INCREASED MATERNAL CORTISOL CONCENTRATION IN LATE GESTATION ADVERSELY AFFECTS FETAL AND NEONATAL DEVELOPMENT

By

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Elevated or prolonged maternal stress during pregnancy can result in poor obstetric outcomes and programming of fetal tissues. The effects of in utero fetal programming are thought to be the contributing factors in the “developmental origins of adult disease” hypothesis. Late in gestation there is an exponential increase in fetal glucocorticoid concentrations, which is a highly regulated process believed to be essential in maturing fetal organs for ex utero survival. Altering this process by advancing the fetus’s exposure to glucocorticoids can have profound effects on the normal trajectory of cardiac development. Experiments in this dissertation were designed to: 1) Test the feasibility of implanting radiotelemetry devices into late gestation ovine fetuses to measure cardiac function; 2) Evaluate the effects of chronically increased maternal cortisol concentration on the cardiac function of the late gestation ovine fetus; 3) Investigate genes and pathways that are differentially regulated in the fetal heart by chronically increased maternal cortisol concentration; 4) Describe the effects of increased maternal cortisol concentrations in late gestation on postnatal growth and metabolism.
This dissertation explains in depth a novel method for the chronic implantation of radiotelemetry devices into late gestation ovine fetus, as well as the normal pattern of maturation for parameters of the fetal ECG, blood pressure, and heart rate. When maternal cortisol concentrations were chronically increased, aortic blood pressure and heart rate were significantly reduced, and the duration of the PR interval and P wave were significantly increased in the fetus immediately before birth. Furthermore, statistical modeling and systems analysis of microarray data from the hearts of these fetuses revealed key genes and pathways that likely contribute to the cardiac dysfunction. Lastly, growth and glucose and insulin regulation were significantly altered in postnatal lambs whose mothers had increased concentrations of cortisol in late gestation.

These results indicate that increased maternal cortisol concentrations in late gestation adversely affect the fetal cardiac conduction system at birth, which may increase the risk for cardiac dysfunction at birth and developing cardiovascular disease in adulthood. These findings further strengthen the idea that long-term exposure to increased glucocorticoid concentrations in utero can have lasting consequences.
CHAPTER 1
BACKGROUND

Development of the Heart

The heart is one of the first organs to develop in the embryo, through a complex yet highly orchestrated process cardiac precursor cells proliferate and differentiate into a muscular heart tube and then into a functional four chambered heart. During gestational week three in the human, surface epiblast cells migrate toward the primitive streak and node of the embryo. At the primitive streak, epiblastic cells change shape, detach from the epiblast and invade the space between the layers of the epiblast and hypoblast. Some of these invading cells displace those of the hypoblast, creating the embryonic endoderm germ layer in the process. Further invasion of epiblast cells become situated between the endoderm and epiblast, forming the mesoderm germ layer.

Cardiac progenitor cells are located on the lateral sides of the primitive streak and ingress through the primitive streak, moving laterally and cranially to reside in the cardiogenic fields of the lateral plate mesoderm. Cells that migrate first, differentiate and form the primary heart field and subsequently the cardiac crescent while cells that migrated last will form the secondary heart field, which acts as a source of progenitors to the developing heart tube. Inductive signals from the underlying endoderm activate genes that are responsible for encoding specific transcription factors for the primary and secondary heart fields.

In the primary heart field, inductive forces cause the expression of Nkx2-5 and GATA4 transcription factors, and Isl1 and FoxH1 in the secondary heart field.
These transcription factors activate the NK2, MEF2, GATA, Tbx, and Hand genes of the evolutionarily conserved core cardiac regulatory network that further activate genes pertaining to the growth and patterning of the specific cardiac regions (185). The primary heart field will become responsible for formation of the left ventricle and parts of the atria while the secondary heart field will give rise to part of the atria, right ventricle, and outflow tract.

Angioblastic blood islands appear in the cardiac crescent splanchnic mesoderm that give rise to a series of vessels that develop into a pair of bilateral endocardial heart tubes and dorsal aortae. Lateral folding of the embryo brings the heart tubes together where they fuse, creating the endocardium of the primitive heart tube. At this stage in development polarity has already been established in the inflow tract, with the major pacemaker region residing in the posterior part of the heart tube, which will become the sinoatrial node (96).

As the cardiac tube gradually grows in length and size, it undergoes cardiac looping, forming a bend to the right of midline, which will eventually become the primitive ventricle. This primitive ventricle continues to enlarge and eventually becomes positioned more midline, over the atrium and venous channels. At this point the primitive heart is a continuous convoluted tube without septal partitioning and the myocardial cells have poorly developed sarcomeres, sarcoplasmic reticula, and spontaneously depolarize (235). The dominant pacemaker area is located caudally within the inflow tract region (257, 113). The heart tube beats in a peristaltic contraction with a slow impulse conduction that is characteristic of slow voltage gated calcium ion channels (54,
In order to create the characteristic four chambers of the mammalian heart, the primitive single chambered heart tube must undergo a series of subdivisions.

The common chamber of the heart tube becomes divided longitudinally as two ridge-like partitions develop simultaneously from the walls of the common heart chamber. Endocardial mesenchyme cell proliferation and invasion causes endocardial cushions to grow from the dorsal and ventral sides of the atrioventricular aperture, eventually fusing together and dividing the atrio-ventricular canal into the atrium and ventricle (1). During this process, different patterns of gene expression begin to differentiate regions of the heart. Primary myocardial cells of the chambers start to show gene expression for gap junction related proteins and fast voltage gated sodium ion channels, allowing for high conduction velocities and action potentials in the atrial and ventricular tissue (77, 235). These fast conducting segments develop within the existing pathways of the working myocardium, creating electrical diversity within the tissue (71, 120). In contrast, the pattern of gene expression of the working myocardium is repressed in the nodal regions, leading to the retention of automaticity, a slow conduction phenotype, and dominant pacemaker activity that is characteristic of these regions (185).

Further separation occurs within the common ventricle as proliferative muscle grows superiorly across the floor along the interventricular ridge towards the base of the heart, but does not fuse, leaving an interventricular foramen. Simultaneously within the atrium, the interatrial septum develops as proliferative muscle grows towards the endocardial cushions; however, a small pore, the foramen primum, develops in the interatrial septum near the endocardial cushions but quickly disappears as a second
pore, the foramen secundum, forms higher up in the interatrial septum. The septum secundum develops from the right atrium near the interatrial septum and extends partly over the foramen secundum (1). This forms the foramen ovale, which acts as a kind of Heimlich valve, allowing unidirectional shunting of blood from the right atrium into the left atrium. The foramen ovale is a fetal cardiac shunt that allows oxygen rich blood coming from the placenta to be preferentially diverted into the left atrium. The oxygen rich blood is then pumped by the left ventricle to oxygen sensitive tissues such as the brain and heart (65).

The outflow tract of the primitive heart tube also undergoes division, creating the aorta and pulmonary artery within the truncus arteriosus. Proliferation of mesenchymal tissue advances down the truncus arteriosus in a spiral manner, completing a 180-degree rotation as it fuses with the interventricular foramen (1). Blood ejected from the left ventricle leaves via the aorta while blood ejected from the right ventricle leaves through the pulmonary artery. Both outflow pathways occupy the same space, thus the spiraling of the septum creates an exquisite pathway for blood from the left ventricle to pass underneath/behind the pulmonary artery and enter the aorta, while blood from the right ventricle is able to pass above/in front on its way to the pulmonary circuitry. In the fetus, blood ejected by the right ventricle is diverted almost entirely from the pulmonary circuitry to the descending aorta by another fetal cardiac shunt, the ductus arteriosus.

The ventricular conduction system is comprised of the bundle of His, left and right bundle branches, and the Purkinje fiber network (82, 38). These cells reside just below the endocardium and function to rapidly conduct the impulse from the atrioventricular node to the contractile myocardium. The cells that make up the
ventricular conduction system are of myocardial origin and arise out of the embryonic ventricular trabeculae through a distinct transcriptional network comprised of Nkx2-5, Tbx5, Tbx3, Irx3, Hopx, and Id2, which directs their differentiation (166, 40, 101). These cells are distinct in their functions and characteristics, having more glycogen, fewer sarcomeres, a poor contractile apparatus, and high concentrations of CX40 and SCN5A compared to the cells of the myocardium (167, 7, 105). The effect of these regional differences in gene expression leads to a primitive heart that contracts synchronously and sequentially between chambers and the emergence of the adult style ECG.

**Growth and Maturation of the Fetal Heart**

Throughout and after this intricate process, the heart grows in order to meet the growing metabolic needs of the fetus and prepare for survival after birth. Many fetal tissues, especially of the heart, are reliant on maternally supplied nutrients. In a normal pregnancy, significant changes occur in the maternal endocrine and metabolic profile that aid in facilitating nutrient delivery to the growing fetus. These adaptations include the elevation of cortisol concentration and insulin insensitivity, resulting in a mild increase of maternal plasma glucose concentrations. Transplacental delivery of glucose from mother to fetus occurs in a concentration dependent manner primarily through the GLUT-1 transporter (100). When maternal plasma glucose concentrations are elevated, such as in cases of maternal diabetes, the increased concentration gradient means more glucose becomes available to the fetus (89). Fetal tissues have a great affinity for glucose due in large part to an increased concentration of insulin receptors present on cells compared to adult tissues (114). As anabolic factors increase, a substantial enlargement in fetal energy stores and tissue growth occurs, especially of the heart and other tissues that are highly insulin sensitive (177, 86). The heart accomplishes this
growth through either hyperplasia (increasing myocyte number) or hypertrophy of terminally differentiated myocytes (increasing cell size). In early to mid-gestation cardiac growth by hyperplasia is dominant, as it is necessary to build an adequate cell population; however, by late gestation and shortly after birth, proliferative mononucleated myocytes arrest in the cell cycle and become multinucleated terminally differentiated tissue, allowing for predominately hypertrophic growth of the myocytes (111).

A number of factors have been identified which contribute to regulating proliferation and differentiation of the fetal cardiac tissue. Circulating factors that stimulate proliferation include angiotensin II (Ang II), cortisol, and insulin-like growth factor-1 (242, 241, 79). Other factors such as atrial natriuretic peptide (ANP) and tri-iodo-L-thronine (T3) have been shown to suppress proliferation (182, 37). Specifically, ANP appears to counteract the stimulatory effects of Ang II by disrupting the ERK signaling cascade, while T3 down regulates the cell cycle promotor cyclin D1 and upregulates the cell cycle suppressor p21. Plasma T3 concentrations increase exponentially before and after birth, supporting the idea that T3 is a key component in tissue differentiation (194). Additionally, hemodynamic forces also influence cardiomyocyte proliferation, with increased arterial pressure being stimulatory and reduced systolic load being suppressive (181, 112). Increased arterial and diastolic pressures increases cell cycle activity and the length of cardiomyocytes; decreased systolic load exerts its suppressive effects on proliferation likely by reducing cell cycle activity, although decreased Ang II concentrations also play a role.
Maturation of the Cardiac Mitochondria and Metabolism

Mitochondria are the main energy producers for the mature cardiomyocyte, occupying about 35 percent of the cellular fraction and generating nearly 90 percent of cellular ATP (9, 98). The mitochondria are organelles that contain a central matrix enclosed by an inner and outer membrane that are separated by the intermembrane space. Cristae from the inner membrane contain the five complexes of the electron transport chain (ETC) responsible for oxidative phosphorylation and project into the matrix. Complexes I and II reduce NADH and FADH$_2$ (substrates generated from oxidation of acetyl-CoA by the tricarboxylic acid cycle in the mitochondrial matrix) passing electrons down the ETC (220). This process is coupled to the transport of protons from the matrix to the intermembrane space, establishing a proton gradient that is used by complex V to generate ATP.

The early embryonic heart likely relies on anaerobic glycolysis for energy production as the ETC activity is relatively low during this developmental stage (145, 156, 236). Although the early embryonic heart is capable of consuming oxygen for metabolism, the relatively low levels of oxygen are thought to inhibit aerobic respiration (156). Between E10-E14 mitochondrial ETC activity increases substantially, particularly in complexes I, II, and V (146, 145, 157). In the human, a similar increase in ETC activity occurs around 9 weeks of gestation (148, 158). These functional changes coincide with ultrastructural maturation of the cardiac mitochondria occurring during this period.

The inner mitochondrial membrane is smooth and lacking defined cristae at E10 in the rat. By E12 small outgrowths from the inner mitochondrial membrane project into the mitochondrial matrix, which are thought to develop over time into lamellar cristae.
characteristic of mature mitochondria (221). The mitochondria also undergo extensive network remodeling during this period, as they become more interconnected and perinuclear (62), suggesting an increased reliance on aerobic metabolism and mitochondrial ATP production between the early and mid-embryonic period.

By the time the embryo reaches the fetal stage of development the cardiac mitochondria are actively undergoing oxidative respiration; however, in addition to glucose, lactate becomes a major carbon source for metabolism (266, 70). Circulating lactate concentrations are relatively high compared to the adult and lactate consumption accounts for most of the myocardial oxygen consumption in the fetal lamb (15). Although the fetal heart is capable of metabolizing fatty acids, the amount delivered to the fetus is considerably low and thus their contribution to myocardial ATP production is <15% (141, 16). In late gestation, the fractional volume of the cardiac mitochondria increases as term approaches, the mitochondria have mature and complex cristae, and become positioned near the sarcoplasmic reticulum (SR) and myofibrils in addition to the nucleus (95, 229, 30). The proximity of the mitochondria to the SR and myofibrils is crucial in providing ATP for ATPases involved in contraction and calcium sequestration (213).

After birth, there are substantial changes in mitochondrial morphometrics and metabolism as the nutrient supply dramatically changes. Immediately after birth, the heart relies almost entirely on glycolysis and lactate oxidation, with minimal contribution from β-oxidation of fatty acids (141, 266, 15). However, in the newborn period, there is a gradual shift in the reliance on glucose and lactate for carbon sources to β-oxidation of fatty acids supplied in the mother’s milk (141, 140, 266, 8). Similarly, within this period
there is a substantial decrease in circulating blood lactate concentration and a concomitant increase in free fatty acids (155, 123, 195). A further increase in mitochondrial volume also occurs in the left and right ventricles in the neonatal period, likely as a response to changes in cardiac work (253, 230).

Furthermore, there are considerable changes in gene expression occurring in the late gestation fetal heart that continue into the postnatal period, reflecting the modulation of metabolism and mitochondrial function. Genes associated with fatty acid metabolism and mitochondrial fission and fusion increased in expression from two weeks before birth to term and from birth to two weeks postnatally (204). Similarly, genes associated with mitochondrial development and carbohydrate metabolism increased from two weeks before birth to term with no further increase or decrease thereafter (204). These findings suggest that the metabolic machinery of the cardiac mitochondria begins to change during the fetal period in preparation for the conversion of metabolic substrate postnatally. These adaptations result in a metabolic profile in which the cardiac mitochondria gradually adapt to the changing nutrient supply, with an increasingly greater reliance on β-oxidation of fatty acid (81).

**Physiological Role of Cortisol in Pregnancy**

In the late 1960s, Liggins began infusing glucocorticoids into pregnant sheep to study their effects on the timing of labor. It was known then that fetuses lacking an intact hypothalamic-pituitary-adrenal axis failed to go into labor at term, suggesting that the fetus, not the mother, initiates labor and parturition (136, 200). Liggins began infusing different concentrations of glucocorticoids into these pregnant sheep and found that parturition could be induced at high doses (133). Furthermore, the premature lambs were alive and breathing, which was remarkable for such immaturity. Liggins and Howie
went on to show that administration of antenatal glucocorticoids to women threatening premature delivery reduced respiratory complications in the premature neonate and perinatal mortality (135). Since then, there has been much interest and research into understanding the maturational effects of glucocorticoids on fetal tissues.

Throughout pregnancy maternal cortisol, the endogenous glucocorticoid, concentration rise approximately three to five-fold as a physiological adaptation. This increase in cortisol is necessary for maintaining maternal cardiovascular function and facilitating delivery of nutrients from the mother to the developing fetus (178). In the placenta, 11β-hydroxysteroid-dehydrogenase 2 (11β-HSD-2) acts as a buffer, inactivating cortisol by converting it to cortisone, thereby producing a gradient in cortisol from mother to fetus and limiting fetal exposure. As a consequence, only 2% of maternal cortisol is estimated to cross the placenta; however, because of the large difference in distribution volume, this accounts for approximately 95% of the fetal plasma cortisol concentration until close to term (58, 91). Synthetic glucocorticoids readily bypass this degradation and maternal hypersecretion of cortisol also allows for considerable glucocorticoid exposure to fetal tissues.

Fetal adrenal production and secretion of cortisol begins at ~90% of gestation in the sheep and human fetus (63, 171). Fetal cortisol concentrations increase exponentially during this period. Although fetal cortisol secretion is essential in the maturation of the fetal organs, including the heart (210), excessive fetal glucocorticoid exposure is associated with alterations in the glucocorticoid regulated trajectory of heart maturation (180), increased heart size (108, 201, 106), and “programmed” susceptibility to cardiovascular disease in adulthood (21, 60, 179, 59).
Effects of Altered Glucocorticoid Signaling on the Fetal Heart

The major physiological effects of glucocorticoids are mediated by the glucocorticoid (GR) and mineralocorticoid (MR) receptors. Previous studies from our lab have indicated that glucocorticoid induced cardiac growth is mediated through the MR (201, 68). The GR is a nuclear hormone receptor that binds various synthetic and endogenous corticosteroids. When in the unoccupied state, GR typically resides in the cytoplasm bound to a complex of stabilizing chaperon proteins. Upon ligand binding with GR, it dissociates from its chaperons and translocates to the nucleus and binds to specific motifs called glucocorticoid response elements (GRE) in the promoter region of genes. Binding to the GRE either activates or represses gene transcription. GR is present in nearly every tissue of the body and is highly conserved across species, underscoring its importance in homeostasis and survival. Likewise, global knockout of the GR results in a phenotype that is not compatible for ex utero survival, likely due to poor and incomplete lung development. However when the GR was conditionally knocked out of cardiomyocytes in the fetal mouse, significant alterations in the macro and micro structure of the compact myocardium were evident along with changes in the expression for genes involved in cardiac calcium handling and metabolism (210, 208).

