The Impact of Prenatal Glucocorticoid Exposure on the Ovine Kidney

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To my darling husband Nigel and mother Pamela,

Thank you both for your love and support throughout this journey.

"The length of your education is less important than its breadth, the length of your life is less important than its depth."

- Marilyn vos Savant

ABSTRACT

In obstetric practice, pregnant women at risk of pre-term delivery between 24 and 34 weeks of gestation are administered synthetic glucocorticoids (betamethasone or dexamethasone) to induce fetal organ maturation. During this gestational period, the fetal kidney is undergoing a phase of rapid organogenesis with an increase in renal growth and active nephrogenesis occurring. The studies comprising this thesis examine the effects of prenatal betamethasone exposure on the fetal and adult ovine kidney. The central hypothesis of these studies was that exposure of the fetal kidney to betamethasone in late gestation would change renal structure and induce long-term alterations in the expression of glucocorticoid-sensitive genes and proteins.

In the fetal studies, pregnant Merino ewes bearing single fetuses received single or repeated-weekly intra-muscular (i.m.) injections of betamethasone (0.5 mg/kg body weight) or saline commencing on day 104 of gestation (term is 150 days). Kidneys were collected from fetuses at 109, 116, 121 and 146 days of gestation (d). Using gold standard unbiased stereological techniques, the physical disector/fractionator method, total glomerular (nephron) number and glomerular volume were determined in 146 d fetal kidneys exposed to repeated maternal saline or betamethasone administration. In the adult study, kidneys were collected from 3.5-year-old sheep that had been exposed to maternal or direct fetal injections of saline or betamethasone in late gestation. Real-time RT-PCR and Western blot analyses were used to determine mRNA and protein levels; immunohistochemistry was used to localise immuno-reactive (IR) glucocorticoid receptor (GR) protein in the kidney.

Maternal betamethasone administration had variable effects on fetal growth. Overall, nephron number was not different between control and betamethasone-exposed fetuses. However, nephron number and total glomerular volume were significantly reduced in fetuses exposed to betamethasone with renal growth restriction. There was no effect of sex on nephron number or kidney size in term fetal sheep. Maternal betamethasone administration did not alter the expression of renal GR, mineralocorticoid receptor (MR), 11β-hydroxysteroid dehydrogenase-2 (11β-HSD-2), sodium/potassium adenosine triphosphatase-α_1 (Na^+/K^+-ATPase-α_1) or angiotensin II receptor type 1 or 2 (AT_1, AT_2

receptor) mRNA in the fetal or adult kidney. Renin mRNA expression was increased in the fetal kidney at 116 and 121 d. There was no difference in the location or intensity of GR-IR cells between groups in the fetal or adult kidneys. Further, there was no effect of prenatal treatment on renal GR protein levels at any age.

This is the first study to examine the effect of late-gestation betamethasone exposure on nephron endowment in fetal sheep. In this thesis I have demonstrated that renal growth restriction as a result of betamethasone exposure is associated with a reduction in fetal nephron endowment. Although betamethasone does not appear to consistently alter nephron number or glomerular size, it may indirectly affect total nephron endowment through effects on renal growth. I have also provided evidence which suggests that late-gestation betamethasone exposure in sheep does not program permanent alterations in the renal expression of genes or proteins involved in glucocorticoid hormone action or components of the renin-angiotensin system. Therefore, exposure of the fetal kidney to betamethasone during nephrogenesis may alter renal structure if kidney growth is perturbed; however, there are no persistent alterations in the expression of glucocorticoid-sensitive genes. These findings are consistent with the preservation of normal basal blood pressure in the adult sheep I studied and with the limited results from human studies of late-gestation maternal glucocorticoid administration.

ABSTRACTS AND AWARDS ARISING FROM THIS THESIS

ABSTRACTS

MEYER, A. J., MORITZ, K. M., SLOBODA, D. M., MOSS, T. J. M., WADDELL, B. J. & NEWNHAM, J. P. (2005). The impact of maternal betamethasone administration on the fetal ovine kidney. *Pediatr Res* **58,** 1023.

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JOHNSTON, A. J., SLOBODA, D. M., MOSS, T. J. M., WADDELL, B. J. & NEWNHAM, J. P. (2002). Antenatal betamethasone treatments increase glucocorticoid receptor and sodium-potassium-adenosine triphosphatase-alpha-1 protein in the kidneys of adult offspring in sheep. In *Proceedings of the Australian Health and Medical Research Congress 2002*, Melbourne, VIC, Australia.

AWARDS

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The University of Western Australia Pro Vice Chancellor, Research, Innovation and Training Prize awarded for oral presentation at The Australian Society for Medical Research 4th Annual Medical Research Symposium, Perth, WA, Australia.

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DECLARATION

I, Amanda Jane Meyer, declare that this thesis is less than 100,000 words in length, exclusive of references. Except where due acknowledgement has been made, this thesis comprises original work that I have performed during my PhD candidature at The University of Western Australia. To the best of my knowledge and belief, this thesis contains no material that has been previously written or published by any other person, except where due reference is given in the text. No part of this thesis has been submitted to any institution for the award of any other degree or diploma.

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Amanda Jane Meyer

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ABBREVIATIONS

♀	female
♂	male
-/-	knock-out gene
° C	degrees Celsius
α	alpha
β	beta
µg	microgram
µL	microlitre
11β-HSD	11-beta-hydroxysteroid dehydrogenase
11β-HSD-1	11-beta-hydroxysteroid dehydrogenase type 1
11β-HSD-2	11-beta-hydroxysteroid dehydrogenase type 2
a(p)	area per grid point
ACE	angiotensin-converting enzyme
ACTH	adrenocorticotrophic hormone
ACTORDS	Australasian Collaborative Trial of Repeated Doses of Corticosteroids for the Prevention of Neonatal Respiratory Disease
AngII	angiotensin II
ANOVA	analysis of variance
A_o	angiotensinogen
AOD	arbitrary optical density
AP-1	activating protein-1
AT_1	angiotensin II type 1
AT_1R	angiotensin II type 1 receptor
AT_2	angiotensin II type 2
ATP	adenosine triphosphate
AVP	arginine vasopressin
BSA	bovine serum albumin
bv	blood vessel
cap	capsule
CBC	corticosteroid-binding capacity
CBG	corticosteroid-binding globulin
cd	collecting duct
cDNA	complementary deoxyribonucleic acid
CIHR	Canadian Institutes of Health Research
CRH	corticotrophin-releasing hormone
cRNA	complementary ribonucleic acid
C_T	cycle of threshold fluorescence
CV	co-efficient of variation
d	days of pregnancy/gestation
DAB	3,3'-diaminobenzidine
dct	distal convoluted tubule
DEPC	diethyl pyrocarbonate

dH_2O	distilled water
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
F1	fetal injection of one dose of betamethasone
F4	fetal injection of four doses of betamethasone
FGF-2	fibroblast growth factor-2
FS	fetal injection saline
FSA	filtration surface area
g	gram
GDNF	glial cell line-derived neurotrophic factor
GFR	glomerular filtration rate
glom	glomerulus
GR	glucocorticoid receptor
GRE	glucocorticoid response element
h	hour
H&E	haematoxylin and eosin
H_2O_2	hydrogen peroxide
hGR	human glucocorticoid receptor
HPA	hypothalamic-pituitary-adrenal
HPD	hypothalamo-pituitary disconnection
HRP	horseradish peroxidase
hsps	heat shock proteins
IgG	immunoglobulin G
i.m.	intra-muscular
i.p.	intra-peritoneal
i.v.	intra-venous
IR	immuno-reactive
IUGR	intra-uterine growth restriction
JGA	juxta-glomerular apparatus
KCl	potassium chloride
kDa	kilo Dalton
kg	kilogram
KH_2PO_4	potassium phosphate, monobasic
L	litre
LIF	leukaemia inhibitory factor
M1	maternal injection of one dose of betamethasone
M4	maternal injection of four doses of betamethasone
M	molar (moles/litre)

MACS	Multiple Courses of Antenatal Corticosteroids for Preterm Birth Study
MAP	mean arterial pressure
mg	milligram
MHz	mega Hertz
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
mmHg	millimeter of mercury
MPA	medroxyprogesterone acetate
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
MS	maternal injection of saline
mtal	medullary thick ascending limb
NaCl	sodium chloride
$NADP^+$	oxidized form of nicotinamide adenine dinucleotide phosphate
Na_2HPO_4	sodium phosphate, dibasic
Na^+/K^+-ATPase	sodium/potassium-adenosine triphosphatase
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
N_{glom}	number of glomeruli
NGS	normal goat serum
nm	nanometer
NICHD MFMU	National Institute of Child Health and Human Development Maternal Fetal Medicine Units
P-$450_{17\alpha}$	17α-hydroxylase
p	probability value
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with 0.1% v/v Tween® 20
PCO_2	partial pressure of carbon dioxide
PCR	polymerase chain reaction
pct	proximal convoluted tubules
P_f	fraction of section area used for counting glomeruli
P_{glom}	number of points overlaying glomeruli
P_{kid}	number of points overlaying kidney tissue
PO_2	partial pressure of oxygen
P_s	sum of grid points that overlaid kidney tissue
PVDF	polyvinylidene difluoride
Q-	actual number of glomeruli counted
R^2	co-efficient of determination
r.p.m.	revolutions per minute
RAS	renin-angiotensin system
RDS	respiratory distress syndrome
RNA	ribonucleic acid
RNase	ribonuclease
RPA	ribonuclease protection assay

RT-PCR	reverse transcription-polymerase chain reaction
s	seconds
SCC	side chain cleavage enzyme ($P\text{-}450_{scc}$)
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
S.E.M.	standard error of the mean
t	mean section thickness
TGF-α	transforming growth factor-alpha
TIMP-2	tissue inhibitor of metalloproteinase-2
tl	thin limb of the loop of Henle
UK	United Kingdom
UPE	umbilico-placental embolisation
US/USA	United States/United States of America
V	volt/s
v/v	volume per volume
V_{corp}	mean renal corpuscle volume
$V_{corp.tot}$	total renal corpuscle volume
V_{glom}	mean glomerular volume
$V_{glom.tot}$	total glomerular volume
V_{kid}	kidney volume
VLBW	very low birth weight
vr	vasa rectae
w/v	weight per volume

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1. Introduction

1.1. GENERAL INTRODUCTION

Increased fetal glucocorticoid exposure and/or low birth weight are associated with the development of adulthood diseases. In obstetric practice, women at risk of pre-term delivery are treated with antenatal glucocorticoids, exposing the fetus to increased levels of glucocorticoids. Repetitive prenatal exposure to synthetic glucocorticoids has been shown to reduce birth weight in some human and animal studies. In addition, late-gestation administration of synthetic glucocorticoids to pregnant rats induces hypertension in adult offspring. Concerns were raised regarding the effect of antenatal glucocorticoid therapy in humans after a study by Doyle *et al*. (2000) reported that blood pressure was increased in adolescents exposed to synthetic glucocorticoids *in utero*.

Impaired renal development, including changes in kidney structure and/or function, is associated with elevated blood pressure. Alterations in kidney structure, such as reduced nephron endowment, are associated with increased blood pressure in a number of animal models. Further, increased fetal glucocorticoid exposure has been shown to reduce nephron endowment in sheep and rats. In obstetric practice, antenatal glucocorticoid therapy is administered to women at risk of pre-term delivery at a time when the fetal kidney is undergoing nephrogenesis. Therefore, antenatal glucocorticoid therapy may reduce nephron endowment in humans that could predispose individuals to chronic renal disease and hypertension in adulthood.

A reduction in nephron endowment may contribute to, but is not the sole cause of, the development of hypertension in adult life. Other changes in renal development (i.e. glucocorticoid sensitivity and expression of the renin-angiotensin system) may be programmed by glucocorticoids during prenatal life and contribute to alterations in renal function and adult disease. Hence, it is important to examine the effects of prenatal glucocorticoid exposure on the fetal and adult kidney. Dissection of the molecular pathways involved in glucocorticoid-mediated programming of the kidney may provide new insights into the pathophysiology and control of disease.

The effects of prenatal glucocorticoid therapy on the human kidney are unknown. In the studies presented in this thesis, pregnant sheep were administered a synthetic

glucocorticoid in late gestation to mimic antenatal glucocorticoid therapy used in obstetric practice. The central hypothesis of this thesis was that exposure of the fetal kidney to synthetic glucocorticoids would change renal structure and cause long-term alterations in the expression of glucocorticoid-sensitive genes and proteins.

In overview, this literature review will first discuss the structure and function of the kidney. Next, sections examining glucocorticoids and their effects on the kidney will be presented. This chapter will then review prenatal glucocorticoid administration in human and animal studies, followed by the developmental origins of health and disease. To conclude, the summary and aims of this thesis will be presented.

1.2. THE KIDNEYS

The kidneys are paired organs that are essential for the maintenance of normal volume and composition of the body fluids. Renal tissue can be separated into two regions: an outer *cortex* and an inner *medulla*. The cortex contains the glomeruli, proximal and distal convoluted tubules, cortical collecting ducts and the peritubular capillaries of the nephrons (Figure 1.1). The straight portions of the proximal (pars recta) and distal (thick ascending limb) tubules, loops of Henle, vasa recta and terminal collecting ducts are located in the renal medulla (Figure 1.1).

Each kidney contains approximately one million nephrons, which are the functional units responsible for regulating the excretory function of the organ. Nephrons can be classified according to the position of their glomeruli in the cortex (superficial, mid-cortical and juxta-medullary) or the lengths of their loops of Henle (cortical loops, short loops and long loops) (Jamison & Kriz, 1982). In rabbit, rat and monkey kidneys, juxta-medullary glomeruli are larger than those in the outer cortical zone (Bankir & Rouffignac, 1976; Artacho-Perula *et al.*, 1993; Skov *et al.*, 1999). The classification of nephrons according to their glomerular position does not coincide with the length of their loops of Henle (Jamison & Kriz, 1982). Cortical loops of Henle descend to variable depths in the medullary rays of the cortex but do not penetrate the renal medulla (Jamison, 1987). Short loops of Henle are found in the kidneys of humans and pigs and form their bend within the outer medulla, whereas long loops infiltrate the inner medulla (Jamison & Kriz, 1982). The extension of the tubules into the medulla is

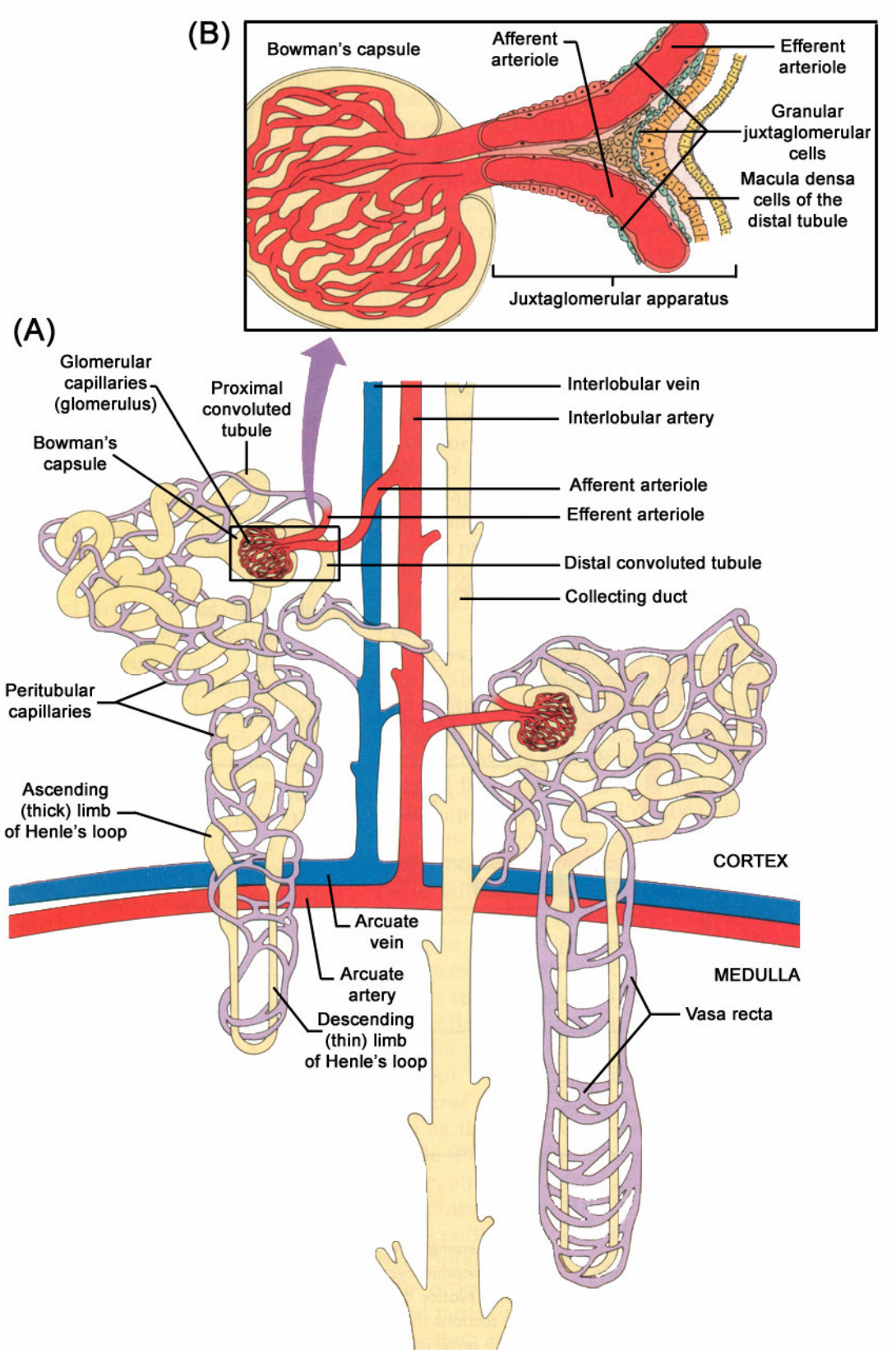


Figure 1.1. Anatomical structure of renal nephrons and their vasculature.

(A) Anatomy of cortical and juxta-medullary nephrons. (B) Juxta-glomerular apparatus of a nephron. Figure adapted from Marieb (1998).

part of the urinary concentrating mechanism, where the permeability of each region of the tubule differs in order to regulate the composition of the tubular fluid that will be excreted by the kidneys as urine (Jamison & Kriz, 1982). The ability of the kidneys to concentrate urine is closely related to the length of the thin loops of Henle and collecting ducts (Schmidt-Nielsen & O'Dell, 1961). The maximal length of the loop of Henle, and hence the maximal concentrating ability of the kidney, is directly related to the total thickness of the medulla (Beuchat, 1990). Nephron heterogeneity is thought to be the consequence of an evolutionary trend to conserve water; the concentrating system is most efficient with complementary short- and long-looped nephrons (Bankir *et al.*, 1987).

1.2.1. Renal structure

Each nephron consists of two major structures: a *glomerulus* and its associated *tubule*. The glomerulus consists of the glomerular tuft of capillaries, which is surrounded by Bowman's capsule. Blood enters each glomerulus via the *afferent arteriole*, which branches from the interlobular arteries that deliver the blood supply to the renal cortex. Podocytes line the visceral layer of the glomerulus and divide into foot processes, which filter the fluid that passes from the glomerular capillaries into *Bowman's space* (the space that exists between the glomerular tuft and the capsule). Approximately 20% of the water in plasma (along with ions and other small solutes) entering the afferent arteriole filters out of the glomerular capillaries into Bowman's space (Best & Taylor, 1985). The remaining 80% of plasma (along with larger molecules such as proteins, lipids and red blood cells) flows from the glomerular capillaries into the *efferent arteriole* that leaves the glomerulus (Best & Taylor, 1985). The efferent arteriole then disperses into a network of *peritubular capillaries* that surround the renal tubules and empty into interlobular veins. The peritubular capillaries deliver substances for tubules to secrete into the tubular fluid and reabsorb water and solutes that are reclaimed by the tubule (Best & Taylor, 1985). In the deepest part of the renal cortex, the efferent arterioles of juxta-medullary nephrons branch into *vasa recta* that descend to varying depths in the medulla and form hair-pin shaped capillaries as they ascend back to the cortex.

Although the nephron is a continuous tubule, differences in histology and function along its length results in its segmentation into three main parts: the *proximal tubule*, the *loop of Henle* and the *distal tubule*. The proximal tubule is continuous with Bowman's space; therefore, the filtrate that passes into Bowman's space first enters the proximal tubule. The epithelial cells of the proximal tubule are cuboidal in shape, contain numerous mitochondria and are lined with densely packed microvilli that project into the tubular lumen, forming a brush border (Best & Taylor, 1985). These characteristics of proximal tubule cells are important for their role in reabsorption and secretion. Approximately two-thirds of the filtered water and sodium ions, and virtually all glucose, amino acids and vitamins, are reabsorbed in the proximal tubule (Best & Taylor, 1985).

The loop of Henle consists of a *descending limb* and an *ascending limb* (Jamison & Kriz, 1982). The thin descending limb of the loop of Henle consists of an epithelium of flat, squamous epithelial cells with a small number of short microvilli and few mitochondria (Best & Taylor, 1985). The thick ascending limb of the loop of Henle begins between the inner and outer zones of the medulla and is characterised by cuboidal epithelial cells that contain numerous mitochondria and lack a luminal brush border (Best & Taylor, 1985). Another 25% of filtered sodium and water is reabsorbed in the loop of Henle (Best & Taylor, 1985). The loop of Henle is the site of urine concentration; water can leave the descending limb of the loop of Henle but not the ascending limb.

The distal nephron is comprised of several anatomically and functionally distinct segments including: the *distal tubule*, *connecting tubule* and *collecting duct* (Jacobson, 1981). The distal tubule commences from the end of the cortical thick ascending limb and continues to the connecting tubule; its epithelial cells are cuboidal and contain numerous mitochondria, but lack a luminal brush border (Best & Taylor, 1985). The thick ascending limb returns to its original glomerulus and contacts at the vascular pole forming part of the juxta-glomerular apparatus. Reabsorption of the remaining 5% of sodium ions and 10% of water are fine-tuned in the distal nephron and collecting duct in accordance with the body's needs (Best & Taylor, 1985).

The juxta-glomerular apparatus (JGA) is a specialised structure located at the point of contact between the distal tubule and the vascular pole of the glomerulus. It is comprised of the macula densa, extra-glomerular mesangial cells and granular juxta-glomerular cells (Best & Taylor, 1985). The macula densa is a row of tightly packed cuboidal epithelial cells lining the distal tubule at the point of contact with the glomerulus. The extra-glomerular mesangial cells extend from the glomerular tuft into the triangular region bounded by the afferent arteriole, efferent arteriole and the macula densa of the distal tubule (Best & Taylor, 1985). The granular juxta-glomerular cells are located in the region of the extra-glomerular mesangial cells and in the walls of the adjacent afferent and efferent arterioles (Best & Taylor, 1985). These specialised smooth muscle cells contain renin, which is released in response to a reduced sodium load at the macula densa, to cause vascular constriction of the arterioles via the renin-angiotensin system. This *tubulo-glomerular* feedback regulates glomerular blood flow and filtration rates in response to physiological demand.

1.2.2. Renal development

Ontogenesis of the vertebrate kidney involves the development of three spatially and temporally different organs: the *pronephros*, *mesonephros* and the *metanephros* (Figure 1.2). In humans and many other animals, pronephric tubules develop and fuse to form the anterior portion of the pronephric duct (Davies, 1951). External glomeruli develop by budding from the coelomic epithelium and are vascularised by vessels from the dorsal aorta (Davies, 1951). The sheep does not develop a pronephros as such; instead a giant glomerulus containing fused glomeruli develops in the area from the 7^{th} to 14^{th} somites, which in many other species is considered a pronephric area (Davies, 1951).

As illustrated in Figure 1.2, the mesonephros grows caudally to the regressing pronephros and exists during a similar period of gestation in humans and sheep: 10 to 40% in humans and 11 to 38% (day 17 to 57 of gestation; term is 150 days) in sheep (Wintour *et al.*, 1998; Moritz & Wintour, 1999). In humans, the mesonephros appears during the fourth week of gestation (term is 40 weeks), is fully developed by the eighth week, and then degenerates prior to the third month of gestation (de Martino & Zamboni, 1966). In male embryos, remnants of the mesonephros persist to form the efferent ductules and vasa deferentia of the testes (Saxen, 1987). In sheep, the

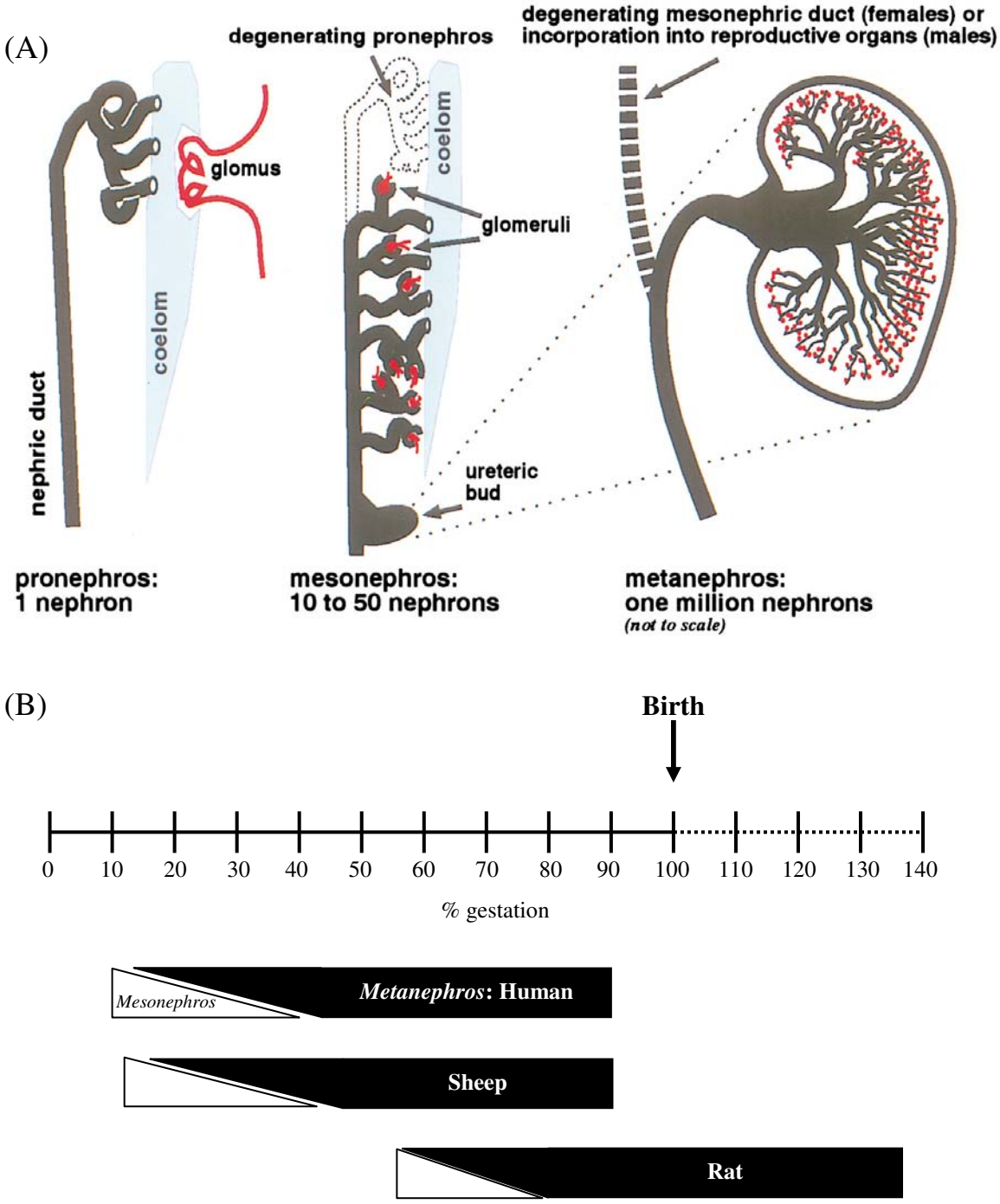


Figure 1.2. Renal development.

(A) Ontogenesis of the vertebrate kidney involves the development of three spatially and temporally different organs: the pronephros, mesonephros and the metanephros. Figure adapted from Vize *et al.* (1997). (B) Comparative development of the mesonephros (white shading) and metanephros (black shading) in humans, sheep and rats. The gestational time-line indicates the percentage of gestation when the organs are present during renal development. Data from Wintour *et al.* (1998).

mesonephros becomes functional at 16 to 18 days of gestation, producing hypotonic urine that drains into the allantoic sac (Davies, 1951). In contrast to humans and sheep, mesonephric development in rodents occurs much later in gestation. The mesonephros is present from 54 to 70% of gestation in mice and from 57 to 81% of gestation in rats (Wintour *et al.*, 1998). The number of mesonephric tubules and glomeruli varies between species from approximately 30 in humans to more than 50 glomeruli in sheep and pigs (Tiedemann, 1976). The mesonephric nephron lacks the loop of Henle and the JGA that are characteristic of the metanephros (Wintour *et al.*, 1999a).

The metanephros is the permanent form of the kidney and its development extends from 12 to 90% of gestation in humans, 18 to 90% of gestation in sheep and 57 to 138% of gestation in rats (Wintour *et al.*, 1998). Nephrogenesis proceeds in a centrifugal pattern, with the oldest, most mature glomeruli located in the deepest layer of the cortex adjacent to the medulla, and immature glomeruli situated in the nephrogenic zone within the subcapsular space (Lumbers, 1995a). Sixty percent of nephrons are formed in the last trimester of gestation in humans (Hinchliffe *et al.*, 1991) and sheep (Wintour *et al.*, 1999a). Nephrogenesis is completed prior to birth in humans and sheep, ceasing by approximately 36 weeks of gestation in humans (Harrison *et al.*, 1982; Hinchliffe *et al.*, 1991; Beckwith, 1992; Bissinger, 1995) and by day 130 of gestation in sheep (Robillard *et al.*, 1981). In contrast to humans and sheep, rats are mostly postnatal developers; they do not complete nephrogenesis until approximately one week after birth (Merlet-Benichou *et al.*, 1994; Ortiz *et al.*, 2001).

In infants born prior to 32 to 36 weeks' gestation, nephrogenesis continues into the postnatal period (Bissinger, 1995). Nephrogenesis ceases after 40 days of postnatal life in pre-term infants born between 24 and 30 weeks' gestation (Rodriguez *et al.*, 2004). At term, the kidneys of humans (Brion *et al.*, 1997) and sheep (A.J. Meyer, *unpublished observation*) have a combined weight of approximately 25 grams. Since the full complement of nephrons is attained prior to birth, the increase in kidney size in postnatal life is the result of elongation of the renal tubules and an increase in the amount of interstitial tissue (Moore & Persaud, 1993).

The successful completion of nephrogenesis requires programmed cell death (apoptosis), proliferation and differentiation to occur in a strategic fashion (Sorenson, 1998). A schematic illustration of nephrogenesis is shown in Figure 1.3. The process of metanephric development involves mutual induction of the ureteric bud and the metanephric mesenchyme. Glial cell line-derived neurotrophic factor (GDNF) secreted by the mesenchyme induces the mesonephric (Wolffian) duct to produce a ureteric bud, which invades the metanephric mesenchyme and branches to form the collecting ducts (Davies & Fisher, 2002). Repeated branching of the ureteric bud gives rise to numerous generations of collecting tubules. As illustrated in Figure 1.4, by 20 weeks of gestation, branching of the collecting duct is complete and one third of the complement of nephrons has been formed in the human fetus (Harrison *et al.*, 1982). The tips of the branching ureteric buds secrete signalling factors, such as leukaemia inhibitory factor (LIF), transforming growth factor-alpha (TGF-α), fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF) and tissue inhibitor of metalloproteinase-2 (TIMP-2), that prevent apoptosis and induce condensation of the metanephric mesenchyme (Perantoni *et al.*, 1995; Barasch *et al.*, 1997; Barasch *et al.*, 1999; Davies & Fisher, 2002). Following condensation, epithelial vesicles are formed, which elongate and differentiate to form comma-shaped and then S-shaped bodies (Saxen, 1987). The lower limb of the S-shaped body develops into the glomerulus whilst the upper limb forms the proximal and distal tubules. The S-shaped body then fuses with the ureteric (collecting) duct at the blind end of the distal tubule forming a continuous uriniferous tubule. Nephrogenesis continues with development of the glomerular tuft of capillaries and differentiation of the epithelial glomerular podocytes. Concurrently, the nephron tubule elongates and differentiates. Metanephric mesenchymal cells that are not induced to differentiate into nephrogenic epithelial cells undergo apoptosis (Araki *et al.*, 1999).

1.2.3. Renal function

The functional role of the fetal kidney is distinctively different to that of the adult. In adult humans and sheep, the kidneys receive 25% of cardiac output; whereas in the fetus, only 3% is distributed to the kidneys (Wintour & Moritz, 1997). During intra-uterine life, the majority of fetal arterial circulation bypasses the kidneys and is directed back to the placenta, which primarily regulates fetal fluid and electrolyte balance.

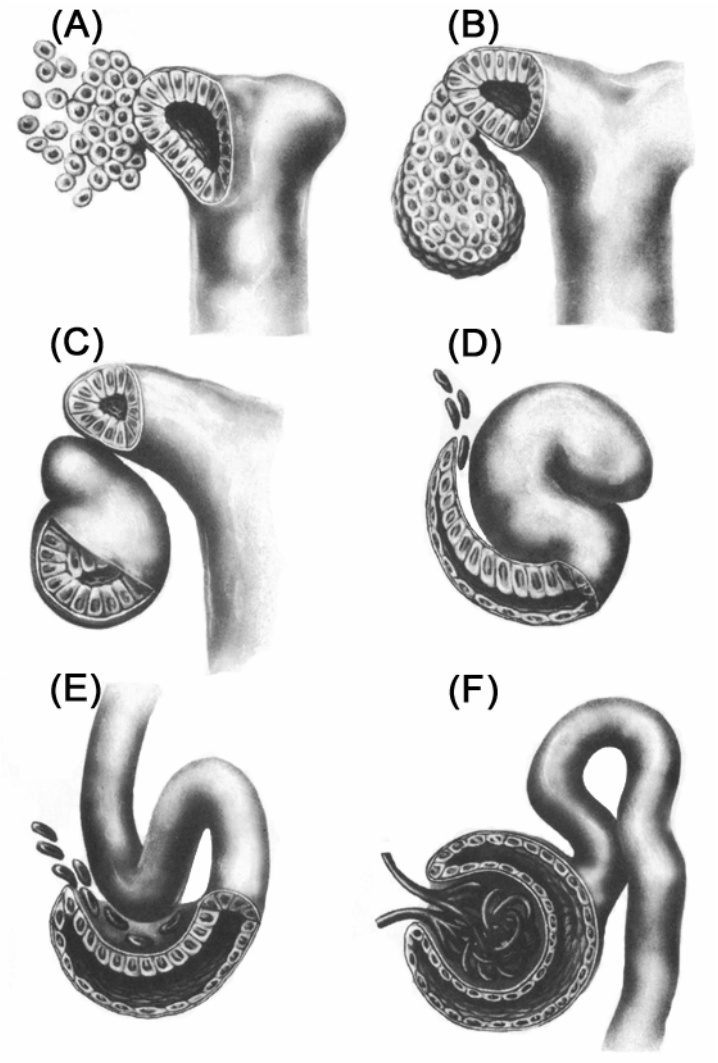


Figure 1.3. Schematic illustration of nephrogenesis.

(A) Induction of metanephric mesenchyme. (B) Condensation of mesenchymal cells. (C) Formation of the comma-shaped body. (D) Differentiation of the tubule into the S-shaped body. (E) Glomerulus develops from the lower limb of the S-shaped body. (F) Invasion of the glomerular tuft of capillaries and differentiation of the glomerular epithelium. Figure adapted from Saxen (1987).

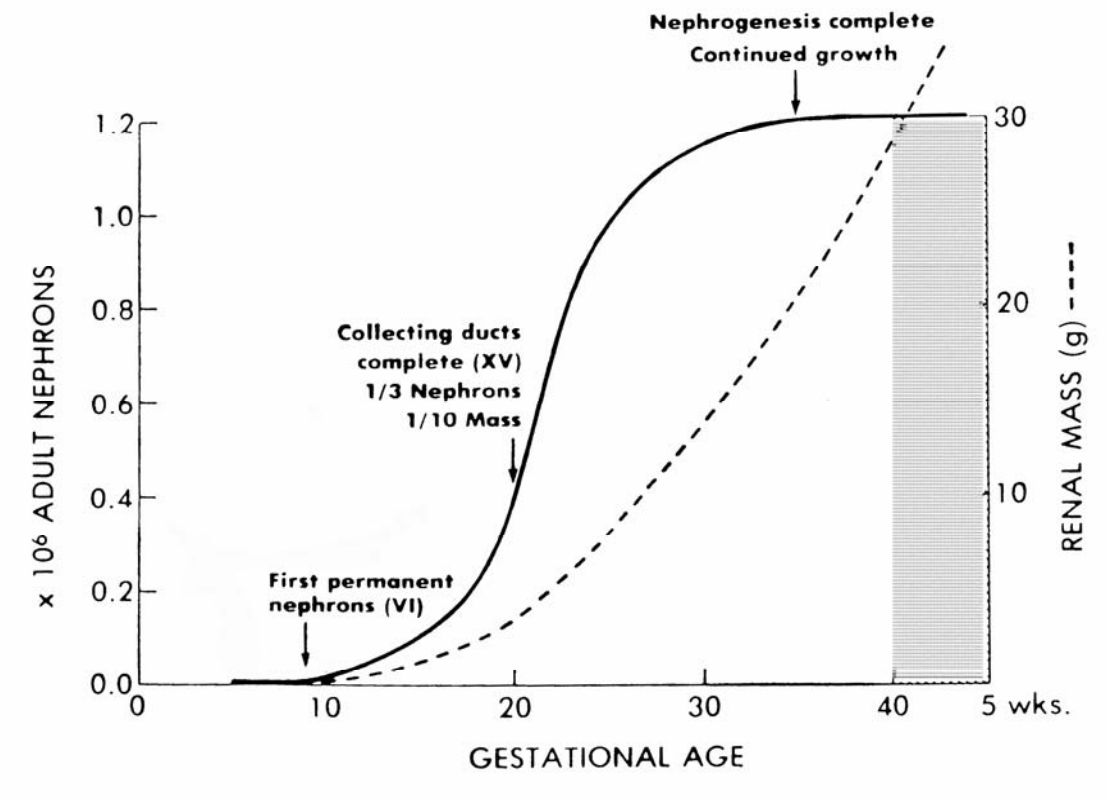


Figure 1.4. Schematic representation of the time course of renal development in humans. The branching of the collecting ducts (15 generations) is complete by 20 weeks of gestation; however the majority of nephrons and most of the functional mass of the cortex form after this time. Nephrogenesis is complete by 35 weeks of gestation, but renal mass increases in postnatal life (grey shading). Figure adapted from Harrison *et al.* (1982).

However, the fetal kidneys play an important role in maintaining fluid and electrolytes in the amniotic cavity. Large volumes of hypotonic urine are produced by the fetal kidney, which is essential for fetal well-being. Fetal urine is a major source of amniotic fluid in late gestation (Wlodek *et al.*, 1988) and urine flow rates in fetal humans and sheep are eight-fold higher in comparison to adults (Wintour & Moritz, 1997) to maintain adequate amniotic fluid and electrolyte levels. Bilateral renal agenesis results in oligohydramnios or anhydramnios because no urine is excreted into the amniotic cavity (Peipert & Donnenfeld, 1991; Vanderheyden *et al.*, 2003). Oligohydramnios and anhydramnios are associated with intra-uterine growth restriction, pulmonary hypoplasia and high rates of perinatal morbidity and mortality (Peipert & Donnenfeld, 1991; Vanderheyden *et al.*, 2003). Glomerular filtration rate (GFR) in the fetal kidney is lower than that of the adult, but the filtration fraction is relatively similar (Hill & Lumbers, 1988). The maintenance of GFR in late gestation is dependent on the operation of the renin-angiotensin system (Lumbers, 1995b).

1.2.3.1. Intra-renal renin-angiotensin system

In the adult, the renin-angiotensin system (RAS) is involved in renal sodium reabsorption through effects on tubular function, GFR and stimulation of aldosterone secretion (Lumbers, 1995a). In the fetus, the RAS is important for kidney development (Harris & Gomez, 1997; Guron & Friberg, 2000; Wolf, 2002; Woolf & Welham, 2002). All components of the system – renin, angiotensinogen, angiotensin-converting enzyme (ACE) and angiotensin II receptors – are present in the fetal kidneys from early in development in humans and sheep (Schutz *et al.*, 1996; Wintour *et al.*, 1996; Butkus *et al.*, 1997; Wintour *et al.*, 1998). GFR was reduced in fetal sheep at 119 to 128 days' gestation after the RAS was blocked using the ACE inhibitor - captopril (Lumbers *et al.*, 1993). In addition, intra-venous (i.v.) administration of an angiotensin II type-1 (AT_1) receptor antagonist reduced blood pressure in fetal sheep at 129 and 140 days of gestation (Forhead *et al.*, 2000a; Forhead & Fowden, 2004). Thus, during late gestation, the fetal RAS is essential for the maintenance of normal GFR and blood pressure (Lumbers, 1995b).

Renin is the initiating enzyme of the RAS cascade where angiotensinogen (A_o) is ultimately converted to angiotensin II (AngII; Figure 1.5). Renin is released from the

myoepithelial cells of the afferent arteriole in response to altered renal sympathetic nerve activity, reduced renal perfusion pressure and/or low sodium composition of the tubular fluid flowing past the macula densa cells (Dzau *et al.*, 1988; Hackenthal *et al.*, 1990). Renin is also produced by mesangial cells (Vidotti *et al.*, 2004) and proximal tubule cells (Moe *et al.*, 1993) in the rat kidney. Briefly, within the proximal tubule renin converts A_o to angiotensin I, which is subsequently cleaved by ACE to form AngII (Harris & Gomez, 1997). AngII binds to its receptors, AT_1 and AngII type-2 receptor (AT_2), to exert the physiological actions of the RAS.

In adults, AngII binds to AT_1 receptors in glomerulosa cells of the adrenal gland to stimulate the production and secretion of aldosterone (Boon *et al.*, 1997). *In vitro*, the ovine fetal adrenal gland is capable of producing and secreting aldosterone from as early as day 40 of gestation (Wintour *et al.*, 1975) and plasma aldosterone levels have been measured in fetal sheep from 60 days of gestation until term (Wintour *et al.*, 1975; Brown *et al.*, 1978). Unlike the adult adrenal, the mid-gestation fetal adrenal gland is unresponsive to AngII. An infusion of AngII (1 μg/h for 3 h or 36 h) into chronically-cannulated ovine fetuses at 75 to 85 days of gestation did not increase plasma aldosterone levels (Moritz *et al.*, 1998). It has been proposed that AngII binding to AT_2 receptors in the fetal adrenal may antagonise the steroidogenic actions of AngII on AT_1 receptors (Moritz *et al.*, 1999). Simultaneous infusion of an AT_2 receptor blocker (PD123319) and AngII significantly increased fetal plasma aldosterone levels in mid-gestation fetal sheep (Moritz *et al.*, 1999). Therefore, the reduction in adrenal AT_2 receptor levels in late gestation (Wintour *et al.*, 1999b) suggests that fetal sheep would become responsive to AngII infusions (Moritz *et al.*, 1999). A high dose infusion of AngII into fetal sheep at 112 to 131 days of gestation produced a modest increase in plasma aldosterone levels (Robillard *et al.*, 1982). AngII stimulation becomes more effective in causing aldosterone release as the fetus approaches term (Giry *et al.*, 1985). The primary function of AngII during late gestation is to regulate GFR and promote salt and water excretion from the fetal kidney, to maintain fluid balance and ensure normal growth and development (Lumbers, 1995b).

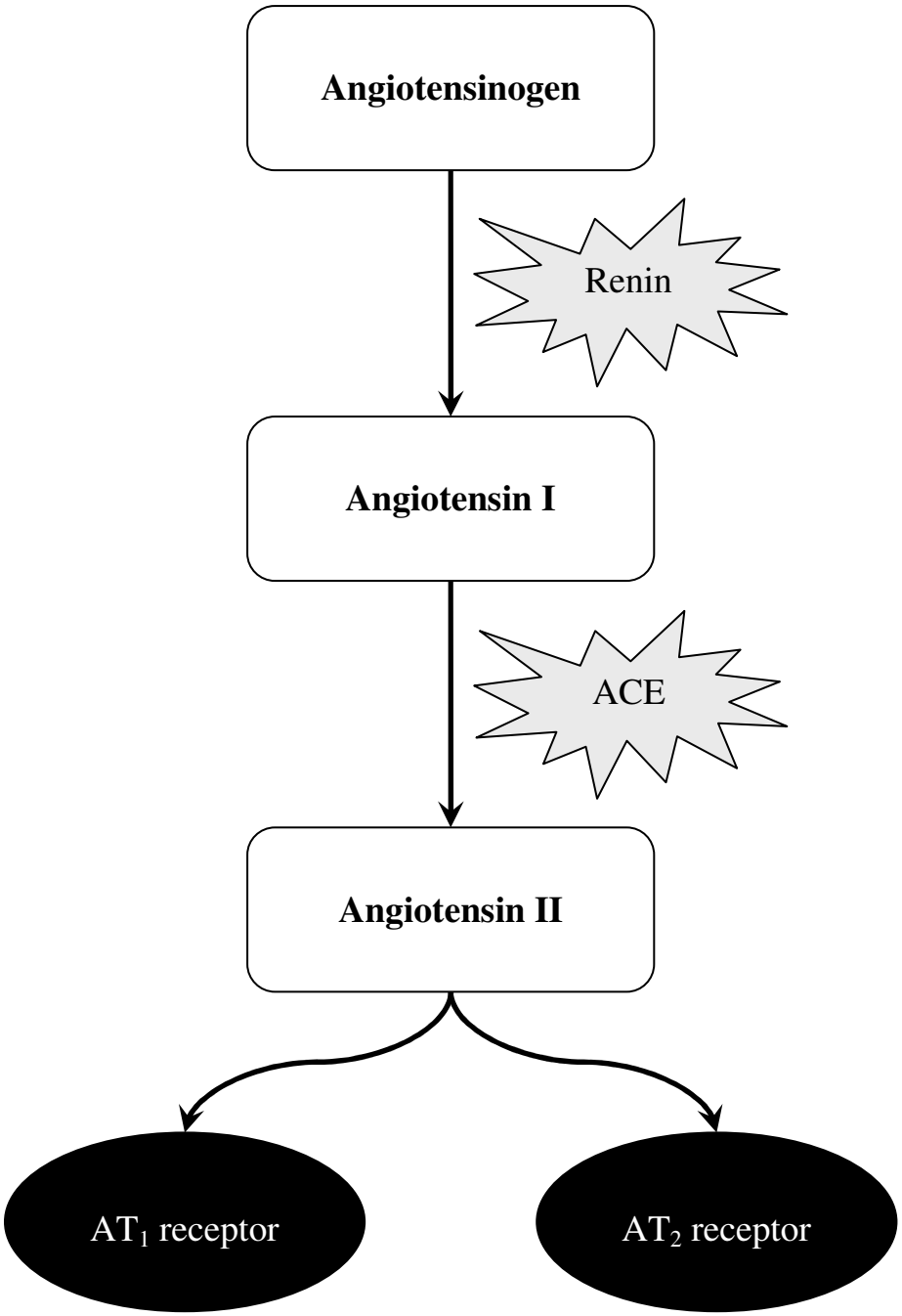


Figure 1.5. The renin-angiotensin system.

Angiotensinogen is ultimately converted to angiotensin II by subsequent enzymatic reactions with renin and angiotensin-converting enzyme (ACE). Angiotensin II binds to its receptors, the AT_1 and AT_2 receptors, to exert the physiological actions of the RAS.

In the human fetal kidney, renin messenger ribonucleic acid (mRNA) is expressed from 28 to 30 days of gestation and has been localised to glomerular capillaries and renal blood vessels of the mesonephros (Schutz *et al.*, 1996). In the metanephros, renin mRNA is present in the JGA and arteries close to glomeruli (Schutz *et al.*, 1996). Renin immuno-reactivity has been detected as early as the fifth gestational week in the mesonephros and at eight weeks of gestation in the metanephros of human fetuses (Celio *et al.*, 1985). The ovine renin sequence was published in 1992 and has a 73% sequence homology with human renin (Aldred *et al.*, 1992). The ontogeny of renal renin expression in the ovine fetus has been well described (Carbone *et al.*, 1993; Kon *et al.*, 1994; Rawashdeh *et al.*, 1996; Wintour *et al.*, 1996; Zhang *et al.*, 1996; Wang *et al.*, 1997). At 41 days of gestation, renin mRNA and immuno-reactive (IR)-cells were detected in blood vessels of mesonephroi and metanephroi of ovine fetuses (Wintour *et al.*, 1996). At 44 days of gestation, renin-IR cells were present in the tunica media of renal, interlobar and afferent blood vessels located in the deep cortex and medulla of fetal sheep kidneys (Kon *et al.*, 1994). From 50 to 63 days of gestation, renin-IR cells were located in all vessels from the interlobar arteries to the afferent arterioles (Kon *et al.*, 1994). As maturation proceeds, the distribution of renin-IR cells and mRNA shifts from the intra-renal blood vessels in the fetus to the classic juxta-glomerular location in the adult kidney (Gomez *et al.*, 1991). Renin–IR cells have been localised in the JGA and the afferent arterioles of fetal sheep aged approximately 105 days of gestation, 2-week-old lambs and adult sheep (Rawashdeh *et al.*, 1996). In mature fetuses at 139 days of gestation, renin–IR cells exhibited a more extensive distribution, being localised to the afferent arteriole and JGA as well as other segments of the arterial vascular tree (Rawashdeh *et al.*, 1996). In sheep, renal renin mRNA and protein levels increased significantly from approximately day 93 to day 140 of gestation, remained elevated in newborns and declined to early gestation levels in adulthood (Carbone *et al.*, 1993). Consistent with this, renal renin mRNA levels were lower in fetal sheep of approximately 91 and 134 days of gestation in comparison to those at 142 days of gestation, and all fetal renin mRNA levels were higher than those of adult sheep (Zhang *et al.*, 1996). In the ovine fetus, the increase in renal renin content in late gestation is the result of an increase in the number of renin-containing cells and the renin content per cell (Wang *et al.*, 1997). These studies suggest that increased renal renin expression may play an important role in preparing the fetus for birth (Rawashdeh *et al.*, 1996).

A_o mRNA has been detected in the proximal portion of primitive tubules of the mesonephros in the embryonic human kidney at days 25 to 30 of gestation (Schutz *et al.*, 1996). A_o mRNA is expressed in proximal tubules of the metanephros from eight weeks of gestation until term in the human fetal kidney (Schutz *et al.*, 1996). Expression of A_o and ACE mRNA has been detected in mesonephroi and metanephroi of ovine fetuses at 41 days of gestation (Wintour *et al.*, 1996). Few proximal tubules showed A_o–IR cells in the metanephros at that time in gestation (Wintour *et al.*, 1996). In the ovine kidney, A_o–IR cells have been localised to cortical proximal tubule cells, where staining intensity was highest in the fetus and lower in adult kidneys (Darby *et al.*, 1994). In fetal sheep, renal A_o mRNA levels increased with increasing gestation, with an overall 170% increase in expression from 80 to 139 days of gestation (Zhang *et al.*, 1998). Similarly, A_o protein levels increased by 44% from 80 to 139 days of gestation (Zhang *et al.*, 1998). Renal A_o mRNA and protein levels were significantly lower in adult sheep when compared to late gestation fetuses at 139 days of gestation (Zhang *et al.*, 1998). Renal ACE concentrations increased significantly from 129 to 143 days of gestation and declined in the neonatal period prior to reaching the elevated levels found in the kidneys of adult sheep, which are comparable to levels found near term (Forhead *et al.*, 2000b). Therefore, in sheep, there is an age-dependent difference in the regulation of renal A_o and ACE.

In human fetal kidneys, ACE protein has been localised in the apical membrane of the mesonephric tubule cells at 30 to 35 days of gestation (Schutz *et al.*, 1996). ACE immuno-reactivity was localised on the apical and basolateral membranes of proximal tubular cells from the early nephron stage of glomerular capillary loop formation and on glomerular endothelial cells as soon as the capillary invades the inferior cleft of the S-shaped body (Mounier *et al.*, 1987). ACE-IR cells were detected in proximal convoluted tubule cells and collecting duct cells in increasing amounts until birth (Schutz *et al.*, 1996). In postnatal kidneys, ACE immuno-reactivity was localised to the brush borders of the proximal tubules where it may regulate tubular reabsorption (Mounier *et al.*, 1987).

In human pregnancy, the use of ACE inhibitors during the second or third trimesters has adverse fetal effects including: intra-uterine growth restriction, neonatal hypertension, renal failure, oligohydramnios and patent ductus arteriosus (Quan, 2006). In reported

cases of maternal ACE inhibitor use during pregnancy, oligohydramnios was noted and presumably due to reduced renal function and fetal urine production (Quan, 2006). On histopathological examination of the fetal kidneys, juxta-glomerular hyperplasia, renal tubular dilatation and increased cortical and medullary fibrosis were observed (Quan, 2006). Therefore, AngII is important for normal renal tubular development.

Two isoforms of the AngII receptors are found in humans and sheep: AT_1 and AT_2. The ovine genomic sequence containing the full-length AT_1 receptor was published in 1998 (Millican & Bird, 1998). In mammalian cells, AngII binds to the AT_1 receptor to stimulate vasoconstriction, vascular cell hyperplasia and hypertrophy, and sodium retention (Tufro-McReddie *et al.*, 1994; Berry *et al.*, 2001). AngII also exerts a tonic inhibition on renin secretion and gene expression via the AT_1 receptor (Tufro-McReddie *et al.*, 1994; Kurtz & Wagner, 1999). The function of the AT_2 receptor is less certain; however, AT_2 receptors are involved in vasodilation and natriuresis (Berry *et al.*, 2001) and have recently been shown to inhibit renin synthesis and AngII formation in rats (Siragy *et al.*, 2005). Renal AT_2 receptors are involved in early kidney development and levels decrease after nephrogenesis is complete in rats (Shanmugam *et al.*, 1995; Norwood *et al.*, 1997), mice (Kakuchi *et al.*, 1995) and sheep (Robillard *et al.*, 1995; Butkus *et al.*, 1997; Gimonet *et al.*, 1998). AT_2 receptors were abundantly expressed during fetal development and their expression was markedly down-regulated after birth, whereas the abundance of AT_1 receptors increased as maturation proceeded (Robillard *et al.*, 1994; Robillard *et al.*, 1995; Butkus *et al.*, 1997; Gimonet *et al.*, 1998; Wolf, 2002). During renal development, AT_1 receptors stimulate growth (Tufro-McReddie *et al.*, 1994) that is antagonised by AT_2 receptors, which inhibit growth and induce apoptosis (Yamada *et al.*, 1996; Wolf, 2002). It has been proposed that alterations in the levels of AT_1 and AT_2 receptors during renal development may alter tubular formation by shifting the balance between growth and apoptosis (Wolf, 2002).

