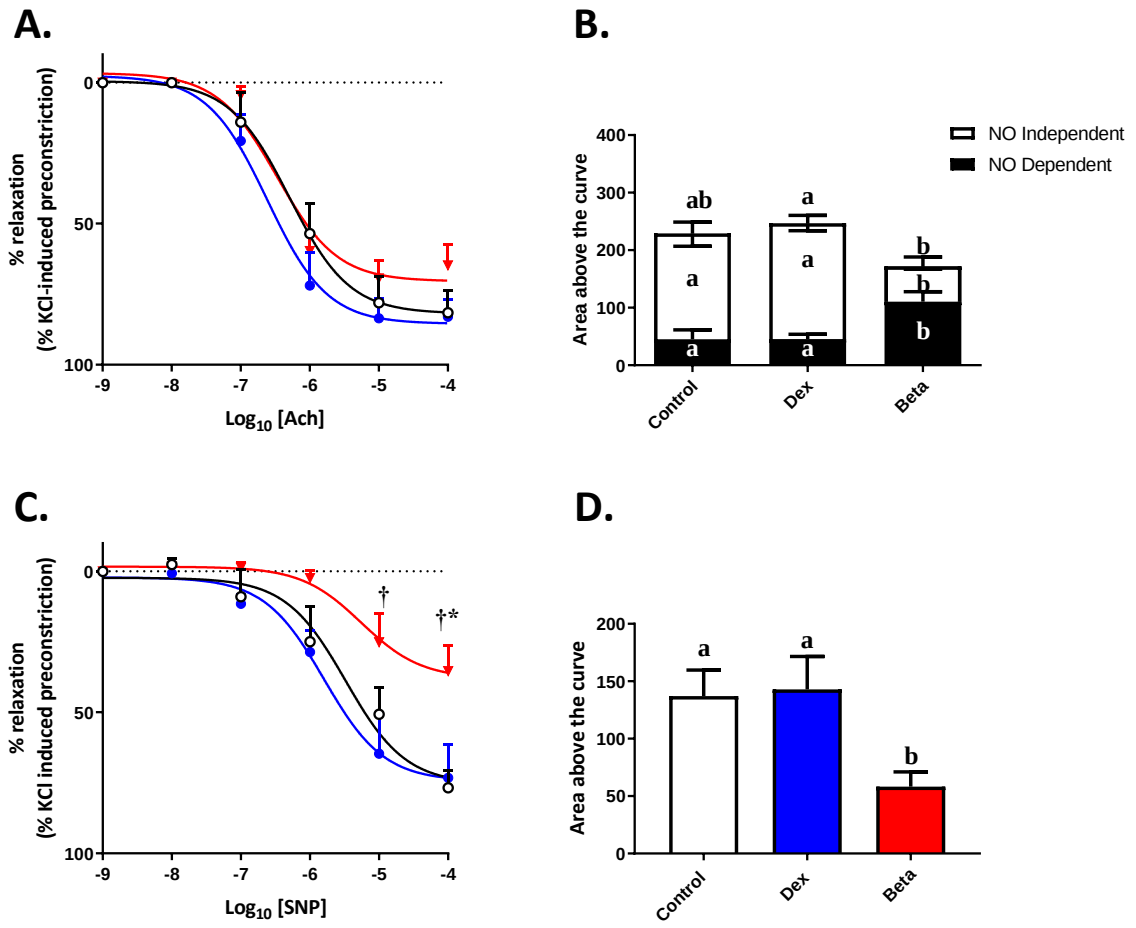


Figure 4.10 Vasodilator responses. A. vascular relaxation from KCl-induced pre-constriction in response to increasing doses of Ach, B. breakdown of the vasodilator response to Ach, by treating vessels with the eNOS inhibitor L-NAME, and assessing the area above the Ach dose-response curve, before and after inhibition. The area above the curve is dependent on eNOS and hence NO can be determined, C. vascular relaxation from KCl-induced pre-constriction in response to NO donor SNP, in control (white, n=10) Dex (blue, n=10) and Beta (red, n=8) treated embryos. AC two-way ANOVA with Tukey post-hoc test where * represents statistically different to control, and † represents statistically different to Dex, B ordinary one-way ANOVA with Tukey test. statistically significant differences denoted by different letters.

4.6 Effects on Molecular Pathways in the Heart

Dex significantly increased protein carbonylation in the heart whereas Beta did not (Fig.4.11). Whilst there is no significant effect of either drug on catalase expression. Both Dex and Beta increased activation of the pERK and pSAPK/JNK pathways, associated with downstream increased expression of hsp60 and PDI, whilst Dex also increased hsp27 and hsp70. Both drugs increased total caspase 3 levels but only Dex increased cleaved caspase-3 levels. Beta resulted in a significant upregulation of p38 and p53 mRNA.

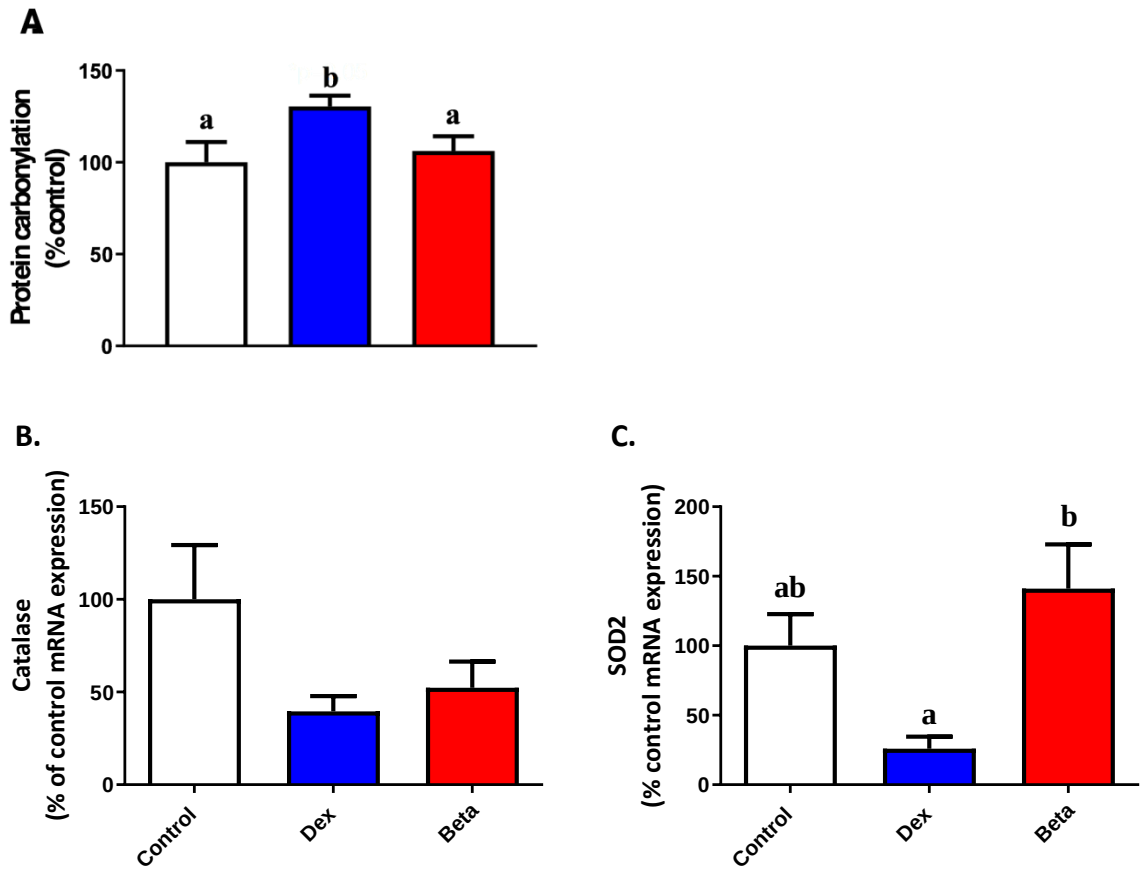


Figure 4.11 Oxidative stress indices. A. Levels of protein carbonylation detected by western blot, B. mRNA expression of Catalase, C. mRNA expression of SOD2 (Mn dependent), in control (n=10) and dexamethasone (n=10) treated embryos. Ordinary one-way ANOVA with Tukey test. statistically significant differences denoted by different letters.

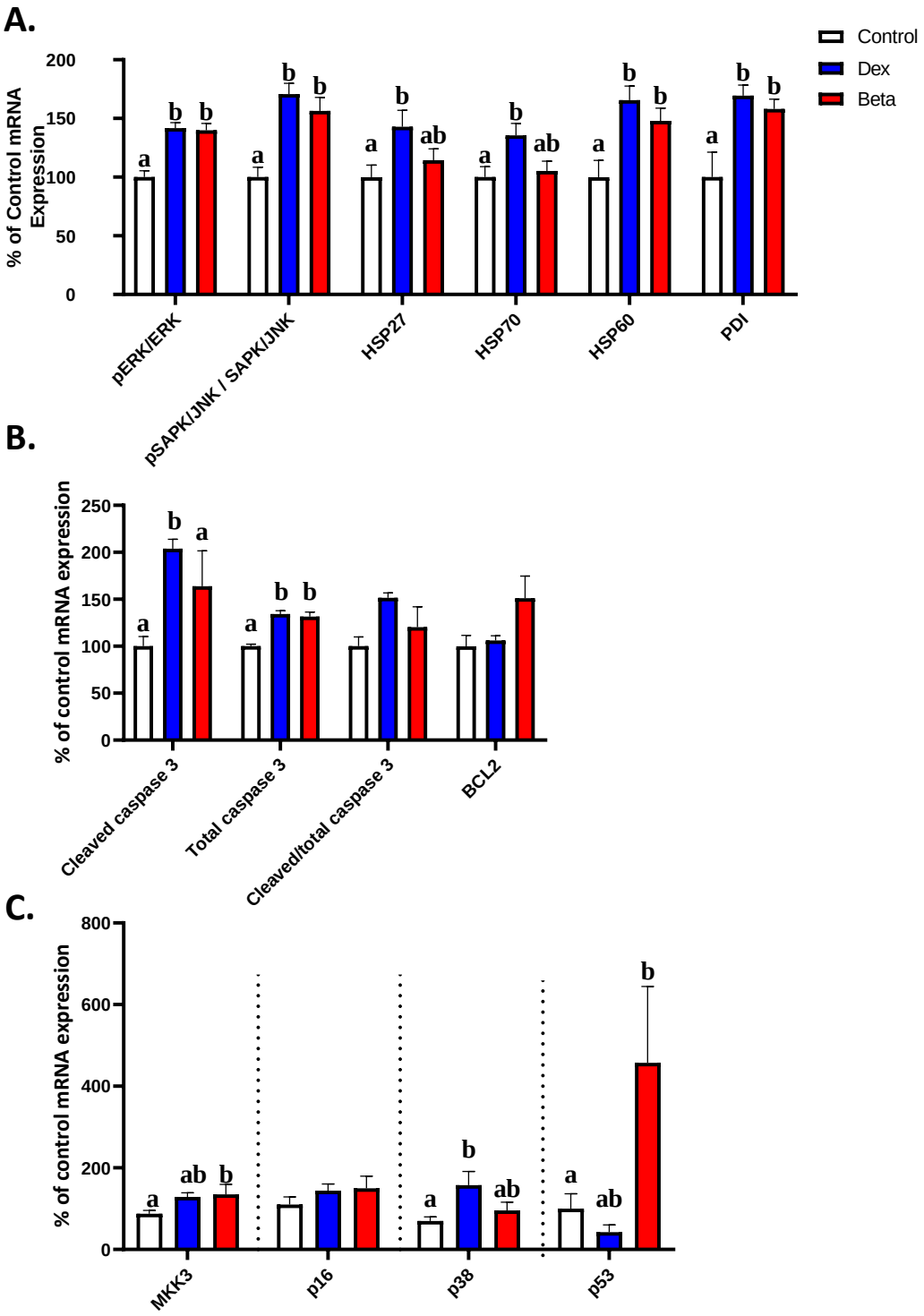


Figure 4.12 Cellular stress, apoptosis, and cell cycle markers. A. phosphorylated ERK protein/non-phosphorylated levels, phosphorylated SAPK/JNk / non-phosphorylated levels, protein expression of chaperones HSP27, HSP70, HSP60, and PDI B.. protein levels of caspase-3 cleaved caspase-3, ratio of cleaved caspase-3 to total caspase 3, BCL, C. mRNA levels relative to control of MKK3, p16, p38, p53, in control (n=6) dexamethasone (n=6) and betamethasone (n=6) treated embryos. Ordinary one-way ANOVA with Tukey test. statistically significant differences denoted by different letters.

When GR mRNA levels were assessed, Dex treated hearts had a significantly lower level than Beta hearts although neither were significantly different to controls (Fig.4.13). In the Dex treated embryos this resulted in a significant decrease in GR protein levels, whilst in Beta embryos the levels were unchanged.

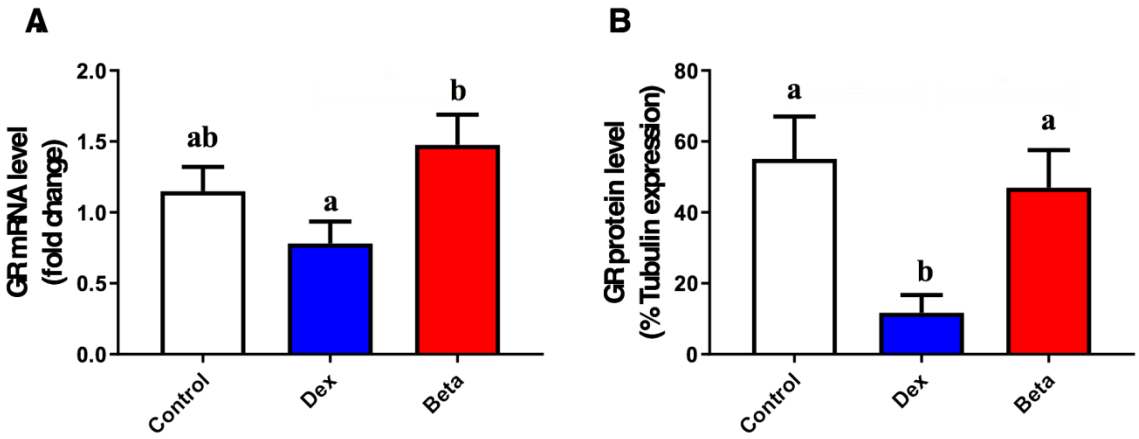


Figure 4.13 GR mRNA and protein levels. A. Levels of GR mRNA (fold change) B. GR protein levels (relative to tubulin expression), in control (n=10) dexamethasone (n=10), and betamethasone (n=8) treated embryos. Ordinary one-way ANOVA with Tukey test. statistically significant differences denoted by different letters.

4.7 Effects on Lung Maturation

Both Dex and Beta increased protein expression of caveolin-1 and SP-B, however only Beta treated hearts had levels significantly elevated relative to controls (Fig.4.14). Both drugs significantly increased parabronchial atrial and air capillary diameter, and decreased diffusion distance (Fig.4.15). However, only Dex significantly increased thickness of the gas exchange zone.

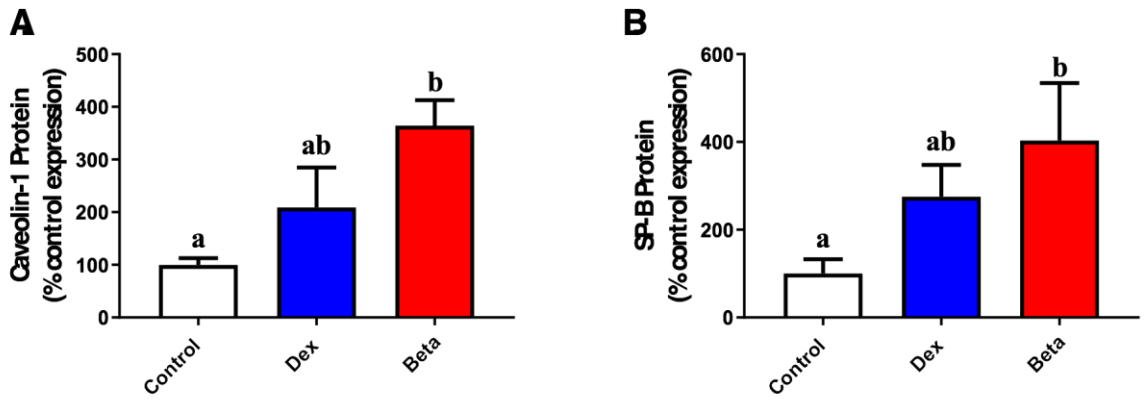


Figure 4.14 Protein markers of lung maturation. A. Expression of caveloin-1 (% control expression), B. expression of SP-B (% control expression), in control (n=6) dexamethasone (n=6) and betamethasone (n=6) treated embryos. Ordinary one-way ANOVA with Tukey test. statistically significant differences denoted by different letters.

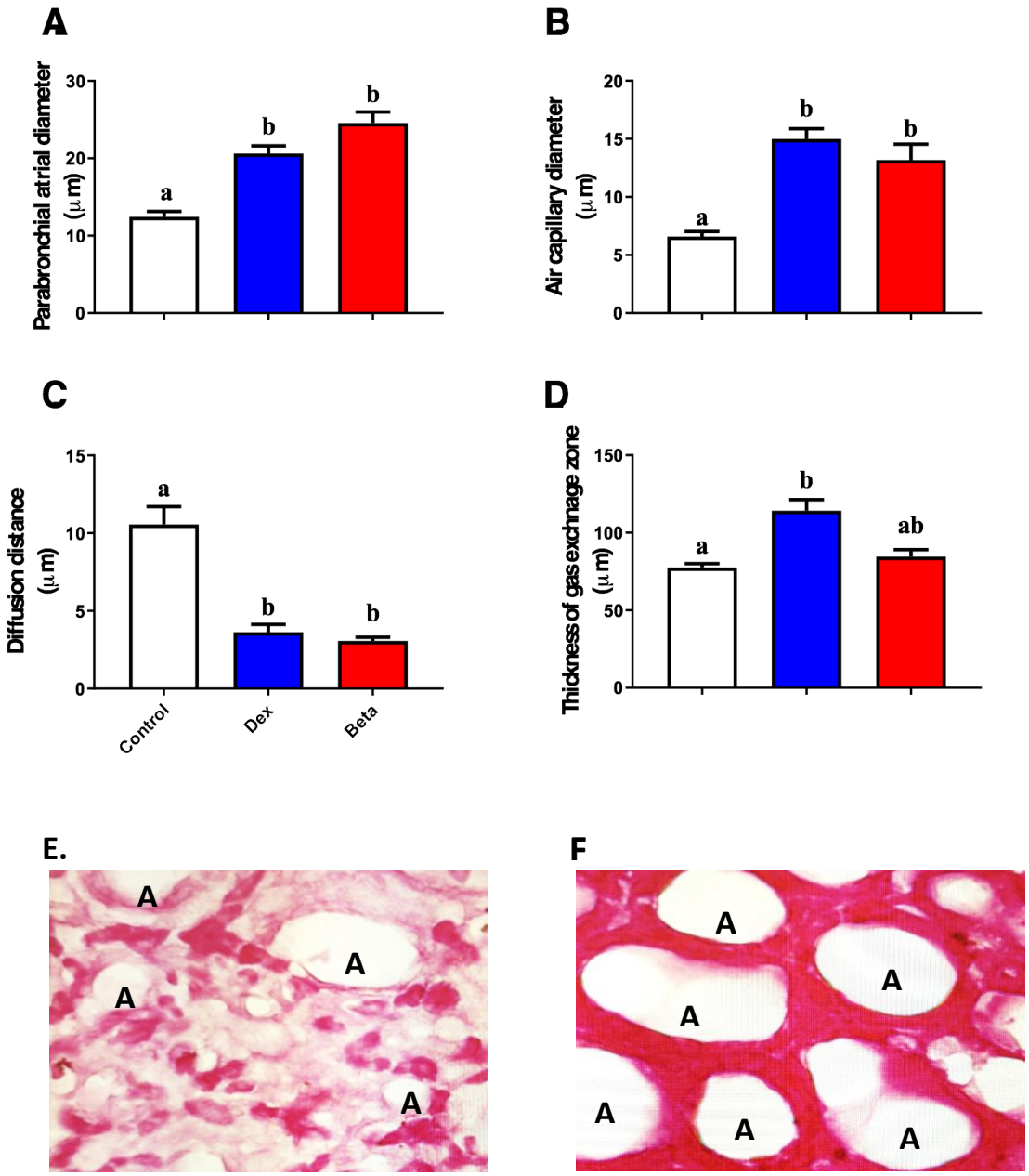


Figure 4.15 Morphological markers of lung maturation. A. parabronchial atrial diameter (µm), B. air capillary diameter (µm), C. diffusion distance (µm), D. thickness of gas exchange region adjacent to parabronchi (µm), E. representative section of control lung showing air capillaries (A), F. representative section of Dex lung showing air capillaries (A), in control (n=8) dexamethasone (n=8) and betamethasone (n=6) treated embryo Ordinary one-way ANOVA with Tukey test. statistically significant differences denoted by different letters.

4.8 DISCUSSION

4.8.1 Summary

The data in this chapter show that Dex and Beta have differential adverse effects on embryonic growth and cardiovascular development. Both Dex and Beta resulted in a significant asymmetric growth restriction at day 19 of incubation, with Beta having a more severe effect. This is echoed in the cardiac function; Beta resulted in a significant systolic dysfunction, whilst both Beta and Dex induced a diastolic dysfunction, with the effects of Beta being more severe. Both glucocorticoids induced left ventricular dilatation, again with Beta being more severe, and Beta also induced a right ventricular dilatation. In terms of individual cardiomyocytes, Beta resulted in a significant reduction in nuclear density whilst not affecting cardiomyocyte volume, whereas Dex resulted in a significant increase in cardiomyocyte volume with only a small reduction in nuclear density.

Dex and Beta also display divergent effects on peripheral vascular function. Whilst Dex increased the femoral constrictor response to KCl, Beta impaired this and the vasoconstrictor response to PE. Both drugs enhanced constriction to ET-1 with Dex having a more profound effect. Beta significantly impaired relaxation in response to NO donor SNP, and to vasodilator Ach, the latter being associated with a significant decrease in NO-independent vasodilatation.