Instances in which overexposure of fetal tissues to GCs due to maternal overproduction are rare. However, one such case is Cushing’s syndrome, a rare disorder characterized by excessive production of cortisol, which affects about 39 per million people (66, 138). Although the hypersecretion of cortisol in Cushing’s syndrome is rare in pregnancy, premature delivery, stillbirth (in utero fetal death occurring after 20 weeks gestation), and intrauterine growth restriction are common complications of this disorder (258). Similar complications have also been reported in pregnancies
complicated by chronic maternal stress or adverse events including violence or loss of a relative during pregnancy (256, 144). In adults with Cushing’s syndrome, left ventricular hypertrophy is a well-known cardiac complication (239), and hypertrophic cardiomyopathy is also observed in neonates receiving antenatal glucocorticoid treatment (285). Obstructive hypertrophic cardiomyopathy has also been reported in the newborn of a mother with Cushing’s syndrome along with increased thickening of the interventricular septum and left ventricle (67). Furthermore, it is known from animal studies, that the late gestation heart is susceptible to remodeling by glucocorticoid exposure (68, 201, 210). During the last 20% of gestation in sheep and humans, the fetal cardiomyocytes transition from mono-nuclear proliferative cells to bi-nucleated terminally differentiated tissue (27), suggesting that the late gestational fetal heart may be particularly susceptible to excessive glucocorticoid exposure.

The glucocorticoid receptor (GR) was the first of the steroid hormone receptors to be discovered in mitochondria and it was later found that multiple glucocorticoid response elements reside in the mitochondrial genome (55). Recently, it has been shown that GRs can translocate from the cytoplasm to the mitochondria by forming protein complexes with anti-apoptotic proteins (61). This suggests that GCs could modulate mitochondrial function via GR signaling. Mice with conditional cardiac GR knockout fail to induce gene expression in genes involved energy metabolism including PGC1α, a transcriptional coactivator that is a central factor in mitochondrial biogenesis (210, 10). Mitochondria are dynamic organelles, capable of responding to changes in cellular homeostasis by altering both their morphology and mitochondrial and nuclear DNA (mtDNA and nDNA) gene expression. These adaptations allow the mitochondria to
fine tune energy production to the specific needs of the cell. During brief periods of stress, glucocorticoids act to bolster mitochondrial function, by enhancing mitochondrial membrane potential, calcium buffering capacity, respiration and the resistance to pro-apoptotic signals (61). This occurs through formation of an integrated mitochondrial network that reduces mitochondrial dysfunction and oxidative damage while maximizing respiratory capacity (252, 150, 186, 80, 209, 104). For example, in neonatal mouse hearts, antenatal dexamethasone treatment accelerates ATP production in a dose dependent manner (160). Cumulatively, acute glucocorticoid exposure increases the energy generating capability of mitochondria, while improving resistance to apoptosis as normal physiologic adaptations to stress.

In contrast to the short-term adaptations of mitochondria to glucocorticoids, chronic exposure detrimentally impacts both mitochondrial structure and function. Long-term glucocorticoid exposure has been found to decrease the activity of complexes I and III, increase ROS production, decrease antioxidant levels and activity, and increases the cells susceptibility to pro-apoptotic signals (61, 159, 246, 147, 69). Whereas acute GC exposure is associated with an integrated mitochondrial network, long-term exposure promotes formation of swollen, poorly organized mitochondria and fragmentation of the mitochondrial pool, processes that are highly associated with apoptosis (76, 104, 102). Additionally, the lack of robust mtDNA repair mechanisms compared to those of nDNA, coupled with its close proximity to the ROS generating structures make mtDNA susceptible to oxidative damage in the form of base deletions, mispairing and point mutations (281, 214, 193). Furthermore, it has been shown in a rodent model that repeated maternal restraint stress results in offspring with higher
levels of oxidative damage in hippocampal mtDNA (233). Similarly, increased oxidative damage in both mtDNA and nDNA as well as reduced activity in complex I have been found in biopsies of skeletal muscle from human patients on long-term GC (159). Therefore, a persistent GC signal results in dysfunctional mitochondria that produce less ATP and contribute to poor cellular homeostasis through excessive ROS production. These maladaptations hamper the cells’ adaptive capability to further insults leaving them vulnerable to apoptosis.

In summary, the heart is the first organ to develop in the embryo, which occurs though an intricate yet orchestrated series of events. As the fetus grows and develops maturational events gradually occur, preparing the fetus for ex utero life. In the fetal heart, differentiation of the cardiac tissue and maturation of the cardiac mitochondria are necessary for survival in the post-natal period. Many endocrine factors influence these maturational processes including glucocorticoids, which appear to be critically involved. Inappropriate glucocorticoid signaling in the fetal heart alters its developmental trajectory, which is influenced in part by changes in the expression of genes involved in cardiac metabolism, architecture, and ion homeostasis. Furthermore, early or excessive glucocorticoid exposure increases the risk for the offspring to develop cardiovascular disease in adulthood.

Recent studies from our lab have suggested that the cardiac conduction system might be particularly vulnerable to increased glucocorticoid exposure, which may be a consequence of mitochondrial dysfunction. Therefore, this thesis aimed to characterize the changes occurring in the cardiac conduction system from normal fetal lambs and those chronically exposed to increased maternal cortisol concentrations in late
gestation. To accomplish this, a novel method was developed for the long-term acquisition and measurement of the fECG (chapter 2). This method was used in chapter 3 to describe functional changes occurring in the fetal heart in late gestation, at birth, and shortly thereafter. In chapter 4, statistical modeling of micro array data was used to reveal changes in gene expression and related pathways that contribute to the functional changes observed in chapter 3. Lastly, the effects of elevated maternal cortisol on postnatal maturation, growth, and metabolism were studied (chapter 5).
CHAPTER 2
MATURATION OF THE ECG OF THE LATE GESTATION OVINE FETUS ASSESSED USING RADIOTELEMETRY

Introduction

The ovine fetus has long been used as a model of fetal development, particularly because of the similar developmental trajectory to the human fetus and the relative quiescence of the ovine uterus, reducing the risks inherent with open fetal surgery. These properties have made the chronically instrumented ovine fetus invaluable in the characterization and manipulation of physiological processes. The ovine fetus has been particularly useful as a model for study of fetal organ maturation, as the ovine fetus has similarities in maturational trajectory to the human, owing in part to the similar surge in fetal cortisol production in the days preceding birth (36, 94). Cortisol contributes to maturation of many fetal tissues including the heart, lung, gastrointestinal tract, liver, brain (134, 25, 209, 224, 191). Our laboratory has been interested in the study of influences of maternal cortisol on fetal cardiac maturation (68, 117, 201, 205), having found a significant impact on fetal cardiac gene expression related to cardiac proliferation and apoptosis, metabolism, and perinatal survival. Many previous studies have documented the late gestation changes in fetal blood pressure and heart rate, as well as responses of fetal heart rate and ECG to manipulations such as hypoxia, cord occlusion, or chronic growth restriction (255, 264, 125, 137, 73, 170). Fetal ECG is suggested as a good measure for assessing fetal brain ischemia and this has been widely studied in that context as an index of fetal stress (223, 173). However, very chronic study of fetal ECG, and study during labor and delivery is complicated by the need to limit maternal movements during signal acquisition. Chronic implantation of radiotelemetry devices in the late gestation ovine fetus has been performed before,
though with limited success (2, 92). The limited success of radiotelemetry in sheep has restricted the measurement of fetal ECG in this animal model to systems requiring catheterization and hardwiring; this protocol may limit maternal movement, possibly altering the physiological control of fetal heart rate. In the current study, we surgically implanted radiotelemetry devices into late gestation ovine fetuses and assess the use of this method to characterize changes in the fECG occurring after surgery, throughout late gestation, and throughout labor and delivery.

Materials and Methods

Ewes of known gestational age and their lambs were studied. Ewes were of mixed breeds, averaging 71.0 ± 7.09 kg at the time of surgery; lambs were 4.18 ± 0.33 kg at birth (143 ± 1; day 139-148 gestation). All animals were housed in a facility with temperature and light controlled (lights on 0700 to 1900) rooms throughout the study period; the University of Florida Institutional Animal Care and Use Committee approved all animal use for this study. Ewes were housed in individual pens of approximately 2.25 m² each throughout the study. Husbandry, including cleaning of the pens and feeding, typically lasted approximately 30 minutes and was completed between 0700 and 0800. An entry sheet was used to record times for husbandry and feeding as well as postoperative care these times were excluded from the analysis.

At approximately day 118 (±1) of gestation, survival surgery was performed. Ewes were induced with isoflurane by mask, intubated and maintained on isoflurane anesthesia with controlled ventilation. The total period of anesthesia, including prep and surgery was 4.5 ± 0.3 hours. Maternal end expiratory PCO2, O2 saturation, blood pressure and heart rate were monitored throughout the procedure. A flow probe (6mm 6PSS; Transonics Inc., Ithaca, NY) was placed on the main uterine artery for
assessment of labor. To study changes in fetal aortic pressure and ECG, a radiotelemetry device (DSI PA-D70 PCTP; Data Sciences International Inc., Minneapolis, MN) was placed in the fetus to allow continuous measurement of fetal aortic pressure, amniotic pressure, fECG and temperature. Briefly, the head and neck of the fetus were located in the uterus and exposed. A midline incision was made in the fetal neck and the telemetry device was placed subcutaneously at the level of the clavicle and sutured in place. The grounding lead for the ECG was attached to the skin of the thorax using a sterilized fishing hook (size 6; Baitholder; Eagle Claw; Denver, CO). The solid tip ECG probe was placed into the right jugular vein of the fetus, advanced into the superior vena cava until a P wave was visualized and optimized in the acquisition software (Ponemah 5.00), and secured at the entry point in the jugular with sterile tissue adhesive (Vetbond, 3M; St. Paul, MN). For placement of the aortic pressure catheter, purse string sutures (5-0 Prolene Suture, Ethicon; Somerville, NJ) were placed in the left carotid artery of the fetus, and the catheter was advanced into the aorta, using the acquisition software to assure placement in the aorta outside of the left ventricle. The catheter was secured in place using the purse string sutures and a drop of tissue adhesive. The other pressure catheter was used to measure amniotic fluid pressure; it was tunneled underneath the fetal skin and sutured to the skin of the fetal neck with the tip exposed. All aortic pressures were corrected by subtraction of amniotic fluid pressure. Therefore, all parts of the telemetry device were implanted within the fetus. Catheters were also placed in the fetal saphenous arteries and veins and advanced to the fetal femoral arteries and veins (118), and in the maternal femoral arteries and veins. At the end of surgery, elastic surgical dressing was placed around
the abdomen of the ewe (size 9 and 10 Surgilast, Derma Sciences; Princeton, NJ) fetal and maternal catheters were placed in a pocket made from sterilization wrap (Kimguard, Kimberly Clark; Roswell, GA) which was placed under the Surgilast on the flank of the ewes. A repeater device for the telemetry device was secured in place on the flank of the ewe by wrapping the unit in bandage tape (Vetwrap, 3M; St. Paul, MN) and fastened with a cable tie to the Surgilast over the maternal abdomen.

Six fetuses died due to surgical complications; all of these deaths appeared to be attributable to cord occlusion. Subsequently we altered the surgery to minimize the extent to which the fetus was displaced during surgery; only the fetal head and neck is exposed and approximately 500mL of sterile saline is infused back into the amniotic cavity.

After surgery ewes were returned to their housing pens (24 ft² each) in which they were allowed to move freely. The ECG, aortic and amniotic pressure signals were continuously acquired from the repeater device by a radioreciever fastened to the front of the pen, which was connected to the DSI exchange matrix and computer. The system used in these studies used a repeater device in order to assure that animals in adjacent pens could have data collected at unique frequencies; the current systems available from DSI do not require use of the repeater, and instead use multiple receivers placed around the pen. Although the signal strength of the telemetry device is sufficient to allow complete implantation into the fetus, the repeater device assured that the rebroadcast signal allowed for uninterrupted communication with the receiver without interference with the signal from adjacent pens. This arrangement allows for the acquisition of data during labor, delivery, and thereafter from multiple animals.
simultaneously. Each signal (aortic pressure, amniotic pressure and ECG) was sampled at 500Hz by the Dataquest ART software. The catheters were used to collect samples for maternal and fetal hormones, glucose and lactate measurements, and for fetal blood gases. These samples were collected without restraint of the ewe and were collected in the morning at least one hour after completion of the daily husbandry activities. Measurements for maternal and fetal cortisol concentrations as well as fetal blood gases are presented in Table 2-1.

Ewes were treated at the end of surgery and for 2 days postoperatively with analgesic (flunixin meglamine; 1mg/kg sid; Merck Animal Health, Germany), and for 5 days postoperatively with antibiotic (Polyflex, 12-15 mg/kg bid; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO); rectal temperature was measured twice a day for 5 days. Ewes were fed a diet of pelleted feed per NRC standards adjusted for the ewe's body weight and fetal gestational age.

One ewe was euthanized in labor due to dystocia, one delivered a live lamb that died shortly following birth after suspected dystocia, and one fetus was hypoxic throughout the pregnancy and died. The chronically hypoxic fetus (PO2 of 10-15 mmHg) was not included in the analysis. In this study, a total of 4 male and 4 female lambs were born to 8 ewes at 143±1 days (range 139-148 days) gestation.

Analysis of the acquired aortic pressure, heart rate (HR) and fECG was performed using analysis modules in DSI Dataquest Open A.R.T 4.31 and Ponemah 5.00 software Blood Pressure Analysis Module and ECG PRO, respectively). For calculation of MAP, amniotic pressure was used as the reference pressure. Hourly means of aortic and amniotic pressures and heart rate were calculated; HR calculation
used the peak to peak intervals in the aortic pressure waveform. For the fECG analysis, data was imported into the Ponemah Analysis modules, and the software assigned elements of the ECG (i.e. start and end of P wave, QRS, T wave peak and end); these template cycles were then finely adjusted by the operator, and added to a library for each animal that was used to match the remaining cycles. Unmatched cycles were excluded from the analysis. About 80% of cycles in each period chosen for analysis could be matched.

**Mean Aortic Pressure, Heart Rate, and fECG Characteristics Following Surgery**

One hour means of MAP, HR, and fECG parameters (P duration (atrial depolarization), PR interval from the start of atrial depolarization to the start of ventricular depolarization), QRS duration (ventricular depolarization), corrected QT interval (depolarization and repolarization of the ventricles, QT interval corrected for RR interval, QTc), and ST interval (isoelectric period between ventricular depolarization and repolarization)) were collected and analyzed over the first 24 hours following the end of surgery. Following the first post-operative day, six-hour means were calculated over the next four post-operative days for statistical analysis.

**Mean Aortic Pressure, Heart Rate, and fECG Characteristics in Late Gestation**

MAP and HR were calculated as 24 hour means over the 14 days before birth; this period was chosen to allow inclusion of data from all fetuses following the 5 days of recovery from surgery. The parameters of the fECG (P and QRS duration; PR, QR, QRS, QTc and ST intervals) were calculated for the one-hour interval between 0600 and 0700 for the 14 days before birth. Fetal heart rate variability (HRV) was used as an index of the relative sympathetic and parasympathetic tone in control of HR. Using Dataquest Open A.R.T 4.31 software, a power spectral density analysis was performed
on 10 minutes of fetal aortic pressure tracing from the same time on each day for 14 days before birth. The analysis used a nonparametric method (fast Fourier transform) in order to estimate the power of the spectrum for very low frequency (VLF; 0.001-0.025 cycles/beat), low frequency (LF; 0.025-0.125 cycles/beat), high frequency (HF; 0.2-0.5 cycles/beat) components (122, 275). The ratio of LF:HF was calculated as an index of relative sympathetic to parasympathetic tone.

**Mean Aortic Pressure, Heart Rate, and fECG Characteristics 24 Hours Before Birth**

In the 24 hours before birth, MAP, HR, and fECG parameters were calculated as hourly mean values; in the last hour before delivery and, when possible, in the 10 minutes after birth data was calculated as one-minute means. In most cases the signal was disrupted by the final process of delivery, although intermittent reading over a number of beats were still possible between maternal “pushes”. Because the repeater was affixed to the ewes abdomen and needs to be within approximately 40 cm of the fetal transmitter, postnatal signals were not reliably collected unless someone was present at the time of birth to move the repeater to the neck of the lamb. However, in many cases the newborn stayed close enough to the ewe to collect data in the immediate postpartum period. In all animals, the time of birth was confirmed using the telemetry record for fetal/neonatal temperature, which in all cases showed a decrease in temperature measured by the telemetry device at the time of birth.

**Analysis of Diurnal Rhythms for Mean Aortic Pressure, Heart Rate, and fECG Characteristics**

On days 11, 10, and 9 before birth, hourly means for MAP, HR, and fECG were calculated across the 72 hours to test for diurnal rhythms. The Cosinor (Michael Sachs. (2014) cosinor: Tools for estimating and predicting the cosinor model. https://CRAN.R-
project.org/package=psych Version = 1.6.9.) and Psych (Revelle, W. (2016) psych: Procedures for Personality and Psychological Research, Northwestern University, Evanston, Illinois, USA, https://CRAN.R-project.org/package=cosinor) packages were used in R software (http://www.R-project.org/) to fit hourly mean data to a cosine function, and depict the 24 hour variation graphically. The Metacycle package was used in R software to test for significance of rhythmicity in individual fetuses, and to determine acrophase (time of the peak of the rhythm), amplitude (the difference between the peak and the mean value), and MESOR (estimate of the average value of the oscillating variable) describing the rhythms (278).

**Statistical Analysis**

Analyses of fetal MAP, HR, HRV, and fECG parameters following surgery and in late gestation were performed using one-way analysis of variance corrected for repeated measures across time in IBM SPSS version 23.0 (IBM Corp.; Armonk, NY). For all analyses, statistical significance was set for p<0.05. Values represent mean±SE unless otherwise stated.

**Results**

**Mean Aortic Pressure, Heart Rate, and fECG Characteristics Following Surgery**

In the 24 hours following the end of surgery, fetal MAP did not significantly differ over time (Figure 2-1 B). The change in HR significantly declined over the first 24 hours following surgery (from 216±10 bpm to 176±5 bpm in the first 13 hours) (Figure 2-1 A); likewise, the duration of the RR interval calculated from the fECG significantly increased (281±14 msec to 335±18 msec in the first 5 hours). The duration of the QRS and ST intervals significantly increased in the hours after the end of surgery (QRS: 24.9±1.8 msec to 27.1±2.1 msec in the first 4 hours, and ST: 139±13 to 160±9 msec in the first 9
Mean Aortic Pressure, Heart Rate, and fECG Characteristics in Late Gestation

Over the last 14 days of fetal life, fetal MAP significantly increased (from 45.2±2.2 mmHg to 55.6±3.4 mmHg) and HR significantly decreased (from 156±16 bpm to 145±15 bpm) (Figure 2-2 A and D). There were no significant changes over time in the measured parameters of the fetal ECG except for the RR interval (Figure 2-3 A,C,E,G,I). There were no significant changes over the last 14 days of fetal life in the measured parameters of the HRV (Table 2-3).