The ontogeny and regulation of AngII receptor expression in the fetal ovine kidney is well described (Robillard *et al.*, 1994; Robillard *et al.*, 1995; Butkus *et al.*, 1997; Gimonet *et al.*, 1998). In sheep, AT_1 receptor gene expression increased between 60 and 100 days of gestation, remained constant between 100 and 140 days of gestation (Robillard *et al.*, 1994; Butkus *et al.*, 1997) but 10 days after birth, renal AT_1 receptor mRNA levels were significantly lower than fetal levels (Robillard *et al.*, 1994). AT_1

receptor mRNA expression was localised in the central mesangial regions of glomeruli and cortical and medullary interstitial cells in the mesonephric and metanephric kidneys of fetal sheep from 27 to 140 days of gestation (Butkus *et al.*, 1997; Gimonet *et al.*, 1998). It has been proposed that the presence of AT_1 receptors in the interstitium may be necessary for the development of the loop of Henle and the maturation of the inner stripe of the outer medulla (Butkus *et al.*, 1997). In the human embryonic mesonephros, AT_1 receptor mRNA was detected in developing glomeruli at 25 to 27 days' gestation (Schutz *et al.*, 1996). Similar to sheep, AT_1 receptor mRNA was localised in presumptive mesangial cells in glomeruli and proximal tubular epithelium of the human fetal metanephros (Schutz *et al.*, 1996). In human pregnancies, the use of AT_1 receptor antagonists during the second and third trimesters results in similar deleterious effects to that observed after maternal ACE inhibitor use: high rates of perinatal mortality, oligohydramnios and neonatal anuria (Quan, 2006). Histological examination of fetal kidneys revealed tubular dilatation, poor or absent proximal tubular differentiation and high renin expression localised to the juxta-glomerular apparatus (Quan, 2006). These histopathological findings were proposed to result from AT_1 antagonist-related hypo-perfusion of the fetal kidney (Quan, 2006).

In the human fetal kidney, AT_2 receptor mRNA has been localised in the undifferentiated mesenchyme of the mesonephros surrounding the developing glomeruli and tubules at 23 to 24 days of gestation (Schutz *et al.*, 1996). AT_2 mRNA expression was highest in the metanephros at eight to nine weeks of gestation and declined after 20 weeks of gestation but remained detectable until birth (Schutz *et al.*, 1996). Similarly, expression of AT_2 receptor mRNA in the fetal sheep kidney is high during the second trimester of gestation (50 to 100 days) and decreases rapidly thereafter (Robillard *et al.*, 1995; Gimonet *et al.*, 1998). AT_2 receptor mRNA expression levels in fetal sheep, measured by RNase protection assay, were comparable from 60 to 100 days of gestation and fell below the limit of detection at 140 days of gestation (Butkus *et al.*, 1997). AT_2 receptor mRNA expression was present in undifferentiated mesenchymal cells surrounding the developing glomeruli at 27 and 40 days of gestation and was also localised in the macula densa at 75 and 131 days of gestation in fetal sheep kidneys (Butkus *et al.*, 1997; Gimonet *et al.*, 1998). AT_2 receptors in macula densae inhibit pro-renin processing in rat juxta-glomerular cells (Ichihara *et al.*, 2003). In sheep, *in situ* hybridisation of AT_2 receptor mRNA was almost undetectable after 120 days of

gestation (Gimonet *et al.*, 1998) and was absent in kidneys from 2-day-old lambs (Butkus *et al.*, 1997); consistent with data from other animal studies demonstrating undetectable levels of AT_2 receptors in rats after postnatal day 14 or 28, respectively (Aguilera *et al.*, 1994; Norwood *et al.*, 1997). AT_2 receptors are involved in apoptosis (Yamada *et al.*, 1996), accounting for their localisation surrounding the developing glomeruli and the reduction in AT_2 receptor expression levels later in development when nephrogenesis is complete (Butkus *et al.*, 1997).

1.2.3.2. Renal Na^+/K^+-ATPase

Sodium/potassium-adenosine triphosphatase (Na^+/K^+-ATPase) is a protein complex that actively transports sodium ions out of cells, and potassium ions into cells, in an adenosine triphosphate (ATP)-dependent manner; for each ATP molecule metabolised, three sodium ions and two potassium ions are exchanged (Feraille & Doucet, 2001). Na^+/K^+-ATPase consists of two subunits, a larger α-subunit that mediates catalytic activity and a smaller regulatory β-subunit (Feraille & Doucet, 2001). The α_1-isoform is ubiquitous and is the predominant isoform in the kidney (Feraille & Doucet, 2001). Na^+/K^+-ATPase actively transports sodium from the tubular cells into the interstitial fluid, which then diffuses into the peritubular capillaries (Sherwood, 1997). Na^+/K^+-ATPase activity is high in the thick ascending limb and distal convoluted tubule, intermediate in the proximal tubule, relatively low in the collecting duct, and virtually absent in the thin segments of the loop of Henle (Feraille & Doucet, 2001). This is consistent with the immunohistochemical localisation of Na^+/K^+-ATPase protein in the rat kidney (Baskin & Stahl, 1982); intense IR-staining was present on the basolateral surfaces of epithelial cells in the distal convoluted tubules, subcapsular collecting tubules, thick ascending limbs and papillary collecting ducts, and minimal staining was observed in the proximal convoluted tubules (Baskin & Stahl, 1982). Immuno-staining was absent in the thin limbs of the loop of Henle, pars recta and outer-medullary collecting ducts (Baskin & Stahl, 1982). The major function of renal Na^+/K^+-ATPase is net sodium reabsorption, and a close relationship exists between the abundance of Na^+/K^+-ATPase and the capacity for sodium reabsorption in various nephron segments (Garg *et al.*, 1981).

Na^+/K^+-ATPase activity in homogenates of guinea pig kidneys increases with maturation, with activity being approximately 3.8-fold higher in newborns, and 6.5-fold higher in adults, than in near-term fetuses (Guillery *et al.*, 1997). Newborn guinea pigs had a seven-fold and 1.4-fold higher abundance of Na^+/K^+-ATPase-α_1 protein and mRNA, respectively, compared to near-term fetuses (Guillery *et al.*, 1997). The maturational increase in renal Na^+/K^+-ATPase activity and abundance in the newborn guinea pig is a result of post-transcriptional events including increased protein synthesis and/or decreased Na^+/K^+-ATPase degradation (Guillery *et al.*, 1997). The ontogeny of Na^+/K^+-ATPase activity and expression in the ovine kidney has been partially described. Renal cortical Na^+/K^+-ATPase activity was similar at 132 and 140 days of gestation but increased by 80% in 1-day-old lambs (Petershack *et al.*, 1999). Similarly, renal Na^+/K^+-ATPase-α_1 mRNA levels were significantly higher at 145 days of gestation and in neonates (1- or 7-day-olds) compared with fetuses at 80, 100, 120 or 130 days of gestation (von Reitzenstein & Keller-Wood, 2004). Renal Na^+/K^+-ATPase protein levels prior to 132 days of gestation in the fetal sheep have not been reported.

1.3. CORTICOSTEROIDS

Adrenal corticosteroids are separated into two functionally distinct classes: *mineralocorticoids* and *glucocorticoids*. In humans and sheep, aldosterone is the principal mineralocorticoid secreted from the outer zona glomerulosa of the adrenal cortex in response to AngII. Aldosterone promotes sodium reabsorption in the renal distal nephron, colon and ducts of sweat and salivary glands (Farman & Bocchi, 2000). Glucocorticoids (cortisol, corticosterone) are synthesised and secreted by the zona fasciculata of the adrenal cortex under the influence of the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1.6). Glucocorticoids maintain metabolic homeostasis and have an important role in the adaptation to stress. They exert a diverse array of physiological actions including the regulation of amino acid and carbohydrate metabolism, maintenance of blood pressure and modulation of stress and inflammatory responses (Munck & Naray-Fejes-Toth, 1992). Glucocorticoids are also necessary to promote organ maturation in the late gestation fetus, which is essential for extra-uterine life (Liggins, 1994).

1.3.1. Synthesis and secretion of glucocorticoids

The synthesis of all steroid hormones in the adrenal gland involves step-wise modification of cholesterol (Figure 1.7). Cortisol is the major glucocorticoid secreted by the adrenal cortex in humans and sheep. Glucocorticoid secretion is regulated by the HPA axis. Corticotrophin-releasing hormone (CRH) and/or arginine vasopressin (AVP) are released from the neurosecretory cells in the hypothalamus in response to stress and diurnal rhythm (there is no circadian rhythm in cortisol secretion in sheep (Simonetta *et al.*, 1991)). These hormones target corticotrophs in the anterior pituitary that synthesise and ultimately release ACTH into the circulation (Dallman *et al.*, 1987). ACTH is trophic to the adrenal zona fasciculata and accordingly stimulates the synthesis and secretion of glucocorticoids (Dallman *et al.*, 1987). Circulating levels of glucocorticoids are maintained through a negative feed-back system involving the hippocampus and HPA axis. Glucocorticoids negatively feed-back by binding to glucocorticoid receptors (GRs) in the hypothalamus and anterior pituitary to inhibit the synthesis and secretion of CRH and ACTH (Figure 1.6).

1.3.2. Transport and metabolism of glucocorticoids

Approximately 90% of endogenous glucocorticoids are bound to the steroid-binding site of plasma corticosteroid-binding globulin (CBG) under normal physiological conditions (Siiteri *et al.*, 1982). CBG facilitates the transport of glucocorticoids by restricting glucocorticoid movement into extra-vascular tissues (only unbound steroids can enter cells) and therefore modulates the enzymatic degradation of steroids and/or their access to target cells.

In sheep, the pre-partum cortisol surge is associated with a concurrent rise in fetal CBG and plasma CBC (corticosteroid-binding capacity) levels (Fairclough & Liggins, 1975; Ballard *et al.*, 1982; Berdusco *et al.*, 1993; Challis *et al.*, 1995). This ensures that the amount of free cortisol in fetal sheep plasma is similar during this period of gestation (Berdusco *et al.*, 1995). The rise in fetal CBG levels has been found to precede elevations in cortisol levels presumably to prevent glucocorticoid-induced premature labour (Fairclough & Liggins, 1975). CBG decreases the amount of unbound cortisol in

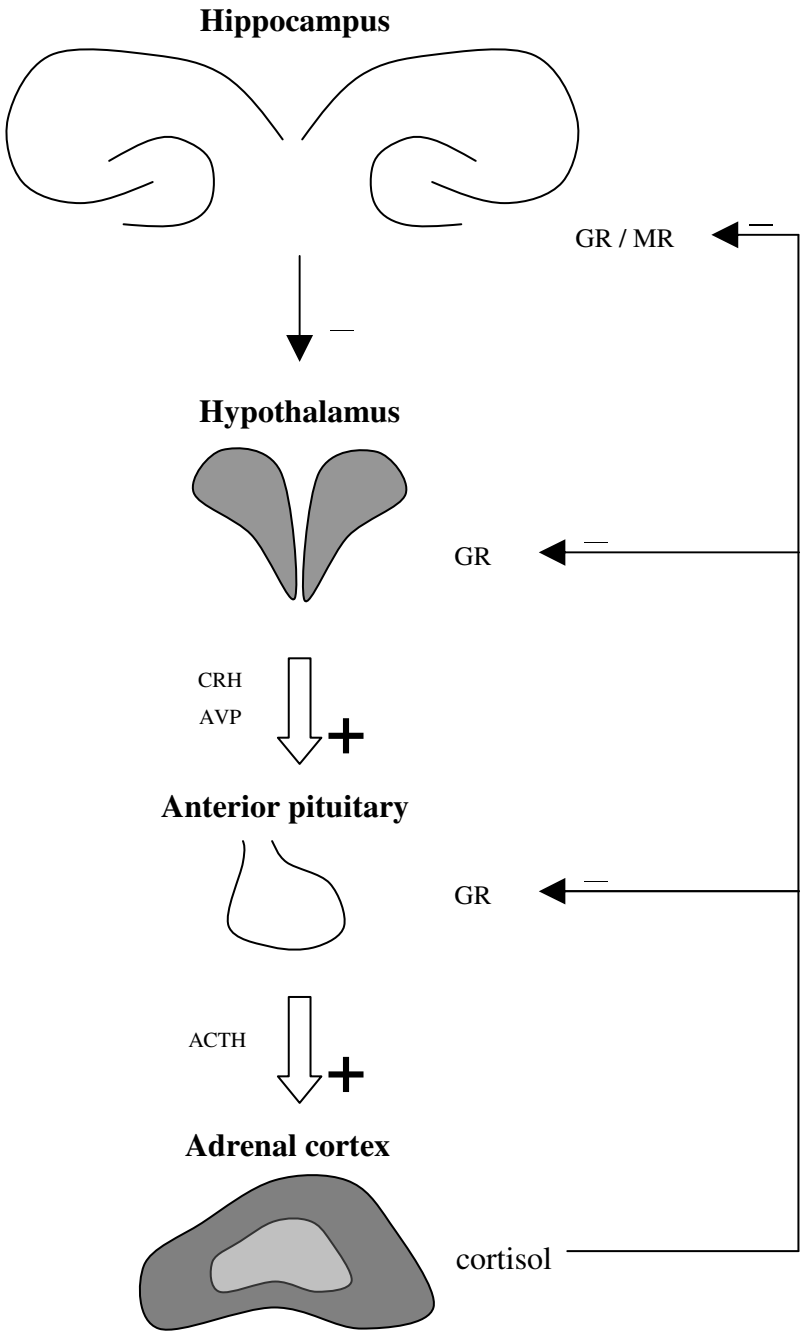
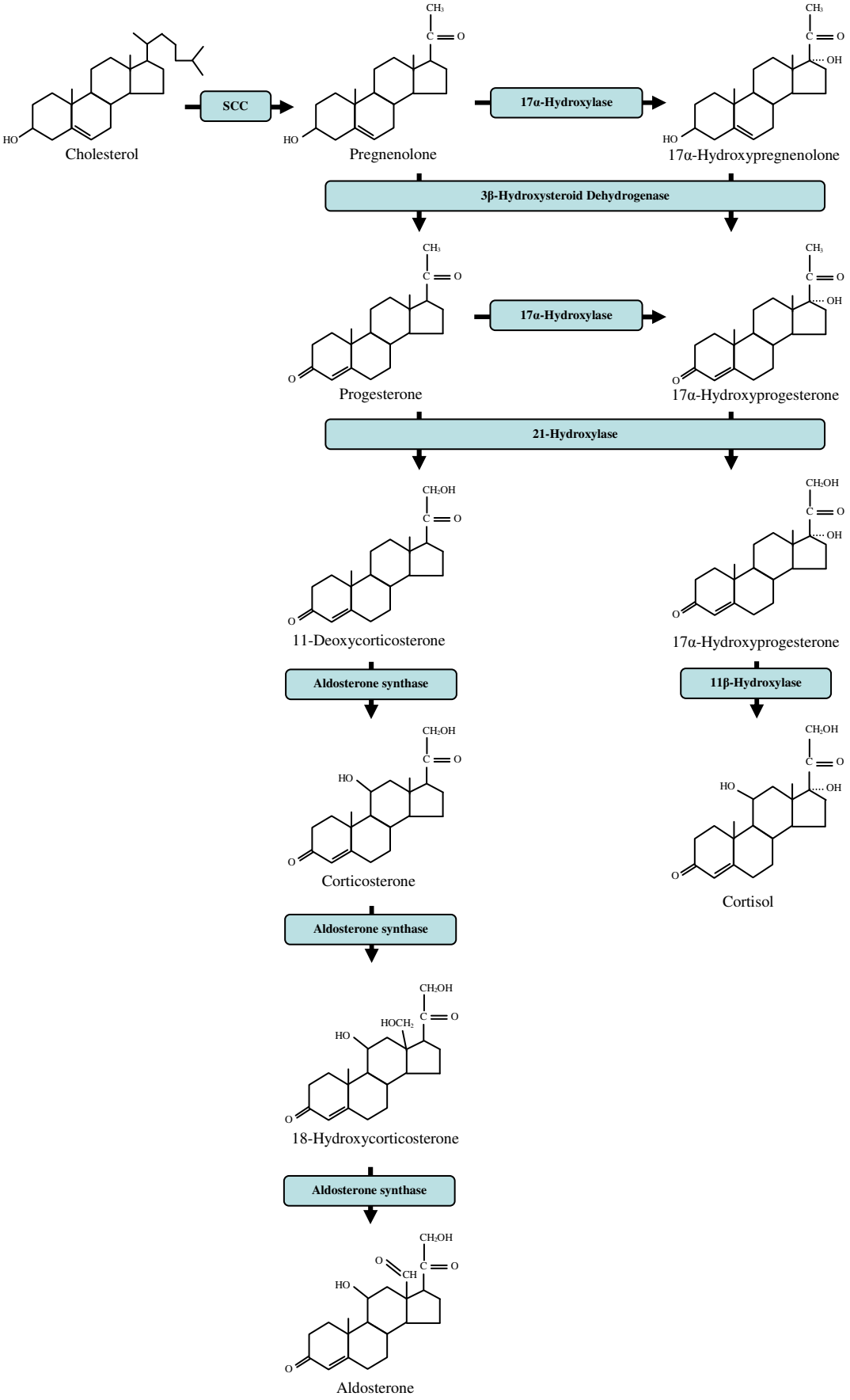


Figure 1.6. The hypothalamic-pituitary-adrenal (HPA) axis and its negative feed-back loop. HPA axis activity is regulated by negative feed-back of glucocorticoids binding to glucocorticoid (GR) and/or mineralocorticoid receptors (MR) in the anterior pituitary, hypothalamus and hippocampus. ACTH: adrenocorticotrophic hormone; AVP: arginine vasopressin; CRH: corticotrophin-releasing hormone. Stimulatory (+) and inhibitory (-) actions. Figure adapted from Sloboda (2001).

Figure 1.7. Corticosteroid biosynthesis. The biosynthesis of corticosteroids (cortisol, aldosterone) involves a series of enzymatic reactions that modify the precursor, cholesterol. SCC: Side Chain Cleavage enzyme.


Cholesterol
SCC
Pregnenolone
17α-Hydroxylase
17α-Hydroxypregnenolone
3β-Hydroxysteroid Dehydrogenase
Progesterone
17α-Hydroxylase
17α-Hydroxyprogesterone
21-Hydroxylase
11-Deoxycorticosterone
17α-Hydroxyprogesterone
Aldosterone synthase
11β-Hydroxylase
Corticosterone
Cortisol
Aldosterone synthase
18-Hydroxycorticosterone
Aldosterone synthase
Aldosterone

plasma. Therefore, the rise in CBG may reduce the efficacy of the negative feed-back action of glucocorticoids at the hypothalamic and pituitary level and may help explain the concurrent rise in fetal plasma ACTH and cortisol in sheep (Fairclough & Liggins, 1975; Challis & Brooks, 1989; Challis *et al.*, 1995). After parturition, plasma CBG, and total and free cortisol levels fall in sheep (Ballard *et al.*, 1982). Therefore, increased plasma CBG levels in late gestation allow normal glucocorticoid-induced maturation of fetal organs whilst protecting the fetus from excessive, and possibly detrimental, levels of the steroid hormones.

In fetal sheep, the liver is the primary site of CBG production; however mRNA transcripts of the gene have also been detected in the pituitary and kidney (Berdusco *et al.*, 1995). CBG–IR cells have been localised in the epithelium of proximal and distal convoluted tubules in the ovine fetal kidney from day 63 of gestation, with the intensity of CBG immuno-staining gradually decreasing with increasing gestation to negligible levels in newborn lambs (Reznikov *et al.*, 2004). It is unknown whether the IR cells correspond to synthesis or uptake of CBG (Berdusco *et al.*, 1995). Tubular cells within the fetal kidney may contain CBG to modulate access of glucocorticoids to corticosteroid receptors during development.

Target tissue bioavailability of glucocorticoids is also regulated by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD). 11β-HSD inter-converts biologically active glucocorticoids (cortisol, corticosterone) with their inactive metabolites (cortisone, 11-dehydrocorticosterone) (Stewart *et al.*, 1994). Two isoforms of the enzyme, 11β-HSD-1 and 11β-HSD-2 have been identified in human and ovine tissues (Tannin *et al.*, 1991; Yang *et al.*, 1992b; Albiston *et al.*, 1994; Stewart *et al.*, 1994; Yang & Yu, 1994). 11β-HSD-1 is a low-affinity $NADP^+$-dependent enzyme expressed in most tissues that converts cortisone to cortisol. 11β-HSD-2 is a high-affinity NAD^+-dependent enzyme that inactivates glucocorticoids and is localised in the kidney, colon, pancreas, placenta, ovary, prostate and testis of humans (Albiston *et al.*, 1994). The major site of glucocorticoid metabolism (cortisone production) is the kidney and glucocorticoid metabolites are excreted in the urine (Whitworth *et al.*, 1989).

1.3.3. Corticosteroid receptors

Corticosteroids, such as aldosterone and cortisol, exert their physiological actions by binding to intra-cellular receptors: the *mineralocorticoid receptor* (MR) and *glucocorticoid receptor* (GR). These receptors are members of the super family of steroid/thyroid/retinoic acid receptor proteins that function as ligand-dependent transcription factors (Evans, 1988). The GR and MR share functional similarities and have a high degree of sequence homology (Hollenberg *et al.*, 1985; Arriza *et al.*, 1987). GRs are expressed in most tissues and cells; however MRs are more discretely localised in mineralocorticoid target tissues such as the kidney.

In the absence of a steroid hormone ligand, the corticosteroid receptors are localised in the cytoplasm of target cells as part of a large multi-protein complex. This protein complex consists of the receptor polypeptide and heat shock proteins (hsps)-90, -70 and -56 (Pratt & Welsh, 1994), and is necessary to activate hormone binding by the receptor (Rajapandi *et al.*, 2000). Hsps activate the hormone binding activity of the receptor by partially unfolding the hormone-binding domain, exposing its steroid-binding site (Rajapandi *et al.*, 2000). Corticosteroids are lipophilic substances that readily cross the cell membrane to interact with intra-cellular GRs and MRs (Bamberger *et al.*, 1996). Ligand binding of corticosteroids to receptors induces a conformational change in the receptor complex that causes dissociation of the receptor from the hsps. The receptor-ligand complex then translocates to the nucleus where it forms GR or MR homodimers or heterodimers (GR/MR) and binds to glucocorticoid response elements (GREs) in the promoter region of glucocorticoid-responsive genes, to regulate gene transcription (Bamberger *et al.*, 1996; Trapp & Holsboer, 1996). The GR-ligand complex can also interact with other transcription factors (i.e. activating protein-1 [AP-1], nuclear factor of kappa light polypeptide gene enhancer in B-cells [NF-κB]) to influence gene transcription (Bamberger *et al.*, 1996). Therefore, corticosteroid receptors enhance or inhibit the transcription of target genes by binding directly to GREs or interacting with other transcription factors.

1.3.3.1. Glucocorticoid receptor

There are two isoforms of the human GR (hGR): hGRα and hGRβ; the hGRα (94 kDa) appears to be the dominant form (Hollenberg *et al.*, 1985). The hGRα and hGRβ isoforms are the product of alternative splicing of the hGR gene (Encio & Detera-Wadleigh, 1991). A partial sequence of the ovine GR was published in 1992 (Yang *et al.*, 1992a); however, α- and β-isoforms of the receptor have not been reported. Gene transcripts and protein of the hGRα are found in most tissues and cells including brain, skeletal muscle, lung, kidney, liver, heart, colon, nasal mucosa, macrophages, eosinophils and neutrophils (Pujols *et al.*, 2002). Human GRβ mRNA was detected in all of these cells and tissues except the colon; however, hGRβ protein was not detected by Western blotting using an antibody raised against epitopes common to both receptor isoforms (Pujols *et al.*, 2002). Human GRβ has been localised in the nucleus of cells independent of glucocorticoid treatment and does not bind glucocorticoids or activate GREs (Hollenberg *et al.*, 1985; Oakley *et al.*, 1996). Previous studies have demonstrated that hGRβ heterodimers (with hGRα or MR) have diminished transcriptional activity (Bamberger *et al.*, 1997; Oakley *et al.*, 1999).

The importance of the GR in fetal development has been demonstrated by targeted disruption of the GR gene in mice (Cole *et al.*, 1995). The majority of GR deficient (-/-) mice die from respiratory failure within a few hours of birth, due to atelectatic lungs (Cole *et al.*, 1995). These mice also have reduced activation of hepatic gluconeogenic enzymes, enlarged and disorganised adrenal glands with deficient development of chromaffin cells, and impaired glucocorticoid regulation via the HPA axis characterised by increased plasma ACTH and corticosterone levels (Cole *et al.*, 1995). Hence, functional GR are required for normal prenatal development and survival after birth.

1.3.3.2. Mineralocorticoid receptor

MRs are expressed in mineralocorticoid target tissues including the kidney, hippocampus, pancreas, salivary glands, mammary glands and sweat glands of rats and humans (Arriza *et al.*, 1987; Sasano *et al.*, 1992). The MR and GR DNA- and hormone-binding regions are highly homologous; therefore, MRs can bind glucocorticoids with high affinity and stimulate transcription by binding to GREs (Arriza *et al.*, 1987). Aldosterone binds preferentially to MRs in the kidney *in vivo* (Sheppard & Funder,

1987), despite the equal affinity of aldosterone and cortisol for the MR *in vitro* (Arriza *et al.*, 1987) and circulating cortisol levels some 1000-fold higher than aldosterone. MRs are selective for aldosterone *in vivo* because of the presence of the enzyme 11β-HSD-2 that inactivates cortisol and corticosterone but not aldosterone (Funder *et al.*, 1988). Aldosterone is not metabolised by 11β-HSD because the 11-hydroxyl group cyclizes with the aldehyde at C18 to form a hemiketal group (Stewart & Krozowski, 1999).

1.4. GLUCOCORTICOIDS AND THE KIDNEY

1.4.1. Renal glucocorticoid receptors

The ontogeny of GR expression in the human and rodent kidney has been well described. Developing glomeruli, central collecting ducts and proximal and distal tubules in the metanephros of human embryos express GR mRNA at eight weeks of gestation (Condon *et al.*, 1998). In embryonic mouse kidneys, GR mRNA has been localised in the mesonephric tubules and duct from 11.5 days of gestation (term is 19 days) and in the developing metanephros from 12.5 days of gestation (Speirs *et al.*, 2004). In adult rat kidneys, GR mRNA expression is found in proximal tubules and cortical collecting tubules, with lower levels in the distal tubules (Roland *et al.*, 1995). GR mRNA levels, measured using polymerase chain reaction (PCR), were two-fold higher in glomeruli, proximal tubules, and thick ascending limb segments than in the collecting duct segments of rat nephrons (Todd-Turla *et al.*, 1993). Similarly, a ribonuclease protection assay (RPA) of micro-dissected tubules of adrenalectomised adult rats found that GR mRNA expression was highest in the glomerulus and was roughly equivalent along the length of the tubule (Escoubet *et al.*, 1996). Differences in the sensitivity of the techniques used to localise and/or measure GR mRNA may explain the variability in the results reported.

GR-IR protein has been localised to the nucleus and cytoplasm of cells lining the renal tubules in human embryos from eight weeks of gestation (Costa *et al.*, 1996) and in mice from 14.5 days of gestation (Thompson *et al.*, 2004). Cells of the proximal, distal and collecting tubules of rat kidneys display strong nuclear and moderate cytoplasmic

staining of GR protein (Teasdale *et al.*, 1986). In rabbit kidneys, intense staining was observed in the medullary thick ascending limb and distal tubule with weaker staining localised in the thin loop of Henle, cortical thick ascending limb, collecting ducts and the papillary epithelium (Farman *et al.*, 1991). In human kidneys, intense staining of GR protein, both nuclear and cytoplasmic, was present in the distal convoluted tubules and collecting ducts, with moderate staining in glomeruli and weak staining in the proximal tubules (Yan *et al.*, 1999). Glomerular GR protein staining was observed in the nuclei and cytoplasm of parietal and visceral epithelial cells, endothelial cells and mesangial cells within the human kidney (Yan *et al.*, 1999). In contrast, GR protein was not detected in the glomeruli of rat or rabbit kidneys (Teasdale *et al.*, 1986; Farman *et al.*, 1991). The failure of these studies to demonstrate glomerular GR protein staining may reflect limitations in the immunohistochemical method used, as more sensitive techniques such as PCR and RPA have detected the presence of GR mRNA within glomeruli of rats (Todd-Turla *et al.*, 1993; Escoubet *et al.*, 1996).

Functional GR are present in the ovine mesonephros at 28 days of gestation and GR mRNA expression was found to be greatest in the proximal and distal tubules, with lower levels observed in collecting duct epithelium (Peers *et al.*, 2001). GR mRNA levels in the kidney remain relatively constant throughout gestation in fetal sheep (Yang, 1992; Hantzis *et al.*, 2002; von Reitzenstein & Keller-Wood, 2004), suggesting that renal GR mRNA may not be regulated by circulating glucocorticoid levels (Yang, 1992; Yang *et al.*, 1992a), which is consistent with studies performed using fetal and neonatal rats (Kalinyak *et al.*, 1989). Renal GR protein levels and localisation in fetal or adult sheep kidneys have not been reported.

1.4.2. Renal mineralocorticoid receptors

The action of aldosterone within the kidney is to promote sodium reabsorption in the distal nephron (Farman & Bocchi, 2000). MR–IR cells have been observed in the distal tubule, connecting tubule and cortical collecting tubules of rat, rabbit and human kidneys (Krozowski *et al.*, 1989; Rundle *et al.*, 1989; Lombes *et al.*, 1990; Farman *et al.*, 1991; Sasano *et al.*, 1992; Hirasawa *et al.*, 1997; Hirasawa *et al.*, 1999). MR immuno-staining was also present in the thin and thick limbs of the loop of Henle and medullary and papillary collecting ducts in human and rabbit kidneys (Lombes *et al.*,

1990; Farman *et al.*, 1991; Sasano *et al.*, 1992; Hirasawa *et al.*, 1997; Hirasawa *et al.*, 1999). In addition, MR-IR cells were localised in the papillary epithelium of rabbit kidneys, although the physiological relevance of this observation remains unknown (Lombes *et al.*, 1990; Farman *et al.*, 1991).

MR mRNA has been localised by *in situ* hybridisation in the distal convoluted tubules, and cortical and medullary collecting tubules of the rat kidney (Roland *et al.*, 1995). Using competitive PCR (a more sensitive technique), MR mRNA was detected in the glomeruli and all nephron segments in the rat kidney; levels of MR mRNA were 10-fold higher in the cortical collecting duct, outer medullary collecting duct and inner medullary collecting duct segments than in the proximal tubule and thick ascending limb segments (Todd-Turla *et al.*, 1993). In human embryos, MR mRNA was expressed in the kidney by six weeks of gestation and was localised to the epithelial cells lining the collecting ducts (Condon *et al.*, 1998). In fetal sheep kidneys, MR mRNA levels have been reported to remain constant throughout days 60 to 140 of gestation (Hantzis *et al.*, 2002). A recent study demonstrated that renal MR mRNA expression peaked in fetal sheep at 145 days of gestation (von Reitzenstein & Keller-Wood, 2004).

1.4.3. Glucocorticoid effects on renal MRs and GRs

Glucocorticoids can alter the expression of MR and GR (Kalinyak *et al.*, 1987; Hagley & Watlington, 1996). *In vitro*, dexamethasone administration has been shown to down-regulate MR number in A6 cells derived from Xenopus kidney (Hagley & Watlington, 1996). Adrenalectomy increased renal GR mRNA levels by 80% in adult rats whilst dexamethasone treatment in intact animals reduced renal GR mRNA levels by 60% (Kalinyak *et al.*, 1987). Previous studies in fetal sheep and rats have reported that GR mRNA was not regulated by circulating endogenous glucocorticoid levels (Kalinyak *et al.*, 1989; Yang, 1992; Yang *et al.*, 1992a).

In sheep, maternal dexamethasone infusion (0.48 mg/h for 48 h) at approximately 27 days of pregnancy significantly increased fetal renal MR and GR mRNA levels at 130 days of gestation; however, the affinity and number of binding sites of fetal renal MR and GR protein were unaffected by the treatment (Hantzis *et al.*, 2002). Maternal cortisol infusion (5 mg/h for 48 h) early in gestation had no effect on fetal renal MR and

GR mRNA expression or receptor affinity or number of binding sites at 130 days of gestation in sheep (Hantzis *et al.*, 2002). Ovine fetal mesonephric GR mRNA expression levels at 29 days of gestation were not altered by dexamethasone or cortisol exposure (Peers *et al.*, 2001). Prolonged low-dose maternal dexamethasone treatment (20 μg/kg ewe weight/d between days 25 and 45 of pregnancy) did not alter GR or MR mRNA levels in the kidneys of fetal sheep at 130 days of gestation (Moritz *et al.*, 2002a). Therefore, in fetal sheep, a short-term exposure to dexamethasone early in gestation increased renal GR and MR mRNA expression late in gestation. The effects of late-gestation synthetic glucocorticoid administration on renal GR and MR mRNA and protein levels are unknown.

1.4.4. Glucocorticoid effects on renal function

Glucocorticoids influence numerous renal functions including haemodynamics, gluconeogenesis and tubular transport. Glucocorticoids regulate sodium-dependent transport of hydrogen (Freiberg *et al.*, 1982; Kinsella *et al.*, 1985; Loffing *et al.*, 1998; Gupta *et al.*, 2001), phosphate (Freiberg *et al.*, 1982; Levi *et al.*, 1995; Xan *et al.*, 2000; Park *et al.*, 2001), sulphate (Sagawa *et al.*, 2000) and bicarbonate (Ruiz *et al.*, 1995; Ali *et al.*, 2000) ions across tubular epithelia within the kidney. Normal GFR and renal plasma flow are maintained by glucocorticoids and renal haemodynamics are augmented when glucocorticoids are administered to humans or animals in pharmacologic doses (Gans, 1975; Kleeman *et al.*, 1975; Hall *et al.*, 1980; Connell *et al.*, 1987; van Acker *et al.*, 1993). A short-duration infusion of cortisol into fetal sheep (at 111 to 120 days of gestation) caused a natriuresis (Wintour *et al.*, 1985) due to an increase in GFR and a reduction in proximal tubular reabsorption of sodium (Towstoless *et al.*, 1989). In older fetuses (mean age of 133 days of gestation) infused with low doses of cortisol, the increased capacity of the distal tubule to reabsorb sodium prevented this natriuresis (Towstoless *et al.*, 1989). Treatment of fetal sheep (aged between 126 and 135 days of gestation) with high doses of cortisol also increased GFR, which was associated with increased renal blood flow, but did not consistently cause a natriuresis (Hill *et al.*, 1988). Prolonged infusion of cortisol into ovine fetuses at 107 days of gestation increased GFR, urine flow rate, free water clearance and the excretion of potassium and creatinine compared to control animals (Dodic & Wintour, 1994). The infusion of cortisol in that study did not cause a natriuresis, indicating that functional

maturation of the kidney had occurred as a result of prolonged exposure to glucocorticoids (Dodic & Wintour, 1994).

Short-term exposure of fetal sheep to synthetic glucocorticoids has also been shown to alter renal function in pre-term lambs (Stonestreet *et al.*, 1983; Ervin *et al.*, 1996; Berry *et al.*, 1997; Smith *et al.*, 2000). Fetal intra-muscular (i.m.) administration of a high dose (18 mg) of betamethasone at 131 days of gestation increased GFR and reduced fractional sodium excretion in pre-term lambs born 40 to 60 hours after treatment (Stonestreet *et al.*, 1983). A single dose of betamethasone (0.5 mg/kg) injected i.m. into the ewe or directly into the fetus resulted in increased mean arterial pressure (MAP), GFR and total renal sodium reabsorption in pre-term (124 to 128 days of gestation) ventilated lambs delivered within 48 hours of treatment (Ervin *et al.*, 1996; Berry *et al.*, 1997; Smith *et al.*, 2000). Increases in MAP, GFR and sodium reabsorption were consistent with changes in renal haemodynamics after the transition from fetal to newborn life at term in sheep (Nakamura *et al.*, 1987). The results suggest that a relatively short-term exposure of the fetus to betamethasone may increase the functional capacity of the pre-term lamb kidney by accelerating glomerular and tubular maturation. Premature maturation of the fetal kidney, as represented by accelerated cellular differentiation, has been observed in rats (Slotkin *et al.*, 1992) and monkeys (Epstein *et al.*, 1977) treated with synthetic glucocorticoids. Therefore, prenatal betamethasone exposure appears to alter renal function in pre-term lambs through precocious maturation of the kidney.

In contrast to the above studies, renal function in term fetal sheep was not affected by prior prenatal betamethasone exposure (Jobe *et al.*, 1996; Smith *et al.*, 2003). At 145 days of gestation, GFR, urine flow rate and urinary sodium excretion were similar to controls in lambs exposed to a single dose (at 104 days of gestation) or multiple doses (at 104, 111 and 118 days of gestation) of betamethasone administered to pregnant ewes (Smith *et al.*, 2003). In addition, it has been shown previously that a single injection of betamethasone to the fetus at 126 days of gestation had no effect on renal function in lambs that were delivered at term (Jobe *et al.*, 1996). The differences in renal function between betamethasone-exposed lambs delivered pre-term or at term have been attributed to the difference in acute versus persistent effects of prenatal glucocorticoid treatment (Smith *et al.*, 2003).

Recently, the effects of early prenatal dexamethasone treatment on renal function and blood pressure responses in late-gestation sheep between 115 and 122 days of gestation were reported (Moritz *et al.*, 2005b). At 130 days of gestation, fetal sheep exposed to dexamethasone at approximately day 27 of gestation had increased renal MR, GR, A_o, AT_1 receptor and AT_2 receptor mRNA expression (Hantzis *et al.*, 2002; Moritz *et al.*, 2002b). Basal blood pressure and renal function were similar in saline- and dexamethasone-exposed fetal sheep, and responses to an intra-fetal i.v. infusion of cortisol (100 μg/h for 4 h) or aldosterone (5 μg/h for 4 h) were similar between the groups (Moritz *et al.*, 2005b). In contrast, on day three of an angiotensin II (10μg/h for 3 d) fetal i.v. infusion, urine flow rate and GFR were significantly lower in dexamethasone-exposed fetal sheep at 130 days of gestation (Moritz *et al.*, 2002b). These results suggest that up-regulation of renal GR and MR (receptors for cortisol and aldosterone) mRNA does not affect renal function in late-gestation fetal sheep exposed to early dexamethasone and highlights the role of the RAS in this model (Moritz *et al.*, 2005b).

1.4.5. Glucocorticoid effects on the renal RAS

GREs have been identified in the 5'-flanking region of the renin gene (Pratt *et al.*, 1988). Increased renal renin gene expression and renin content occurs in late gestation in conjunction with elevated fetal plasma cortisol concentrations in sheep (Carbone *et al.*, 1993; Rawashdeh *et al.*, 1996; Zhang *et al.*, 1996). However, a recent study using hypothalamo-pituitary disconnection (HPD) at 120 days of gestation prevented the pre-partum cortisol surge in late-gestation fetal sheep but did not alter renal renin mRNA, protein content, nor plasma renin levels compared to sham-operated animals (Chen *et al.*, 2005). HPD did not prevent the age-related increase in plasma renin concentration from 120-125 to 135-139 days' gestation (Chen *et al.*, 2005). These data suggest that the increases in renal renin mRNA and plasma renin concentration close to term in fetal sheep are not regulated by augmented fetal plasma cortisol levels (Chen *et al.*, 2005).

The literature contains conflicting observations concerning the effect of exogenous glucocorticoids on renal renin gene expression in fetal sheep. Maternal infusion of cortisol (5 mg/h for 48 h) or dexamethasone (0.48 mg/h for 48 h) into pregnant sheep at

approximately 27 days of gestation did not alter fetal kidney renin mRNA levels at 130 days of gestation (Moritz *et al.*, 2002b). Similarly, renal renin mRNA levels were not altered by a six-day i.v. infusion of cortisol (0.86 μg/min/kg) into fetal sheep between 94 and 100 days of gestation (Carbone *et al.*, 1995). However, an i.v. infusion of hydrocortisone succinate (2-3 mg/24 h) into fetal sheep between days 109 and 116 of gestation significantly reduced renal renin mRNA levels (Williams *et al.*, 2004) as did an intra-peritoneal (i.p.) infusion of cortisol (3 mg/h for 48 h) at 130 days of gestation (Segar *et al.*, 1995). In sheep, maternal betamethasone administration (two doses of 0.17 mg/kg, 24 h apart) on days 80 and 81 of gestation reduced renal renin production within 24 h but produced a subsequent up-regulation of renal renin production at 135 days of gestation (Connors *et al.*, 2005). These data suggest that in the fetal sheep kidney, renin-responsiveness to exogenous glucocorticoid administration is greater in late gestation and may involve the presence of transcription factors which first appear during this period (Carbone *et al.*, 1995).

A_o mRNA is up-regulated in response to glucocorticoid administration (Campbell & Habener, 1986; Chan *et al.*, 1992; Deschepper, 1994). Glucocorticoids increase transcription of A_o by direct interaction of the GRE in the A_o gene and the DNA-binding domain of the GR (Deschepper, 1994). Dexamethasone administration (0.48 mg/h for 48 h) to pregnant ewes early in gestation (at 26 to 28 days of gestation) significantly increased fetal renal A_o mRNA at 130 days of gestation; however, maternal cortisol infusion (5 mg/h for 48 h) did not alter fetal renal A_o mRNA levels (Moritz *et al.*, 2002b). Prolonged low-dose maternal dexamethasone administration (20 μg/kg ewe weight/d for 20 d) early in gestation did not affect fetal renal A_o gene expression at 130 days of gestation (Moritz *et al.*, 2002a). In fetal sheep, renal A_o mRNA expression was unaffected by an intra-fetal i.p. infusion of cortisol (3 mg/h for 48 h) at 130 days of gestation (Segar *et al.*, 1995). Therefore, the effect of glucocorticoid administration on fetal renal A_o mRNA expression is dependent on the steroid used, the dose administered and the timing of the glucocorticoid exposure.

A GRE has been identified in the regulatory region of the ACE gene (Shai *et al.*, 1990) and glucocorticoids have been shown to stimulate ACE gene expression and protein synthesis in cultured rat aortic smooth muscle cells (Fishel *et al.*, 1995) and bovine pulmonary artery endothelial cells (Dasarathy *et al.*, 1992). In contrast, an i.v. infusion

of cortisol (2-3 mg/kg/d for 5 d) into fetal sheep did not affect renal ACE concentration at 113 or 129 days of gestation (Forhead *et al.*, 2000b). Similar findings were reported in another study where an i.v. infusion of dexamethasone (45-60 µg/kg/d), administered for 48 hours into fetal sheep at approximately 129 days of gestation, failed to alter renal ACE concentration – a proxy measure of renal ACE activity (Zimmermann *et al.*, 2003). Therefore, the rise in renal ACE near term in sheep may be independent of the pre-partum cortisol surge (Forhead *et al.*, 2000b).

The effect of glucocorticoids on renal AT_1 receptor expression varies with gestational age at exposure. Maternal infusion of cortisol (5 mg/h for 48 h) or dexamethasone (0.48 mg/h for 48 h) into pregnant sheep early in gestation increased fetal renal AT_1 receptor mRNA levels at 130 days of gestation (Moritz *et al.*, 2002b). Prolonged maternal low-dose dexamethasone (20 µg/kg ewe weight/d) administration at 25 to 45 days of gestation did not affect fetal renal AT_1 receptor mRNA expression at 130 days of gestation (Moritz *et al.*, 2002a). Maternal betamethasone administration (two doses of 0.17 mg/kg, 24 h apart) on days 80 and 81 of gestation did not alter renal AT_1 receptor mRNA expression measured at 82 or 135 days' gestation in fetal sheep (Zhang *et al.*, 2005). Chronically-instrumented twin fetal sheep at 130 days of gestation, receiving a continuous i.p. infusion of cortisol (3 mg/h for 48 h), had increased plasma cortisol concentrations and significantly reduced renal AT_1 receptor mRNA expression relative to controls infused with saline (Robillard *et al.*, 1994; Segar *et al.*, 1995). Further, plasma cortisol levels were reduced and renal AT_1 receptor mRNA and protein levels were increased in fetal sheep at 135 to 139 days of gestation that had undergone HPD surgery two weeks earlier (Chen *et al.*, 2005). Therefore, acute exposure to glucocorticoids early in gestation increases renal AT_1 receptor mRNA; however, in late-gestation fetal sheep, elevated plasma cortisol levels attenuate renal AT_1 receptor expression.

Renal AT_2 receptor mRNA levels do not appear to be regulated by endogenous glucocorticoids in late-gestation fetal sheep. AT_2 receptor mRNA levels in kidneys of fetal sheep were unaffected by an i.p. infusion of cortisol at 130 days of gestation (Robillard *et al.*, 1995). HPD, which prevented the late gestation cortisol surge, also had no effect on fetal renal AT_2 receptor mRNA levels in sheep at 135 to 139 days of gestation (Chen *et al.*, 2005). Maternal cortisol infusion during early gestation did not

alter fetal renal AT_2 receptor mRNA at 130 days of gestation (Moritz *et al.*, 2002b). However, AT_2 receptor mRNA levels were reduced in the kidneys of fetal sheep at 82 and 135 days' gestation after mid-gestation maternal betamethasone administration (Zhang *et al.*, 2005). In contrast, maternal dexamethasone infusion early in pregnancy increased AT_2 receptor mRNA expression in the macula densae of dexamethasone-exposed fetal sheep at 130 days of gestation (Moritz *et al.*, 2002b). The timing of glucocorticoid administration may be critical in determining the effects on renal AT_2 receptor expression as mRNA levels of this receptor are much higher in early gestation and are virtually undetectable by 140 days of gestation in sheep (Butkus *et al.*, 1997).

1.4.6. Glucocorticoid effects on renal Na^+/K^+-ATPase

Glucocorticoids modulate renal Na^+/K^+-ATPase mRNA in the developing kidney. Cortisol infusion (3 mg/h for 48 h) into fetal sheep significantly increased renal cortical mRNA and protein levels of Na^+/K^+-ATPase-α_1 and -β_1 at 132 days of gestation (Petershack *et al.*, 1999). In the infant (10-day-old) rat kidney, glucocorticoids directly stimulated the transcription of Na^+/K^+-ATPase α_1- and β_1-subunits (Celsi *et al.*, 1991; Wang *et al.*, 1994); a GRE has been identified in the α_1-subunit gene in rats (Yagawa *et al.*, 1990). In rats, betamethasone administration on postnatal day 10 or 20 increased renal Na^+/K^+-ATPase α_1- and β_1-mRNA; however this treatment failed to affect the expression of these transcripts when administered to fetal, neonatal (5-day-old) or adult rats (Celsi *et al.*, 1991; Celsi *et al.*, 1993). Therefore, glucocorticoid induction of Na^+/K^+-ATPase mRNA appears to be age-dependent and the glucocorticoid-sensitive period coincides with the physiologic need for renal maturation (Celsi *et al.*, 1993).

Prenatal glucocorticoid exposure increased renal total sodium reabsorption (Ervin *et al.*, 1996; Berry *et al.*, 1997; Smith *et al.*, 2000) and was associated with a two- to three-fold increase in renal Na^+/K^+-ATPase activity in pre-term (127 days of gestation) lambs (Berry *et al.*, 1997) and a two-fold increase in term newborn lambs (Jobe *et al.*, 1996). Glucocorticoids have been shown to increase Na^+/K^+-ATPase activity in developing proximal tubular cells (Aperia *et al.*, 1981; Igarashi *et al.*, 1983) and thick ascending limbs of the rat kidney (Rane & Aperia, 1985), rat kidney homogenates (Dobrovic-Jenik & Milkovic, 1988), cultured rabbit proximal tubular cells (Lee *et al.*, 1995) and rat renal epithelial (NRK-52E) cells (Stewart & Whorwood, 1994) and in kidney homogenates

from fetal sheep (Jobe *et al.*, 1996; Berry *et al.*, 1997; Petershack *et al.*, 1999). In rabbits, adrenalectomy (glucocorticoid withdrawal) reduces Na^+/K^+-ATPase activity in the proximal convoluted tubule (Garg *et al.*, 1985), thick ascending limb (El Mernissi & Doucet, 1983; Garg *et al.*, 1985) and distal convoluted tubule by 40 to 50% (El Mernissi & Doucet, 1983; Garg *et al.*, 1985). Na^+/K^+-ATPase activity was restored to control levels in these tubules by the administration of dexamethasone (Garg *et al.*, 1985). In cortical and medullary collecting tubules of rabbit kidneys, adrenalectomy reduced Na^+/K^+-ATPase activity by 75%, which was restored by the administration of dexamethasone or aldosterone (El Mernissi & Doucet, 1983). Therefore, renal Na^+/K^+-ATPase activity appears to be regulated by mineralocorticoids and glucocorticoids.

1.4.7. Renal 11β-hydroxysteroid dehydrogenase

The kidney is a major site of cortisol metabolism (Jenkins, 1966). In the kidney, 11β-HSD-2 modulates access of glucocorticoids to GR, and protects the non-selective MR from occupation by glucocorticoids (Albiston *et al.*, 1994). Intense 11β-HSD-2 immuno-reactivity has been localised in cortical distal convoluted tubules, medullary thick ascending limbs of the loop of Henle and collecting ducts of human (Krozowski *et al.*, 1995; Hirasawa *et al.*, 1997) and rat kidneys (Roland *et al.*, 1995; Smith *et al.*, 1997). *In situ* hybridisation studies have demonstrated intense staining in renal cortical and medullary collecting ducts in human adult kidneys probed with 11β-HSD-2 complementary ribonucleic acid (cRNA) (Whorwood *et al.*, 1995). Immunohistochemical studies have demonstrated the presence of 11β-HSD-2 protein in the cytoplasm of cells in distal convoluted tubules and collecting ducts and in visceral epithelial and glomerular endothelial cells of the glomerulus in adult human kidneys (Kataoka *et al.*, 2002). 11β-HSD-2 protein has also been localised to the connecting tubule, cortical collecting duct and outer medullary collecting duct of rabbit kidneys; 11β-HSD-2 immuno-staining was cytoplasmic, consistent with its biological function of inactivating intra-cellular glucocorticoids before they can occupy the MR (Naray-Fejes-Toth & Fejes-Toth, 1998).

Renal 11β-HSD-2 is an autocrine protector of the MR (Edwards *et al.*, 1988; Stewart *et al.*, 1991; Kenouch *et al.*, 1994; Hirasawa *et al.*, 1997; Shimojo *et al.*, 1997). 11β-HSD-2 activity along the nephron has been examined in several species including rats, mice,

rabbits and humans (Kenouch *et al.*, 1994). The highest enzyme activity was found in the mineralocorticoid-sensitive distal nephron, including the thick ascending limb and cortical collecting duct (Kenouch *et al.*, 1994). From 14 weeks of gestation to term, both 11β-HSD-2 and MR immuno-reactivity have been detected in distal tubules, collecting tubules and the thick ascending limb of the loop of Henle in human fetal kidneys (Hirasawa *et al.*, 1999). The mRNA for these genes was localised to the renal collecting ducts by 16 weeks of gestation (Condon *et al.*, 1998). Postnatal kidneys have similar 11β-HSD-2 staining patterns, with immuno-reactivity and mRNA expression localised to the cortical and medullary collecting ducts (Whorwood *et al.*, 1995). Approximately 40% of 11β-HSD-2 staining is nuclear; therefore, the enzyme confers specificity upon the MR both in the nucleus and cytoplasm of a cell (Shimojo *et al.*, 1997).

11β-HSD-2 expression also regulates glucocorticoid access to renal GRs during prenatal life (Whorwood *et al.*, 1992), with co-localisation of these proteins present in developing glomeruli, central collecting duct, and proximal and distal tubules of the metanephros of the human fetus from eight weeks of gestation (Condon *et al.*, 1998). 11β-HSD-2 and GR mRNA have been localised in the kidneys of developing mice, where the enzyme is thought to regulate glucocorticoid-mediated maturational events during embryonic development (Thompson *et al.*, 2004).

The sheep kidney is unique in that 11β-HSD-1 mRNA and activity are absent and 11β-HSD-2 works as a uni-directional enzyme converting active glucocorticoids to their inactive metabolites (Langlois *et al.*, 1995; Yang, 1995). A third isoform of the enzyme, 11β-HSD-3, was reported to be present in the sheep kidney (Gomez-Sanchez *et al.*, 1997). 11β-HSD-3 is a uni-directional, high-affinity nicotinamide adenine dinucleotide phosphate $(NADP)^+$-dependent enzyme that has oxidative activity lower than that of 11β-HSD-2 (Gomez-Sanchez *et al.*, 1997). Therefore, in the sheep kidney, 11β-HSD-2 and 11β-HSD-3 may modulate glucocorticoid binding to corticosteroid receptors.

The ontogeny of 11β-HSD in the ovine fetal kidney has been well characterised (Yang *et al.*, 1992b; Kim *et al.*, 1995; Langlois *et al.*, 1995; Wood & Srun, 1995; McMillen *et al.*, 2000). 11β-HSD-2 mRNA and activity are present in the ovine kidney by day 85 of gestation and increase progressively until term (Langlois *et al.*, 1995; Wood & Srun, 1995; McMillen *et al.*, 2000). This increase in 11β-HSD-2 activity in the fetal sheep

kidney was thought to parallel the rise in fetal plasma cortisol levels in late gestation and protect the renal MR from illicit occupancy (Wood & Srun, 1995). However, more recently it has been demonstrated that renal 11β-HSD-2 activity was not altered during the last 15 to 20 days of gestation when endogenous cortisol levels rise (Clarke *et al.*, 2002). Fetal renal 11β-HSD-2 activity was similar at 128-132 days' gestation compared to 140-142 days' gestation, when plasma cortisol levels increased 5.5-fold (Clarke *et al.*, 2002). Furthermore, a premature elevation in fetal plasma cortisol to pre-partum levels, by a five-day intra-fetal i.v. infusion of cortisol (1-3 mg/d) at 123-127 days' gestation, did not affect renal 11β-HSD-2 activity (Clarke *et al.*, 2002). These results suggest that renal 11β-HSD-2 activity increases throughout nephrogenesis, which is complete by day 130 of gestation (Robillard *et al.*, 1981), but is not affected by fetal plasma cortisol levels (Clarke *et al.*, 2002).