Beta did not show any significant increase in protein carbonylation (associated with oxidative stress) whilst Dex did, however both treatments resulted in up-regulation of cell stress kinase

pathways pERK and pSAPK/JNK. Both resulted in activation of downstream mitochondrial and ER chaperones hsp60 and PDI, and Dex also activated cytosolic chaperones hsp27 and hsp70. Both treatments induced an increase in total caspase-3 levels, however only Dex increased cleaved caspase-3 levels. Beta also resulted in significant upregulation of cell cycle mediator p53. Dex demonstrated negative feedback at the level of the cardiac GR, which was absent in Beta-treated hearts.

Both glucocorticoids resulted in similar histological maturation of embryonic lungs, and both significantly increased expression of caveolin-1 and SPB, with Beta having a greater effect.

In this chapter we addressed the hypothesis that Dex and Beta exposure in late cardiovascular development will result in cardiac and peripheral vascular dysfunction and that the severity and nature of this dysfunction will differ depending on which steroid is administered. The data presented here support this hypothesis.

4.8.2 Effects on embryonic growth

Both glucocorticoids resulted in a significant asymmetric growth restriction. This is a common side effect of glucocorticoids seen in many animal models (Newnham & Moss, 2001). It is likely that this is at least in part due to a switch away from tissue accretion and towards maturation, resulting in reduced body mass accumulation over the final period of development than in control embryos (Fowden & Forhead, 2015). The asymmetric nature of

this growth restriction is interesting as it reflects what is seen in various other antenatal insults such as fetal hypoxia or fetal nutrient restriction (Giussani, 2016; Elias et al., 2016). This is likely due to differential effects of glucocorticoids on the vascular perfusion and metabolism of the brain versus other organs, and is a key component of the additional role of glucocorticoids as a stress hormones; in times of stress when oxygen or nutrient delivery may be compromised it is essential to protect the developing brain even if at the expense of other organ systems.

Plausible explanations for the more severe effect of Beta compared with Dex on late stage embryonic growth may relate to differences in binding affinity or clearance. It is possible that the Beta stereoisomer binds to the GR with a higher affinity than Dex overall leading to greater levels of activation. Alternatively, the Na^+-acetate Beta formulation is known to have a longer plasma half-life than the Na^+-phosphate formulations and hence Beta, which contains both formulations, may be active in the fetal circulation for longer. Most pharmacological studies suggest a similar glucocorticoid potency of Dex and Beta of around 25-30x greater than hydrocortisone (Buttgereit et al., 1999; Parente, 2017). However, there is some evidence for subtle differences. For example, Beta has been shown to induce apoptosis in a lymphoblast cell line at lower concentrations than Dex (Longui et al., 2005). Conversely, data in the present study which show a fall in cardiac GR after Dex but not Beta treatment in the day 19 chicken embryo arguses against Beta having a greater affinity for GR compared with Dex. There is however extensive evidence for the Beta acetate drug formulation remaining in the fetal circulation longer than the phosphate formulation (Samtani et al., 2005; Salem and Najib, 2012). Schmidt et al., (2016) demonstrated that the

combined Beta treatment was more effective at inducing lung maturation than either Dex or Beta phosphate, which had similar effects to each other. However in that study, Dex and Beta phosphate administered at the same dose did display subtle differences. For example, Beta phosphate resulted in significant upregulation of surfactant proteins -A, -B, and -C as well as ATP-binding cassette family member A3 (ABCA3), suggesting that drug formulation is not the only difference (Schmidt et al., 2016). In order to determine whether drug formulation or nature of the stereoisomers is the cause of the differential effects observed in this model, a further study directly comparing Dex Phosphate with both Beta phosphate and Beta acetate would be valuable.

4.8.3 Effects on Cardiac Structure and Function

Both Dex and Beta induced cardiac diastolic dysfunction with an increased LVEDP and Tau (the time constant of ventricular relaxation), and further Beta significantly impaired both LVDP, contractility index, CFR and the systolic duration of the cardiac cycle. This was also associated with a significant left ventricular dilatation and divergent effects on isolated cardiomyocytes, with Dex inducing hypertrophy, whilst Beta significantly reducing nuclear density whilst not affecting mono-nucleation.

The severe dysfunction observed in the Beta treated hearts may result from the pronounced decrease in cardiomyocyte number leading to markedly thinner ventricle walls and a lower number of total contractile units. This has ultimately resulted in a dilated cardiac phenotype,

worryingly resembling dilated cardiomyopathy, reminiscent of end stage cardiac failure in adults (Fig.4.16). The additional dilatation observed in the right ventricle in the Beta- but not Dex-treated embryos may also be due to the severe reduction in cardiomyocyte number, although this was not studied directly in the right ventricle. Conversely, it appears that Dex treatment has resulted in the more traditional switch from tissue accretion to tissue maturation in the heart, accelerating a change from cardiomyocyte hyperplasia towards hypertrophy, an effect typically seen towards term (Oparil et al., 1984; Christoffels et al., 2000; Corrigan et al., 2010). These hypertrophic effects of Dex on the heart without significant reduction in cardiomyocyte number may have compensated in part for the ventricular dilatation, thereby better maintaining systolic and diastolic function in Dex compared with Beta treated hearts. Even in the absence of dilatation, significant cardiomyocyte hypertrophy would still adversely affect cardiac function in Dex-treated hearts compared to controls, as there is evidence that premature hypertrophic maturation impairs electrical conductance and contractility, affecting contractility and relaxability normally (Fig.4.16; Severinova et al., 2019; Frey & Olson, 2003).

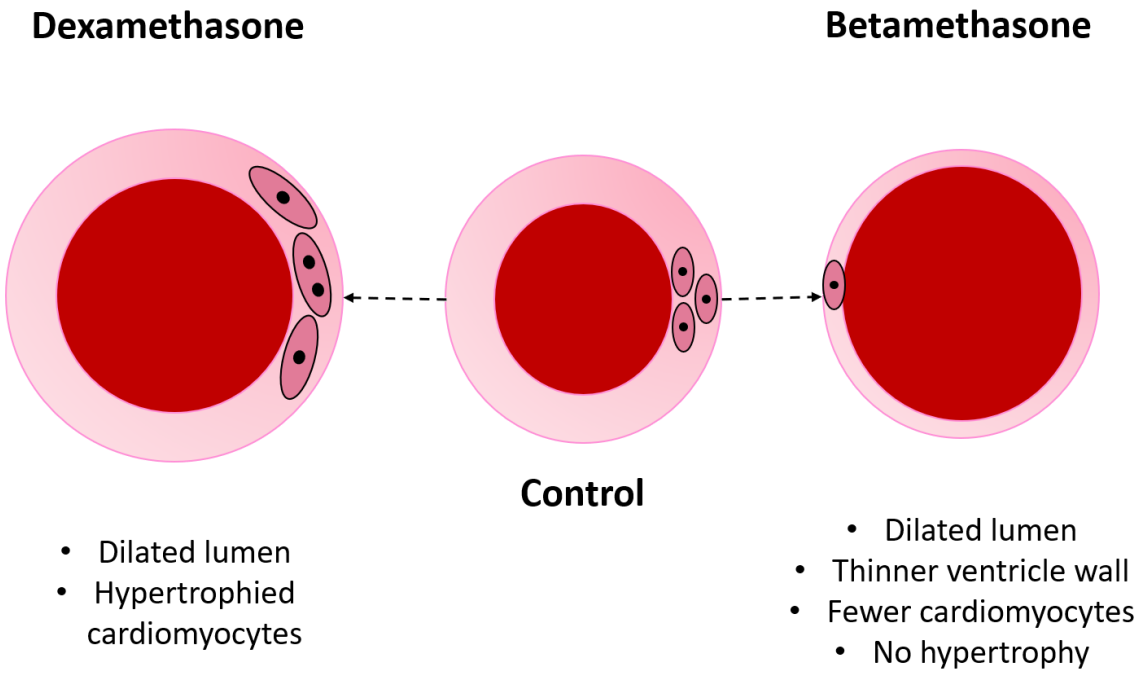


Figure 4.16 Effects of Dex and Beta on cardiac structure.

4.8.4 Effects on peripheral vascular function

Divergent effects of Dex and Beta are also reflected in the *ex vivo* femoral vascular function. Dex treatment resulted in a switch towards a vasoconstrictor phenotype with an enhanced KCl and ET-1 response, whilst vasodilator responses were not affected. This may be secondary to increased hyperplasia or hypertrophy of vascular smooth muscle cells (vSMCs; McLellan et al., 1992; Volk et al., 2010), and alterations to either ET-1 receptor densities (e.g. up-regulation of ET_A) or downstream contractile pathways (Kutzler et al., 2003; Lee et al., 2014) resulting in an enhanced vascular response.

Beta treatment however results in a distinct vascular phenotype. KCl-induced constriction is slightly reduced compared to controls but is significantly different to the enhanced Dex response. This suggests that the hyperplasia/hypertrophy of the vSMCs seen following Dex treatment is absent when Beta is given. In addition, there is a significant impairment of the vascular response to PE, even when corrected for individual vessel contractility. PE is a selective α_1-adrenergic receptor agonist that activates phospho-lipase C induced IP_3 production, downstream Ca^{2+} release and myosin light chain kinase (MLCK) activation, resulting in increased vascular constriction (Brozovich et al., 2016). This is the mechanism largely activated in the peripheral circulation by the sympathetic nervous system, which controls vascular tone via synapses with vSMCs, which mainly release noradrenaline (NA). However, NA is a non slective adrenergic agonist and so vascular responses will differ to those induced by PE if other adrenergic receptors are also present (Smith & Maani, 2019). Decreased PE responsiveness could be secondary to a decrease in α_1-adrenergic receptor density in the femoral vascular bed. Whilst there is little evidence for glucocorticoids modulating α-adrenergic signalling in the vasculature, cross-talk between the glucocorticoid and α-adrenergic pathways has been demonstrated in the heart (Lister et al., 2006). It is also possible that there is downregulation in the downstream contractile pathway. This possibility is made less likely by the fact that ET-1 responses are also slightly up-regulated relative to control embryos following Beta treatment, and ET-1-induced vasoconstriction is also primarily mediated by the ET_{-A} receptor stimulating IP_3 production (Davenport et al., 2016). Vascular responses to ET-1 in offspring exposed to antenatal Beta have indeed been shown to be enhanced secondary to activation of the cyclic ADP ribose (cADPR)/ryanodine receptor pathway (Lee et al., 2014). cADPR is an intracellular secondary messenger which regulates ryanodine receptor opening allowing Ca^{2+} release from the sarcoplasmic reticulum. Activation

of the ET_{-B} receptor can activate cADPR induced vasoconstriction in the vSMCs, whilst ET_{-A} can act via cADPR or the classic IP_3 pathway (Barone et al., 2002). Hence, it is possible that the enhanced ET-1 response is due to increased cADPR signalling, and that a downstream inhibition of the IP_3 pathway is present. Further investigation into the effects of inhibiting cADPR production or blocking specific ET-1 receptors would help illuminate the specific altered mechanisms.

Beta treatment also induces an impaired vasodilator phenotype associated with endothelial dysfunction. Overall relaxation to muscarinic agonist Ach was significantly impaired in Beta treated embryos. Ach is known to induce vasodilatation by several concurrent mechanisms, including the production of NO by endothelial NO synthase (eNOS), production of prostacyclins by cyclo-oxygenase-1 (COX-1), and production of other vascular mediators, such as H_2S, termed endothelial derived hyperpolarising factors (EDHF; Liberman et al., 2018). To investigate which pathway is impaired following Beta treatment we also assessed vascular responses to NO donor SNP, which was also significantly impaired. NO produced in the endothelium in response to various receptor agonists (such as Ach and CGRP), or in response to shear stress, rapidly diffuses into the vSMCs whereby it activates soluble guanylate cyclase (sGC) leading to increased production of cyclic guanine monophosphate (cGMP) which in turn induces vasodilatation by enhancing Ca^{2+} reuptake, and opening hyperpolarising K^+ channels (Chen et al., 2008). NO may also directly interact with sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) to reduce cytosolic Ca^{2+} levels (Zhao et al., 2015). The impaired response to SNP suggests downregulation of one or more of these pathways in the vSMCs themselves. Most evidence from adult vascular exposure to glucocorticoids suggests that impairments in NO

vasodilatation are secondary to decreased production via eNOS or iNOS (induced NO synthase), which is at odds with the results presented here (Whitworth et al., 2002). In addition, when vasodilatation in response to ACh was repeated in the presence of an eNOS inhibitor, relaxation was more severely affected in the Beta treatment group than either control or Dex embryos. This suggests that femoral vessels isolated from Beta-treated embryos are more reliant on NO-dependent vasodilatation to maintain appropriate relaxation, and indeed they have a significantly enhanced NO-dependent and impaired NO-independent vasodilator component of their relaxation capacity (see Fig.4.10B). The impaired response to the NO-donor SNP in the vSMCs results in an overall dilator dysfunction despite the upregulation of NO-dependent vasodilator mechanisms in response to ACh. It is unclear which NO-independent pathways are altered in these vessels; further investigation using specific enzyme inhibitors would help to further elucidate the dysfunctional phenotype. The differential vascular effects of Dex and Beta are summarised in Figure 4.17 below.

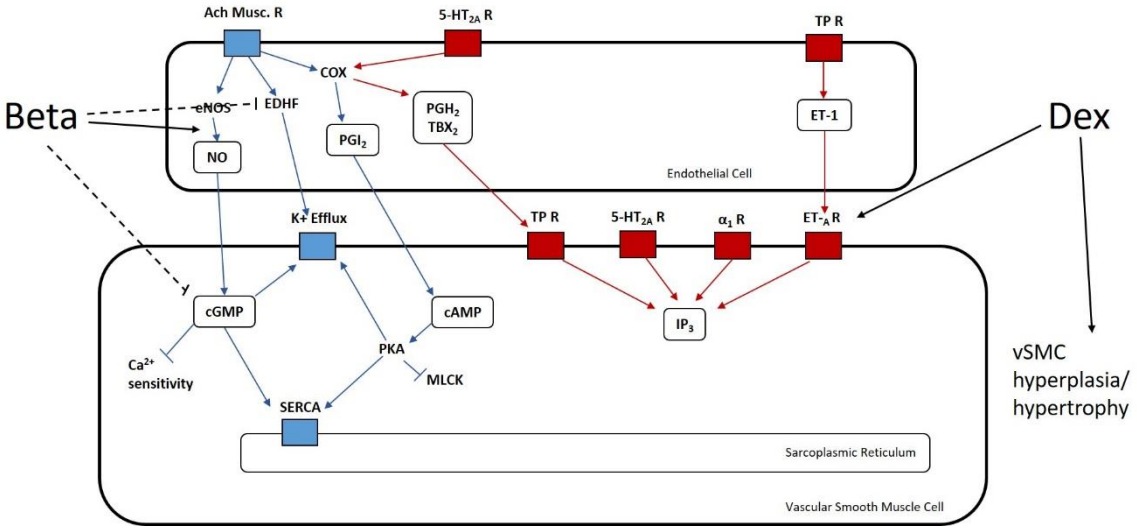


Figure 4.17 Vascular effects of Dex and Beta. Dashed arrows represent upregulation of a pathway, whilst dashed lines with flat ends represent suppression of a pathway. Interactions of vasodilators (blue) Ach which activates nitric-oxide synthase (eNOS), cyclo-oxygenase (COX) and endothelium derived hyperpolarising factors (EDHF). Production of NO and prostacyclins (PGI_2) leads to production of secondary messengers cAMP and cGMP in the vSMCs, which activate K^+ efflux channels, protein kinase A (PKA), SERCA, and inhibit myosin light-chain kinase (MLCK) and reduced Ca^{2+} sensitivity of sarcomeres. Vasoconstrictors (red) acting on 5-HT_{2A} receptors or thromboxane receptors (TP Rs) on the endothelial cells stimulate production of ET-1, thromboxane (TBX_2) and PGH_2. Activation of 5-HT_{2A}, TP, ET_{-A}, or α_1-adrenergic receptors leads to production of secondary messenger IP_3 and a subsequent increase in cytosolic Ca^{2+}.

4.8.5 Effects on molecular pathways in the heart

Whilst Dex treatment results in induction of oxidative stress in the embryonic heart, evidenced by increased protein carbonylation, and decreased levels of antioxidants (Mn)-SOD, and Catalase, Beta-treated hearts do not display these changes. However, it is evident in both cases that there is significant cellular stress as both pERK and pSAPK/JNK pathways are up-regulated, as are downstream chaperone proteins (mitochondrial and ER associated in Beta hearts, whilst also being associated with the cytosol in Dex hearts). In addition, it appears that apoptosis may be up-regulated in Dex-treated embryos, but not in those exposed to Beta, which challenges our previous observations of drastically reduced

cardiomyocyte numbers following Beta treatment. The lack of a fall in apoptotic markers in the Beta-treated embryos may reflect observations in other studies, in which Beta can drastically reduce cell proliferation without inducing caspase-3 mediated apoptosis (Scheepens et al., 2003). It is also possible that Beta exposure is reducing cardiomyocyte number by other pathways, such as autophagy (Wang et al., 2015).

The link between Dex and cellular stress in various adult tissues has been well documented, and in many cases this is related to induction of oxidative stress (Mutsaers & Tofighi, 2012; Lv et al., 2018; Suwanjang et al., 2016; Yazıcı et al., 2018). This has also been associated with ER stress which matches our results of up-regulation of ER chaperone hsp60 (Liu et al., 2018). Oxidative stress is known to induce cardiomyocyte hypertrophy, which we see in these hearts (Kim et al., 2016). Whilst there is clearly cellular stress in the Beta-exposed hearts, it seems to be predominantly mitochondrial and there are no clear markers of oxidative stress. In other studies, Beta has been associated with oxidative stress in the fetal brain, umbilical vein blood, and the kidneys of adult offspring exposed antenatally (Bi et al., 2014; Miller et al., 2007; Verhaeghe et al., 2009). It is unclear in this model if a transient oxidative stress was induced following Beta treatment that had resolved by day 19 of incubation, and therefore it was not detected in this study. This however does not explain why markers of oxidative stress are still present in Dex-exposed embryos at day 19, if the oxidative stress is transient. It has been observed that Dex has a higher activation capacity for the non-canonical GR pathway, and hence this pathway may be responsible for some of the divergent effects between the two steroids (Buttgereit et al., 1999). Non-genomic effects of glucocorticoids can be categorised as membrane effects, effects mediated by the canonical cytosolic GR (cGR), and effects

mediated by the membrane bound GR (mGR) (Stahn & Buttgereit, 2008). Glucocorticoids may intercalate into cell membranes altering some of their biological properties, for example impairing ATP production by increasing proton leak which may be important in reducing function of immune cells (Buttgereit & Scheffold, 2002). Effects mediated by the cGR are secondary to release of protein mediators upon ligand binding, and include inhibition of arachidonic acid release (Croxtall et al., 2000). mGR is thought to be a splice isoform of the cGR, however its cellular role and production is unclear. Dex has however been shown to act via the mGR to inhibit T-cell signalling in the immune system, reducing inflammation (Löwenberg et al., 2006). A method to compare relative activation of the non-canonical GR pathway would be able to further elucidate the origin of the differences in oxidative and cellular stress observed here.

If indeed no oxidative stress is present following Beta treatment, then other factors must be activating the cellular stress responses, potentially by direct activation via ligand-bound cGR. We observed that the negative feedback on the endogenous glucocorticoid axis, at the level of the cardiac GR was absent in Beta-treated embryos. Synthetic glucocorticoids are known to exert negative feedback via the GR, resulting in down-regulation of tissue GR levels (Paragliola et al., 2017). Down-regulation of GR at the level of transcription, results in lower protein levels and hence lower activation of GR pathways. Therefore, following Dex treatment there is a rapid peak of activation, followed by down-regulation of the cardiac GR. However, following Beta treatment this is not seen and hence GR-activated responses may be triggered and maintained. Additionally, the Na^+-acetate prodrug formulation of Beta means Beta remains in the fetal circulation for longer compared to Dex, so overall activation of the GR

pathway may be greater and last for a longer period of development (Samtani et al., 2005). This may be critical in explaining the more severe effects of Beta on embryonic growth and cardiac function and structure. It may also provide a further explanation for the divergent molecular pathways activated in the embryonic heart; essentially a dose-dependent effect may be seen whereby the effective dose of glucocorticoid is greater due to longer exposure and maintained GR-mediated responses following Beta compared with Dex administration.

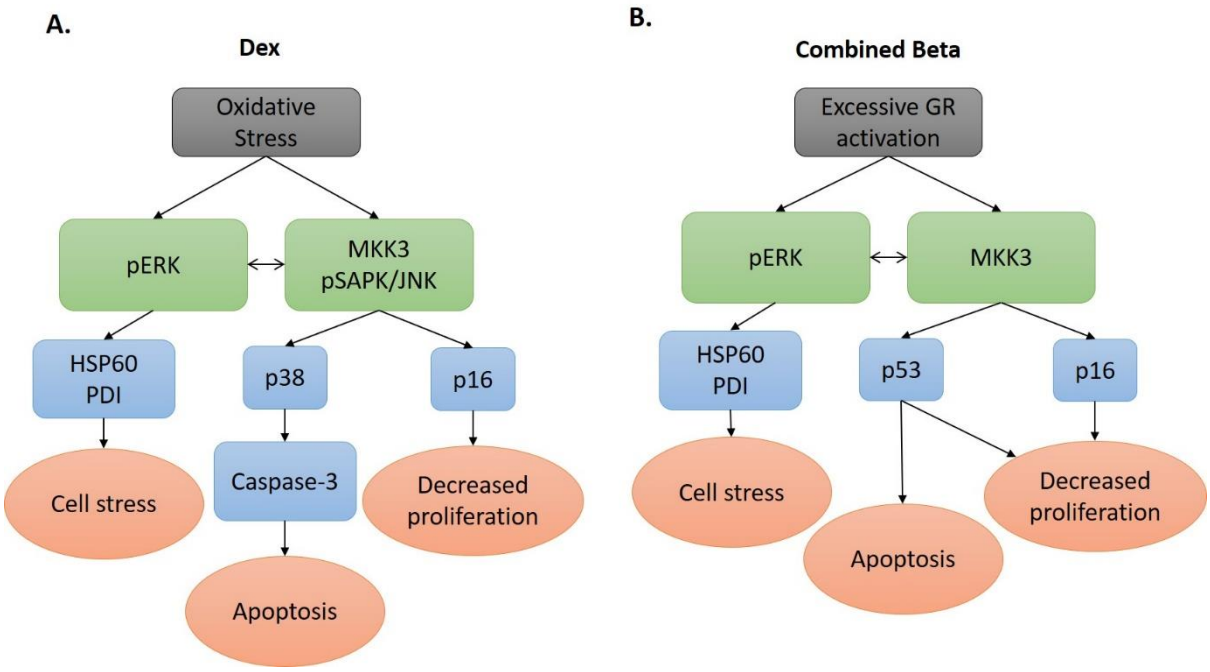


Figure 4.18 Molecular changes in hearts of Dex and Beta treated embryos. A. Dex treatment results in oxidative stress, activating cellular stress pathways and downstream chaperone proteins, caspase-3 mediated apoptosis, and p16 mediated decreases in cell proliferation, B. Beta treatment reaults in excessive GR activation, cell stress pathway activation, p53 mediated apoptosis, and p53/p16 mediated decreases in cell proliferation.