Mean Aortic Pressure, Heart Rate, and fECG Characteristics Before and After Birth

In the final 24 hours before birth, the hourly mean in aortic pressure significantly increased (from 51.8±3.3 mmHg to 56.6±2.1 mmHg) and the hourly mean HR significantly increased (from 154±4 bpm to 160±6 bpm) as labor progressed (Figure 2-2 B and E). The PR and RR intervals were the only parameters of the ECG to significantly change over this period (Figure 2-3 B,D,F,H,J). The PR interval initially decreased as labor progressed, with a small increase shortly before birth (Figure 2-3 B). In the immediate perinatal period, the fetal HR decreased in the final hour before birth from 173 bpm to 144 bpm at birth, and averaged 217 bpm over the first 10 minutes of extrateral life (Figure 2-2 C). Similarly, the fetal blood pressure rose from 58 mmHg to 66 mmHg in the final hour, and averaged 68 mmHg in the early postnatal period (Figure 2-2 F).
Analysis of Diurnal Rhythms for Mean Aortic Pressure, Heart Rate, and fECG Characteristics

Analysis for 24 hour patterns over three consecutive days in late gestation indicated significant patterns in HR and the RR interval in 4/7 fetuses, in the duration of the ST interval in 3/7 fetuses, in the duration of the QRS, PR, and QTc intervals in 2/7 fetuses, but in MAP in only 1/7 fetuses (Figure 2-4 A-F; Acrophases, amplitudes, and MESOR for each parameter are presented in Table 2-2).

Discussion

Our results using telemetry agree with already published data on MAP and HR using other methods in “control” fetuses. We found that fetal MAP and HR increased over the final day as birth approached, with a concomitant decrease in the PR as well as in the RR interval, though no other parameters of the fECG were changed. In the ovine fetus, a gradual rise in fetal blood pressure normally occurs in the hour before parturition, which is associated with the occurrence of uterine contractions (43). In these control fetuses, aortic pressure steadily rose over the last hour of life, including the immediate postpartum period. These findings reflect the cardiovascular adaptations associated with birth and the transition to ex utero life. In the sheep, cardiac output approximately doubles following birth, as the ventricular circulation transitions from a parallel (fetal) to in-series circuit (infant/adult) (93). Systolic blood pressure also rises at birth in part due to the removal of the low resistance placental unit, increased systemic vascular resistance, and circulating vasoactive hormones including cortisol and catecholamines (253, 94). These adaptations are thought to provide for an increased metabolic demand and processes regulating breathing and thermogenesis, which is reflected by changes in blood flow to certain organs during this transition (18). Comline
and colleagues also showed that fetal heart rate during labor varied, but decreased overall in the last hour before birth. Our data also reveals a gradual increase in HR; we observed a variable increase in heart rate at the time of birth that was not significant overall. Others have shown that heart rate increases immediately following birth in humans (53) and in mice is accompanied by a decrease in the duration of the QRS and QTc intervals (172).

This methodology also allowed us to examine the recovery of the fetus from our surgical manipulation, which involved fairly extensive manipulation of the fetus. Although there was no significant change in MAP in the hours following surgery, ST interval and QRS duration increased, while the HR (and RR interval) decreased after surgery and stabilized after several hours. These findings indicate that ventricular activation and relaxation times are affected by surgery; however, these changes were transient and there was a rapid recovery during the first post-operative day. These post-operative changes likely reflect the fetuses intraoperative exposure to isoflurane, which is known to decrease blood pressure and HR in the fetus, and to alter the ECG in adolescents and adults (116, 184, 222, 237).

We confirmed the increase in arterial pressure and decrease in heart rate during late gestation that was reported by Unno and colleagues (255); Unno and colleagues showed in their study that the fetal heart rate increases between 140-143 days of gestation, corresponding to the exponential rise in fetal cortisol concentrations. However, we found little change in the parameters of the fECG occurring over the same period. This indicates that while the cardiac tissue undergoes maturational changes throughout late gestation, the dynamics of the fetal cardiac action potentials and the
conduction system are mature by the last 0.10 of gestation, suggesting that cardiac ion channels are present and mature by this time. This is consistent with our previous observation in transcriptomic analyses, that genes associated with Purkinje fiber and ion channel maturation are not differentially expressed between 0.90 and 0.97 gestation in the ovine fetus (204). We found only 11 genes that were related to voltage gated ion channels, ligand gated ion channels, or other ion channels and significantly changed between 0.90 of gestation and postnatal day 14 (234); however, the change in expression of these genes occurred postnatally. Similar studies in the mouse have shown that although expression of various cardiac ion channel genes changes considerably from E17.5 to adulthood, less change occurs from E17.5 to 1 day postnatal, as a relatively mature conduction system is likely necessary for survival at birth (88). Although studies using fECG and magnetocardiography indicate that considerable change in some parameters occur between mid and late gestation in the human fetus (particularly increasing durations of the P and T waves, PR, QRS, and QTc intervals), less change occurs during late gestation (39). It has been proposed that this is due to the growing myocardial mass during development, which creates a larger surface area that the electrical signal must traverse (39). In premature human neonates (26-37 weeks of gestation) the duration of the P wave, QRS and QT intervals are shorter compared to the full-term neonate, and this is consistent with an association of the size of the chambers to the conduction times through the heart (249, 250). However, estimates of the duration of the P wave and QRS and PR intervals in the human fetus at ≥37 weeks of gestation, were considerably longer than measured in the sheep fetus from our study (P: 53ms vs 32ms; PR:110ms vs 78ms; QRS duration: 53ms vs 28ms
These discrepancies are likely not related to differences in heart size since it is similar in both species at birth (this study: 25.5g vs 24.5g (83, 149)). Instead, this might reflect differences in methodology, proteins involved in cardiac conduction, or perhaps most likely, differences in autonomic activity between the human and sheep fetus. Measurements of the PR interval in our study are similar to those made invasively in other studies in the sheep fetus (267). Our study also is consistent with the studies by Koome and colleagues showing no significant change in LF/HF ratio between 0.7 and 0.8 gestation despite a progressive decrease in heart rate over the period from 0.6 to 0.8 gestation in the fetal sheep (125); these data are consistent with the lack of changes in PR interval.

Significant diurnal rhythms in HR were observed in the majority of the fetuses in the current study. Studies in several species, including the late gestation fetal baboon, sheep, and human have shown significant 24 hour rhythms in HR, HRV, and MAP (28, 29, 189, 255). These variations can be influenced by behavioral state, rest and activity cycles, and body/breathing movements (151, 244, 192, 152, 143). The autonomic nervous system (ANS) of the fetus also considerably influences HR and HRV. Sympathetic tone predominates in the fetus, but the relative contribution declines as gestation advances and parasympathetic tone increases; however, neither sympathetic nor parasympathetic input alone are essential for the diurnal rhythms of HR and HRV (50, 107, 260). Unno and colleagues had previously found an acrophase in HR occurring around 2330, using 14 hour light and 10 hour dark periods, compared to around 0200 in our study that used 12 hour light and 12 hour dark periods. In our study, there was no observed rhythm in MAP. Others have found a significant rhythm in MAP,
with peak times occurring around the time of the heart rate peak (255). Maternal factors including daylight, feeding, and melatonin are known to contribute to the entrainment of fetal circadian rhythms, which could contribute to differences between studies (183, 203, 254). The absence of significant variation in mean pressure could reflect an absence of rhythm in fetal cortisol. In previous studies from our laboratory (20) we found no diurnal rhythm in fetuses in which ewes were fed ad libitum; in contrast, others have found a rhythm in animals when fed for a limited time in the morning (154, 225, 227).

We also found there to be significant 24 hour rhythms for several parameters of the fECG occurring in some of the fetuses, including the duration of the PR, RR, ST, QTc, and QRS intervals. These might be driven in part by the autonomic nervous system as it contributes to the regulation of the ECG through innervation of the sinoatrial and atrioventricular nodes, and the ventricular myocardium. Intrinsic factors that might influence the rhythms of the fECG include circadian expression of cardiac ion channels. Circadian patterns of expression for the repolarizing cardiac K+ channels and Ito current have been found in the adult rat (282). Circadian oscillations in expression of clock component genes have previously been described in both adult human and rodent hearts (128, 212). These expression patterns are known to be associated with circadian changes in HR (99, 284) and metabolism (124).

There are some pitfalls that we encountered while implementing this method that should be considered, particularly instances of unexpected and sudden fetal death occurring in the first 48 hours of implantation. These are easily detected posthoc as acute increases in aortic pressure and heart rate, followed by severe bradycardia and progressive hypotension until death. Several of these instances could be attributed to
umbilical cord occlusion resulting from manipulation during surgery or insufficient replacement of the amniotic fluid with sterile saline. Because of these issues, we adjusted our protocol in order to minimize fetal manipulation during surgery and to infuse sterile saline into the amniotic cavity. Implementing these strategies greatly improved the likelihood of a successful preparation.
Figure 2-1. Fetal heart rate (A), aortic blood pressure (B), and fECG parameters (C-G) in the 24 hours following the end of surgery (n=6). Data is depicted as mean±SEM * Indicates a significant change over time.
Figure 2-2. Fetal heart rate (A-C), aortic blood pressure (D-F) in the final 14 days before birth (A,B), in the final 24 hours (B,E), and from 60 minutes prior to birth to 10 minutes after birth (C,F). Data is depicted as mean±SEM of one hour of data in the morning in panels A and D (n=8): one hour data over 24h in B and E (n=8), and one minute of data in C and F (n=8). Note that the Y axis range in panel C is different than panels A and B. * Indicates a significant change over time.
Figure 2-3. fECG parameters in the final 14 days before birth (A,C,E,G,I) and 24 hours prior to birth shown as hourly means (B,D,F,H,J). Data are shown as mean ±SEM (n=8) * Indicates a significant change over time.
Figure 2-4. 24 Hour Diurnal patterns in fetal HR, BP, and fECG parameters in fetuses (n=7). Circles indicate the hourly mean values on the 3 consecutive days (days 11, 10 and 9 prior to birth); lines indicate the cosine fit of these mean values; black bars on x-axis indicate period of lights off.
Table 2-1. Maternal and fetal plasma cortisol and fetal blood gases. Data are shown as mean±SE (n) Ns are the same for all fetal measurements. *indicates a significant change over time.

<table>
<thead>
<tr>
<th>Day of Birth</th>
<th>Maternal Plasma Cortisol (ng/ml)</th>
<th>Fetal* P O2</th>
<th>Fetal P CO2</th>
<th>Fetal pH*</th>
</tr>
</thead>
<tbody>
<tr>
<td>125d</td>
<td>10.4±3.3 (8)</td>
<td>4.5±1.5 (8)</td>
<td>49.5±3.0</td>
<td>7.36±0.02</td>
</tr>
<tr>
<td>130d</td>
<td>5.6±1.4 (8)</td>
<td>6.5±2.2 (8)</td>
<td>54.5±1.5</td>
<td>7.37±0.02</td>
</tr>
<tr>
<td>135d</td>
<td>4.9±0.9 (7)</td>
<td>16.4±4.9 (7)</td>
<td>54.0±1.6</td>
<td>7.35±0.01</td>
</tr>
<tr>
<td>138d</td>
<td>7.4±1.9 (8)</td>
<td>24.6±6.4 (7)</td>
<td>52.4±1.6</td>
<td>7.37±0.01</td>
</tr>
<tr>
<td>140d</td>
<td>10.3±3.1 (7)</td>
<td>33.6±13.3 (7)</td>
<td>54.4±1.4</td>
<td>7.35±0.02</td>
</tr>
<tr>
<td>Day of Birth</td>
<td>17.4±3.8 (7)</td>
<td>72.3±12.6 (7)</td>
<td>59.2±2.8</td>
<td>7.32±0.02</td>
</tr>
</tbody>
</table>
Table 2-2. Values of parameters of the 24 Hour patterns in the late gestation ovine fetus; data are mean values for 7 fetuses calculated using the Metacycle Package for R.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Amplitude</th>
<th>Acrophase</th>
<th># of Fetuses With Significant Rhythm</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>172</td>
<td>3.9</td>
<td>0159</td>
<td>4/7</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>46</td>
<td>0.8</td>
<td>2241</td>
<td>1/7</td>
</tr>
<tr>
<td>RR Interval (msec)</td>
<td>356</td>
<td>6.7</td>
<td>2236</td>
<td>4/7</td>
</tr>
<tr>
<td>ST Interval (msec)</td>
<td>173</td>
<td>3.4</td>
<td>1909</td>
<td>3/7</td>
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<tr>
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<tr>
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Table 2-3. Frequency measurements of the fetal heart rate variability in 7 late gestation fetuses. Values represent group mean ± standard error.

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<tr>
<td>MF</td>
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<td>VHF</td>
<td>0.013±0.003</td>
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<tr>
<td>LF:HF</td>
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<td>3.54±0.415</td>
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CHAPTER 3
CHRONIC MATERNAL HYPERCORTISOLEMIA IN LATE GESTATION ALTERS FETAL CARDIAC FUNCTION AT BIRTH

Introduction

Glucocorticoids are essential for maturation of the fetal heart in late gestation (210). Nevertheless, experimental data from animal studies and clinical data from human studies indicate that the late gestation fetal heart may be especially susceptible to either early or excessive glucocorticoid signaling (21, 169). Overexposure to maternal glucocorticoids has been extensively studied in the context of antenatal glucocorticoid therapy for premature delivery; however, increased maternal secretion of endogenous corticosteroids can also contribute to the overexposure of fetal tissues (163, 176).

Although Cushing’s syndrome is rare in pregnancy, the incidence of complications such as premature delivery, stillbirth, and intrauterine growth restriction are increased in these pregnancies, as well as those complicated by chronic maternal stress caused by adverse events such as violence or loss of a relative during pregnancy (256, 144, 258, 139).

In previous studies using an ovine model of pregnancy, we have shown that modest, chronic increases in maternal cortisol concentration beginning at 115 days of gestation increases the thickness of the right ventricular and left ventricular free walls and increases fetal heart weight relative to fetal body weight at 130 days of gestation (term ~145 days) (201). These changes were not accompanied by an increase in fetal blood pressure or cardiac fibrosis, and could be blocked by MR antagonism. Cortisol significantly increased activated caspase-3 staining of Purkinje fibers in the intraventricular septum and subendocardial layers of the left ventricle; this effect of cortisol could be blocked by GR antagonism, suggesting that chronically increased fetal
cortisol concentrations increased apoptosis of the fetal cardiac conduction system (68). Statistical modeling of gene expression in the 130-day fetal lamb septa indicated overexpression of pathways related to cellular calcium homeostasis and apoptosis (specifically muscle cell apoptosis) (205). Expanding on these studies, we found that cortisol treatment dramatically increased the incidence of stillbirth, particularly in the immediate perinatal period, when administered from 115 days of gestation to term (117). Transcriptomic analysis of septa collected during labor indicated changes in cardiac metabolism and mitochondrial function. Nearly one fifth of the genes differentially regulated by cortisol in these hearts were associated with the mitochondria, and qRT-PCR of mitochondrial DNA (mtDNA) from the left ventricle revealed significantly less mtDNA in cortisol compared to control fetuses (205). These results suggested that cardiac oxidative metabolism was adversely impacted by the chronic exposure to increased cortisol in these fetuses.

Together, our prior studies suggest that a modest yet chronic elevation in maternal cortisol concentration throughout late gestation might induce pathophysiological alterations in the late gestation fetal heart and cardiac conduction system. These deleterious changes appeared to become most apparent during the perinatal period, a time in which the fetal heart is normally subject to changes in metabolism, afterload, and ultimately cardiac work (253). For this reason, we investigated the effect of chronic maternal hypercortisolemia throughout late gestation on fetal blood aortic pressure, heart rate and electrocardiogram.

**Materials and Methods**

Rambouillet ewes and their lambs were studied. The University of Florida Institutional Animal Care and Use Committee approved all animal use for this study. All
animals were housed individually in pens (24 ft$^2$) within a temperature and light (lights on 0700 to 1900) controlled facility throughout the study period. Ewes were assigned to one of two groups of ewes at 115 days: a control group (n=7 singleton pregnancies) with no infusion, and a group of ewes treated with cortisol (CORT; n=5 singleton pregnancies; 1 mg·kg$^{-1}$·day$^{-1}$ of Solu-Cortef; hydrocortisone sodium succinate in sodium phosphate; Pfizer, New York, NY). An ambulatory infusion pump (3D Micro Infusion Pump; Strategic Applications Inc.; Lake Villa, IL) was used to deliver the infusion at a rate of 0.16 ml/h. This pattern of cortisol infusion was designed to mimic the chronic increases in maternal plasma cortisol concentrations occurring with chronic stress or Cushing’s syndrome during pregnancy.

At approximately day 118 (±1 SE) days of gestation, surgery was performed under isoflurane anesthesia. A flow probe (6mm 6PSS; Transonics Inc., Ithaca, NY) was placed on the main uterine artery to allow observation of the uterine artery pressure waveform as an index of the progression of labor. To study changes in fetal aortic pressure and electrocardiogram (ECG), a radiotelemetry device (DSI PA-D70 PCTP; Data Sciences International Inc., Minneapolis, MN) was placed in the fetus to allow continuous measurement of fetal aortic pressure, amniotic pressure, fetal ECG, and temperature. Briefly, the head and neck of the fetus were located in the uterus and exposed. A small midline incision was made in the fetal neck, and the telemetry device was placed subcutaneously at the level of the clavicle and sutured in place. The solid tip ECG probe was placed into the right jugular vein of the fetus and advanced into the superior vena cava, with the tip just outside the right atrium. The aortic pressure catheter was inserted into the left carotid artery of the fetus and advanced into the aorta.
outside of the left ventricle. The other pressure catheter was used to measure amniotic fluid pressure; it was tunneled underneath the fetal skin and sutured to the skin with the tip exposed. The grounding lead for the ECG was attached to the skin of the thorax with a sterilized fishing hook (size, 6; Baitholder; Eagle Claw; Denver, CO). Catheters were also placed in the fetal tibial arteries and the maternal femoral arteries and veins. Ewes were treated at the end of surgery and for 2 days postoperatively with analgesic (flunixin meglamine; 1 mg/kg sid; Merck Animal Health, Germany) and for 5 days postoperatively with antibiotic (Polyflex, 12-15 mg/kg bid; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO). Rectal temperature was measured twice a day for 5 days. Ewes were fed a diet of pelleted feed according to NRC standards adjusted for the ewe’s body weight and fetal gestational age.