Renal 11β-HSD is an important pharmacokinetic determinant of the activity of synthetic glucocorticoids. Fluorination of synthetic glucocorticoids increases the reductase activity and reduces oxidation, thus leading to a strong shift to the active form of the steroid (Diederich *et al.*, 2002). In human kidney slices and microsomes, the NAD^+-dependent reduction of 11-dehydro-dexamethasone to dexamethasone is favoured over oxidation due to 11β-HSD-2 activity (Diederich *et al.*, 1996; Diederich *et al.*, 2002). Therefore, due to reduced local inactivation of synthetic steroids by 11β-HSD-2, the fetal kidney may be targeted by prenatal glucocorticoid administration. The renal effects of prenatal synthetic glucocorticoid administration during late gestation are unknown.

1.4.8. Glucocorticoids: Preparing the fetus for birth

Glucocorticoids induce maturation of fetal tissues in preparation for extra-uterine life (Liggins, 1994). Circulating plasma levels of cortisol increase in the fetus towards term in the majority of mammalian species, as illustrated in Figure 1.8 (Fowden *et al.*, 1998). In most mammals, rising fetal plasma cortisol levels are associated with the initiation of parturition (Bassett & Thorburn, 1969; Magyar *et al.*, 1980; Norman *et al.*, 1985).

The transition of the fetus from intra-uterine to postnatal life is marked by a rapid increase in renal tubular sodium reabsorption (Nakamura *et al.*, 1987). The urinary fractional excretion of sodium is approximately 7.5% at term in the sheep fetus and

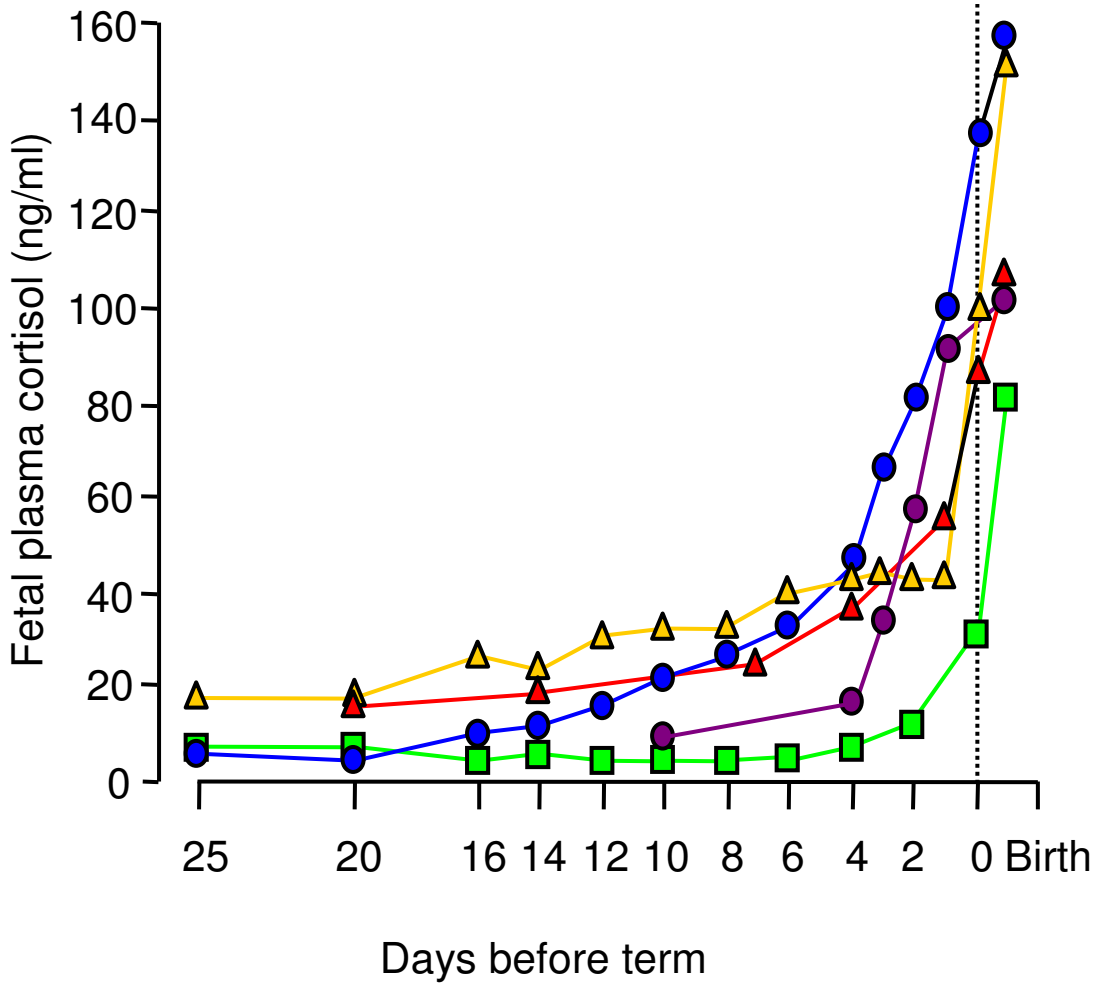


Figure 1.8. Fetal plasma cortisol concentrations in various species prior to birth. Mean fetal plasma cortisol concentrations in sheep (●), pig (▲), humans (▲), guinea pig (●) and horse (■), with respect to days prior to delivery at term. Figure adapted from Fowden *et al.* (1998).

declines to around 1% within 24 hours of birth (Nakamura *et al.*, 1987). During this period, the expression and activity of renal tubular sodium transporters (Na^+/H^+, Na^+/K^+-ATPase) is up-regulated (Guillery *et al.*, 1995; Guillery *et al.*, 1997; Petershack *et al.*, 1999). Glucocorticoids are thought to be responsible for the maturation of the renal sodium transporters as fetal cortisol levels are elevated immediately prior to birth and exogenous glucocorticoid administration has been demonstrated to increase the expression and activity of the transporters in sheep (Guillery *et al.*, 1995; Jobe *et al.*, 1996; Berry *et al.*, 1997), rats (Dobrovic-Jenik & Milkovic, 1988; Celsi *et al.*, 1991; Wang *et al.*, 1994; Gupta *et al.*, 2001) and baboons (Smith *et al.*, 2004).

In the neonate, sodium conservation is important for growth and maturation. Premature infants born at less than 33 weeks of gestation have a reduced capacity for renal sodium conservation (Al-Dahhan *et al.*, 1983). In humans, maternal dexamethasone administration reduced absolute and fractional sodium excretion during the first week of life in premature infants born at less than 35 weeks' gestation (al-Dahan *et al.*, 1987; Zanardo *et al.*, 1990). Therefore, glucocorticoid-induced maturation of renal tubular transport systems is important for renal electrolyte balance and postnatal growth in the neonate.

1.5. PRENATAL GLUCOCORTICOID ADMINISTRATION

1.5.1. Betamethasone

Betamethasone (9α-fluoro-16β-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione) is a synthetic glucocorticoid used in clinical practice to mimic the actions of cortisol. Fluorinated synthetic glucocorticoids (betamethasone, dexamethasone; Figure 1.9) are used for antenatal glucocorticoid therapy as they have the lowest inactivating capacity by 11β-HSD-2 (Blanford & Murphy, 1977; Diederich *et al.*, 2002) and do not bind to CBG (Peets *et al.*, 1969; Ballard *et al.*, 1975a). Therefore, they can readily cross the placenta and enter fetal tissues in their biologically active form. In obstetric practice, betamethasone preparations such as Celestone® Chronodose® (Schering-Plough Pty Ltd) and Celestone® Soluspan® (Schering-Plough Pty Ltd) that contain a mixture of betamethasone phosphate and betamethasone acetate are employed. Rapid exposure to

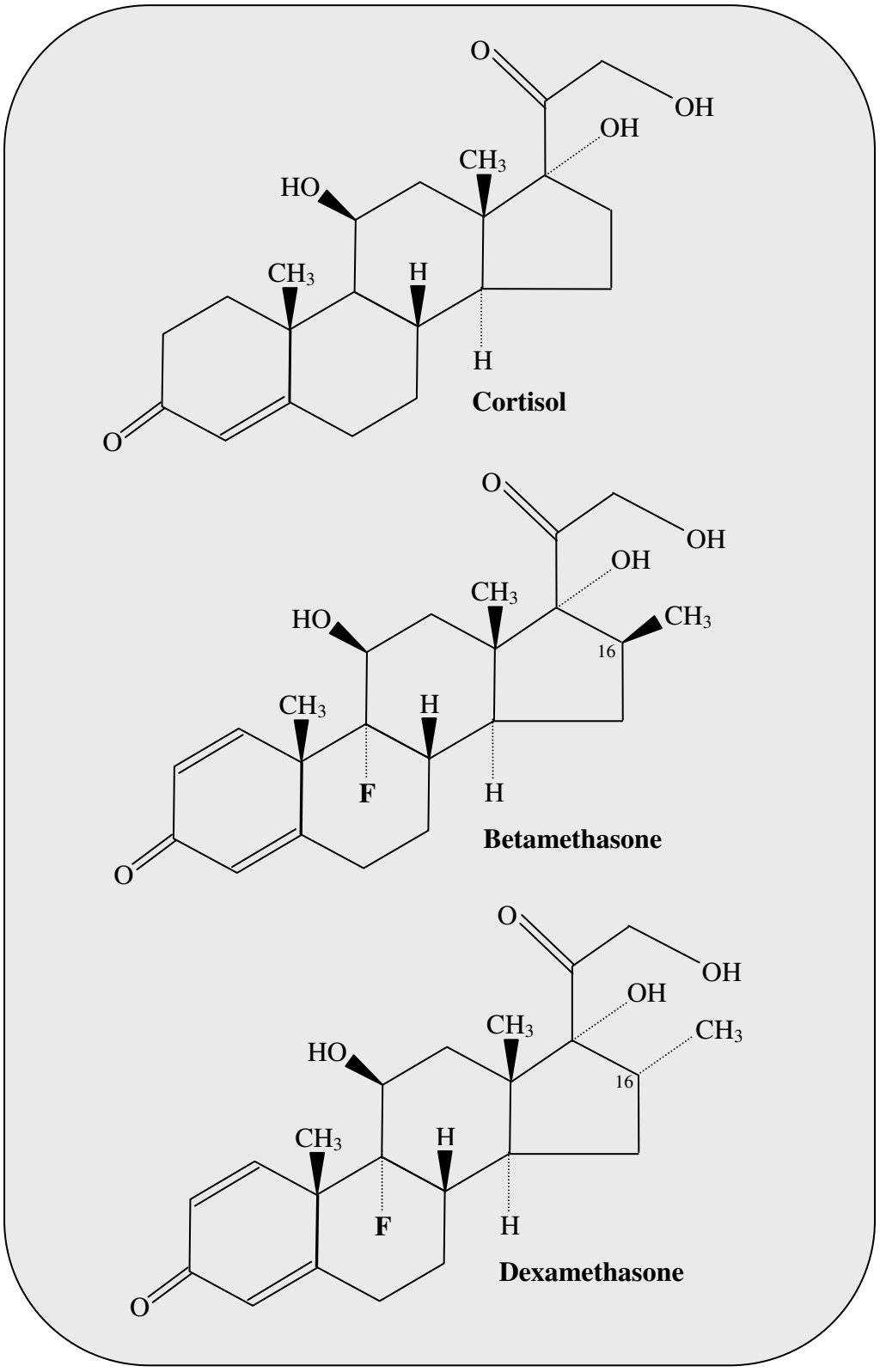


Figure 1.9. Molecular structure of cortisol and the synthetic analogues, betamethasone and dexamethasone. An extra double bond and fluorination at the 9α position increases glucocorticoid activity, decreases the rate of metabolism and prolongs the half-life of betamethasone and dexamethasone.

betamethasone is provided by hydrolysis of the phosphate ester and sustained activity is provided by the acetate ester, which is only slightly soluble and affords a repository for slow absorption (Ballard & Ballard, 1995).

In comparison to cortisol, betamethasone exerts a longer duration of action and is devoid of mineralocorticoid activity (NIH, 1995). The glucocorticoid potency (transcriptional activity) of betamethasone is 45 times greater than cortisol in CV-1 cells (African green monkey kidney cells) transfected with a plasmid containing the coding sequence for human GRα (Diederich *et al.*, 2004). In addition, betamethasone has 5.4-fold higher affinity for GRs compared to cortisol (Ballard & Ballard, 1995). Betamethasone has negligible mineralocorticoid potency compared to aldosterone in CV-1 cells transfected with human MR (Diederich *et al.*, 2004). Thus, betamethasone binds to GRs to stimulate increased glucocorticoid transcriptional activity without eliciting mineralocorticoid side-effects. Since betamethasone is only weakly oxidised by 11β-HSD-2, is not bound to CBG and does not bind to MR, it binds to GR virtually unhindered to elicit potent glucocorticoid effects.

1.5.2. Animal studies of glucocorticoid administration

Experimental studies in sheep have been used to mimic antenatal glucocorticoid administration in humans. In these studies, betamethasone was administered to pregnant ewes at gestational time-points equivalent to those at which antenatal glucocorticoid therapy is used in clinical practice. Administration of a dose of 0.5 mg/kg of betamethasone to the pregnant ewe or fetus results in improved postnatal lung function after pre-term delivery (Rebello *et al.*, 1996; Rebello *et al.*, 1997). However, the route and the amount administered are important when considering glucocorticoid effects on fetal growth. Direct fetal administration of single or repeated doses (0.5 mg/kg estimated fetal body weight) of glucocorticoids does not alter fetal growth (Jobe *et al.*, 1998a; Newnham *et al.*, 1999; Moss *et al.*, 2001; Moss *et al.*, 2003b). However, maternal betamethasone administration (0.5 mg/kg ewe body weight) in sheep reduces fetal body weight (Ikegami *et al.*, 1997; Jobe *et al.*, 1998a; Jobe *et al.*, 1998b; Newnham *et al.*, 1999; Sloboda *et al.*, 2000; Moss *et al.*, 2001; Moss *et al.*, 2003b). A single maternal dose of betamethasone administered at 104 days of gestation reduced fetal body weight by 11 to 20% at 125 days of gestation (Ikegami *et al.*, 1997; Jobe *et al.*,

1998a; Jobe *et al.*, 1998b; Newnham *et al.*, 1999) and by 14% at 145 days of gestation (Jobe *et al.*, 1998b). Repetitive maternal glucocorticoids, administered on a weekly basis for three weeks beginning at 104 days of gestation, significantly reduced fetal weight at 125 days of gestation by 23 to 27% (Ikegami *et al.*, 1997; Jobe *et al.*, 1998a; Jobe *et al.*, 1998b; Newnham *et al.*, 1999; Sloboda *et al.*, 2000), at 146 days of gestation by 19 to 22% (Jobe *et al.*, 1998b; Sloboda *et al.*, 2000; Moss *et al.*, 2003b) and by 34% after spontaneous delivery at approximately 154 days of gestation (Moss *et al.*, 2001).

Assuming linear growth in fetal sheep between 104 and 125 days of gestation (Barbera *et al.*, 1995), it has been calculated that the growth restriction observed after a single dose of maternal betamethasone is equivalent to four days of growth arrest and after repeated doses is equal to a period of growth arrest of approximately nine days (Jobe *et al.*, 1998b). Growth arrest is associated with the cessation of cell division, which has been demonstrated by the reduction in thymidine incorporation into DNA in the brain, lungs, kidneys, heart and adrenals of fetal guinea pigs treated with dexamethasone (Sanfacon *et al.*, 1977). The DNA content (g/kg body weight) was reduced in the liver and lungs of betamethasone-exposed sheep at 125 days of gestation, consistent with the notion that maternal betamethasone administration causes growth arrest in fetal sheep (Jobe *et al.*, 1998b).

In sheep, a single maternal dose of betamethasone is sufficient to induce lung maturation, improve cardiovascular outcome and enhance renal function in pre-term lambs (Rebello *et al.*, 1996; Berry *et al.*, 1997; Smith *et al.*, 2000). However, exposure to repeated doses of maternal betamethasone did not further improve renal and cardiovascular responses compared to a single dose in pre-term sheep (Smith *et al.*, 2003). Repeated prenatal exposure to maternal betamethasone in sheep reduces brain and organ weights (Jobe *et al.*, 1998b; Quinlivan *et al.*, 1998a; Huang *et al.*, 1999; Newnham *et al.*, 1999; Moss *et al.*, 2005), delays axonal myelination (Dunlop *et al.*, 1997) and retinal maturation (Quinlivan *et al.*, 2000), induces postnatal insulin resistance (Moss *et al.*, 2001) and alters postnatal HPA axis responsiveness (Sloboda *et al.*, 2002a). Prenatal exposure to betamethasone or dexamethasone also leads to vascular dysfunction in fetal and postnatal sheep (Derks *et al.*, 1997; Anwar *et al.*, 1999; Molnar *et al.*, 2002; Molnar *et al.*, 2003). These studies highlight the necessity of research into the long-term effects of antenatal glucocorticoid treatment in humans.

1.5.3. Antenatal glucocorticoid administration

Pre-term delivery occurs in 6 to 15% of all pregnancies (Slattery & Morrison, 2002). Pre-term infants have a high risk of developing respiratory distress syndrome (RDS), which is associated with increased neonatal morbidity and mortality. In 1969 Liggins demonstrated that intra-fetal dexamethasone infusions at 117 to 123 days of gestation caused persistent partial lung expansion in sheep born pre-term, suggesting premature functional and morphological maturation (Liggins, 1969). These findings prompted the first clinical trial of antenatal glucocorticoid therapy for pulmonary maturation to prevent RDS in premature infants, which began in 1969 (Liggins & Howie, 1972). Betamethasone treatment (two 12 mg doses of betamethasone administered i.m. 24 h apart) significantly reduced the incidence of RDS and neonatal mortality (Liggins & Howie, 1972).

Since the pioneering work of Liggins and Howie, many clinical trials have assessed the effects of antenatal glucocorticoid therapy on neonatal outcome (Liggins & Howie, 1972). In the 2003 Cochrane meta-analysis by Crowley, of the randomised trials published between 1972 and 1994, antenatal glucocorticoid therapy resulted in an overall 50% reduction in the odds of contracting neonatal RDS (Crowley, 1995). Babies delivered between 24 hours and seven days post-treatment showed a 65% reduction in RDS (typical odds ratio of RDS, 0.35; 95% confidence interval, 0.26-0.46) and neonatal mortality was also substantially reduced (typical odds ratio, 0.60; 95% confidence interval, 0.48-0.76; (Crowley, 1995)). The Cochrane meta-analysis provided conclusive evidence that antenatal glucocorticoid therapy reduced the rates of RDS, bronchopulmonary dysplasia and intra-ventricular haemorrhage in premature neonates (Crowley, 1995). In 1994, The National Institutes of Health Consensus Development Panel recommended that all fetuses between 24 and 34 weeks' gestation at risk of pre-term delivery should be considered for antenatal glucocorticoid therapy (NIH, 1995).

A single course of antenatal glucocorticoids has not been associated with any significant adverse maternal or fetal effects in humans (Penney & Cameron, 2004). Results from the follow-up study of the Auckland Steroid Trial (randomised controlled trial) reported that systolic and diastolic blood pressure were not different in 6-year-old children exposed to betamethasone compared to those exposed to placebo (Dalziel *et al.*, 2004). A small follow-up study of a randomised, double-blind, placebo-controlled trial of

betamethasone found that systolic blood pressure was significantly lower and diastolic blood pressure was not different in 20-year-olds exposed to a single course of prenatal betamethasone (Dessens *et al.*, 2000). Most recently, the 30-year follow-up study of the Auckland Steroid Trial reported that there was no difference in systolic or diastolic blood pressure in adults exposed to prenatal betamethasone or placebo (Dalziel *et al.*, 2005).

1.5.4. Repetitive antenatal glucocorticoid therapy

Previous studies have suggested that the efficacy of antenatal glucocorticoid therapy in humans is reduced seven days after the initial treatment (Liggins & Howie, 1972; Ballard *et al.*, 1975b). Liggins and Howie found no difference in the incidence of RDS in betamethasone-exposed and control infants (2.2% compared with 9.4%) delivered seven or more days after the initial glucocorticoid therapy (Liggins & Howie, 1972). A further study found that betamethasone levels were undetectable in maternal and cord serum by two days after glucocorticoid treatment (Ballard *et al.*, 1975b). A systematic review of seven randomised controlled trials reported that 40% of women treated with a single dose of glucocorticoids remain undelivered more than seven days after treatment allocation (McLaughlin *et al.*, 2003). Despite unknown safety and efficacy, the use of repeated weekly-doses of antenatal glucocorticoids for treatment of women with persistent threatened pre-term delivery became common practice world-wide (Planer *et al.*, 1996; Wing *et al.*, 1996; Quinlivan *et al.*, 1998b; Brocklehurst *et al.*, 1999; McLaughlin & Crowther, 2003).

Ninety-six percent of survey respondents from the USA Society of Perinatal Obstetricians (Planer *et al.*, 1996) and 98% of respondents surveyed in obstetric units in the UK (Brocklehurst *et al.*, 1999) prescribed repeated doses of antenatal glucocorticoids. A survey of fellows, members and trainees of the Royal Australian College of Obstetricians and Gynaecologists conducted in 1997 found that 85% of obstetricians prescribed repeated courses of antenatal glucocorticoids in cases with persistent or recurrent risk of pre-term delivery and 50% prescribed weekly doses in cases with persisting risk of pre-term birth (Quinlivan *et al.*, 1998b). In 2001, The National Institutes of Health Consensus Development Panel recommended that due to the lack of data regarding safety and efficacy, repeat courses of glucocorticoids should

not be used routinely and only reserved for patients enrolled in randomised controlled trials (NIH, 2001). A subsequent survey of the Royal Australian and New Zealand College of Obstetricians and Gynaecologists in 2001 found that 44% of obstetricians still recommended repeated antenatal glucocorticoids be given to women who remained at risk of pre-term delivery (McLaughlin & Crowther, 2003). Therefore, there exists a large population of individuals who have been exposed to repeated doses of antenatal glucocorticoids.

Concerns have been raised regarding the potential adverse effects of repetitive antenatal glucocorticoids in humans (Newnham & Moss, 2001). Non-randomised cohort studies in humans have reported reductions in birth weight (Reinisch *et al.*, 1978; Banks *et al.*, 1999; French *et al.*, 1999; Bloom *et al.*, 2001; Thorp *et al.*, 2002) and head circumference (French *et al.*, 1999; Abbasi *et al.*, 2000; Thorp *et al.*, 2002) in pre-term infants exposed to multiple doses of prenatal glucocorticoids. In contrast, the 2003 Cochrane review by Crowther and Harding, using data from two randomised controlled trials (Guinn *et al.*, 2001; McEvoy *et al.*, 2002), reported that there was no statistically significant difference in birth weight (or other primary outcomes excluding the incidence of severe lung disease and use of surfactant) in pre-term infants exposed to single or repeated prenatal glucocorticoids (Crowther & Harding, 2003). Crowther and Harding concluded that "there is insufficient evidence on the benefits and risks to recommend repeat dose(s) of prenatal corticosteroids for women at risk of preterm birth for the prevention of neonatal respiratory disease" (Crowther & Harding, 2003).

Several large multi-centred randomised controlled trials examining the effectiveness and safety of repeated prenatal glucocorticoids are currently in progress. The MACS (Multiple Courses of Antenatal Corticosteroids for Preterm Birth Study) trial, funded by the Canadian Institutes of Health Research (CIHR), has recruited patients from 20 countries including Canada, Argentina, Poland and Israel (MIRU, 2002). In the USA, the National Institute of Child Health and Human Development (NICHD) Maternal Fetal Medicine Units (MFMU) Network has recently published the first paper from their randomised placebo-controlled trial of antenatal corticosteroid regimens (Wapner *et al.*, 2006). The NICHD MFMU Network reported that there was no significant difference in mean birth weight or head circumference; however, the repeat group had a reduction in multiples of the birth weigh median by gestational age and more neonates weighing less

than the 10th percentile (Wapner *et al.*, 2006). In Australia and New Zealand, the ACTORDS (Australasian Collaborative Trial of Repeated Doses of Corticosteroids for the Prevention of Neonatal Respiratory Disease) trial has recently published their neonatal outcomes (Crowther *et al.*, 2006). In the ACTORDS trial, mean birth weight and head circumference did not differ between placebo and repeat corticosteroid groups; however, z-scores for weight and head circumference at birth were lower in the babies exposed to repeated corticosteroids (Crowther *et al.*, 2006). The results of these trials will provide the obstetric and scientific communities with high quality data that will help elucidate the short- and long-term effects of repeated prenatal glucocorticoids in humans.

1.6. DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE

Exposure to an adverse intra-uterine environment, characterised by low birth weight, has been shown to be associated with an increased risk of developing adult cardiovascular and metabolic disorders. Numerous epidemiological studies have demonstrated associations between low birth weight and an increased risk of developing hypertension (Curhan *et al.*, 1996a; Curhan *et al.*, 1996b), cardiovascular disease (Rich-Edwards *et al.*, 1997), insulin resistance (Fall *et al.*, 1995; Lithell *et al.*, 1996), hyperglycaemia (Levitt *et al.*, 2000), diabetes mellitus (Hales *et al.*, 1991; Curhan *et al.*, 1996b; Lithell *et al.*, 1996) and hyperlipidemia (Fall *et al.*, 1995) in adult life. Furthermore, many studies have found that low birth weight is associated with increased activity of the HPA axis in adult life, characterised by elevated plasma levels of cortisol (Levitt *et al.*, 2000; Phillips *et al.*, 2000; Reynolds *et al.*, 2001).

1.6.1. Fetal programming and glucocorticoids

The term “fetal programming” was coined to describe the process by which fetal adaptations to a sub-optimal intra-uterine environment during a critical period of development leads to adult disease (Seckl, 1998). It is hypothesised that the programming of fetal tissues results in permanent changes in gene expression, tissue structure and/or organ function that contribute to, or predisposes the individual to, the development of adult-onset diseases.

In the early 1990s, follow-up studies of children and adults born in England revealed for the first time that the intra-uterine environment contributed to elevated blood pressure (Barker *et al.*, 1990; Law *et al.*, 1991; Law *et al.*, 1993; Law & Barker, 1994), impaired glucose tolerance (Hales *et al.*, 1991), and an increased risk of developing coronary heart disease (Barker, 1996) and syndrome X (diabetes mellitus, hypertension and hyperlipidemia) (Barker *et al.*, 1993). Barker and colleagues reported that the highest blood pressures occurred in men and women who had been small babies with large placentae (Barker *et al.*, 1990). Recently, a systematic review of 80 studies (including more than 444,000 people aged 0- to 84-years of age) found that blood pressure fell by 2 mmHg with each 1 kg increase in birth weight (Huxley *et al.*, 2000). Therefore, birth weight is inversely related to systolic blood pressure. Law and co-authors reported that the relationship between systolic blood pressure and birth weight is established within the first few months of life and becomes stronger with increasing age, suggesting that high blood pressure is initiated by processes associated with reduced growth *in utero* and becomes amplified in later life (Law *et al.*, 1993).

Increased fetal glucocorticoid exposure may underpin the link between low birth weight and adult disease (Seckl, 1997; Seckl *et al.*, 1999; Seckl *et al.*, 2000; Seckl, 2001, 2004). Normally during gestation, placental 11β-HSD-2 protects the fetus from high levels of maternally-derived glucocorticoids (Beitins *et al.*, 1973; Benediktsson *et al.*, 1997). Studies in rats (Benediktsson *et al.*, 1993; Lindsay *et al.*, 1996; Langley-Evans, 1997b), humans (Benediktsson *et al.*, 1995; Stewart *et al.*, 1995; McTernan *et al.*, 2001) and sheep (Kerzner *et al.*, 2002) have demonstrated that placental 11β-HSD-2 activity is inversely related to birth weight. These studies suggest that increased fetal exposure to maternal glucocorticoids, due to reduced placental 11β-HSD-2 activity, may contribute to the reduction in birth weight (Edwards *et al.*, 1996). Furthermore, animal experiments using maternal low-protein diet (Langley & Jackson, 1994; Langley-Evans *et al.*, 1996; Bertram *et al.*, 2001), synthetic glucocorticoid administration (Benediktsson *et al.*, 1993; Levitt *et al.*, 1996; Celsi *et al.*, 1998; Wyrwoll *et al.*, 2006) or 11β-HSD-2 inhibition (Lindsay *et al.*, 1996; Langley-Evans, 1997b) have demonstrated reduced birth weight and hypertension in adult offspring. The common link between these models is increased fetal glucocorticoid exposure.

Antenatal glucocorticoid therapy in humans has been associated with reduced birth weight in a number of non-randomised studies (Reinisch *et al.*, 1978; Banks *et al.*, 1999; French *et al.*, 1999; Bloom *et al.*, 2001; Thorp *et al.*, 2002). The long-term effects of prenatal glucocorticoid exposure on adult-onset diseases, such as hypertension, are uncertain. The literature contains conflicting data concerning the effect of antenatal glucocorticoid therapy on blood pressure in humans. Blood pressure is higher in infants (during the first three days of life) exposed to a single course of dexamethasone (Kari *et al.*, 1994) and in adolescents at 14-years of age born pre-term (with a birth weight less than 1501 g) after maternal treatment with a single course of betamethasone (Doyle *et al.*, 2000). More recently, randomised controlled trials of single course antenatal glucocorticoid therapy reported that blood pressure was not higher in 6-year-old (Dalziel *et al.*, 2004), 20-year-old (Dessens *et al.*, 2000) and 30-year-old humans (Dalziel *et al.*, 2005). The effect of prenatal glucocorticoid exposure on blood pressure in older adults has not been reported. Further, the effect of repeated prenatal glucocorticoid exposure on blood pressure in humans remains unknown. It is possible that repeated antenatal glucocorticoid therapy may program postnatal blood pressure in human populations.

1.6.2. Nephron number and blood pressure

Nephrogenesis ceases prior to birth in humans and sheep and therefore at term, each kidney contains its full complement of nephrons (Robillard *et al.*, 1981; Brion *et al.*, 1997). A recent stereological study of nephron number at autopsy in human kidneys from Australian Aborigines, Australian non-Aborigines, US blacks and US whites at ages from birth to 84-years-old found that nephron number ranged from 210,332 to 1,825,380 and was independent of age (Hoy *et al.*, 2003). Using unbiased stereological techniques, previous investigators have reported that nephron number in sheep ranges from 365,672 ± 36,016 (mean ± S.E.M.) at 130 days of gestation in Merinos (Douglas-Denton *et al.*, 2002) to 559,000 ± 198,000 (mean ± S.D.) at 140 days of gestation (Mitchell *et al.*, 2004) and 333,832 ± 69,560 (mean ± S.D.) at eight weeks of postnatal life (Brown *et al.*, 2002) in Border Leicester X Merinos. Based on the above observations, there appears to be a large degree of variability in total nephron number within the normal population and between species.

In 1988, Brenner and colleagues proposed that an inverse relationship existed between total nephron number and the subsequent risk of developing hypertension (Brenner *et al.*, 1988). They hypothesised that a decreased filtration surface area (FSA), due to a reduced number of nephrons and/or a decrease in FSA per glomerulus, lead to renal sodium retention that increased mean arterial blood pressure and resulted in glomerular hypertension and eventual glomerular sclerosis (Brenner *et al.*, 1988). Furthermore, it was hypothesised that low birth weight increased the risks for systemic and glomerular hypertension based on observations suggesting: (1) a direct relationship between birth weight and nephron number; (2) an inverse relationship between birth weight and adult hypertension; and (3) an inverse relationship between nephron number and blood pressure (Brenner & Chertow, 1994). In addition, it was proposed that adult blood pressure could be programmed *in utero* by altering fetal nephron endowment (Mackenzie *et al.*, 1996).

A recent study of nephron number in kidneys of patients with primary hypertension has provided strong evidence that a congenital nephron deficit contributes to the development of essential hypertension (Keller *et al.*, 2003). Using unbiased stereological methods, Keller and colleagues compared the number and volume of glomeruli in 10 middle-aged white patients with a history of primary hypertension with those of 10 normotensive subjects that were matched for sex, age, height and weight (Keller *et al.*, 2003). They found that patients with hypertension had 46.6% fewer glomeruli that were 133% larger than matched normotensive controls (Keller *et al.*, 2003). Few obsolescent glomeruli were observed suggesting a congenital nephron deficit in the hypertensive patients (Keller *et al.*, 2003), supporting Brenner's hypothesis.

Congenital nephron deficit (oligonephropathy) at birth is also associated with the development of hypertension in mice. GDNF-heterozygous mice have approximately 30% fewer nephrons than wild-type mice at age 1 and 14 months (Cullen-McEwen *et al.*, 2001; Cullen-McEwen *et al.*, 2003). MAP was significantly higher in GDNF-heterozygous mice than wild-type mice; however, GFR and renal blood flow were similar between the groups (Cullen-McEwen *et al.*, 2003). GDNF-heterozygous mice had hypertrophied glomeruli and a much greater calculated single nephron glomerular filtration rate (SNGFR) compared with wild-type mice (Cullen-McEwen *et al.*, 2003).

Glomerular hypertrophy and increased SNGFR are compensatory mechanisms to maintain normal GFR in the presence of a reduced nephron endowment (Cullen-McEwen *et al.*, 2003). These adaptations, however, are associated with increased glomerulosclerosis, leading to further loss of nephrons and a self-perpetuating vicious cycle of hypertension and progressive renal injury (Hostetter *et al.*, 2001).

Uni-nephrectomy of fetal sheep (Moritz *et al.*, 2002c) and neonatal rats (Woods, 1999) reduces nephron endowment and causes hypertension in adult offspring. Removal of one kidney in sheep at 100 days of gestation resulted in the animals having significantly higher MAP and lower GFR at age 6 and 12 months compared with sham-operated controls (Moritz *et al.*, 2002c). Similarly, uni-nephrectomy of newborn rats led to reduced GFR and increased MAP at age 22 weeks (Woods, 1999). Compensatory renal growth has been observed in fetal sheep at 130 days of gestation after unilateral nephrectomy some 30 days earlier (Douglas-Denton *et al.*, 2002). The weight of the remnant kidney increased by 34% in uni-nephrectomised fetal sheep compared to controls and contained 45% more nephrons (365,672 in controls versus 530,763 in uni-nephrectomised animals) (Douglas-Denton *et al.*, 2002). However, assuming that each kidney contained the same number of nephrons (< 3% variability exists between the left and right kidney (Hinchliffe *et al.*, 1991)), uni-nephrectomised sheep still had 27% fewer nephrons overall compared to controls. Further, alterations in the RAS may have contributed to the development of hypertension as plasma renin activity was significantly lower in uni-nephrectomised sheep at age 12 months (Moritz *et al.*, 2002c). Low-renin hypertension is associated with increased sodium retention (Pratt, 2000) and uni-nephrectomised sheep tended to excrete less sodium at age 6 and 12 months (Moritz *et al.*, 2002c). Thus, alterations in nephron endowment and the RAS may contribute to the development of high blood pressure in experimental studies of uni-nephrectomy in sheep.

Compared to fetal uni-nephrectomy, a reduction in nephron number in adult life, such as the removal of a kidney after trauma or for donation, is generally not related to the onset of hypertension in humans, contradicting Brenner's hypothesis. A 25-year follow-up of patients aged between 39- and 64-years-old, who had undergone donor nephrectomy, found no evidence of renal functional deterioration or increased prevalence of hypertension (Goldfarb *et al.*, 2001). Similarly, a 45-year follow-up of United States

Army personnel, who underwent uni-nephrectomy after kidney trauma, found that the prevalence of hypertension was not increased (Narkun-Burgess *et al.*, 1993). A cross-sectional follow-up of 348 patients aged between 28- and 94-years of age, who had donated a kidney two to 33 years earlier, found that although hypertension was present in 38% of donors, the age-adjusted prevalence was not higher than the general population (Fehrman-Ekholm *et al.*, 2001). Mean 24-hour blood pressure values were similar in uni-nephrectomised children compared to age-, height- and weight-matched controls; however, children born with unilateral renal agenesis had elevated mean 24-hour systolic blood pressure compared to their matched controls (Mei-Zahav *et al.*, 2001). Therefore, the timing of the reduction in nephron number is critical in determining the impact on blood pressure. It appears that if the reduction is the result of impaired nephrogenesis *in utero* (i.e. renal agenesis), compensatory mechanisms in the remnant kidney may contribute to the development of hypertension in adult life.

1.6.3. Programming of nephron number and blood pressure

Previous studies in humans have demonstrated a direct relationship between total nephron number and birth weight (Hinchliffe *et al.*, 1992; Manalich *et al.*, 2000; Hughson *et al.*, 2003; Hughson *et al.*, 2006). Low birth weights are associated with reduced nephron endowment and increased risk of developing hypertension (Brenner & Chertow, 1994; Mackenzie *et al.*, 1996; Amann *et al.*, 2004). Furthermore, various animal models have demonstrated that fetal nephron endowment is influenced by maternal nutrition, intra-uterine growth restriction (IUGR) and glucocorticoid administration (Kett & Bertram, 2004).

Fetal over-exposure to glucocorticoids may be the common pathway by which maternal low-protein diet and IUGR could influence nephron endowment. Maternal low-protein diets increase fetal glucocorticoid exposure by down-regulating placental 11β-HSD-2 (Langley-Evans *et al.*, 1996; Bertram *et al.*, 2001; Lesage *et al.*, 2001). IUGR is also associated with increased fetal glucocorticoid exposure due to reduced placental 11β-HSD-2 activity (Lindsay *et al.*, 1996; Shams *et al.*, 1998; McTernan *et al.*, 2001). Placental 11β-HSD-2 mRNA levels were significantly reduced in human IUGR pregnancies when compared to placentae from gestationally-matched, appropriately grown infants (Shams *et al.*, 1998; McTernan *et al.*, 2001). In animal experiments,

maternal low-protein diet and IUGR increase fetal glucocorticoid exposure, and are associated with reduced nephron endowment.

1.6.3.1. Intra-uterine growth restriction

Naturally occurring intra-uterine growth restriction (IUGR) is associated with reduced nephron endowment at birth in humans (Hinchliffe *et al.*, 1991), sheep (Bains *et al.*, 1996; Mitchell *et al.*, 2004) and pigs (Bauer *et al.*, 2002). Human fetuses with type II (asymmetrical) IUGR have 35% fewer nephrons than controls (Hinchliffe *et al.*, 1991). Piglets with birth weights between the fifth and tenth percentiles had 27% fewer nephrons than piglets with weights above the 40th percentile (Bauer *et al.*, 2002). A stereological study to estimate the absolute number of glomeruli in the kidneys of lambs found that IUGR reduced nephron number by 35% (Bains *et al.*, 1996). However, the design of this study precluded the authors from separating the effects of IUGR and maternal under-nutrition on nephron number, and all animals used in the study died from natural causes within 96 hours of birth (Bains *et al.*, 1996). IUGR in sheep, due to natural twinning or induced by umbilico-placental embolisation (UPE) with microspheres from days 120 to 140 of gestation, reduced body weight compared with controls by 34% in the UPE fetuses and by 28% in the twins (Mitchell *et al.*, 2004). The total number of nephrons in the kidneys of twin sheep was 40% lower than in controls, but UPE failed to affect nephron endowment (Mitchell *et al.*, 2004). These findings demonstrate that the timing and duration of IUGR are important determinants of nephron endowment in fetal sheep.

In rats, IUGR induced by partial uterine artery ligation on day 17 of gestation (term is 21.5 days) or maternal low-protein diet (5% protein) from days 8 to 21 of gestation reduced birth weight by approximately 30% and reduced total nephron number by 30% and 17% (respectively) in offspring at age 2 weeks (Merlet-Benichou *et al.*, 1994). A similar reduction in nephron endowment (22%) was seen after bilateral uterine artery ligation in pregnant rats on day 19 of gestation, which was associated with increased renal apoptosis (Pham *et al.*, 2003). Futhermore, ligation of 25 to 30% of the utero-placental vessels in pregnant rabbits during the last third of gestation induced type II IUGR and resulted in fetuses that were approximately 25% smaller with approximately

28% fewer nephrons than controls (Bassan *et al.*, 2000). Thus, experimental IUGR reduces nephron endowment in many species.

1.6.3.2. Maternal low-protein diet

Fetal adaptations to a sub-optimal intra-uterine environment, such as that induced by maternal under-nutrition, may contribute to an acquired nephron deficit. Feeding pregnant rats low-protein diets (6 to 9% protein) has been associated with reduced birth weight (Zimanyi *et al.*, 2000; Vehaskari *et al.*, 2001; Woods *et al.*, 2001), reduced (by 16 to 41%) nephron endowment (Merlet-Benichou *et al.*, 1994; Langley-Evans *et al.*, 1999; Zimanyi *et al.*, 2000; Vehaskari *et al.*, 2001; Woods *et al.*, 2001; Welham *et al.*, 2002; Almeida & Mandarim-de-Lacerda, 2005) and increased blood pressure in rat offspring (Langley-Evans *et al.*, 1999; Vehaskari *et al.*, 2001; Woods *et al.*, 2001; Almeida & Mandarim-de-Lacerda, 2005). In sheep, maternal nutrient restriction during early gestation (days 30 to 70 of gestation) was demonstrated to reduce nephron endowment by approximately 25% in 14-day-old lambs (Langley-Evans *et al.*, 2003a). Welham and colleagues demonstrated that a maternal low-protein diet during pregnancy was associated with increased apoptosis in mesenchymal cells at the start of metanephrogenesis in rat offspring (Welham *et al.*, 2002). Therefore, increased deletion of mesenchymal precursors at the start of nephrogenesis may lead to the generation of significantly fewer nephrons in the maternal low-protein diet model (Welham *et al.*, 2002).

Recent evidence suggested that the under-nutrition-related reduction in nephron number was not causally linked to increased blood pressure at age 4 weeks in rats (Langley-Evans *et al.*, 2003b). Systolic blood pressure was increased in offspring exposed to a maternal low-protein diet, and to a maternal low-protein diet supplemented with urea or alanine; although supplementation of the low-protein diet with glycine prevented the appearance of high blood pressure in offspring (Jackson *et al.*, 2002). The acquired nephron deficit in offspring exposed to a maternal low-protein diet was normalised by the addition of urea, alanine or glycine supplements to the pregnant rats' feed (Marchand *et al.*, 2001; Langley-Evans *et al.*, 2003b). Therefore, rat offspring exposed to maternal low-protein diets supplemented with urea or alanine had a normal nephron complement but increased blood pressure. These data suggest that factors aside from a

reduction in nephron endowment must contribute to the pathogenesis of hypertension in this model.

The RAS has been implicated in the elevation of blood pressure in rat offspring exposed to maternal low-protein diet. Postnatal treatment with losartan (a specific AngII receptor antagonist) between age 2 and 4 weeks prevented increased systolic blood pressure in 4- and 12-week-old rats that had been exposed to a maternal low-protein diet *in utero* (Sherman & Langley-Evans, 2000). Similarly, captopril (an ACE inhibitor) treatment of rats between age 2 and 4 weeks, or age 10 to 12 weeks, significantly reduced the elevation in blood pressure in offspring at age 4 and 12 weeks whose mothers were fed a low-protein diet during pregnancy (Sherman & Langley-Evans, 1998). Therefore, components of the RAS may mediate the hypertensive effects of prenatal exposure to a maternal low-protein diet.

1.6.3.3. Glucocorticoid administration

Fetal exposure to elevated levels of glucocorticoids accelerates renal maturation leading to growth arrest and premature termination of differentiation (Epstein *et al.*, 1977; Sanfacon *et al.*, 1977) that may result in reduced nephron endowment. Synthetic glucocorticoid administration in pregnant sheep (Wintour *et al.*, 2003; Figueroa *et al.*, 2005) and rats (Celsi *et al.*, 1998; Ortiz *et al.*, 2001; Ortiz *et al.*, 2003) has been shown to reduce nephron endowment and increase blood pressure in adult offspring. Dexamethasone administration to pregnant ewes on approximately day 27 of pregnancy resulted in offspring that were hypertensive at age 4, 10 and 19 months (Dodic *et al.*, 1998). At 7-years of age, those sheep exposed to dexamethasone *in utero* had 38% fewer nephrons and higher MAP compared with control animals (Wintour *et al.*, 2003). These hypertensive sheep also had altered cardiovascular haemodynamics and baroreceptor-heart rate reflexes, impaired cardiac functional reserve and left ventricular hypertrophy (Dodic *et al.*, 1999; Dodic *et al.*, 2001). Prenatal exposure to a single course of betamethasone at 80 days of gestation in sheep reduced nephron number by approximately 25% at 135 days of gestation and increased MAP at age 6 months, but was not associated with changes in GFR, total renal plasma flow or heart rate (Figueroa *et al.*, 2004; Figueroa *et al.*, 2005).

In rats, maternal administration of dexamethasone (0.1 mg/kg) throughout gestation resulted in pups that were 30% smaller at birth, had 60% fewer nephrons at age 20 days and had a lower GFR and elevated MAP at age 60 days compared with control rats (Celsi *et al.*, 1998). Another study using maternal dexamethasone treatment (0.1 mg/kg) throughout gestation reported a 13% reduction in nephron number in adult male rats at 65- to 75-days-old; however this was not associated with alterations in MAP, renal vascular resistance, renal blood flow, filtration fraction or GFR (Martins *et al.*, 2003). Dexamethasone (0.2 mg/kg), when administered on days 15 and 16 of gestation, caused a 30% reduction in nephron number and hypertension but did not alter GFR in rat offspring at 60 to 70 days of age (Ortiz *et al.*, 2001). Similarly, adult rats that were exposed to dexamethasone on days 17 and 18 of gestation had an approximate 20% reduction in nephrons but no change in GFR compared with controls and the males were hypertensive (Ortiz *et al.*, 2001). Hypertension was also observed in adult male rats administered dexamethasone on days 13 and 14 of gestation although they did not have a reduction in nephron number (Ortiz *et al.*, 2003). In contrast, maternal dexamethasone administration (125 μg/kg for 60 h) in pregnant spiny mice from days 20 to 23 of gestation (term is 40 d) significantly reduced nephron number in adult offspring but did not induce hypertension (Dickinson *et al.*, 2005b). It appears then that a reduction in nephron endowment may be only one factor that contributes to the development of hypertension in offspring of animals exposed to maternal glucocorticoid treatment.

The timing of the glucocorticoid treatment is critical in determining the degree of reduction in nephron endowment. In rats, nephrogenesis commences at 12 to 13 days of gestation and continues until approximately one week after birth (Merlet-Benichou *et al.*, 1994; Ortiz *et al.*, 2001). Prenatal dexamethasone exposure has been shown to reduce nephron endowment and increase blood pressure when given during a discrete window (days 15 to 18) of gestation in rats (Ortiz *et al.*, 2001). Treatment before (days 11 to 14 of gestation) or after (days 19 to 21 of gestation) this period did not affect nephron number in adult rats (Ortiz *et al.*, 2001). Dexamethasone administration to pregnant ewes on approximately day 27 of pregnancy reduced nephron number in adult offspring by 38% (Wintour *et al.*, 2003). The metanephros of the sheep fetus does not contain glomeruli and the ureteric bud has branched only once at 27 days of gestation (Wintour *et al.*, 2003). Glucocorticoid administration early in gestation reflects a developmental disruption that permanently alters nephron endowment prior to the

development of the functioning metanephros. The effect of glucocorticoid treatment in late gestation on nephron endowment in fetal sheep is unknown.

Despite animal studies demonstrating reduced fetal nephron endowment after synthetic glucocorticoid administration, there have been no studies examining nephron number after prenatal glucocorticoid treatment in humans. Pregnant women may be treated with dexamethasone (20 μg/kg/day) if suspected of carrying a fetus with virilising congenital adrenal hyperplasia, to suppress fetal adrenal androgen production (White & Speiser, 2000). In obstetric practice, synthetic glucocorticoids are routinely administered to women at risk of pre-term delivery between 24 and 34 weeks' (60 – 85%) gestation to promote maturation of the fetus in preparation for extra-uterine life (NIH, 1995). Previous studies have demonstrated that prenatal glucocorticoids induce growth arrest through the premature termination of cellular differentiation (Epstein *et al.*, 1977; Sanfacon *et al.*, 1977; Slotkin *et al.*, 1992; Jobe *et al.*, 1998). Further, antenatal glucocorticoid therapy is administered during the last trimester when 60% of nephrons are formed (Hinchliffe *et al.*, 1991). Therefore, prenatal glucocorticoid exposure during development may alter kidney structure or function that could contribute to renal abnormalities that predispose individuals to developing chronic kidney disease or hypertension in later life.

1.7. SUMMARY AND THESIS AIMS

The studies comprising this thesis examine the effects of late-gestation, prenatal betamethasone exposure on the ovine kidney. The short- and long-term renal outcomes after prenatal glucocorticoid exposure, in relation to structure and/or the expression of glucocorticoid-sensitive factors, were assessed in the kidneys of fetal and adult sheep. The overall aim was to advance our understanding of the effects of late-gestation glucocorticoid exposure on the kidney, specifically to elucidate the impact on renal development and the possible programming of hypertension in adult life. The specific aims of the studies in this thesis are addressed in each of the experimental chapters.

Chapter 3: **Nephron endowment in fetal sheep: the effect of sex and repeated maternal betamethasone administration.**

This chapter examines the effects of repeated late-gestation maternal betamethasone administration on nephron number and glomerular size in term fetal sheep. In sheep, early-gestation maternal administration of dexamethasone reduced nephron number and increased glomerular size in the kidneys of adult offspring (Wintour *et al.*, 2003); however, the effect of late-gestation maternal betamethasone administration on these parameters is unknown. It is important to examine the effect of late-gestation maternal betamethasone administration on nephron endowment as this treatment is used in obstetric practice and the effects on the human kidney remain unknown.

A further study examined whether sex differences existed in nephron number or glomerular size in late-gestation fetal sheep. The effect of sex on renal structure in Merino sheep is unknown.

Chapter 4: **The effects of maternal betamethasone administration on the fetal kidney.**

This chapter examines the effect of maternal betamethasone administration on the expression of various glucocorticoid-sensitive factors in the fetal kidney. Pregnant Merino ewes were administered betamethasone on days 104, 111 and 118 of pregnancy and kidneys were collected from male fetal sheep at 109, 116, 121 and 146 days of gestation. The collection of kidneys at these specific time-points enabled examination of the effects of single or multiple doses of maternal betamethasone on the developing kidney. Western blot analysis was used to measure protein levels of GR and Na^+/K^+-ATPase-α_1 and immunohistochemistry was used to localise GR-IR cells within sections of fetal kidneys. Relative gene expression of GR, MR, 11β-HSD-2, Na^+/K^+-ATPase-α_1, A_o, renin, AT_1 and AT_2 receptors were measured in the fetal sheep kidneys using real-time RT-PCR. These studies enabled examination of the effect of late-gestation glucocorticoid exposure on the expression of factors involved in renal glucocorticoid hormone action and components of the RAS.

Chapter 5: **The effects of prenatal betamethasone exposure on the adult kidney.**

Increased fetal glucocorticoid exposure has been implicated in the development of adulthood diseases. The aim of this chapter was to investigate the effect of prenatal, maternal or fetal, glucocorticoid administration on the expression of glucocorticoid-sensitive factors in the adult kidney. Betamethasone was administered to pregnant ewes or directly to the fetus on days 104, 111, 118 and 125 of gestation. Kidneys were collected from adult offspring at age 3.5 years for molecular analyses. Protein levels of GR and Na^+/K^+-ATPase-α_1 were measured by Western blot analysis and GR-IR cells were localised within sections of adult kidneys using immunohistochemistry. Real-time RT-PCR was used to measure relative gene expression of GR, MR, 11β-HSD-2, Na^+/K^+-ATPase-α_1, A_o, renin and AT_1 receptor in the adult sheep kidneys. These studies enabled assessment of whether there were any long-term effects of late-gestation glucocorticoid exposure on the adult kidney and if the route of administration further affected these results.

2. General Methods

2.1. SHEEP HUSBANDRY

All experimental procedures were approved by the Animal Experimentation Ethics Committee of The Western Australian Department of Agriculture. Pregnant date-mated Merino ewes were bred at the Western Australia Department of Agriculture's Mt Barker Research Support Unit. Ultrasonography was performed at approximately 50 days of pregnancy (d; term is 150 days) to confirm that the ewes were pregnant with a single fetus; ewes pregnant with twins were not used. Prior to the commencement of treatment, ewes were transported to the Western Australia Department of Agriculture's Medina Research Support Unit. Ewes were maintained in a field environment and brought into an adjacent shed or pen for injections. After the injections, pregnant ewes were returned to the paddocks. Some ewes were allowed to deliver spontaneously in a field environment (Moss *et al.*, 2001) and other animals were scheduled for operative delivery of fetal sheep.

Two cohorts of sheep are described in the studies within this thesis. The first cohort consisted of 3.5-year-old adult sheep, born after maternal or fetal injections of saline or betamethasone at 104, 111, 118 and 125 d (Chapter 5). These adult sheep were enrolled in a long-term study conducted by Dr Timothy Moss and Prof. John Newnham that examined the postnatal effects of prenatal glucocorticoid exposure (Moss *et al.*, 2001; Sloboda *et al.*, 2002a; Moss *et al.*, 2005; Sloboda *et al.*, 2005). The second cohort consisted of fetal sheep, delivered at 109, 116, 121 or 146 d after maternal injections of saline or betamethasone at 104, 111 and 118 d (Chapter 4). These fetal sheep were part of an ontogeny study, conducted by Dr Deborah Sloboda, which examined the effects of prenatal glucocorticoids on the fetal sheep pancreas.

2.2. TREATMENT PROTOCOL

A synthetic derivative of progesterone, medroxyprogesterone acetate (MPA; Depo-Provera®; Upjohn, Rydalmere, NSW, Australia), was administered intra-muscularly (i.m.) to pregnant ewes at a dose of 150 mg at approximately 100 d. This standard treatment is used in sheep to prevent subsequent spontaneous abortion after betamethasone administration (Jenkin *et al.*, 1985; Nathanielsz *et al.*, 1988; Moss *et al.*, 2001; Sloboda *et al.*, 2002a). In the adult study, MPA injections were administered by

Dr Timothy Moss. Mr Shaofu Li administered MPA to pregnant ewes in the fetal studies. After MPA treatment, ewes were randomised into control (saline) or betamethasone-treated groups. Ewes or fetuses in the control groups were injected i.m. with 5 mL of normal saline (0.9% NaCl) at each injection time-point.

2.2.1. Celestone® Chronodose®

Celestone® Chronodose® (Schering-Plough Pty Ltd, Baulkham Hills, NSW, Australia) is an injectable suspension consisting of soluble and slightly soluble esters of betamethasone. Each millilitre of the preparation contains 5.7 mg betamethasone; with 3.9 mg betamethasone sodium phosphate and 3 mg betamethasone acetate (Schering-Plough Pty Ltd). Of the two esters, betamethasone sodium phosphate is freely soluble while betamethasone acetate is practically insoluble. Therefore, betamethasone sodium phosphate is absorbed rapidly into tissues and provides prompt glucocorticoid action whereas the acetate ester provides sustained activity with a slow absorption rate.