4.8.6 Effects on lung maturation

Addtional results presented in this chapter demonstrate that both Dex and Beta accelerate maturation of the embryonic lungs. Histological markers of lung maturation such as increased parabronchial atrial diameter and air capillary diameter, are similarly enhanced by both glucocorticoids. However, Beta appears to have a greater effect in up-regulating lung proteins caveolin-1 and SP-B, both markers of late development lung maturation in the chicken embryo (Bjørnstad et al., 2014). This demonstrates that the beneficial pulmonary maturational effects associated with AGT are also seen in our avian model. There is some evidence in other animal models or clinical studies that Beta may be more potent in stimulating lung maturation and reducing risk of various pulmonary consequences of preterm birth, such as RDS and BPD (Jobe & Soll, 2004). It is not entirely clear if our model supports this difference, but what the data show is that Dex and Beta have both maturational effects on the lungs but they achieve this via differential mechanisms once again.

4.8.7 Conclusions

Adopting an integrative approach, combining functional experiments in isolated organs with those at the cellular and molecular levels, this chapter has isolated the direct effects of Dex compared with Beta on growth and the developing cardiovascular system using a chicken embryo model system. We show that human clinically relevant doses of Dex or Beta result in significant asymmetric growth restriction, as well as cardiac and peripheral vascular dysfunction. However, the mechanisms underlying these detrimental changes are highly divergent. Dex treatment promotes oxidative stress, cardiomyocyte hypertrophy, enhanced

peripheral constrictor responses to K and activation of GR-induced negative feedback pathways. Conversely, Beta does not promote oxidative stress but induces a marked reduction in cardiomyocyte number, while depressing vasoactive responses to K, PE and SNP, and also lacking any negative feedback at the level of the cardiac GR. Additional data show that both Dex and Beta accelerate lung maturation. The more severe effects of Beta on embryonic growth and the developing cardiovascular system with similar maintenance of accelerated lung maturational responses, provide evidence that Dex compared with Beta may be safer for the treatment of the preterm baby.

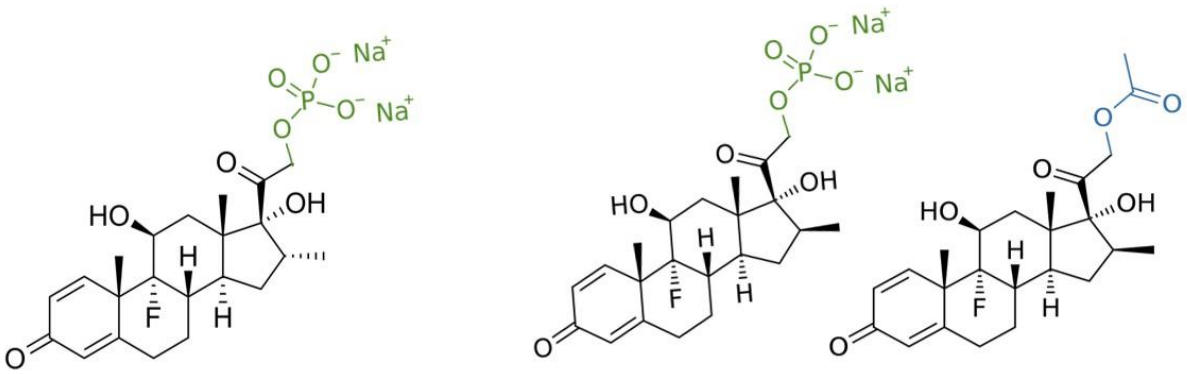
CHAPTER 5: BETAMETHASONE PRODRUG FORMULATIONS

5.1 INTRODUCTION

Antenatal glucocorticoid therapy (AGT) has been used extensively in the clinic for the treatment of threatened pre-term birth since the 1990's (NIH, 1994). It has been shown that AGT prior to preterm birth drastically reduces the risk of mortality and several severe morbidities such as Broncho-pulmonary dysplasia (BPD) and necrotising enterocolitis (NEC; Roberts et al., 2017). However, there are remaining serious questions as to how to optimise this therapy. Not only are two different synthetic steroids Dexamethasone (Dex) and Betamethasone (Beta), utilised for this therapy, but the dosing regimen and the formulations in which they are given (prodrugs) also differs, despite no clear evidence as to which has the greatest efficacy.

Beta and Dex are stereoisomeric synthetic glucocorticoids with a glucocorticoid potency greater than cortisol, but with very little mineralocorticoid activity (He et al., 2014; Buttgereit et al., 1999). Typically, both are administered via intramuscular injection to the mother following presentation with threatened preterm birth (Ballard, 2000). In order to solubilise the steroids, so they can be administered in this manner, a polar group is linked to the carbon backbone via an ester linkage, forming a biologically inactive pro-drug (Jornada et al., 2015). Dex is typically administered as a Na^+-phosphate pro-drug, whilst Beta is given as a 50:50 mixture of Na^+-phosphate and acetate forms (the most common brand being Celestone Soluspan, produced by Merck; Fig.5.1; Schmidt et al., 2019). Dex is administered as 4x6mg

doses over a 48-hour period, whereas Beta is administered as 2x12mg doses, also over a 48-hour period (Fig.5.1; Ballard, 2000).



Dexamethasone	Betamethasone
Na^+-phosphate formulation	50:50 mixture of Na^+-phosphate and acetate formulations
4 x 6mg every 12 hours	2 x 12mg every 24 hours
24mg in 48 hours	24mg in 48hours

Figure 5.1 Drug regimens used for AGT. Molecular structures are shown above text, the Na+-phosphate moiety is shown in green, and the acetate moiety is shown in orange.

For the steroids to become active in tissues they must be hydrolysed by endogenous enzymes, resulting in cleavage of the non-functional phosphate and acetate moieties (Miyabo et al., 1981; Jornada et al., 2015). The rate of cleavage of the phosphate moiety is greater than that of the acetate moiety, leading to the acetate drug formulation having a longer half-life in plasma (Samtani et al., 2005). Indeed, this is the reason for only 2 doses of Beta being administered for AGT whereas Dex is given as 4 doses at shorter time intervals (Ballard, 2000). Evidence from maternal glucocorticoid administration in sheep has demonstrated that Beta phosphate administration results in a rapid peak of active glucocorticoid (9.3ngmL^{-1}) in the fetal circulation at 2.4 hours, that is undetectable by 24 hours (Schmidt et al., 2018).

Conversely, maternal administration with Beta acetate doesn't cause a rapid large peak of active glucocorticoid (3.13ngmL^{-1} is the maximal serum concentration), but a sustained smaller increase that is maximal at 8.5 hours and is still detectable after 24 hours (Schmidt et al., 2018).

Despite these clear pharmacological differences, there are few clinical studies that have compared the efficacy of glucocorticoid prodrug formulations when treating threatened preterm birth. Some small clinical trials have suggested that Beta treatment has a greater efficacy in preventing neonatal death and BPD, whilst others have suggested that Dex phosphate is superior at preventing intra-ventricular haemorrhage (IVH) thus leading to better neurological outcomes (Lee et al., 2006; Elimian et al., 2007; Lee et al., 2008). Other studies have reported no observable differences between the two drugs (Bar-Lev et al., 2004). Schmidt et al., (2018) demonstrated in fetal sheep that a single 0.125mgkg^{-1} dose of Beta acetate was as effective at physiological lung maturation as 2 doses of 0.25mgkg^{-1} combined Beta. Further, sheep experiments have also suggested that combined Beta treatment is more consistently effective at increasing the expression of lung surfactant proteins than either Dex or Beta phosphate (Schmidt et al., 2016), suggesting that the acetate prodrug has a greater effect on lung maturation. As well as different efficacies in preventing morbidities associated with preterm birth, there is also evidence in animal studies for differences in adverse outcomes on other neonatal systems. For example, combined Beta may have a more pronounced acute effect on fetal heart rate and fetal body movements (Rotmensch et al., 1999), and induces an increase in fetal femoral vascular resistance (Derks et al., 1997).

One of the well-known mechanisms leading to gluccocorticoid-induced hypertension following adult or fetal Dex exposure, is the generation of oxidative stress (Safaeian & Zabolian, 2014; Tain et al., 2014). Dex is known to be a potent inducer of oxidative stress in cell culture, and treatment of dex-induced hypertensive animals with antioxidants can restore normal physiological function (Zhang et al., 2004; Tai et al., 2016; Lin et al., 2018). Beta has also been associated with increases in oxidative stress in fetal animal models (Verhaeghe et al., 2009; Stojanovska et al., 2018). Therefore, it is possible that molecular changes involved in AGT-induced cardiovascular dysfunction relate to the generation of oxidative stress and the associated cellular responses, including activation of stress kinase pathways, cell cycle changes and apoptosis (Kim et al., 2016).

The lack of any clear rationale for the choice of glucocorticoid, drug formulation, and dosing regimen used for AGT is concerning and in urgent need of further research. As preterm birth rates have not fallen over the past decade, a vast number of children will be exposed to these treatments (Harrison & Goldenberg, 2016). Both in terms of ensuring positive neonatal outcomes, and in reducing any potential negative side effects of this treatment, it is therefore necessary to have a better understanding of how all these factors influence outcomes. The chicken embryo model provides us with a unique opportunity to compare the direct effects of Dex phosphate vs combined Beta vs Beta phosphate vs Beta acetate, of clinically relevant doses on the developing cardiovascular system. This model has the additional benefit of removing any confounding influence of the mother and placenta, as the embryo develops in isolation within the egg. Studies in our laboratory have previously demonstrated that it is possible to dose the chicken embryo directly via application of aqueous solution to the

chorioallantoic membrane (Itani et al., 2015). Therefore this model allowed us to establish any differences between Dex and Beta drug formulations on embryonic growth, cardiovascular function and lung maturation.

In this chapter we tested the hypothesis that differences in the cardiovascular dysfunction induced by Dex or Beta exposure in late cardiovascular development are dependent on the prodrug formulation administered, with combined and acetate prodrug formulations having a different severity and nature of dysfunction compared to the Na^+-phosphate prodrug formulation. To address this hypothesis, there were 5 objectives:

1. To investigate the effects of Dex phosphate, combined Beta, Beta phosphate, and Beta acetate treatment on embryonic survival and growth of the chicken embryo;
2. To investigate the effects of Dex phosphate, combined Beta, Beta phosphate, and Beta acetate treatment on the cardiac function and structure of the chicken embryo;
3. To investigate the effects of Dex phosphate, combined Beta, Beta phosphate, and Beta acetate treatment on peripheral vascular function in the chicken embryo;
4. To investigate the molecular pathways induced by Dex phosphate, combined Beta, Beta phosphate, and Beta acetate treatment in the chicken embryo;
5. To investigate the effects of dexamethasone phosphate, combined betamethasone, betamethasone phosphate, and betamethasone acetate on lung maturation in the chicken embryo.

5.2 METHODS

5.2.1 Experimental Protocol

Detailed experimental procedures are shown in Section 2. In brief, all procedures were performed under the U.K. Animals (Scientific Procedures) Act 1986 and were approved by the Ethical Review Board of the University of Cambridge. Fertilised Bovans Brown chicken eggs were weighed, irradiated with UV light, and placed into an incubator at 38°C and 45% humidity for 19 days (see Section 2.1.). Eggs were dosed with Dexamethasone phosphate or Betamethasone phosphate/acetate/combined (Celestone, Merck, USA), or Dexamethasone sodium-phosphate, Betamethasone sodium-phosphate, Betamethasone acetate (Sigma, UK) in aqueous solution, or water on day 14 of development via injection through a small hole in the shell (situated above the air sac) onto the chorioallantoic membrane, as previously described (Itani et al., 2015).

The glucocorticoid dose used in this study was 0.1mgkg^{-1}. AGT was administered on day 14 of incubation, which is equivalent to 26/27 weeks in human pregnancy; a relevant, early period at which glucocorticoids can realistically be administered in the clinical setting (Roberts & Dalziel, 2006).

5.2.2 Assessment of Embryonic Growth

On Day 19 of incubation embryonic growth was assessed using a high precision calliper (Mitutoyo 500-181-20, UK) to determine embryonic lengths. Embryo mass and mass of the eggshell, yolk, extra tissues, and any remaining liquid was measured and recorded. The ratio

of partitioning of resources was calculated as embryonic tissue mass/extra-embryonic tissue mass. Crown-rump length, head diameter, head length were recorded (Section 2.2). Individual organs were also dissected and weighed.

5.2.3 Assessment of Cardiac Structure & Function

A cohort of 10 embryos per group at day 19 were anaesthetised with sodium pentobarbitate (0.3mL of Pentoject (Animalcare Ltd., York, UK), intraperitoneal) then perfusion fixed using paraformaldehyde (Section 2.5) at 2.66kPa (embryonic day 19 blood pressure) inserted into the left ventricle via a cannula. Fixed hearts were sectioned into 1mm slices and photographed. Areas of cardiac compartments in each section were counted, and a mid-cardiac section was chosen for measurement. Volumes were then calculated as per Section 2.5.

Cardiomyocytes were also isolated from hearts of day 19 embryos (Section 2.6) by perfusing the coronary vasculature with collagenase and protease solution. After 10 minutes, the hearts were perfused with Krebs buffer to remove enzymes, and then suspended in buffer for 40 minutes. Cells were stored in 4% PFA. Cardiomyocyte suspensions were mixed with 10% methylene blue in a 1/1 ratio and visualised under a light microscope (Section 2.6). Cell nuclearity of 300 random cardiomyocytes per heart was assessed, and measurements of length and width were taken from 50 random cardiomyocytes. Cell volumes were then calculated (Section 2.6).

To assess *ex vivo* cardiac function hearts were rapidly excised and placed into icy Krebs Buffer solution. The aorta was cannulated onto a 19-gauge blunt needle and mounted onto the Langendorff apparatus. Pressure recordings were subsequently made (Section 2.3). In brief; following a period of normalisation, a small non-distensible balloon made from saran wrap, and connected to a pressure transducer, was introduced through the left atrium into the left ventricle. The balloon was then filled with water in 5µL increments until a smooth baseline recording was established. Baseline recordings were made and Coronary Flow Rate (CFR) was assessed. In vitro responses to the sympathetic agonist Isoprenaline (Sigma-Aldritch, in the range 10^{-9} – 10^{-7}) and the parasympathetic agonist Carbachol (Sigma-Aldrich, in the range 10^{-8} – 10^{-6}) were then determined by administering via the perfusing cannula. Analysis of the Langendorff experiments was conducted on the LabChart software (Version 7, ADInstruments).

5.2.4 Assessment of Peripheral Vascular Function

A second order branch of the femoral artery was dissected into cold Krebs solutions and then 2mm sections were mounted onto a wire myograph (Section 2.4). Vessels were maintained at 38°C in Krebs solution, and were aerated with 95% O_2/5% CO_2 gas. To assess vasoconstrictor responses vessels were exposed to increasing doses of NE, 5-HT, PE, and ET-1 at 2-minute intervals. To assess vasodilator function vessels were pre-constricted with K^+, then exposed to increasing doses of Ach, or SNP. To assess the NO dependent component of the Ach response, vessels were treated with 5µm L-NAME (Section.2.4).

5.2.5 Assessment of Molecular Pathways in the Heart

Frozen heart tissues were powdered on dry ice, and separated into 50mg aliquots, then stored at -80°C. Extraction of proteins was conducted in TK lysis buffer (Section 2.7.1). For Western blot analysis, protein extracts were separated on a 10% agarose gel, then stained with primary antibody and secondary antibodies (Table 2.2). Gel bands were then quantified as described in Section 2.7.2. To compare all 5 treatment groups, it was necessary to perform multiple blots per protein of interest. To control for intra-gel variability a set of control samples was run on each gel and the expression in all treatment groups was calculated relative to the mean of all control values.

A further set of frozen tissue aliquots were used to conduct RNA extraction using a Qiagen RNEasy mini kit (Qiagen, NL; Section 2.7.3). RNA samples were then converted into cDNA libraries using Qiagen Reverse Transcriptase kit (Qiagen, NL). DNA primers to various mRNAs of interest were designed and tested (only primers that gave a single peak, with a clear dose dependent relationship were used further; Table 2.3). mRNA expression analysis was then conducted as per Section 2.7.4.

5.2.6 Assessment of embryonic lung maturation

Perfusion fixed lungs were embedded in paraffin, then sectioned into 8µm sections with an ultramicrotome (Section 2.8). Slides were stained with haematoxylin and eosin (Section 2.6), then imaged. Measurements of parabronchial atrial diameter (n=20), atrial septum thickness (n=20), air capillary diameter (n=20), and distance between air capillary and nearest blood

vessel (diffusion distance) (n=20), and thickness of gas exchange zone (n=5) were taken per animal.

5.2.7 Statistics

All data except for dose-response curves were analysed using a one-way ANOVA with Tukey post-hoc test, conducted on GraphPad Prism 7 (Graphpad Software, CA, USA) software. Statistical differences ($p<0.05$) are demonstrated by different letters. Dose-response curves were assessed using a mixed-model two-way ANOVA. In this case, significant differences to control values are indicated by * , whilst significant differences between Dex and Beta values are indicated by †. For all comparisons, significance was taken as $P<0.05$.

5.3 Effects on Embryonic Growth

All pro-drug formulations resulted in a significant embryonic growth restriction, with Beta prodrugs having a more severe effect than Dex (Fig.5.2 A). In terms of both absolute and relative embryo mass, Beta phosphate had less of an effect than either combined Beta or Beta acetate (Fig.5.2 A&B). Whilst the ratio of embryonic to extra-embryonic tissues was decreased in the Dex group relative to controls, it was decreased even more in Beta treatment groups, with combined Beta having the most severe effect (Fig.5.2 C). All Beta treatments increased liquid mass in the day 19 egg (Fig.5.2 D). Whilst CRL was unchanged, relative head diameter was increased in all Beta treatment groups (Fig.5.2 E&F). All treatments increased relative heart mass (Fig.5.2 H), however only combined Beta significantly increased relative brain mass (Fig.5.2 G).

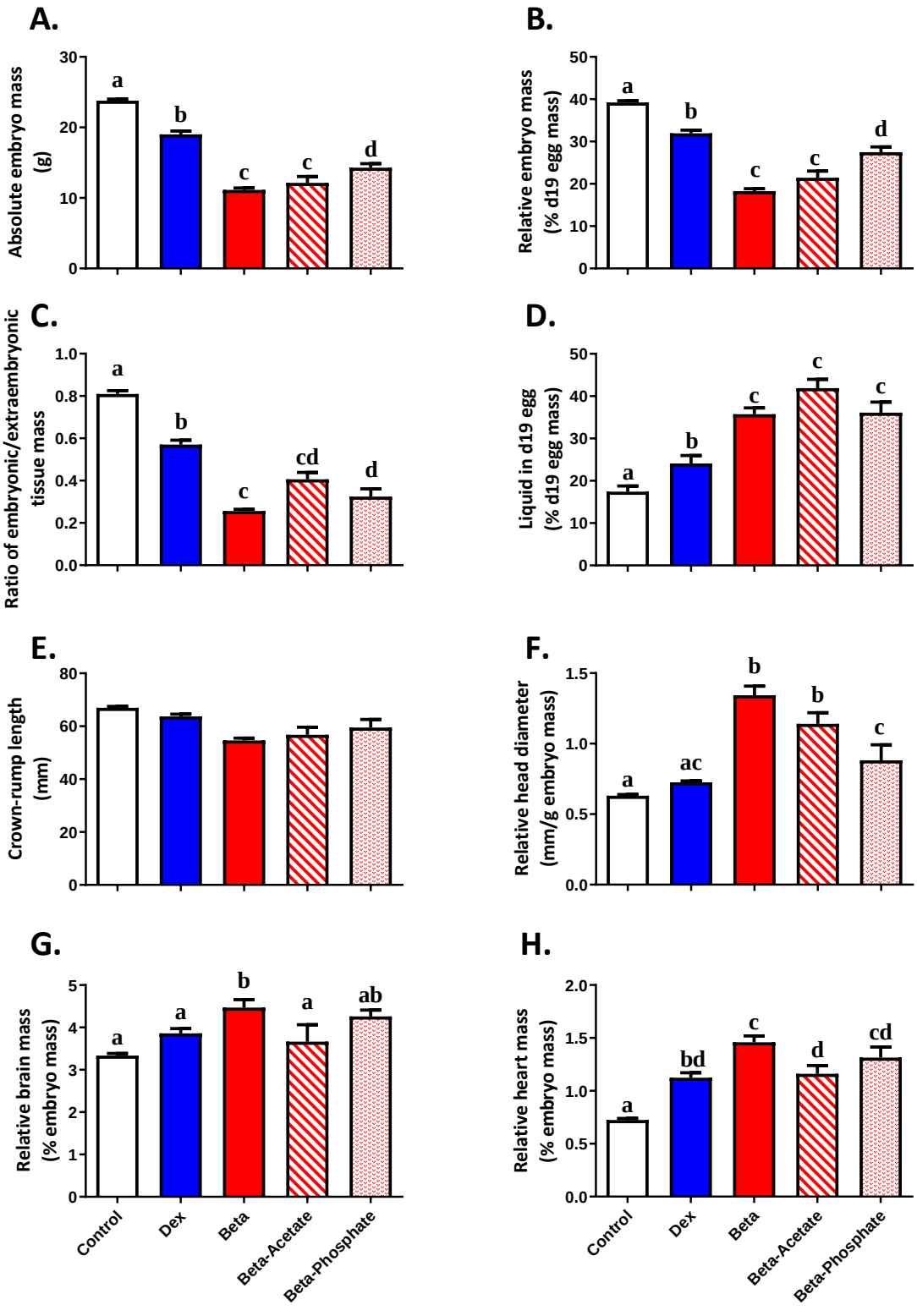


Figure 5.2 Day 19 biometry. A. absolute embryo mass (g), B. relative embryo mass (% of d19 egg mass), C. ratio of embryonic to extraembryonic tissue mass, D. liquid mass in d19 egg (% d19 egg mass), E. crown-rump length (mm), F. relative head diameter (mm/g d19 embryo mass), G. relative brain mass (%d19 embryo mass), H. relative heart mass (% d19 embryo mass), in control (n=10), Dex (n=10), combined Beta (n=10), Beta phosphate (n=10), and Beta acetate (n=10) treated embryos. One-way ANOVA with Tukey post-hoc test, $P<0.05$ significant differences represented by different letters.