Blood samples (8 ml) were collected from the ewe and fetus on approximately 125, 130, 135, 138, and 140 days of gestation and on the day of delivery if possible. Fetal blood samples were analyzed for fetal blood gases \( P_aO_2 \), \( P_aCO_2 \), pH, (iSTAT Handheld; Abbott Point of Care Inc.; Princeton, NJ). Plasma was analyzed for maternal and fetal electrolytes (Roche Electrolyte Analyzer 9180), glucose and lactate (YSI Model 2700 Glucose/Lactate Analyzer, Yellow Springs, OH), cortisol (EA65; Oxford Biomedical, Oxford, MI) and insulin (Alpco ovine insulin kit, Alpco Diagnostics, Salem, NH) as previously described. Uterine blood flow was measured for at least 30 minutes using an ambulatory Bluetooth based acquisition system (Transonics Physiogear; Transonics Inc., Ithaca, NY) in ewes on day 130, 135, 140 and then continuously until birth to monitor uterine contractions.

Two fetuses were excluded from the analysis. In the control group, we excluded
one fetus that was hypoxic throughout the pregnancy. In the cortisol-infused group we excluded one ewe that delivered a weak lamb at 135 days of gestation. In the control group, we included one ewe that was euthanized in labor due to dystocia, and one ewe that delivered a live lamb that died shortly after birth. In the cortisol group, we included a ewe that delivered a live lamb that died within 20 minutes of birth. In this study, a total of 3 male and 4 female lambs were born to 7 control ewes at 143±1 days (range 139-148 days) gestation, and 4 male lambs and 1 female lamb were born to 5 cortisol-infused ewes at 144±2 days (range 140-150 days) gestation.

Ewes and their lambs were euthanized as soon as possible after birth, between 10 and 480 minutes after birth (Euthasol; Fort Worth, TX) and measurements of the body and weights of the heart, liver, adrenal, lung, kidneys, perirenal fat, pancreas, and brain were performed. Aortic pressure and ECG data were collected telemetrically using an acquisition rate of 500 Hz. Analysis of the fetal aortic blood pressure and ECG was performed using DSI Dataquest Open A.R.T 4.31 and Ponemah 5.00 software. Aortic pressure was corrected by subtraction of the amniotic fluid pressure. Mean aortic pressure (MAP), systolic pressure (SP), diastolic pressure (DP) and heart rate (HR) were calculated from the aortic pressure waveform as 24-hour means measured for 14 days before birth and 1 hour means over the final 24 hours of fetal life. ECG parameters (P duration, PR, QR, QRS, corrected QT (QTc), and ST intervals) were calculated for the one-hour interval between 0600 and 0700 for the 14 days before birth, and for each hourly interval over the final 24 hours before birth. In all animals, the time of birth was confirmed using the telemetry record of fetal/neonatal temperature, which in all cases revealed a rapid decrease at the time of delivery. Data from the control animals has
been previously reported (in review at AJP)

The harmonics of fetal heart rate variability (HRV) was used as an index of the relative sympathetic and parasympathetic tone in control of HR. Power spectral density analysis was performed using Dataquest Open A.R.T 4.31 software using the interbeat interval derived from 10 minutes of fetal aortic pressure data occurring at the same time on each day for 14 days before birth, or inter-beat interval derived from 2 hours of fetal ECG taken in the 2 hour period before birth. The analysis used a nonparametric method (fast Fourier transform) to estimate the power of the spectrum for very low frequency (VLF; 0.001-0.025 cycles/beat), low frequency (LF; 0.025-0.125 cycles/beat), medium frequency (MF; 0.125-0.2 cycles/beat), high frequency (HF; 0.2-0.5 cycles/beat), very high frequency (VHF; 0.5-1.0 cycles/beat) components, the standard deviation of the normal to normal R-R intervals (SDNN), and the root mean square of successive heartbeat interval differences (RMSSD) (261, 122, 275). The ratio of LF:HF and SDNN:RMSSD were calculated as indices of sympathetic tone relative to parasympathetic tone.

**Statistical Analysis.** Analyses of fetal and maternal glucose, cortisol, lactate, electrolytes, fetal blood gases, MAP, SP, DP, HR, and ECG parameters were performed using two-way analysis of variance corrected for repeated measures across time. Between treatment comparisons at individual time points was performed by posthoc t-test with Bonferroni adjustment. Because of the unequal variance across time points in fetal cortisol concentrations as a consequence of the steep increase in values near term, the values were log transformed and analyzed using two-way analysis of variance corrected for repeated measures over time. Two-way analysis of variance was
used to analyze the fetal insulin concentration because of missing samples from some animals. Between group comparison of fetal organ weights and morphometrics at necropsy was performed using Student’s t-test.

**Results**

**Maternal Blood Samples in Late Gestation**

Infusion of 1mg/kg/d cortisol (CORT) significantly increased maternal cortisol concentration (mean concentrations over the study: 16.4±1.7 ng/ml in cortisol infused ewes vs 7.4±1.4 ng/ml in control ewes (Figure 3-1 A). Maternal cortisol infusion did not significantly alter maternal plasma electrolytes, plasma proteins (PP) or insulin (Table 3-1); although plasma glucose concentrations tended to be higher in the CORT ewes, this increase was not significant (Figure 3-1 B). Cortisol infusion did significantly increase maternal lactate concentrations (Figure 3-1 C).

**Effects of Maternal Cortisol on Fetal Blood Samples in Late Gestation**

The fetal cortisol concentration was significantly increased by infusion of cortisol to the ewe. As expected, fetal cortisol concentrations significantly increased over time in both groups (Figure 3-1 D). Fetal plasma electrolytes, PP (Table 3-1) and packed cell volume (PCV) were not changed by maternal cortisol infusion; PCV did increase in both groups as gestation progressed (data not shown). Fetal plasma glucose, lactate concentrations (Figure 3-1 E and F), $P_aO_2$, $P_aCO_2$, pH (Figure 3-2), and plasma insulin (Table 3-1) were also not significantly changed in the CORT fetuses compared to the control fetuses.
Effects of Maternal Cortisol on Fetal Organ Weight and Body Morphometrics at Necropsy

Birth weight, girth, and hindlimb measurements were all significantly greater in CORT fetuses, however ponderal index was not different between CORT and control fetuses (Table 3-2). Heart weight was not significantly different between groups; however, left ventricular free wall thickness relative to tibial length and interventricular septal thickness relative to tibial length were both significantly greater in CORT newborns (Table 3-2). In the immediate postnatal period, CORT newborns tended to be weak (4/5), unable to stand (3/5), had evidence of respiratory distress with incomplete inflation of lungs (3/5), and/or had a considerable amount of meconium staining (2/5) at birth. In contrast, only one of the control newborns was weak, shivering, and unable to stand after 30 minutes; all other control newborns were healthy, standing shortly after birth, and with little or no meconium staining evident.

Effects of Maternal Cortisol on Fetal Blood Pressure, Heart Rate, and ECG in Late Gestation

Maternal cortisol infusion did not alter the pattern of change of MAP throughout the final 14 days of fetal life, but did significantly reduce MAP on the day of birth (43.4±4.7 mmHg in CORT fetuses vs 56.9±4.0 mmHg in control fetuses Figure 3 A). Maternal cortisol significantly altered the pattern of change in SP and DP over the 14d. DP, SP and PP all increase over time in both groups of fetuses, but the DP and SP did not increase further on the day of birth in the CORT fetuses, as it did in the control fetuses (Figure 3-3 B and C). Fetal HR significantly decreased over the last 14 days in utero in both groups (Figure 3-3 D). Although maternal cortisol infusion did not significantly alter fetal HR over this period, the CORT fetuses had significantly lower HR on the day of birth (140±7 BPM in CORT fetuses vs 159±6 BPM in control fetuses).
Overall, the PR interval was significantly increased by maternal cortisol infusion (overall mean interval 83.0±2.0 msec in CORT fetuses vs 77.5±1.7 msec in control fetuses; Figure 3-4 A). There was no significant effect of CORT in late gestation on the duration of the P wave; although the P wave duration tended to be higher in the CORT fetuses, this was only significant 4 days prior to birth (Figure 3-4 B). The time course for the QR-interval was also significantly altered by maternal cortisol infusion over late gestation; the overall mean interval in control fetuses was decreased from 13.1±0.8 msec in control fetuses to 12.2±1.1 msec, whereas in CORT fetuses the overall mean interval increased from 10.7±0.91 msec to 12.9±1.35 msec.

Analysis of fetal heart rate harmonics indicated that there were no significant differences in VLF, LF, MF, HF, VHF power, or the LF:HF ratio between the two groups when data for each morning of the final 14 days of fetal life was analyzed (Table 3-3), suggesting no major changes in relative contributions of sympathetic or parasympathetic tone to HR over this period in either group of fetuses.

Effects of Maternal Cortisol on Fetal Blood Pressure, Heart Rate, and ECG in the 24 Hours Before Birth

Fetal MAP dropped significantly in the final hour before birth in the fetuses of cortisol-infused ewes (41.5±5.6 mmHg in CORT fetuses vs 56.9±4.7 mmHg in control fetuses; Figure 3-3 E). The time courses for SP and DP were significantly altered by CORT in the final 24 hours before birth (Figure 3-3 F and G); although overall the SP and DP were not different between the groups, diastolic and systolic pressures were significantly reduced in the CORT ewes as compared to control ewes in the hour before birth (SP: 52.5±5.3 mmHg in CORT fetuses vs 69.4±4.5 mmHg in control fetuses; DP: 29.6±5.7 in CORT fetuses vs 47.1±4.8 mmHg in control fetuses). HR was also
significantly lower in the final hour before birth (129±8 bpm in CORT fetuses vs 161±7 BPM in control fetuses; Figure 3-3 H). The reduction in heart rate that occurred before birth in CORT fetuses persisted in the 10 minutes immediately after birth (86±20 bpm in CORT newborns vs 185±29 bpm in control newborns; Figure 3-3 L). Although the mean aortic pressure, as well as systolic and diastolic pressures were not significantly different between groups immediately after birth (Figure 3-3 I-K), 2 of the weakest CORT newborns had extremely low aortic pressures (10 and 15mmHg; Figure 3-3 I).

The duration of the fetal P wave and the PR-interval were significantly prolonged by maternal cortisol infusion over the last 24 hours of fetal life (mean P wave duration over 24h: 44.0±2.4 msec in CORT fetuses vs 35.2±2.1 msec in control fetuses; PR interval: 87.0±2.9 msec in CORT fetuses vs 76.9±2.4 msec in control fetuses; Figure 3-4 C,D). Differences between groups for both of these variables were increased as the fetuses neared birth. Although the ST-interval was not overall significantly different in this period, it was significantly increased in the final hour before birth in CORT fetuses (174±6 msec in CORT fetuses vs 157±5 msec in control fetuses). Both groups of fetuses had similar patterns of decelerations occurring in the two hours before birth and measurements of the inter-beat interval were not different (Table 3-3). Importantly, no fetus had a sinusoidal pattern or absent heart rate variability occurring during this period. However, on the day of birth, and particularly in the immediate peripartal period, abnormalities in the ECG were evident in the CORT fetuses (Figure 3-5), including AV block (Figure 3-5 A), notched P waves (Figure 3-5 B), atrial fibrillation (Figure 3-5 C), and abnormal ST segments (Figures 3-5 A and 3-5 D). In the hour before birth, AV block were evident in all the CORT fetuses (5 of 5), with the most affected fetus.
displaying 44 instances, and with prominent atrial fibrillation after birth in that same lamb (as illustrated in Figure 3-5 C); In comparison only 2 of 7 control fetuses had AV block. Notching or bifurcation of P waves were identified in 4 of 5 CORT fetuses compared to 3 of 7 fetuses in the control group. Abnormal ST segments were identified in 2 of 5 CORT fetuses with a “tombstone” like T wave shape, indicative of myocardial hypoxemia (Figure 3-5 D) (270, 269).

Discussion

In the present study, we demonstrated that as pregnancy progresses in late gestation, chronically elevated maternal cortisol concentrations detrimentally impact fetal cardiac function, particularly shortly before and after birth. Although there was no long-term effect of the maternal cortisol infusion on the normal decrease in fetal HR, nor the normal increase in fetal blood pressure over late gestation, the increase in heart rate and blood pressure during labor and delivery was blunted. Furthermore, there was a dramatic and precipitous decline in fetal MAP and HR that became more pronounced as labor progressed and immediately after birth. The decline in fetal HR on the day of birth corresponded with slowing of atrial and atrioventricular (AV) conduction as both atrial (P wave duration) and AV (PR Interval) conduction times were prolonged in the cortisol fetuses. A parallel increase in the QR interval also suggests that conduction through the Purkinje fibers to the intraventricular septum is also slowed. Importantly, the decrease in heart rate was not associated with alterations in sympathetic or parasympathetic cardiac tone, as measurements of heart rate variability were not significantly different from the control fetuses. Our findings indicate that chronic exposure to excess cortisol in utero can detrimentally impact cardiac conduction not only immediately before birth, but also shortly thereafter. We suspect that this cardiac phenotype is a function of cortisol
signaling through the glucocorticoid receptor (GR), altering cardiac ion channel
expression and metabolic homeostasis in cardiomyocytes, and in particular in those in
the cardiac conduction pathway.

Adrenal glucocorticoids and the potent synthetic glucocorticoid dexamethasone
are known to contribute to prolongation of the cardiac action potential through
decreases in \( I_{\text{to}} \) potassium channel density and increases in L-type calcium channel
density (268, 263). Similar alterations have also been observed in the mouse when GR
is conditionally overexpressed in cardiomyocytes; moreover, alterations in the ECG are
evident including elongated PQ, QRS, and QTc intervals, and a substantially greater
incidence of AV block (211). This suggests that the changes observed in the fetal ECG
during late gestation and at birth are in part due to excessive and prolonged signaling
through the GR, altering the ion homeostasis in the cardiac tissue. In contrast, when the
GR was conditionally knocked out of cardiomyocytes in the fetal mouse, significant
alterations in the macro- and micro- structure of the compact myocardium were evident,
along with changes in the expression for genes involved in calcium handling and
cardiac metabolism (210, 208). Additionally, Purkinje fibers of the adult human heart
have been found to have high concentrations of corticosteroid-binding globulin (CBG)
(217). Plasma CBG functions as a transporter of systemic cortisol. The function of CBG
in Purkinje fibers is not well understood, but could potentially confer a protective effect
or alternatively act as a delivery mechanism. Taken together, these findings highlight
the critical importance of GR signaling in the normal development and maturation of key
electrophysiologic, metabolic, and structural pathways in the fetal heart. The observed
arrhythmias, including prolonged P and PR intervals of the ECG therefore might result,
in part, through excessive GR signaling and the downstream disturbances of cardiac calcium and potassium ion currents.

Previously, we showed that CORT fetuses at term had significantly less mitochondria, and gene array data from the hearts indicated that many of the genes differentially regulated by maternal cortisol infusion were associated with changes in cardiac metabolism and mitochondrial function (205). In late gestation, the fetal cardiac mitochondria are in a dynamic state of maturational change as the number and volume of mitochondria increase as it prepares to make the switch from primarily utilizing carbohydrates in utero, to fatty acids ex utero as the main energy sources for ATP production (30, 16). In cardiomyocytes, ATP is primarily consumed by ATPases that support the contractile machinery, Ca\textsuperscript{2+} reuptake, ion channels that maintain the membrane potential, and anabolic reactions (213). Therefore, changes that affect mitochondrial ATP production could dramatically alter cardiomyocyte function. Discovery of glucocorticoid response elements in the mitochondrial DNA and that cytoplasmic GR are capable of translocating into the mitochondria have suggested steroid modulation of mitochondrial function (61, 55). In tissues with a high metabolic demand, such as the fetal heart and neuromuscular system, changes in ATP production could be exceptionally devastating. Mitochondrial diseases in children and adults are associated with dilated and hypertrophic cardiomyopathies, heart failure, and ECG abnormalities including prolonged QTc interval and AV block (129, 11, 5).

The changes in ECG, MAP, and HR occurring in the immediate perinatal period suggest that the fetal hearts chronically exposed to higher than normal cortisol may struggle to maintain efficient function, increasing the likelihood of failure when
confronted with the considerable physiological challenges, and further increase in cortisol, occurring within labor and delivery. Nevertheless, the entirety of the cardiac effects observed in this study may not be exclusively due to the direct effect of maternal hypercortisolemia on the fetal heart as there are likely other metabolic and endocrine changes secondary to the excess cortisol that are contributing factors. Ultimately, we believe that alterations in cardiac ion and metabolic homeostasis are key factors in precipitating these outcomes, which are brought on by early and persistent glucocorticoid signaling; however, further studies will be necessary to identify specific cellular changes that contribute to the phenotype.
Figure 3-1. Plasma cortisol (A,D), glucose (B,E), and lactate (C,F) concentrations in control ewes and their fetuses (open circles) or ewes that were infused with cortisol during pregnancy (1.0 mg·kg\(^{-1}\)·day\(^{-1}\)) and their fetuses (CORT: filled circles). * Indicates a significant overall effect of time; ** Indicates a significant overall effect of maternal cortisol treatment. † Indicates values significantly different between groups at the indicated time point.
Figure 3-2. Blood pO\textsubscript{2} (A), pCO\textsubscript{2} (B), and pH (C) in fetuses of control ewes (open circles) or fetuses of ewes that were infused with cortisol during pregnancy (1.0 mg·kg\textsuperscript{-1}·day\textsuperscript{-1}) (filled circles).
Figure 3-3. Mean aortic pressure (A, E, I), systolic Pressure (B, F, I), diastolic Pressure (C, G, K) and heart rate (D, H, L) during late gestation (A-D), the immediate perinatal period (E-H), and 10 minutes after birth (I-L) in fetuses of control ewes (open circles) or fetuses of ewes infused with cortisol during pregnancy (1.0 mg·kg⁻¹·day⁻¹) (filled circles). * Indicates a significant effect of time; ***Indicates a significant interaction between the effects of maternal cortisol treatment and time. † Indicates values significantly different between groups at the indicated time point.
Figure 3-4. The duration of the PR interval (A, C) and the P wave (B, D) during late gestation (A, B) and the immediate perinatal period (C, D) in fetuses of control ewes (open circles) or fetuses of ewes infused with cortisol during pregnancy (1.0 mg·kg⁻¹·day⁻¹) (filled circles). * Indicates a significant effect of time. **Indicates a significant overall effect of maternal cortisol treatment. ***Indicates a significant interaction effect of maternal cortisol treatment and time. † Indicates values significantly different between groups at the indicated time point.
Figure 3-5. Examples of ECG abnormalities identified in 4 CORT treated fetus or lamb at the time of birth. A, AV block (P wave with no associated QRS complex) and elevated ST segment in a fetus during active labor, B, notched (or split) P wave in a newborn lamb; C, atrial fibrillation in a newborn lamb who later succumbed; D, abnormal ST segment and notched P waves in a newborn lamb. Note that the shape of the ECG varies with the positioning of the lead on the chest wall relative to the lead secured within the jugular. Thus, the shape can vary between fetuses, and also within fetus depending on the orientation of the fetus.
Table 3-1. Maternal and fetal plasma electrolyte, protein, and insulin concentrations. Values represent group mean ± standard error.