2.2.2. Maternal betamethasone administration

Maternal betamethasone administration in sheep has been described previously (Jobe *et al.*, 1993; Ikegami *et al.*, 1997; Jobe *et al.*, 1998b; Newnham *et al.*, 1999; Moss *et al.*, 2001; Sloboda *et al.*, 2002a). Briefly, pregnant ewes were weighed at each treatment time-point and then injected in the rump with betamethasone (Celestone® Chronodose®; Schering-Plough Pty Ltd) at a dose of 0.5 mg/kg body weight. Each dose of betamethasone was approximately 27 mg, which correlates with the total dose of betamethasone used in clinical practice (Liggins & Howie, 1972). Betamethasone doses of 0.5 mg/kg (maternal or fetal weight) are the minimal doses required to consistently improve pre-term lung function in sheep (Ikegami *et al.*, 1997) and have been shown previously to cause growth restriction if injected maternally, but not directly to the fetus (Newnham *et al.*, 1999; Moss *et al.*, 2001).

Adult study

Adult sheep in the M1 group had been exposed to a single maternal i.m. injection of betamethasone at 104 d. Sheep in the M4 group had been exposed to repeated maternal injections of betamethasone at 104, 111, 118 and 125 d. Maternal i.m. betamethasone injections in the adult study were performed by Dr Timothy Moss.

Fetal study

In the fetal study, pregnant ewes received one, two or three doses of betamethasone. Ewes in the single-dose group were injected at 104 d. Ewes in the two-dose group received i.m. betamethasone injections at 104 and 111 d, and those in the three-dose group received injections at 104, 111 and 118 d. Maternal betamethasone injections were performed by Mr Shaofu Li with assistance from myself and Dr Deborah Sloboda.

2.2.3. Direct fetal betamethasone administration

Direct fetal injections were performed using an established technique (Jobe *et al.*, 1993; Newnham *et al.*, 1994; Newnham *et al.*, 1999; Moss *et al.*, 2001). In brief, the pregnant ewe was held in a sitting position and the fetus was imaged with a 3.5 MHz sector transducer (Echo Camera SSD-500; Aloka Co Ltd, Tokyo, Japan) using 70% ethanol as a coupling medium. Betadine solution (Faulding, Salisbury South, SA, Australia) was applied to the maternal abdomen before inserting a 21 gauge 9 cm spinal needle (Terumo, Macquarie Park, NSW, Australia) into the fetal shoulder or rump. Betamethasone (0.5 mg/kg estimated fetal body weight: 1.4 kg at 104 d, 1.9 kg at 111 d, 2.2 kg at 118 d and 2.5 kg at 125 d) was injected whilst continually observing the needle tip using ultrasonography (Moss *et al.*, 2001).

Direct fetal injections were only used in the adult cohort study and were performed by Dr Timothy Moss and Prof. John Newnham. Adult sheep in the F1 group had received an i.m. injection of betamethasone at 104 d and saline injections at 111, 118 and 125 d. Those in the F4 group had received weekly injections of betamethasone at 104, 111, 118 and 125 d.

2.2.4. Pharmacokinetics of betamethasone after maternal or fetal intra-muscular injections in sheep

The pharmacokinetics of betamethasone after maternal or fetal intra-muscular administration in sheep has been published previously (Moss *et al.*, 2003a). Similar to results obtained in humans (Ballard *et al.*, 1975b), betamethasone levels peaked in maternal plasma within 1 h of maternal administration and had a half-life of 4.75 h as illustrated in Figure 2.1 A (Moss *et al.*, 2003a). Betamethasone rapidly crosses the sheep placenta, and fetal plasma levels of betamethasone peaked between 1 and 4 h, with a half-life of approximately 8.5 h, after maternal administration (Moss *et al.*, 2003a). In contrast, as illustrated in Figure 2.1 B, fetal plasma betamethasone concentrations peaked rapidly (within 30 min) and had a much shorter half-life (approximately 1 h) after direct fetal injection (Moss *et al.*, 2003a). This study demonstrated that direct fetal injections of betamethasone resulted in 10-fold higher fetal plasma betamethasone concentrations which were rapidly cleared from the fetal circulation; whereas, maternal betamethasone administration resulted in lower fetal plasma betamethasone concentrations that were present in the circulation for a greater length of time. The duration of exposure to glucocorticoids is thought to be directly related to the amount of betamethasone administered (Moss *et al.*, 2003a). A maternal dose of 0.5 mg/kg equates to approximately 27 mg of betamethasone in our sheep, whereas the same dose for a fetus assumed to weigh 1.4 kg at 103 d equals only 0.7 mg of betamethasone (Moss *et al.*, 2003a). Moss and colleagues suggest that the "maternal circulation likely acts as a "reservoir" from which transplacental passage of betamethasone to the fetus continues" (Moss *et al.*, 2003a). Incorporating data from another study (Fowden *et al.*, 1996), the authors suggest that it is the sustained exposure to betamethasone that causes fetal growth restriction after maternal glucocorticoid administration (Franco *et al.*, 2003; Moss *et al.*, 2003a).

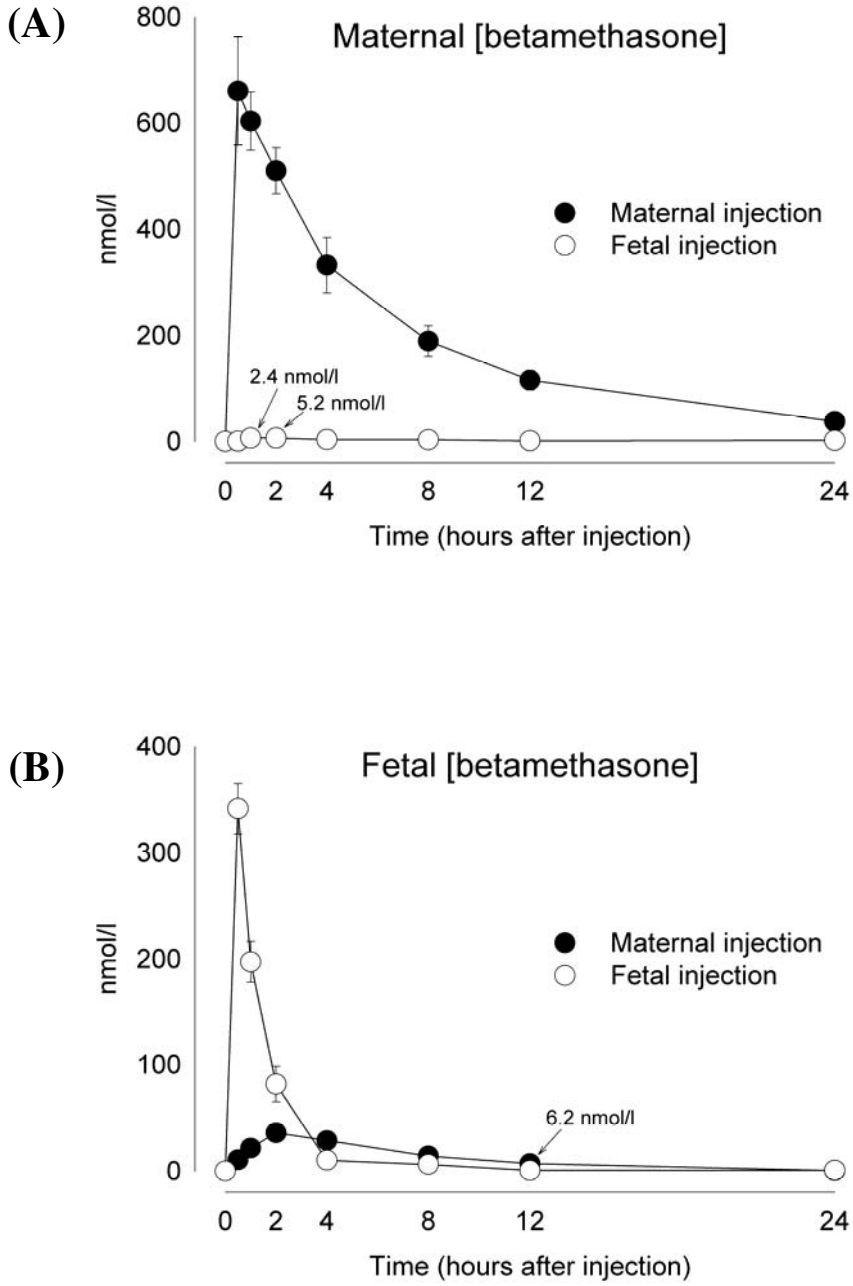


Figure 2.1. Pharmacokinetics of betamethasone after maternal or fetal intramuscular administration. (A) Maternal plasma betamethasone concentration (n = 5) after maternal (●) or direct fetal (○) i.m. betamethasone (0.5 mg/kg body weight) administration. (B) Fetal plasma betamethasone concentration (n = 5) after maternal or fetal injection. Figure adapted from Moss *et al.* (2003).

2.3. POST MORTEM TISSUE COLLECTION

Adult sheep (3.5-years-old) in the cohort study were transported to the Large Animal Facility at The University of Western Australia and housed for 4 months prior to euthanasia. After overnight fasting, the sheep were weighed and then killed with an overdose of pentobarbital sodium (Lethabarb®; Arnolds of Reading Pty Ltd, Boronia, VIC, Australia). Organs were removed quickly, weighed and stored for subsequent analyses.

In the fetal study, pregnant ewes were brought into an adjacent shed and fasted overnight prior to operative delivery of fetal sheep. The next day, pregnant ewes were humanely killed using a captive-bolt gun and fetuses were delivered by Caesarean section. Umbilical arterial blood pH, partial pressure of carbon dioxide (PCO_2) and partial pressure of oxygen (PO_2) were measured at the time of delivery using a Rapidlab 360 Blood Gas Analyser (Bayer Australia Limited, Bentley, WA, Australia). Measurements were made at 37 ºC and values were corrected for a fetal body temperature of 39 ºC. Fetuses were weighed prior to decapitation and exsanguination. Fetal organs were removed quickly, weighed and then stored for subsequent analyses.

I collected, weighed and stored all adult and fetal kidneys for use in my thesis studies. Tissue collection of other organs was performed with the assistance of Dr Deborah Sloboda, Dr Timothy Moss, Dr Ilias Nitsos, Mr Adrian Jonker, Mrs Alana Winsor and Mr Shaofu Li.

2.4. TISSUE PROCESSING

At *post-mortem*, portions of kidney containing both cortex and medulla were removed from the right kidney of all animals and were snap frozen in liquid nitrogen. In the adult study, a 5 mm transverse slice at the level of the renal hilux was removed from the left and right kidney of each animal and immersion-fixed in 4% w/v paraformaldehyde (ICN Biomedicals, Inc., Aurora, OH, USA) in phosphate-buffered saline (PBS; 0.137 M NaCl, 10.1 mM Na_2HPO_4, 2.68 mM KCl, 1.76 mM KH_2PO_4, pH 7.4) for 24 h. In the fetal study, slices were taken from the right kidney only and fixed as above. After

fixation, the kidneys were washed twice in PBS for 24 h and then stored in 70% ethanol prior to processing.

During tissue processing, kidney slices (in labelled histocassettes) were dehydrated in alcohol (5 times in 100% ethanol or once in 70% and 90% ethanol and 3 times in 100% ethanol, depending on the protocol), cleared by immersion in xylene or toluene and then infiltrated with paraffin wax. The adult sheep kidney samples were processed for 13 h (Appendix 1A) using a Shandon Pathcentre (Life Sciences International Europe, England). The fetal kidneys were processed for 14 h using a Shandon Citadel 1000 Tissue Processor (Appendix 1B). After processing, the kidney slices were embedded in paraffin wax and then sectioned at 5 μm using a rotary microtome (Leica Microsystems, Gladesville, NSW, Australia). Kidney sections were mounted on SuperFrost Plus glass microscope slides (Menzel-Glaser, Germany) and dried at 37 ºC overnight.

One section from each kidney was stained with haematoxylin and eosin (H&E; Appendix 2) for routine histology. Where abnormal histology was evident, Masson's trichrome stain (Appendix 3) was performed on another section to identify the pathology. Abnormal histology included renal cystic change, fibrotic cortex, sclerotic glomeruli, infiltration of inflammatory cells, thickened arteries or "thyroidisation" of tubules. Dr Adrian Charles, a consultant Paediatric and Perinatal Pathologist from the Women and Children's Pathology at King Edward Memorial and Princess Margaret Hospitals, confirmed and diagnosed the histopathology in abnormal kidney sections.

2.5. IMMUNOHISTOCHEMISTRY

To localise glucocorticoid receptors (GRs) within fetal and adult sheep kidneys, paraffin-embedded sections of the left (adult) or right (fetal) kidney were stained with an affinity-purified rabbit polyclonal antibody raised against the amino terminus of the GRα of mouse origin (GR (M-20); Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) using standard immunohistochemical techniques. The immunohistochemical method was optimised by trialling various peroxidase blocks, non-specific blocks, diluent compositions and primary antibody concentrations (data not shown).

Sections were de-waxed using Histo-Clear II solution (National Diagnostics, Atlanta, GA, USA) and rehydrated through a graded series of alcohols (100%, 90%, 70% ethanol) to fresh PBS. Endogenous peroxidase activity within the sections was blocked via incubation in 3% v/v hydrogen peroxide (H_2O_2; Chem-Supply, Gillman, SA, Australia) in PBS for 10 min at room temperature. After 3 PBS washes, 5 min each, sections were treated with 1% v/v normal goat serum (NGS; VECTASTAIN® ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA) in diluent (0.1% v/v Tween® 20 [Sigma-Aldrich Pty Ltd., Sydney, NSW, Australia]/ 1% w/v bovine serum albumin [BSA; Trace Biosciences NZ Ltd, Hillcrest, Hamilton, New Zealand]/ PBS) and incubated in a humidity chamber for 60 min at room temperature to prevent sections from drying out. Tween® 20 was added to the diluent as it increases cell membrane permeability. NGS treatment blocks non-specific binding of the secondary antibody to antigens present in the sections. After incubation with NGS, sections were washed with PBS (3 x 5 min) and then incubated with Avidin D blocking solution for 15 min, rinsed with PBS, and then incubated with biotin blocking solution for 15 min and then rinsed again (Blocking kit; Vector Laboratories, Inc.). Kidney sections contain high levels of endogenous biotin. Therefore, it is essential to use the Avidin/Biotin Blocking Kit to reduce high background. The GR antibody was prepared in diluent (at a concentration of 4 μg/mL (1:50) for fetal sections and 2 μg/mL (1:100) for adult sections) and incubated on the sections overnight at 4 ºC in a humidity chamber.

After overnight incubation with the primary antibody, sections were washed for 5 min in PBS, 3 times, and then incubated with the secondary biotinylated anti-goat IgG antibody (1:200 in diluent) for 1 h. During the secondary incubation, the ABC reagent (VECTASTAIN® ABC Kit, Vector Laboratories, Inc.) was prepared by mixing reagent A (Avidin DH) and reagent B (biotinylated horseradish peroxidase H) at dilutions of 1:100 in PBS. The ABC mix was left to stand for at least 30 min prior to use to permit sufficient fusing of the two reagents. Non-bound secondary antibody was removed from sections by washing in PBS 3 times, 5 min each, and then the ABC reagent was incubated on sections for 1 h, followed by further washes (3 x 5 min) in PBS. The antigen-antibody complexes were visualised by incubating sections with the chromogen 3,3'-diaminobenzidine (DAB; ICN Biomedicals, Inc) for 10 min at a concentration of 0.5 mg/mL in dH_2O following activation with 0.1% v/v H_2O_2. DAB produces a brown precipitate that is insoluble in alcohol and clearing agents, allowing sections to be

permanently mounted. The DAB reaction was terminated by incubating the sections in distilled water (dH_2O) for 10 min. Sections were then lightly counterstained with haematoxylin (Gill's Haematoxylin Solution Number 1; Electron Microscopy Services, Washington, WA, USA), dehydrated through graded alcohols (70%, 90%, 100% ethanol), cleared and cover-slipped using Permount mounting medium (Electron Microscopy Services).

Sections of sheep liver were used as positive and negative controls and were run in each assay to ensure specific staining of the antibodies. Negative controls were performed by incubating the antibody with its peptide (section 2.6.7) or omitting the primary antibody prior to application to the section. All kidney sections from control and betamethasone-exposed animals were treated simultaneously to ensure uniform experimental conditions. Sections were viewed under an Olympus BX51TF light microscope (Olympus Optical Co. Ltd, Japan) and images were captured using a SPOT digital camera and SPOT RT software (v 3.5.5; Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

2.6. WESTERN BLOTTING

2.6.1. Protein extraction and quantification

Kidney samples were homogenised (POLYTRON® PT 1200; Kinematica AG, Lucerne, Switzerland) in RIPA lysis buffer (~ 100 mg/mL; 150 mM NaCl, 50 mM Tris-HCl, 1% v/v Triton® X-100, 0.5% w/v deoxycholic acid, 0.1% w/v SDS, pH 8.0) with 100 µM sodium orthovanadate and 1 Complete, EDTA-free Protease Inhibitor Cocktail Tablet (Roche Diagnostics GmbH, Penzberg, Germany) in 50 mL lysis buffer. Following homogenisation, the samples were centrifuged at 10,000 revolutions per minute (r.p.m.) for 15 min at 4 ºC (Centrifuge 5810R; Eppendorf South Pacific Pty Ltd, North Ryde, NSW, Australia) to pellet cellular debris. The supernatant containing the extracted protein was transferred to a new tube and then centrifuged at 10,000 r.p.m. for a further 15 min at 4 ºC. Once more the supernatant was transferred to a new tube and a protein assay was performed to calculate the protein concentration of each sample. Samples were stored at -80 ºC.

Bio-Rad's colorimetric protein assay (Bio-Rad Laboratories, Hercules, CA, USA) was used to determine the concentration of protein in the supernatant of each kidney sample. This assay is based on the Bradford dye-binding procedure that involves the binding of Coomassie Brilliant Blue G-250 to protein (Bradford, 1976). Upon binding of the dye to protein, the dye changes in colour from red to blue (Reisner *et al.*, 1975), shifting the absorption maximum of the dye from 465 to 595 nm (Bradford, 1976).

A standard curve was constructed using seven duplicate BSA (Bio-Rad protein assay standard II lyophilised bovine serum albumin; Bio-Rad Laboratories) standards ranging in concentration from 0 mg/mL to 1 mg/mL. The linear range of the Bio-Rad protein assay for BSA is 0.2 mg/mL to 0.9 mg/mL (Bio-Rad Laboratories). The dye reagent was prepared by diluting Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories) 1:5 in dH_2O and filtering. Each standard was prepared to a volume of 25 µL in dH_2O in a semi-micro cuvette (1 cm path, 1.5 mL volume; Bio-Rad Laboratories) and an equal volume (975 µL) of diluted dye reagent was added. Protein samples were diluted 1:10 in dH_2O then 10 µL of this diluted protein was added to 15 µL dH_2O and 975 µL of dye in the cuvette. All samples were mixed and then incubated at room temperature for 5 min to allow sufficient time for colour development. Protein concentrations in the unknown samples were determined by the mean of the duplicates of the samples in reference to the standard curve multiplied by the dilution factor (250). A SmartSpec 3000™ Spectrophotometer (Bio-Rad Laboratories) was used to measure the absorbance at 595 nm for the protein assay.

2.6.2. SDS-PAGE

Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) is a technique that is used to separate proteins through a polyacrylamide gel, under denaturing conditions, according to their size. Equal amounts of protein from each sample were prepared to a volume of 15 µL in PBS; 4 µL of loading buffer (0.313 M Tris, 50% v/v glycerol, 10% w/v SDS, 0.05% w/v bromophenol blue) and 1 µL of reducing agent (2 M dithiothreitol [DTT]) were added to each protein sample and mixed. The loading buffer contained SDS to solubilise the proteins and DTT to denature the proteins by reducing disulphide bonds. Samples were then heated at 95 ºC for 5 min to further denature the proteins under reducing conditions. After heating, the samples

were briefly micro-centrifuged for 8 s at a maximum speed of 13,400 r.p.m. (MiniSpin® Microcentrifuge; Eppendorf South Pacific Pty Ltd) and then loaded into a 7.5% Tris-HCl Criterion™ Precast Gel (Bio-Rad Laboratories) assembled in a Criterion™ Cell (Bio-Rad Laboratories). The first lane in every gel was loaded with 20 µL of Precision Plus Protein™ Prestained Standard (Bio-Rad Laboratories) as a molecular weight marker. The running buffer consisted of a 10-fold dilution of 10x Tris/Glycine/SDS buffer (Bio-Rad Laboratories), which resulted in a solution of the following composition: 25 mM Tris (pH 8.3), 192 mM glycine and 0.1% SDS. Protein samples were separated by SDS-PAGE run at 80 V for 30 min and then 150 V for 1 h.

2.6.3. Protein transfer to membrane

Following SDS-PAGE, proteins were electrophoretically transferred from the gel to an Immun-Blot® polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories). This was performed using the Criterion™ Blotter Tank (Bio-Rad Laboratories) according to the manufacturer's instructions. A Tris/Glycine buffer (0.025 M Tris, 0.192 M Glycine; ICN Biomedicals, Inc.) with 20% v/v methanol was used during the transfer. Prior to assembling the gel-membrane sandwich, the hydrophobic PVDF membrane was soaked in 100% methanol to facilitate equilibration in the transfer buffer and to prepare the membrane for protein binding. The transfer was run for 1 h at 100 V, with the tank surrounded by ice to prevent overheating. After the transfer was complete, the membrane was lightly stained with Ponceau S staining solution (0.1% w/v Ponceau S, 5% v/v acetic acid; Sigma-Aldrich, Pty Ltd., Saint Louis, MO, USA) to visualise the protein bands (protein bands after loading less than 20 µg could not be visualised by this technique). The stain was removed by gently agitating the membrane in repeated washes of PBS with 0.1% v/v Tween® 20 (PBST). The membrane was then incubated in blocking solution (5% w/v Diploma Instant Skim Milk Powder; Bonland Dairies Pty Ltd, Rowville, VIC, Australia; in PBST) for 1 h at room temperature to prevent non-specific binding of the antibodies to the membrane.

2.6.4. Antibody incubation

The primary antibody was diluted in blocking solution and incubated with the membrane in a sealed plastic bag overnight at 4 ºC atop a mechanical shaker. The

following day the membrane was washed five times, 5 min each, with PBST to remove any unbound primary antibody and then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody incubated for 1 h at room temperature. After incubation with the secondary antibody, the membrane was washed six times, 5 min each, in PBST.

2.6.5. Enhanced chemiluminescence technique

Protein-antibody complexes were visualised using the SuperSignal® Substrate System (Pierce Biotechnology, Inc., Rockford, IL, USA). The SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc.) is a highly sensitive, enhanced substrate for detecting HRP on Western blots. The substrate was prepared by mixing equal volumes of Luminol/Enhancer and Stable Peroxide solutions; and membranes were gently agitated with 0.125 mL of the substrate per square centimetre of membrane for 5 min at room temperature. Membranes were then drained and placed inside a plastic sleeve within a hypercassette (Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, England) and exposed to high performance chemiluminescence film (Hyperfilm™ ECL™; Amersham Pharmacia Biotech UK Ltd) for 5 s to 5 min, depending upon the strength of the signal. Films were processed using a Scopix LR 5200 wet imager (Agfa-Gevaert Ltd, Nunawading, VIC, Australia) or an automatic film processor (Type 9432/131; Agfa-Gevaert, Germany).

2.6.6. Densitometric analysis of Western blots

Western blot films were placed atop a light box and images were captured using a SPOT digital camera and SPOT RT software (v 3.5.5; Diagnostic Instruments, Inc.). Image files were resized to 800 x 600 pixels and saved in ".tif" format in order to perform image analysis using Scion Image software (v Beta 4.0.2; Scion Corporation, Frederick, MD, USA). Densitometric analysis was performed on the GR blots by measuring the integrated area under the curve for each GR protein band. On blots probed with Na^+/K^+-ATPase-α_1, the pixel density of each band was calculated using the same size measuring frame, equal in area to the largest band on the blot, and reported as the arbitrary optical density (AOD) units. The integrated area under the curve analysis was unable to be performed on these blots due to the close proximity of another band.

2.6.7. Specificity of primary antibody

Peptide neutralisation of the primary antibody was performed to examine the specificity of the GR primary antibody binding on the Western blot. A 10-fold excess of peptide (GR (M-20)-P; Santa Cruz Biotechnology, Inc.) was mixed with a small volume of undiluted GR (M-20) antibody and incubated at room temperature for 60 min, then at 4 ºC overnight atop a mechanical shaker. The mixture was centrifuged at 10,000 r.p.m. for 15 min at 4 ºC to pellet immune-complexes. The supernatant was carefully removed and diluted in blocking buffer to a final dilution of 1:200. A Western blot was performed and a strip of membrane with samples from each group was probed with the peptide-antibody mixture in place of the primary antibody (Figure 2.2). The blot was processed simultaneously with a GR blot of adult kidneys to ensure both membranes were exposed to the same experimental conditions.

A single strongly hybridising band was present on the GR blot film. The peptide neutralisation blot was devoid of bands, suggesting that the bands were specific to the GR in protein extracted from adult sheep kidneys. Further, direct re-probing of the blot with an anti-β-actin antibody was used as an internal control for Western blot analysis and ensured that protein had been transferred from the gel to the peptide neutralisation blot (Liao *et al.*, 2000). Following exposure to film, membranes were stored at 4 ºC in PBS for at least 24 h to ensure that the chemiluminescent substrate had ceased light emission. Membranes were then washed three times, for 5 min, in PBST and directly re-probed using a mouse monoclonal anti-β-actin primary antibody (Clone AC-15; Sigma-Aldrich, Inc.) diluted in blocking solution and incubated overnight at 4 ºC whilst gently shaking. The membranes were then washed five times, 5 min each, in PBST to remove any unbound anti-β-actin antibody and then incubated with a sheep anti-mouse IgG HRP-conjugated secondary antibody (NA 931; Amersham Biosciences UK Ltd, Buckinghamshire, England) diluted in blocking solution and incubated for 1 h at room temperature. After six washes in PBST, 5 min each, the membranes were incubated with SuperSignal® Substrate, exposed to film and analysed (as described in 2.6.5 and 2.6.6).

Direct re-probing of all Western blots used in this thesis was performed using the anti-β-actin antibody. However, a recent study demonstrated that β-actin was developmentally-regulated in the rat kidney (Madsen *et al.*, 2004). Analyses of my data

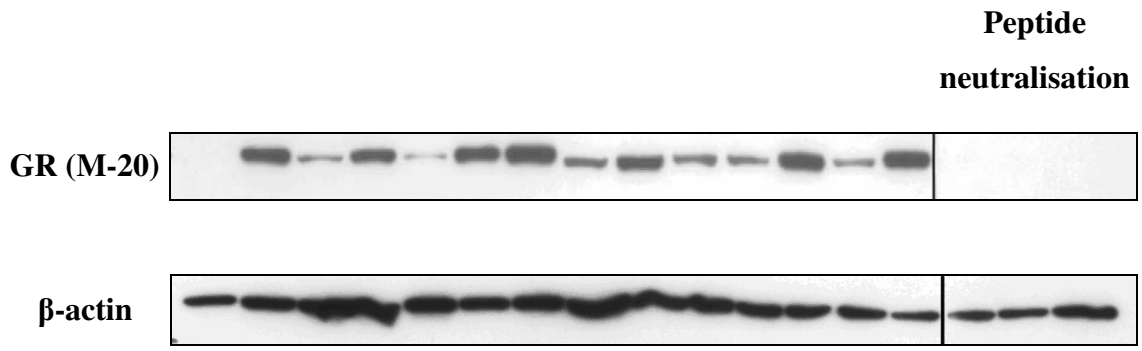


Figure 2.2. GR (M-20) antibody specificity.

Western blot analysis, incorporating peptide neutralisation, was used to determine the specificity of the GR (M-20) antibody. A Western blot of adult sheep kidney protein was probed with the antibody to produce a strongly hybridising band. The peptide neutralisation blot on the right was completely clear of bands, suggesting that the band detected by GR (M-20) antibody on the left is specific. Further, the blot was directly re-probed with an anti-β-actin antibody to ensure that protein had been transferred from the gel to the peptide neutralisation blot.

found that β-actin protein levels were developmentally-regulated and were affected by betamethasone in the sheep kidney (data not shown). Therefore, all β-actin data were removed from analyses of Western blot data in this thesis.

Specificity experiments were not performed for the Na^+/K^+-ATPase-α_1 (MA3-929) primary antibody as the immunogen for the antibody was sheep kidney.

2.6.8. Optimisation of amount of protein loaded

Preliminary Western blots were performed to optimise the amount of protein loaded into the gel for GR and Na^+/K^+-ATPase-α_1 protein bands. The amount of protein loaded on the blots was optimised to ensure that the density of the bands did not exceed the linear range of the band intensity-protein abundance relationship that was detectable by the film.

2.6.8.1. GR blot optimisation

A Western blot was performed (as described above 2.6.2 – 2.6.6) using fetal kidney protein with the amount of protein loaded into each gel well ranging from 12.5 μg to 200 μg (Figure 2.3). The immunoblot was probed with a GR primary antibody (GR (M-20); Santa Cruz Biotechnology, Inc.) at a concentration of 1 μg/mL (1:200) and a donkey anti-rabbit IgG HRP-conjugated secondary antibody (NA 934; Amersham Biosciences UK Ltd) diluted 1:10,000. The AOD of the GR blot was plotted versus the amount of protein loaded per well (Figure 2.3) and 50 μg was chosen as the desired amount and fell within the linear range of AOD for the protein.

2.6.8.2. Na^+/K^+-ATPase-α_1 blot optimisation

Western blots with 50 μg of adult kidney protein probed with various dilutions of primary and secondary antibodies were found to be too intense; therefore, the amount of protein for adult and fetal kidney protein blots was optimised by running a blot with protein amounts ranging from 5 μg to 40 μg. Blots of adult and fetal kidney proteins were incubated with a 1:40,000 dilution (0.19 μg/mL) of Na^+-K^+-ATPase-α_1

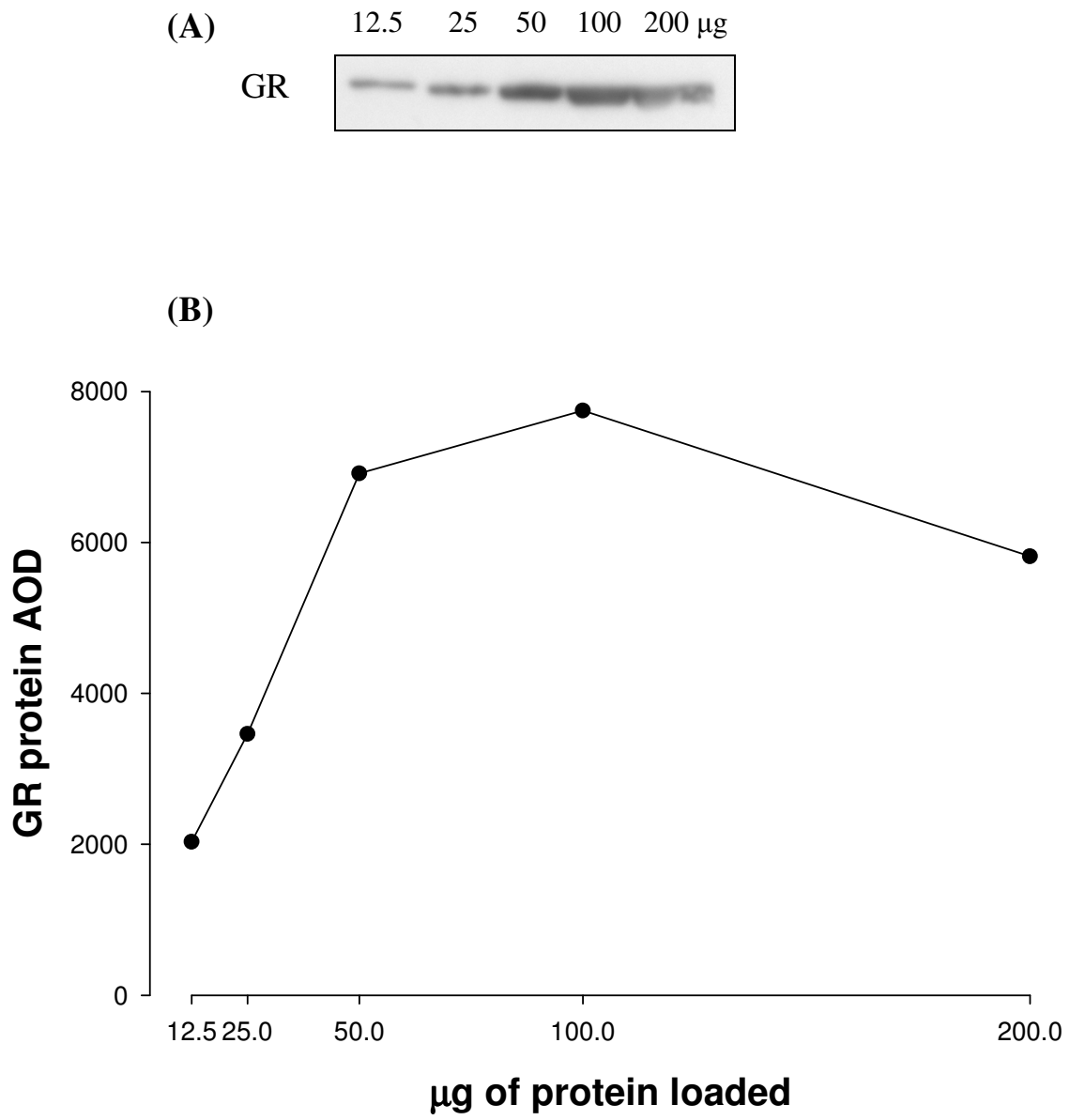


Figure 2.3. Optimisation of the amount of protein loaded for GR Western blot.
(A) Digital image of a Western blot probed for the GR with µg of protein for each band labelled above. (B) Plot of GR band intensity (AOD) versus µg protein loaded per lane. The amount of protein chosen to load for each sample was 50 µg.

primary antibody (MA3-929; Affinity BioReagents, Golden, CO, USA) and a 1:20,000 (fetal blots) or 1:80,000 dilution (adult blots) of rabbit anti-mouse IgG peroxidase conjugate secondary antibody (A-9044; Sigma-Aldrich, Inc.). Bands on fetal kidney blots were optimal with 20 μg of protein loaded (Figure 2.4); whereas adult kidney blots were optimal with 5 μg of protein (Figure 2.5).

2.6.9. Determination of the molecular weight of the band

The molecular weights of the immuno-reactive protein bands on Western blots were calculated by plotting the migration distance of the molecular weight markers against the $\log_{10}$ of the molecular weight (Figure 2.6). A linear plot was then fitted to the data and the equation of the line was used to predict the molecular weight of the protein band based on the measured migration distance of this band. Several markers were run on gels for GR and Na^+/K^+-ATPase-α_1 protein and the average of the calculated molecular weight according to each marker was used as the estimated molecular weight of the protein band (Appendix 4). The average molecular weight was calculated to be 42 kDa for β-actin, 74 ± 1 kDa for GR, and 101 ± 1 kDa for Na^+/K^+-ATPase-α_1.

2.7. REAL-TIME REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION

Real-time reverse-transcriptase (RT)-polymerase chain reaction (PCR) was used to quantitate gene expression (mRNA) in ribonucleic acid (RNA) extracted from the kidneys of sheep exposed to prenatal saline or betamethasone administration.

2.7.1. RNA isolation

Two methods of RNA isolation were used in the studies comprising this thesis. RNA-Bee™ (Tel-Test, Inc., Friendswood, Texas, USA) was used to extract total RNA from adult sheep kidneys. Generally, this technique yielded good RNA; however, a small number of samples had to be re-extracted due to poor quality. The RNeasy® Midi Kit (QIAGEN Pty Ltd, Clifton Hill, VIC, Australia) was used to extract RNA from all fetal kidneys and those samples of adult kidney that had to be re-extracted.

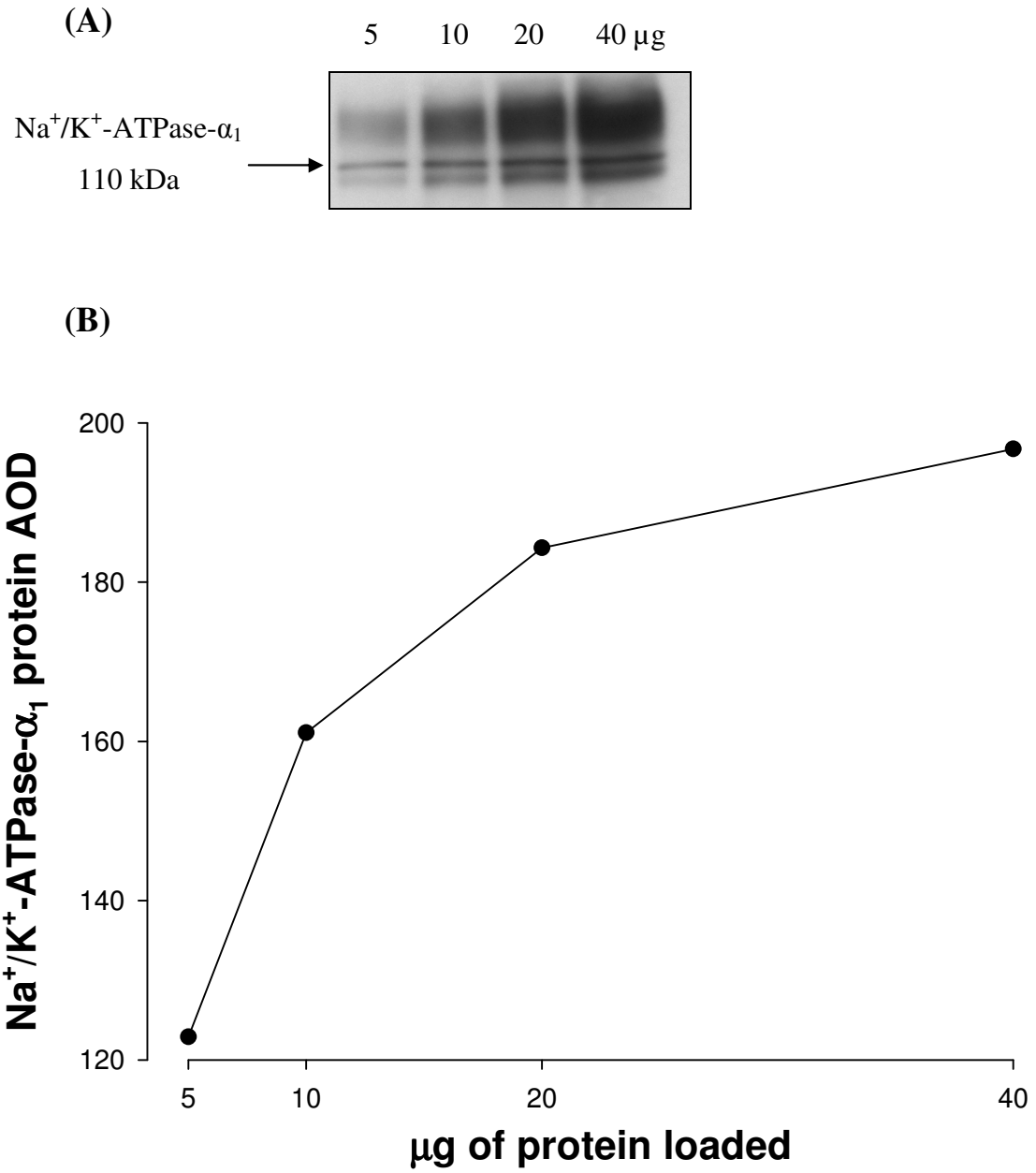


Figure 2.4. Optimisation of the amount of protein loaded on a fetal kidney Na^+/K^+-ATPase-α_1 Western blot. The Western blot of Na^+/K^+-ATPase-α_1 is shown in (A) with various amounts of protein loaded. (B) The AOD of the blot was plotted against the amount of protein loaded. The amount chosen to load for each sample was 20 µg.

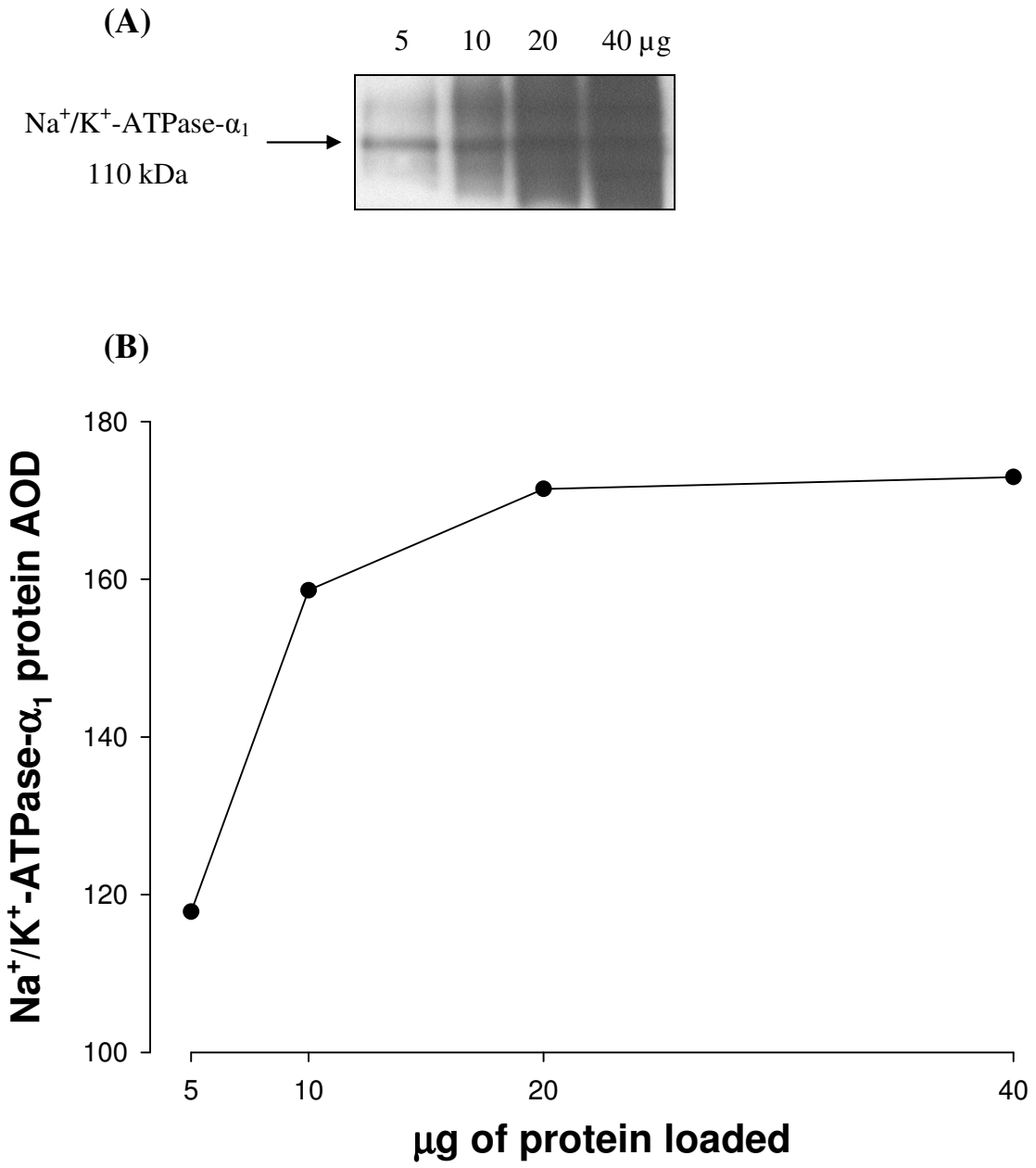


Figure 2.5. Optimisation of the amount of protein loaded on an adult kidney Na^+/K^+-ATPase-α_1 Western blot. (A) The amount chosen to load for each sample was 5 µg, as it gave the clearest band signal. The AOD of the Na^+/K^+-ATPase-α_1 band was plotted against the amount of protein loaded (B).

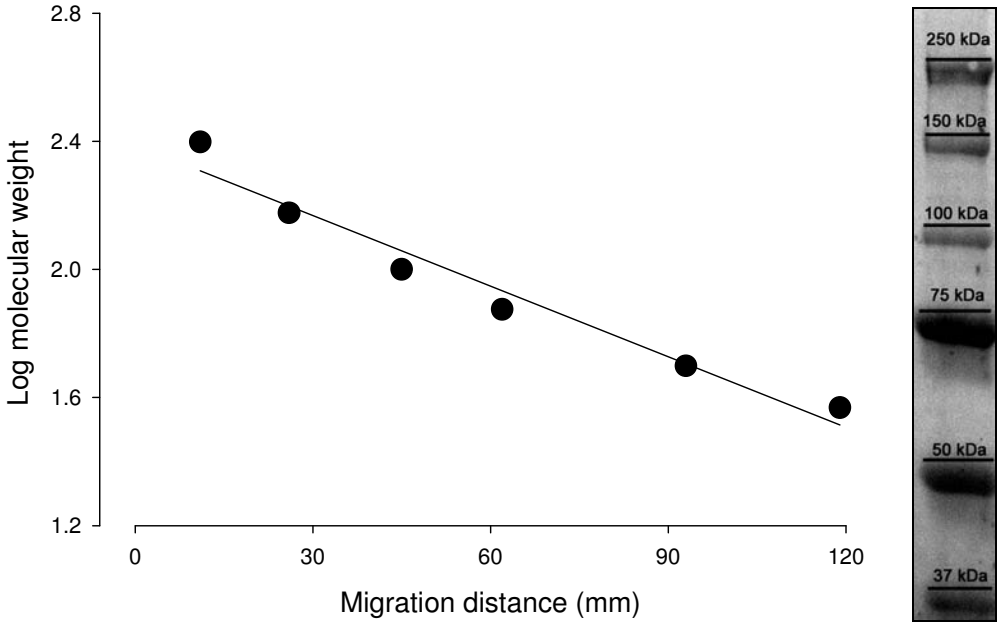


Figure 2.6. Standard curve for Precision Plus Protein™ Standards All Blue.

The $\log_{10}$ of the known molecular weight standards (Precision Plus Protein™ Standards All Blue; Bio-Rad Laboratories) was plotted against the migration distance of the markers to generate a standard curve. The molecular weight of the target band was then determined by measuring the migration distance and inserting it into the equation of the standard curve (i.e. y = -0.0073x + 2.3884). A digital micrograph of the Precision Plus Protein™ Standards All Blue ladder is shown on the right.

2.7.1.1. RNA-Bee™

Total RNA was extracted from adult sheep kidneys using RNA-Bee™ (Tel-Test, Inc.) according to the manufacturer's protocol. Briefly, kidney samples containing both medulla and cortex were crushed in liquid nitrogen using a mortar and pestle and homogenised in RNA-Bee™ solution (1 mL/50 mg tissue) using a POLYTRON® PT 1200 (Kinematica AG) for approximately 45 s in sterile homogenising tubes. The homogeniser probe and mortar and pestle were cleaned thoroughly between each sample with 100% ethanol (Merck, Germany), then rinsed with 0.1% v/v diethyl pyrocarbonate (DEPC)-treated dH_2O (Appendix 5) and treated with RNaseZap® RNase decontamination solution (Ambion, Inc., Austin, Texas, USA) and rinsed again. Phase separation was achieved by the addition of 200 µL of chloroform (Sigma-Aldrich, Inc.) per 1 mL of RNA-Bee™. The tube was capped and vigorously shaken for approximately 30 s and then stored on ice for 5 min. The homogenate was transferred to RNase-free 1.5 mL microfuge tubes (Ambion, Inc., Austin, Texas, USA) and centrifuged at 10,629 r.p.m. for 15 min at 4 ºC. After centrifugation, RNA remains exclusively in the upper colourless aqueous phase whereas the deoxyribonucleic acid (DNA) and proteins are in the interphase and lower blue-phenol phase. The aqueous phase was transferred to a clean tube and 500 µL of isopropanol (Sigma-Aldrich, Inc.) was added. The samples were stored at room temperature for 5 to 10 min and then overnight at 4 ºC to enhance RNA precipitation. The RNA was precipitated by centrifuging the samples at 10,629 r.p.m. for 5 min at 4 ºC, the supernatant was discarded and the RNA pellet was washed with 1 mL of 75% v/v ethanol, vortexed and then centrifuged at 8,403 r.p.m. for 5 min at 4 ºC. The ethanol wash was repeated to improve the 260/280 ratio (described in section 2.7.3). The RNA pellet was air-dried for 10 min and then dissolved in 50 µL DEPC-treated dH_2O and incubated for 15 min at 55 ºC. Total RNA concentration was determined as described in section 2.7.2.

2.7.1.2. RNeasy® Midi Kit

Total RNA was isolated from fetal and adult sheep kidneys using an RNeasy® Midi Kit (QIAGEN Pty Ltd, Clifton Hill, VIC, Australia) according to the manufacturer's instructions. Between 150 and 250 mg of frozen kidney tissue (from the right kidney of each animal, containing both cortex and medulla) was placed in 4 mL of Buffer RLT (supplied in kit, prepared by the addition of 10 µL of β-mercaptoethanol per 1 mL of Buffer RLT) within an ice-cold sterile tube and homogenised for 45 s. The lysate was then transferred to a 15 mL Falcon tube and centrifuged at 4,000 r.p.m. for 10 min. The supernatant was transferred to a clean tube and 4 mL of 70% v/v ethanol was added to the tube, to make a total volume of approximately 8 mL, which was mixed immediately by shaking vigorously. The sample was then applied to an RNeasy® midi column (with a maximal loading volume of 4 mL; supplied in kit) and centrifuged at 4,000 r.p.m. for 5 min, then repeated with the remaining lysate, discarding the flow-through each time. Buffer RW1 (4 mL; supplied in kit) was added to each column, centrifuged at 4,000 r.p.m. for 5 min, and washed twice with 2.5 mL of Buffer RPE (supplied in kit, prepared by adding 4 volumes of absolute ethanol) and centrifuged at 4,000 r.p.m. for 2 min and then 5 min. The RNeasy® column was transferred to a new collection tube and total RNA was eluted by the addition of 250 µL of RNase-free dH_2O that was pipetted directly onto the silica-gel membrane. The sample was left to stand for 1 min prior to centrifuging at 4,000 r.p.m. for 3 min. The elution was repeated with a second volume of RNAse-free dH_2O to obtain approximately 500 µL of total RNA in solution. Total RNA concentration was determined as described in section 2.7.2.

2.7.2. RNA quantitation

The amount of RNA in each sample was measured in a quartz cuvette using the SmartSpec 3000™ Spectrophotometer (Bio-Rad Laboratories). For each sample, 2 µL of the stock total RNA was added to 998 µL of DEPC-treated dH_2O, producing a total volume of 1000 µL. The total RNA concentration was calculated using the formula:

$$A_{260} \times \text{conversion factor (40)} \times \text{dilution factor (500)} = \mu g/mL$$

where A_{260} is the absorbance at 260 nm; the mass/absorption conversion factor for A_{260} = 1.000 is 40 µg/mL; and the dilution factor is 500 (2 µL of RNA in 1000 µL total volume). Absorbance readings at 260 nm and 280 nm were collected and used to calculate the A_{260}:A_{280} ratio (A_{260}/A_{280}). Pure RNA preparations have an A_{260}/A_{280} of 2.0. Many samples exhibited an A_{260}/A_{280} of 1.4 to 1.8, suggesting possible DNA contamination, and consequently underwent RNA purification.

2.7.3. RNA purification

Total RNA samples were purified using the RNeasy® MinElute™ Cleanup Kit (QIAGEN Pty Ltd) according to the manufacturer's instructions. The kit contains: RNeasy® MinElute™ Spin Columns in collection tubes, 1.5 mL and 2.5 mL collection tubes, Buffer RLT, Buffer RPE and RNase-free water; enough to process 50 samples. Buffer RLT was prepared by adding 10 µL of β-mercaptoethanol (ICN Biomedicals, Inc.) per 1 mL of Buffer RLT. Buffer RPE working solution was prepared by adding 4 volumes of 100% ethanol. Briefly, RNA samples were adjusted to a volume of 100 µL with RNase-free water and 350 µL of Buffer RLT was added and mixed thoroughly. To each sample, 250 µL of 100% ethanol was added and mixed by pipetting. The sample (700 µL) was then applied to an RNeasy® MinElute™ Spin Column in a 2 mL collection tube and centrifuged at 10,000 r.p.m. for 15 s. The flow-through was discarded; and the spin column was transferred to a new 2 mL collection tube and 500 µL of Buffer RPE was pipetted onto the spin column. The tubes were centrifuged for 15 s at 10,000 r.p.m. to wash the column and the flow-through was discarded. To dry the silica-gel membrane, 500 µL of 80% v/v ethanol was added to each RNeasy® MinElute™ Spin Column and centrifuged at 10,000 r.p.m. for 2 min. The RNeasy® MinElute™ Spin Columns were then transferred into new 2 mL collection tubes and centrifuged with caps open at 14,000 r.p.m. for 5 min. To elute the RNA, the spin columns were transferred to new 1.5 mL collection tubes and 14 µL of RNase-free water was pipetted directly onto the centre of each silica-gel membrane. The tubes were closed and centrifuged at 14,000 r.p.m. for 1 min. The dead space volume of the RNeasy® MinElute™ Spin Column is 2 µL; therefore, elution with 14 µL of RNase-free dH_2O results in 12 µL of purified RNA in solution. After RNA purification, the concentration of RNA was measured once more using the SmartSpec 3000™

Spectrophotometer (Bio-Rad Laboratories) as described in 2.7.2 and then samples were stored at -80 ºC until use.

2.7.4. Reverse transcription

Reverse transcription and real-time RT-PCR were performed in the Department of Anatomy and Cell Biology at Monash University, Melbourne, Australia under the instruction of, and with technical assistance from, Mrs Debbie Arena and Dr Karen Moritz.

Prior to use in real-time RT-PCR, RNA from each sample was reverse-transcribed in a 50 µL reaction containing 30.75 µL of master-mix: 5 µL 10 x Taqman® RT Buffer, 11 µL 25 mM $MgCl_2$, 10 µL DNTPs, 2.5 µL 50 mM random hexamers, 1 µL of RNase inhibitor and 1.25 µL of MultiScribe™ Reverse Transcriptase (TaqMan® Reverse Transcription Reagents; Applied Biosystems, Foster City, CA, USA) and 19.25 µL of total RNA diluted to 1 µg in RNase-free dH_2O.