5.4 Effects on Cardiac Structure & Function

Combined Beta resulted in a significant decrease in left ventricle wall volume, and an increased in left lumen to wall area and volume ratio (Fig.5.3 A&C). Dex phosphate treatment resulted in a significant increase in left lumen volume (Fig.5.3 B), but no significant increase in lumen to wall area or volume ratio (Fig.5.3 C&D). Combined Beta treatment resulted in a decrease in right ventricle wall volume, an increase in right ventricle lumen volume whilst the other treatments did not (Fig.5.3 E&F). This resulted in an increased right ventricle wall to lumen area and volume ratio in the combined Beta treatment group (Fig.5.3 G&H).

Only Dex phosphate significantly increased cardiomyocyte volume, and significantly reduced the percentage of mono-nucleated cardiomyocytes (Fig.5.4 B&C). Both combined Beta and Beta phosphate treatment reduced cardiomyocyte width (Fig.5.4 A), although this had no significant effect on cardiomyocyte volume. All Beta treatments resulted in a significant reduction in the cardiomyocyte nuclear density in the left ventricle (Fig.5.4 D).

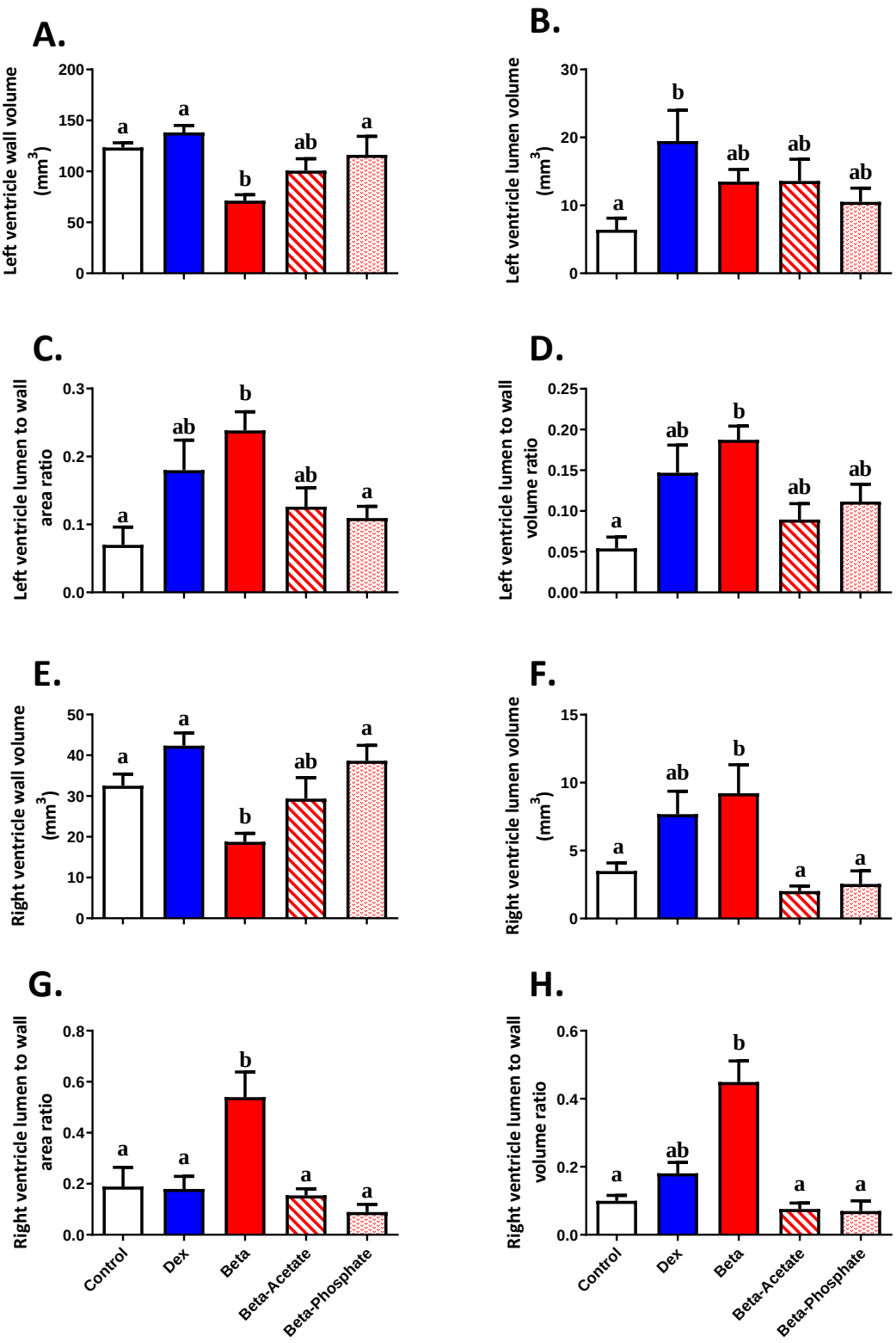


Figure 5.3 Cardiac stereology. A. left ventricle wall volume (mm^3), B. left ventricle lumen volume (mm3), C. left ventricle lumen to wall area ratio, D. left ventricle lumen to wall volume ratio, E. right ventricle wall volume (mm^3), F. right ventricle lumen volume (mm3), G. right ventricle lumen to wall area ratio, H. right ventricle lumen to wall volume ratio, in control (n=10), Dex (n=10), combined Beta (n=10), Beta phosphate (n=10), and Beta acetate (n=10) treated embryos. One-way ANOVA with Tukey post-hoc test, $P<0.05$ significant differences represented by different letters.

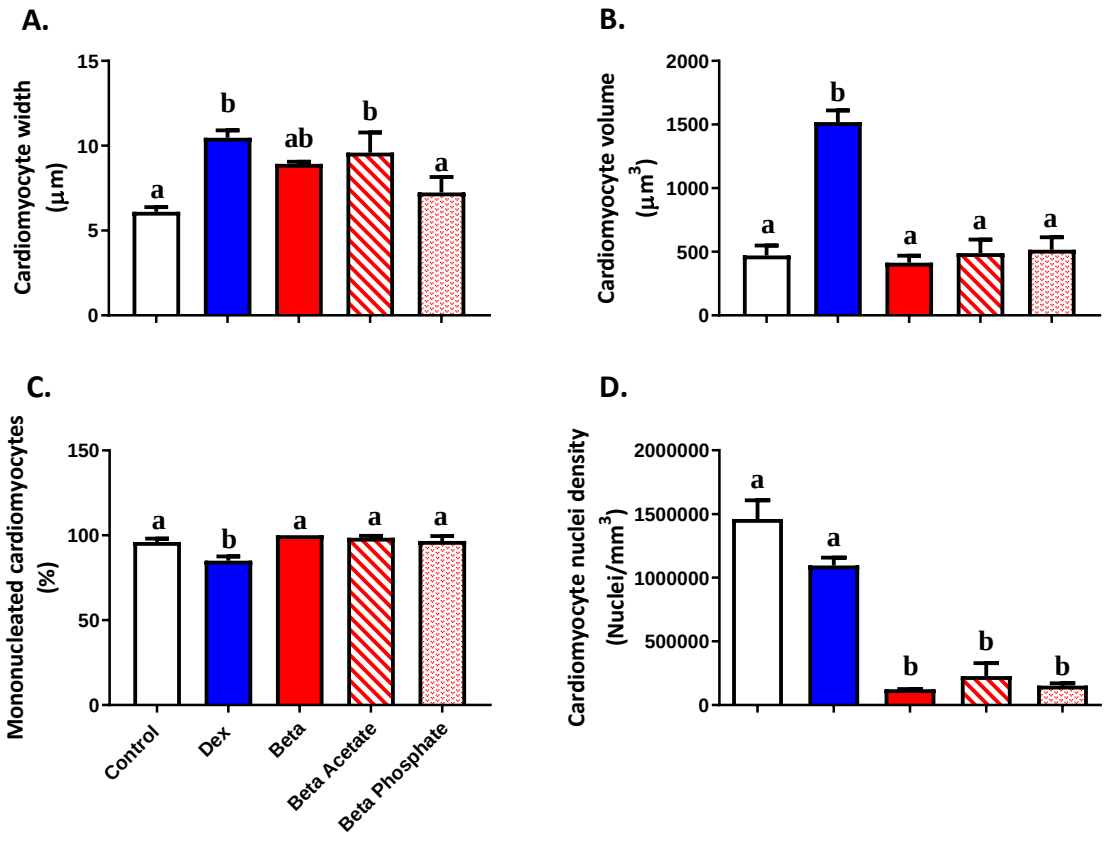


Figure 5.4 Cardiomyocyte analysis. A. cardiomyocyte width (µm), B. cardiomyocyte volume (µm^3), C. % mononucleated cardiomyocytes, D. cardiomyocyte nuclear density (nuclei/mm3), in control (n=8), Dex (n=8), combined Beta (n=10), Beta phosphate (n=10), and Beta acetate (n=10) treated embryos. One-way ANOVA with Tukey post-hoc test, P<0.05 significant differences represented by different letters.

All Beta treatments resulted in a significant impairment of LVDP. All treatment groups had a significantly greater LVEDP and Tau than the control treated embryos (Fig.5.5 A-D). Only combined Beta treatment significantly decreased CFR and total cycle duration, due to a significant depressive effect on the systolic component of the cardiac cycle (Fig.5.6 A&B). In terms of cardiac response to autonomic agonists, all Beta treatments increased the HR response ratio to isoprenaline relative to carbachol (Fig.5.7 B).

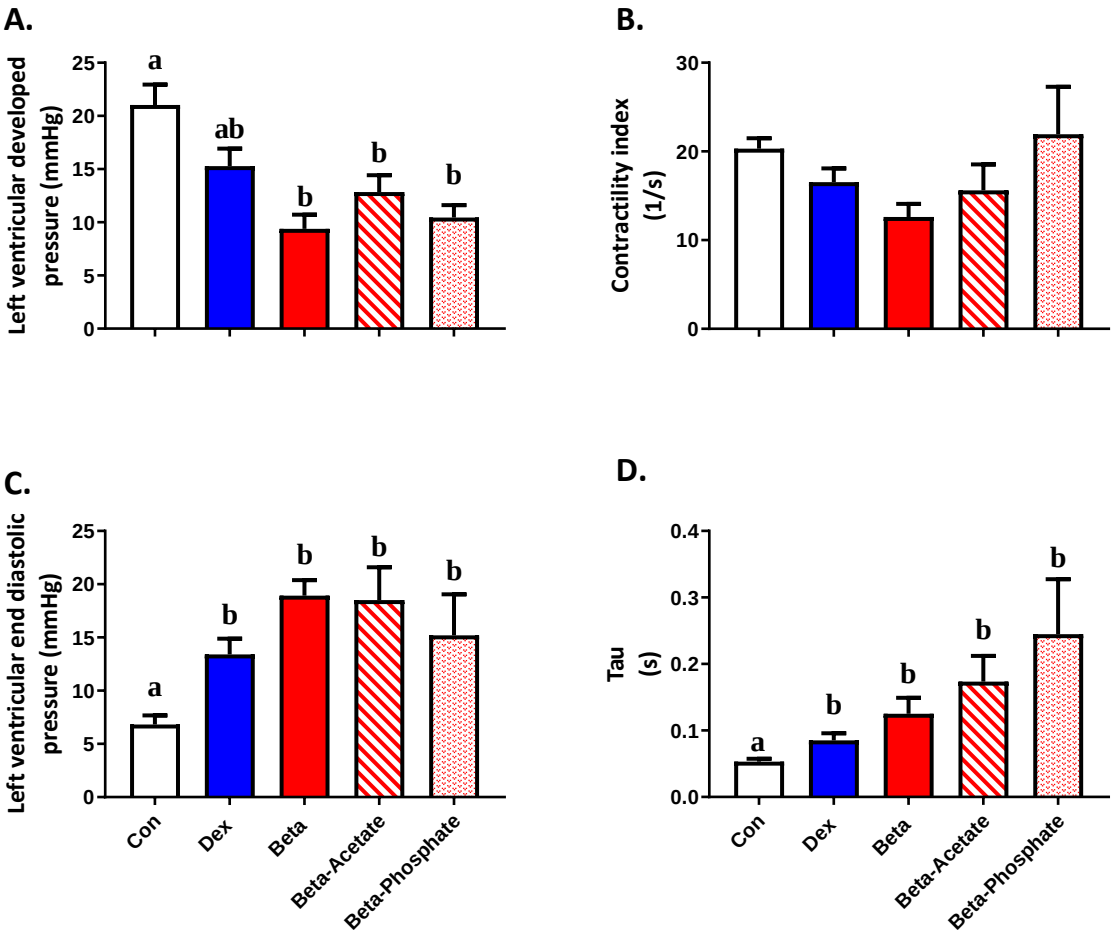


Figure 5.5 Systolic and diastolic function A. left ventricular developed pressure (mmHg), B. contractility index (1/s), C. left ventricular end diastolic pressure (mmHg), D. tau (s), in control (n=10), Dex (n=10), combined Beta (n=10), Beta phosphate (n=10), and Beta acetate (n=10) treated embryos. One-way ANOVA with Tukey post-hoc test, $P<0.05$ significant differences represented by different letters.

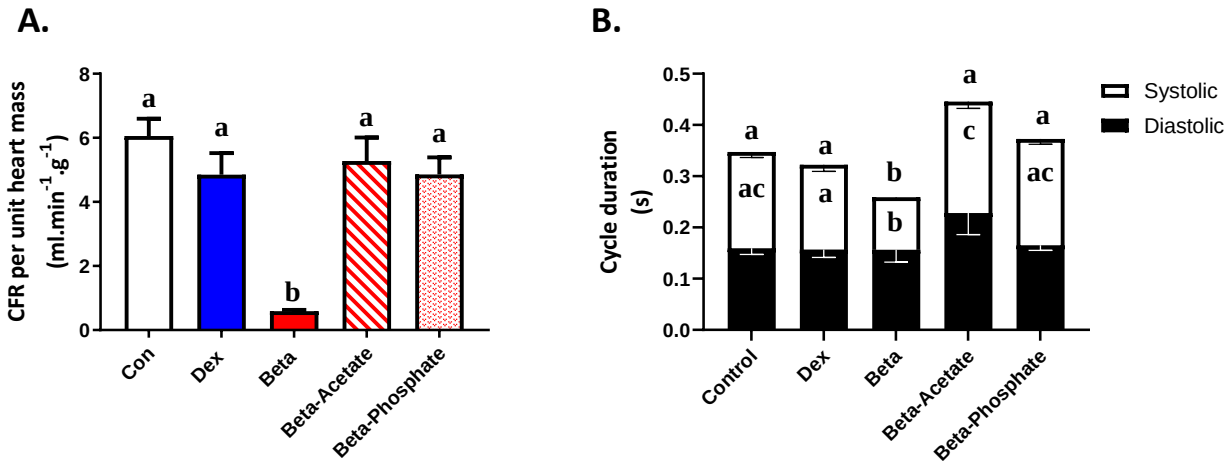


Figure 5.6 Coronary flow rate & cycle duration. A. coronary flow rate per unit cardiac mass ($mlmin^{-1}g^{-1}$), B. cycle duration (s), in control (n=8), Dex (n=8), combined Beta (n=10), Beta phosphate (n=6), and Beta acetate (n=6) treated embryos. One-way ANOVA with Tukey post-hoc test, P<0.05 significant differences represented by different letters.

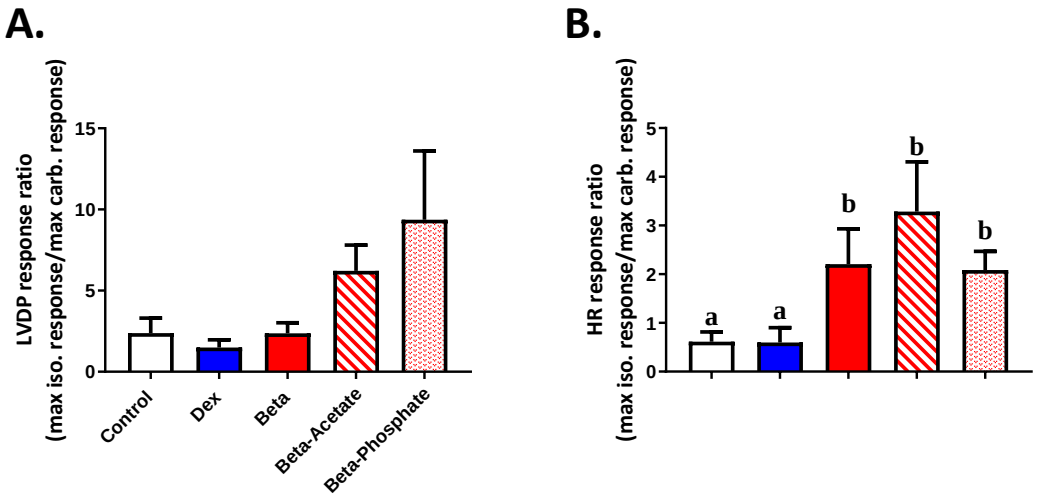


Figure 5.7 Cardiac response to autonomic agonists. A. ratio of maximal LVDP isoprenaline response to maximal LVDP carbachol response, B. ratio of maximal HR isoprenaline response to maximal HR carbachol response, in control (n=8), Dex (n=8), combined Beta (n=10), Beta phosphate (n=6), and Beta acetate (n=6) treated embryos. CF One-way ANOVA with Tukey post-hoc test, p<0.05 significant differences represented by different letters, ABDE two-way ANOVA with Tukey post-hoc test, P<0.05 *significantly different to control, † significantly different to Dex.

5.5 Effects on Peripheral Vascular Function

All Beta treatments impaired the contractile response to KCl compared to the Dex treatment group (Fig.5.8 A&B). However, only Beta acetate significantly reduced KCl response relative to control embryos (Fig.5.8 B). All Beta treatments also impaired the contractile response to PE compared to both control and Dex treatment groups, in terms of response to higher doses but not in terms of area under the curve (Fig.5.8 C&D). Beta phosphate and Beta acetate significantly impaired contractile response to 5-HT whilst neither Dex or combined Beta had any effect (Fig.5.8 E&F). Dex, combined Beta, and Beta acetate significantly increased the contractile response to ET-1 compared to control embryos. Beta phosphate increased the response to an even greater extent (Fig.5.8 G&H).

All Beta treatments significantly impaired relaxation in response to SNP relative to Dex embryos, whilst combined Beta and Beta phosphate also significantly impaired relaxation relative to control embryos, however there was no significant difference in area above the curve (Fig.5.9 C&D). All Beta treatments also significantly impaired NO-independent vasodilatation compared to Dex treatment (Fig5.9 B). Beta phosphate also impaired the response to Ach in the centre of the dosing curve (Fig.5.9 A).

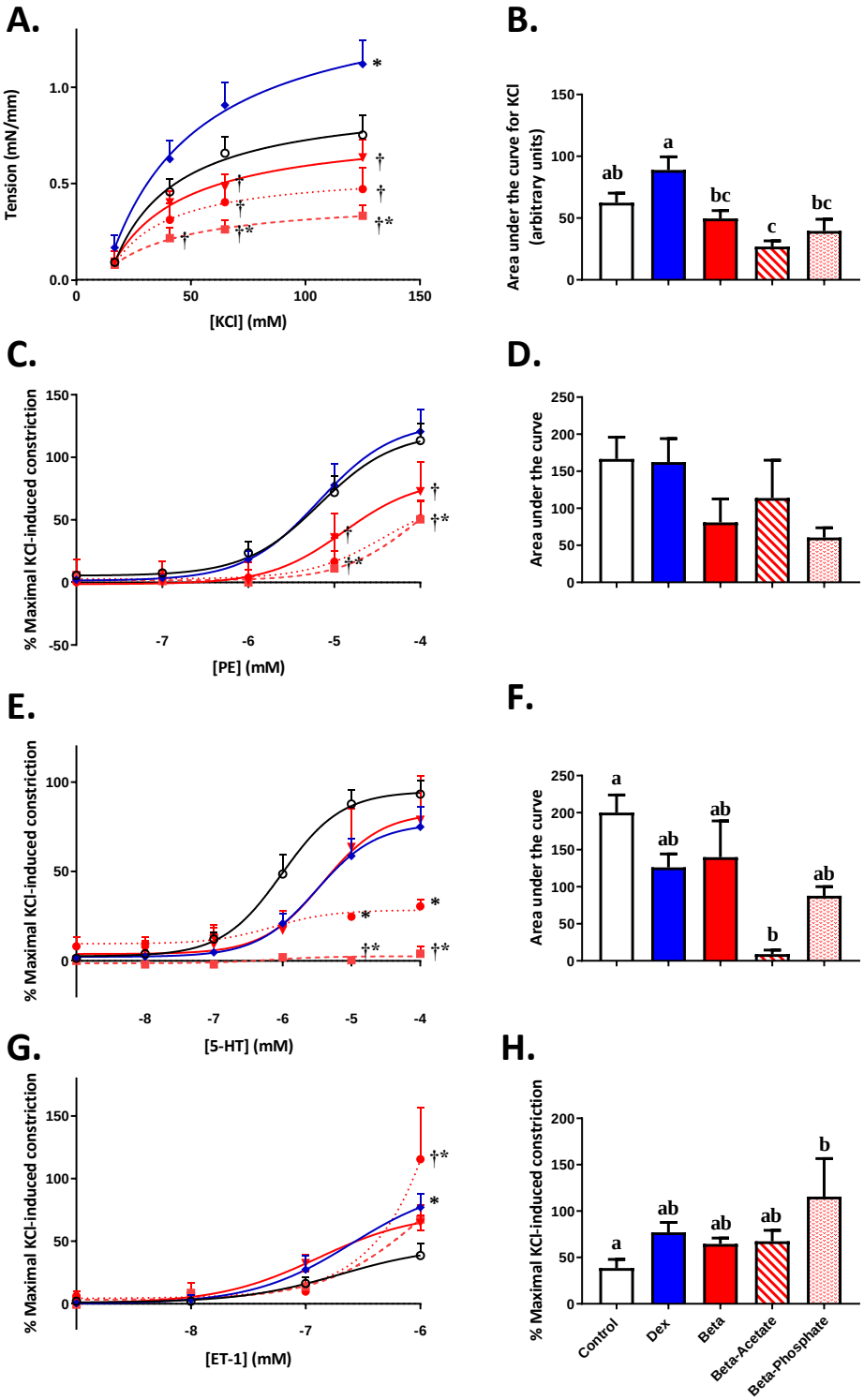


Figure 5.8 Vasoconstrictor responses. A. tension generated in response to KCl (mM), B. Area under the curve of the KCL response (arbitrary units), C. response to PE as a % of maximal KCl response, D. pD2 of PE response, E. response to 5-HT as a % of maximal KCl response, F. area under the curve of 5-HT response (arbitrary units), H. response to ET-1 as a % of maximal KCl response, H. maximal ET-1 response as a % of maximal KCl response, in control (white, n=10) Dex (blue, n=10) Beta (red triangles, n=10), Beta acetate (red squares, n=8) and Beta phosphate (red circles, n=8) treated embryos, ACEG two-way ANOVA with Tukey post-hoc test where $P<0.05$ * represents statistically different to control, and † represents statistically different to Dex, BDFH ordinary one-way ANOVA with Tukey test. statistically significant differences denoted by different letters.