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<td>Plasma K⁺ (mEq/L)</td>
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<tr>
<td>Plasma Ca²⁺ (mEq/L)</td>
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<td>5.31±0.10</td>
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<tr>
<td>Plasma protein (mg/ml)</td>
<td>6.29±0.20</td>
<td>6.82±0.24</td>
</tr>
<tr>
<td>Plasma Insulin (ng/ml)</td>
<td>1.47±0.100</td>
<td>1.78±0.091</td>
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Table 3-2. Relative organ weights, cardiac wall thicknesses, and body morphometrics at necropsy in control and cortisol exposed lambs. Values represent group mean ± standard error.

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<th>Cortisol</th>
<th>p-value</th>
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<tr>
<td>Body Weight (g)</td>
<td>3892±258</td>
<td>5528±504*</td>
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</tr>
<tr>
<td>Crown to rump (cm)</td>
<td>54.2±2.08</td>
<td>58.9±2.25</td>
<td></td>
</tr>
<tr>
<td>Girth (cm)</td>
<td>34.9±1.1</td>
<td>39.2±1.0*</td>
<td></td>
</tr>
<tr>
<td>Rear leg hock to hoof (cm)</td>
<td>19.2±0.60</td>
<td>22.0±0.71*</td>
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<td>Ponderal Index (g/cm³)</td>
<td>2.53±0.11</td>
<td>2.48±0.11</td>
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</tr>
<tr>
<td>Relative weight (g/kg)</td>
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<td></td>
<td></td>
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<tr>
<td>Brain</td>
<td>1.19±0.09</td>
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<td>Pituitary</td>
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<td>0.0271±0.0047</td>
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<tr>
<td>Lung</td>
<td>21.4±2.2</td>
<td>1.73±2.2</td>
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</tr>
<tr>
<td>Liver</td>
<td>28.8±5.1</td>
<td>24.3±1.0</td>
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<tr>
<td>Kidney</td>
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<td>2.57±0.09</td>
<td></td>
</tr>
<tr>
<td>Adrenal</td>
<td>0.0944±0.0060</td>
<td>0.0872±0.0011</td>
<td></td>
</tr>
<tr>
<td>Perirenal adipose</td>
<td>3.68±0.44</td>
<td>3.39±0.21</td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>1.01±0.10</td>
<td>0.76±0.07</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>6.89±0.48</td>
<td>6.35±0.24</td>
<td></td>
</tr>
<tr>
<td>Relative cardiac wall thickness (mm/cm tibial length)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left ventricular free wall</td>
<td>0.383±0.015</td>
<td>0.449±0.016*</td>
<td></td>
</tr>
<tr>
<td>Septum wall thickness</td>
<td>0.445±0.016</td>
<td>0.520±0.024*</td>
<td></td>
</tr>
<tr>
<td>Right ventricular free wall</td>
<td>0.332±0.011</td>
<td>0.315±0.041</td>
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</tr>
</tbody>
</table>

*indicates values significantly different between lambs of cortisol-infused ewes as compared to control lambs (p<0.05).
Table 3-3. Frequency and time measurements of the fetal heart rate variability in late gestation and in the immediate perinatal period. Values represent group mean ± standard error. (SDNN: Standard Deviation of NN Intervals; RMSSD: Root Mean Square of Successive Differences)

<table>
<thead>
<tr>
<th></th>
<th>Late Gestation</th>
<th>Perinatal Period</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Cortisol</td>
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<tr>
<td>Very Low Frequency</td>
<td>0.315±0.010</td>
<td>0.297±0.012</td>
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<tr>
<td>Low Frequency (LF)</td>
<td>0.135±0.011</td>
<td>0.141±0.013</td>
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<tr>
<td>Medium Frequency</td>
<td>0.088±0.010</td>
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<tr>
<td>High Frequency (HF)</td>
<td>0.047±0.006</td>
<td>0.060±0.007</td>
</tr>
<tr>
<td>Very High Frequency</td>
<td>0.028±0.009</td>
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<tr>
<td>LF:HF</td>
<td>3.48±0.319</td>
<td>2.88±0.378</td>
</tr>
<tr>
<td>SDNN</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>RMSSD</td>
<td>NA</td>
<td>NA</td>
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</tbody>
</table>
CHAPTER 4
CHRONIC EXPOSURE TO CORTISOL IN UTERO ALTERS THE CARDIAC TRANSCRIPTOME OF THE NEWBORN

Introduction

Adverse obstetric outcomes including premature delivery, stillbirth (in utero fetal death occurring after 20 weeks gestation), and intrauterine growth restriction are associated with pregnancies complicated by chronic maternal stress, violence, or loss of a relative during pregnancy (144, 256). The underlying cause of these outcomes in pregnancies complicated by maternal stress is still under investigation. In our lab, we have found a marked increase in fetal and neonatal death at term as well as changes in cardiac structure and function in an ovine model of pregnancy in which the ewe is chronically infused with 1mg/kg/d cortisol (CORT) to mimic concentrations produced during stress (117). In sheep and human fetuses, cardiomyocytes undergo transition from mono-nuclear proliferative cells to bi-nucleated terminally differentiated tissue during the last 20% of gestation, suggesting that the fetal heart is susceptible to remodeling by glucocorticoid exposure (68, 201, 210). In late gestation and early postnatal period, the cardiac mitochondria also undergo a period of maturation as they increase in size and volume to meet the metabolic needs of the cardiomyocytes and changing nutrient supply (30, 16). We have previously used systems modeling on microarray data from hearts of fetuses exposed to CORT to discover key genes and pathways involved in the pathological alterations.

After 15 days of CORT (from 115-130 days of gestation) the genes differentially regulated by CORT indicated alterations in pathways relating to muscle cell apoptosis and cellular calcium homeostasis, consistent with the histological findings from these hearts (205, 68). When the treatment was carried out until term (~15 additional days),
over-representation of genes in pathways related to cardiac metabolism and mitochondrial function were present. Furthermore, approximately one-fifth of the genes differentially regulated by CORT were associated with the mitochondria and further analysis revealed significant decreases in cardiac mitochondrial content (205).

Recently, we have used radiotelemetry devices chronically implanted into the fetus found that CORT decreases fetal aortic pressure and heart rate, and prolongs the duration of the P wave and PR interval in the hour before birth. In the current study, we sought to model the patterns of change in gene expression in the left ventricle and interventricular septum that might contribute to the changes in cardiac function observed from these newborn telemeterized lambs.

**Materials and Methods**

**Animal Treatment**

All animal use in this study was approved by the Institutional Animal Care and Use Committee of the University of Florida. Tissues used in the experiments of this study came from tissues recovered at necropsy of the newborn lambs in Chapter 2. In short, ewes were continuously infused with 1mg/kg/d cortisol (cortisol sodium succinate; Pfizer, New York, NY) beginning at day 115 of gestation until the end of the study period (birth of the lamb); control ewes were not infused and underwent similar surgical and sampling procedures. The newborn lambs (n=5 control and n=5 cortisol lambs) were euthanized via intravenously administered Euthasol solution between 10 and 480 minutes after birth (Euthasol; Fort Worth, TX) and their hearts removed and dissected using sterile instruments and gloves. Tissue samples taken from the interventricular septum and left ventricle were then snap frozen using liquid nitrogen and stored at -80°C until extraction of the RNA.
Messenger RNA from homogenized samples of lamb left ventricle and septa were extracted using Trizol and then purified by on-column DNase digestion (Qiagen RNeasy Plus kits; Qiagen Sciences, Germantown, MD). RNA integrity numbers for the RNA samples were between 7.2-9.1 (septum) and 7.5-9.1 (LV). RNA (500 ng) was labeled with Cy5 using the Agilent QuickAmp Labeling kit to generate cRNA, hybridized to the Agilent-019921 Sheep Gene Expression Microarray 8x15k (G4813A GPL14112), and scanned at the Interdisciplinary Center for Biotechnology Research core at the University of Florida. Our group has previously described the annotation for this array platform in previous publications (197).

Microarray Data Analysis

Raw microarray data was processed using the limma package in R software (RCoreTeam), to perform background correction and data normalization using the quantile method (248, 207, 232). Probes with low expression, microarray control probes, and probes that were less than 10% brightness of the negative controls were excluded. The remaining probes were then processed using a moderated t test that employs an empirical Bayes method for small sample size (P < 0.05) (231). The effect of cortisol was analyzed by comparing the cortisol group to the control group.

Network and Enrichment Analysis

WebGestalt is a web based gene ontology (GO) software that uses a hypergeometric test to compare the gene set of interest to a reference gene set in order to evaluate the significance of enrichment in GO terms and pathways. We used WebGestalt to identify overrepresented biological processes, molecular functions, and cellular components associated with the differentially regulated genes using P=0.05 as the criterion for statistical significance after Benjamini-Hochberg multiple test correction.
of the P value, and accepting at least two genes per category (286, 262). The ClueGO (v2.3.3) plugin was used in Cytoscape software to perform network inference and gene ontology analysis of the differentially regulated genes (23, 219, 161).

**Immunoblot Analysis**

Tissue samples from the septum (~50mg) were kept on dry ice and homogenized in protein extraction buffer (50mM Tris, 150mM NaCl, 1% NP-40, 1mM Na3VO4, phosphatase inhibitor cocktail 2 and 3, and protease inhibitor cocktail (#P5726, #P0044, and #P8340; Sigma-Aldrich). Aliquots of protein extracts were stored at -80°C until use. Equal amounts of protein were loaded into gels for immunoblotting. Immunoblotting analysis was carried out using a Li-COR Odyssey system. Proteins detected were against mitochondrial and metabolic proteins and were evaluated using a fluorochrome-conjugated secondary antibody followed by detection at 700 or 800 nm with the Odyssey Infrared Imaging System (LICOR Biosciences). We evaluated the appropriate protein loading conditions for each protein by increasing protein concentrations and working within the linear range for each. The signal band for each protein was normalized to each animal’s total lane protein. The antibodies used were against: cytochrome c oxidase subunit 4 ((COX4), #4850 Cell; Signaling), pyruvate dehydrogenase E1a (#AB110334; Abcam), phosphorylated pyruvate dehydrogenase E1a (#AB92696; Abcam), oxidative phosphorylation antibody cocktail (#AB110411; Abcam) (against Complex I subunit NDUFB8 (#AB110242), Complex II subunit 30kDa (#AB14714), Complex III subunit Core 2 (#AB14745), Complex IV subunit II (#AB110258), and ATP synthase subunit alpha (#AB14748)) and cytochrome c (#AB13575; Abcam) and cytochrome c (#AB13575; Abcam).
Histology and Immunofluorescence Analysis

An approximately 6mm thick short axis section of the fetal heart was fixed at necropsy in 4% phosphate-buffered paraformaldehyde for 24 hours before long-term storage in 70% reagent alcohol. The tissues were embedded in paraffin wax and 5μm thick sections were cut for histological analysis. Collagen deposition in the hearts was determined using picrosirius red staining as previously described (201), imaged using a circular polarized light, and analyzed using ImageJ. Five images were taken at x10 magnification from the left ventricular free wall of each animal and the average area stained per tissue area was compared by Student’s t-test. The collagen spectra of the circular polarized light images were isolated and measured using the color threshold and binary tools in Image J. The total tissue area was measured after enhancing the image contrast by 5% and making the image binary. All images were subjected to the same threshold and correction criteria.

Assessment of mitochondrial area was performed using an antibody against cytochrome c oxidase subunit 4 (COX4; Cell Signaling #4850) as previously described (204), with the addition of wheat germ agglutinin (Wheat Germ Agglutinin (WGA), Oregon Green® 488 Conjugate; Invitrogen #W6748) and DAPI (Invitrogen #D1306) used as directed by the supplier. Five images were taken at x40 magnification through each filter (350, 488, 647 nm) from the left ventricular free wall of each animal and the average area stained per tissue area was compared by Student’s t-test. The merged red, blue, green image was adjusted to decrease the blue intensity by 50% to correct for the difference in intensity. The mitochondrial stained area was measured by converting the images taken at 647nm to an RGB stack in Image J. The “R” (red) channel was then measured using the Triangle thresholding parameter. The nuclear area was measured
by converting the images taken at 350nm to an RGB stack in Image J. The “B” (blue) channel was then measured using the Renyi Entropy thresholding parameter. The cell area was measured by converting the images taken at 488nm to an RGB stack in ImageJ. The “G” (green) channel was then measured using the Huang thresholding parameter. The nuclear (DAPI) and cell (WGA) areas were combined as a measure for tissue area. All images were subjected to the same threshold and correction criteria. The average area stained per tissue area was compared by Student’s t-test.

**Results**

CORT induced significant changes in gene expression in both the LV and septum of the newborn lambs. There were 386 genes that were differentially regulated by CORT, 166 genes were upregulated and 220 genes were downregulated in the LV (Figure 4-1 A). In the septum, 185 genes were differentially regulated by CORT, 61 genes were upregulated and 124 genes were downregulated (Figure 4-1 B).

**Pattern of Change in Gene Expression from the Newborn Lamb LV**

Gene ontology analysis of the 166 genes upregulated by CORT from the LV revealed biological processes relating to response to chemical stimulus (48 genes; adj. P=0.0108), response to organic substances (36 genes; adj. P=0.0108), response to endogenous stimulus (23 genes; adj. P=0.0271), response to hormone stimulus (19 genes; adj. P=0.0271), and SMAD protein signal transduction (3 genes; adj. P=0.0331). Molecular functions associated with the upregulated genes included oxidoreductase activity (19 genes; adj. P= 0.0084), BMP receptor binding (2 genes; adj. P=0.0271), and glutathione peroxidase activity (3 genes; adj. P=0.0162). The cytoplasm (108 genes; adj. P=6.82E-05) and intracellular membrane-bound organelle (111 genes; adj. P=6.82E-05) were the major cellular components that were associated with the genes.
upregulated by CORT. Pathways in cancer (10 genes; adj. P=2.49E-05), wnt signaling (6 genes; adj. P=0.0006), insulin signaling (5 genes; adj. P=0.0033), and TGF-beta signaling (4 genes; adj. P=0.0037) were the major KEGG pathways associated with the genes upregulated by CORT in the LV (Table 4-1).

Analysis of the 220 genes downregulated by CORT in the LV identified C-terminal protein amino acid modification (5 genes; adj. P=0.0292) as the only biological process affected by CORT. ATPase activity coupled to transmembrane movement of substances (6 genes; adj. P=0.0396), hydrolase activity acting on acid anhydrides, catalyzing transmembrane movement of substances (6 genes; adj. P=0.0396), and primary active transmembrane transporter activity (6 genes; adj. P=0.0396) were among the molecular functions associated with the genes downregulated by CORT. Intracellular membrane-bounded organelle (138 genes; adj. P=6.00E-04), the nucleoplasm part (24 genes; adj. P=6.00E-04), and the nuclear lumen (54 genes; adj. P=6.00E-04) were among the cellular components associated with the genes downregulated by CORT. KEGG pathways associated with these downregulated genes included metabolic pathways (19 genes; adj. P=2.00E-04, hypertrophic cardiomyopathy (4 genes; adj. P=0.0037), and lysosome (5 genes; adj. P=0.0026) (Table 4-2).

Analysis of the differentially regulated genes using ClueGo revealed similar results, albeit with more descriptive terms. 378 unique gene names were assigned to the 386 genes differentially regulated by CORT in the LV. 370 of these genes were recognized as having functional annotations by the ClueGO software. 207 significant nonredundant gene ontology terms and pathways were then assigned to 67 of these
genes. BMP4 (26 terms), SMAD4 (12 terms), and PNPT1 (11 terms) were the genes with the most associated terms, reflecting their enrichment significance.

**Patterns of Change in Gene Expression from the Newborn Lamb Septum**

Gene ontology analysis of the 61 genes upregulated in the septum identified protein localization to organelle (9 genes; adj. P=0.0219), protein retention in endoplasmic reticulum (ER) lumen (2 genes; adj. P=0.0343), and protein complex disassembly as biological processes affect by CORT. ER retention sequence binding (2 genes; adj. P=0.0053) was the only significant molecular function associated with the upregulated septal genes. The cytoplasm (45 genes; adj. P=0.0004), troponin complex (2 genes; adj. P=8.40E-03), and non-membrane-bounded organelle (23 genes; 8.40E-03) were cellular components associated with the septal genes upregulated by CORT. KEGG pathways associated with the upregulated genes of the septum included the insulin signaling pathway (4 genes; adj. P=7.00E-04), amino sugar and nucleotide sugar metabolism (2 genes; adj. P=8.10E-03), and starch and sucrose metabolism (2 genes; adj. P=8.10E-03) (Table 4-3).

There were no significant biological processes nor molecular functions associated with the 124 genes downregulated by CORT in the septum. Membrane-bounded organelle (87 genes; adj. P=2.47E-05), nucleus (61 genes; adj. P=2.00E-04), and nucleoplasm (23 genes; adj. P=1.10E-03) were among the cellular components associated with the downregulated genes in the septum. The KEGG pathways associated with the downregulated septal genes included insulin signaling pathway (4 genes; adj. P=1.40E-02), adipocytokine signaling pathway (3 genes; adj. P=1.40E-02), and lysosome (3 genes; adj. P=0.0253) (Table 4-4).
Analysis in ClueGo identified 183 unique gene names which were assigned to the genes differentially regulated by CORT in the septum. 181 of these genes were recognized as having functional annotations by the ClueGO software. 60 significant nonredundant gene ontology terms and pathways were then assigned to 31 of these genes. EIF2AK2 (8 terms), NCOA1 (5 terms), and SLC35D1 (4 terms) were the genes with the most associated terms.