To ensure there was no contaminating genomic DNA, half-volume control reactions (that did not include the reverse transcriptase) were included in a separate reverse transcription reaction with all the total RNA samples. The reverse transcription was performed in an iCycler™ Thermal Cycler (Bio-Rad Laboratories) at 25 ºC for 10 min, 48 ºC for 30 min and 95 ºC for 5 min. Upon completion, all complementary DNA (cDNA) samples were stored at -20 ºC until use.

2.7.5. Real-time RT-PCR

Real-time RT-PCR was performed using an ABI PRISM® 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Validation experiments for each gene of interest had been performed prior to these studies to optimise primer and probe concentrations, determine the relative efficiencies of each gene and to ensure that the C_T (cycle of threshold fluorescence) value obtained for each gene was the same in a separate reaction as in the multiplex reaction. All but one of the sequences of the primers and probes used in the real-time RT-PCR studies had been published previously (Moritz *et al.*, 2000; Dodic *et al.*, 2002; Hantzis *et al.*, 2002).

A multiplex comparative C_T method was used to determine relative mRNA expression levels of GR, MR, 11β-HSD-2, Na^+/K^+-ATPase-α_1, AT_1, AT_2, A_o and renin in the sheep kidney. Multiplex PCR uses more than one primer pair in the same tube; one primer pair amplifies the target gene and another primer pair amplifies the endogenous active reference gene (18S ribosomal RNA). Different reporter dyes (which fluoresce at distinct emission wavelengths) were attached to each target (FAM™) and reference (VIC™) TaqMan® probes. Competition between the two primer pairs was avoided by limiting the 18S primers. The primer and TaqMan® probe sequences for all genes are shown in Table 2.1. The 18S probe and primers were supplied in the TaqMan® Ribosomal RNA Control Reagents kit (Applied Biosystems). Real-time RT-PCR reactions were carried out in 25 μL volumes consisting of 1x TaqMan® Universal PCR Master Mix (including passive reference dye ROX™; Applied Biosystems), 50 nM TaqMan® 18S probe, 20 nM 18S forward primer, 80 nM 18S reverse primer, the appropriate concentration of TaqMan® probe and primers for each target gene as listed in Table 2.1 and 5 μL of cDNA template. All reactions were amplified at 50 ºC for 2 min and 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min.

The accession numbers (NCBI) of the nucleotide sequences of the target genes are as follows: GR: S44554, MR: AF349768, Na^+/K^+-ATPase-α_1: X02813, AT_1 receptor: AF056308, AT_2 receptor: S81979, renin: L43524 and A_o: D17520.

2.7.6. Quantitation of mRNA

The comparative C_T method uses arithmetic formulas for relative quantitation of gene expression. The C_T value is the cycle number at which cDNA amplification was first detected above the background in each sample. Firstly, the C_T value of 18S was subtracted from the C_T value of the target gene, to normalise quantitation of target mRNA for differences in the amount of total RNA added to each reaction and produce the delta (Δ) C_T value. This is represented by the equation below:

$$\mathbf{C_T\ (target) - C_T\ (18S) = \Delta\ C_T}$$

Table 2.1. Probe and primer sequences and concentrations used in real-time PCR.

GENE	SEQUENCE 5' – 3' (NUCLEOTIDE POSITION)	CONCENTRATION (nM)
TaqMan® PROBE		
GR	AAAGAAGATTTTATCGAACTCTGCACCCCTGG (424 – 455)	75
MR	TCCTCATTTCTTCAAACGCAGCCTGG (486 – 511)	100
11β-HSD-2	TGCTTGGCTGCCTATGGGACCTCC (681 – 705)	125
Na^+/K^+-ATPase-α_1	TGCTGGGCATCCGAGTGACCTG (2906 – 2927)	50
AT_1	ACCGCTGGCCCTTCGGCAA (596 – 614)	150
AT_2	TGGCTTCCCTTCCATGTTCTGACCTTC (664 - 690)	75
Angiotensinogen	CCACGGACCCAAATCTCGCTGC (723 – 744)	75
Renin	TCTGTGTGACTGTGATCCCGCCCA (543 – 520)	225
FORWARD PRIMER		
GR	ACTGCCCCAAGTGAAAACAGA (402 – 422)	900
MR	TCCAAAGGATGGCCTCAAAA (465 – 484)	900
11β-HSD-2	AGCAGGAGACATGCCGTTTC (659 – 679)	300
Na^+/K^+-ATPase-α_1	AACGGCTTCCTCCCTAATCAC (2884 – 2904)	300
AT_1	GGGCTGTCTACACTGCTATGGAA (572 – 594)	900
AT_2	TGTTCTGGCGTTCATCATTTG (642 – 662)	300
Angiotensinogen	CTCTCCCACGCTCACTAGACTTG (699 – 721)	300
Renin	TGAGCCAGGACCTGGTGACT (499 – 518)	900
REVERSE PRIMER		
GR	ATGAACAGAAATGGCAGACATTTTA (552 - 528)	900
MR	ATCTTTCTCAGCTCCTTGATGTAATTT (539 – 513)	900
11β-HSD-2	GCAATGCCAAGGCTGCTT (725 -707)	300
Na^+/K^+-ATPase-α_1	GTCGTTGATCCAACGGTCATC (2949 - 2929)	300
AT_1	CCGGAAGCGATCTTACATAGGTA (638 – 616)	900
AT_2	CCATCCAAGCTAGAGCATCCA (712 – 692)	300
Angiotensinogen	ATGCATGAACCTGTTGATCTTCTC (769 – 746)	300
Renin	TCCGTGACCTCGCCAAAG (562 – 545)	50

The normalised amount of target (ΔC_T) is a unit-less number that can be used to compare the relative amount of target in different samples by using a calibrator. The mean of the ΔC_T of the saline (control) group (MS, FS for adult study; 109 d saline for fetal study) was used as the calibrator in these experiments. Subsequently, the value of the calibrator was subtracted from the normalised amount of target to give the $\Delta \Delta C_T$ value (formula below).

$$\mathbf{\Delta C_T - C_T \text{ (calibrator)} = \Delta \Delta C_T}$$

The amount of target normalised to the endogenous reference (18S) and relative to the calibrator (saline group) is given by:

$$\mathbf{2^{-\Delta \Delta CT}}$$

2.7.7. Intra-assay coefficient of variation

The intra-assay coefficient of variation (CV) for each gene in the real-time RT-PCR experiments was determined by running one sample three to five times in one assay. The mean of the ΔC_T values of the CV samples was subtracted from each individual sample to give a $\Delta \Delta C_T$ value which was then put into the $2^{-\Delta \Delta CT}$ formula. The mean and standard deviation of the mean (S.D.) of the $2^{-\Delta \Delta CT}$ values were used to calculate the CV (S.D./mean) which was then expressed as a percentage.

2.8. STATISTICAL ANALYSIS

All data were statistically analysed using SigmaStat software (Version 3.00, SPSS Inc., Chicago, IL, USA) and are presented as mean ± S.E.M. One-way analysis of variance (ANOVA) tests were used to analyse data from three or more groups. Where only two sets of data samples were obtained, unpaired *t*-tests were performed. Two-way ANOVA tests were used to examine the effect of two variables on data. A probability value (p) less than 0.05 was deemed to be statistically significant. Specific statistical analyses are described in detail within the experimental chapters.

3. Nephron Endowment in Fetal Sheep: The Effect of Sex and Repeated Maternal Betamethasone Administration

3.1. INTRODUCTION

The gestational timing of nephrogenesis differs between mammalian species. Animals such as the guinea pig, spiny mouse, sheep and humans complete nephrogenesis prior to birth (Moritz & Wintour, 1999; Guron & Friberg, 2000; Dickinson *et al.*, 2005a). In other species, including the mouse, rat, rabbit, pig and dog, nephrogenesis continues into the postnatal period (Moritz & Wintour, 1999; Guron & Friberg, 2000; Dickinson *et al.*, 2005a).

In all mammals, the intra-uterine environment plays an important role in determining nephron endowment. Intra-uterine growth restriction (Hinchliffe *et al.*, 1992; Merlet-Benichou *et al.*, 1994; Bassan *et al.*, 2000; Mitchell *et al.*, 2004) and fetal exposure to maternal low-protein diet (Merlet-Benichou *et al.*, 1994; Langley-Evans *et al.*, 1999; Zimanyi *et al.*, 2000; Vehaskari *et al.*, 2001; Langley-Evans *et al.*, 2003b; Gilbert *et al.*, 2005), iron restriction (Lisle *et al.*, 2003), mild vitamin A deficiency (Lelievre-Pegorier *et al.*, 1998) and maternal dexamethasone administration (Celsi *et al.*, 1998; Ortiz *et al.*, 2001; Martins *et al.*, 2003; Ortiz *et al.*, 2003; Wintour *et al.*, 2003; Figueroa *et al.*, 2005; Moritz *et al.*, 2005c) reduces nephron endowment in offspring. Further, reduced nephron endowment has been linked to the development of hypertension in adulthood (Brenner *et al.*, 1988; Brenner & Chertow, 1994; Wintour *et al.*, 2003; Figueroa *et al.*, 2005). Hence, a healthy prenatal environment is critical for normal renal development and function.

An inverse relationship has been demonstrated between birth weight and the development of hypertension in adult life (Barker *et al.*, 1990; Law *et al.*, 1993; Law & Shiell, 1996). Increased fetal exposure to maternal glucocorticoids may underlie the link between low birth weight and adult hypertension (Edwards *et al.*, 1993). Prenatal exposure to elevated levels of glucocorticoids restricts fetal growth (Langley-Evans, 1997b; Celsi *et al.*, 1998; Jobe *et al.*, 1998b; Quinlivan *et al.*, 1998a; Newnham *et al.*, 1999; Sloboda *et al.*, 2000; Bloomfield *et al.*, 2001; Moss *et al.*, 2001; Newnham & Moss, 2001; Moss *et al.*, 2002) and is associated with increased blood pressure in animal studies of maternal low-protein diet (Langley-Evans *et al.*, 1996; Langley-Evans *et al.*, 1999), carbenoxolone treatment (Lindsay *et al.*, 1996; Langley-Evans, 1997b) and synthetic glucocorticoid administration (Benediktsson *et al.*, 1993; Celsi *et al.*, 1998;

Dodic *et al.*, 1998; Ortiz *et al.*, 2001; Ortiz *et al.*, 2003; Figueroa *et al.*, 2005). Impaired renal development is thought to be a consequence of intra-uterine growth restriction (IUGR); reduced nephron endowment and filtration surface area in the kidney leads to glomerular and systemic hypertension (Brenner & Chertow, 1994).

Maternal administration of betamethasone in doses sufficient to induce lung maturation results in fetal growth restriction in sheep (Jobe *et al.*, 1998b; Newnham *et al.*, 1999; Sloboda *et al.*, 2000; Moss *et al.*, 2001; Moss *et al.*, 2002). Maternal dexamethasone administration early in gestation (day 27 of pregnancy; (Wintour *et al.*, 2003; Moritz *et al.*, 2005c)) and betamethasone administration in mid-gestation (day 80 of pregnancy; (Figueroa *et al.*, 2005)) have been shown to reduce nephron number and increase blood pressure in sheep offspring. In rats, late-gestation administration of synthetic glucocorticoids reduced nephron endowment (Ortiz *et al.*, 2001; Ortiz *et al.*, 2003) and increased blood pressure (Levitt *et al.*, 1996; Ortiz *et al.*, 2001; Ortiz *et al.*, 2003) in adult offspring. Antenatal glucocorticoid therapy in humans has been associated with higher systolic and diastolic blood pressures in adolescents who were born pre-term (Doyle *et al.*, 2000); however, the effect on nephron number is unknown.

In recent years, concerns have been raised regarding the long-term effects of antenatal glucocorticoid therapy in humans. Synthetic glucocorticoids are administered to women at risk of pre-term delivery between 24 and 34 weeks of gestation to promote fetal organ maturation (NIH, 1995). As illustrated in Figure 3.1, during this gestational period, organs such as the kidney are undergoing rapid organogenesis (Harrison *et al.*, 1982; Hinchliffe *et al.*, 1991; Brion *et al.*, 1997) and developmental disruptions could be detrimental to the structure and/or function of fetal organs. Previous studies have suggested that fetal exposure to synthetic glucocorticoids promotes cell differentiation and slows development resulting in growth restriction (Epstein *et al.*, 1977; Slotkin *et al.*, 1992). The effect of late-gestation synthetic glucocorticoid administration on the fetal kidney is unknown.

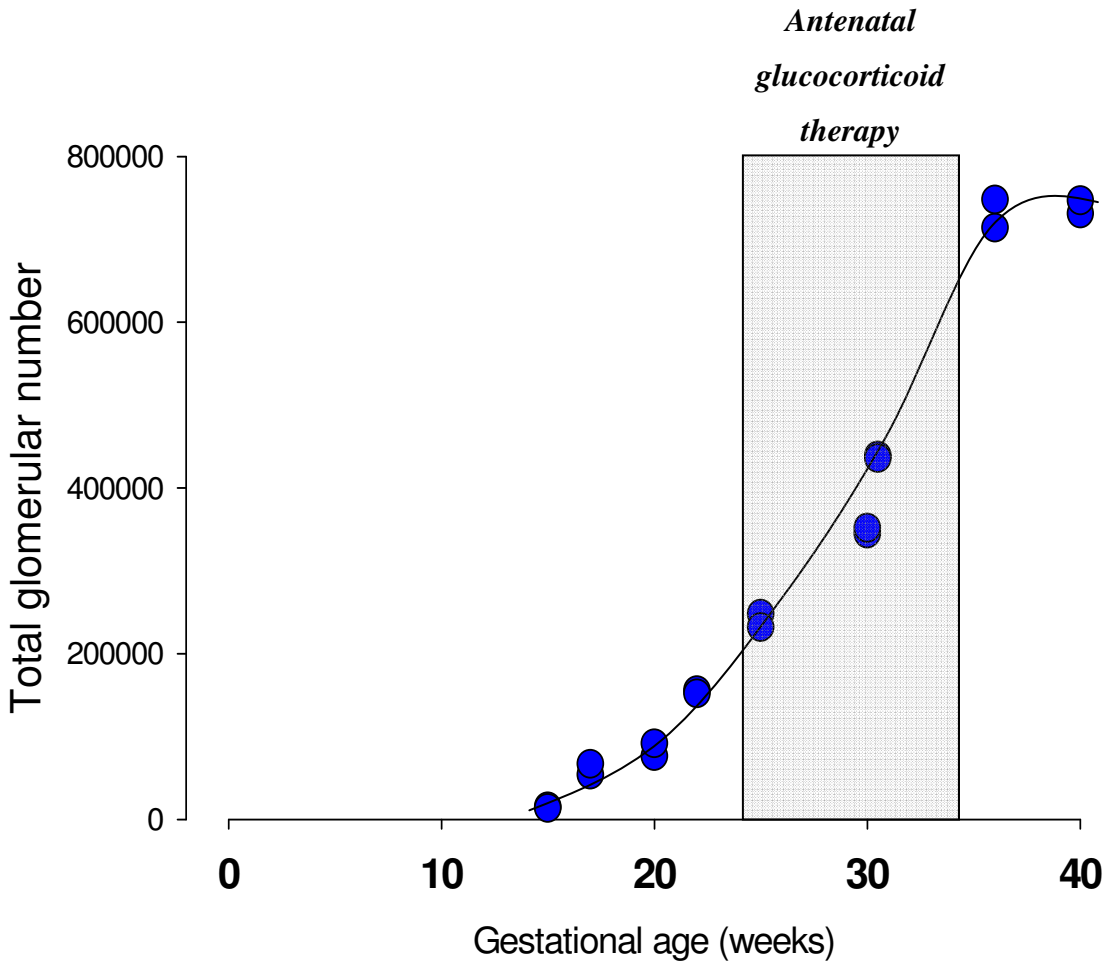


Figure 3.1. Antenatal glucocorticoid therapy and the timing of nephrogenesis in normal human infants. Two-thirds of nephrons are formed in the last trimester of pregnancy, which is the same period of gestation (24 to 34 weeks' gestation) when antenatal glucocorticoids are administered to women at risk of pre-term delivery. Data extracted from Hinchliffe *et al.* (1991). Total glomerular number was determined in 18 human fetal kidneys from nine singleton, non-growth-retarded, non-congenitally malformed, spontaneous second trimester abortions and stillbirths. The unbiased physical disector method was used to calculate total glomerular number.

Until recently, women who failed to deliver and remained at risk of pre-term delivery were commonly treated with repeated doses of glucocorticoids at seven-day intervals (Quinlivan *et al.*, 1998b). Therefore, there exists a population of individuals who have been exposed to repeated doses of glucocorticoids in mid-to-late gestation. Table 3.1 illustrates the percentage of obstetricians who prescribed repeated doses of glucocorticoids. The long-term effects of this treatment remain unclear. To date, there are no published reports describing the effects of clinically-relevant repeated doses of glucocorticoid administration in late gestation on nephron number in any species.

The gestational timing of renal development is similar in sheep and humans (Moritz & Wintour, 1999). The initial aim of the present study was to determine the effect of maternal glucocorticoid administration on fetal nephron endowment and glomerular size in term fetal sheep. It was hypothesised that increased fetal glucocorticoid exposure would restrict fetal growth and accelerate renal maturation, thereby inhibiting the induction of new nephrons and reducing nephron endowment at birth. In this study, unbiased stereological techniques were used to estimate glomerular (nephron) number and glomerular size in term fetal sheep that had been exposed to repeated doses of maternal saline or betamethasone. Unbiased stereological techniques are generally accepted as the "gold standard" for the estimation of total nephron number and glomerular volume (Bertram, 1995; Nyengaard, 1999; Kett & Bertram, 2004).

Stereological studies of human kidneys, using the unbiased physical disector/fractionator technique, have found that nephron number does not vary between the sexes (Nyengaard & Bendtsen, 1992; Hoy *et al.*, 2003). No sex difference exists in the number of glomeruli per kidney, determined using the acid maceration technique, in Sprague-Dawley and Wistar rats varying in age from approximately 5- to 100-days-old (Solomon, 1977) or adult Munich-Wistar rats (Munger & Baylis, 1988). Determining total nephron number in rats using the acid maceration technique has been demonstrated to yield comparable results to those obtained using the physical disector/fractionator combination (Bertram *et al.*, 1992; Lelievre-Pegorier *et al.*, 1998). A recent study of nephron number in fetal sheep (determined using acid maceration) at 135 days of gestation reported that estimated total nephron number was independent of sex (J.P. Figueroa, *personal communication*). Therefore, studies using gold standard unbiased

Table 3.1. Use of repeated antenatal glucocorticoid therapy in clinical practice.

YEAR	% OF OBSTETRICIANS PRESCRIBING REPEATED DOSES OF GLUCOCORTICOIDS	COUNTRY	REFERENCE
1996	96% (92% weekly)	USA	(Planer *et al.*, 1996)
1997	85% (50% weekly)	Australia	(Quinlivan *et al.*, 1998b)
1997	98% (74% weekly)	UK	(Brocklehurst *et al.*, 1999)
1999	75%	USA	(Erickson *et al.*, 2001)
2001	44%	Australia New Zealand	(McLaughlin & Crowther, 2003)

stereological techniques suggest that total nephron number may be independent of sex.

Other stereological studies have reported variable results in regard to the effect of sex on nephron number per unit area or per unit volume. In human infants (Manalich *et al.*, 2000) and neonatal (aged 1- to 11-days-old) Sprague-Dawley rats (Kavlock & Gray, 1982) nephron number per unit area is not different between the sexes. The total number of glomeruli counted in a transverse section of kidney from adult Munich-Wistar rats did not differ with sex (Remuzzi *et al.*, 1988). In contrast, the number of glomeruli counted in a sagittal section through the middle of a kidney of adult BALB/c Han mice differs with sex, with females reported to have 10% more glomeruli in a central section compared with males (Messow *et al.*, 1980). Similarly, adult male Wistar rats had fewer but larger glomeruli per unit area compared with female animals (Lisle *et al.*, 2003). A recent review by Kett and Bertram (2004) highlighted that estimates of nephron number per unit area or volume do not necessarily equate with total nephron number in the kidney. Therefore, unbiased stereological techniques should be employed to ascertain whether differences exist in nephron endowment between the sexes.

Due to an unfortunate sex-bias in the experimental groups in the initial study, a second study was performed. An analysis of unbiased stereological data obtained from Merino sheep kidneys was conducted to determine if sex differences in renal morphology were present. I hypothesized that there would not be an effect of sex on nephron number or glomerular size in kidneys from male and female Merino sheep. This study is important because if differences do not exist in renal morphology between the sexes, then data on nephron number and glomerular size in male and female Merino sheep could be pooled to increase the power of future analyses.

3.2. METHODS

3.2.1. STUDY 1:

3.2.1.1. Animals

All experimental procedures were approved by the Animal Experimentation Ethics Committee of The Western Australia Department of Agriculture. Date-mated Merino ewes bearing single fetuses were managed in a field environment with *ad libitum* access to food and water. At 100 days of pregnancy (d), all ewes received an intra-muscular (i.m.) injection of 150 mg medroxyprogesterone acetate (MPA; Depo Provera; Upjohn, Rydalmere, NSW, Australia; section 2.2) to prevent glucocorticoid-induced pregnancy loss (Moss *et al.*, 2001; Sloboda *et al.*, 2002a; Moss *et al.*, 2003b). Ewes were then randomised to receive i.m. injections of 0.5 mg/kg betamethasone (Celestone Chronodose; Schering-Plough, Baulkham Hills, NSW, Australia) or saline solution (5 mL, 0.9% NaCl) at 104, 111 and 118 d (term is 150 days; section 2.2.2). Ewes were weighed at the time of each injection. Injections were performed by Mr Shaofu Li with assistance from Dr Deborah Sloboda and myself. Ewes received a total dose of approximately 27 mg of betamethasone in a single injection, comparable to the total dose of 24 mg of betamethasone (2 x 12 mg) used in obstetric practice. The dose of betamethasone administered to pregnant ewes in this study was the minimal dose that is required to achieve maximal lung maturation in pre-term lambs (Rebello *et al.*, 1996). Betamethasone was given to ewes at gestational ages equivalent to those at which human antenatal glucocorticoid therapy is used (i.e. 24 to 34 weeks' gestation (NIH, 1995)).

3.2.1.2. Tissue Collection

Fetuses were delivered by Caesarean section at 146 ± 1 d, after ewes were humanely killed by captive bolt. Umbilical arterial blood pH, PCO_2 and PO_2 were measured at the time of delivery (section 2.3). Immediately after delivery, fetuses were weighed and killed by decapitation. Major fetal organs were removed, weighed and collected for use in other studies by Dr Deborah Sloboda, Dr Timothy Moss, Dr Ilias Nitsos, Mr Shaofu Li and myself. Adrenal weight was recorded as an index of the effect of betamethasone.

In the present study, kidneys were collected from five control and six betamethasone-exposed fetuses for nephron counting. Right kidneys were removed from the fetuses and weighed. Portions of right kidney containing both cortex and medulla were fixed in 4% paraformaldehyde or frozen in liquid nitrogen for subsequent analysis (Chapter 4). The left kidney was left *in situ* and a cannula was secured in the renal artery with assistance from Dr Timothy Moss. The kidney was then removed, weighed, and perfused with 50 mL of 0.9% NaCl, prior to perfusion-fixation with 60 mL of 10% neutral buffered formalin (pH 6.9) using a gravity-fed perfusion apparatus. The perfusate level was maintained at a height of 77.5 cm above the kidney. This height was determined to be the correct fluid level needed to generate a normal fetal MAP of 50 mmHg, which was measured with a pressure transducer at kidney height (D.M. Burrage, *personal communication*). The gravity-fed perfusion apparatus is illustrated in Figure 3.2. Each fixed kidney was cut in half longitudinally and immersed in 250 mL of 10% neutral buffered formalin for 24 h; kidneys were then immersed in fresh formalin for two weeks until sampling and processing.

3.2.1.3. Kidney sampling

Kidney sampling and processing were performed as previously described (Douglas-Denton *et al.*, 2002) by Mrs Rebecca Douglas-Denton and Mrs Sue Connell in the Department of Anatomy and Cell Biology at Monash University, Melbourne, VIC, Australia. Each kidney was cut into quarters and sliced at a thickness of 1 mm to generate an average of 211 (ranging from 124 to 296) pieces of kidney. Systematic sampling was used to select every n^{th} piece of kidney for further processing, starting with a random number in the interval 1 to *n*. Between nine and twelve pieces of kidney were sampled for each animal. Most of the renal medulla was removed and then each cortical piece was embedded in glycolmethacrylate resin (Technovit 7100; Heraeus Kulzer GmbH, Germany; Appendix 6). Each kidney block was then exhaustively sectioned at 15 µm using a fully motorized rotary microtome fitted with glass knives (Leica RM 2165; Leica Microsystems, Gladesville, NSW, Australia). Every 10^{th} and 11^{th} section were collected (with the first section chosen at random) and stained with periodic acid-Schiff reagent (Appendix 7).

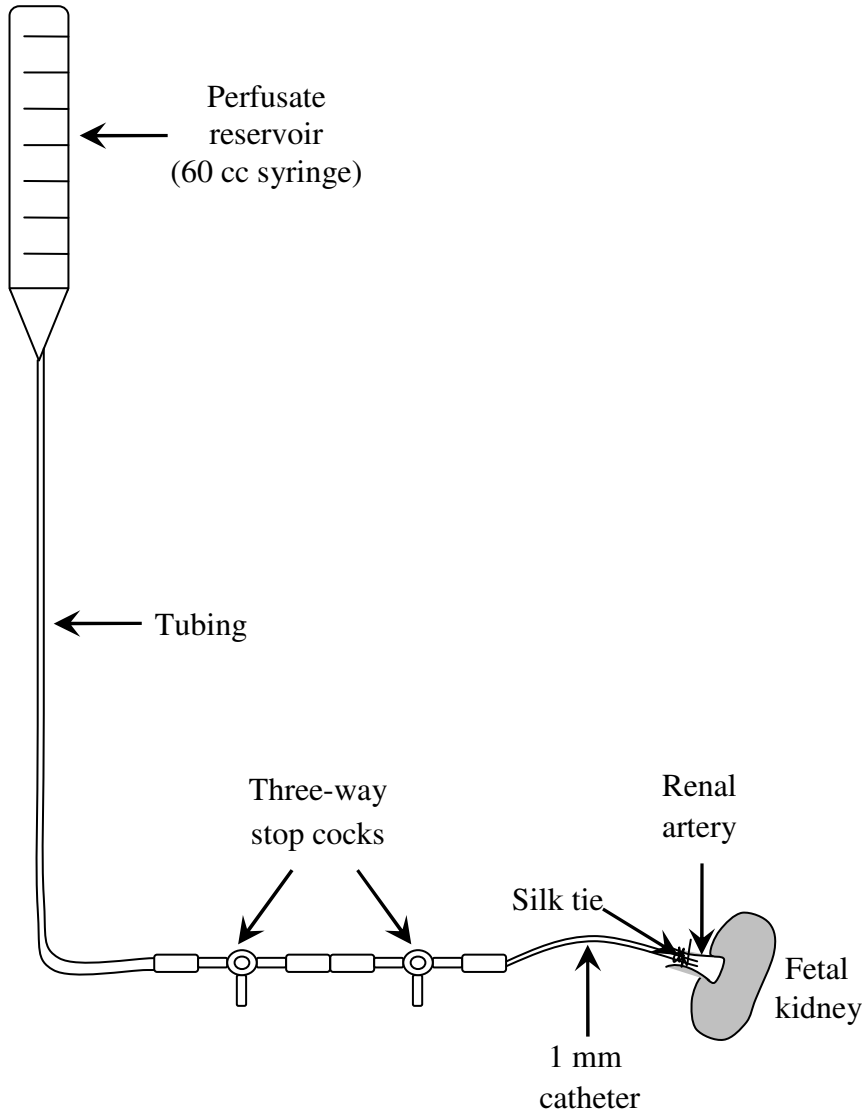


Figure 3.2. Schematic diagram of a simple gravity-fed perfusion system.

The perfusion system was constructed using readily available tubing, stop cocks and a syringe. A 1 mm catheter was inserted into the renal artery of the isolated kidney, which was flushed with 50 mL of 0.9% NaCl prior to fixation with 60 mL of 10% neutral buffered formalin (pH 6.9). The perfusate level was maintained at a height of 77.5 cm above the kidney. Once the perfusion was complete, the catheter was removed and external vessels were dissected from the fetal kidney. The kidney was then re-weighed, and immersed in fixative prior to sampling for stereological analysis.

3.2.1.4. Estimating kidney volume

Left kidney volume was estimated using the Cavalieri principle (Douglas-Denton *et al.*, 2002). The Cavalieri method provides an unbiased estimate of the volume of a structure of arbitrary shape and size (Gundersen & Jensen, 1987; Bertram *et al.*, 1992; Bertram, 1995; Roberts *et al.*, 2000). Briefly, every 10th section was viewed on a Fuji Minicopy Reader (Model RF3A; microfiche) at a final magnification of 24.25x. An orthogonal stereological grid (30 mm) with an area per grid point ($a(p)$) of 1.53 mm^2 (corrected for magnification) was placed on the microfiche screen and grid points that overlaid kidney tissue were counted. Approximately 160 sections (ranging from 129 to 198 sections) were point counted per kidney. Kidney volume was then estimated using the following equation:

$$V_{kid} = n \times 10 \times t \times a(p)_{microfiche} \times P_s$$

where:

- n = inverse of the kidney piece sampling fraction;
- 10 = inverse of the section sampling fraction (every 10th section counted);
- t = average section thickness (0.015 mm);
- $a(p)_{microfiche}$ = area per grid point (1.53 mm^2); and
- P_s = sum of grid points that overlaid kidney tissue.

3.2.1.5. Estimating total nephron number

In this study, nephron number was estimated in the left kidney of fetal sheep using the physical disector/fractionator combination (Sterio, 1984; Nyengaard & Bendtsen, 1990; Bertram, 1995; Douglas-Denton *et al.*, 2002; Wintour *et al.*, 2003). This technique uses a three-dimensional probe, composed of an unbiased counting frame and a parallel plane a know distance apart, to obtain unbiased estimates of the number of arbitrary particles in a specimen (Sterio, 1984). Briefly, every 10th (reference) and 11th (comparison or "look-up") section pair containing complete kidney sections were projected side-by-side onto a desktop in a darkened room using two adjacent light microscopes (Olympus BX50, Olympus Optical Co. Ltd, Japan; as illustrated in Figure 3.3). One microscope was fitted with a motorised stage and the other was fitted with a

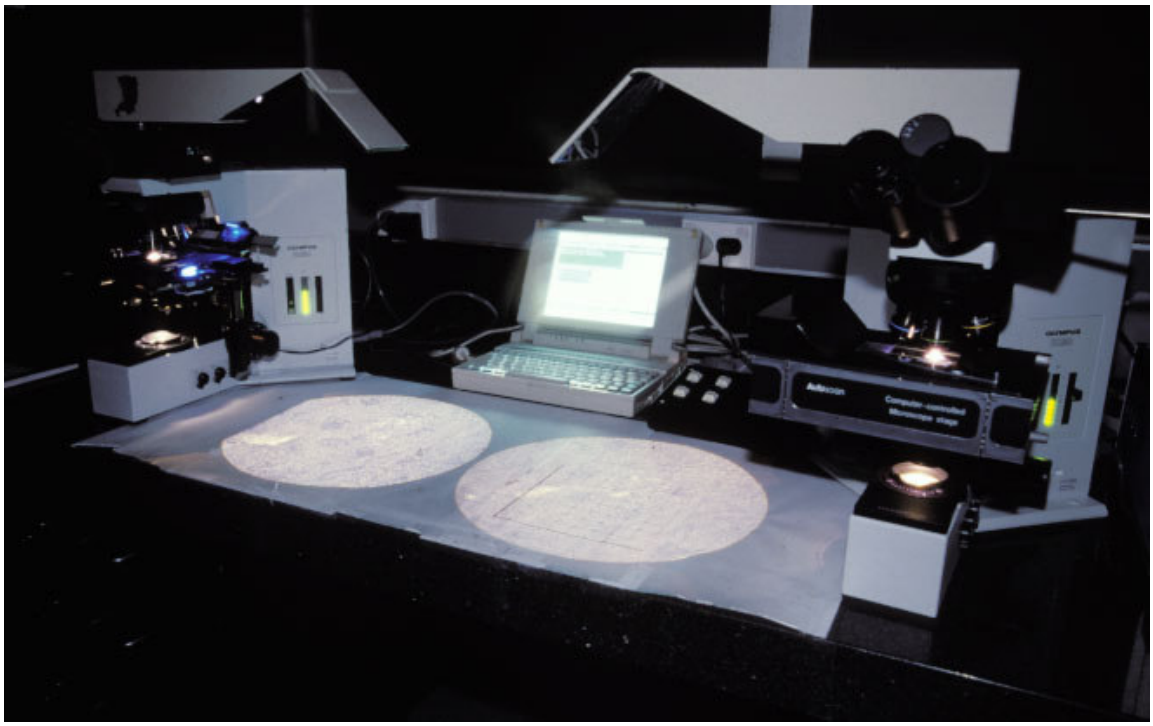


Figure 3.3. The physical disector apparatus.

Two adjacent Olympus BX50 light microscopes project section images onto the desk-top in a semi-darkened room. The left microscope has a rotatable stage to facilitate section alignment, and the right microscope has a motorised stage to allow systematic "stepping" across the section at a constant distance. The reference section was placed under the right microscope and the comparison section was placed under the left microscope. Orthogonal grids were placed on the desk-top in the fields of view in order to point-count various parameters in the reference section (P_f, and points overlaying kidney tissue, glomerular tufts and renal corpuscles). Glomeruli were counted if they were present in one field of view, but not the other, and did not intersect the exclusion edges of the counting frame (the bottom and left edges). The photo above is courtesy of Mrs Rebecca Douglas-Denton at the Department of Anatomy and Cell Biology, Monash University.

rotatable stage to allow section alignment. Sections were projected at a final magnification of 298x. The fraction of the section area used for counting glomeruli was denoted P_f and was calculated by counting the number of boxes, within an orthogonal grid, that were completely filled with kidney tissue. The exception to this rule was where the centre point of the 6 x 6 cm box fell on kidney tissue and a natural border (i.e. kidney capsule) was inside the box. A stereological grid (3 x 3 cm) was placed on each field of view and the number of points overlaying kidney tissue (P_{kid}), glomerular tufts (P_{glom}) and renal corpuscles (P_{corp}) on the reference section were scored. Glomerular tufts were defined as including the glomerular mesangium and intra-capsular glomerular capillaries (Figure 3.4). Renal corpuscles were defined as including the glomerular tuft, Bowman's space and Bowman's capsule (Figure 3.4). Glomerular profiles that were sampled by the grid intersections in the unbiased counting frame in the field of view of the reference section but not in the comparison section were counted. Also, those glomerular profiles present in the comparison section but not in the reference section were counted to double the efficiency of the technique. However, in accordance with the stereological rules reported by Gundersen, glomerular profiles that were intersected by the upper and right borders (inclusion edges) were counted but those that intersected the lower and left borders (exclusion edges) of the counting frame were disregarded (Gundersen, 1977). Also, glomerular profiles that fell on an artificial edge (i.e. where the tissue was cut) were excluded (Gundersen, 1986). The formula used to estimate total nephron number in the left kidney was:

$$N_{glom} = n \times 10 \times (P_s \times a(p)_{microfiche} / 2 \times P_f \times a(p)_{microscope}) \times Q^-$$

where:

- n = inverse of the kidney piece sampling fraction;
- 10 = inverse of the section sampling fraction;
- $P_s \times a(p)_{microfiche}$ = total area of kidney sections;
- $2 \times P_f \times a(p)_{microscope}$ = fraction of the section area used for counting glomeruli in each direction; and
- Q^- = actual number of glomeruli counted.

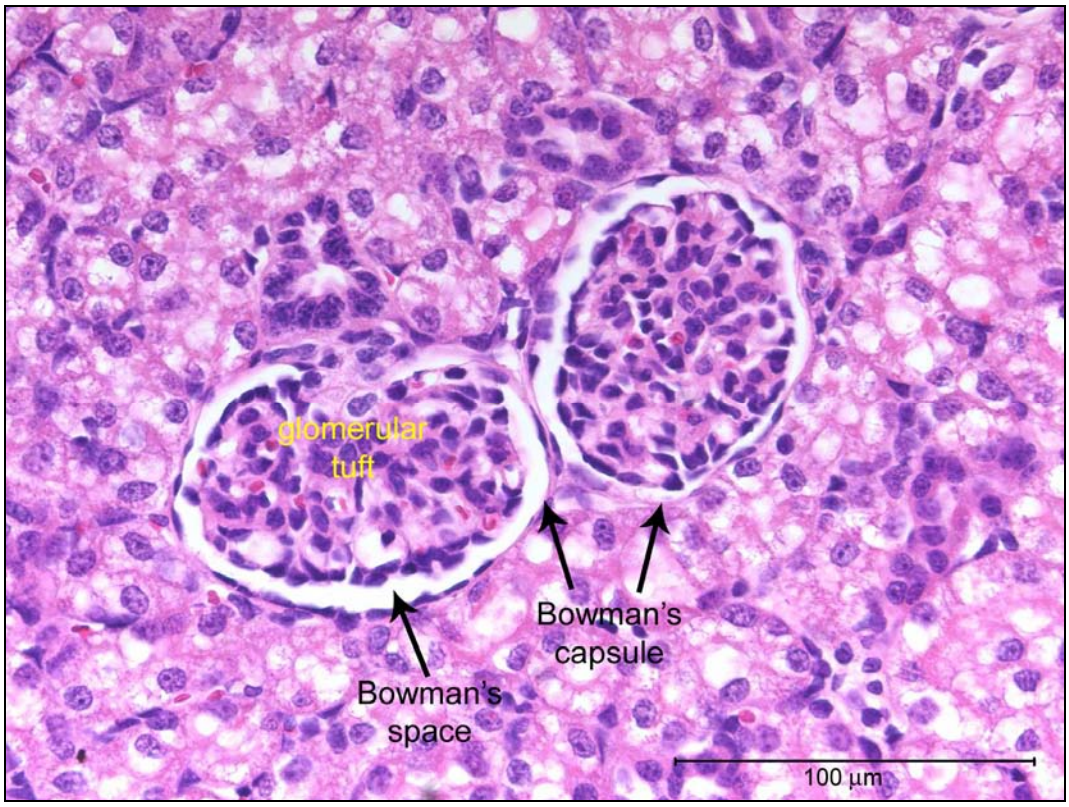


Figure 3.4. Glomerular histology.

Representative digital micrograph of a renal cortical section from a saline-exposed fetal sheep at 146 days' gestation. The glomerular tuft is comprised of the glomerular mesangium and intra-capsular capillaries. Renal corpuscles are defined as including the glomerular tuft, Bowman's space and Bowman's capsule. The kidney section was stained with haematoxylin and eosin. The digital micrograph was captured at 400x magnification; scale bar is 100 µm.

The area per point on the microscope was calculated by:

$$a(p)_{microscope} = (P_f \text{ grid size})^2 / (\text{magnification})^2 = 60 \text{ mm}^2 / 298^2 = 0.0405 \text{ mm}^2.$$

Between 10 and 33 fields of view were analysed per section. Systematic uniform sampling of vision fields was performed in incremental movements of 1600 µm along the *x*-axis and then 1600 µm down the *y*-axis from a random starting point at the top left-hand corner of each section. The constant stepping was facilitated by a motorized stage connected to a computer using the Trakscan program (v7.0.0; Autoscan Systems Pty Ltd, Gardenvale, VIC, Australia). For each kidney, between 26 and 39 pairs of sections were analysed (478 to 793 fields of view) and between 200 and 270 glomeruli were counted. Each kidney took me between 16 and 20 hours to count.

I performed all glomerular counts blinded to treatment. One kidney was counted three times to ensure reproducibility of estimates; the repeated estimates of total nephron number were 344,396, 345,460 and 321,853 (mean: 340,236; S.D.: 16,697). Thus the intra-observer co-efficient of variation for nephron counting was 5%. To validate my counting technique, the reliability of the counts was determined by consecutive pairs of counting trials (n = 3) with an experienced stereologist (Mrs Rebecca Douglas-Denton). The typical error in the reliability from the trials was 4%.

3.2.1.6. Estimating glomerular and renal corpuscle volumes

Mean glomerular volume was estimated using the equation:

$$V_{glom} = (P_{glom} / P_{kid}) / (N_{glom} / V_{kid}).$$

The total volume of all glomeruli in the kidney was estimated using:

$$V_{glom.tot} = V_{glom} \times N_{glom}.$$

Mean renal corpuscle volume was estimated using the equation:

$$V_{corp} = (P_{corp} / P_{kid}) / (N_{glom} / V_{kid}),$$

and total renal corpuscle volume was estimated by:

$$V_{corp.tot} = V_{corp} \times N_{glom}.$$

3.2.2. STUDY 2:

Due to unbalanced sex ratios in the experimental groups, a second study was designed to investigate the effect of sex on nephron endowment in control and betamethasone-exposed fetal sheep. Data from four female (aged 140 ± 1 d) and one male (aged 137 d) control Merino sheep were provided by Dr Karen Moritz. These five control sheep had been exposed to a maternal i.v. infusion of saline from 26 to 28 d. Kidney volume and nephron number had been determined in the right kidney of these control sheep by Dr Karen Moritz using the same techniques as described in Study 1. The estimated inter-observer co-efficient of variation in nephron counting between Dr Karen Moritz and myself was approximately 3%.

3.2.3. Statistical Analysis

In Study 1, data from five (male) saline-exposed fetuses and six (1 male, 5 females) betamethasone-exposed fetuses were compared. One unusually large female lamb from the saline group, with a birth weight of 7.15 kg, was excluded from the analysis; the birth weight of this animal was greater than two S.D. above the mean of the other fetuses.

Study 2 consisted of two separate analyses. Firstly, data from male (n = 6) and female (n = 4) control fetal sheep were compared. Secondly, data from female control (aged 140 ± 1 d) and female betamethasone-exposed (aged 147 d; n = 5 from Study 1) fetal sheep were compared. In the second analysis, the gestational age of each group was significantly different ($p < 0.001$); therefore, body weight and left kidney weight were predicted at 147 d for control fetuses as kidney weight and body weight increase with gestational age (Harrison *et al.*, 1982). Body weight and left kidney weight were predicted using a regression equation based on data from 18 control sheep ranging in age from 126 to 147 d (data not shown). Nephrogenesis is complete in sheep by 130 d (Robillard *et al.*, 1981); therefore, because the female fetuses were aged between 138 and 142 d, and there was no evidence of a nephrogenic zone in histological sections, actual nephron numbers as determined using the physical disector/fractionator were used and not adjusted for a gestational age of 147 d.

Data were normally distributed and are reported as mean ± S.E.M. SigmaStat (v 2.03, SPSS Inc., Chicago, IL, USA) and SPSS (v 12.0, SPSS Inc.) software were used to perform univariate (*t*-tests) and linear regression analyses on body weight and various renal parameters. Data that were not normally distributed were transformed to achieve normality. Differences between saline and betamethasone-exposed groups were deemed statistically significant at $p < 0.05$. Dr Dorota Doherty (Senior Biostatistician, Women's and Infants' Research Foundation) and Mrs Liz Nathan (Biostatistician, Women's and Infants' Research Foundation) provided assistance with statistical analyses and advice on the study data.

3.3. RESULTS

3.3.1. STUDY 1:

3.3.1.1. Delivery data, fetal body and kidney weights

At delivery, there were no differences in umbilical arterial blood pH, PCO_2 and PO_2 between fetuses exposed to maternal betamethasone and controls (Table 3.2). However, exposure to repeated doses of betamethasone significantly reduced fetal body weight by 28% ($p = 0.02$) at 146 ± 1 d (Table 3.2). Repeated maternal betamethasone administration tended to lower total kidney weight ($p = 0.08$) compared with controls (Table 3.3). Kidney to body weight ratios were similar between the groups ($p = 0.63$; Table 3.3). Maternal betamethasone administration had variable effects on fetal body and kidney weights. Four of six fetuses exposed to maternal betamethasone were growth-restricted (< 2 S.D. below mean of controls: body weight < 4202 g), and of these, three had growth-restricted kidneys (< 2 S.D. below mean of controls: < 22 g total weight). The co-efficient of variation for body weight was 11% in the saline group and 25% in the betamethasone group. Similarly, the co-efficient of variation in total kidney weight was 33% in the betamethasone group compared with 9% in controls.

Table 3.2. Delivery data: fetal body weight and umbilical arterial blood gases.
Fetal sheep exposed to repeated maternal saline or betamethasone (0.5 mg/kg) administration in late gestation were delivered 28 days later, at 146 ± 1 d.

	SALINE	BETAMETHASONE
Body weight (kg)	5.40 ± 0.27	3.88 ± 0.40 *
pH	7.40 ± 0.01	7.38 ± 0.01
PCO_2 (mmHg)	50.9 ± 2.1	55.5 ± 1.6
PO_2 (mmHg)	16.5 ± 1.4	12.9 ± 0.5

Values are mean ± S.E.M. of saline (n = 5) and betamethasone (n = 6) groups.
* $p < 0.05$, groups compared by *t*-test.

Table 3.3. Renal parameters in 146 ± 1 d fetal sheep exposed to maternal saline or betamethasone administration.

	SALINE	BETAMETHASONE	p VALUE
Left kidney weight (g)	12.9 ± 0.7	10.4 ± 1.4	0.16
Total kidney weight (g)	26.8 ± 1.1	20.6 ± 2.7	0.08
Kidney weight/body weight (g/kg)	5.0 ± 0.3	5.3 ± 0.4	0.63
Left kidney volume (cm^3)	12.4 ± 0.7	11.3 ± 1.7	0.58
Total nephron number	486,362 ± 23,222	444,278 ± 43,114	0.44
Mean glomerular volume ($x10^{-4}$ mm^3)	7.14 ± 0.70	7.02 ± 0.43	0.88
Total glomerular volume (cm^3)	0.346 ± 0.034	0.316 ± 0.038	0.58
Mean corpuscle volume ($x10^{-4}$ mm^3)	10.84 ± 1.22	10.48 ± 0.71	0.79
Total corpuscle volume (cm^3)	0.528 ± 0.065	0.475 ± 0.065	0.59

Values are mean ± S.E.M.

Saline: n = 5 males; Betamethasone: n = 1 male, n = 5 females.

3.3.1.2. Kidney volume

Overall, left kidney volume was not different between saline and betamethasone-exposed fetuses (Table 3.3). However, left kidney volume was 40% smaller in betamethasone-exposed fetuses with renal growth restriction compared to controls and normal kidney weight betamethasone fetuses ($p = 0.003$)

3.3.1.3. Total adrenal weight

Total adrenal weight was similar in saline and betamethasone-exposed fetuses with normal kidney weight (saline: 0.462 ± 0.029 g; betamethasone normal kidney weight: 0.479 ± 0.029 g; $n = 3$; $p = 0.71$). In contrast, total adrenal weight was 27% lower ($p = 0.01$) in betamethasone-exposed fetuses with renal growth restriction (0.343 ± 0.040 g; $n = 3$) compared to fetuses with normal kidney weight (saline and betamethasone-exposed; Figure 3.5).

3.3.1.4. Other organ weights

Organ weights of the liver ($p = 0.04$) and total brain ($p = 0.01$) were reduced in fetuses exposed to repeated doses of maternal betamethasone compared to controls. Further, weights of the kidneys ($p < 0.001$), brain ($p = 0.001$), body ($p = 0.01$), adrenals ($p = 0.01$), liver ($p = 0.02$) and placenta ($p = 0.04$) were reduced in betamethasone-exposed animals with adrenal/renal growth restriction compared to fetuses (control or betamethasone-exposed) with normal kidney and adrenal weight.

3.3.1.5. Nephron number

Eleven kidneys were used for stereological analysis (saline: $n = 5$; betamethasone: $n = 6$). Nephron numbers ranged from 413,621 to 547,129 in control animals and from 340,236 to 574,506 in betamethasone-exposed fetuses. The co-efficient of variation in nephron numbers within the groups was 11% in controls and 24% in betamethasone-exposed fetal sheep. There was a significant positive correlation between left kidney weight and total nephron number ($R^2 = 0.64$, $p = 0.003$; Figure 3.6) in all animals. Nephron number was also significantly correlated with total glomerular tuft volume

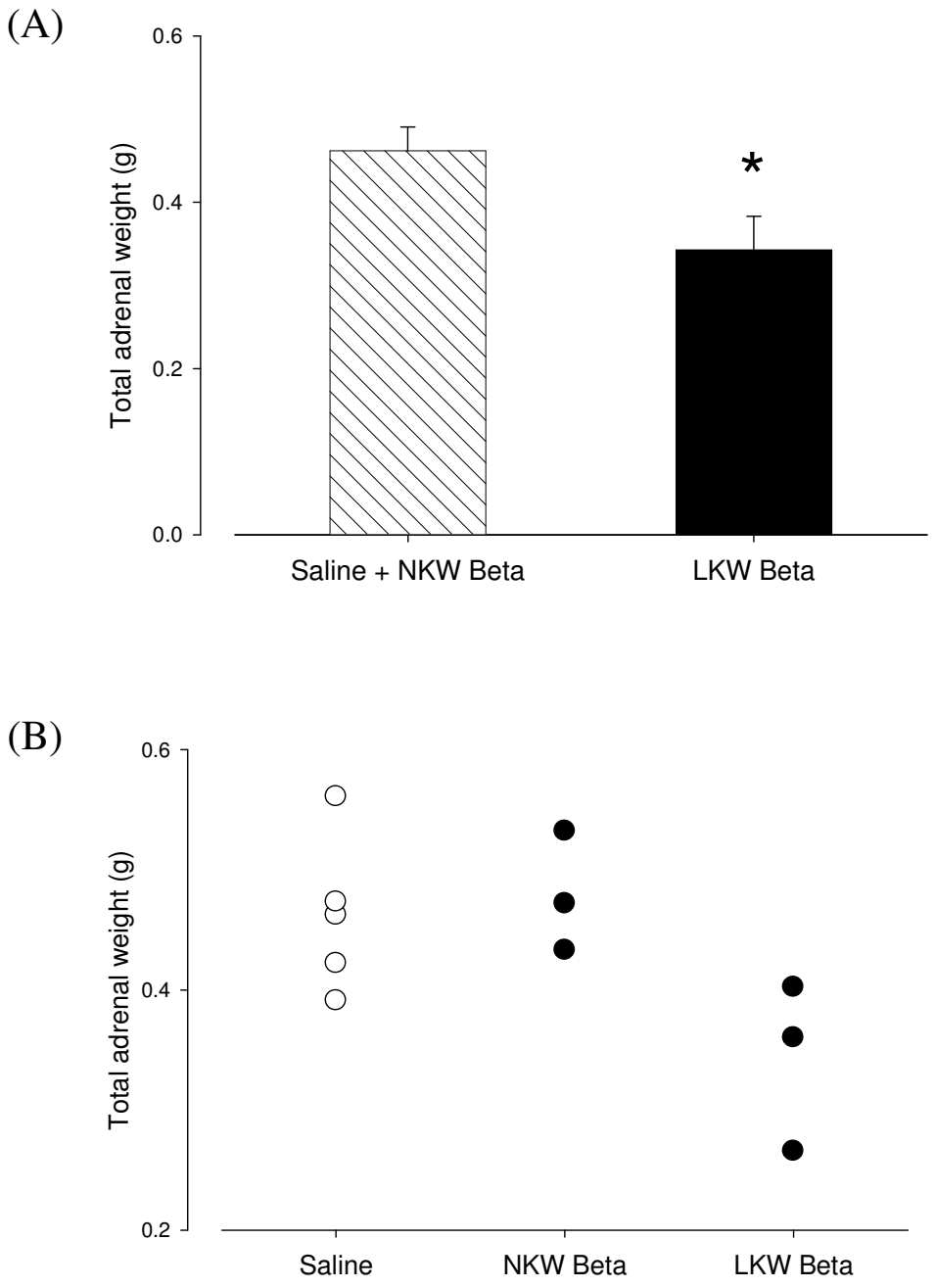


Figure 3.5. Total adrenal weight.

(A) Graph illustrates mean ± S.E.M. of saline + normal kidney weight (NKW) betamethasone and low kidney weight (LKW) betamethasone groups. (B) Graph illustrates individual data points for all animals. White circles represent saline animals; black circles represent betamethasone-exposed animals.

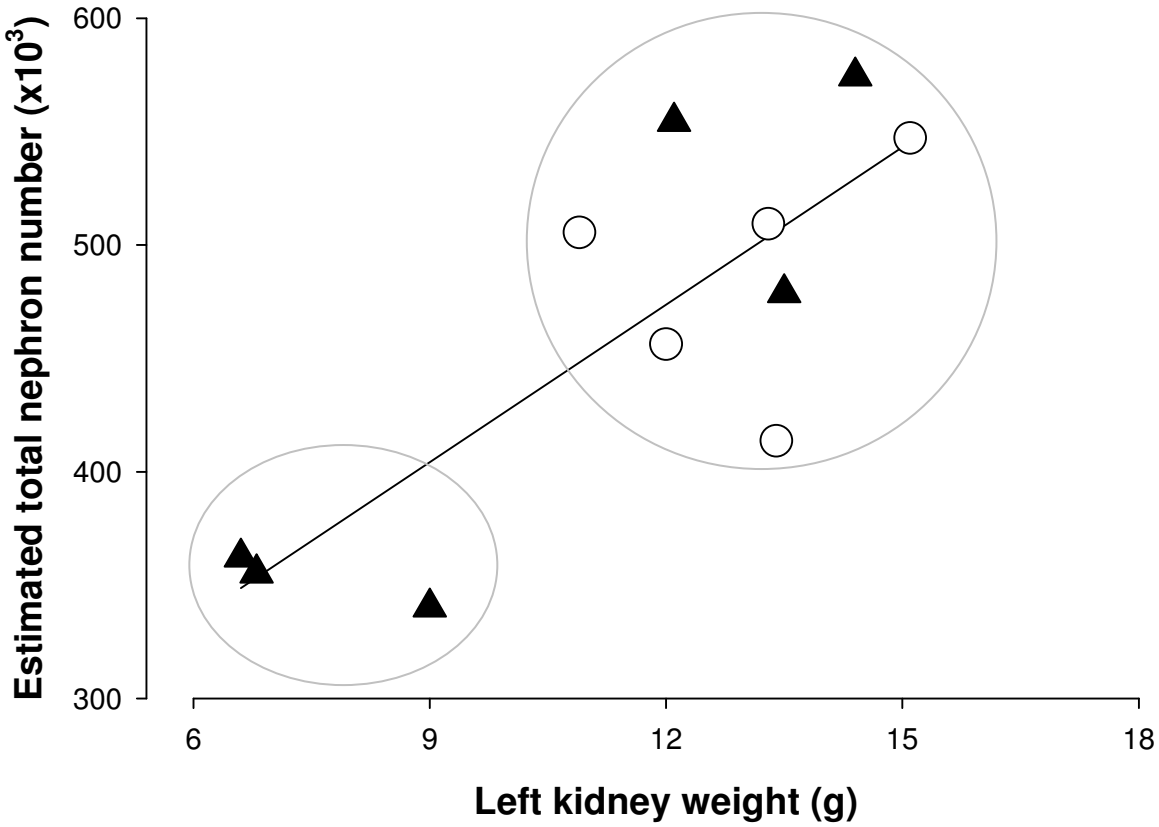


Figure 3.6. Relationship between estimated total nephron number and left kidney weight. Fetuses were exposed to repeated maternal saline (O) or betamethasone (▲) administration in late gestation and delivered 28 days later at 146 ± 1 d. The linear regression line is plotted (R^2 = 0.64, p = 0.003) and predicts an increase of 23,153 nephrons per gram of left kidney weight. There appears to be two distinct sub-sets within the betamethasone group: one group with low left kidney weight and reduced nephron number, and another group similar to controls, with normal kidney weight and nephron number.