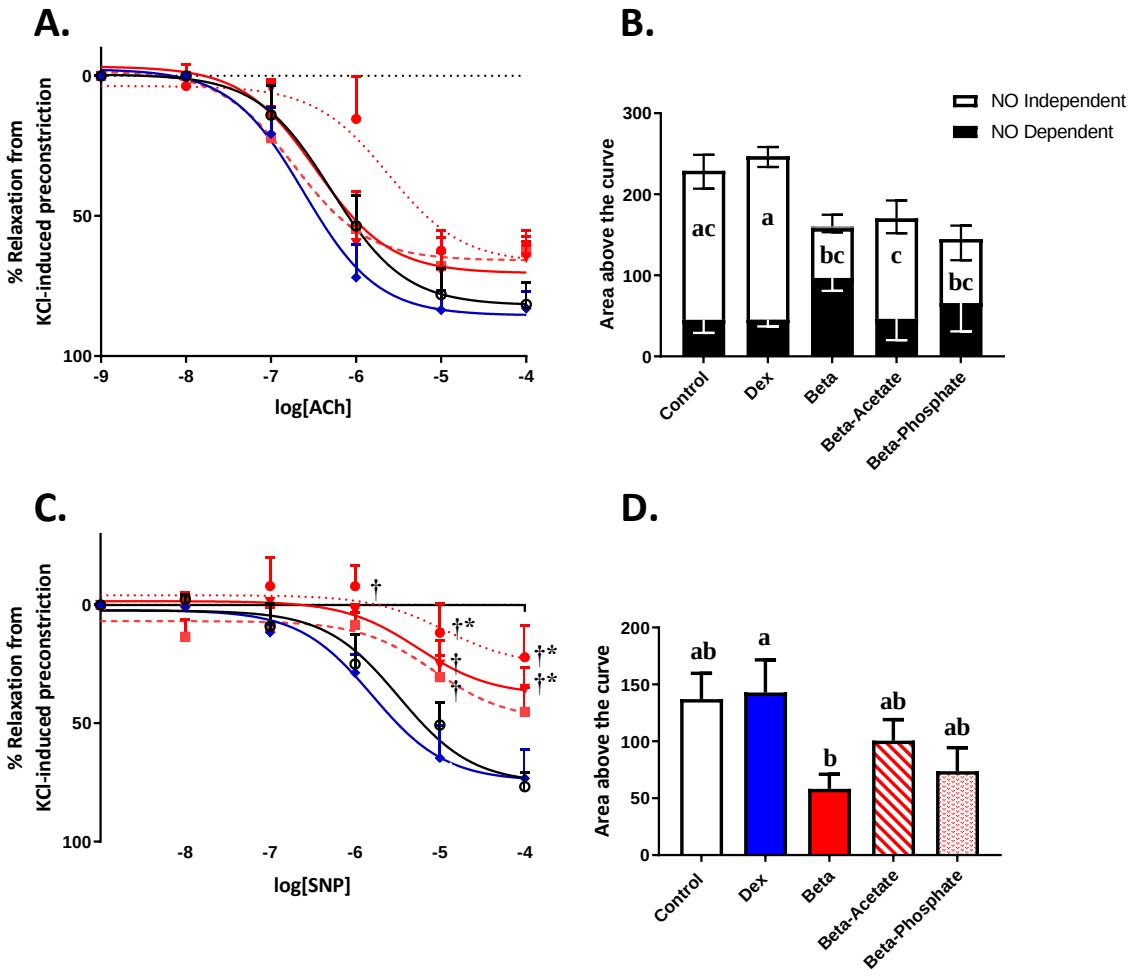


Figure 5.9 Vascular vasodilator responses. A. vascular relaxation from KCl-induced pre-constriction in response to increasing doses of Ach, B. breakdown of the vasodilator response to Ach, by treating vessels with the eNOS inhibitor L-NAME, and assessing the area above the Ach dose-response curve, before and after inhibition, the area under the curve dependent on eNOS and hence NO can be determined, C. vascular relaxation from KCl-induced pre-constriction in response to NO donor SNP, in control (white, n=10), Dex (blue, n=10), Beta (red triangles, n=10), Beta acetate (red squares, n=8) and Beta phosphate (red circles, n=8) treated embryos. AC two-way ANOVA with Tukey post-hoc test where $P<0.05$ * represents statistically different to control, and † represents statistically different to Dex, B ordinary one-way ANOVA with Tukey test. Statistically significant differences denoted by different letters.

5.6 Effects on Molecular Pathways in the Heart

Beta phosphate treatment resulted in a significant decrease in protein carbonylation levels (Fig.5.10 A). No treatment resulted in a significant change in expression of (Mn)-SOD or Catalase relative to control levels (Fig.5.10 B&C). Both Dex and combined Beta treatment significantly up-regulated pERK/ERK levels whilst Beta phosphate and Beta acetate significantly reduced them (Fig.5.11 A). Dex treatment also significantly raised the pSAPK/JNK / SAPK/JNK protein expression (Fig.5.11 A). Both Dex and combined Beta treatment significantly raised levels of chaperones hsp60 and PDI, whilst Beta acetate increased expression of hsp27 (Fig.5.11 A). Whilst all glucocorticoid treatments significantly increased total caspase-3 levels, only Dex treatment significantly increased cleaved caspase-3 levels and the ratio of cleaved to total caspase-3 protein (Fig.5.11 B). Combined Beta treatment increased p16 mRNA expression, and Beta acetate increased p53 expression (Fig.5.11 C).

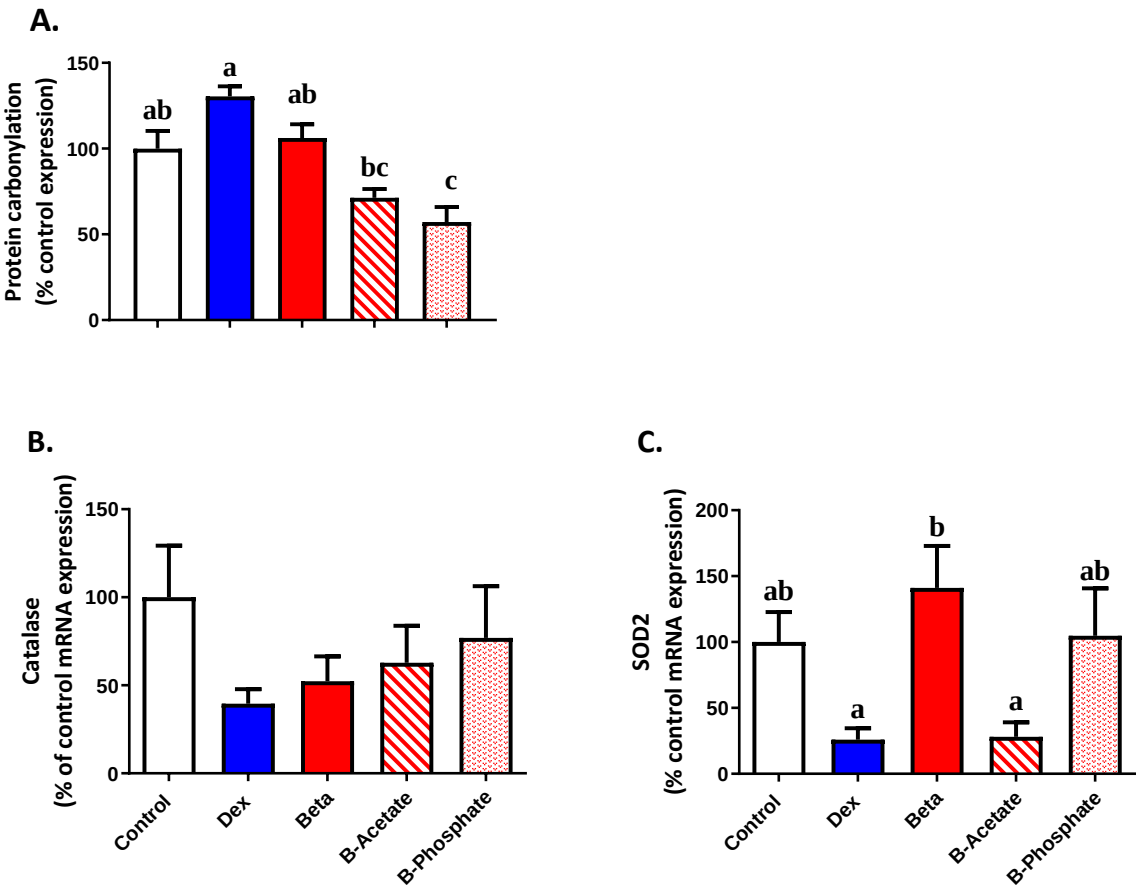


Figure 5.10 Oxidative stress indices. A. Levels of protein carbonylation detected by western blot, B. mRNA expression of Catalase, C. mRNA expression of SOD2 (Mn dependent), in control (n=6), Dex (n=6), Beta (n=5), Beta acetate (n=5) and Beta phosphate (n=5) treated embryos. Ordinary one-way ANOVA with Tukey test. $P<0.05$, statistically significant differences denoted by different letters.

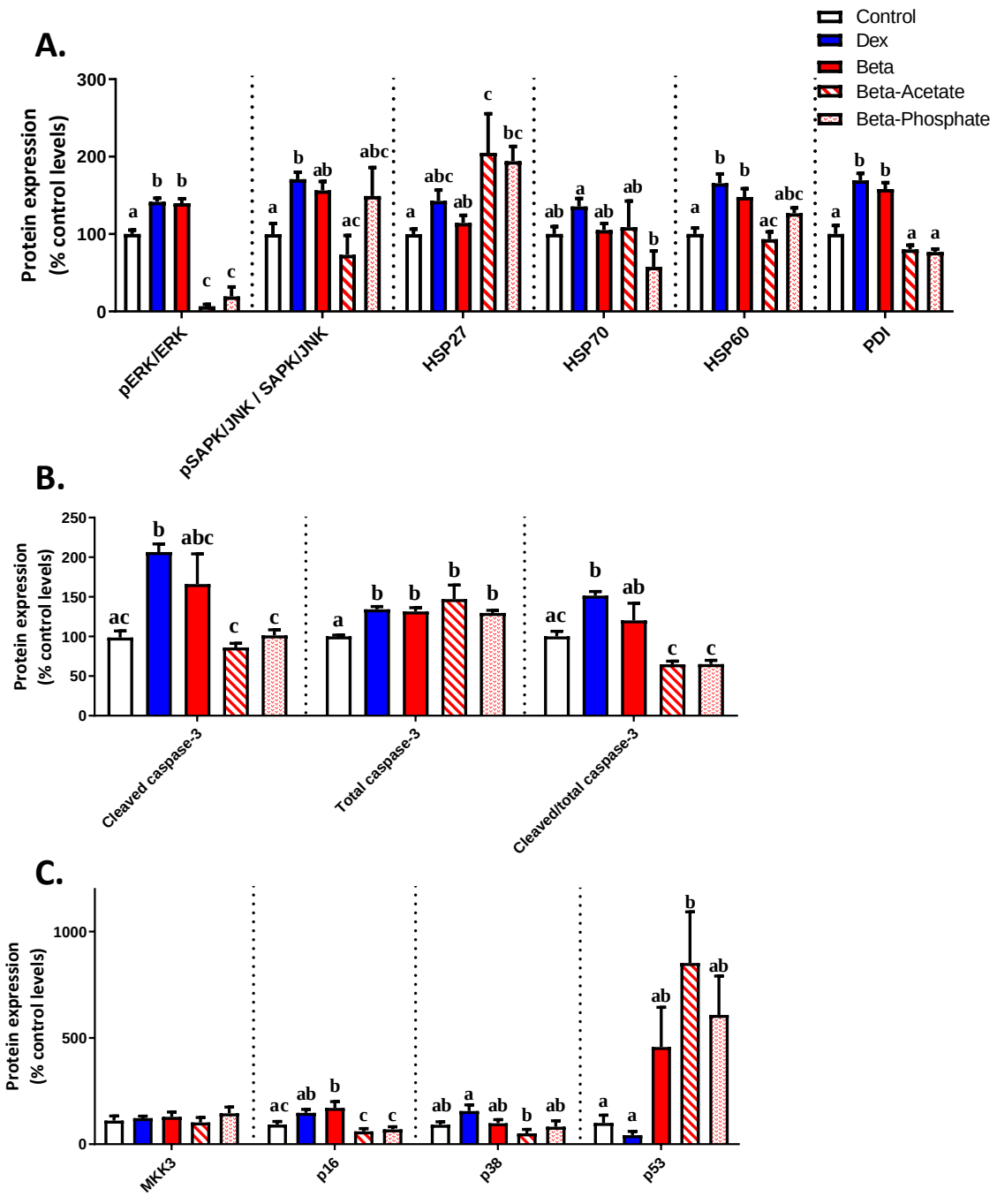


Figure 5.11 Cellular stress, apoptosis, and cell cycle markers. A. phosphorylated ERK protein/non-phosphorylated levels, phosphorylated SAPK/JNK / non-phosphorylated levels, protein expression of chaperones HSP27, HSP70, HSP60, and PDI B. mRNA levels relative to control of MKK3, p16, p38, p53 C. protein levels of caspase-3 cleaved caspase-3, ratio of cleaved caspase-3 to total caspase 3, BCL, in control (n=6), Dex (n=6)(only in B.), Beta (n=5), Beta acetate (n=5) and Beta phosphate (n=5) treated embryos. Ordinary one-way ANOVA with Tukey test. $P<0.05$, statistically significant differences denoted by different letters.

Both Dex and Beta phosphate treatment groups resulted in a significant reduction in GR protein levels relative to control levels, however Combined Beta and beta acetate had no effect (Fig.5.12).

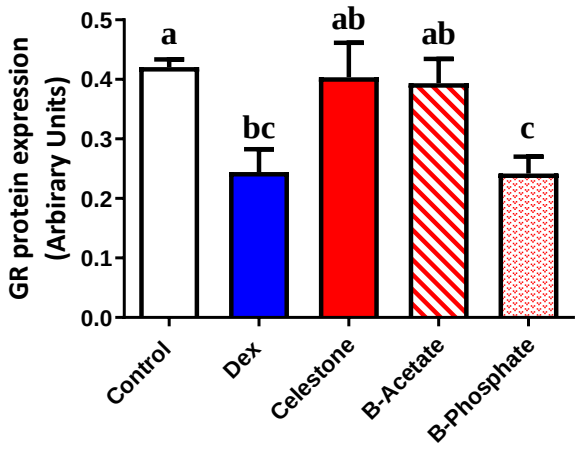


Figure 5.12 GR protein levels. GR protein levels (relative to tubulin expression), in control (n=16), Dex (n=6), Beta (n=5), Beta acetate (n=5) and Beta phosphate (n=5) treated embryos. Ordinary one-way ANOVA with Tukey test. P<0.05, statistically significant differences denoted by different letters.

5.7 Effects on Lung Maturation

Only combined Beta treatment resulted in a significant increase in protein expression of caveolin-1 and SP-B (Fig.5.13 A&B). All treatments resulted in significantly increased parabronchial atrial diameter, air capillary diameter, and reduced diffusion distance (Fig.5.14 A-C). Dex treatment also significantly increased the thickness of gas exchange zone in the embryonic lungs, relative to control embryos (Fig.5.14 D).

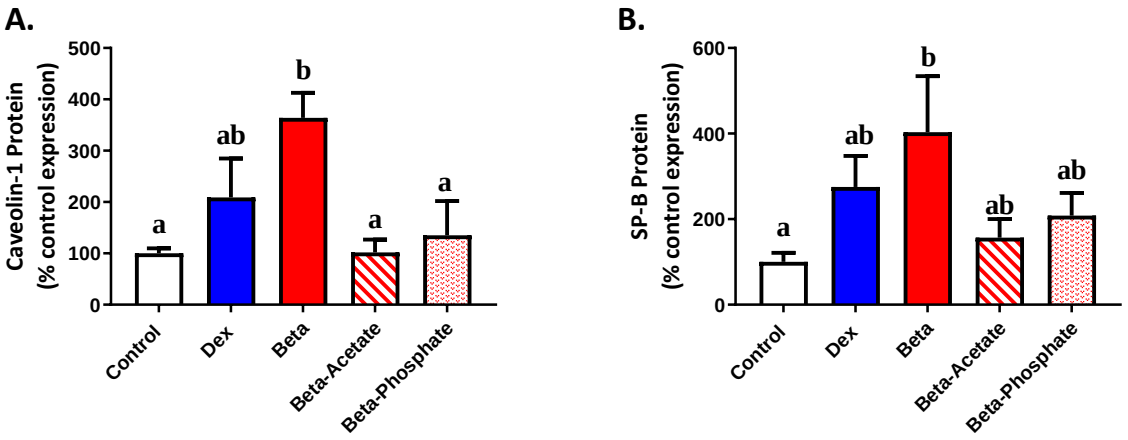


Figure 5.13 Protein markers of lung maturation. A. Expression of caveloin-1 (% control expression), B. expression of SP-B (% control expression), in control (n=6), Beta (n=6), Beta acetate (n=5), and Beta phosphate (n=5) treated embryos. Ordinary one-way ANOVA with Tukey test. P<0.05, statistically significant differences denoted by different letters.

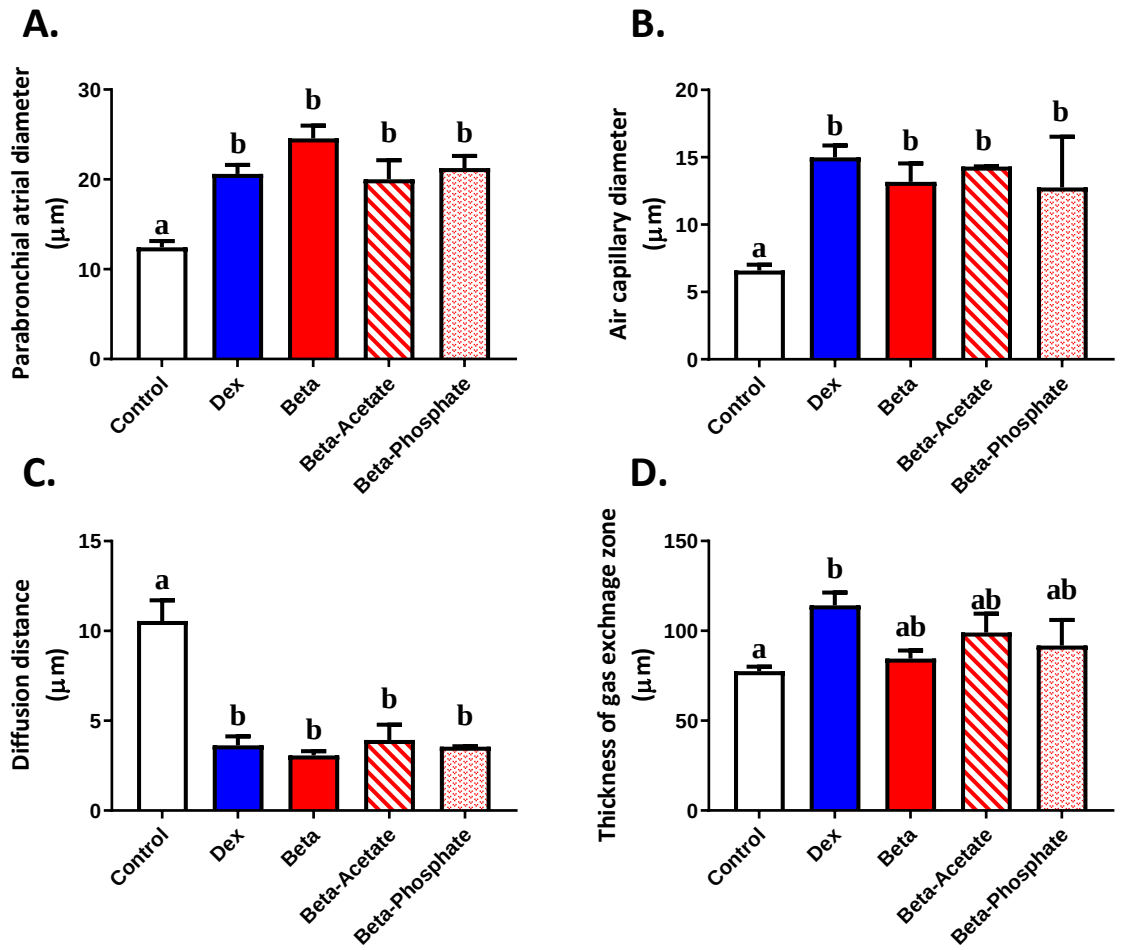


Figure 5.14 Morphological markers of lung maturation. A. parabronchial atrial diameter (µm), B. air capillary diameter (µm), C. diffusion distance (µm), D. thickness of gas exchange region adjacent to parabronchi (µm), in control (n=8) Dex (n=8) Beta (n=6) Beta acetate (n=6) and Beta phosphate (n=6) treated embryo Ordinary one-way ANOVA with Tukey test. P<0.05 statistically significant differences denoted by different letters.