**Immunoblotting and Histology of Structural, Mitochondrial, and Metabolically Related Proteins**

The patterns of change in gene expression suggested that there could be considerable changes to pathways involved in metabolism and the TGF-β pathway. We used immunoblotting and histological techniques to assess some of these pathways. CORT significantly increased collagen deposition in the fetal left ventricle after histological staining with picrosirius red (1.5±0.3% stained area in control fetuses vs 3.6±0.9% stained area in CORT fetuses) (Figure 4-2). CORT also significantly reduced COX4 stained area relative to total tissue area in the left ventricle (23.3±7.0% stained area in control fetuses vs 5.3±0.8% stained area in CORT fetuses) (Figure 4-3). Similarly, COX4 protein concentration was significantly reduced by CORT in the fetal septum (2250±585 arbitrary fluorescent units (AFU) in control fetuses vs 1008±286 AFU in CORT fetuses) (Figure 4-4 E). All of the other proteins tested by immunoblotting were similar between groups.

**Discussion**

We investigated the effect of CORT on the patterns of change in gene expression from the LV and septum of newborn lambs. The results from the current study indicate that chronically higher-than-normal maternal cortisol concentrations in
late gestation enacts significant changes in gene expression within pathways involved in maintaining normal cardiac architecture and metabolism in the newborn heart. These findings are particularly interesting given the cardiac dysfunction that was present in these lambs around the time of birth.

Glucocorticoids are essential for the maturational development of fetal tissues in preparation for ex utero survival (134). A growing body of evidence suggests that improper exposure of fetal tissues to glucocorticoids, particularly the heart, can be detrimental to the normal trajectory of organ development (210, 21, 169). Our group has previously shown, using similar methods that CORT alters gene expression in pathways relating to cardiac metabolism and reduces mitochondrial content in the unborn term fetal heart (205). In these unborn term lambs, the most upregulated gene by CORT in the fetal septum was PDK4, a glucocorticoid target and key negative regulator of pyruvate metabolism (44). We had anticipated to find similar results in the newborn lambs of the current study, albeit with additional results that might reflect the lambs’ exposure to labor, delivery, and tissue oxygenation. Instead, there were only three genes (FBXW11, MAF1, ING4) that were significantly changed by CORT in both the unborn term and newborn lamb septa. PDK4 was similar between groups in the newborn lamb LV and septum. Protein analysis of pyruvate dehydrogenase E1a (target for PDK4) and its phosphorylated form in the septum further suggest that this pathway is not affected by CORT in these newborn lambs. Nevertheless, other genes differentially regulated by CORT were associated with metabolic pathways in the LV and septum including the insulin signaling pathway.
In the unborn term lambs from our previous study, about 20% of the genes differentially regulated by CORT were associated with the mitochondria and further analysis showed a significant reduction in mitochondrial content (205). Others have shown that chronic glucocorticoid exposure significantly reduces the activities of complexes I and III, increases ROS production, decreases antioxidant levels and activity, and increases susceptibility to pro-apoptotic signals (61, 159, 147, 246). We predicted that CORT would affect similar aspects of the electron transport chain, which might contribute to mitochondrial dysfunction. Analysis of the microarray indicated that COX4 was significantly decreased in the fetal septum and was the only mitochondrial protein tested to be significantly changed by CORT in the fetal septum. We also performed immunofluorescence against COX4 and found similar results in the fetal LV. Interestingly, qPCR gene expression of COX4 was similar between groups in the LV and septum (data not shown) suggesting that there are post transcriptional or post translational changes that might contribute to the reductions. Cytochrome c oxidase is the terminal complex in the electron transport chain and is composed of nuclear and mitochondrial derived proteins. Subunit 1 of cytochrome c oxidase is a mitochondrial DNA derived protein and the catalytic core of the complex and subunit 4 is a critical assembly protein encoded by nuclear DNA (14, 32, 132). Decreased levels of COX4, in vitro, have been shown to severely compromise the assembly of cytochrome c oxidase, leading to impairment of oxidative phosphorylation, reduced ATP production, and sensitizes the cell to apoptosis (132, 206). RNAi knockdown of COX4 in nematodes has also been shown to reduce the lifespan, fecundity, respiration, and electron transport activity (243). These findings are particularly interesting given the results from our
previous studies and further implicates mitochondrial dysfunction in the cardiac phenotype.

In the LV, SMAD protein signaling was a significantly upregulated process associated with SMAD4 and BMP4 genes. Similar results were found using the ClueGo plugin in Cytoscape, suggesting that increased SMAD-BMP signaling could be important in contributing to the dysfunction observed in these hearts. BMPs are part of the TGF-β superfamily of proteins, accounting for about two-thirds of the known members. BMPs signal through SMAD dependent and independent pathways. SMAD dependent pathways converge at the SMAD4 protein as a common mediator of BMP signaling that regulates target gene transcription (90). Although BMP4 and SMAD4 are essential for cardiac development, enhanced expression has been implicated as a major factor in pathological remodeling of the myocardium (109, 196, 165). BMP signaling is highly conserved and glucocorticoid receptor (GR) knockdown reduces the expression of several BMPs, including BMP4, in zebrafish embryos, (175, 174). In contrast, maternal treatment with the synthetic glucocorticoid dexamethasone upregulates the expression of BMP4 in the fetal mouse kidney (57). BMP4 is also upregulated in pathological cardiac hypertrophy, acute myocardial infarction, chronic ischemic heart disease, and cardiomyocyte apoptosis (240, 279, 188). Increased SMAD4 protein expression and collagen deposition occurs in the boarder and scar tissue following myocardial infarction (87). Increased collagen deposition is also associated with myocardial stiffness and impairment of diastolic and systolic function (64). Interestingly, the lambs from this study had impaired diastolic and systolic pressure in the hour before birth; however, the amount of collagen deposition following
myocardial infarction is greater than observed in the lambs of the present study (41). This suggests that excessive glucocorticoid signaling through the GR can potentiate BMP-SMAD signaling in the fetal heart, leading to increased cardiac collagen deposition, and impairing cardiac function.

The findings from this study indicate that chronically elevated maternal cortisol concentrations during late gestation can alter cardiac gene expression in pathways relating to metabolism and collagen deposition. The newborn CORT lambs of this cohort had significantly reduced heart rate and blood pressure in the hour before birth along with changes in their ECG, which may have been influenced by the changes in gene expression. Although it is unlikely that the cardiac dysfunction observed in these lambs is the direct result of changes to a single gene, changes in gene expression within multiple pathways likely plays a larger role in the cardiac dysfunction observed around birth. We speculate that impaired mitochondrial function alters cardiac metabolism and along with increased collagen deposition underlie the cardiac phenotype observed in these newborn lambs.
Figure 4-1. Volcano plots illustrating probes upregulated (green), downregulated (red), and unchanged (black) in newborn left ventricle of control and CORT fetuses. The x-axis describes the difference in expression between cortisol and control, changes in the positive direction being greater in cortisol than control. The y-axis is the negative log of the P value.
Figure 4-2. Representative images of picrosirius red staining using circular polarized light from control (A) and CORT fetuses (B). Quantification of stained area relative to tissue area (C). * Indicates statistical difference (p<0.05) between control and CORT fetuses.
Figure 4-3. Representative images of COX4 staining from control (A) and CORT fetuses (B). Quantification of stained area relative to tissue area (C). * Indicates statistical difference (p<0.05) between control and CORT fetuses.
Figure 4-4. Quantification of proteins related to the mitochondria and metabolism in the fetal septum from control fetuses (white bars) and CORT fetuses (black bars) by western blot analysis. The y-axis represents arbitrary fluorescence units (AFU). * Indicates statistical difference (p<0.05) between control and CORT fetuses.
Table 4-1. Top 10 enriched KEGG pathways, gene ontology biological processes, molecular functions, and cellular components that were significantly upregulated by CORT in the newborn lamb left ventricle.

<table>
<thead>
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<th>Analysis</th>
<th>Pathways, processes, functions, components</th>
<th>No. of Genes involved</th>
<th>Adjusted P values</th>
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</thead>
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<td>Pathways in cancer</td>
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<td>Wnt signaling pathway</td>
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<td>6.00E-04</td>
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<td>Insulin signaling pathway</td>
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</tr>
<tr>
<td></td>
<td>TGF-beta signaling pathway</td>
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<td>3.70E-03</td>
</tr>
<tr>
<td></td>
<td>Protein processing in endoplasmic reticulum</td>
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<td>4.00E-03</td>
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<td></td>
<td>Melanogenesis</td>
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<td>Response to organic substance</td>
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<td>Positive regulation of metabolic process</td>
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<td>Negative regulation of programmed cell death</td>
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<td>Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen</td>
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<td>Glutathione peroxidase activity</td>
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<td><strong>Cellular Components</strong></td>
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<td>BMP receptor binding</td>
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<td>Unfolded protein binding</td>
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Table 4-2. Top 10 enriched KEGG pathways, gene ontology biological processes, molecular functions, and cellular components that were significantly downregulated by CORT in the newborn lamb left ventricle.

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<td>NOD-like receptor signaling pathway</td>
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<td>Circadian rhythm - mammal</td>
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<td>Collecting duct acid secretion</td>
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<td>Lysosome</td>
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<td>Glycerophospholipid metabolism</td>
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<td>Cysteine-type peptidase activity</td>
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<td>ATPase activity, coupled to transmembrane movement of substances</td>
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<td>ATPase activity, coupled to movement of substances</td>
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Table 4-3. Top 10 enriched KEGG pathways, gene ontology biological processes, molecular functions, and cellular components that were significantly upregulated by CORT in the newborn lamb septum.

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<td>Neurotrophin signaling pathway</td>
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<td>Phototransduction</td>
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<td>Oocyte meiosis</td>
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<td>Vascular smooth muscle contraction</td>
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<td>Starch and sucrose metabolism</td>
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<td>Striated muscle thin filament</td>
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Table 4-4. Top 10 enriched KEGG pathways, gene ontology biological processes, molecular functions, and cellular components that were significantly downregulated by CORT in the newborn lamb septum.

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<td>Progesterone-mediated oocyte maturation</td>
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<td>GnRH signaling pathway</td>
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<td>Leukocyte transendothelial migration</td>
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CHAPTER 5
INCREASED MATERNAL NIGHTTIME CORTISOL CONCENTRATIONS IN LATE GESTATION ALTER GLUCOSE AND INSULIN IN THE NEONATAL LAMB

Introduction

A growing body of clinical, experimental and epidemiological evidence suggests that chronic maternal stress during pregnancy is a major risk factor for poor obstetric outcomes (45, 51). The timing of prenatal exposure to maternal cortisol and psychosocial stress is associated with human infant cognitive development. Activation of the hypothalamo-pituitary-adrenal (HPA) axis by psychological or physiological stressors induces the synthesis and release of the glucocorticoid hormone cortisol. Cortisol’s actions include the transrepression and activation of genes involved in adaptive mechanisms to stress, such as the stimulation of glucose production and support of the cardiovascular system; however, prolonged or excessive exposure is associated with deleterious effects in cells and tissues (162). Throughout pregnancy maternal cortisol concentrations rise, producing a period of physiological hypercortisolemia. The increased maternal cortisol facilitates delivery of nutrients to the developing fetus (31). Placental 11β-hydroxysteroid-dehydrogenase 2 (11β-HSD-2) (19) acts as a partial buffer through conversion of cortisol to cortisone. As a consequence, it is estimated that about 2% of maternal cortisol crosses the placenta to the fetus; however, because of the differences in volume of distribution for cortisol, this represents nearly all of the fetal plasma cortisol concentration until very close to term when fetal adrenal production of cortisol increases (58, 91). Synthetic glucocorticoids are able to bypass this degradation, but maternal hypersecretion of cortisol also results in increased cortisol exposure to the fetus. Although the normal increase in fetal cortisol production near term is essential for organ maturation (134), overexposure of the
developing fetus to maternal glucocorticoids has been proposed as a mechanism for fetal programming (163). Increased exposure to maternal glucocorticoids has primarily been studied in the context of antenatal glucocorticoid therapy, rather than increased maternal secretion of the endogenous adrenal corticosteroid, cortisol. In humans and in most animal species, glucocorticoid exposure increases the incidence of decreased body weights at birth or produces small for gestational age offspring (13, 21, 110, 176, 202). Antenatal glucocorticoid therapy also alters HPA responses in the newborn (259) and has been implicated in adverse cardiac and renal growth (22).

Little is known about effects of increased maternal cortisol secretion near term on early postnatal growth and metabolism. In order to investigate the effects of chronic exposure to increased maternal cortisol on neonatal growth, metabolism and organ maturation, we developed a model of chronic stress during pregnancy in which the ewe is infused with cortisol for twelve hours overnight beginning at gestational day 115 (term is ~145 days). In previous studies, we had found that chronic continuous infusion of cortisol to the ewe resulted in chronic increase in maternal glucose, but also resulted in an increased incidence of peripartum fetal death (117). Therefore, a model using 12h of cortisol infusion, producing half the daily total dose, but similar increase in nighttime plasma cortisol concentrations, was used in the present study. This method mimics conditions similar to those seen in patients with Cushing’s syndrome and chronic depression, in which there is a loss of the normal circadian rhythm of cortisol, resulting in elevated nighttime cortisol concentrations (127, 198) (215). We hypothesized that increased cortisol exposure in utero and withdrawal from the excess exposure at birth would result in an impaired HPA reactivity in the neonate and decreased neonatal
growth, with impaired glucose metabolism in the early postnatal period.

**Materials and Methods**

Two groups of black-faced ewes and their lambs were studied. All animals were housed in a facility with temperature and light (lights on 0700 to 1900) and temperature controlled rooms throughout the study period; all animal use was approved by the University of Florida Institutional Animal Care and Use Committee. Ewes were fed a diet of pelleted feed according to NRC standards for the ewe’s body weight and gestation; feeding was further supplemented with alfalfa hay beginning at day 140 of gestation. Ewes were assigned to one of two groups of ewes at 115 days: a control group (n= 7; 6 singleton and 1 twin pregnancies) with no infusion, and a group of ewes treated with cortisol (0.5mg/kg/d infused from 2100 to 0900, infusion rate 1.4 ml/h, n=8; 7 singleton and 1 twin pregnancies). The two groups of ewe had similar weights at surgery (control: 92 kg ± 5; cortisol: 82 kg ± 5) and body condition scores at term (control: 3.2 ± 0.1; cortisol 2.9 ± 0.1).

At approximately day 115 (±1) of gestation, surgery was performed under isoflurane anesthesia. A flow probe (6mm 6PSS; Transonics Inc., Ithaca, NY) was placed on the main uterine artery. Catheters were also placed in both maternal femoral arteries and veins; the uterus was not opened and the fetuses were not instrumented. Ewes were treated at the end of surgery and for two days postoperatively with flunixin meglamine (1mg/kg; Merck Animal Health, Germany) as an analgesic, and were treated with antibiotic (Polyflex, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) for 3-5 days postoperatively. Rectal temperature was measured twice a day for 5 days.

A programmable timer was used to control the infusion pump (Chronotrol, ChronTrol Corporation, San Diego, CA). This pattern of cortisol infusion was designed
to mimic the effect of Cushing’s disease in humans, which results in higher than normal evening concentrations of cortisol. Although sheep do not have a circadian rhythm in cortisol (20), the activity associated with normal husbandry and feeding, which occurs in the morning after lights on, would be expected to increase morning cortisol concentrations in the ewes, and since most ewes finish the feed before the timing of feeding (just after lights on) this would also be expected to increase maternal cortisol near the time of refeeding each morning (225). The timing of the cortisol infusion was designed to allow adequate time after lights on to allow for collection of blood sampling and measurement of uterine blood flow while the infusion is still ongoing.

Blood samples (8 ml) were collected on approximately days 125, 130, 135, 140 days gestation and on day 145 if the lamb had not yet been delivered. Blood samples were analyzed for maternal electrolytes (Roche Electrolyte analyzer 9180), glucose and lactate (YSI Model 2700 glucose/lactate analyzer, Yellow Springs, OH), cortisol (Siemens Coat-a Count kit; Los Angeles, CA) and insulin (Alpco ovine insulin kit, Alpco Diagnostics, Salem, NH) as previously described (68). After the blood sample was collected, uterine blood flow was measured for at least 30 minutes using an ambulatory Bluetooth based acquisition system (Transonics Physiogear; Transonics Inc., Ithaca, NY). A glucose tolerance test was also performed in each ewe at approximately day 133 of gestation (0.4 g/kg iv) as previously described (68).

In both groups one ewe was euthanized in labor due to dystocia, and two lambs were stillborn in the cortisol-exposed group. One set of twins was born in each group of ewes. A total of 4 male and 4 female lambs were born to 7 control ewes at 145±1 days (range 144-149 days) gestation, and 4 male and 5 female lambs were born to 8 cortisol-
infused ewes at 145±1 days (range 142-146 days) gestation.

The lambs were sampled after birth (after the ewe had licked off the lamb and the lamb was standing; control: 1.4 ± 0.5 hours; cortisol: 1.6 ± 0.5 hours, and subsequently at approximately 12, 24, 36, 48 hours after birth and on postnatal day 3, 6, 9, 12 and 14 for measurement of plasma cortisol, insulin, glucose, and lactate concentrations and packed cell volume. Plasma electrolyte, plasma protein and hematocrit were also measured at birth and days 3, 6, 9, 12 and 14 after birth. Plasma ACTH (20) and plasma renin activity (274) were measured on days 0, 3, 6, 9, 12 and 14 after birth; aldosterone (Aldosterone EIA 501090; Cayman Chemical, Ann Arbor, MI) was measured in samples collected on days 0, 3, 6 and 14 days and plasma creatinine (Calorimetric Assay kit 700460; Cayman Chemical) was measured in samples collected on days 0, 3, 6, 9 and 14 days; all samples in the study were analyzed at the same time to eliminate between assay variability. The sample near the time of birth was collected by percutaneous venipuncture of the jugular; subsequently an indwelling venous catheter (Intracath, 17g needle and 19G catheter; The Deseret Company, Sandy, UT) was placed and used for sampling and for injections of glucose or insulin. Body weight and temperature was also measured daily. On postnatal day 3-5 and on day 10-12 glucose tolerance tests (0.25 g/kg) were performed; blood samples were collected at -5, 0, 2, 5, 10, 20, 30, 40, 50, 60 and 90 minutes after injection of glucose to determine the plasma insulin response to increases in plasma glucose. On postnatal day 5-8 an insulin tolerance test (0.25U/kg) was performed; samples were collected at -5, 0, 5, 10, 15, 20, 30, 40, 50, 60 and 90 minutes after insulin injection to determine the plasma ACTH and cortisol response to decreased plasma glucose concentration. Plasma
glucose and lactate concentrations were measured in all samples collected after both the glucose or insulin tolerance test.