($V_{glom.tot}$; $R^2 = 0.58$, $p = 0.006$) and total renal corpuscle volume ($V_{corp.tot}$; $R^2 = 0.60$, $p = 0.005$) in all animals.

Although there was no statistically significant effect of maternal betamethasone administration on nephron number between groups overall ($p = 0.44$, Table 3.3), it appeared that nephron number was reduced in growth-restricted fetuses. Growth-restricted fetuses (n = 4 betamethasone) weighed 37% less than those with normal birth weights (n = 5 saline; n = 2 betamethasone) and had 19% fewer nephrons ($p = 0.07$). Two distinct sub-sets within the betamethasone group are illustrated in Figure 3.6. Univariate analysis of nephron number according to left kidney weight (normal kidney weight: n = 5 saline, n = 3 betamethasone; low kidney weight: n = 3 betamethasone) revealed that betamethasone-exposed fetuses with adrenal and renal growth restriction (mean kidney weight 43% lower, $p < 0.001$) had 30% fewer nephrons ($p = 0.001$).

3.3.1.6. Glomerular tuft and renal corpuscle volume

Mean glomerular tuft (V_{glom}) and renal corpuscle (V_{corp}) volumes were similar in betamethasone-exposed and control fetuses (Table 3.3). V_{glom} ranged from 5.14 to 9.11 x 10^{-4} mm^3 and V_{corp} varied from 7.51 to 13.7 x 10^{-4} mm^3. There was a strong correlation between mean glomerular tuft and renal corpuscle volumes in all animals ($R^2 = 0.90$, $p < 0.001$). $V_{glom.tot}$ ranged from 0.186 to 0.452 cm^3. $V_{corp.tot}$ varied from 0.272 to 0.746 cm^3. In all animals, $V_{glom.tot}$ and $V_{corp.tot}$ were correlated with left kidney weight ($V_{glom.tot}$: $R^2 = 0.80$, $p < 0.001$, Figure 3.7; $V_{corp.tot}$: $R^2 = 0.69$, $p = 0.002$); but overall were unaffected by betamethasone exposure (Table 3.3). However, $V_{glom.tot}$ was reduced by 34% ($p = 0.02$) and $V_{corp.tot}$ was 37% lower ($p = 0.03$) in betamethasone-exposed fetuses with renal growth restriction compared to fetuses with normal kidney weight.

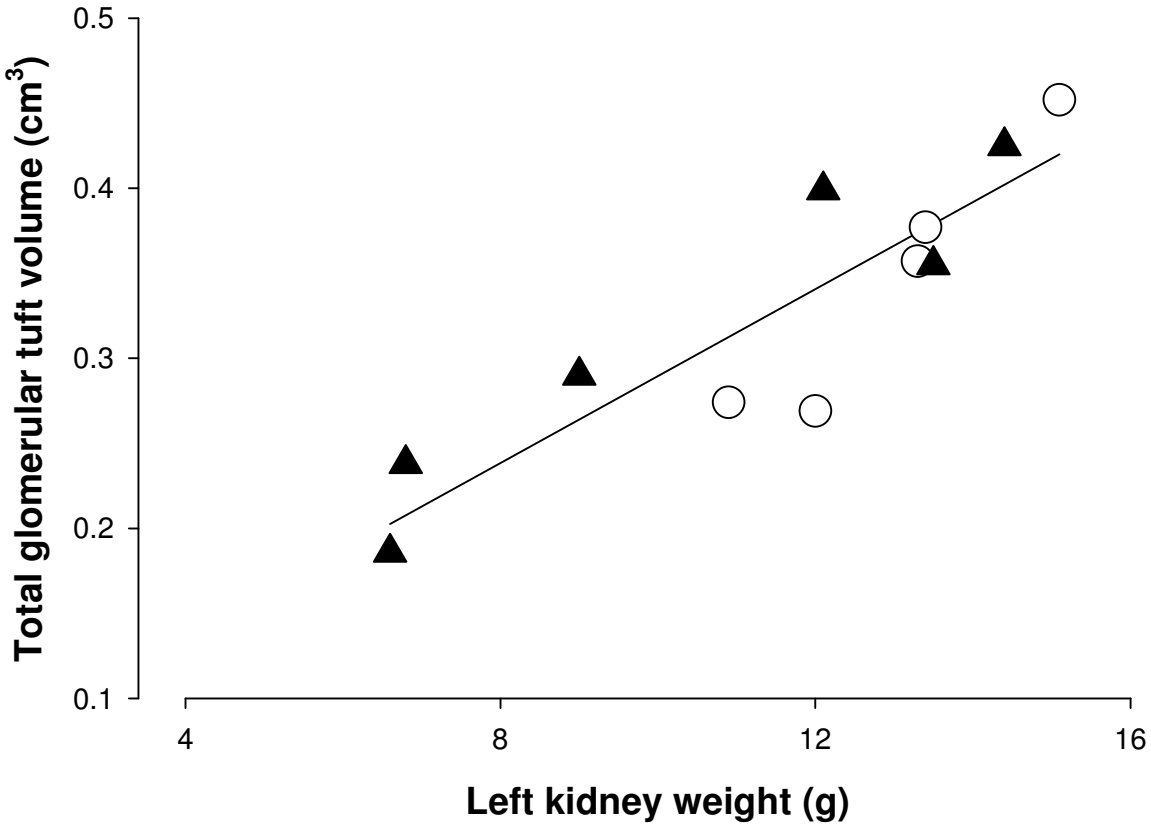


Figure 3.7. Correlation between total glomerular tuft volume and left kidney weight. Total glomerular tuft volume was significantly positively correlated with left kidney weight in late-gestation fetal sheep exposed to maternal saline (O) or betamethasone (▲). The linear regression line is plotted ($R^2 = 0.80$, $p < 0.001$).

3.3.1.7. Maternal body weight

There was no difference in maternal body weight between groups at 104 (saline: 50 ± 1 kg; betamethasone: 54 ± 4; p = 0.42), 111 (saline: 53 ± 1 kg; betamethasone: 58 ± 3 kg; p = 0.21) or 118 d (saline: 55 ± 0 kg; betamethasone: 59 ± 4 kg; p = 0.32). Thus, maternal weight gain from day 104 to 118 of pregnancy (saline: 5 ± 1 kg; betamethasone: 6 ± 1 kg; p = 0.64) was not different between groups.

3.3.2. STUDY 2:

3.3.2.1. Part 1: Male versus female controls

The results of univariate analyses of fetal characteristics by sex are illustrated in Table 3.4 and demonstrate no sex difference in gestational age, body weight, left kidney weight, kidney volume or nephron number in late-gestation fetal sheep. Nephron number was significantly positively correlated with body weight ($R^2 = 0.44$, p = 0.04) in all sheep. However, nephron number was not correlated with left kidney weight (p = 0.15).

3.3.2.2. Part 2: Female sheep: control versus betamethasone

The gestational age of the fetuses was significantly different (p < 0.001) between control and betamethasone-exposed groups; therefore, body weight and kidney weight were predicted for 147 d. The mean fetal body weight of betamethasone-exposed fetuses was 27% lower than predicted values for controls (p = 0.04; Table 3.5). Three out of five female fetuses in the betamethasone group were growth-restricted; three out of five (two had growth-restricted body weights) also had growth-restricted kidneys. Left kidney weight was reduced by 33% (p = 0.04; Table 3.5) in female fetuses exposed to betamethasone compared to predicted values for fetuses exposed to maternal saline infusion.

Table 3.4. Univariate analysis of fetal sheep characteristics by sex.

	FEMALES	MALES	p VALUE
Gestational age (d)	140 ± 1	144 ± 1	0.11
Body weight (kg)	4.10 ± 0.31	5.08 ± 0.26	0.11
Left kidney weight (g)	13.7 ± 1.08	12.5 ± 0.56	0.37
Kidney volume (cm^3)	11.1 ± 0.53	12.0 ± 0.48	0.40
Nephron number	454,895 ± 11,562	468,774 ± 17,570	0.69

Values are mean ± S.E.M. Females: n = 4; Males: n = 6.

Table 3.5. The effect of maternal betamethasone administration on female fetal sheep parameters at 147 d.

	SALINE	BETAMETHASONE	p VALUE
Gestational age (d)	140 ± 1	147 ± 0	< 0.001 *
Left kidney weight (g)	14.4 ± 1.1 †	9.6 ± 1.4	0.04 *
Body weight (kg)	5.05 ± 0.35 †	3.66 ± 0.41	0.04 *
Nephron number	454,895 ± 11,562	418, 233 ± 42,080	0.48

Values are mean ± S.E.M.

† denotes predicted values. * denotes a statistically significant difference between saline- (n = 4) and betamethasone-exposed (n = 5) groups ($p < 0.05$).

Nephron number was significantly correlated with left kidney weight ($R^2 = 0.52$; $p = 0.03$) in all female fetuses. Overall, nephron number was not different between 147 d female fetuses exposed to betamethasone or 140 ± 1 d female controls ($p = 0.48$; Table 3.5). However, nephron number was approximately 26% lower in betamethasone-exposed fetuses with renal growth restriction (352,608 ± 6,506; $n = 3$; $p < 0.001$) compared to fetuses with normal kidney weight (475,486 ± 17,844; saline: $n = 4$; betamethasone: $n = 2$).

3.4. DISCUSSION

This is the first study to examine the effect of late-gestation synthetic glucocorticoids on nephron number in fetal sheep. The recommended gold standard method of unbiased stereology (the physical disector/fractionator method) was used to estimate total nephron number and determine renal corpuscle and glomerular tuft volume in one kidney from each animal studied. Previous studies of nephron number in sheep kidneys have used this method (Douglas-Denton *et al.*, 2002; Wintour *et al.*, 2003; Mitchell *et al.*, 2004; Moritz *et al.*, 2005c). My data indicates that a broad spectrum in nephron numbers exist among fetal sheep at term: total nephron number ranged from 340,236 to 574,506. Consistent with data from a previous study of fetal sheep, nephron number was significantly correlated with fetal kidney weight (Douglas-Denton *et al.*, 2002). $V_{glom.tot}$ and $V_{corp.tot}$ were strongly correlated with kidney weight and the estimated total nephron number in all animals, in agreement with data obtained in multi-racial studies of human kidneys at autopsy (Hoy *et al.*, 2003; Hughson *et al.*, 2003). In the present study, V_{glom} and V_{corp} were tightly related, as reported previously in data from humans (Hoy *et al.*, 2003) and sheep (Douglas-Denton *et al.*, 2002).

I have confirmed that late-gestation maternal administration of betamethasone to pregnant sheep has variable effects on fetal growth (Jobe *et al.*, 1998a; Jobe *et al.*, 1998b; Newnham *et al.*, 1999; Sloboda *et al.*, 2000). In the present study, total adrenal weight was used to assess the outcome of betamethasone exposure. Previous studies have demonstrated that maternal glucocorticoid administration results in reduced fetal adrenal gland weight (Challis *et al.*, 1974; Hristic *et al.*, 1995; Hristic *et al.*, 1997) and adrenal gland suppression (Pratt *et al.*, 1999; Sloboda *et al.*, 2003; Kutzler *et al.*, 2004). Adrenal gland weights were significantly reduced in three out of six betamethasone-

exposed fetuses; these same three fetuses also exhibited renal growth restriction. Such variable action of betamethasone may be consistent with only a 50% reduction in the incidence of respiratory distress syndrome (RDS) observed in clinical practice after antenatal glucocorticoid therapy (Crowley, 1995). This paradigm is also observed in selected fetal lung maturation responses to maternal betamethasone in sheep (Jobe, 2003). It must be noted that growth restriction is not evident in all neonates exposed to prenatal betamethasone without RDS. The mechanisms that underpin the variable responses observed after maternal glucocorticoid administration in late gestation are currently unknown; however, a recent study suggests one mechanism may involve cytokines (Newnham *et al.*, 2003). Concurrent cytokine expression, due to inflammation, augments the effect of maternal betamethasone on fetal lung maturation in sheep (Newnham *et al.*, 2003). Cytokine expression after maternal betamethasone administration was not measured in the studies comprising this thesis.

Although repetitive maternal betamethasone administration in late gestation significantly reduced fetal body weight, there was no consistent effect of treatment on nephron number or glomerular tuft and renal corpuscle volume measured at term. Overall, fetuses exposed to betamethasone did not have significantly fewer nephrons compared to controls due to a large range in nephron numbers within the betamethasone group. However, a *post-hoc* finding was that were two distinct populations within the betamethasone group. It appeared that only 50% (3/6) of the fetuses in the betamethasone group were grossly affected by the treatment, with those affected exhibiting reduced body, kidney and adrenal gland weights, fewer nephrons, and lower total glomerular and renal corpuscle volumes. These data are consistent with studies that have shown that IUGR is associated with a nephron deficit in humans (Hinchliffe *et al.*, 1992), pigs (Bauer *et al.*, 2002), rabbits (Bassan *et al.*, 2000), rats (Merlet-Benichou *et al.*, 1994) and sheep (Bains *et al.*, 1996). In future studies, by increasing the number of animals in the groups, a clearer picture into the renal effects of maternal betamethasone may be gained.

Fetal growth is paramount in the last trimester of gestation. Sixty percent of fetal kidney growth occurs between 100 and 130 d (Wintour *et al.*, 1999a) and nephrogenesis ceases between 120 and 130 d in sheep (Robillard *et al.*, 1981; Gimonet *et al.*, 1998). Repeated betamethasone administration prior to this time (104 to 118 d) significantly reduced

body weight by 28% and, in some cases, lowered total kidney weight. In sheep, three doses of maternal betamethasone at weekly intervals have been shown to induce IUGR equivalent to a period of growth arrest of approximately 9 days (Jobe *et al.*, 1998b). Therefore, in the present study, fetuses affected by betamethasone may have experienced a period of growth arrest during a critical window of renal development that ultimately reduced fetal kidney weight and nephron endowment. The importance of the gestational timing of IUGR and the impact on fetal nephron endowment was highlighted in a study conducted by Mitchell *et al.* (2004) who found that IUGR during late gestation using umbilico-placental embolisation between 120 and 140 d resulted in fetuses that had 34% lower body weight with 27% lighter kidneys but similar nephron numbers to controls.

Maternal glucocorticoid administration has been shown to result in reduced nephron endowment in offspring of rats (Celsi *et al.*, 1998; Ortiz *et al.*, 2001; Ortiz *et al.*, 2003) and sheep (Wintour *et al.*, 2003; Figueroa *et al.*, 2005; Moritz *et al.*, 2005c). Mid-gestation maternal betamethasone administration (on days 80 and 81 of pregnancy) reduced nephron endowment by 25% in fetal sheep at 135 d (Figueroa *et al.*, 2005). Studies by Wintour *et al.* (2003) and Ortiz *et al.* (2001, 2003) examined the effect of maternal dexamethasone administered at the start of development of the permanent kidney (metanephros) in the sheep (day 27, term 150 days) or rat (day 15, term 23 days), respectively. Dexamethasone infused early in gestation in sheep reduced nephron number in adult offspring by 38% and was associated with hypertension throughout life (Dodic *et al.*, 1998; Wintour *et al.*, 2003). Offspring of pregnant rats injected with dexamethasone on days 15 and 16 had 30% fewer nephrons, and when injected on days 17 and 18 of gestation had 20% fewer nephrons than controls (Ortiz *et al.*, 2001). Prenatal dexamethasone administration on days 15 to 16 or 17 to 18 increased systolic blood pressure at 60 to 70 days of age in rat offspring (Ortiz *et al.*, 2001). There was no reduction in nephron number or effect on blood pressure in offspring of pregnant rats administered with dexamethasone later in gestation (Ortiz *et al.*, 2001). The gestational timing of the exposure to increased levels of glucocorticoids in those studies was earlier than in the present study. Betamethasone was administered during late gestation to mimic the period of gestation when the majority of women who present with pre-term labour would receive antenatal glucocorticoid therapy. Therefore, it appears that in sheep and rats, a developmental disruption during the early stages of metanephric

development has a greater impact on nephron endowment at birth than late gestation insults. The mechanisms involved in the reduction of nephron numbers after early glucocorticoid administration are probably distinct from those which cause a reduction in nephron number in late gestation.

There was an uneven sex distribution between the treatment groups as a result of random allocation. In order to exclude the possibility that this sex bias influenced the experimental outcomes, a second study was performed to examine: (1) the effect of sex on nephron number and (2) the effect of maternal betamethasone administration on nephron endowment in female sheep. As I was physically unable to study more animals, I used data from control animals from another study performed by Dr Karen Moritz. Using the unbiased physical disector/fractionator method, we have found that nephron number in the kidneys of fetal Merino sheep is unaffected by sex (A.J. Meyer & K.M. Moritz, *unpublished observation*). Similarly, total nephron number was not different between male and female fetal sheep using the acid maceration technique at 135 d (J.P. Figueroa*, personal communication*). Several studies using unbiased stereological techniques have not shown a difference in nephron number between males and females in the normal human population (Neugarten *et al.*, 2002; Hoy *et al.*, 2003; Hughson *et al.*, 2003). Manalich *et al*. (2000) found that glomerular number was independent of sex in their study of low birth weight and normal birth weight infants. Previous studies using rats have demonstrated that the number of nephrons does not differ between the sexes (Silbiger & Neugarten, 1995; Neugarten *et al.*, 2002). Based on these findings, I do not expect that the different sex ratios between the groups would alter the conclusions of this study.

In part two of the second study, the effect of maternal betamethasone on nephron number in female fetal sheep was investigated. Using regression analysis, values for body and kidney weight were predicted at a specific gestational age based on data from a population of control fetal sheep. I have demonstrated that overall there was no effect of betamethasone on nephron endowment in female fetal sheep; however, betamethasone-exposed female lambs with renal growth restriction had 26% fewer nephrons than lambs with normal kidney weight.

To date, there are no published studies of the effect of antenatal glucocorticoid therapy on nephron number in humans; however, blood pressure has been reported in several studies of individuals exposed to a single course of glucocorticoids. Doyle *et al.* (2000) reported that very low-birth weight adolescents who had been exposed to antenatal glucocorticoids had higher systolic and diastolic blood pressures at age 14 years compared with adolescents who had not been exposed. A follow-up study of 20-year-old adults whose mothers had received a single course of betamethasone during pregnancy found that their systolic blood pressure was significantly lower and their diastolic blood pressure similar to those individuals in the placebo group (Dessens *et al.*, 2000). More recently the 30-year prospective follow-up of the Auckland Antenatal Steroid Trial (1969-1974) found that there was no difference in blood pressure between betamethasone- and placebo-exposed groups (Dalziel *et al.*, 2005). Therefore, the elevated blood pressure reported by Doyle *et al.* (2000) may be a transient effect that does not persist to adult life and is probably not associated with altered renal structure.

Currently, several large multi-centred randomised controlled trials examining the effects of repeated doses of prenatal glucocorticoids in humans are in progress. When surveyed in 2001, the use of repeated prenatal glucocorticoid therapy was still recommended by 44% of obstetricians in the Royal Australian and New Zealand College of Obstetricians and Gynaecologists despite the uncertainty of the safety and efficacy of this treatment (McLaughlin & Crowther, 2003). The 2003 Cochrane review by Crowther and Harding, using data from two randomised controlled trials (Guinn *et al.*, 2001; McEvoy *et al.*, 2002), reported that there was no statistically significant difference in birth weight in pre-term infants exposed to single or repeated prenatal glucocorticoids (Crowther & Harding, 2003). Recent randomised controlled trials have shown that there were more neonates with birth weights below the 10^{th} percentile in the repeat group; however, mean birth weight was not reduced after repeated corticosteroids (Crowther *et al.*, 2006; Wapner *et al.*, 2006).

In the present study, I observed variable effects of repeated betamethasone exposure on fetal body and kidney weights. Late-gestation repeated maternal betamethasone administration reduced fetal growth but did not consistently affect nephron endowment or glomerular size in term fetal sheep. Betamethasone-exposed fetuses with renal growth restriction had significantly fewer nephrons, consistent with data from other

studies of IUGR (Hinchliffe *et al.*, 1992; Merlet-Benichou *et al.*, 1994; Bains *et al.*, 1996; Bassan *et al.*, 2000; Bauer *et al.*, 2002). Therefore, I speculate that human babies exposed to repeated doses of prenatal glucocorticoids, with normal birth weights, are likely to have normal nephron endowment; although those infants who are growth-restricted with small kidneys may have a nephron deficit. Inter-species variability in corticosteroid-sensitivity may underpin part of the difference observed between prenatal glucocorticoids in sheep and humans. Humans are reported to be glucocorticoid-resistant (Claman, 1972); therefore, the data presented in this chapter do not support major concern for renal effects of repeated glucocorticoids in obstetric practice.

4. The Effects of Maternal Betamethasone Administration on the Fetal Kidney

4.1. INTRODUCTION

Intra-uterine exposure to excess glucocorticoids has been associated with low birth weight and the development of adult-onset diseases such as hypertension and type II diabetes (Edwards *et al.*, 1993; Langley-Evans, 1997a; Seckl, 1997; Seckl *et al.*, 2000; Seckl, 2004). Normally, the fetus is protected from glucocorticoids of maternal origin by placental 11β-HSD-2, an enzyme that metabolises endogenous glucocorticoids to their inactive forms (Krozowski *et al.*, 1995). However, synthetic glucocorticoids used in obstetric practice to treat women at risk of pre-term delivery have low placental inactivation and rapidly enter the fetal compartment to induce organ maturation (Blanford & Murphy, 1977). The short- and long-term effects of antenatal glucocorticoid therapy on the developing kidney remain to be elucidated. In animal experiments, fetal exposure to glucocorticoids during discrete periods of development has been demonstrated to induce the programming of organs, which may impact on adult health and disease.

Previously in obstetric practice, women with recurrent risk of pre-term delivery were frequently treated with repeated doses of glucocorticoids despite the unknown safety and efficacy of this therapy (Wing *et al.*, 1996; Quinlivan *et al.*, 1998b; McLaughlin & Crowther, 2003). The beneficial effects of antenatal glucocorticoids were thought to diminish after seven days, based on data showing no difference in the incidence of RDS in glucocorticoid- and placebo-exposed infants delivered more than a week after treatment (Liggins & Howie, 1972; Crowley, 1995). Repetitive antenatal glucocorticoid therapy has been associated with reduced birth weight in non-randomised studies (Reinisch *et al.*, 1978; Banks *et al.*, 1999; French *et al.*, 1999; Bloom *et al.*, 2001; Thorp *et al.*, 2002). Recent randomised controlled trials have reported no difference in mean birth weight in infants exposed to placebo or repeated corticosteroids; however, there were more neonates weighing less than the 10^{th} percentile in the repeat group (Crowther *et al.*, 2006; Wapner *et al.*, 2006). The long-term effects of repeated corticosteroids on adult health and disease are unknown but will be assessed in ongoing investigations by the randomised controlled trials. In 1994, the National Institutes of Health Consensus Development Panel highlighted the need for research into the short- and long-term effects of repetitive prenatal administration of glucocorticoids (NIH, 1995). In 2000, the

Panel concluded that current data were insufficient to support the use of repeated doses of antenatal glucocorticoids in clinical practice (NIH, 2001).

Experiments in sheep have been useful for determining the short-term and long-term effects of repetitive prenatal glucocorticoid administration on fetal and postnatal animals (Dunlop *et al.*, 1997; Ikegami *et al.*, 1997; Jobe *et al.*, 1998a; Jobe *et al.*, 1998b; Quinlivan *et al.*, 1998a; Newnham *et al.*, 1999; Quinlivan *et al.*, 2000; Sloboda *et al.*, 2000; Moss *et al.*, 2001; Moss *et al.*, 2002; Sloboda *et al.*, 2002a; Sloboda *et al.*, 2002b; Smith *et al.*, 2003; Moss *et al.*, 2005). In these experiments, betamethasone was administered to pregnant ewes at weekly intervals, beginning at 70% of gestation, to mimic antenatal glucocorticoid therapy in obstetric practice. These studies showed that repeated doses of maternal betamethasone reduced fetal body weight. Renal function in pre-term and term lambs exposed to single or repeated doses of betamethasone has been measured previously (Berry *et al.*, 1997; Smith *et al.*, 2003). Basal mean arterial pressure (MAP), glomerular filtration rate (GFR), total renal sodium reabsorption and renal Na^+/K^+-ATPase activity were increased and plasma renin activity (PRA), angiotensin II (AngII) and aldosterone were significantly reduced in 127 d pre-term lambs after maternal betamethasone administration 24 h before delivery (Berry *et al.*, 1997). In contrast, basal MAP, GFR, sodium excretion, urine flow, osmolar clearance, PRA and AngII were similar in pre-term (125 d) or term (145 d) lambs exposed to saline or single (104 d) or repeated doses (104, 111, 118 d) of maternal betamethasone (Smith *et al.*, 2003). Plasma aldosterone levels were attenuated in pre-term lambs exposed to maternal betamethasone (single or multiple doses), but were unaltered in term lambs (Smith *et al.*, 2003). The difference in the findings between these two studies (Berry *et al.*, 1997; Smith *et al.*, 2003) has been attributed to the acute versus persistent effects of antenatal glucocorticoids (Smith *et al.*, 2003). The short- and long-term effects of late-gestation maternal betamethasone administration on the expression of mRNA and protein of glucocorticoid-sensitive genes in the fetal sheep kidney have not been examined.

Reduced fetal growth impairs kidney development and may lead to alterations in gene expression that underlie changes in renal structure or function that could predispose the kidney to disease later in life. Maternal administration of betamethasone during late gestation, a period when the fetal kidney is undergoing rapid organogenesis, reduces

fetal kidney weight in sheep (Jobe *et al.*, 1998b; Newnham *et al.*, 1999). Further, maternal glucocorticoid administration early in gestation (26 to 28 d) increased fetal kidney expression of GR, MR, A_o, AT_1 receptor and AT_2 receptor mRNA at 130 d (Hantzis *et al.*, 2002; Moritz *et al.*, 2002b) and Na^+/K^+-ATPase-α_1 and –γ mRNA at 140 d (Moritz *et al.*, 2005c), and programmed high blood pressure in postnatal life (Dodic *et al.*, 1998; Wintour *et al.*, 2003). The aim of the present study was to assess the effects of single or repeated doses of maternal betamethasone in late gestation on the renal expression of GR and Na^+/K^+-ATPase-α_1 protein and GR, MR, 11β-HSD-2, Na^+/K^+-ATPase-α_1, A_o, AT_1 receptor, AT_2 receptor and renin mRNA in sheep at four gestational ages (109, 116, 121 and 146 d; term is 150 d).

4.2. EXPERIMENTAL PROCEDURES

4.2.1. Animals

All experimental procedures were approved by the Animal Experimentation Ethics Committee of The Western Australian Department of Agriculture. Pregnant Merino ewes bearing single male fetuses were used; sex-typing of the fetuses was performed by Mr Shaofu Li after amniocentesis of pregnant ewes at 60 d. The fetus was imaged using ultrasonography; 10 mL of amniotic fluid was withdrawn from the amniotic sac using a spinal needle and was transferred to a sterile tube. PCR was performed, on DNA extracted from cells in the amniotic fluid, targeting the male (SRY and ZFX/ZFY) and female (ZFX/ZFY) gene bands (Appendix 8). The SRY gene is male-specific and the ZFX/ZFY gene was run as a control for the PCR. Female fetuses and twins were excluded from this study.

4.2.2. Experimental Protocol

Pregnant ewes were injected i.m. with 150 mg of medroxyprogesterone acetate (MPA) at 100 d and then randomised to receive saline or betamethasone (0.5 mg/kg ewe weight) injections (section 2.2; Figure 4.1). Ewes received a total dose of approximately 28 mg of betamethasone in a single injection, based on a mean body weight of 55.3 ± 1.0 kg at 104 d. Treatments began at 104 d (70% of gestation) and occurred at weekly

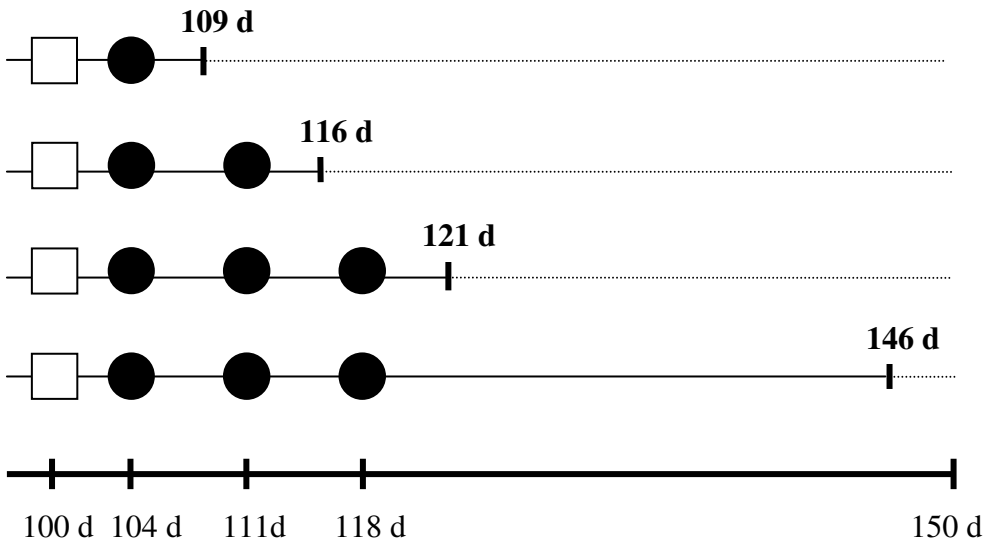


Figure 4.1. Injection and tissue collection time-line for the fetal study.

Solid lines represent a gestational time-line for injections and tissue collection. White squares signify maternal i.m. MPA (150 mg) injection at 100 d; black circles symbolise maternal saline or betamethasone i.m. injections (0.5 mg/kg ewe weight) at 104, 111 or 118 d (term is 150 d). Two groups of animals, the control group (that received saline only) and the treatment group (that received betamethasone only), were euthanised for fetal tissue collection at each gestational age, highlighted in bold.

intervals, to mimic single or repeated dosing schedules used in obstetric practice (Ikegami *et al.*, 1997). The gestational timing of glucocorticoid administration is similar to that used clinically because women at risk of pre-term delivery are treated with glucocorticoids between 24 and 34 weeks' (60 to 85%) gestation (NIH, 1995).

Groups of ewes and their fetuses were euthanised at 109 d (saline: n = 6; betamethasone: n = 6), 116 d (saline: n = 6; betamethasone: n = 6), 121 d (saline: n = 6; betamethasone: n = 4) or 146 d (145 to 147 d; saline: n = 7; betamethasone: n = 3) for tissue collection (section 2.3; Figure 4.1). Ten ewes had been injected with betamethasone for the 146 d collection; however, at delivery it was found that one ewe had aborted and six were pregnant with female fetuses and were therefore excluded leaving only three male betamethasone-exposed fetuses at 146 d. Tissue was collected from fetuses three to five days after being exposed to one, two or three doses of saline or betamethasone to assess the short-term effects of glucocorticoids on the kidney. Tissue collection from fetuses at 146 d, four weeks after the third weekly maternal injection of saline or betamethasone at 118 d, was used to examine the persistent effects of glucocorticoids on the fetal kidney.

4.2.3. Molecular analyses

4.2.3.1. Histology

Qualitative analysis of renal histology was performed on a single transverse section from the mid-hilar region of the right kidney of each fetal sheep. Sections (5 µm) were routinely stained with haematoxylin and eosin (H&E; Appendix 2) and examined under a light microscope (Olympus BX51TF; Olympus Optical Co. Ltd, Japan).

4.2.3.2. Immunohistochemistry

Immunohistochemical detection of GR-immuno-reactive (IR) staining was performed on a single 5 µm transverse section from the right kidney of each animal. Detailed methodology is described in section 2.5. Briefly, sections were incubated overnight at 4°C with the polyclonal primary antibody, GR (M-20) (Santa Cruz Biotechnology, Inc.), at a dilution of 1:50 (4 µg/mL). The biotinylated anti-goat IgG secondary antibody

(VECTASTAIN® ABC Kit, Vector Laboratories, Inc.) was applied for 1 h at a dilution of 1:200. The ABC reagent (VECTASTAIN® ABC Kit, Vector Laboratories, Inc.) was prepared at dilutions of 1:100 and incubated on the sections for 1 h. The chromogen reaction with DAB (ICN Biomedicals, Inc) was terminated at 5 min for 121 d sections, but incubated for 10 min for all other ages. Sheep liver sections were used as positive and negative (primary antibody omitted) controls. Sections from all fetal sheep were processed in a single experiment.

4.2.3.3. Western blot analysis

Western blotting for GR and Na^+/K^+-ATPase-α_1 protein was performed on total protein extracted from the right kidney of fetal sheep as described in section 2.6. GR Western blots were probed with the primary antibody, GR (M-20) (Santa Cruz Biotechnology, Inc.), at a dilution of 1:200 (1 μg/ml) and the secondary antibody, ECL anti-rabbit IgG peroxidase-linked species-specific whole antibody (from donkey) (NA934; Amersham Biosciences UK Ltd), at a dilution of 1:10,000. Na^+/K^+-ATPase-α_1 blots were probed with the primary antibody MA3-929 (Affinity BioReagents) at a concentration of 0.19 μg/mL (1:40,000) and secondary antibody, anti-mouse IgG (whole molecule) peroxidase conjugate (A9044; Sigma-Aldrich, Inc.), at a dilution of 1:20,000.

4.2.3.4. Real-time RT-PCR

Total RNA was extracted from the right kidney of fetuses at 109, 116, 121 and 146 d (section 2.7.1.2) exposed to maternal saline or betamethasone. Real-time RT-PCR (section 2.7.5-6) was used to measure relative expression levels of GR, MR, 11β-HSD-2, Na^+/K^+-ATPase-α_1, AT_1, AT_2, A_o and renin mRNA in these samples. Gene expression levels were calculated relative to 109 d saline. Some samples were excluded from analysis on the basis that they gave abnormal results as a consequence of contaminated or degraded RNA. All fetal samples were run in the same assay for each gene and the intra-assay co-efficient of variation for each gene was less than 10%.

4.2.3.5. Cortisol Radio-Immuno-Assay (RIA)

Fetal plasma cortisol levels were measured by Mr Shaofu Li using a Clinical Assays™ GammaCoat™ Cortisol ^{125}I RIA kit (DiaSorin Inc., Stillwater, MN, USA). This assay has been previously validated for use in sheep (Sysyn *et al.*, 2001; Kerzner *et al.*, 2002). The GammaCoat™ antiserum exhibits 100% cross-reactivity with cortisol. All samples were run in a single assay; the sensitivity of the assay was 1.2 ng/mL and the intra-assay co-efficient of variation was 2.3%.

4.2.4. Statistics

All data are shown as mean ± S.E.M.; statistical analyses were performed with SigmaStat (Version 3.00, SPSS Inc.) software. Student's *t*-tests were performed to compare data from saline and betamethasone groups at each time-point. Two-way analysis of variance (ANOVA) was used to analyse data from both groups across gestation. Where appropriate, a post-hoc test (Holm-Sidak method) was used to examine all pair-wise multiple comparisons. Data that were not normally distributed were transformed to achieve normality.

4.3. RESULTS

4.3.1. Fetal body weight and umbilical blood gases

Umbilical arterial blood gases and pH were not different between groups at any gestational age (Table 4.1). Fetal body weight increased with age ($p < 0.001$) and varied significantly with treatment ($p < 0.001$; Figure 4.2). Fetal body weight at 109 d, after a single dose of maternal betamethasone or saline at 104 d, was not different between groups (Table 4.1). At 116 d, after two doses of maternal betamethasone or saline, body weight of betamethasone-exposed fetuses was 14% lower than that of saline animals ($p = 0.02$; Table 4.1). Two out of six betamethasone-exposed fetuses (33%) were growth-restricted (> 2 S.D. below saline mean) at 116 d. Fetal body weight was reduced by 28% in betamethasone-exposed fetuses at 121 d, after three doses, compared to controls ($p < 0.001$; Table 4.1). In the betamethasone group, three out of four fetuses (75%) were growth-restricted at 121 d. Near-term fetuses (146 d) exposed to three doses of maternal betamethasone were 14% lighter than saline fetuses; however due to low numbers of

Table 4.1. Fetal body weight and umbilical arterial pH, PO_2 and PCO_2 values.

Values are mean ± S.E.M.

	109 d		**116 d**		**121 d**		**146 d**	
Group *n*	**Saline** (n = 6)	**Beta** (n = 6)	**Saline** (n = 6)	**Beta** (n = 6)	**Saline** (n = 6)	**Beta** (n = 4)	**Saline** (n = 7)	**Beta** (n = 3)
Body weight (kg)	1.43 ± 0.05	1.42 ± 0.08	2.13 ± 0.07	**1.83 ± 0.09 ***	2.78 ± 0.14	**2.01 ± 0.15 ****	5.36 ± 0.20	4.62 ± 0.18
pH	7.38 ± 0.01	7.39 ± 0.01	7.39 ± 0.00	7.39 ± 0.01	7.34 ± 0.01 [a]	7.36 ± 0.02	7.39 ± 0.01	7.40 ± 0.01
PO_2 (mm Hg)	14.8 ± 2.28	15.8 ± 2.84 [a]	17.2 ± 0.64	17.4 ± 0.53	13.8 ± 0.67 [a]	14.5 ± 0.68	15.6 ± 1.14	15.9 ± 1.37
PCO_2 (mm Hg)	52.3 ± 1.90	52.7 ± 2.34	48.7 ± 1.70	52.1 ± 0.72	56.8 ± 0.82 [a]	54.3 ± 2.52	53.4 ± 2.30	56.4 ± 2.80

[a] (n = 5). * $p < 0.05$, ** $p < 0.01$, Student's *t*-test saline versus betamethasone at each gestational age.

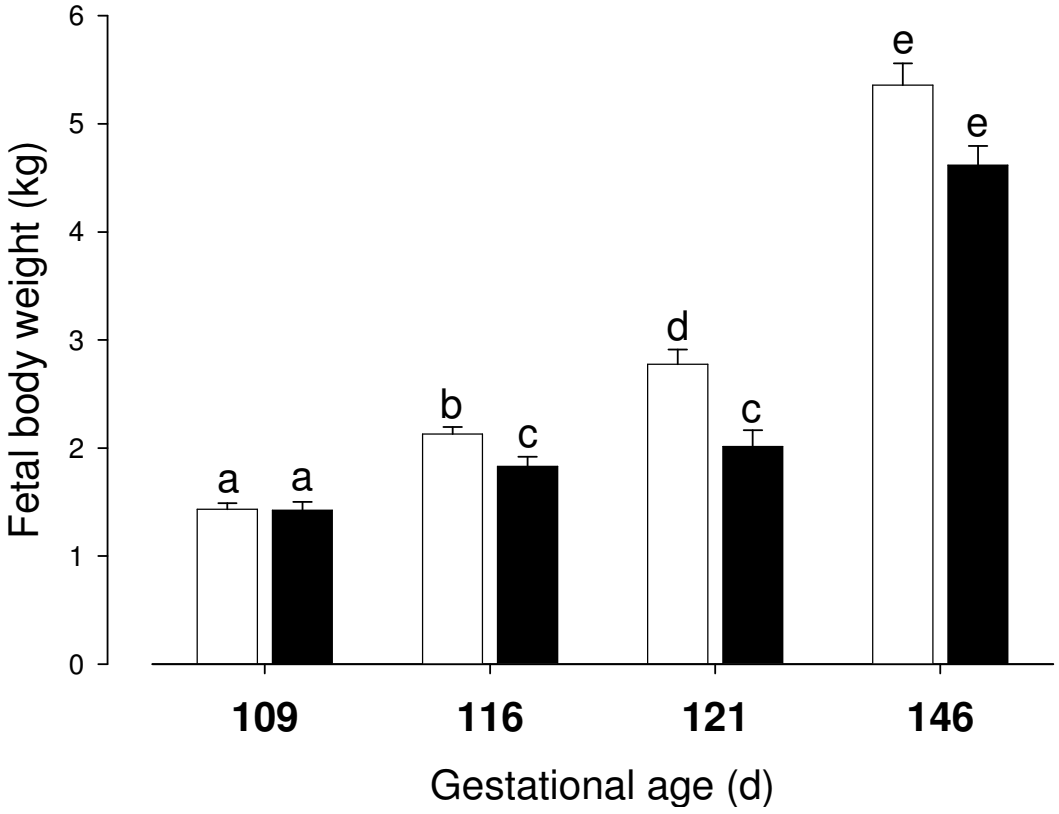


Figure 4.2. Delivery body weight of fetal sheep exposed to saline or betamethasone. Bars show group mean ± S.E.M. for control (white) and betamethasone-exposed (black) fetuses. Different letters above bars indicate statistically significant differences between groups and/or ages ($p < 0.05$). Body weight increased with age ($p < 0.001$) and was significantly affected by betamethasone exposure ($p < 0.001$). There was a statistically significant interaction between age and treatment ($p = 0.02$). Fetal body weight increased significantly at each gestational age in control animals; however, body weight was not different between betamethasone-exposed fetuses at 116 and 121 d.

animals in the treatment group, this difference did not reach statistical significance ($p = 0.06$; Table 4.1).

4.3.2. Fetal total kidney weight

Total kidney weight as a proportion of body weight was not different between the groups at any gestational age. Fetal total kidney weight was significantly affected by age ($p < 0.001$) and treatment ($p = 0.01$; Figure 4.3). Total kidney weight at 109 d was not different between the two groups (saline: 12.11 ± 0.51 g; betamethasone: 12.66 ± 0.70 g; Figure 4.3), but was 15% lower at 116 d after maternal betamethasone (saline: 17.90 ± 0.56 g; betamethasone: 15.20 ± 1.15 g; $p = 0.03$; Figure 4.3). At 121 d, total kidney weight was 27% lower in betamethasone-exposed fetuses than in controls (saline: 18.53 ± 0.82 g; betamethasone: 13.59 ± 0.78 g; $p < 0.001$; Figure 4.3). Total kidney weight was not different between groups at 146 d (saline: 26.80 ± 0.92 g; betamethasone: 26.10 ± 1.66 g; Figure 4.3). Total kidney weight did not change significantly between 116 d and 121 d in fetuses from both groups; total kidney weight was significantly greater at 116 d than at 109 d, and was significantly increased at 146 d than at 121 d.

4.3.3. Fetal plasma cortisol levels

Overall, fetal plasma cortisol levels were affected by gestational age ($p < 0.001$) but not treatment ($p = 0.86$). Plasma cortisol levels in saline- and betamethasone-exposed fetuses were higher at 146 d compared with other time-points ($p < 0.001$; Figure 4.4.). There was a significant interaction between age and treatment ($p = 0.03$); at 146 d, plasma cortisol levels were attenuated in betamethasone-exposed fetuses compared to saline controls (saline: 39.5 ± 4.80 ng/mL; betamethasone: 24.9 ± 12.9 ng/mL; $p = 0.03$). Fetal plasma cortisol levels close to term were variable in the betamethasone-exposed group: two animals (euthanised at 145 d) had plasma cortisol levels of 9.70 ng/mL and 14.5 ng/mL but the third animal (euthanised at 147 d) had a plasma cortisol level of 50.6 ng/mL.

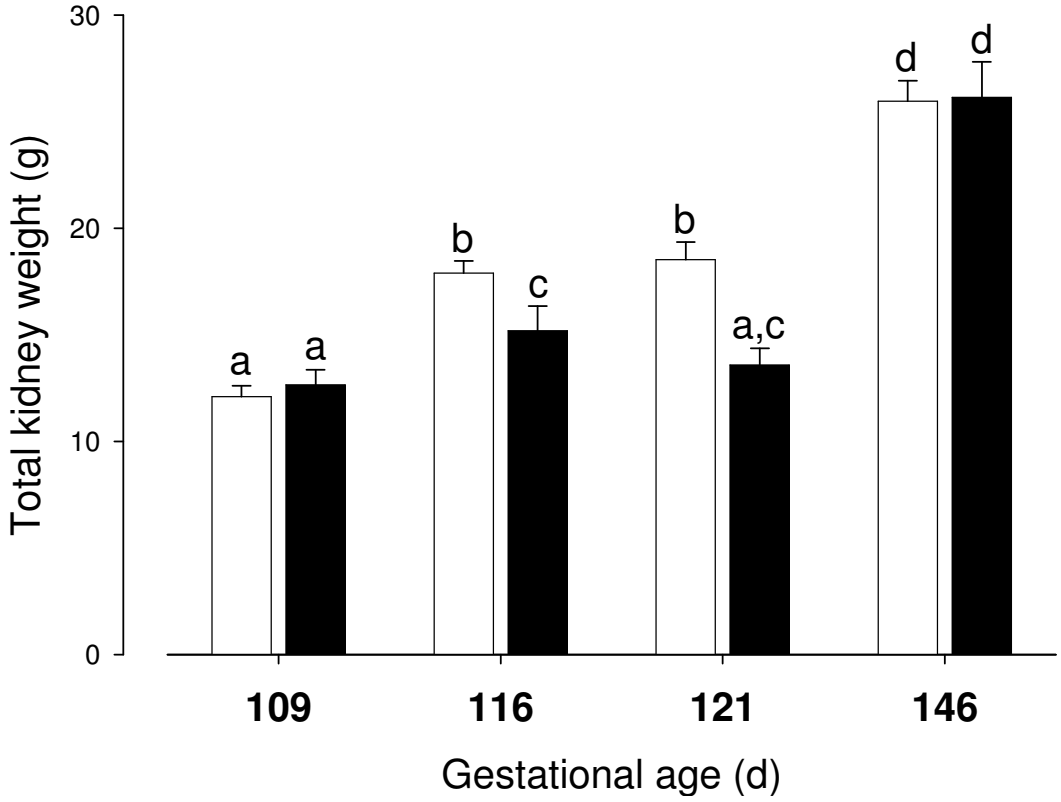


Figure 4.3. Total kidney weight of fetal sheep exposed to saline or betamethasone. Bars show group mean ± S.E.M. for control (white) and betamethasone-exposed (black) fetuses. Different letters above bars indicate statistically significant differences between groups and/or ages ($p < 0.05$). Total kidney weight was significantly affected by age ($p < 0.001$) and treatment ($p = 0.01$) and a significant interaction existed between the two variables ($p = 0.02$). Total kidney weight at 116 d was not different from that at 121 d; however, at these ages, kidney weight was different to that at 109 and 146 d. Betamethasone-exposed fetuses had lower total kidney weights compared to controls at 116 and 121 d. At 121 d, kidney weight in betamethasone-exposed fetuses was not different compared to 109 d and 116 d.

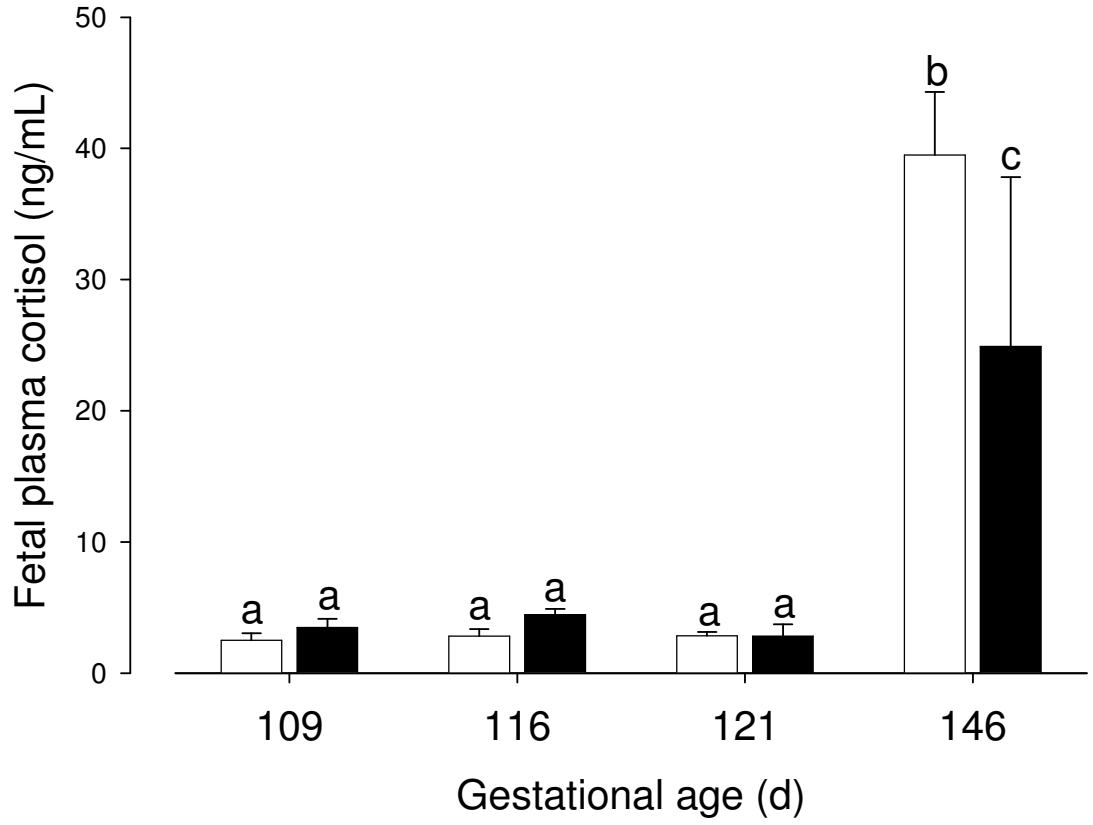


Figure 4.4. Fetal plasma cortisol levels.

Bars show the mean ± S.E.M. of plasma cortisol levels in saline (white) and betamethasone-exposed (black) fetal sheep in late gestation. Common letters denote statistical similarity; different letters denote significant difference ($p < 0.05$). Fetal plasma cortisol levels were significantly higher in both groups at 146 d compared to other gestational ages ($p < 0.001$); however, cortisol levels were attenuated, relative to controls, in betamethasone-exposed fetuses at this age ($p = 0.03$).

4.3.4. Histology

All fetal kidney sections stained with H&E appeared to have normal histology when examined under the light microscope. Representative sections at each gestational age are shown in Figure 4.5.

4.3.4.1. Glomerular maturation and growth

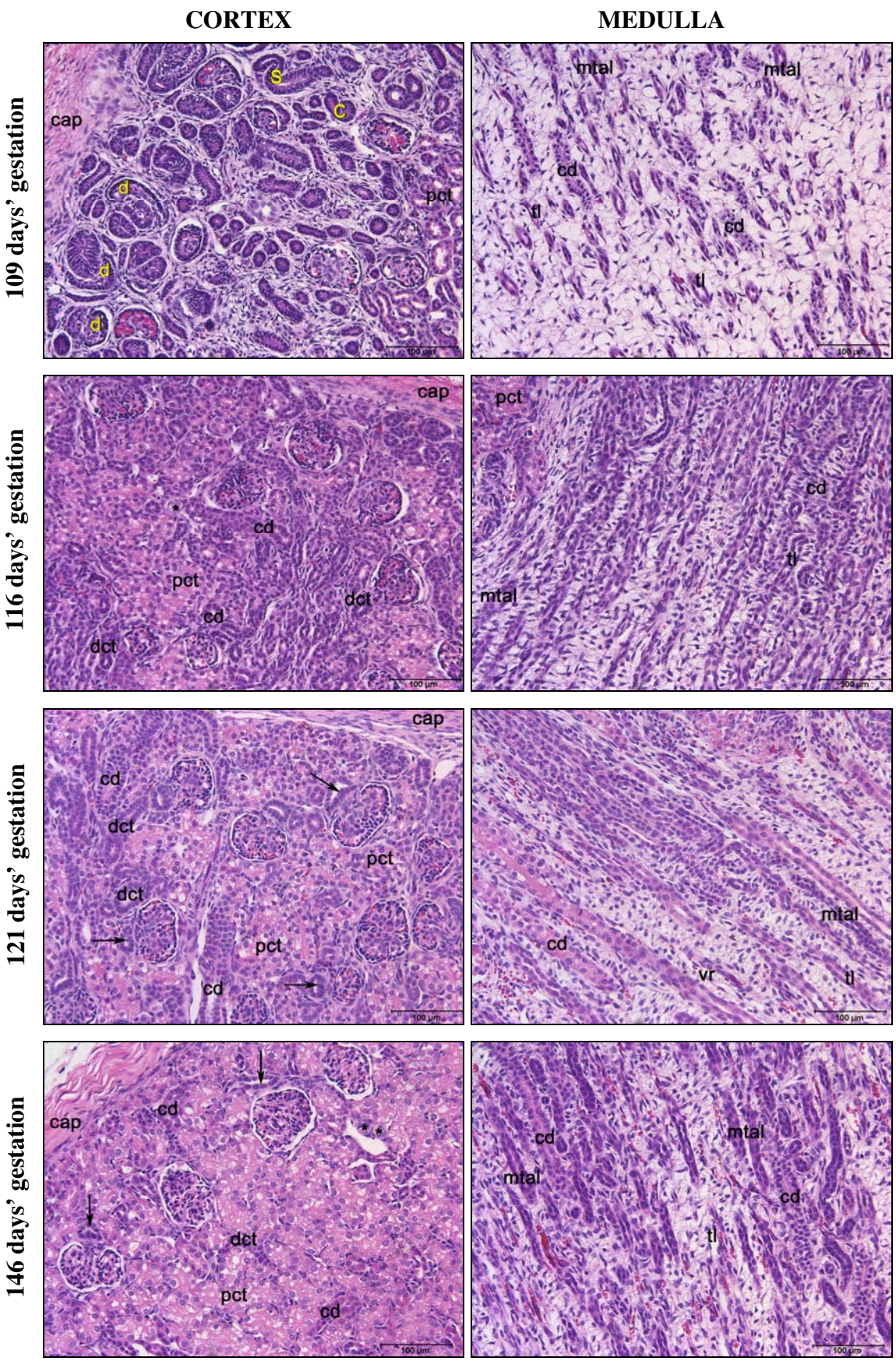
Various maturational states of glomeruli are illustrated in Figure 4.5. At 109 d, an active nephrogenic zone was evident in all animals. The nephrogenic zone contained condensed mesenchymal cells, comma-shaped bodies, S-shaped bodies and developing glomeruli. Glomeruli that were mature in appearance were found in the juxta-medullary region and immature glomeruli were present in the mid-cortical area. In some developing glomerular tufts, the invasion of capillaries was marked by the presence of clusters of red blood cells. At 116 d, the nephrogenic zone was still evident, although it appeared narrower than at 109 d. By 121 d, the nephrogenic zone had disappeared but developing glomeruli and tubules were still present in the subcapsular space. In one control animal, S-shaped bodies were still present in the outer cortex at 121 d. A continuous layer of cuboidal cells lining the glomerular tufts was present in developing glomeruli in sections of 109, 116 and 121 d kidneys. By 146 d, the layer of cells lining the visceral layer of Bowman's capsule was discontinuous, and the cells appeared to be dispersed over the surface of the developing capillary tufts. The size, cellularity and number of capillary loops within the glomerular tufts appeared to increase with gestational age.