5.8 DISCUSSION

5.8.1 Summary

The data in this chapter show that prodrug formulations resulted in a significant asymmetric embryonic growth restriction at day 19 of incubation. Combined Beta and Beta acetate resulted in a more severe asymmetric growth restriction (in terms of relative embryo mass, and relative head diameter), compared with either Dex or Beta Phosphate. However, combined Beta had a more severe effect promoting asymmetric growth restriction than the other treatments as relative brain mass was also increased. Relative heart mass was significantly increased by all drug treatments, with a more severe effect when combined Beta was given. In terms of cardiac structure, only combined Beta had a significant left ventricular dilatation compared to control embryos. Dex treatment resulted in a significant hypertrophy of individual cardiomyocytes and a decrease in the percentage of mononucleation. All Beta treatments did not affect cardiomyocyte volume but did significantly reduce nuclear density. All drug treatments impaired cardiac diastolic function, whilst all Beta treatments also impaired systolic function. Only combined Beta treatment resulted in a decrease in CFR and cardiac cycle duration, whist all Beta treatments resulted in a significant increase in the heart rate response ratio of the maximal heart rate change to isoprenaline relative to carbachol, indicating sympathetic dominance.

In the peripheral vasculature, Beta treatment impaired responses to vasoconstrictors KCl, PE and 5-HT, with Beta acetate being the most severely affected. All drug treatments increased vascular responses to ET-1, with Beta phosphate having the largest effect. All Beta treatment

groups had an impaired vasodilator response to NO donor SNP and had a reduced NO-independent vasodilator response to Ach.

There was no evidence for oxidative stress in any Beta treatment group as protein carbonylation levels were unchanged relative to controls and decreased by Beta phosphate. Beta acetate and Beta phosphate treatment appeared to reduce cellular stress by reducing the pERK/ERK whilst not affecting pSAPK/JNK / SAPK/JNK protein ratios. Beta phosphate also resulted in a decrease of downstream chaperone PDI, whilst not affecting cellular chaperones hsp27, hsp70, or hsp60. Beta acetate and Beta phosphate also reduced the ratio of cleaved caspase-3 to total caspase-3 protein. GR protein was down-regulated in hearts from Dex and Beta phosphate treated embryos, suggesting active negative feedback.

In the embryonic lungs combined Beta treatment alone significantly increased levels of surfactant proteins caveolin-1 and SP-B. All Beta treatment groups significantly increased histological measures of pulmonary maturation to the same extent as Dex treatment. Dex treatment alone increased the thickness of the pulmonary gas exchange zone.

In this chapter we addressed the hypothesis that differences in the cardiovascular dysfunction induced by Dex or Beta exposure in late cardiovascular development is dependent on the prodrug formulation administered, with combined and acetate prodrug formulations having a different severity and nature of dysfunction compared to Na^+-phosphate prodrug formulations. The evidence here partially support the hypothesis that the prodrug

formulation administered affects the severity and nature of growth restriction and the induced cardiovascular dysfunction.

5.8.2 Effects on Embryonic Growth

As with the Dex and combined Beta treatments, treatment of the developing chicken embryo with either Beta acetate or Beta phosphate prodrug formulation resulted in a significant asymmetric growth restriction. The severity of the growth restriction is greater following combined Beta treatment than Dex treatment. Beta acetate resulted in a similar severity of growth restriction as the combined Beta treatment, whereas Beta phosphate acted more like Dex treatment. The observation that Beta acetate seems to be responsible for the worst growth restriction is likely related to the different pharmacokinetics of the prodrugs. There is extensive evidence for the Beta acetate drug formulation remaining in the fetal circulation for longer than the phosphate formulation, following maternal administration (Samtani et al., 2005; Salem & Najib, 2012). Therefore, it is likely that embryos exposed to the acetate prodrug have an overall greater activation of the GR pathways over time, which are known to stimulate the switch from tissue accretion to maturation (Fowden & Forhead, 2015; Fowden et al., 2016). However, we also observed subtle differences between Dex phosphate and Beta phosphate prodrug formulations (for example in liquid mass in the day 19 egg), suggesting that the nature of the stereoisomer itself is also partly responsible for nutrient resource distribution and conversion into fetal mass. Most pharmacological studies suggest a similar glucocorticoid potency of Dex and Beta of around 25-30x greater than hydrocortisone (Buttgereit et al., 1999; Parente, 2017). However, there is some evidence for subtle

differences, for example Dex and Beta phosphate administered at the same dose display differences in molecular markers of lung maturation (Schmidt et al., 2016).

5.8.3 Effects on the Embryonic Heart

Effects of the various glucocorticoid treatments on the embryonic heart were more divergent than those on growth. All treatments resulted in a significant increase in relative heart mass, however combined Beta had a more severe effect than Dex. Similarly, only combined Beta treatment resulted in a significant left and right ventricle dilatation in the heart, associated with decreases in left and right ventricle wall volumes. At the level of individual cardiomyocytes, Dex treatment induced a significant increase in cell volume, and a decrease in the % mononucleation. All Beta treatments induced a significant decrease in nuclear density in the left ventricle, without affecting cardiomyocyte volume or nuclearity. Whilst all drug treatments resulted in a significant increase in LVEDP and Tau, indicative of diastolic dysfunction, only Beta treatment groups impaired LVDP, indicative of systolic dysfunction.

It appears that Dex treatment has resulted in a premature switch away from cardiomyocyte hyperplasia towards hypertrophy (Oparil et al., 1984; Christoffels et al., 2000; Corrigan et al., 2010). Significant cardiomyocyte hypertrophy, and a decrease in % mono-nucleation in the Dex treated hearts may explain the diastolic dysfunction as premature maturation may impair electrical conductance and contractility, leading to an inability of the left ventricle to relax normally (Fig.5.15; Severinova et al., 2019). It may be that the Dex hearts have undergone hypertrophy as an adaptive response to prevent further ventricle dilatation, and this is why

hearts in this group are less severely affected. The severe dysfunction observed in the Beta treated hearts appears to be a result of the significant decrease in cardiomyocyte number leading to thinner ventricle walls and lower myofibril density. This has ultimately resulted in a dilated cardiac phenotype (see Fig.5.15; Bollen et al., 2017). The additional dilatation observed in the right ventricle in the Beta treated embryos may also be due to the severe reduction in cardiomyocyte number, or the increased dilatation in the left ventricle leading to a greater resistance in the pulmonary circulation and via the foramen ovale (Kurmani and Squire, 2017). The observation that combined Beta treatment is substantially more severe in terms of cardiac dilatation than either acetate or phosphate prodrugs alone, suggests that exposure to both prodrug forms has additional negative effects. Whilst the phosphate prodrug results in a rapid peak of free glucocorticoid in the circulation, the acetate form results in a lower sustained release (Samtani et al., 2005). The combination of the two may lead to a greater overall exposure; essentially the embryo is receiving a 'double hit' of activated glucocorticoid (Samtani et al., 2005). However, other studies have suggested that fetal exposure to active Beta is actually greater when the phosphate form is given compared to the combined form (Schwab et al., 2006). Clearly there are difficulties associated with measuring active glucocorticoid in the circulation, and our lack of knowledge of movement between the prodrug and active drug pools makes interpretation difficult.

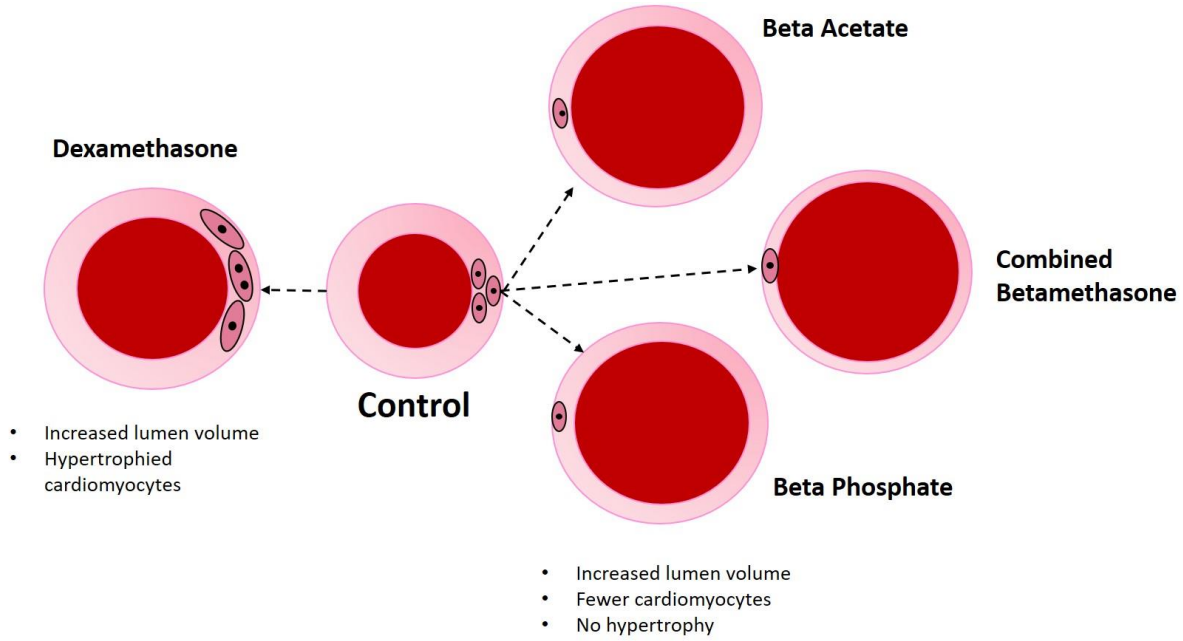


Figure 5.15 Summary of effects of glucocorticoids administered as different prodrug formulations on left ventricle structure. Dexamethasone results in a significant hypertrophy of individual cardiomyocytes, whilst Beta treatment drastically reduces cardiomyocyte number without altering cardiomyocyte volume. This is most severe in the combined Beta treatment group where a significant left ventricle dilatation is present.

5.8.4 Effects on the Peripheral Vasculature

Dex treatment results in a switch towards a vasoconstrictor phenotype with an enhanced KCl and ET-1 response, whilst vasodilator responses are not affected. This may be secondary to increased hyperplasia or hypertrophy of vascular smooth muscle cells (vSMCs; McLellan *et al.*, 1992; Volk *et al.*, 2010), and alterations to either ET-1 receptor densities (e.g. upregulation of ET_A) and/or downstream contractile pathways (Kutzler et al., 2003; Lee et al., 2014).

Beta treatments however resulted in distinct vascular phenotypes. KCl induced constriction is slightly reduced compared to controls thereby significantly impaired when compared to the enhanced Dex response in all Beta treatment groups, with Beta acetate being most severely

affected. All Beta treatments also resulted in a significant impairment of the vasoconstrictor response to higher doses of α_1-adrenergic receptor agonist PE, and this effect is similar between the different prodrug formulations. This decreased PE responsiveness is likely secondary to a decrease in α_1-adrenergic receptor density, or decreased receptor binding affinity in the femoral vascular bed (Brozovich et al., 2016; Ullian, 1999). ET-1 induced vasoconstriction, like PE induced vasoconstriction, is also primarily mediated by stimulating IP_3 production, downstream Ca^{2+} release and myosin light chain kinase (MLCK) activation, resulting in increased vascular constriction (Brozovich et al., 2016; Davenport et al., 2016). There is also evidence that enhanced ET-1 responses may be secondary to activation of the cyclic ADP ribose (cADPR)/ryanodine receptor pathway (Lee *et al.*, 2014). cADPR is an intracellular secondary messenger which regulates ryanodine receptor opening allowing Ca^{2+} release from the sarcoplasmic reticulum. Activation of the ET_{-B} receptor can activate cADPR induced vasoconstriction in the vSMCs, whilst ET_{-A} can act via cADPR or the classic IP_3 pathway (Barone et al., 2002). Hence, it is possible that the enhanced ET-1 response is due to increased cADPR signalling, and that a downstream inhibition of the IP_3 pathway is present in the Beta treatment groups. Clearly, further investigation into the effects of inhibiting cADPR production or blocking specific ET-1 receptors would help illuminate the specific altered mechanisms.

An additional vasoconstrictor dysregulation is seen in the Beta phosphate and acetate treatment groups, as both have a severely attenuated response to 5-HT. Serotonin (5-HT) is known to act via a multitude of receptors and to have divergent effects in different vascular system (Watts, 2016). The dose-dependent vasoconstriction is thought to mainly be due to

action of the 5-HT-$_{2A}$ receptor (Watts, 2005), and excess 5-HT has long been associated with hypertension in the adult (Huzoor-Akbar et al., 1989; Watts & Thompson, 2004; Nichols, 2009). The reduced response in Beta phosphate and acetate exposed embryos could be secondary to a variety of factors including distribution and identity of 5-HT receptors in the femoral artery. Other studies have demonstrated that glucocorticoids can attenuate 5-HT mediated vasoconstriction (Angeles et al., 2006; Selli & Tosun, 2016). A further study of 5-HT receptors sub-types would help elucidate the mechanism behind the altered 5-HT vascular constriction reported here, and why this dysfunction is not seen in the combined Beta treatment group.

A similar effect on vasodilator function was seen in all Beta treatment groups. Overall relaxation to muscarinic agonist Ach was not significantly altered, however the NO-independent component of the response was down-regulated. Ach is known to induce vasodilatation by several concurrent mechanisms, including production of NO by endothelial NO synthase (eNOS), production of prostacyclins by cyclo-oxygenase-1 (COX-1), and production of other vascular mediators, such as H_2S, termed endothelial derived hyperpolarising factor (EDHF; Liberman et al., 2018). This suggests that Beta femoral vessels are more reliant on NO-dependent vasodilatation to relax. We also observed a significant impairment in the response to NO delivered by NO donor SNP. NO produced in the endothelium in response to various receptor agonists (such as Ach), or in response to shear stress, rapidly diffuses into the vSMCs whereby it activates soluble guanylate cyclase (sGC) leading to increased production of cyclic guanine monophosphate (cGMP). This in turn induces vasodilatation by enhancing Ca^{2+} reuptake, and opening hyperpolarising K^+ channels

(Chen et al., 2008). NO may also directly interact with sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) to reduce cytosolic Ca^{2+} levels (Zhao et al., 2015). The impaired response to SNP in femoral vessels from all Beta groups suggests down-regulation of one or more of these pathways in the vSMCs themselves. Hence the down-regulation of NO-independent vasodilatation, and the impaired response to SNP in the Beta treated embryos is evidence of an impaired vasodilator function at the level of the vascular smooth muscle and the endothelium. It is unclear which NO-independent pathways are altered in these Beta-exposed vessels; further investigation using selective enzyme inhibitors would help to further elucidate the dysfunctional phenotype. Nevertheless, it appears broadly that the vascular dysfunction mirrors the cardiac dysfunction in glucocorticoid treated embryos. Whilst Beta treatment in these embryos seems to impair cardiac and vascular function, Dex treatment does not.

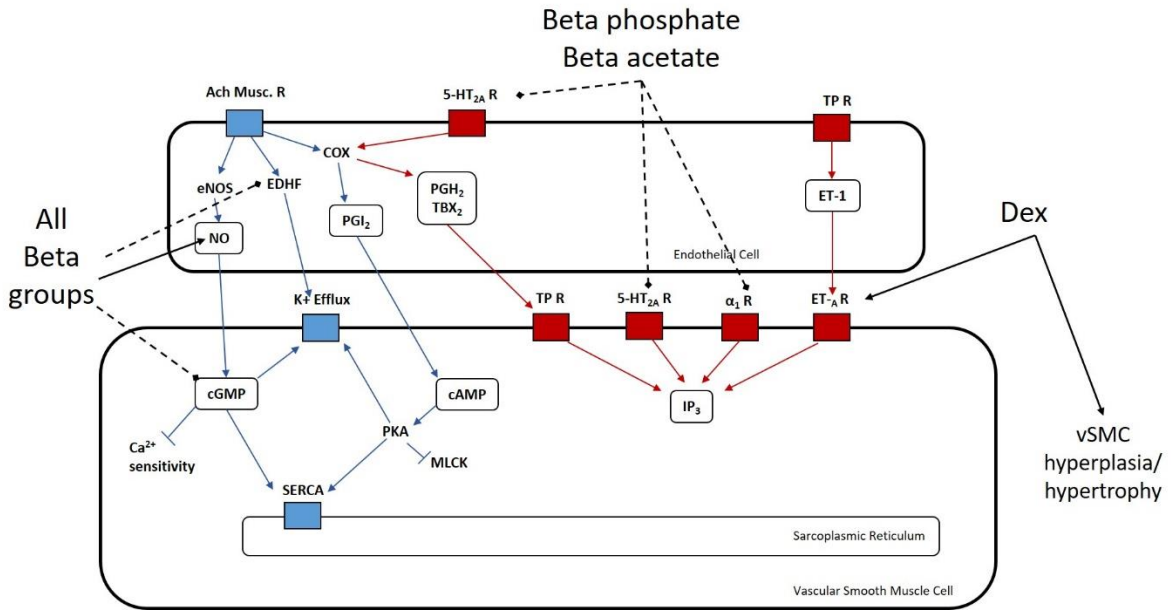


Figure 5.16 Vascular interactions following glucocorticoid exposure. Arrows represent upregulation of a pathway, whilst dashed lines represent suppression of a pathway. Interactions of vasodilators (blue) Ach which activates nitric-oxide synthase (eNOS), cyclo-oxygenase (COX) and endothelium derived hyperpolarising factors (EDHF). Production of NO and prostacyclins (PGI_2) leads to production of secondary messengers cAMP and cGMP in the vSMCs, which activate K^+ efflux channels, protein kinase A (PKA), SERCA, and inhibit myosin light-chain kinase (MLCK) and reduced Ca^{2+} sensitivity of sarcomeres. Vasoconstrictors (red) acting on 5-HT_{2A} receptors or thromboxane receptors (TP Rs) on the endothelial cells stimulate production of ET-1, thromboxane (TBX_2) and PGH_2. Activation of 5-HT_{2A}, TP, ET_{-A}, or α_1-adrenergic receptors leads to production of secondary messenger IP_3 and a subsequent increase in cytosolic Ca^{2+}.

5.8.5 Activation of molecular pathways in the heart

Unlike Dex-treated hearts, none of the Beta treatment groups demonstrated any evidence of oxidative stress, and Beta phosphate has a significantly decreased level of protein carbonylation. Interestingly, whilst our previous results in Chapter 4 suggested an up-regulation of cellular stress in both Dex and Beta treated embryos, there was no up-regulation in stress kinase pathways or downstream chaperones seen in the Beta acetate or Beta phosphate groups. In fact, we measured a significant decrease in the pERK/ERK ratio and in chaperone protein PDI in the Beta phosphate group, which suggests that cell stress pathways

are less likely to be active in these cells. Whilst Dex treated hearts had an increased ratio of cleaved caspase-3 to total caspase-3 protein, this was not present in any of the Beta treatment groups, suggesting that caspase-3 mediated apoptosis is not active in these embryos. p53 was significantly up-regulated in the Beta acetate group which is known to be a vital mediator of cellular senescence and known to be involved in architectural changes in the heart (p53 cardiac-specific knock-out in mice results in significant hypertrophy; Mak et al., 2017).

It is well known in the adult that glucocorticoids have an immunosuppressive effect, reducing cellular stress signalling (Sapolsky et al., 2000). The pERK and pSAPK/JNK pathways are both highly associated with immune signalling, as both up-regulate production of NFκB and cytokine production (Meyerovich et al., 2016). There is also evidence in specific cell lines of Beta down-regulating pERK/ERK protein ratio, whilst initiating cellular differentiation (Jin et al., 2015). The difference in cell stress markers seen between the combined and individual Beta treatments may be linked to a dose-dependent effect of GR activation, with the combined treatment having a greater overall dose of GR activation leading to cellular stress, whereas lower levels of GR activation may reduce cellular stress pathway activation. There is indeed evidence in many different cell types of biphasic or more complex dose-dependent glucocorticoid responses (Richardson & Dodge, 2003; Yokoyama et al., 2008; Scheschowitsch et al., 2017).

We also assessed negative feedback at the level of the GR protein in the embryonic heart. Synthetic glucocorticoids are known to exert negative feedback via the GR, resulting in down-

regulation of tissue GR levels (Paragliola et al., 2017). Down-regulation of GR at the level of transcription, results in lower protein levels and hence lower activation of GR pathways. Therefore, following Dex treatment there is a rapid peak of activation, followed by down-regulation of the cardiac GR. However, following combined Beta, and Beta acetate treatment this is not seen. Interestingly, the negative feedback response is still present in the Beta phosphate treatment group, suggesting that the prolonged exposure of the Beta acetate prodrug is responsible for the lack of GR downregulation. Whilst a loss of GR negative feedback may explain the more serve effects of combined Beta and Beta acetate treatment through greater overall GR activation, it doesn't explain why Beta phosphate has divergent effects on the embryonic heart when compared to Dex phosphate. Combined, the molecular data presented in this chapter suggest that following Dex exposure, oxidative stress activates cell stress pathways, the caspase-3 mediated apoptotic pathway, and altered cell cycle dynamics, whilst combined Beta treatment also induced cellular stress, decreased cell proliferation and non-caspase-3 mediated apoptosis secondary to greater activation of the GR pathways. Beta acetate also stimulates the excessive GR activation and increased p53 expression, although without the associated cell stress. Conversely, Beta phosphate restores GR negative feedback and reduces cellular stress, but still leads to a significant decrease in cardiomyocyte number via pathways that remain to be identified.