Crown to rump and hock to hoof length of the rear legs were measured on days 0, 7 and 14. On day 14-15 after birth, ewes and their lambs were killed (Euthasol; Fort Worth, TX) and measurements of the weights of the heart, liver, adrenal, lung, kidneys, perirenal fat, pancreas, and brain were performed.

**Statistical Analyses.** Between group differences in plasma hormone, glucose, lactate and electrolyte concentrations over age in late gestation ewes, postpartum ewes or postnatal lambs were compared by 2-way analysis of variance (ANOVA) corrected for repeated measures across time (SPSS, IBM Corp., Armonk, NY). The effect of the sex of the lamb on these variables was analyzed using 3 way ANOVA, also corrected for repeated measures over time. Responses to glucose in the pregnant ewes or to insulin or glucose injection in the postnatal lambs were also analyzed by two way ANOVA. The maternal glucose responses were also fit to a 5 parameter fit \( y=y_0+a(\text{exp} \cdot b t)+c(\text{exp} \cdot d t) \) and the neonatal glucose responses were fit to a 4 parameter fit \( y= a(\text{exp} \cdot b t)+c(\text{exp} \cdot d t) \) as the samples in the lamb were only collected until 90 minutes in order to minimize the total sampled volume and therefore the estimates of baseline glucose at the end of the test were not possible to fit. The parameters were compared between the 2 groups by Student’s t test. Differences in tissue weights between the two groups of lambs were also analyzed by t-tests.

**Results**

**Effects of Cortisol Exposure During Pregnancy on the Pregnant and Postpartum Ewe**

As expected, the infusion of cortisol increased plasma cortisol concentrations
during the infusion period in the treated ewes (Figure 5-1 A). Plasma glucose concentrations were not significantly increased in the ewes during the infusion of cortisol, except at the first-time point (120d, after 5 days of cortisol infusion; Figure 5-1 B). There were no effects of cortisol on plasma insulin, sodium, potassium, protein, or lactate concentrations, or packed cell volume (PCV) (Figure 5-1 C; Figure 5-2 A, B, D, E; lactate not shown). PCV significantly decreased with gestational age (Figure 5-2 E). Plasma Ca++ concentrations were significantly increased in the cortisol infused group at days 130-140 of gestation (Figure 5-2 C). During the maternal glucose tolerance test, although there was no overall treatment effect, there was a significant effect of cortisol administration on both the glucose and insulin responses over time (Figure 5-3 A and D). When the glucose response was fit to a 5-parameter fit (y = y0 + a*exp-bt +c*exp-dt), the a parameter was significantly greater in the cortisol-treated ewes, indicating that the glucose distribution volume is reduced in the cortisol-treated ewes (Table 5-1).

There were no differences in maternal cortisol, glucose or insulin after delivery, when the cortisol infusion was terminated (Figure 5-1 D-F). However, the plasma potassium concentration was significantly higher in the postpartum ewes that had previously received the overnight cortisol infusion (Figure 5-2 G). Plasma Ca++ significantly decreased after delivery; however, this decrease over time was attenuated in ewes that had previously been treated with cortisol during pregnancy (Figure 5-2 H). Plasma Na+, protein and PCV were not significantly different postpartum in ewes that had previously received overnight cortisol treatment as compared to those that were untreated during pregnancy (Figure 5-2 F, I, J). Plasma glucose, lactate, and PCV decreased and plasma protein increased after delivery in both groups (Figures 5-1 E
Effects of Maternal Cortisol on Neonatal Growth and Organ Weights

Maternal cortisol infusion also did not alter body temperature or body weight at birth, or the change in body weight or temperature over the first 14d of life (Figure 5-5); there were no significant effects of sex of the lamb on either of these variables. Ponderal index was reduced overall in the lambs of cortisol-treated ewes (Figure 5-5), although this effect of time was restricted to the male fetuses. There was a significant effect of sex on the change in ponderal index with postnatal age. In male lambs the ponderal index at birth was reduced in the cortisol group, and in the females the ponderal index was significantly reduced in the cortisol group at 14d.

At postnatal day 14, organ weights were similar in the two groups of lambs (Table 5-), with the exception of average kidney weight per g body weight, which was significantly lower in the lambs after previous maternal cortisol infusion. Although the relative heart weight to body weight ratio was not different between groups, the left ventricular wall thickness was increased in the lambs exposed to higher in utero cortisol concentrations (control: 6.5±0.3 mm; cortisol: 7.4±0.3 mm), and this effect was also significant when the wall thickness was corrected for tibial length (Figure 5-5). In contrast, right ventricular free wall thickness (control: 3.5±0.3 mm; cortisol: 3.8±0.1 mm) and septal thicknesses (control: 7.5±0.5 mm; cortisol: 7.9±0.3 mm) were not significantly increased in these lambs. There was no overall effect of sex of the lamb on any of the organ weights or measures of cardiac size.

Effects of Maternal Cortisol on Neonatal Cortisol, ACTH, Glucose and Insulin Metabolism

Plasma concentrations of ACTH, cortisol, glucose, and lactate were all highest in
the samples collected at birth in both groups of lambs (Table 5-3; Figure 5-1, G and H, lactate data not shown). There was no overall effect of treatment on the neonatal cortisol concentration. However, both plasma glucose and plasma insulin concentrations were altered by the in utero maternal cortisol treatment. Overall glucose was significantly increased, and plasma insulin was significantly decreased, after maternal cortisol infusion (Figure 5-1, H and I). This resulted in a significantly reduced insulin to glucose ratio in the neonates of cortisol-treated ewes over the course of the study (control: 48±13; cortisol: 25±6 pmol/mmol).

In both glucose challenge studies, the plasma glucose concentration was similar between the groups of lambs (Figure 5-3 B and C); however, there was a significant effect of maternal cortisol on the glucose response to glucose injection at 10-12 days of age, when the second glucose challenge test was performed (Figure 5-3 C). Parameter a in the glucose disappearance curve was also significantly higher in the cortisol treated lambs during both glucose tolerance tests (Table 5-1); this reflects decreased glucose distribution volume in the offspring of the cortisol-treated ewes. The insulin response to glucose injection at either age was not significantly affected overall by the maternal cortisol infusion (Figure 5-3 E and F), although in male lambs in the cortisol group, plasma insulin concentrations at 40-90 minutes were significantly lower than in the male lambs in the control group during the glucose challenge at 10-12 days of age. There was no significant difference between the sexes in the insulin response in either the control or cortisol treatment groups.

As a test of HPA axis responsiveness, the ACTH and cortisol responses to a standard insulin tolerance test of 0.25 U insulin/kg body weight were determined. As the
lambs were not fasted prior to insulin injection, the glucose response to insulin was relatively small; the overall glucose response was not significantly different between the two groups of lambs (p=0.059), nor was the overall area under the curve for plasma glucose after injection of insulin different between groups. However, the cortisol exposed lambs had significantly lower plasma glucose concentrations at 15 and 20 minutes after the injection of insulin (Figure 5-4 A), suggesting that insulin sensitivity is increased in the offspring of the cortisol-treated ewes. The ACTH and cortisol responses to this mild hypoglycemic challenge were not different between the groups of lambs, though the response was highly variable among the individual lambs within each group (Figure 5-4 B and C).

**Effects of Maternal Cortisol on Neonatal Measures of Renal Function**

In both groups of lambs there were significant effects of postnatal age on plasma Na⁺, K⁺, and packed cell volume, with the highest concentrations in the sample collected shortly after birth (Figure 5-2 K,L,O); conversely, plasma protein and plasma Ca²⁺ significantly increased with postnatal age (Figure 5-2 M and N). Plasma Na⁺, Ca²⁺ and protein were also significantly altered by maternal cortisol infusions, with greater plasma Na⁺ and lower plasma Ca²⁺ and plasma protein concentrations in the cortisol-exposed lambs (Figure 5-2 K,M,N). The decrease in packed cell volume was also greater overall in the cortisol-exposed lambs (Figure 5-2 O). As a result of these changes, we further assessed volume regulation by measuring plasma aldosterone, renin activity and plasma creatinine concentrations (Table 5-3). In both groups of lambs, plasma renin activity and plasma aldosterone were highest at birth and significantly decreased over time after birth. Plasma aldosterone concentrations also were not different. In both groups of lambs, plasma creatinine concentrations were
higher at birth than on postnatal days 3-14. There was no overall effect of cortisol exposure, although plasma creatinine concentrations were significantly higher in the cortisol-exposed lambs on day 14. There was no effect of sex on the effect of cortisol on creatinine, although female lambs in the control group had significantly higher plasma creatinine at birth than female lambs of cortisol-treated ewes, and overall males in the control group had higher plasma creatinine concentrations than females in the control group.

**Discussion**

The results of this study indicate that a modest increase in maternal cortisol concentrations each night during late gestation raises maternal plasma cortisol and alters glucose and insulin regulation in the neonate. This occurs without a chronic increase in maternal glucose or insulin. The overnight increase in maternal cortisol concentrations also appears to alter both left ventricular wall thickness and renal size in the two-week-old lamb, suggesting there could be longer term effects on both metabolic and cardiovascular health as the neonate matures.

The overnight increase in maternal plasma cortisol had a minor effect on maternal glucose and insulin as compared to our previous study in which cortisol was continuously infused in late gestation (117). Continuous maternal cortisol infusion resulted in chronically elevated maternal glucose and insulin concentrations, and slowed glucose disappearance after administration of a glucose bolus intravenously. In this model, there was a dramatic increase in fetal/neonatal death in the peripartal period. Overnight infusion of cortisol, administering half the daily total dose of cortisol, also resulted in two incidences of unexplained fetal death at term, suggesting that effects on maternal glucose metabolism and on fetal metabolism/survival are cortisol-
The endogenous glucocorticoid, cortisol, has been found to reduce the growth of the axial skeleton in fetal sheep, and the prepartum cortisol surge has been suggested to be responsible for the normal decline in the rate of growth towards the end of gestation (75). Higher doses of glucocorticoids administered to the mother have been found to reduce growth (110), as does maternal Cushing’s syndrome (139). We hypothesized that chronic maternal cortisol treatment might mimic the effect of Cushing’s syndrome and reduce fetal growth rate; however, this hypothesis was disproved because there was no effect of the maternal hypercortisolemia on body weight at birth or on rate of growth in terms of either neonatal girth or body weight. In a previous study of constant maternal cortisol infusion, the rate of increase in fetal girth was slowed, but the current study suggests that this effect requires either a higher total dose, or constantly increased concentrations throughout the day in order for there to be decreased skeletal growth. Indeed, the change in plasma cortisol expected in the fetus as a result of this infusion of cortisol to the ewe is small relative to the normal increase in cortisol that occurs during the parturient surge in fetal cortisol production. Instead, we found that there was a significant overall effect of cortisol on ponderal index. In particular, ponderal index of the cortisol group was reduced in the males at birth and in the females at day 14. These results indicate that the male and female lambs of the cortisol group are shorter relative to their weight at birth and day 14 respectively, and suggest that body composition may be altered. Although the weight of the perirenal fat pad was not altered, the contribution of subcutaneous and mesenteric fat increases postnatally, and may contribute to differences in body composition. However, fetal
cortisol appears to have few stimulatory effects in promoting white adipose tissue growth at birth (168), although cortisol alters the metabolic function of adipocytes in the adult. In the fetal sheep, an intact adrenal is necessary for the late gestational rise in brown adipose tissue activity, and cortisol regulates the concentration of brown adipose tissue-specific UCP1, the mitochondrial protein involved in thermogenesis, allowing for maintenance of core body temperature after birth (168). The infusion of cortisol in the ewes did not depress the normally high cortisol concentrations in the lambs at birth, and newborn body temperatures were also not altered by the maternal infusion of cortisol.

Although we had hypothesized that increased maternal cortisol concentrations would produce a relative hypoadrenal state in the neonate, there were no significant effects on postnatal ACTH or cortisol concentrations. The maintenance of low fetal cortisol concentrations throughout pregnancy is important for the normal developmental progression of fetal organs, especially the fetal adrenal (277). Prior to the surge in cortisol at birth, the fetal HPA is sensitive to feedback signals from increased maternal cortisol (276), though at the time of birth the HPA appears to be resistant to cortisol-induced suppression of ACTH (273). In humans, antenatal glucocorticoid therapy appears to reduce the HPA responses in the newborn. Infants exposed to antenatal betamethasone treatment had suppressed HPA responses to stressors commonly encountered in the neonatal intensive care unit (52). Effects of prenatal betamethasone exposure on regulation of stress physiology in healthy premature infants (218). In this study, the 12h daily infusions of cortisol did not impact adrenal maturation or responsiveness, indicating that higher maternal glucocorticoid concentrations are required for the chronic suppression of adrenal maturation.
Although differences in adrenocortical activity in the adult are known to impact glucose homeostasis, the impact of excess in utero glucocorticoids on neonatal glucose metabolism is not well characterized. In spite of similar postnatal cortisol concentrations in the two groups of lambs, neonatal plasma insulin concentrations were significantly decreased with a concomitant increase in neonatal plasma glucose concentrations, resulting in decreased insulin to glucose ratio. As compared to their mothers, the neonatal glucose concentrations were relatively high by 3 days of age, and in the control group the plasma insulin concentrations also higher than in the ewes; in contrast the lambs of cortisol-treated ewes have relatively low insulin concentrations relative to the postnatal glucose concentrations. The active isoform of GR is expressed in humans around the time of islet formation and could modulate development of the pancreas, reducing the number of available β cells later in life (190). Although islet formation begins on gestational day 24 in the fetal sheep (42), islet remodeling continues until after birth in the sheep (at least 10 days of age), as well as in the mouse (to 2-3 weeks of age) and human (to 4-6 months of age) (85, 115, 251). In vitro studies have demonstrated that glucocorticoids not only reduce islet mass, by decreasing β-cell proliferation and increasing apoptosis, but also reduce insulin content and secretion (199, 265). In the rat, there is a negative correlation between fetal corticosterone concentrations and insulin content, supporting the idea that glucocorticoids have a negative effect on β-cell development (24). In fact, antenatal glucocorticoid exposure significantly reduces the HOMA-B score in young adults, which is driven by higher glucose concentrations but lower insulin concentrations (119). It is not possible to test for fasting concentrations of glucose or insulin in the lambs without also increasing
plasma cortisol concentrations, as fasting would require that they be separated from the ewes. The results of the glucose tolerance tests suggest that glucose distribution volume may be decreased in the lambs of cortisol-infused ewes, indicated by the higher a parameter in the glucose disappearance curve (72±13 in control lambs vs 104±8 in cortisol lambs 5-7 days after birth; 85±4 in control lambs vs 106±3 in cortisol lambs 10-12 days after birth), which may reflect reduced glucose uptake into tissues. This may also contribute to the small increase in the basal plasma glucose concentrations in these lambs. The insulin responses to the glucose challenges were not different between the groups of neonates. This indicates that the pancreatic response to glucose is not impaired, despite reduced basal values of plasma insulin in the lambs and reduced insulin to glucose ratio at normal glucose concentrations. As these lambs have ready access to milk and are therefore likely to be in a postprandial state, the lower insulin concentrations pre-glucose challenge suggest that there are decreases in the action of other insulin-stimulatory mechanisms such as gut hormones or increased inhibition by alpha-adrenergic activity. The changes observed in the lambs from this study suggest that there could be long-term consequences to the increase in cortisol during late gestation on glucose disposal and on islet responses to basal, although not hyperglycemia-stimulated, insulin secretion. These effects could contribute to the reduced ponderal index, and may influence long-term nutrient storage.

Neonates from cortisol-exposed ewes had thicker left ventricles of the heart. The normal increase in fetal glucocorticoid production by the ovine and human fetus normally coincides with the maturation of the heart, as cardiomyocytes transition from mono-nuclear proliferative cells to bi-nucleated terminally differentiated tissue (74, 111).
Late gestation may therefore be a particularly vulnerable period for the fetal heart. Excessive or premature exposure of the fetus to glucocorticoids, either synthetic or naturally occurring, is often associated with alterations in fetal heart development (210). Moreover, obstructive hypertrophic cardiomyopathy, with increased thickening of the interventricular septum and left ventricle, has been reported in the newborn of a mother with Cushing’s syndrome (67). In previous studies in the fetus, shorter-term chronic maternal cortisol infusion increased the thickness of all three walls of the preterm fetal heart without a significant increase in fetal blood pressure (108, 201), although longer-term infusion until birth did not significantly increase wall thickness later in gestation (117). Higher doses of glucocorticoids increase fetal blood pressure, resulting in hypertrophy of the left ventricle (142, 247). As the focus of the present study was metabolic alterations in the newborns, we did not measure blood pressure postnatally in these neonates, and therefore we do not know what role increased blood pressure might play in the wall thickness of these hearts.

The newborns of cortisol-infused ewes had smaller kidneys, and increased plasma sodium concentration. At necropsy plasma creatinine concentrations were also slightly higher in this group, suggesting that there may be physiologic effects, and possibly impaired renal function, that would be evident later in life. Nephrogenesis in the sheep is complete at about 130 days of gestation (271) and glucocorticoid exposure prior to the completion of nephrogenesis in the rat or sheep reduces postnatal nephron number (187, 226, 271). Glucocorticoids also promote renal tubule cellular differentiation, accelerating functional development and altering the renal sodium handling (228). Treatment of the pregnant dam with dexamethasone from day 1 of
Pregnancy until parturition produces pups with reduced kidney weight, glomerular filtration rate, urinary sodium excretion rate and fractional sodium excretion (34). Similarly, antenatal betamethasone treatment reduces GFR and increases sodium uptake in male, but not female, renal proximal tubule cells, likely through suppression of nitric oxide (238, 245). The natural rise in fetal glucocorticoids is associated with an increase in the activity of tubular sodium/hydrogen exchanger (NHE) and (Na\(^+\)K\(^+\)) ATPase during the transition from fetal to neonatal life (35, 84). Perinatal dexamethasone administered to the pregnant rat programs an increase in the expression of the Na\(^+\)K\(^+\)2Cl\(^-\) (NKCC2) and Na\(^+\)Cl\(^-\) cotransporters in the renal cortex at 2 months, NHE3 in the proximal tubule at 7-8 weeks, and the α1 subunit of (Na\(^+\)K\(^+\)) ATPase from whole kidney at 6 months (48, 49, 280). Thus, the increased in utero exposure to cortisol in this ovine model may have reduced renal maturation, but increased expression of renal sodium transporters, increasing sodium retention in the neonates.