4.3.4.2. Tubular maturation and growth

At 109 d, the renal cortex appeared to be thinner than the medulla. Interstitial tissue occupied most of the medulla and renal tubules were immature. Medullary rays had started to form in the deep cortex and extended into the outer medulla. The papilla contained few tubules but papillary collecting ducts were evident. At 116 d, there were more tubules present in the medulla in the outer zone (inner and outer stripes) compared with 109 d. There also appeared to be more vasa rectae supplying blood to the papilla. Medullary rays had enlarged, containing increased numbers of tubules. At 121 d, the

Figure 4.5. Renal histology.

Representative digital micrographs of transverse cortical and medullary sections of fetal sheep kidneys stained with haematoxylin and eosin. Four gestational time-points (109, 116, 121 and 146 d) are shown. C = comma-shaped body; cap = renal capsule; cd = collecting duct; d = developing glomeruli; dct = distal convoluted tubule; mtal = medullary thick ascending limb; pct = proximal convoluted tubule; S = s-shaped body; tl = thin limb of the loop of Henle; vr = vasa recta; * = arteriole; → = macula densa. All digital micrographs were captured at 200x magnification; a 100 μm scale bar is indicated.


CORTEX
MEDULLA
109 days' gestation
116 days' gestation
121 days' gestation
146 days' gestation
cap
S
C
d
pct
mtal
cd
tl
dct
vr
100 µm

4.3.5. Glucocorticoid receptor mRNA

There was no effect of age ($p = 0.24$) or treatment ($p = 0.12$) on fetal renal GR mRNA expression after late-gestation maternal saline or betamethasone administration (Figure 4.6).

4.3.6. Glucocorticoid receptor protein

The anti-GR antibody used for Western blotting recognised a single 74 ± 1 kDa band in protein extracted from fetal sheep kidneys. The specificity of this antibody was confirmed by pre-absorption with its peptide sequence (section 2.6.7).

There was no effect of prenatal betamethasone exposure on renal GR protein levels (Figures 4.7, 4.9, 4.11 and 4.13) or localisation (Figures 4.8, 4.10, 4.12 and 4.14) in fetal sheep at 109, 116, 121 or 146 d. Fetal exposure to a single dose of maternal betamethasone at 104 d, did not affect renal GR protein levels at 109 d ($p = 0.13$; Figure 4.7). There was minimal GR immuno-staining in sections of 109 d fetal sheep kidneys. There were no differences in the localisation or intensity of GR immuno-staining in sections of kidney from saline- or betamethasone-exposed fetuses at 109 d. In sections that demonstrated staining, nuclear and cytoplasmic GR immuno-staining was localised to the smooth muscle layer underneath the renal capsule, tunica media of blood vessels, the capillary tuft of developing glomeruli and some surrounding tubules, and in the interstitial cells in the medulla (Figure 4.8 A to F).

Maternal betamethasone administration did not affect fetal renal GR protein levels ($p = 0.19$; Figure 4.9) or alter the localisation (Figure 4.10) of GR immuno-staining at 116 d. GR immuno-staining was evident in the cytoplasm and nuclei of cells in the tunica media of renal blood vessels. Light cytoplasmic GR immuno-staining was also found in the capillary tuft of developing glomeruli within the nephrogenic zone (Figure 4.10. A and B) and their associated tubules and in collecting ducts deep in the medulla and papilla (Figure 4.10 C to F).

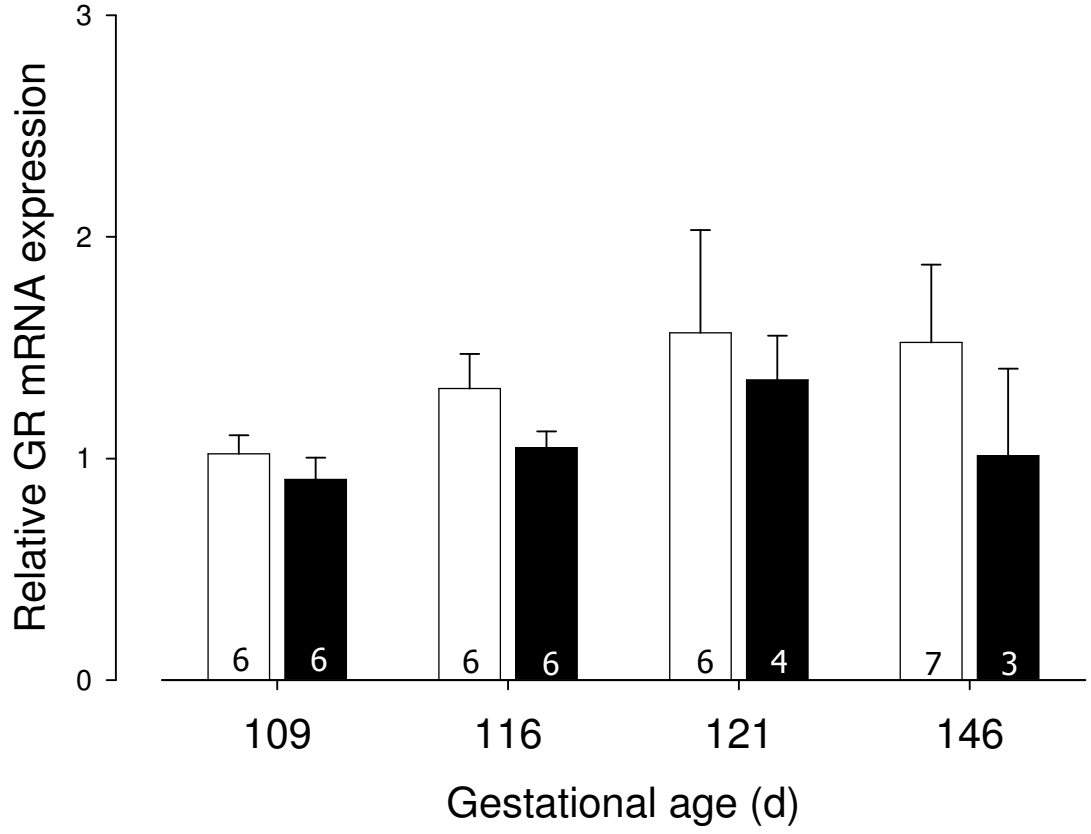


Figure 4.6. GR mRNA expression in the fetal kidney.

Bars show mean ± S.E.M. of relative GR mRNA expression in fetal kidneys after maternal saline (white) or betamethasone (black) administration. Numbers of fetuses in each group are illustrated within each bar. There was no effect of age ($p = 0.24$) or treatment ($p = 0.12$) on relative fetal renal GR mRNA expression.

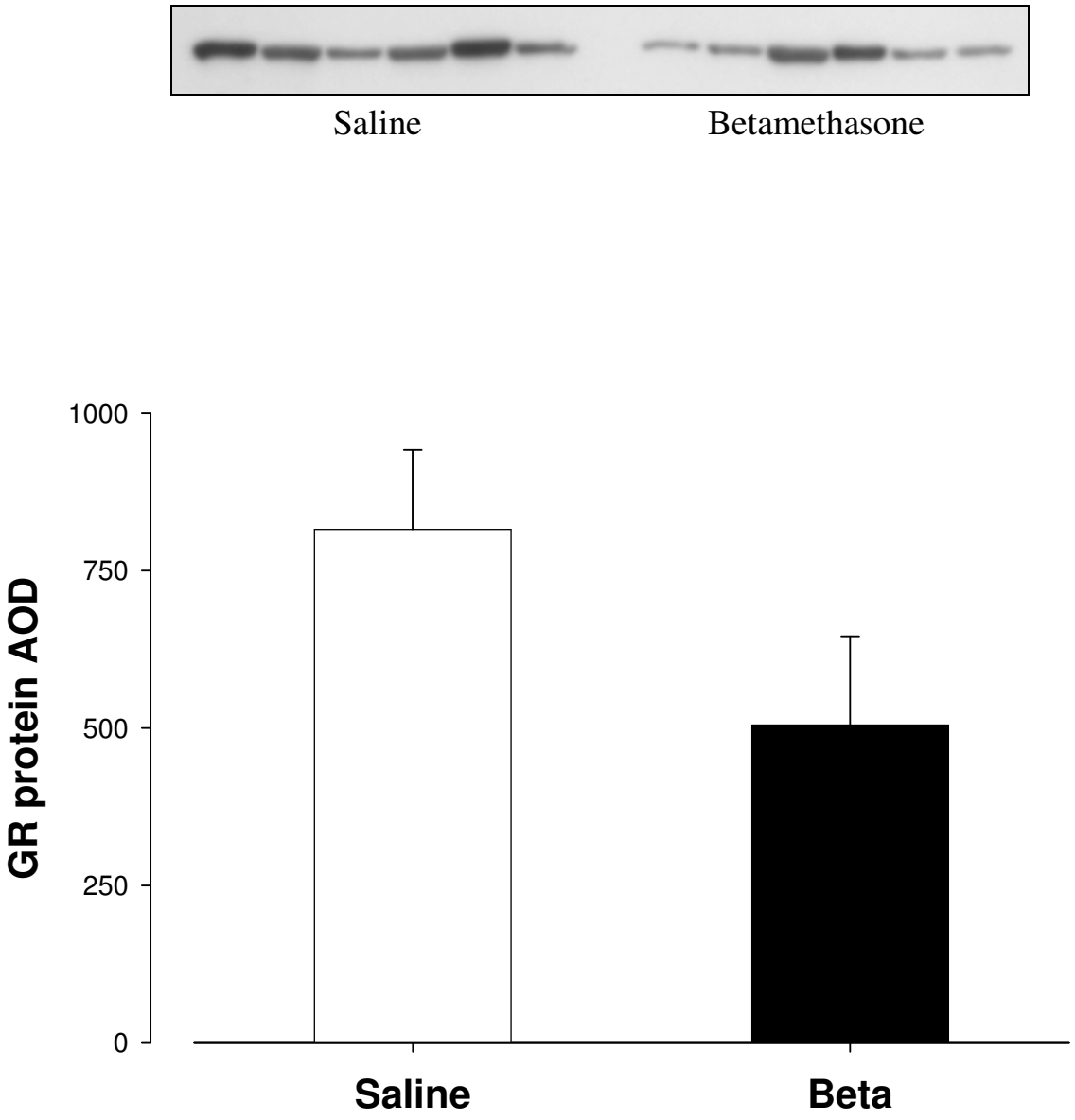
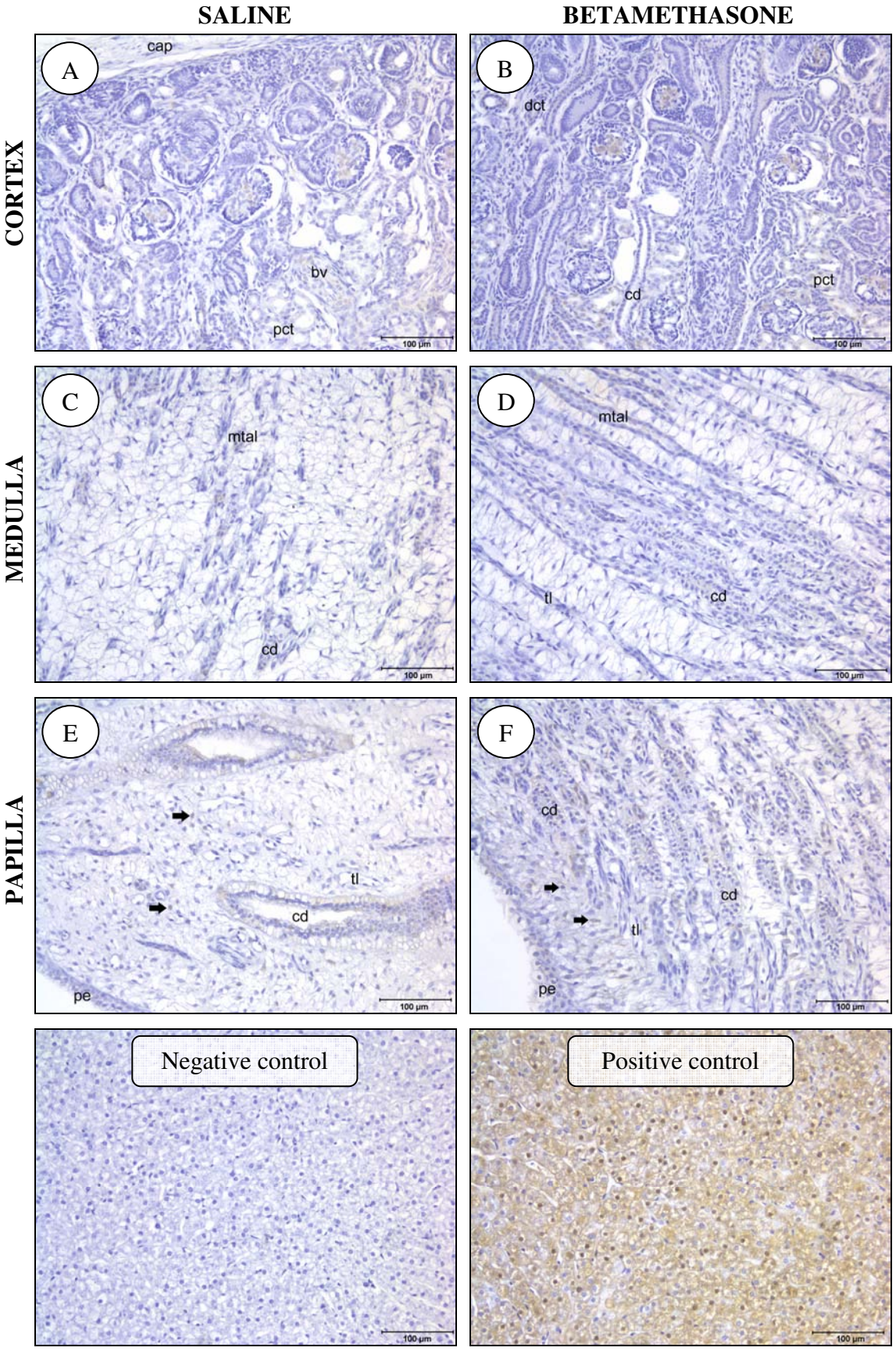


Figure 4.7. Renal GR protein levels at 109 days' gestation.

Bars show mean ± S.E.M. of renal GR protein levels at 109 d after maternal saline (white; n = 6) or betamethasone (black; n = 6) administration. A digital photo of the Western blot is shown above and the AOD of the GR band is plotted below. There was no statistical difference between the groups (p = 0.13).

Figure 4.8. GR immuno-staining in fetal sheep kidneys at 109 days' gestation.
Representative digital micrographs of GR immuno-staining (brown) in kidney sections (A to F) from fetal sheep at 109 d, after exposure to maternal saline or betamethasone at 104 d. There was no difference in the renal localisation or intensity of GR immuno-staining between saline- and betamethasone-exposed fetal sheep. Moderate GR immuno-staining was present in the tufts of developing glomeruli, arterioles and some tubules within the cortex (A and B). In the medulla, light staining was evident in collecting ducts, thick ascending limbs and some thin limbs of the loop of Henle (C and D). Light GR immuno-staining was localised to collecting duct and interstitial cells within the papilla (E and F). bv = blood vessel; cd = collecting duct; mtal = medullary thick ascending limb; pe = papillary epithelium; tl = thin limb of loop of Henle; vr = vasa recta; ➡ = papillary interstitial cell. Sheep liver was used as the negative (primary antibody omitted) and positive control. All digital micrographs were captured at 200x magnification; scale bar illustrates 100 μm.


SALINE
BETAMETHASONE
CORTEX
MEDULLA
PAPILLA
A
cap
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Negative control
Positive control
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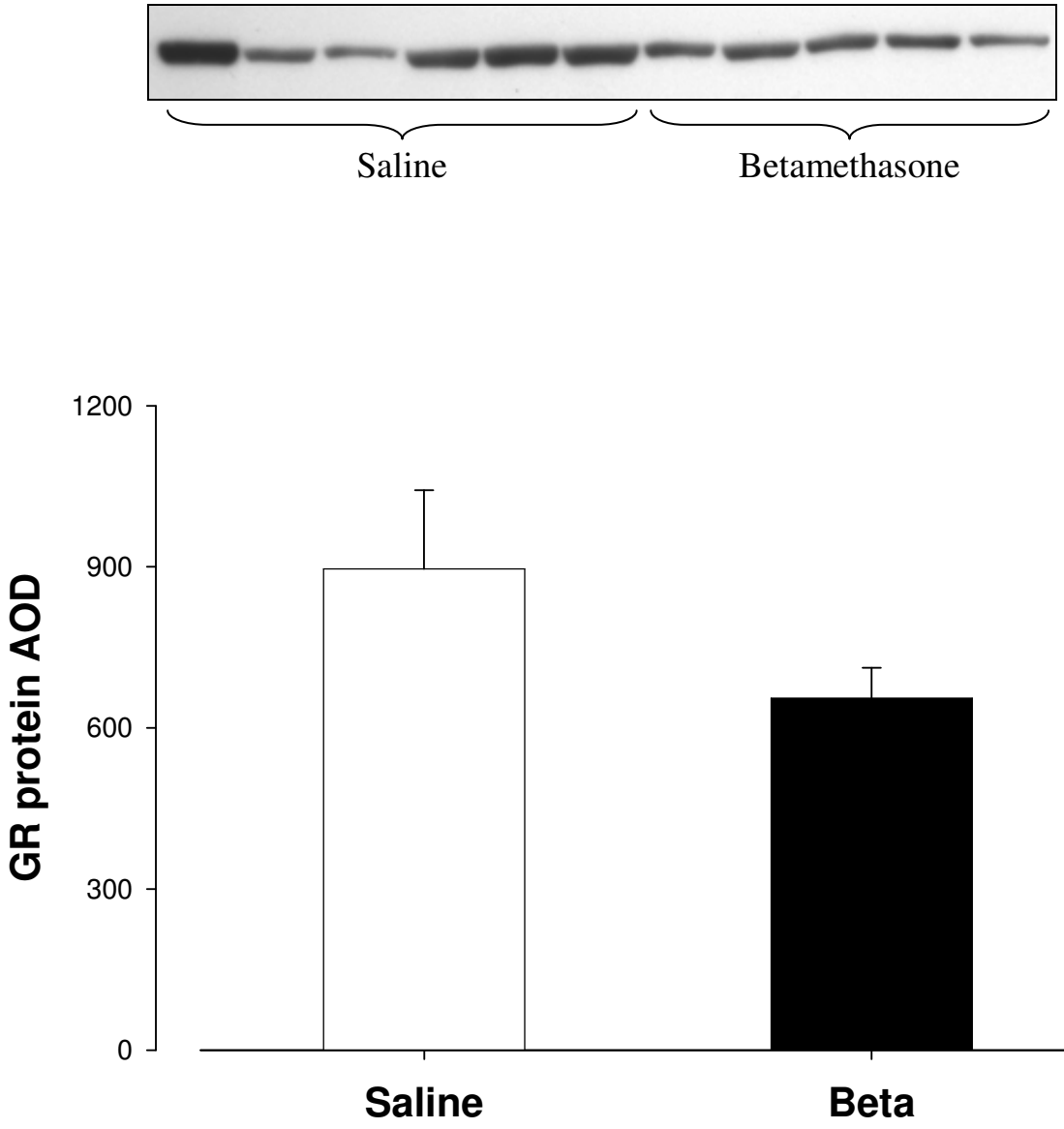
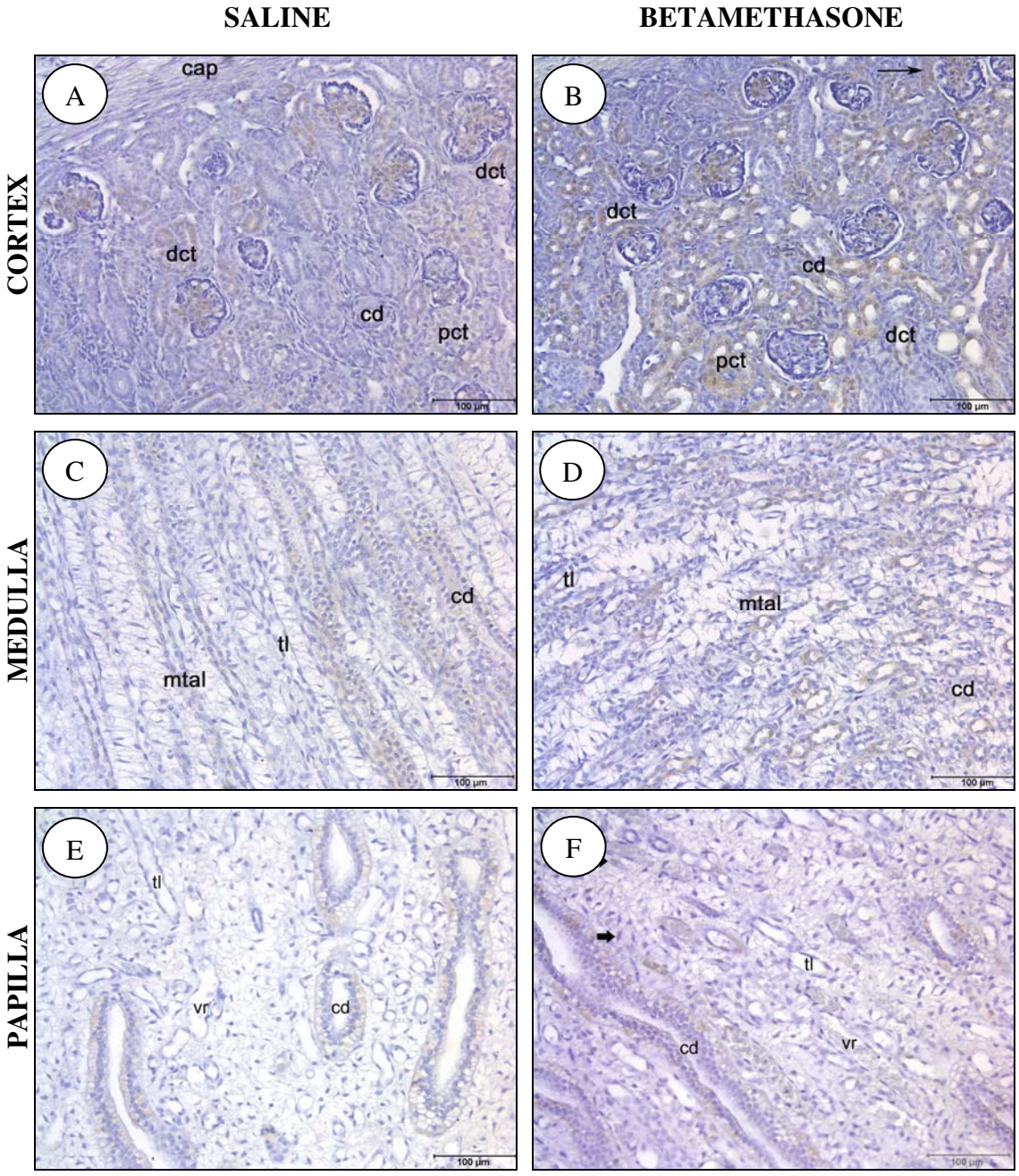


Figure 4.9. Renal GR protein levels at 116 days' gestation.

Bars show mean ± S.E.M. of renal GR protein levels at 116 d after maternal saline (white; n = 6) or betamethasone (black; n = 5) administration at 104 and 111 d. There was no difference between treatment groups (p = 0.19).

Figure 4.10. GR immuno-staining in fetal sheep kidneys at 116 days' gestation.
Representative digital micrographs of GR immuno-staining in kidney sections from fetal sheep at 116 d after exposure to maternal saline or betamethasone at 104 and 111 d. There was no difference in the renal localisation or intensity of GR immuno-staining between groups. Moderate GR immuno-staining was present in glomerular tufts, collecting ducts and proximal and distal tubules within the cortex (A and B). In the medulla, light staining was evident in collecting ducts, thick ascending limbs and some thin limbs of the loop of Henle (C and D). Light GR immuno-staining was localised to collecting duct and interstitial cells within the papilla (E and F). Cap = capsule; cd = collecting duct; dct = distal convoluted tubule; mtal = medullary thick ascending limb; pct = proximal convoluted tubule; tl = thin limb of loop of Henle; vr = vasa recta; ➞ = macula densa; ➧ = papillary interstitial cells. All digital micrographs were captured at 200x magnification; scale bar indicates 100 μm.


SALINE
BETAMETHASONE
CORTEX
MEDULLA
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A
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cd
vr

Renal GR protein levels at 121 d were not different between control and betamethasone-exposed fetal sheep ($p = 0.27$; Figure 4.11). Sections of fetal kidney at 121 d showed more intense GR immuno-staining than any other gestational age (Figure 4.12). Nuclear and cytoplasmic GR immuno-staining was present in the smooth muscle cell layer beneath the renal capsule, tunica media of blood vessels, capillary tufts of developing glomeruli, distal tubules, macula densae, proximal tubules, loop of Henle, medullary thick ascending limbs and the papillary epithelium (Figure 4.12 A to F). The papillary epithelium consisted of stratified cuboidal cells and the most intense nuclear and cytoplasmic GR immuno-staining was observed in the basal layer (Figure 4.12. F). Nuclear immuno-staining was also present in a number of medullary and papillary interstitial cells (Figure 4.12 C to F). Less intense cytoplasmic GR immuno-staining was evident in the collecting ducts. Darker GR immuno-staining was evident in the more distal regions of the nephron including the distal convoluted tubule, macula densa and medullary thick ascending limb. The most intense GR immuno-staining was localised to the tunica media of major renal blood vessels (Figure 4.12. G).

GR protein levels were not different between groups at 146 d ($p = 0.48$; Figure 4.13). GR immuno-staining at 146 d was similarly located, but less intense, to that observed at 121 d (Figure 4.14).

4.3.7. Na^+/K^+-ATPase-α_1 mRNA and protein expression

There was no difference in the relative expression level of renal Na^+/K^+-ATPase-α_1 mRNA between saline or betamethasone-exposed fetal sheep at any gestational age ($p = 0.19$; Figure 4.15). Overall, renal Na^+/K^+-ATPase-α_1 mRNA levels were similar at 109 and 116 d but renal Na^+/K^+-ATPase-α_1 mRNA expression was higher at 121 d than at 116 d ($p = 0.01$) and higher at 146 d than at 109 d ($p < 0.001$) or 116 d ($p < 0.001$). Renal Na^+/K^+-ATPase-α_1 mRNA levels at 146 d were not significantly correlated with fetal plasma cortisol levels ($R^2 = 0.06$; $p = 0.51$).

The anti-Na^+/K^+-ATPase-α_1 antibody used for Western blotting recognised a distinct protein band at 101 ± 1 kDa. Renal Na^+/K^+-ATPase-α_1 protein levels were not different between saline- and betamethasone-exposed fetuses at 109 d ($p = 0.70$; Figure 4.16),

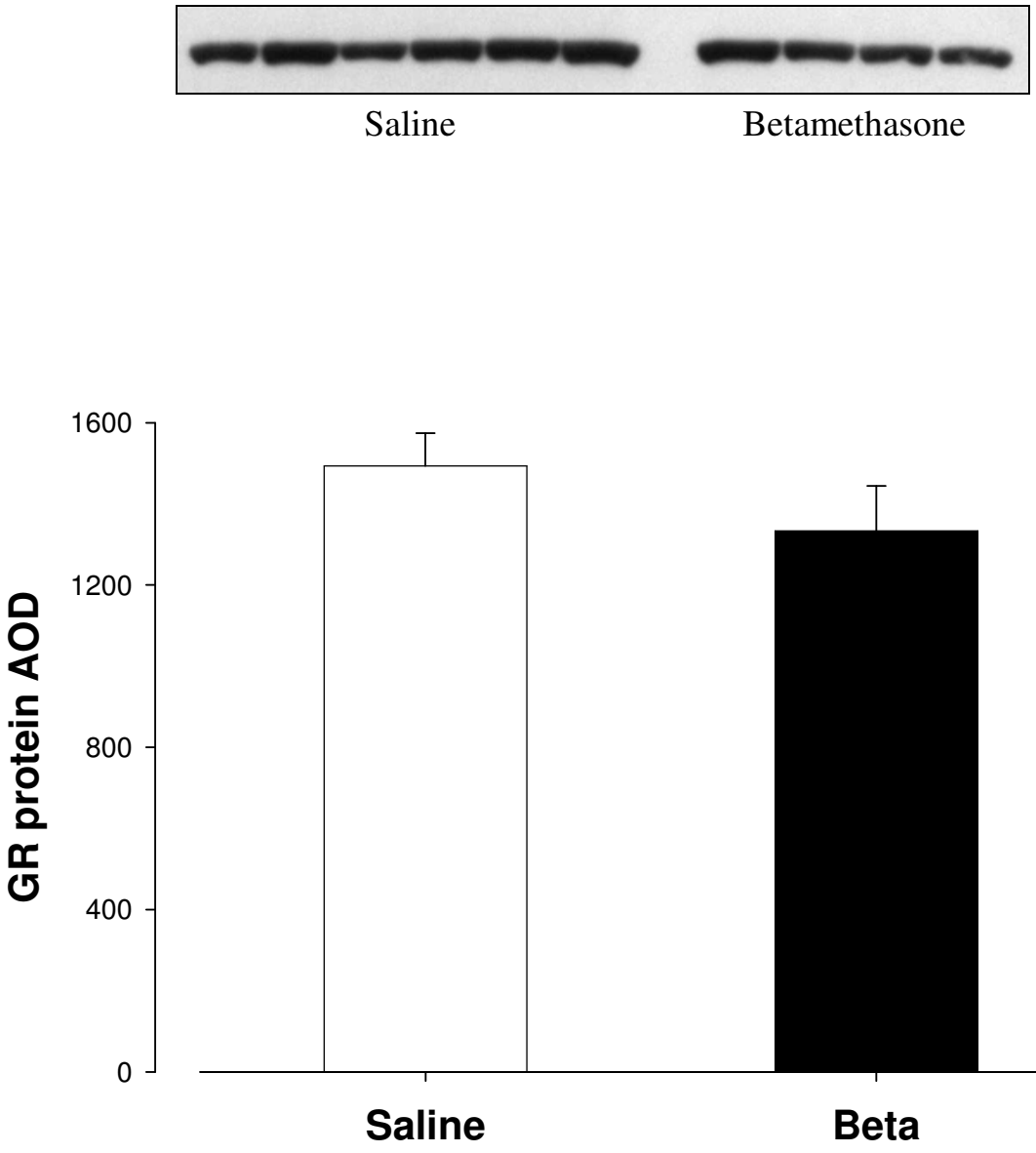
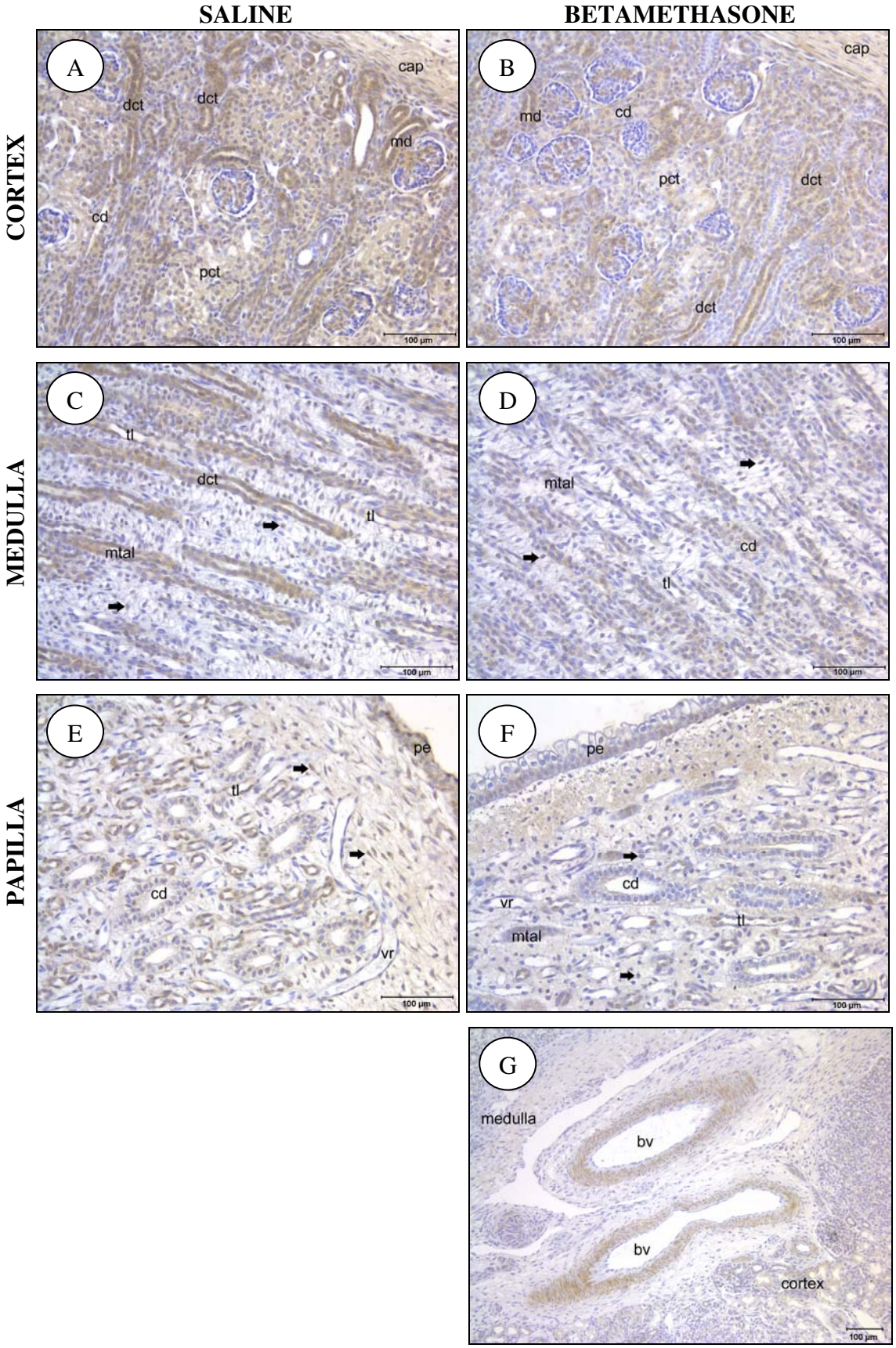


Figure 4.11. Renal GR protein levels at 121 days' gestation.

Bars show mean ± S.E.M. of renal GR protein levels at 121 d after maternal saline (white; n = 6) or betamethasone (black; n = 4) administration at 104, 111 and 118 d. There was no difference in renal GR protein levels between groups (p = 0.27).

Figure 4.12. GR immuno-staining in fetal sheep kidneys at 121 days' gestation.
Representative digital micrographs of GR immuno-staining in kidney sections from fetal sheep at 121 d, after exposure to maternal saline or betamethasone at 104, 111 and 118 d. There was no difference in the renal localisation or intensity of GR immuno-staining between groups. Intense GR immuno-staining was present in glomerular tufts and distal tubules, with moderate staining in collecting ducts and proximal tubules within the cortex (A and B). In the medulla and papilla, moderate staining was evident in cells of the thick and thin limbs of the loop of Henle and interstitial cells with lighter staining in the collecting ducts (C to F). Intense GR immuno-staining was observed in the tunica media of the arcuate blood vessels (G). Cap = capsule; cd = collecting duct; dct = distal convoluted tubule; md = macula densa; mtal = medullary thick ascending limb; pct = proximal convoluted tubule; pe = papillary epithelium; tl = thin limb of loop of Henle; vr = vasa recta; ➡ = interstitial cell. All digital micrographs were captured at 200x magnification; scale bar illustrates 100 μm.


SALINE
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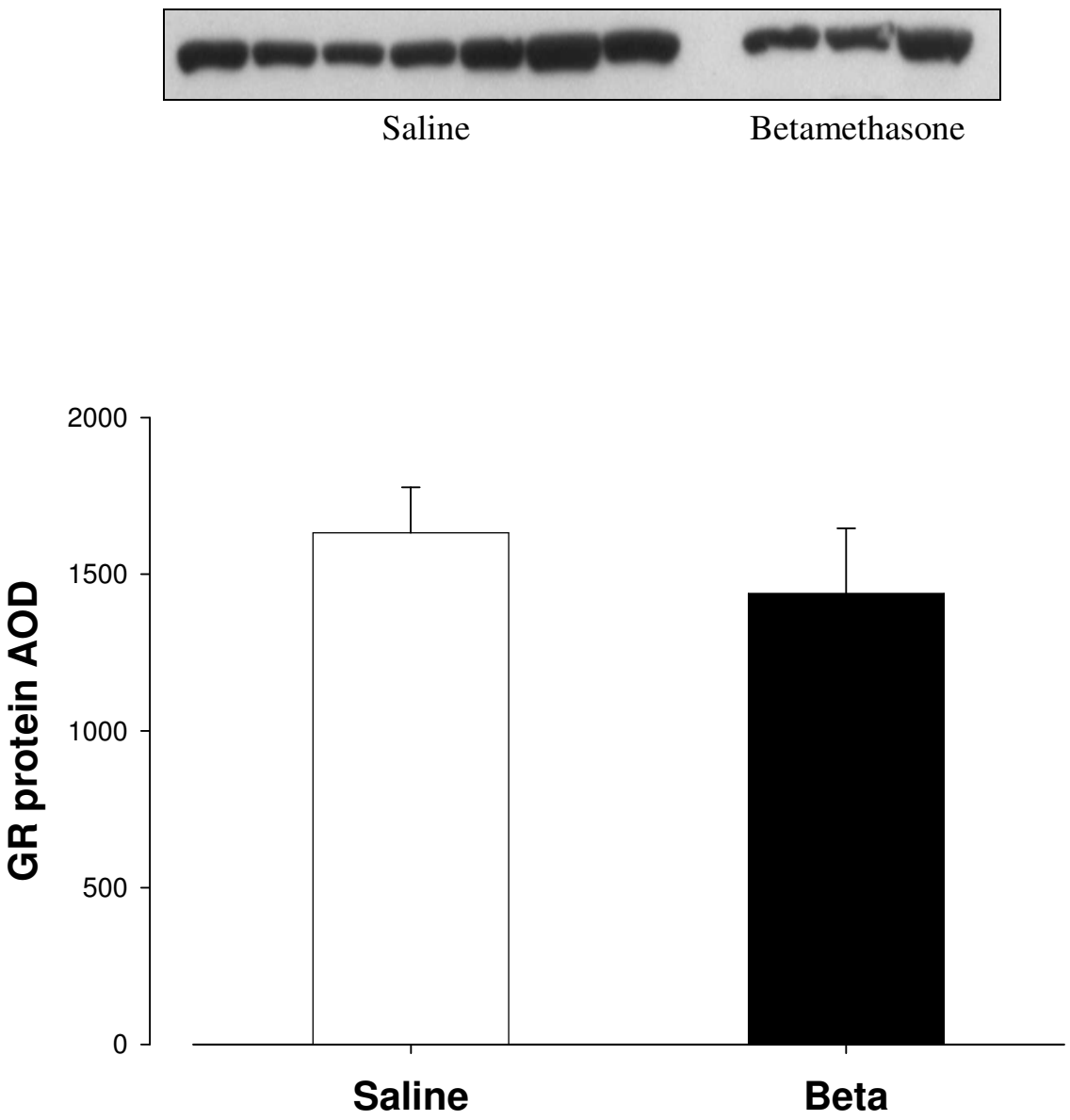
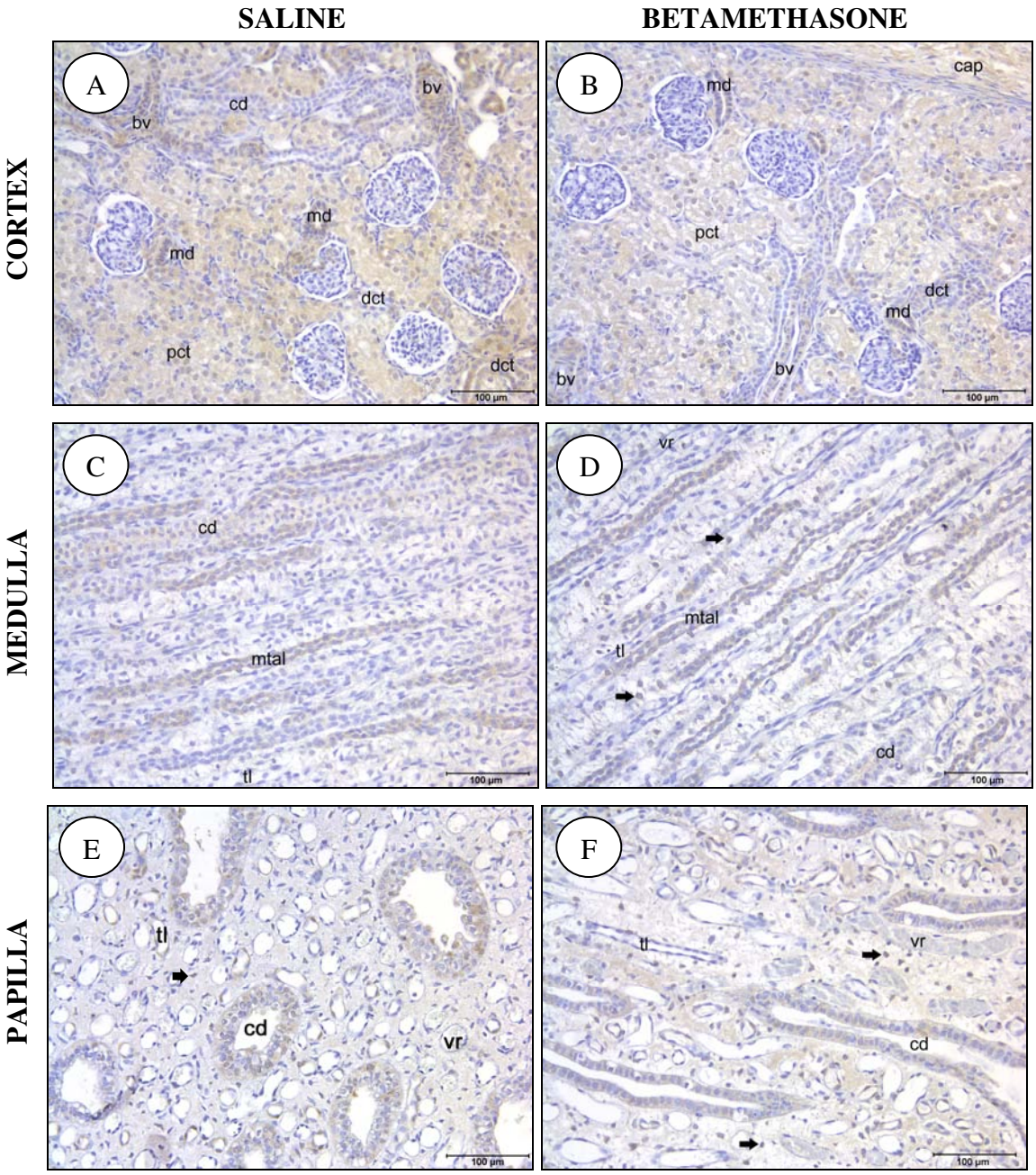


Figure 4.13. Renal GR protein levels at 146 days' gestation.

Bars show mean ± S.E.M. of renal GR protein levels at 146 d after maternal saline (white; n = 7) or betamethasone (black; n = 3) administration at 104, 111 and 118 d. There was no statistical difference between groups (p = 0.48).

Figure 4.14. GR immuno-staining in fetal sheep kidneys at 146 days' gestation.
Representative digital micrographs of GR immuno-staining in kidney sections from fetal sheep at 146 d after exposure to maternal saline or betamethasone at 104, 111 and 118 d. There was no difference in the renal localisation or intensity of GR immuno-staining between groups. Light GR immuno-staining was present in glomerular tufts, collecting ducts and proximal tubules (A and B). Moderate GR immuno-staining was evident in the distal tubules and macula densae cells within the cortex (A and B). In the medulla and papilla, light GR immuno-staining was evident in cells of the thick and thin limbs of the loop of Henle, interstitial cells and collecting ducts (C to F). Bv = blood vessel; cap = capsule; cd = collecting duct; dct = distal convoluted tubule; md = macula densa; mtal = medullary thick ascending limb; pct = proximal convoluted tubule; tl = thin limb of loop of Henle; vr = vasa recta; ➡ = interstitial cell. All digital micrographs were captured at 200x magnification; scale bar indicates 100 μm.


SALINE
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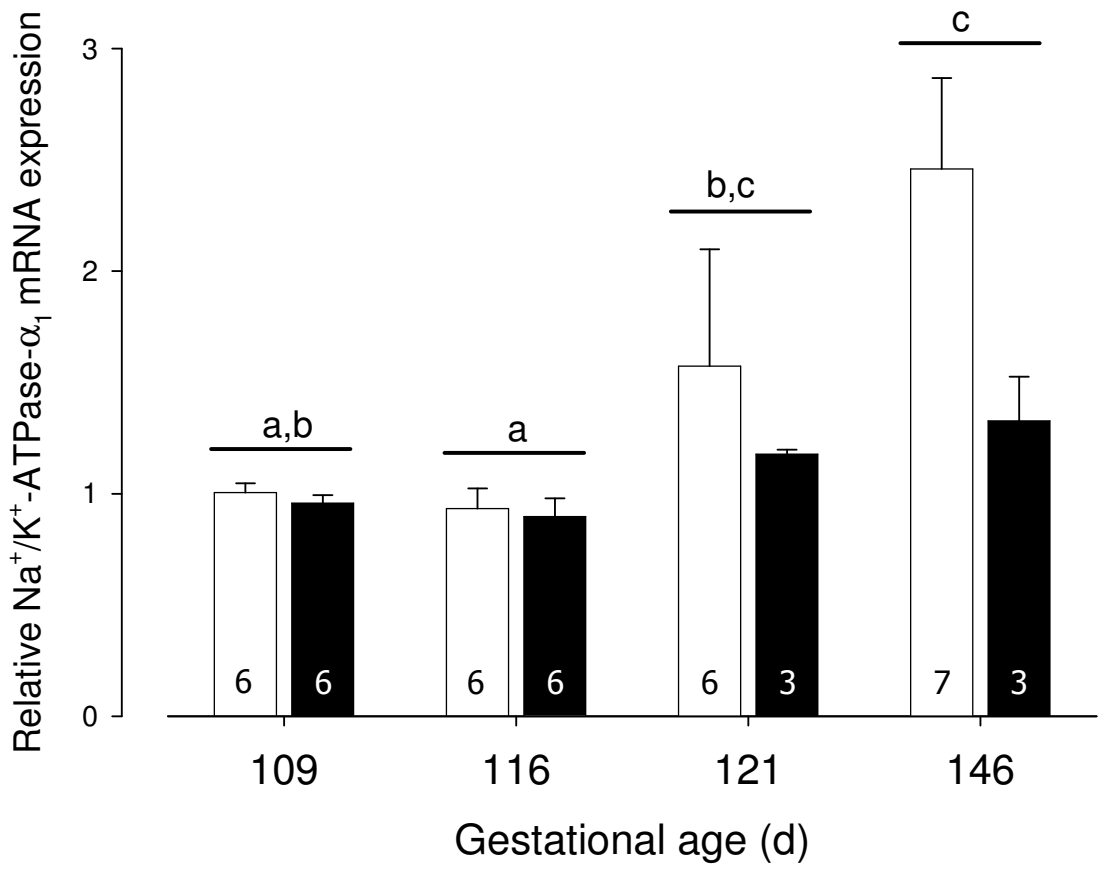


Figure 4.15. Na^+/K^+-ATPase-α_1 mRNA expression in the fetal kidney

Bars show mean ± S.E.M. of relative Na^+/K^+-ATPase-α_1 mRNA expression in fetal kidneys after maternal exposure to saline (white) or betamethasone (black). Numbers of fetuses in each group are listed within each bar. Different letters denote significant statistical difference ($p < 0.05$). There was an effect of age ($p < 0.001$), but not treatment ($p = 0.19$), on relative fetal renal Na^+/K^+-ATPase-α_1 mRNA levels.

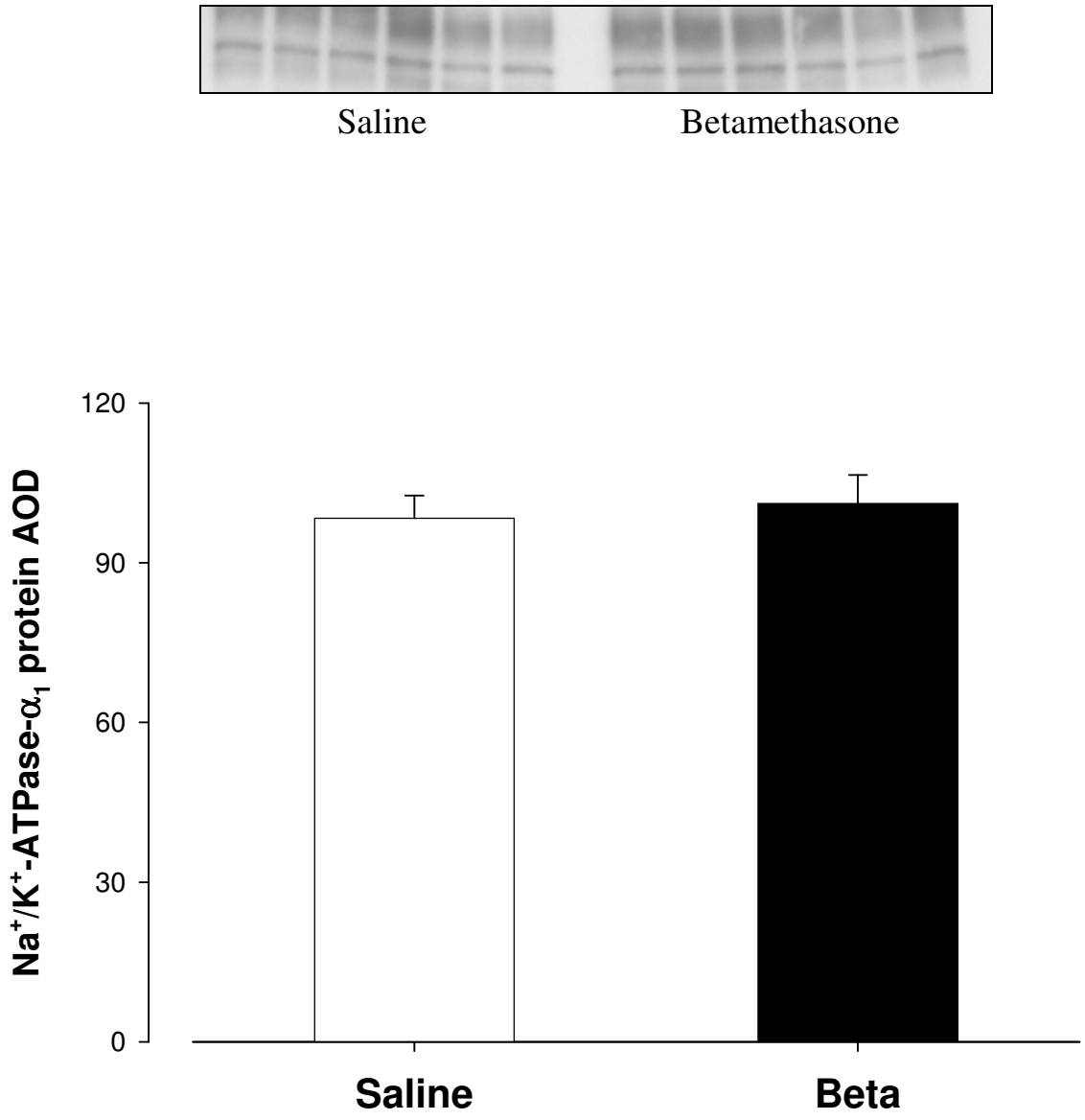


Figure 4.16. Renal Na^+/K^+-ATPase-α_1 protein levels at 109 days' gestation.

Bars show mean ± S.E.M. of renal Na^+/K^+-ATPase-α_1 protein levels at 109 d after maternal saline (white; n = 6) or betamethasone (black; n = 6) administration at 104 d. A digital photo of the Western blot is shown above and the AOD of the Na^+/K^+-ATPase-α_1 band (at 101 ± 1 kDa) is plotted. There was no significant difference between groups (p = 0.70).

121 d ($p = 0.14$; Figure 4.18) and 146 d ($p = 0.75$; Figure 4.19). However, renal Na^+/K^+-ATPase-α_1 protein levels at 116 d were significantly higher in betamethasone-exposed fetuses (170 ± 13 arbitrary optical density (AOD) units) compared to controls (108 ± 21 AOD units; $p = 0.03$; Figure 4.17).

4.3.8. Mineralocorticoid receptor mRNA expression

There was no effect of gestational age ($p = 0.07$), or prenatal treatment ($p = 0.37$), on relative fetal renal MR mRNA expression levels (Figure 4.20).

4.3.9. 11β-HSD-2 mRNA expression

Relative fetal renal 11β-HSD-2 mRNA levels were increased at 121 and 146 d compared to mRNA levels at 109 and 116 d ($p < 0.01$; Figure 4.21). However, there was no effect of maternal betamethasone exposure on fetal renal 11β-HSD-2 mRNA levels ($p = 0.90$).

4.3.10. Renin mRNA expression

Maternal betamethasone exposure increased renal renin mRNA levels relative to control animals at 116 ($p = 0.02$) and 121 d ($p = 0.003$) and relative to betamethasone-exposed 109 d animals (Figure 4.22).

4.3.11. Angiotensinogen mRNA expression

There was no effect of gestational age ($p = 0.87$), or prenatal treatment ($p = 0.60$), on relative fetal renal A_o mRNA expression levels (Figure 4.23).