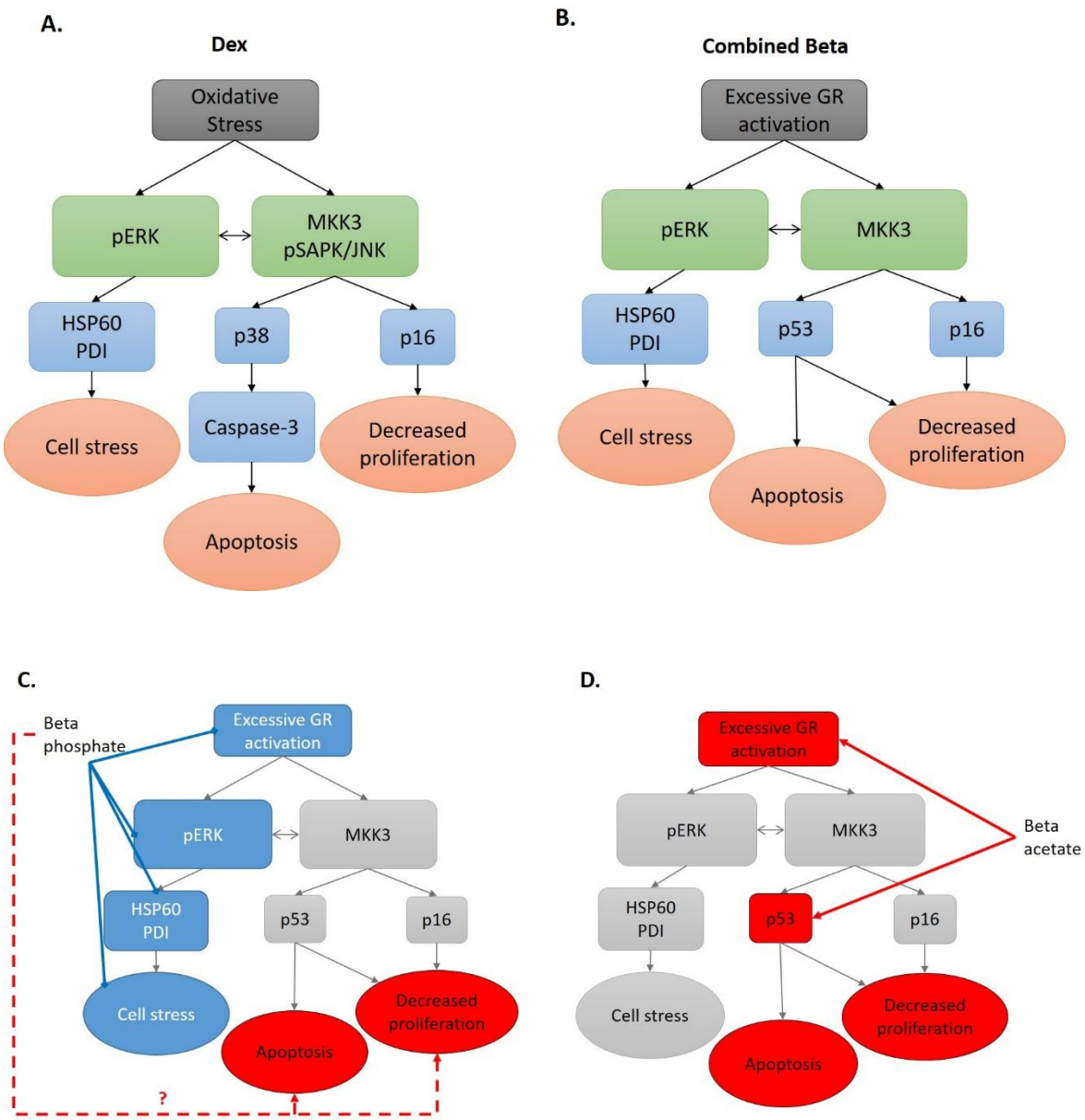


Figure 5.17 Molecular changes in hearts of Dex and Beta treated embryos. A., Dex treament results in oxidative stress which activates cell stress pathways, caspase-3 mediated apoptosis and decreased cellular proliferation, B. combined Beta treatment results in excessive GR activation, cellular stress, apoptosis and decreased cellular proliferation, C. Beta phosphate treatment reduces GR activation, and activation of cell stress pathways (blue arrows), whilst resulting in apoptosis and decreased proliferation via as of yet uninvestigated pathways (dashed red arrow), D. Beta acetate also results in excessive GR activation and p53 mediated apoptosis and decreased cell proliferation.

5.8.6 Effects on Lung Maturation

In our study both Dex and Beta induced maturation of the embryonic lungs. Histological markers of lung maturation such as increased parabronchial atrial diameter and air capillary diameter, are similarly enhanced by all glucocorticoid prodrugs. However, Beta acetate and phosphate appear to have little effect in up-regulating lung proteins caveolin-1 and SP-B (both markers of late development lung maturation in the chicken embryo; Bjørnstad et al., 2014), whereas combined Beta significantly up-regulated these proteins. There is some evidence in other animal models or clinical studies that Beta may be more potent in promoting lung maturation and reducing risk of adverse pulmonary consequences of preterm birth than Dex, such as RDS and BPD (Jobe and Soll, 2004).

5.8.7 Conclusions

The data in this chapter show that the severe growth restriction resulting from combined Beta treatment is likely secondary to the use of the slow-release acetate prodrug formulation. However, the severe effects of combined Beta on the embryonic heart and circulation appear to be caused by exposure to the combined acetate and phosphate drug forms, with the combined treatment having worse effects than either individual prodrug form alone. It is unclear how the pharmacokinetics of the two prodrug formulations interact to induce the cellular stress seen in the combined treatment group, but it appears unlikely that oxidative stress is responsible. We also observed sufficient components of embryonic lung maturation in all glucocorticoid treatment groups. Therefore, based on this evidence, to avoid the more severe effects of any Beta drug formulation on the developing cardiovascular system, it may be safer to use Dex for AGT clinically.

CHAPTER 6: GENERAL DISCUSSION

6.1 HISTORICAL PERSPECTIVE

Antenatal glucocorticoid therapy (AGT) for treatment of preterm birth is widely regarded as one of the most successful translations of basic science to clinical practise in recent history. The series of events leading up to the eventual adoption of AGT by the wider obstetric community is an example of both serendipitous scientific discovery and also of the relative inertia of obstetric medicine. Graham Liggins, an obstetrician in New Zealand, was funded by the Wellcome Trust in 1969 to conduct experiments in sheep to elucidate the mechanisms that trigger parturition. The ultimate goal of this research was to create a clinical therapy that could halt the normal mechanisms inducing labour in humans to try and prevent preterm birth. Liggins would go on to administer pregnant sheep with cortisol, and other synthetic glucocorticoids in attempts to initiate the parturition cascade. In the seminal 1969 paper, he reported not only that he had successfully induced preterm labour in his sheep model, but that the preterm born lambs had partial aeration of the lungs despite their prematurity (Liggins, 1969):

> *'Gross examination of the lungs of ten lambs delivered spontaneously at 117-123 days after dexamethasone infusions showed that in six, none of which had been artificially ventilated, there was partial aeration. In most instances the aeration consisted of patchy expansion in the upper lobes, but in one lamb delivered at 123 days the lower lobes were also partly expanded. This lamb was found alive at least an hour after being born and survived for a further hour until killed.'*

Liggins correctly saw these observations as evidence that the pre-natal cortisol surge in sheep was not only responsible for initiating parturition, but also for maturing fetal organs ready for the neonatal transition. Indeed, it is now understood in sheep that the cortisol surge itself

directly stimulates labour by inducing high expression of the placental enzyme 17$_\alpha$-hydroxylase which promotes placental oestrogen biosynthesis (Mason et al., 1989). Additionally, cortisol acts as a maturational signal for all physiological systems in the fetus, switching tissues from a state of accretion to that of differentiation and maturation (Fowden et al., 2016). Some of the most well-known effects of the prepartum cortisol surge are on the fetal lungs whereby cortisol, both directly and indirectly, promotes surfactant production, lung liquid removal, and increased lung compliance; all helping to ensure the neonate can ventilate when born (Ballard, 2000).

The human situation however is dissimilar from that of the sheep in that the cortisol surge itself does not directly stimulate parturition; rather androgen of adrenal origin serves as the precursor for placental oestrogen synthesis (as the primate placenta does not express 17$_\alpha$-hydroxylase; Mecenas et al., 1996; Nathanielsz et al., 1998). This difference means that the maturational potential of antenatal glucocorticoid treatment can be harnessed clinically without also directly inducing preterm labour. Liggins' observations rapidly led to the initiation of a clinical trial in 1972, in which women presenting at hospital with threatened preterm labour were administered with the synthetic glucocorticoid betamethasone (Beta). Beta treatment significantly reduced instances of respiratory distress syndrome (RDS) in the neonates, and neonatal death (Liggins & Howie, 1972). Initially Beta was chosen because synthetic glucocorticoids Beta and dexamethasone (Dex) can readily cross the placenta as they are not inactivated by endogenous enzyme 11β-HSD2, and they have a longer plasma half-life than cortisol, therefore maximising the likelihood that maturational changes in the fetus will be initiated. A number of other clinical trials followed, all reporting the same

beneficial effects of AGT. Despite the wealth of evidence demonstrating the life-saving potential of this treatment, it was not until the 1990's that AGT was adopted as standard by the wider obstetric community. In 1994 the National Institutes of Health (NIH, 1994) released a consensus statement, purporting that:

> *'Antenatal corticosteroid therapy is indicated for women at risk of premature delivery with few exceptions and will result in a substantial decrease in neonatal morbidity and mortality, as well as substantial savings in health care costs.'*

In the decades since this statement, AGT has become standard obstetric practise and most developed countries have high coverage rates with over 80% of women threatened with preterm labour receiving treatment. However, there is increasing evidence that whilst AGT is clearly lifesaving, it may have undesired detrimental effects on other organ systems. Indeed, the glucocorticoid receptor (GR) is ubiquitously expressed in the developing fetus, and its activation has a myriad of diverse tissue specific effects. Hence, while accelerated maturation of the lungs may be beneficial, the same may not be true for other organ systems. The work in this thesis has isolated direct adverse effects of Dex and Beta and their formulation of administration on the developing cardiovascular system.

6.2 SUMMARY OF RESULTS

Outcome		Dex	Combined Beta	Beta Acetate	Beta Phosphate
Embryo Growth	Embryo mass (%)	↓	↓↓↓	↓↓↓	↓
	Brain mass (%)	↑	↑	↑	↑
Cardiac Structure & Function	Left Ventricle Dilatation	↑	↑↑↑	↑	↑
	Systolic Function	↓	↓↓↓	↓↓↓	↓↓↓
	Diastolic Function	↓	↓	↓	↓
	Cardiomyocyte Size	↑↑↑	-	-	-
	Cardiomyocyte Number	↓	↓↓↓	↓↓↓	↓↓↓
Peripheral Vascular Function	Constriction	↑	↓	↓	↓
	Dilatation	-	↓↓↓	↓↓↓	↓↓↓
Cardiac Molecular Changes	Oxidative Stress	↑↑↑	-	-	↓
	Cell Stress	↑↑↑	↑↑↑	-	-
	Caspase-3 mediated apoptosis	↑↑↑	-	-	-
	Cell proliferation	↓	↓↓↓	↓↓↓	↓
	GR negative feedback	-	↓↓↓	↓↓↓	-
Lung Maturation	Surfactant Proteins	↑	↑↑↑	-	-
	Histological indices	↑↑↑	↑	↑	↑

Table 6.1 Summary of results. Blue arrows represent an increase, red arrows a decrease, and dashes no change.

The data in this thesis show that direct treatment of the chicken embryo with a human clinically relevant dose of dexamethasone (Dex) or betamethasone (Beta) at a developmental stage equivalent to the 26-week-old human fetus promotes asymmetric growth restriction and adversely affects cardiovascular structure and function (key results shown in Table 6.1). Specifically, hearts isolated from Dex-treated embryos had biventricular lumenal dilatation, and evidence of systolic and diastolic dysfunction. Hearts from Beta-treated embryos had a more severe dilated phenotype, impaired systolic and diastolic function, as well as impairments to coronary flow and heart rate. Mechanisms underlying the cardiovascular dysfunction in Dex-treated embryos included blunted inotropic responses to autonomic receptor agonists and cardiomyocyte hypertrophy with reduced proliferation. Enhanced *in vivo* generation of mitochondria-derived ROS, protein carbonylation and activation of ROS-induced cardiomyocyte senescence pathways provide a molecular link between dexamethasone treatment with adverse cardiovascular changes in the chicken embryo. In Beta treated embryos, a severe reduction in cardiomyocyte proliferation and increased cellular stress without evidence for oxidative stress was determined, suggesting a key divergence in mechanisms of cardiac dysfunction between the two synthetic glucocorticoids.

Further, we aimed to understand the role of different Beta prodrugs in inducing the severe cardiovascular dysfunction witnessed in these embryos. We have demonstrated that the acetate prodrug is primarily responsible for the more severe effects of Beta on embryonic growth, likely secondary to loss of GR negative feedback. However, we have also demonstrated that a combined prodrug treatment is significantly worse for the developing cardiovascular system than either phosphate or acetate prodrug alone; potentially delivering

a ‘double-hit’ of both short-term and prolonged active glucocorticoid exposure. The molecular pathways activated by Beta acetate seem to largely result in up-regulation of p53, and severe impairment of cell proliferation without activation of cell stress pathways. It is unclear how Beta phosphate also results in the significant reduction in cardiomyocyte number observed, as none of the pathways we assessed appeared to be activated, and indeed it appears as though Beta phosphate acts to reduce cellular stress.

Finally, we demonstrated that combined Beta had the most beneficial effects on the embryonic lungs, leading to significant up-regulation of surfactant proteins caveolin-1 and SP-B. Dex also increased protein expression of these maturational markers although to a lesser extent. Neither Beta acetate or phosphate significantly altered expression of either protein relative to controls. Histological indices of lung maturation were similarly altered in all glucocorticoid treatment groups except for thickness of gas exchange region which was only increased following Dex treatment.

Therefore, despite maturational beneficial effects on the lungs, the data provide robust evidence for direct adverse effects on the cardiovascular system of the developing offspring independent of effects on the mother and/or placenta of synthetic glucocorticoids in doses relevant to those used in human clinical practise. Further, these effects are dependent on which drug and formulation is used, with Dex possibly being the safer choice. It is of great importance that we further assess the mechanisms behind these negative effects such that clinical therapy can be adjusted to reduced negative effects on the cardiovascular system whilst maintaining maturational effects on the lungs.

6.3 MODEL OF AGT

As with any study on the effects of AGT, interpretation of results depends on the dose and timing of glucocorticoid administration, as well as the specific drug utilised. Here, we used a dose of 0.1mg.kg_{Emb}^{-1}. This is lower than the maternal clinical dose of 24mg, which, accounting for placental expulsion of glucocorticoid, equates to a fetal dose of glucocorticoid in the order of 8mg (Ballard, 2000). The average fetus at 27 weeks is 0.875kg which would give a fetal dose of 9.1mg.kg_{Fet}^{-1} which is 91 times higher than the dose used in these experiments. Therefore, we might expect that if we were to use a higher dose, of closer resemblance to the human clinical dose, the effects describe in this study may be significantly more severe. Additionally, it is now commonplace for fetuses to experience multiple courses of AGT, if after 48 hours of the initial treatment delivery has not occurred (Bonanno et al., 2007) and/or due to steroid exposure in the neonatal period. Therefore, the cumulative exposure in the clinic may be even greater, and thereby, of greater concern. The chicken embryo model allows for the opportunity to conduct a dose-response trial to determine the effective fetal dose at which beneficial lung maturation occurs, and the dose at which detrimental cardiovascular and growth effects occur. Repeating measurements of fetal biometry, cardiac outcomes (e.g. stereology and isolated organ function), and lung maturation at initial doses of 0.01$mgkg^{-1}$, 1$mgkg^{-1}$, and 10$mgkg^{-1}$ would allow us to establish the order of magnitude over which these effects occur.

We chose day 14 of chicken embryo development to give our glucocorticoid treatment as this is the equivalent to roughly 26/27 weeks of human pregnancy, which is an early preterm time-point at which majority large proportion of threatened preterm fetuses would receive AGT

(Roberts & Dalziel, 2006). However, the administration of AGT clinically runs through a large spectrum of gestational ages including late preterm or even term babies (Romejko-Wolniewic, et al., 2014). There is evidence that AGT differs in efficacy depending on gestational age of the fetus at exposure (Porto et al., 2011; Gyamfi-Bannerman et al., 2016). Similarly, in animal models it is likely that the reported effects of AGT will be dependent on the timing of administration. Therefore, we cannot directly extrapolate results from this study to AGT administration at other developmental time-points.

Another limitation to our model of AGT administration is the associated pharmacokinetics in the fetal plasma. In the clinic AGT is administered over 48 hours, as combined Beta treatment has an estimated half-life of 12.5 hours, this may result in up to 60.5 hours of glucocorticoid exposure, or 0.9% of gestation (Salem & Najib, 2012). Assuming a similar half-life in our model, the chicken embryo following a single dose may have up to 12.5 hours of exposure, which is 2.5% of incubation, thereby equivalent to 1 week exposure to synthetic glucocorticoid for the human fetus. Therefore, it is possible that in our chicken embryo model the developing cardiovascular and other physiological systems are exposed for a comparatively greater percentage of development. This may be offset by the exposure of a comparatively much lower dose of glucocorticoid in the chicken embryo than in the human situation. Nevertheless, these differences need to be taken into account. Additionally, the profile of exposure of the specific synthetic glucocorticoid used needs to be taken into consideration. Differences in pharmacokinetics between the different drug formulations in the chicken embryo compared with the human fetus may also result in differential effects.

Clearly, it is essential in further studies to assess the pharmacokinetic profile of both Dex and Beta in this model. It is possible to measure synthetic glucocorticoid concentrations in plasma with a variety of methods including mass spectrometry, and radioactivity assays (Fletcher et al., 2000; Samtani et al., 2005). However due to the small volume of plasma in the embryonic chicken (Barnes & Jensen, 1959) it is not possible to take serial measurements from the same animal over a time course. As such it will be necessary to conduct a cross-sectional study whereby embryos are sacrificed at a series of time points post glucocorticoid administration (e.g. 1 hour, 3 hours, 6 hours, 12 hours, 24 hours, as plasma half-life has been measured to be within this time frame; Schmidt et al., 2018), such that plasma concentrations can be measured. In this study it would also be possible to assess GR pathway activation in specific tissues (e.g. the embryonic heart) by assessing the ratio of nuclear to cytoplasmic GR via western blot (Barrientos et al., 2015), phosphorylated GR levels (relative to total GR) also via western blot, or assessing early transcripts known to be up-regulated following GR canonical pathway activation by RT-QPCR (Gupta et al., 2007).

6.4 SEX DIFFERENCES

There is evidence in both human clinical data and animal studies that the effects of AGT may differ depending on fetal gender (Stevenson et al., 2000). For example, following AGT in the sheep, females are protected from renal oxidative stress whilst males are not (Bi et al., 2014), however females also display impaired glucose tolerance whilst males do not (Massmann et al., 2017). In both guinea pigs and mice, significant differences in brain development based on fetal sex have been reported (McArthur et al., 2016; Constantinof et al., 2019). In general

it is well established that fetal sex may have a significant effect on programming effects of various developmental insults, for example males may be more susceptible to hypertension (Aiken & Ozanne, 2013).

In this study we have not provided any sex-specific data. This is due to the difficulty in sexing chicken embryos at day 19 of incubation. Whilst morphological sexing by eye based on intra-abdominal gonad structure is possible, it is practically difficult and not reliable (González-Morán, 2011). Therefore, during this project we have additionally aimed to develop a method for genetically sexing frozen embryonic chicken tissues. Female chickens possess the ZW sex chromosome configuration, whilst the males have the homogeneous ZZ configuration (Hirst et al., 2018). Basing our protocol on previous work by other groups (Wan et al., 2016), we generated oligo primers (custom, Sigma, UK) for a genetic sequence on the W chromosome, and a control ribosomal sequence present in both genders (Fig.6.2). DNA was extracted from frozen liver samples using a DNeasy blood & tissue kit (Qiagen, NL). A PCR reaction was then set up using ReadyStart PCR Master Mix (Sigma, U.K.), 10ng DNA extract, 0.5µM of W and R primers, and distilled H_2O. The following reaction cycle was then used:

- 94°C 1 minute
- 72°C 2 minutes
- 54°C 3 minutes
- 30 cycles

Once PCR was completed, the products were separated on a 1% agarose gel (Agarose, Sigma, UK, Varigel electrophoresis chamber, Scie-plas, UK). DNA bands were then visualised using ethidium bromide (EtBr) (Sigma, UK, GelDoc-It, UVP, USA). Females possess both a W and an R band, whilst males only have an R band.

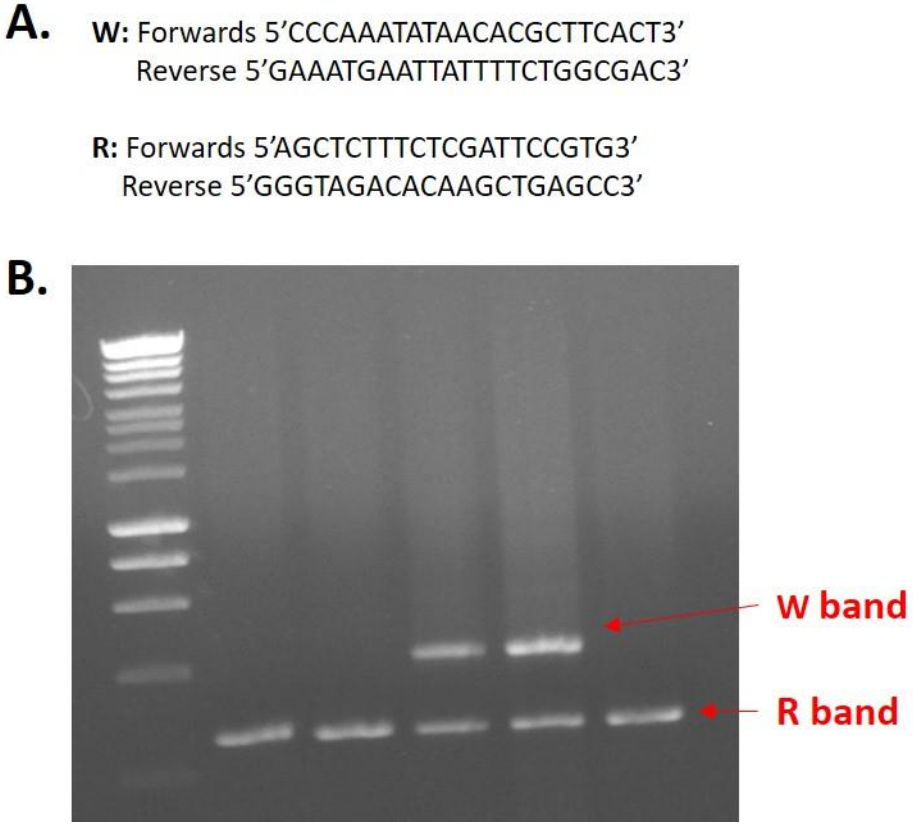


Figure 6.1 PCR-based sexing of chicken embryos. A. Primer sequences, B. Gel visualised under fluorescence light after exposure to EtBr, with PCR product bands labelled.