A limitation to this study is that the effects of cortisol on mammary gland characteristics and milk production were not evaluated. Glucocorticoids are essential in establishing the necessary structural components for milk synthesis and secretion, regulating the gene expression of the milk proteins, and prolonging lactation by delaying mammary involution (33). Since the lambs in this study were reared on ewes, there exists the possibility that milk production and content could have been altered in the ewes exposed to the cortisol treatment, leading to altered neonatal growth and nutrient state. However, as maternal plasma cortisol concentrations were not different in the postpartum period, we do not anticipate differences in milk composition.
Figure 5-1. Plasma cortisol (A, D, G), glucose (B, E, H) and insulin (C, F, I) concentrations in studies in control ewes (white circles) or ewes that were infused with cortisol during pregnancy (0.5 mg·kg⁻¹·day⁻¹ for 12h) (black circles). Values are shown for ewes during pregnancy (A-C), or in the postpartum period (D-F) and for their postnatal lambs (G-I). **Indicates a significant overall effect of maternal cortisol treatment. *Indicates values significantly different between groups at the indicated time point.
Figure 5-2. Plasma sodium (A, F, K), potassium (B, G, L), calcium (C, H, M) protein (D, I, N), and packed cell volume (PCV) (E, J, O) concentrations in studies in control ewes (white circles) or ewes that were infused with cortisol during pregnancy (0.5 mg·kg^{-1}·day^{-1} for 12h) (black circles). Values are shown for ewes during pregnancy (A-E) or in the postpartum period (F-J), and for their lambs during the postnatal period (K-O). **Indicates a significant overall effect of maternal cortisol treatment.
Figure 5-3. Plasma glucose (A-C) and plasma insulin (D-F) concentrations in response to intravenous injection of 0.40 g glucose/kg (Maternal GTT; A, D) in control ewes (white circles) or ewes infused overnight with cortisol (0.5 mg·kg⁻¹·day⁻¹) (black circles) and in response to intravenous injection of 0.25 g glucose/kg in their postnatal lambs at 5-7 days of age (Neonatal GTT1; B, E) or 10-12 days of age (Neonatal GTT2; C, F).
Figure 5-4. Plasma glucose (A), ACTH (B), and cortisol (C) concentrations in response to intravenous injection of 0.25 U insulin/kg in lambs of control ewes (white circles) and cortisol-infused ewes (black). *Indicates values significantly different between groups at this time point.
<table>
<thead>
<tr>
<th>Age</th>
<th>Body Weight (g)**</th>
<th>Crown to Rump (cm)**</th>
<th>Ponderal Index (g/cm³)**</th>
<th>Body Temperature (°F)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>cortisol</td>
<td>control</td>
<td>cortisol</td>
</tr>
<tr>
<td></td>
<td>(all)</td>
<td></td>
<td>(all)</td>
<td></td>
</tr>
<tr>
<td>birth</td>
<td>5178±589</td>
<td>5471±290</td>
<td>54.2±1.6</td>
<td>56.6±1.8</td>
</tr>
<tr>
<td>D3</td>
<td>6165±615</td>
<td>6383±383</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>D6</td>
<td>7181±593</td>
<td>7371±454</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>D7</td>
<td>7580±631</td>
<td>7781±501</td>
<td>62.6±3.3</td>
<td>66.5±17</td>
</tr>
<tr>
<td>D9</td>
<td>8180±614</td>
<td>8481±525</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>D12</td>
<td>9106±666</td>
<td>9436±578</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>D14</td>
<td>9690±702</td>
<td>10185±618</td>
<td>71.3±1.9</td>
<td>75.1±1.6</td>
</tr>
</tbody>
</table>

Figure 5-5. Postnatal measures of growth and body temperature in control and cortisol exposed lambs

*Indicates significant effect of maternal treatment with cortisol; ** indicates significant effect of postnatal age
Table 5-1. Glucose tolerance tests: results of 5 parameter fit (ewes) or 4 parameter fit (lambs) of glucose disappearance curve from 2 minutes to 180 min (ewe) or 2-90 min (lamb).

<table>
<thead>
<tr>
<th></th>
<th>Pregnant Ewes 5 parameter fit: $y = y_0 + (a \cdot e^{b-t}) + (c \cdot e^{d-t})$</th>
<th>Lamb 4 parameter fit: $y = + (a \cdot e^{b-t}) + (c \cdot e^{d-t})$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>cortisol</td>
</tr>
<tr>
<td>y0</td>
<td>40.4±4.4</td>
<td>42.1±6.2</td>
</tr>
<tr>
<td>a</td>
<td>112±9</td>
<td>150±9*</td>
</tr>
<tr>
<td>b</td>
<td>0.294±0.075</td>
<td>0.438±0.127</td>
</tr>
<tr>
<td>c</td>
<td>235±15</td>
<td>220±9</td>
</tr>
<tr>
<td>d</td>
<td>0.0205±0.0035</td>
<td>0.0173±0.0025</td>
</tr>
</tbody>
</table>

*indicates significant difference in values between cortisol and control group (p<0.05, one-tailed Student t test)
Table 5-2. Relative organ weights and cardiac wall thicknesses at necropsy in control and cortisol exposed lambs.

<table>
<thead>
<tr>
<th>Organ Weight / Cardiac Wall Thickness</th>
<th>Control</th>
<th>Cortisol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain/body weight (x 10^2)</td>
<td>0.691±0.044</td>
<td>0.662±0.042</td>
</tr>
<tr>
<td>Lung/body weight (x 10^2)</td>
<td>2.34±0.15</td>
<td>1.95±0.16</td>
</tr>
<tr>
<td>Liver/body weight (x 10^2)</td>
<td>2.85±0.13</td>
<td>2.60±0.08</td>
</tr>
<tr>
<td>Kidney/body weight (x 10^2)</td>
<td>0.325±0.019</td>
<td>0.259±0.009*</td>
</tr>
<tr>
<td>Adrenal/body weight (x 10^5)</td>
<td>5.95±0.77</td>
<td>5.12±0.33</td>
</tr>
<tr>
<td>Perirenal adipose/body weight (x 10^2)</td>
<td>0.429±0.057</td>
<td>0.353±0.032</td>
</tr>
<tr>
<td>Heart/body weight (x 10^2)</td>
<td>0.614±0.021</td>
<td>0.634±0.025</td>
</tr>
<tr>
<td>Left ventricular free wall/tibia length</td>
<td>0.445±0.017</td>
<td>0.496±0.019*</td>
</tr>
<tr>
<td>Septum wall thickness/tibial length</td>
<td>0.512±0.030</td>
<td>0.530±0.021</td>
</tr>
<tr>
<td>Right ventricular free wall thickness/tibial length</td>
<td>0.235±0.018</td>
<td>0.253±0.010</td>
</tr>
</tbody>
</table>

*indicates values significantly different between lambs of cortisol-infused ewes as compared to control lambs (p<0.05).
Table 5-3. Postnatal values of plasma ACTH, renin activity, aldosterone and creatinine concentrations.

<table>
<thead>
<tr>
<th></th>
<th>Plasma ACTH (pg/ml)**</th>
<th>Plasma Renin Activity (ng/ml/h)**</th>
<th>Plasma Aldosterone (pg/ml)**</th>
<th>Plasma Creatinine (mg/100 ml)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control cortisol</td>
<td>control cortisol</td>
<td>control cortisol</td>
<td>control cortisol</td>
</tr>
<tr>
<td>Birth</td>
<td>279±23 304±41</td>
<td>19.8±4.2 10.8±2.5</td>
<td>239±45 233±36</td>
<td>3.7±0.6 2.7±0.3</td>
</tr>
<tr>
<td>D3</td>
<td>158±34 162±23</td>
<td>16.5±4.4 12.2±3.5</td>
<td>204±86 123±20</td>
<td>1.1±0.1 1.1±0.1</td>
</tr>
<tr>
<td>D6</td>
<td>158±33 246±37</td>
<td>16.6±5.9 10.1±2.6</td>
<td>174±95 110±12</td>
<td>0.9±0.1 1.2±0.2</td>
</tr>
<tr>
<td>D9</td>
<td>136±24 208±53</td>
<td>12.7±5.2 10.5±3.3</td>
<td>--- ---</td>
<td>1.1±0.2 1.4±0.2</td>
</tr>
<tr>
<td>D12</td>
<td>158±22 175±27</td>
<td>11.8±4.5 9.6±3.7</td>
<td>--- ---</td>
<td>--- ---</td>
</tr>
<tr>
<td>D14</td>
<td>128±14 173±22</td>
<td>12.2±4.1 6.7±2.7</td>
<td>80±14 96±13</td>
<td>1.0±0.1 1.4±0.2 *</td>
</tr>
</tbody>
</table>

*Indicates significantly different between groups at this time point; ** indicates significant effect of postnatal age (p<0.05)
CHAPTER 6
CONCLUSIONS

The fetal ECG (fECG) was first measured over a century ago using a galvanometric apparatus (46). Since then, there have been significant advancements in its detection and processing that have improved the feasibility and implementation in clinical practice. It is now becoming routine in many parts of the world as a perinatal diagnostic tool that clinicians use as a surrogate for the health status of the fetus. Although this has greatly increased fetal surveillance in human pregnancy, particularly during labor and delivery, obstacles continue to exist that complicate the acquisition, filtering, and processing of the fECG. For instance, the low fECG signal (measured through electrodes placed on the maternal abdomen) is masked by the greater maternal ECG, electromyogram, and utero electromyogram, creating a low signal-to-noise ratio (SNR). Recently, there has been clinical interest in producing novel methods that improve acquiring and processing the fECG from maternal background noise, especially for evaluation of the fECG prior to the perinatal period (121).

Magnetocardiography and magnetic resonance imaging techniques have shown promise for identifying and quantifying changes in the magnetic fields produced by electrical activity of the fetal heart; however, these techniques are not practical for long-term study in humans and large animals or for routine evaluation of the fECG prior to labor and delivery (130). Therefore, there are limited clinical tools available to researchers interested in long-term studies of the fECG in utero. Previous attempts to measure the fECG in ovine models of pregnancy required methods that involved catheterization and physical tethering of the leads, often restricting the movement of the ewe: experimental paradigms that may disturb the physiological control of fetal heart
rate. Thus, developing a method that uses implantable radiotelemetry devices to mitigate these restrictions could give large animal researchers the ability to measure the fECG and fetal pressure in long-term studies. The implantation of similar devices has been attempted before in the late gestation ovine fetus, though with limited success and without characterizing cardiac parameters (92, 2).

The method and findings described from chapter 2 demonstrate that the use of implanted radiotelemetry devices is a powerful tool that can be used in the long-term uninterrupted acquisition, processing, and analysis of fECG and aortic pressures in the chronically instrumented “normal” late gestation ovine fetus. This methodology allows the study of the ovine fetus without maternal restraint, as required by other methods (26), and therefore with minimal stress to the ewe. Using the data collected from the radiotelemetry devices, we characterized the fetal HR, MAP, and fECG following surgery, during late gestation, and in the immediate perinatal period. These results will help establish baseline measures of ECG parameters in the late gestation ovine fetus, as well as characterize changes in HR and MAP in the fetuses of unrestrained and freely moving ewes. Except for the changes following surgery and during labor, there were minimal deviations in the parameters of the fECG in late gestation, suggesting that the fetal cardiac conduction system is mature by 115 days of gestation. In fact, there were only 11 genes related to voltage gated ion channels, ligand gated ion channels, or other ion channels that associated with development between 0.90 of gestation and postnatal day 14, which were mainly driven by postnatal maturation (data from (204)).

Fetal adrenal production of cortisol begins at approximately 80% of gestation in both humans and sheep (153, 74, 17) as part of a tightly regulated process that is
integral to tissue maturation and ex utero survival (134). During this last 20% of gestation the ovine fetal heart undergoes a critical period of cardiac maturation as cardiomyocytes transition from primarily mononucleated proliferative cells to binucleated terminally differentiated tissue (111), suggesting that cardiac tissue may be susceptible to remodeling by either early, elevated, or prolonged glucocorticoid exposure during this time. Other studies from the lab (201), others (67), and the results from the studies contained here have shown that increases in maternal cortisol concentration increase the thickness of the fetal ventricular free walls and interventricular septum indicating that these areas are particularly vulnerable to glucocorticoid overexposure during this period. Previous findings found an increase in the incidence of perinatal stillbirth in CORT pregnancies, an increase in apoptosis in Purkinje fibers of fetal cardiac conduction system, and alterations in the pattern of cardiac gene expression relating to metabolism and mitochondria (68, 117). These findings prompted the use of the method developed in chapter 2 to evaluate the adverse effects of chronically elevated maternal cortisol concentrations on the fetal MAP, HR, and cardiac conduction system.

The results from chapter 3 add to our knowledge of how early and persistent glucocorticoid signaling can detrimentally impact the fetal heart. These findings further support the idea that the cardiac conduction system of the fetus is vulnerable, particularly in the immediate perinatal period. Although CORT fetuses had similar cardiac function during late gestation, it appears that exposure to CORT predisposes the fetal heart to greater bradycardia in labor as well as a lower HR immediately after birth. Furthermore, it appears that pathways involved in atrial and atrioventricular conduction are most affected by CORT and manifest primarily in the immediate
perinatal period. The relevance of this is important considering the increase in perinatal
death occurring around the same time in another cohort of fetuses, which implicates
that alterations in these conduction pathways as contributing factors (117).
Nevertheless, it is clear that glucocorticoid actions on cardiac function, while essential,
are dose dependent, with adverse effects at high or prolonged elevated concentrations.
Similar results have been observed in the adult hippocampus, with corticosterone
having protective effects at low doses and adverse effects at high doses (47, 4).
This biphasic relationship also holds true for the effects of cortisol on the developing heart,
with effects that could contribute to the increase in stillbirth rates associated with chronic
maternal stress in late pregnancy (144, 272).
In other fetal studies, glucocorticoids have been shown to significantly increase fetal
blood pressure (216, 56, 142), as well as increase the increase in arterial pressure,
bradycardia and acidemia during a hypoxic episode (126, 72, 103).

Although the cardiac dysfunction developed primarily around the time birth, it is
likely that the changes in gene expression and proteins occurred earlier. The fetuses of
the CORT group were able to maintain cardiac function under basal conditions in late
gestation; however, the physiological hurdles inherent to labor and delivery were likely
the driving forces behind the cardiac dysfunction. Systems modeling of genes
differentially regulated by CORT indicated that pathways relating to metabolism, the cell
cycle, and fibrosis were most affected. Further analysis suggested that the physiological
changes observed in these lambs around birth are due to changes in mitochondrial
function and increased collagen deposition in the fetal heart. This further implicates the
cardiac mitochondria as being key factors in the pathology, along with the findings from
our previous studies (205). Studies have shown that altered glucocorticoid signaling can negatively affect mitochondrial function (61), alter the ultrastructure of the heart and metabolic gene expression (208), and impair cardiac conduction and function (211). Others have also shown that mitochondrial dysfunction increases ROS production and cardiac fibrosis, which can be alleviated by antioxidant therapy (131). Taken together, it appears that CORT induces programming events within the cardiac tissue, targeting the mitochondria and increasing cardiac fibrosis, yet a “second hit” is required to induce cardiac dysfunction. The process of parturition likely represents the “second hit” as the fetus is subjected to various physiological stressors including cardiovascular challenges as well as a further increase in cortisol concentrations during this process. Under normal circumstances, these challenges and stressors occurring are likely beneficial to the fetal heart, acting to bolster the mechanisms and systems involved in maintaining cardiac function; however, these adaptive mechanisms appear to be impaired by chronic stimulation, limiting the adaptive capability of the fetal heart in this critical period.

Changes in the intrauterine environment have been suggested as a mechanism for programming the offspring to develop diseases that manifest later in life (12). The changes observed in the neonates of chapter 5 could represent permanent modifications in the mechanisms governing neonatal growth, glucose metabolism, and sodium regulation due to steroid-induced in utero effects. However, what is not clear yet, is whether these changes alter the ability to respond to other stimuli and contribute to the development of adult onset diseases. Additional studies that focus on cardiac function, growth, and glucose metabolism will be useful in evaluating the effects of CORT later in adolescence and adulthood.
At present, utilization of radiotelemetry devices (similar to those used in these studies) for monitoring peripartal cardiac function in the human fetus is not possible for clinical practice; however, devices that utilize a scalp electrode are currently available and aid in monitoring fetal status during the peripartal period. Although the devices used in the current studies were effective in acquiring data from the fetal lamb in late gestation and in the peripartal period, devices that are smaller, more energy efficient, can be monitored remotely, and utilize technology that reduces signal loss will improve feasibility for investigators in the future.

Overall, the findings from the studies described in this thesis will advance the overall knowledge of the mechanisms behind, and the consequences of, excessive glucocorticoid exposure to the fetal heart. The results from these studies could be useful in clinical practice as measurements for fetal monitoring, particularly during the peripartal period, and especially in patients that have had stressful pregnancies. Ultimately, understanding these mechanisms and outcomes will be important for designing treatment strategies for affected fetuses, neonates, and adults.
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BIOGRAPHICAL SKETCH

Andrew Antolic was born in 1987 in White Plains, New York. He grew up in Lake Mary, Florida where his passion for biological sciences began while attending Lake Mary High School. Afterwards, he enrolled at the Florida State University and later transferred to the University of Florida. He moved to Saint Louis, Missouri where he attained a master’s degree from Saint Louis University. In 2012 Andrew started his Ph.D. in the Department of Pharmacodynamics in the College of Pharmacy at the University of Florida. He received his Ph.D. from the University of Florida in the summer of 2017 and moved to Tucson, Arizona where he began his postdoctoral training under the guidance of Dr. Sean Limesand.