It is not possible to retrospectively separate all results in this study by gender due to tissues not being frozen for embryos from which functional cardiovascular data were generated. However, it has been possible to retrospectively analyse embryonic growth data by fetal sex (Fig.6.3). Whilst embryo mass, relative head diameter, and relative heart mass are only significantly affected by Dex treatment, relative brain mass has a secondary main effect of gender with females having a greater relative brain mass than males. For future studies sex specific analysis will be conducted on all outcome variables.

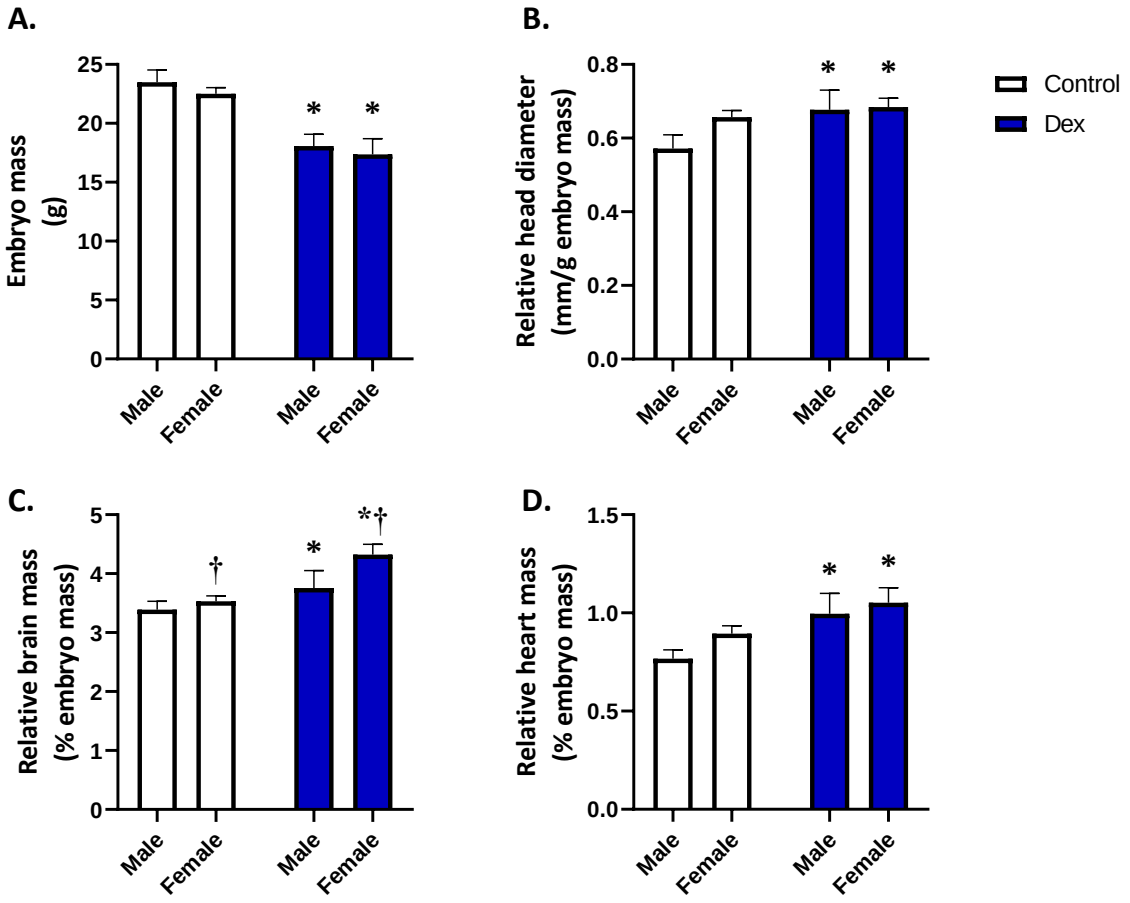


Figure 6.2 Sex-specific embryonic biometry following Dex treatment. A. absolute embryo mass (g), B. relative head diameter (mm/g embryo mass), C. relative brain mass (% embryo mass), D. relative heart mass (% embryo mass), in male and female control (white) (n=13) and Dex (blue) (n=10) treated embryos. 2-way ANOVA with Tukey post-hoc test. * main effect of Dex treatment, $p<0.05$, † main effect of sex, $p<0.05$.

6.5 Translation of Findings to the Clinical Situation

6.5.1 Mammalian Models

Whilst the cardiovascular changes we have documented in this study are linked to negative effects on the developing embryo prior to hatching, we have not demonstrated whether the cardiovascular dysfunction extends into adult life, and if it results in overt disease. It is becoming increasingly apparent that insults experienced during development may result in

physiological changes that confer increased disease risk in adulthood (Fowden et al., 2016). In terms of the clinical situation, as well as immediate neonatal outcomes, it is also important that we investigate any lifelong dysfunction induced by AGT exposure. The chicken model has been used to assess the long-term programming effects of e.g. incubation in hypoxia, demonstrating altered cardiovascular function at adulthood (Itani et al., 2018). Therefore, it would be valuable to allow glucocorticoid-treated embryos to hatch, and to follow up their growth, and cardiovascular functioning at various adult ages (such as young adults at 6 months, and an aged adult at 3 years). Our laboratory has also previously demonstrated that it is possible to assess *in vivo* cardiovascular function in the chicken via insertion of arterial and venous catheters, and arterial flow probes (Skeffington et al., 2018).

In order to establish long-term effects of AGT that are of greater translational importance, it will also be necessary to repeat these experiments in a mammalian model. Whilst the chicken embryo provides a good model for isolating direct adverse effects on cardiovascular development, the lack of placental and maternal physiological changes means it is not possible to extrapolate these results directly to human clinical practise. However, there is already a wealth of evidence demonstrating long-term dysregulation of cardiovascular physiology following glucocorticoid exposure in various mammalian models, predominantly in rodents, sheep and primates. In rats exposed postnatally to human clinically-relevant dose of Dex, there is significant cardiac and peripheral vascular dysfunction at adulthood, with systolic function decreasing more severely with increasing age (Herrera et al., 2010; Bal et al., 2008). In the sheep model there is also evidence for altered vascular function, hypertension, altered cardiac function (including impaired cardiac reserve), and changes in the renin-

angiotensin system (RAS) at adulthood following prenatal glucocorticoid exposure (Dodic et al., 1998, 2001; Pulgar & Figueroa, 2006; Moritz et al., 2011). Nevertheless, much of the literature in mammalian animal models is complicated by use of varying doses and timing of administration of AGT, again making it difficult to relate this work to human clinical practise. There is some human clinical data also available demonstrating altered long term cardiovascular outcomes following AGT. For instance, adult men and women born to mothers who were given AGT have been shown to have increased aortic arch stiffness, elevated blood pressure, altered cardiac structure, right ventricle dysfunction and stress-induced left ventricle dysfunction (Kelly *et al.*, 2012; Lewandowski *et al.*, 2013b, 2015; Huckstep *et al.*, 2018). However, there is a lack of consensus on the long-term cardiovascular implications of AGT as there is also clinical data that suggests no alterations to blood pressure at age 30 following fetal exposure to AGT (Dalziel et al., 2005). The difficulty in interpreting much of the human clinical work is that it is not possible to disentangle the effects on long-term outcomes in the offspring of being born preterm vs being exposed to AGT, as one rarely occurs without the other, and preterm birth itself is known to increase risk of cardiovascular disease in late life (Luu et al., 2017).

In order to better assess the impact of glucocorticoid exposure alone on long-term cardiovascular outcomes, our laboratory has developed an ongoing programme of work in a sheep model in which we can isolate these effects, independent from preterm birth. In this model, pregnant sheep are treated with 2x12mg doses of intramuscular Dex (total 24mg) on days 115 and 116 of gestation, which is the equivalent of 30 weeks of human pregnancy. In order to prevent the initiation of premature labour following the Dex administration, the

ewes are also treated with progesterone at 80 days of gestation, with an additional progesterone only group to control for possible effects of progesterone alone. Using this model, data thus far show that, aat term, in dexamethsone-treated pregnancies, the fetal heart demonstrated impaired systolic and diastolic function independent of effects of progesterone treatment; similar to our results in the chicken embryo (Botting et al., 2017; Fig.6.1 A). Another cohort of lambs were born at term and then raised until 9 months of age. Crucially, birth mass, blood pressure, and heart rate were not different between Dex-exposed and control lambs (Fig.6.1 B). However, *ex vivo* cardiac function was still impaired, and the left ventricle and aorta showed significantly increased fibrosis (Fig.6.1 A&C). *In vivo* functional echocardiography in live animals demonstrated an impaired ejection fraction and left atrial dilatation, associated with impaired compliance of the left ventricle wall (Fig.6.2 D). These results demonstrate that in the absence of preterm birth, exposure to a clinically-relevant model of AGT still results in significant cardiac dysfunction in a mammal in which temporal cardiovascular development also matches the human situation. Many of the outcomes we measured are themselves associated with increased risk of negative cardiovascular outcomes (Bouzas-Mosquera et al., 2011; Gulati et al., 2013; Cikes & Solomon, 2016). It also demonstrates an *in vivo* method with greater clinical translatability to detect these cardiac changes. These data show that using functional echocardiography, it is possible to assess cardiac dysfunction in adults exposed to AGT *in utero*, even in the absence of hypertension or preterm birth.

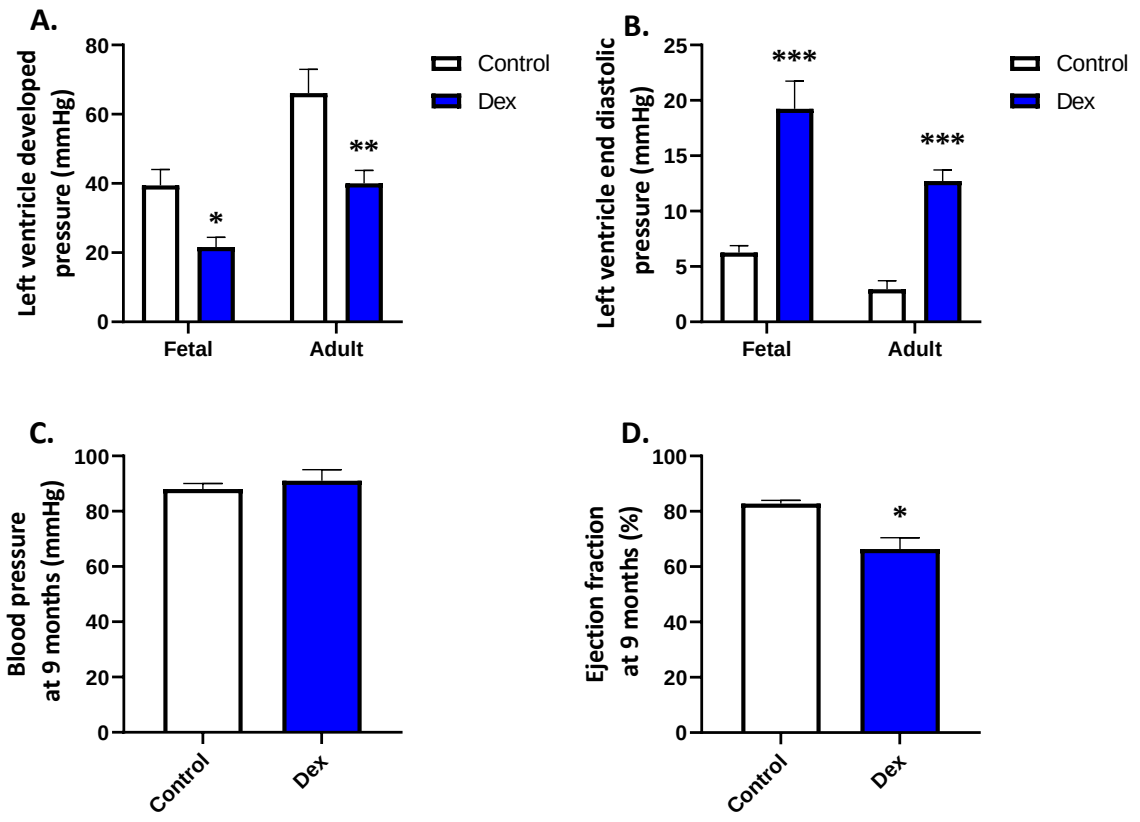


Figure 6.3 Cardiovascular outcomes in fetal and adult sheep exposed to antenatal Dex. A. Left ventricular developed pressure in foetuses and 9 month old lambs, B. Left ventricular end diastolic pressure in foetuses and 9-month-old lambs, C. Blood pressure in 9-month-old lambs, D. Left ventricle ejection fraction in 9-month-old lambs, from control (n=7) or Dex treated (n=6) pregnancy, all born at term. All stats are parametric t-test, $P<0.05$ *, $P<0.01$ **< $P<0.001$ ***. Data from (Botting et al., 2017).

6.5.2 Measurable Outcomes in Clinical Practice

The observations in our sheep model of AGT that adult cardiac dysfunction can be detected by use of *in vivo* functional echocardiography demonstrates it is possible to measure predictive cardiac outcomes in humans, even without the presence of hypertension. In order to translate our findings to human clinical practise it would first be necessary to confirm the observations of impaired cardiac function seen in the fetal sheep in babies and children

exposed to AGT, by use of the echocardiography technique. Whilst blood pressure has been assessed in neonates and children exposed to AGT, surprisingly there are no clinical data assessing cardiac function in these individuals (McKinlay et al., 2015; Cartwright et al., 2018).

In order to be able to dissect the effects of AGT vs prematurity it would be necessary to recruit a large enough human cohort such that a suitable number of pregnancies are covered that are born preterm following AGT, born at term following AGT, or born at term with no AGT. Increasing numbers of children are being born at term or late preterm who have been exposed to AGT, making it possible to compare this group to those born preterm following AGT (Saccone & Berghella, 2016). The ASTECs trial reported outcomes of neonates exposed to Betamethasone who were delivered at term suggesting no negative outcomes in an initial cohort of 937 children (Stutchfield et al., 2005). As AGT is routine treatment, it does not pose great ethical problems, and recruiting a large enough population will not be difficult. To establish whether the fetal cardiac dysfunction seen in the sheep is present in humans, it would be necessary to conduct functional echocardiography on the neonate to record primary outcomes of ejection fraction, atrial diameter, and the ventricular E to A wave ratio. Echocardiography is a readily available technique in most maternity units, as it is already employed for assessments of various fetal and neonatal morbidities, such as detection of fetal heart defects (Liu et al., 2016). Further, it has been demonstrated that this technique has an excellent level of reproducibility, which would limit any differences in results collected from different collaborating institutions (Kou et al., 2014).

6.5.3 Refining Current Clinical Therapy

If it can be demonstrated that a functional cardiac impairment is present in children exposed to AGT, then this opens the door for further clinical trials aimed at fine-tuning current clinical practise to maintain the benefits of AGT but to diminish the negative cardiac effects. The results in this thesis demonstrate that in our chicken embryo model of AGT, Beta administration has more severe negative effects on growth, cardiac, and vascular function than Dex administration. These results therefore suggest that it may be safer to use Dex rather than Beta for AGT in clinical practise. However, there is no current consensus on which steroid should be utilised, largely owing to lack of clear experimental data, and the relatively few clinical studies that directly compare the two drugs (WHO, 2015; Schmidt et al., 2019). The choice of which steroid to use is largely based on availability, price, and in some countries historical usage of one over the other. Dex is currently the primary steroid used for AGT in most countries, likely due to the fact that it is both cheaper (price per unit estimated at $0.22-1.56 for injectible Dex phosphate, $9.51 for Celestone Soluspan (Celestone Soluspan Prices, 2019; Dexamethasone Prices, 2019) and more widely available than Beta (Lawn et al., 2012). The countries that continue to rely on Beta for AGT, such as New Zealand, tend to have done so historically. Due to these factors and that intramuscular Dex is used for many other clinical treatments, thishas led to it being named an essential medicine by the WHO (Lawn & Althabe, 2013). Using the echocardiography techniques and outcomes from an initial clinical study to assess cardiac dysfunction in children following AGT, it would then be possible to conduct a follow-up study to directly compare the cardiac implications of antenatal exposure to either Dex or Beta on cardiac function in babies and children. It would also be vital to measure

otheroutcomes, such as pulmonary or neurological complications, to test whether the beneficial outcomes of AGT are also promoted to the same extent by both steroids.

A further way in which clinical therapy can be fine-tuned to reduce negative cardiac effects is to give a co-treatment at the time of AGT administration that can help negate the damaging changes to the cardiovascular system, whilst allowing the beneficial effects of AGT to remain. Accumulating evidence suggests that in mammals one pathway by which glucocorticoids may promote their deleterious effects is through the inappropriate generation of reactive oxygen species (ROS). Once generated, ROS may subsequently damage cellular components, affect signalling pathways, and alter the oxidant tone of the vasculature by decreasing nitric oxide (NO) bio-availability (Chen & Keaney, 2004; Chen et al., 2008). Indeed, there is evidence of oxidative stress in the heart and brain in mammalian models of antenatal glucocorticoid exposure (Antonow-Schlorke et al., 2009; Camm et al., 2011; Niu et al., 2013; Tijsseling et al., 2013). In a series of studies using neonatal rat pups treated with a tapering course of Dex, it has been demonstrated that co-treatment with the antioxidants vitamins C and E (ingested via drinking water) reduced oxidative stress and helped prevent the detrimental effects of Dex treatment on the brain, heart and peripheral vasculature (Herrera et al., 2010; Camm et al., 2011; Niu et al., 2013). A second series of studies tested whether agents that are not antioxidants *per se* but are known to increase NO bioavailability could also be used to diminish the adverse side-effects of postnatal glucocorticoid therapy. Statins are known to have pleiotropic effects on the vasculature that include increased NO bioavailability. Dex combined with pravastatin treatment restored circulating NOx and prevented the adverse effects of dexamethasone on the developing brain at weaning (Camm EJ, et al. 2018; Tijsseling et al.,

2013). In a separate study it was also demonstrated that pravastatin normalized placental vascular defects, fetal growth and cardiac function in a murine model of glucocorticoid excess (Wyrwoll et al., 2016).

These experiments demonstrate that it is possible to ameliorate the negative cardiac side effects of AGT whilst maintaining the clinical benefits. It therefore is of interest to pursue a further clinical study in which a co-treatment (either an antioxidant, or a statin) is administered concurrently with AGT and cardiac outcomes in children are assessed. Various antioxidants have been used in clinical trials for several complications of pregnancy with varied success (Polyzos et al., 2007; Ardalić et al., 2014). Generic Antioxidants, such as vitamin C, have been used in human pregnancy complicated with poor fetal growth, pre-eclampsia, or preterm birth, without any beneficial effects being observed (Rumbold et al., 2015). However, it is difficult to establish high plasma levels of vitamin C without increasing risk of other complications such as kidney stones in the expectant mother (Ferraro et al., 2016). A statin may be a better choice for clinical use as fewer contra-indications and side effects have been observed and various clinical trials administering statins in pregnancy are ongoing (Karalis et al., 2016). In order to maximise the potential of the statin treatment to successfully reduce oxidative stress following Dex administration, it is necessary to chose a drug with a relatively long half-life in plasma (which can vary significantly from 1-9 hours, depending on the specific compound), which is also highly lipophilic and may cross the placenta readily (Schachter, 2005; Costantine & Cleary, 2013). Pravastatin can readily cross the placenta, and so may be a good choice for future clinical studies (Nanovskaya et al., 2013).

6.6 Closing Remarks

Whilst the initial clinical trial of Liggins and Howie would kick start a change in obstetric medicine that has likely saved the lives of thousands of premature children, the resistance of the wider community meant that it was not until 22 years and 12 trials (involving an additional 2138 infants) later that the NIH consensus statement recommending AGT for threatened preterm labour was released (Ballard & Ballard, 1995; Roberts & Dalziel, 2006). Clearly if the available evidence had been adopted into clinical practise sooner, then thousands of preterm infants would not have suffered unnecessarily. Similarly, we have had accumulating evidence for many years of the potential unwanted side effects of AGT on other developing physiological systems, yet many aspects of this therapy still have not been optimised. Clearly, this is an ethically sensitive topic, as AGT has been demonstrated over and over again to be life-saving for premature infants (Roberts et al., 2017). However, as our knowledge of how factors in early life can alter future risk of disease increases, it is necessary to reflect on and review current clinical practise such that we can fine tune AGT to maintain its benefits whilst eliminating the adverse side effects on other organ systems. The plastic physiology of the fetus makes it susceptible to adaptive changes that can turn detrimental and last into adult life, however it widens the window of opportunity for preventative treatment and correct these changes. Increasingly, we are aware that the earlier a healthcare intervention is made, the greater the return in terms of economic production at a societal level, and health and wellbeing at an individual level (Heckman, 2012). It seems that in the case of prematurely born individuals, we can be doing better than simply ensuring survival.

APPENDIX

Publications arisen from this thesis thus far

Topical review:

- **Garrud, TA** & Giussani, DA, 'Combined Antioxidant and Glucocorticoid Therapy for Safer Treatment of Preterm Birth', *Trends Endocrinol Metab*, 30(4):258-269, 2019.

Conference abstracts accepted for oral presentation:

- **Garrud TA**, Teulings NE, Conlon FG, Ford SG, Niu Y, Nicholas LM, Derks JB, Ozanne SE, and Giussani DA. 'Adverse Effects of Antenatal Glucocorticoids on the Fetal Heart: A Study in the Chick Embryo.' 44th Annual Meeting of the Fetal & Neonatal Physiology Society, Osaka, Japan, Sep 6-10, 2017.

- **Garrud TA**, Teulings NE, Conlon FG, Ford SG, Niu Y, Nicholas LM, Derks JB, Ozanne SE, and Giussani DA. 'Adverse Effects of Antenatal Glucocorticoids on the Fetal Heart: A Study in the Chick Embryo.' 65th Annual Meeting of the Society of Reproductive Investigation, San Diego, California, USA, March 6-10, 2018. **Young investigator travel award.**

Conference abstracts accepted for poster presentation:

- **Garrud TA**, Tuelings NE, Conlon FG, Ford SG, Niu Y, Nicholas LM, Derks JB, Ozanne SE, and Giussani DA. 'Adverse Effects of Antenatal Glucocorticoids on Fetal Heart Structure & Function.' 66th Annual Meeting of the Society of Reproductive Investigation, Paris, France, March 12-16, 2019. **Young investigator travel award.**

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