4.3.12. AT_1 receptor mRNA expression

There was an effect of gestational age ($p < 0.001$), but not treatment ($p = 0.51$), on fetal renal AT_1 receptor mRNA levels (Figure 4.24). Relative renal AT_1 receptor mRNA levels were higher at 116 d than at 109 d ($p = 0.01$). At 121 d, relative fetal renal AT_1

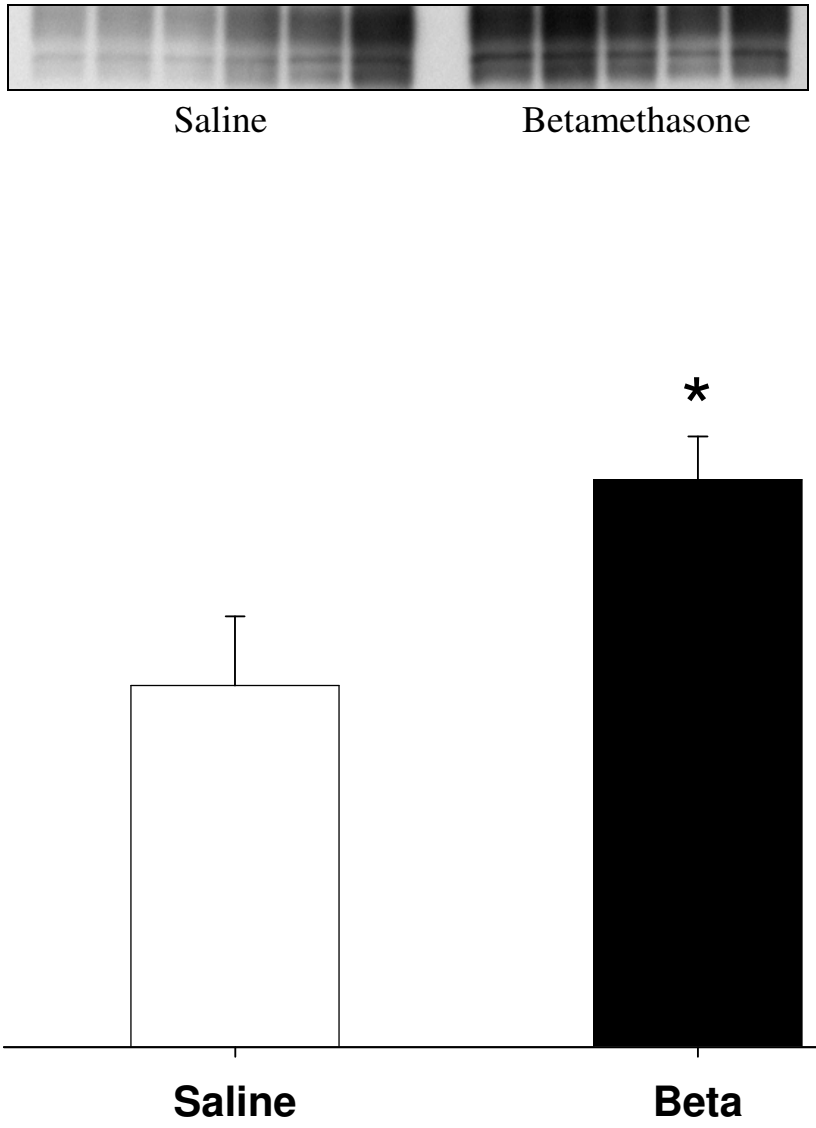


Figure 4.17. Renal Na^+/K^+-ATPase-α_1 protein levels at 116 days' gestation.

Bars show mean ± S.E.M. of renal Na^+/K^+-ATPase-α_1 protein levels at 116 d after maternal saline (white; n = 6) or betamethasone (black; n = 5) administration at 104 and 111 d. Na^+/K^+-ATPase-α_1 protein levels were significantly higher in betamethasone-exposed fetuses than in controls ($p = 0.03$). * $p < 0.05$.

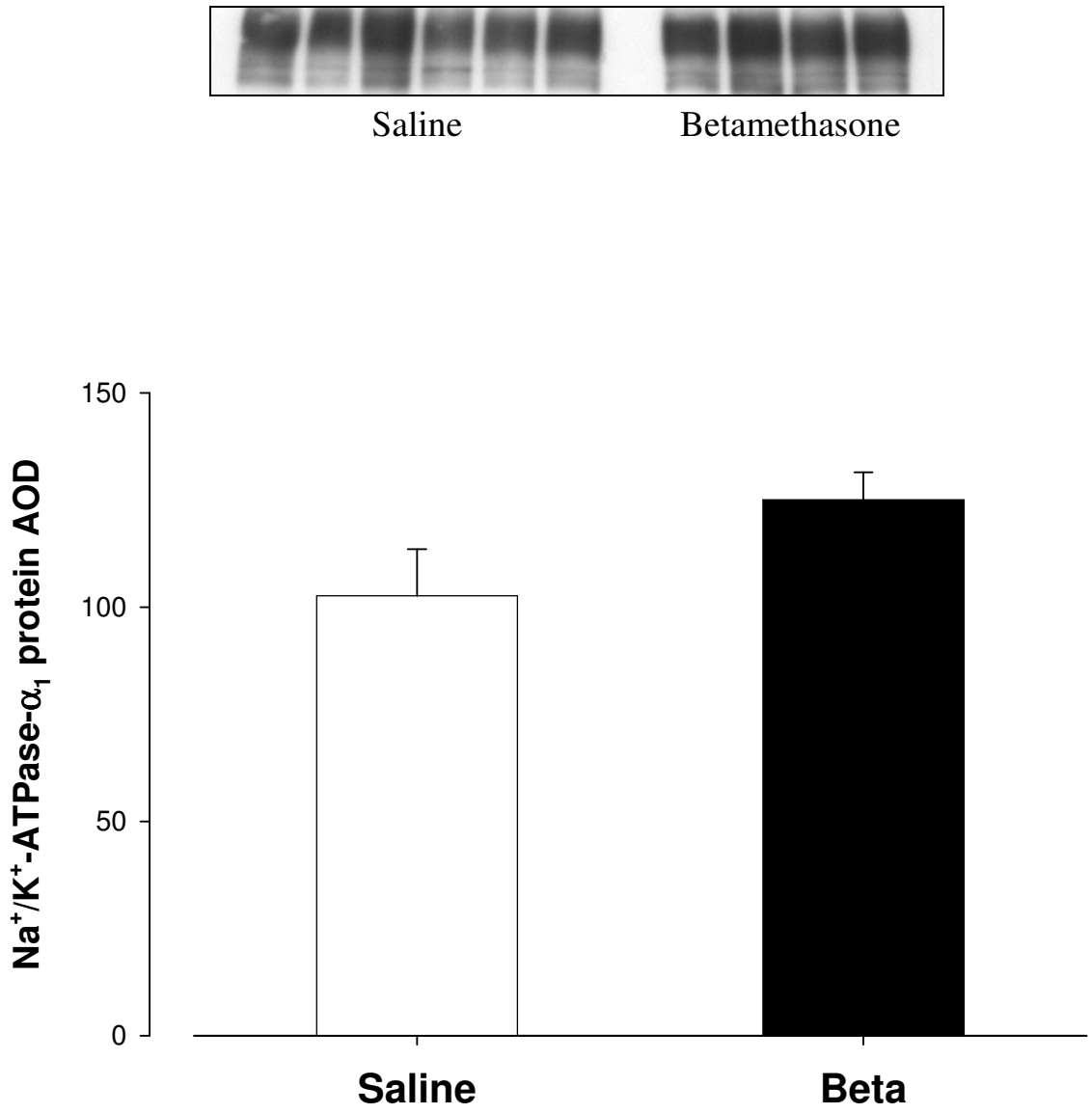


Figure 4.18. Renal Na^+/K^+-ATPase-α_1 protein levels at 121 days' gestation.

Bars show mean ± S.E.M. of renal Na^+/K^+-ATPase-α_1 protein levels at 121 d after maternal saline (white; n = 6) or betamethasone (black; n = 4) administration at 104, 111 and 118 d. Renal Na^+/K^+-ATPase-α_1 protein levels were not different between saline- and betamethasone-exposed fetuses (p = 0.14).

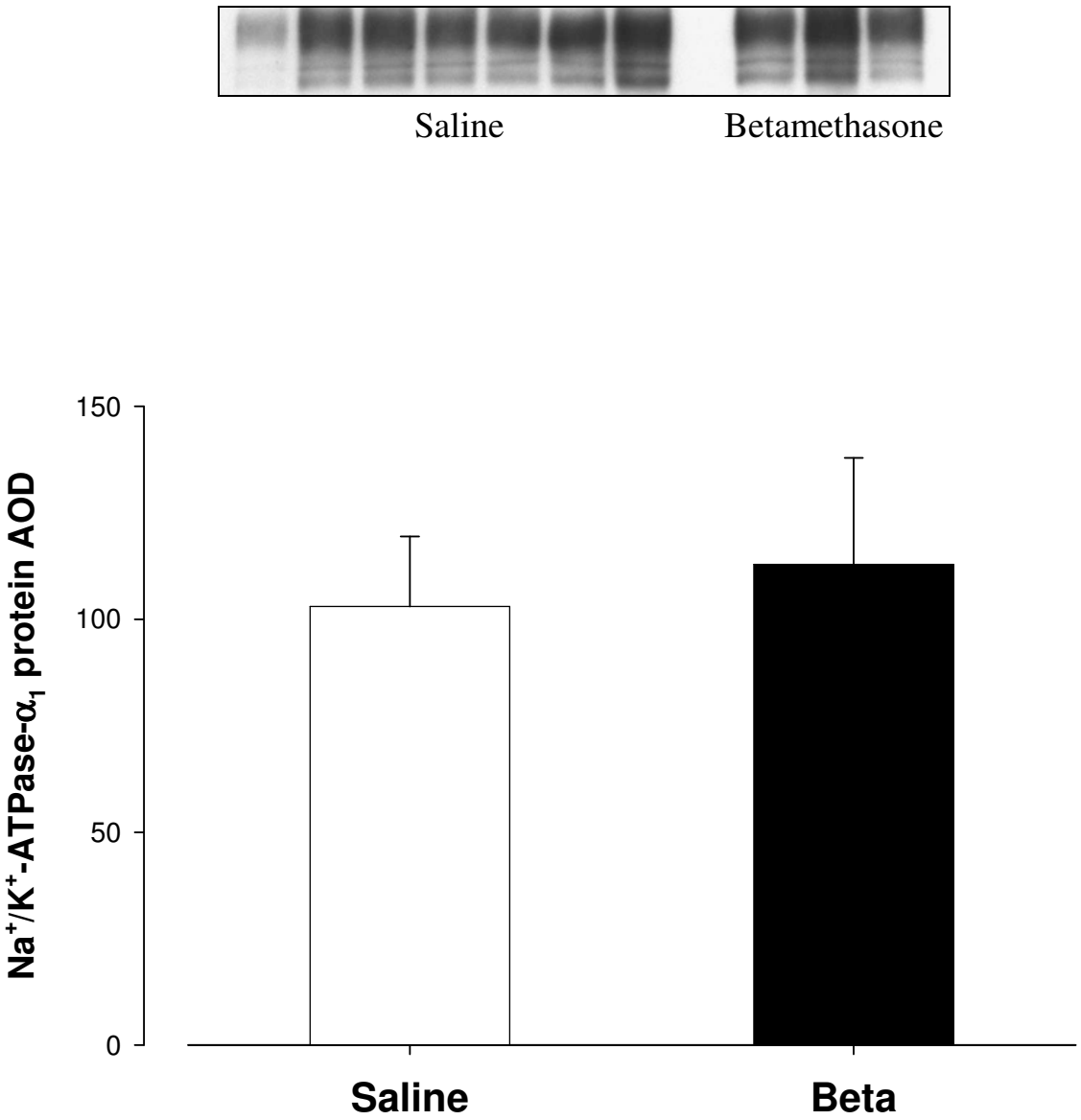


Figure 4.19. Renal Na^+/K^+-ATPase-α_1 protein levels at 146 days' gestation.

Bars show mean ± S.E.M. of renal Na^+/K^+-ATPase-α_1 protein levels at 146 d after maternal saline (white; n = 7) or betamethasone (black; n = 3) administration at 104, 111 and 118 d. There was no difference between groups (p = 0.75).

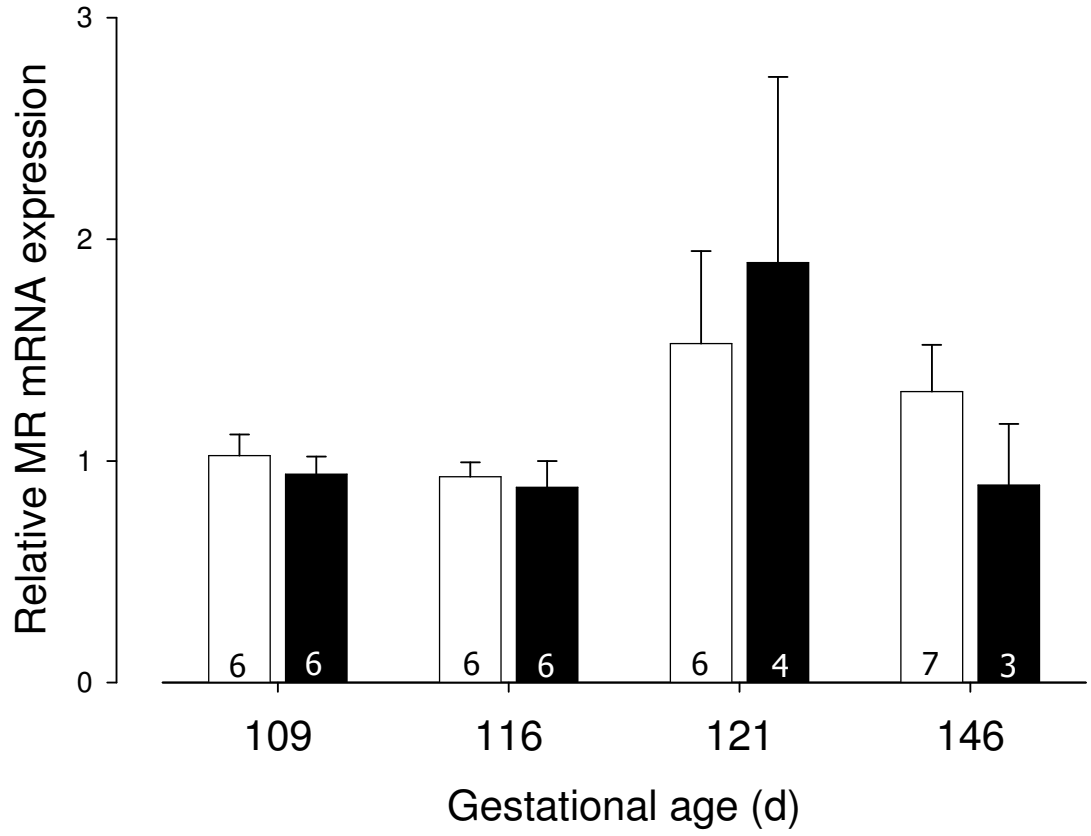


Figure 4.20. MR mRNA expression in the fetal kidney.

Bars show mean ± S.E.M. of relative MR mRNA expression in fetal kidneys after maternal saline (white) or betamethasone (black) administration. Numbers of fetuses in each group are labelled within the bars. There was no effect of age ($p = 0.07$) or group ($p = 0.37$) on relative fetal renal MR mRNA expression.

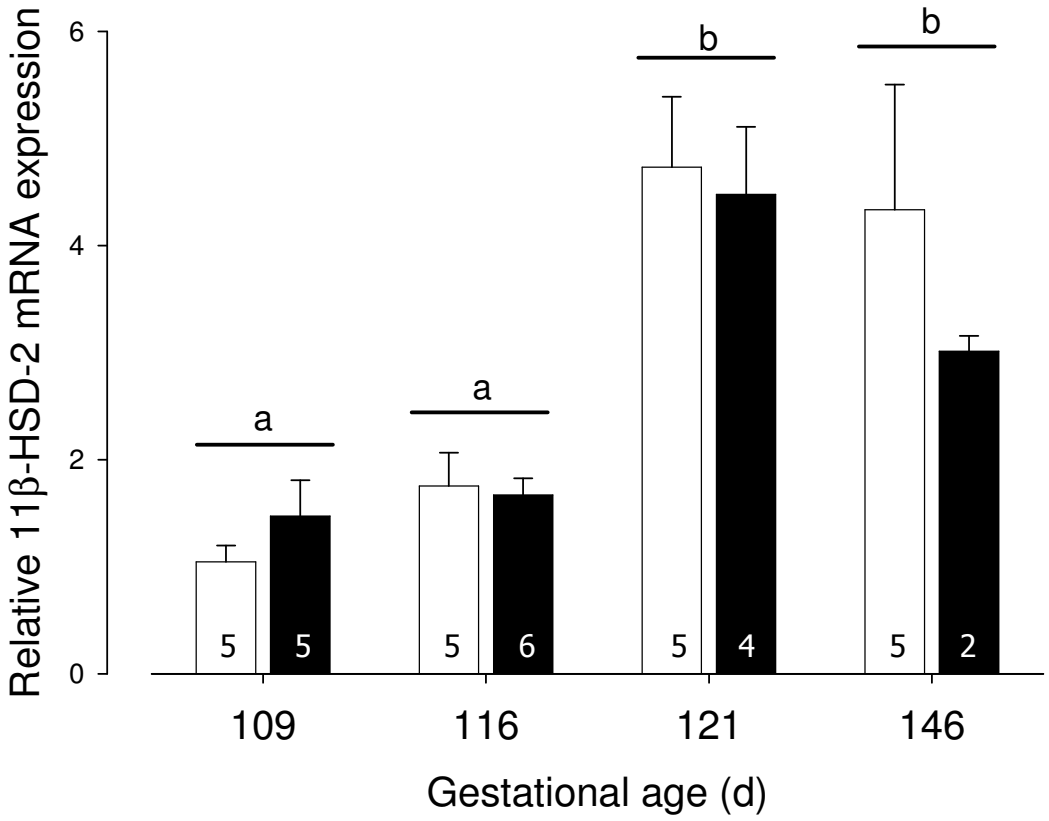


Figure 4.21. 11β-HSD-2 mRNA expression in the fetal kidney.

Bars show mean ± S.E.M. of relative 11β-HSD-2 mRNA expression in fetal kidneys after maternal saline (white) or betamethasone (black) administration. Numbers of fetuses in each group are labelled within the bars. Different letters denote significant statistical differences ($p < 0.01$). There was an effect of gestational age ($p < 0.001$), but not treatment ($p = 0.90$), on relative fetal renal 11β-HSD-2 mRNA levels.

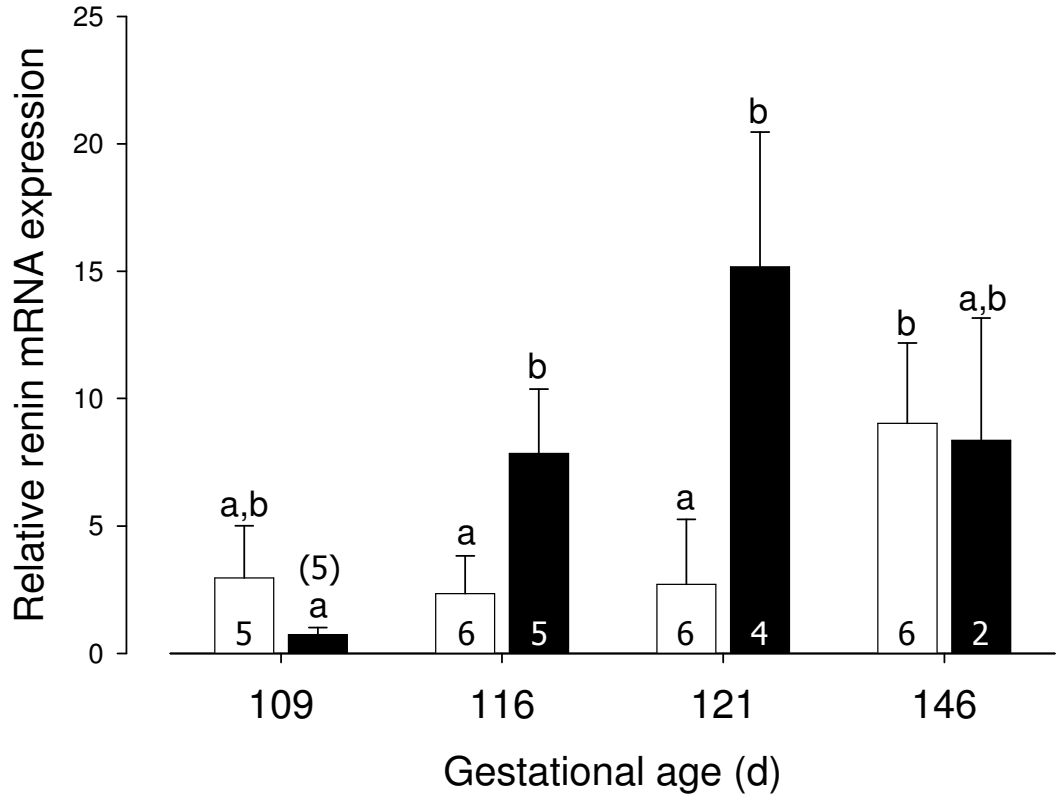


Figure 4.22. Renin mRNA expression in the fetal kidney.

Bars show mean ± S.E.M. of relative renin mRNA expression in fetal kidneys after maternal saline (white) or betamethasone (black) administration. Numbers of fetuses in each group are labelled within the bars or above in parenthesis. Different letters denote significant statistical differences ($p < 0.05$). There was an effect of prenatal treatment ($p = 0.03$), but not gestational age ($p = 0.10$), on relative fetal renal renin mRNA levels.

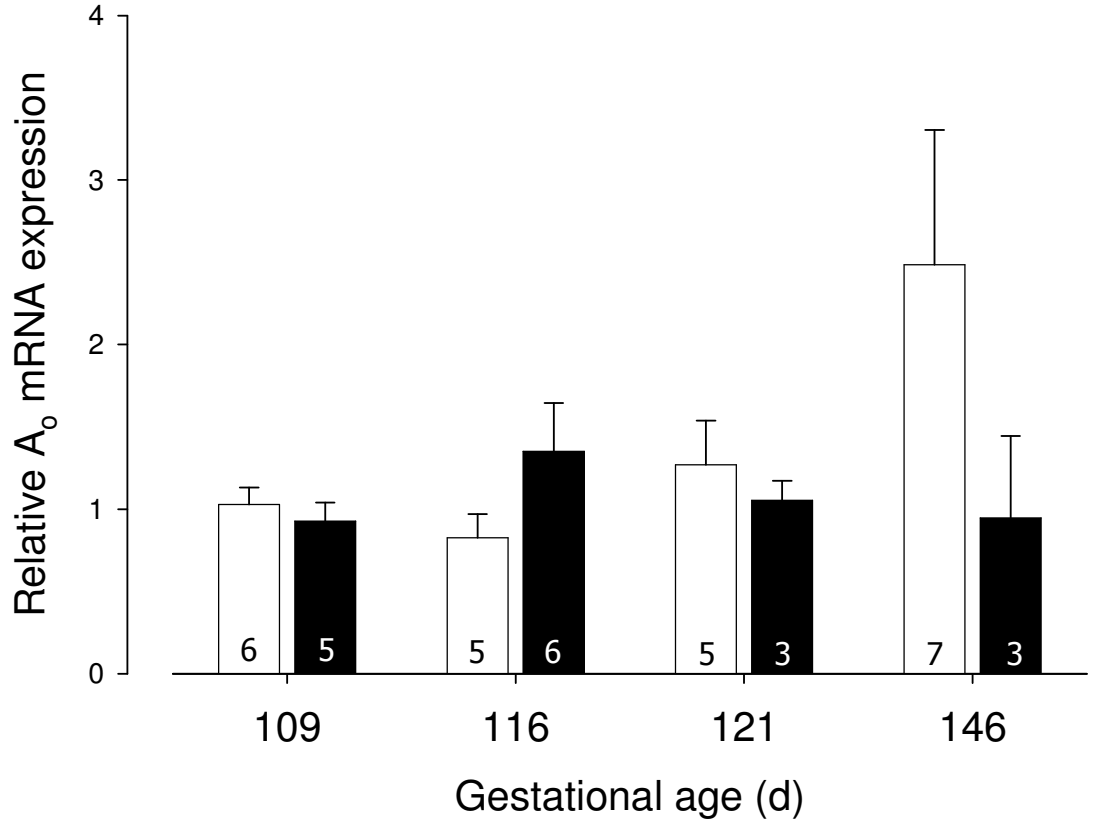


Figure 4.23. A_o mRNA expression in the fetal kidney.

Bars show mean ± S.E.M. of relative A_o mRNA expression in fetal kidneys after maternal saline (white) or betamethasone (black) administration. Numbers of fetuses in each group are labelled within the bars. There was no effect of gestational age ($p = 0.87$), or prenatal treatment ($p = 0.60$), on relative fetal renal A_o mRNA expression.

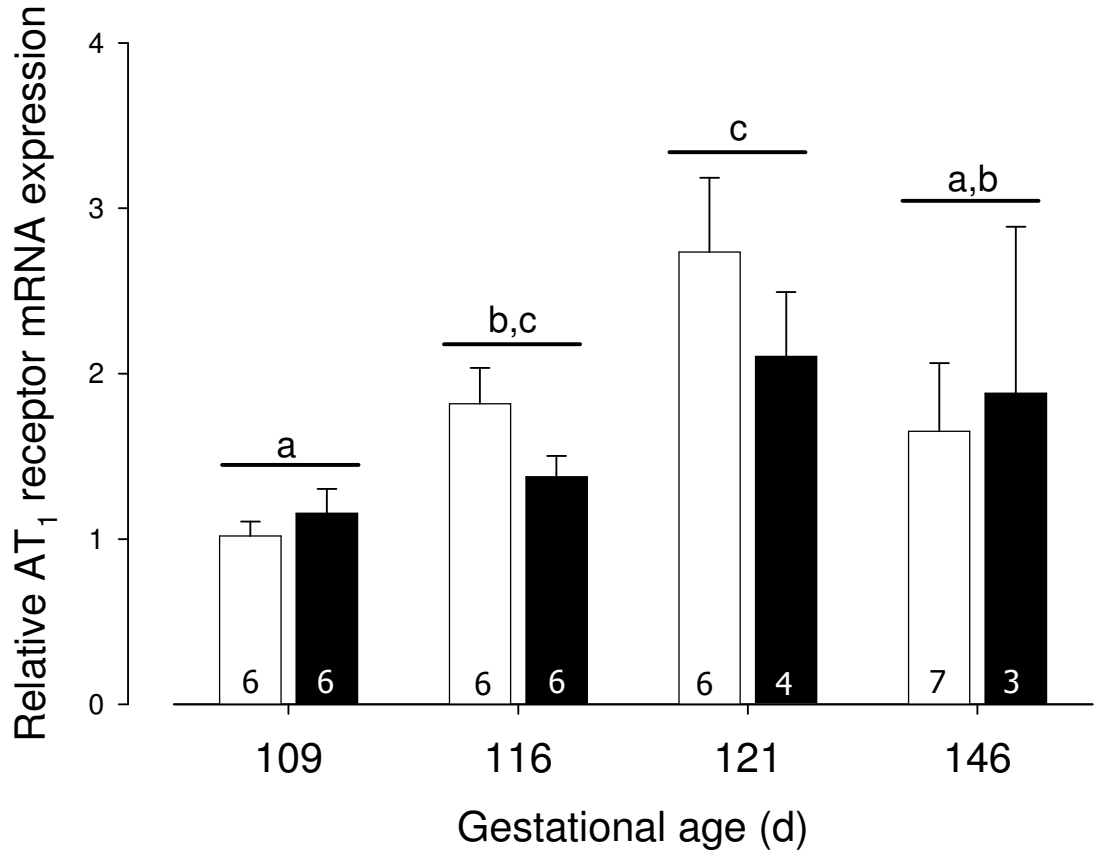


Figure 4.24. AT_1 receptor mRNA expression in the fetal kidney.

Bars show mean ± S.E.M. of relative AT_1 receptor mRNA expression in fetal kidneys after maternal saline (white) or betamethasone (black) administration. Numbers of fetuses in each group are labelled within the bars. Different letters denote significant statistical differences ($p < 0.05$). There was an effect of gestational age ($p < 0.001$), but not treatment ($p = 0.51$), on relative fetal renal AT_1 receptor mRNA levels.

receptor mRNA levels tended to be higher than at 116 d ($p = 0.05$) and were significantly higher than at 109 ($p < 0.001$) and 146 d ($p = 0.007$).

4.3.13. AT_2 receptor mRNA expression

Relative fetal renal AT_2 receptor mRNA levels were attenuated at 146 d compared to other gestational ages ($p < 0.01$; Figure 4.25). There was no difference in relative fetal renal AT_2 receptor mRNA levels between betamethasone-exposed fetuses and controls ($p = 0.23$).

4.4. DISCUSSION

Maternal betamethasone administration had variable effects on fetal growth in late gestation. The incidence of growth restriction in betamethasone-exposed fetal sheep was 2/6 at 116 d and 3/4 at 121 d. Late-gestation maternal betamethasone administration significantly reduced fetal body weight and total kidney weight at 116 and 121 d; however, the kidney-to-body weight ratio was unaltered by prenatal treatment at all gestational ages. The degree of IUGR induced by maternal betamethasone administration at these ages was consistent with previous studies in sheep (Ikegami *et al.*, 1997; Jobe *et al.*, 1998b; Newnham *et al.*, 1999). A prior study has suggested that the IUGR induced by three weekly-doses of maternal betamethasone in sheep was equivalent to a period of growth arrest of approximately nine days (Jobe *et al.*, 1998b). In the present study, fetal body weight did not increase significantly from day 116 to 121 of gestation in betamethasone-exposed fetal sheep, but increased 650 g in controls. Fetal total kidney weight also remained constant from 116 to 121 d in both groups, possibly reflecting a reduction in growth velocity of the kidney as nephrogenesis concludes. This observation is consistent with unbiased stereological studies in human fetuses, which have reported that the fetal kidney undergoes a "lag period" during 25 to 36 weeks of gestation where nephron formation slows as overall fetal growth accelerates (Hinchliffe *et al.*, 1991). Fetal total kidney weight was similar in saline- and betamethasone-exposed sheep at 146 d, indicating accelerated fetal growth between 121 and 146 d in betamethasone-exposed fetuses.

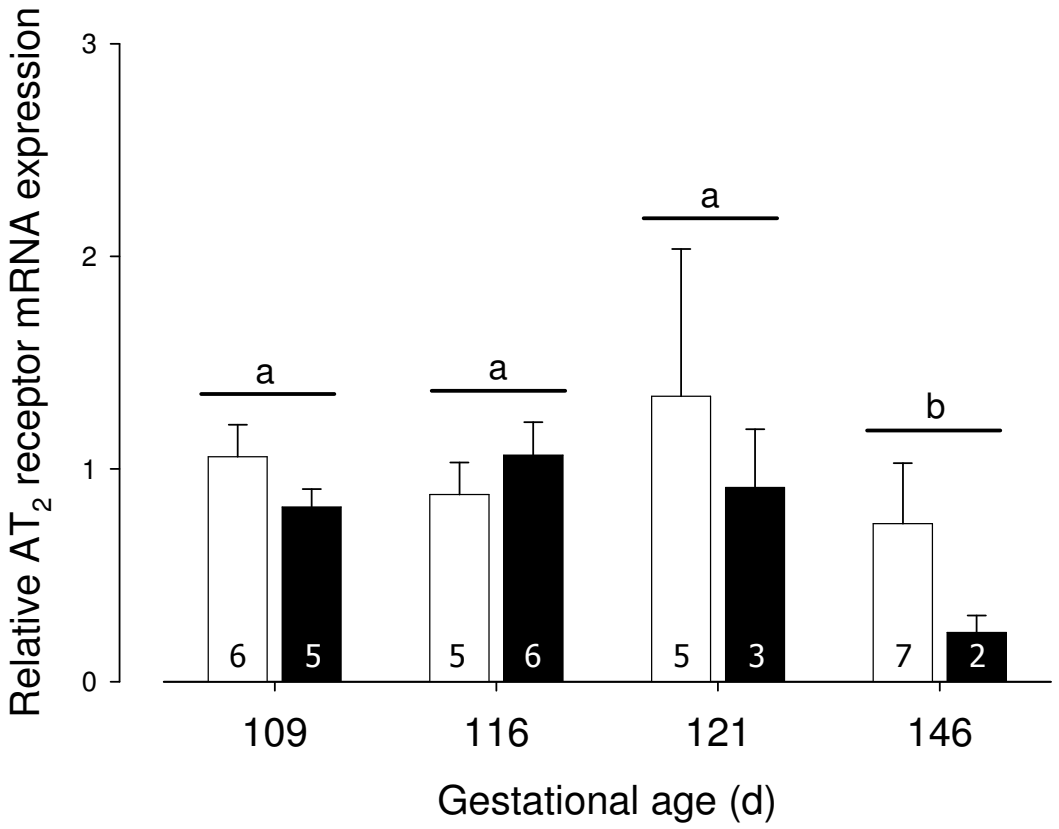


Figure 4.25. AT_2 receptor mRNA expression in the fetal kidney.

Bars show mean ± S.E.M. of relative AT_2 receptor mRNA expression in fetal kidneys after maternal saline (white) or betamethasone (black) administration. Numbers of fetuses in each group are labelled within the bars. Different letters denote significant statistical differences ($p < 0.01$). There was an effect on age ($p = 0.007$), but not prenatal treatment, on fetal renal AT_2 receptor mRNA levels.

In sheep, parturition is initiated by increased fetal cortisol secretion in late gestation (Magyar *et al.*, 1980). In the present study, fetal plasma cortisol levels were low and unaffected by maternal betamethasone at 109 to 121 d. However, fetal plasma cortisol levels increased at 146 d, but were attenuated in betamethasone-exposed fetal sheep. This is consistent with data from a previous cohort of sheep that were exposed to repeated, late-gestation maternal betamethasone (Moss *et al.*, 2004). Fetal plasma cortisol levels at 144 d were lower in animals that had been exposed to three weekly-doses of maternal betamethasone than in control animals (Moss *et al.*, 2004). Further, parturition in ewes injected with four weekly-doses of betamethasone was delayed by three days (Moss *et al.*, 2001). These data are consistent with previous studies that have demonstrated that antenatal glucocorticoid administration prolongs gestation in rhesus macaques (Novy & Walsh, 1983), rats (Chatterjee *et al.*, 1993), guinea pigs (Dean *et al.*, 2001) and sheep (Moss *et al.*, 2001; Kutzler *et al.*, 2004).

11β-HSD-2 mRNA and activity is present in the ovine kidney by day 85 of gestation and increases progressively until term (Langlois *et al.*, 1995; Wood & Srun, 1995; McMillen *et al.*, 2000). In the present study, relative fetal renal 11β-HSD-2 mRNA expression was similar at 121 and 146 d and increased compared to 109 and 116 d. These results are consistent with previous data of renal 11β-HSD-2 mRNA in fetal sheep; 11β-HSD-2 mRNA levels at 141-145 d were higher than at 90 d, but similar to expression levels at 125 d (McMillen *et al.*, 2000). Further, fetal renal 11β-HSD-2 activity was similar at 128-132 days' gestation compared to 140-142 days' gestation, when plasma cortisol levels increased 5.5-fold (Clarke *et al.*, 2002). In addition, an intra-fetal cortisol infusion that prematurely elevated fetal plasma cortisol to pre-partum values did not affect renal 11β-HSD-2 activity (Clarke *et al.*, 2002). Similarly, I have demonstrated that maternal betamethasone exposure in late gestation did not affect renal 11β-HSD-2 mRNA expression in fetal sheep. These results suggest that renal 11β-HSD-2 activity increases throughout nephrogenesis, which is complete by day 130 of gestation (Robillard *et al.*, 1981), but is not affected by glucocorticoids (Clarke *et al.*, 2002) in late gestation. Therefore, increased 11β-HSD-2 activity during nephrogenesis may protect the developing kidney from excess glucocorticoid exposure.

Corticosteroid receptors, GR and MR, are expressed in the fetal sheep kidney from as early as 60 d (Hantzis *et al.*, 2002). Previous studies have demonstrated that renal GR

mRNA levels remain relatively constant throughout gestation in fetal sheep (Yang, 1992; Hantzis *et al.*, 2002; von Reitzenstein & Keller-Wood, 2004), suggesting that renal GR mRNA expression may not be regulated by circulating glucocorticoid levels (Yang, 1992; Yang *et al.*, 1992a). Renal cortical MR mRNA expression peaks at 145 d in sheep, consistent with increased aldosterone effects on the kidney near-term (von Reitzenstein & Keller-Wood, 2004). In the present study, late-gestation maternal betamethasone administration did not affect the expression of renal GR and MR mRNA in fetal sheep. In contrast, early-gestation maternal dexamethasone infusion (0.48 mg/h for 48 h at 26 to 28 d) significantly increased fetal renal GR mRNA levels at 130 d in sheep (Hantzis *et al.*, 2002). The total dose of synthetic glucocorticoids was similar between the two studies, with 23 mg of dexamethasone administered in the early-gestation study and approximately 28 mg of betamethasone administered in the present study. Therefore, the timing of the exposure to synthetic glucocorticoids determines the effect on the expression of renal GR mRNA in fetal sheep.

This is the first study to measure renal GR protein levels and localise GR immuno-staining in fetal sheep kidneys. Consistent with the mRNA results, renal GR protein levels were not affected by maternal betamethasone administration in late-gestation fetal sheep. The localisation and intensity of GR immuno-staining in the kidney was similar in control and betamethasone-exposed sheep. As demonstrated in previous immunohistochemical studies of rat (Teasdale *et al.*, 1986), rabbit (Farman *et al.*, 1991) and human kidneys (Yan *et al.*, 1999), nuclear and cytoplasmic GR immuno-staining was observed in cells of the proximal, distal and collecting tubules in fetal sheep kidneys. GR immuno-staining was also localised to the loop of Henle, medullary and papillary interstitial cells and the papillary epithelium as previously reported in rabbit kidneys (Farman *et al.*, 1991). A recent study of developing rat kidneys observed nuclear GR immuno-staining in medullary interstitial cells, which was co-localised with COX-2 expression (Madsen *et al.*, 2004). Glucocorticoids are known to modulate prostaglandin synthesis in renal medullary interstitial cells (Zusman & Keiser, 1980). Strong GR immuno-staining was also detected in the capillary tufts of developing glomeruli within the fetal sheep kidney. This is consistent with GR mRNA localisation to the vascular tufts of developing glomeruli in human fetal kidneys at 8 to 16 weeks' gestation (Condon *et al.*, 1998). Further, glomerular GR immuno-staining was observed in the nuclei and cytoplasm of parietal and visceral epithelial cells, endothelial cells and

mesangial cells within the human kidney (Yan *et al.*, 1999). A novel finding of the present study was the localisation of intense GR immuno-staining in the tunica media of major renal blood vessels. This finding implies that glucocorticoids may directly alter vascular tone in blood vessels within the kidney to affect renal blood flow and GFR. Future studies could explore the role of the GR in renal vasculature with *in vitro* experiments.

Other studies using Western blot analysis of GR protein levels in sheep have used the PA1-511 (Clone 57) GR antibody from Affinity BioReagents (Saoud & Wood, 1996; Roesch & Keller-Wood, 1999; Sloboda *et al.*, 2002b; Richards *et al.*, 2003). This antibody binds to amino acids 346 to 367 of the human GR and detects a band at 97 kDa (Cidlowski *et al.*, 1990). The peptide sequence is 95% homologous to the ovine GR sequence. Western blot analysis of cytosolic proteins extracted from fetal sheep hypothalamus and pituitary detected a full-length (97 kDa) and half-length (45 kDa) GR protein band (Saoud & Wood, 1996). A second study using Western blot analysis of cytosols from adult sheep hypothalamus, pituitary, hippocampus and kidney revealed a GR protein band between 106 and 113 kDa (Roesch & Keller-Wood, 1999). Most recently, Western blot analysis using the PA1-511 antibody detected a 97 kDa GR protein in adult sheep hippocampus and brainstem (Richards *et al.*, 2003). During initial Western blot experiments performed before the present study, I found that this antibody detected numerous bands on Western blots of freshly-extracted protein from adult sheep kidneys. The three most prominent bands were: 90, 71 and 49 kDa, ranging from the least to most immuno-reactive GR band. These studies, all using the same antibody, create doubt about the actual size of the GR protein in sheep. However, the disparity in the GR size may reflect differences in the state of the receptor or the processing of the receptor protein.

Further Western blot experimentation led me to use the GR (M-20) primary antibody from Santa Cruz Biotechnology, Inc. This antibody was raised against the N-terminus of the GR of mouse origin and was reported to detect the α- (95 kDa) and β- (90 kDa) isoforms of the GR in mouse, rat and human tissues. The NCBI BLAST program predicted that the sheep and mouse GR protein sequences are 85% homologous. My Western blots of freshly-extracted total protein from fetal sheep kidneys revealed a single protein band at 74 ± 1 kDa, which differed from the expected size. I performed a

specificity experiment and demonstrated that this band at approximately 74 kDa was pre-absorbed (band intensity completely eliminated) with the antibody's peptide supplied by the manufacturer. A previous study using an anti-human GR antibody from Santa Cruz Biotechnology reported that Western blots of fetal sheep liver showed GR bands at 45, 57 and 95 kDa (Gupta *et al.*, 2003). The different GR protein sizes in fetal sheep tissues may reflect tissue-specific post-translational GR metabolism.

Sodium reabsorption by renal collecting duct epithelia plays an important role in renal fluid and electrolyte balance during the early postnatal period (Nakamura *et al.*, 2002). In sheep, the expression of the basolateral sodium pump, Na^+/K^+-ATPase, increases in the pre-partum period (Petershack *et al.*, 1999; von Reitzenstein & Keller-Wood, 2004). In this study, renal Na^+/K^+-ATPase-α_1 mRNA levels were elevated in control and betamethasone-exposed fetal sheep at 121 and 146 d, compared to levels at 116 d. Further, late-gestation maternal betamethasone administration did not alter the relative expression of Na^+/K^+-ATPase-α_1 mRNA in the fetal sheep kidney. Previous studies in late-gestation fetal sheep have demonstrated that glucocorticoids are involved in the maturation of renal Na^+/K^+-ATPase activity (Jobe *et al.*, 1996; Berry *et al.*, 1997; Petershack *et al.*, 1999). In the present study, elevated renal Na^+/K^+-ATPase-α_1 mRNA levels at 146 d were not correlated with fetal plasma cortisol levels in late-gestation fetal sheep. Renal Na^+/K^+-ATPase-α_1 protein levels were transiently increased in betamethasone-exposed fetal sheep at 116 d, but not at other gestational ages. This finding is consistent with a recent study that reported that maternal betamethasone administration on days 80 and 81 of gestation did not alter fetal renal Na^+/K^+-ATPase-α_1 protein expression at 135 d (Massmann *et al.*, 2004). The transient elevation in the α_1-subunit protein at 116 d could reflect transcriptional, translational or post-translational events. Alterations in Na^+/K^+-ATPase-α_1 mRNA were not observed at 109 or 116 d, but may have occurred during the five-day period from the second dose of betamethasone at 111 d until tissue collection at 116 d. A previous study of fetal sheep kidneys has also shown a discrepancy between mRNA and protein results (Hantzis *et al.*, 2002). The authors suggested that the difference between mRNA and protein expression may be attributed to the rate of receptor turnover (Hantzis *et al.*, 2002).

Renal Na^+/K^+-ATPase activity was not measured in the present study. A previous study demonstrated that renal Na^+/K^+-ATPase activity was significantly increased in 128 d

fetal sheep delivered 24 h after maternal betamethasone administration (Berry *et al.*, 1997). An intra-fetal cortisol infusion from 130 to 132 d resulted in a 2.2-fold increase in renal Na^+/K^+-ATPase activity in fetal sheep; however, only a modest increase was observed in α_1-subunit protein and mRNA expression (Petershack *et al.*, 1999). The increase in Na^+/K^+-ATPase activity after late-gestation cortisol administration in fetal sheep was attributed to a redistribution or activation of existing protein subunits (Petershack *et al.*, 1999). Therefore, although Na^+/K^+-ATPase-α_1 mRNA and protein levels were not consistently altered by prenatal betamethasone exposure in late gestation in the present study, activity of the enzyme may have been elevated. Future studies should determine the effect of maternal betamethasone administration on renal Na^+/K^+-ATPase activity in the fetal sheep kidney at term.

In the present study, maternal betamethasone administration increased renal renin mRNA levels at 116 and 121 d. Fetal body and kidney weights were significantly reduced at 116 and 121 d after maternal betamethasone administration. A previous study in human infants found that the mean umbilical vein active plasma renin concentration at delivery was significantly higher in the small-for-gestational-age group (Konje *et al.*, 1996). Further, a significant inverse correlation between renin concentration and the anterior-posterior diameter of the kidney was reported (Konje *et al.*, 1996). Therefore, increased renal renin expression may be a consequence of IUGR. A previous study in fetal sheep has demonstrated that kidney size plays an important role in determining GFR; fetal growth restriction (reduced kidney size) is associated with reduced GFR (Cock & Harding, 1997). Reduced renal perfusion pressure is associated with reduced GFR and has been demonstrated to increase renal renin mRNA expression in late-gestation fetal sheep (Rosnes *et al.*, 1998; Rosnes *et al.*, 2001). Hence, renal growth restriction may reduce renal perfusion pressure, which then results in increased renin mRNA expression, as I observed in late-gestation fetal sheep exposed to maternal betamethasone administration.

Renal renin levels do not appear to be regulated by fetal plasma cortisol levels in late-gestation fetal sheep. Recently, it has been shown that hypothalamo-pituitary disconnection (HPD) in fetal sheep blocked the late gestation cortisol increase but did not alter renal renin mRNA, renin or prorenin protein content, nor plasma renin levels (Chen *et al.*, 2005). In contrast, exogenous glucocorticoid administration in late

gestation has been shown to alter renin levels. Maternal betamethasone administration from 104 to 118 d (weekly injections of 0.5 mg/kg body) reduced fetal plasma cortisol levels, but increased renal renin concentration in fetal sheep at 148 d (T.J.M. Moss, *unpublished observation*). Maternal administration of betamethasone on 80 and 81 d reduced renal renin concentration within 24 h, but then resulted in a significant up-regulation of renal renin concentration at 135 d in fetal sheep (Connors *et al.*, 2005). The increase in renal renin mRNA at 116 and 121 d in the present study may precede the increase in renal renin concentration observed in 148 d fetal sheep exposed to the same treatment protocol (T.J.M. Moss, *unpublished observation*). Plasma renin activity (PRA) in 125 or 145 d fetal sheep was not affected by weekly maternal betamethasone from 104 to 118 d, but plasma AngII was reduced in 125 d sheep after exposure to single or multiple maternal betamethasone administration (Smith *et al.*, 2003). PRA, AngII and aldosterone levels were suppressed in newborn lambs delivered 15 or 24 h after maternal betamethasone administration on 122 or 127 d, respectively (Berry *et al.*, 1997; Smith *et al.*, 2000). Therefore, maternal betamethasone administration may briefly reduce the negative feedback effect of AngII on renin production resulting in the transient elevation in renal renin mRNA expression.

Maternal betamethasone administration did not affect the relative renal expression of A_o mRNA in late-gestation fetal sheep; A_o mRNA expression was unaltered over the last third of gestation. Similarly, fetal renal A_o mRNA expression was unaltered by an intra-fetal cortisol infusion at 130 d (Segar *et al.*, 1995). In contrast, early-gestation maternal dexamethasone, but not cortisol, infusion significantly increased fetal renal A_o mRNA at 130 d (Moritz *et al.*, 2002b). Therefore, late-gestation renal A_o mRNA expression appears to be sensitive to synthetic glucocorticoid administration in early, but not late, gestation.

In the present study, relative renal AT_1 receptor mRNA levels in both groups were significantly higher at 121 d than at 109 and 146 d and tended to be higher than at 116 d. There was no difference in relative renal AT_1 receptor mRNA levels between 109 or 116 d and 146 d, which is similar to results from previous studies that demonstrated that renal AT_1 receptor mRNA levels are not different between 100 or 115 d and 140 d (Robillard *et al.*, 1994; Butkus *et al.*, 1997). Maternal betamethasone administration did not alter renal AT_1 receptor mRNA expression in late-gestation fetal sheep. In contrast,

maternal cortisol or dexamethasone infusion in early pregnancy (~ 27 d) increased fetal renal AT_1 receptor mRNA expression at 130 d in fetal sheep (Moritz *et al.*, 2002b). My findings are consistent with a recent study that demonstrated that mid-gestation (80-81 d) maternal betamethasone administration did not alter renal AT_1 receptor mRNA expression at 82 or 135 d in fetal sheep (Zhang *et al.*, 2005). An intra-fetal cortisol infusion at 130 d significantly reduced renal AT_1 receptor mRNA in fetal sheep (Robillard *et al.*, 1994; Segar *et al.*, 1995). On the contrary, renal AT_1 receptor mRNA and protein levels were increased in fetal sheep at 135 to 139 d with reduced fetal plasma cortisol levels due to HPD surgery (Chen *et al.*, 2005). Therefore, in fetal sheep, renal AT_1 receptor mRNA expression appears to be developmentally-regulated.

The timing of glucocorticoid administration appears to be critical for determining the effects on fetal renal AT_2 receptor expression. In sheep, renal AT_2 receptors are involved in early kidney development and levels decrease after nephrogenesis is complete (Robillard *et al.*, 1995; Butkus *et al.*, 1997; Gimonet *et al.*, 1998). Early-gestation (~ 27 d) maternal dexamethasone administration increased renal AT_2 receptor mRNA expression in fetal sheep at 130 d (Moritz *et al.*, 2002b). Mid-gestation (80-81 d) maternal betamethasone administration reduced renal AT_2 receptor mRNA levels in fetal sheep at 82 and 135 d (Zhang *et al.*, 2005). Renal AT_2 receptor mRNA levels were not different between control and cortisol-infused twin fetal sheep at 130 d (Robillard *et al.*, 1995). In the present study, late-gestation (104 to 118 d) maternal betamethasone administration did not alter fetal renal AT_2 receptor mRNA levels at 109 to 146 d. Relative renal AT_2 receptor mRNA expression was reduced at 146 d, in control and betamethasone-exposed fetuses, compared to earlier gestational time-points. This finding is consistent with the normal ontogeny of reduced renal AT_2 receptor expression after the completion of nephrogenesis (Robillard *et al.*, 1995; Butkus *et al.*, 1997; Gimonet *et al.*, 1998). Therefore, in sheep, renal AT_2 receptor mRNA expression does not appear to be regulated by glucocorticoids in late gestation. The rapid decline in renal AT_2 receptor expression between 90 and 120 d in fetal sheep (Robillard *et al.*, 1995) may explain why glucocorticoids have little effect on AT_2 receptor gene expression during late gestation.

The early-gestation fetal sheep kidney appears to be highly susceptible to the programming effects of maternal glucocorticoid administration. At 26 to 28 d in the

fetal sheep, the metanephros is starting to develop with the first branching of the ureteric bud (Wintour & Moritz, 1997). In comparison to late-gestation administration, early-gestation maternal dexamethasone programs the up-regulation of several glucocorticoid-sensitive genes involved in glucocorticoid hormone action and the RAS in the fetal sheep kidney (Hantzis *et al.*, 2002; Moritz *et al.*, 2002b). Tissue sensitivities to glucocorticoids may be altered by increasing fetal plasma cortisol levels in late gestation. Effectively, specific glucocorticoid-sensitive receptors may become relatively resistant to the acute effects of glucocorticoids in late gestation.

Few human studies have examined the effect of antenatal glucocorticoid therapy on renal function in infants. Maternal dexamethasone administration reduced absolute and fractional sodium excretion during the first five days of life in premature neonates born at less than 35 weeks' gestation (al-Dahan *et al.*, 1987; Zanardo *et al.*, 1990). The accelerated renal maturation observed in the premature infants was proposed to be induced by increased Na^+/K^+-ATPase activity (al-Dahan *et al.*, 1987). The effects of maternal dexamethasone administration on renal function in premature neonates were no longer apparent at age 6 to 10 days (al-Dahan *et al.*, 1987). Therefore, the effects of prenatal antenatal glucocorticoid therapy on the human kidney appear to be transient and were only observed in the first few days of life (Jahnukainen *et al.*, 2001).

In conclusion, while there were some short-term effects of late-gestation maternal betamethasone administration on the fetal kidney, there were no long-term effects observable 28 days after the last betamethasone injection. These findings are consistent with recent physiological studies that demonstrated that there was no effect of maternal betamethasone administration on basal renal function or plasma renin activity, AngII or aldosterone levels in term fetal sheep (Smith *et al.*, 2003).

5. The Effects Of Prenatal Betamethasone Exposure On The Adult Kidney

5.1. INTRODUCTION

Numerous epidemiological studies have reported an association between low birth weight and the subsequent development of adult-onset diseases including hypertension, type 2 diabetes and cardiovascular disease (Barker *et al.*, 1990; Fall *et al.*, 1995; Barker, 1996; Curhan *et al.*, 1996a; Curhan *et al.*, 1996b; Law & Shiell, 1996; Lithell *et al.*, 1996; Rich-Edwards *et al.*, 1997). Hence, factors that reduce fetal growth may permanently alter the structure and/or function of organs that program the individual for adult-onset diseases. Data from animal studies have demonstrated that increased fetal glucocorticoid exposure restricts growth and induces programming that predisposes the animal to disease later in life (Benediktsson *et al.*, 1993; Levitt *et al.*, 1996; Langley-Evans, 1997a; Dodic *et al.*, 1998; Edwards *et al.*, 2001; Nyirenda *et al.*, 2001; Aghajafari *et al.*, 2002; Ortiz *et al.*, 2003). Late-gestation dexamethasone administration to pregnant rats reduced birth weight and programmed hypertension in adult offspring (Levitt *et al.*, 1996; Ortiz *et al.*, 2001; Ortiz *et al.*, 2003; O'Regan *et al.*, 2004). In sheep, a late-gestation fetal infusion of cortisol increased basal systolic and diastolic blood pressure (Tangalakis *et al.*, 1992). Therefore, in animal studies, increased fetal glucocorticoid exposure may program hypertension in adulthood.

Over the last few years, concerns have been raised about the long-term effects of antenatal glucocorticoid therapy in humans. Non-randomised studies in humans have demonstrated that birth weight was reduced in infants exposed to repeated doses of prenatal glucocorticoids (Reinisch *et al.*, 1978; Banks *et al.*, 1999; French *et al.*, 1999; Bloom *et al.*, 2001; Thorp *et al.*, 2002). A non-randomised cohort study of pre-term, low birth weight adolescents (at 14 years of age) reported that mean systolic and diastolic blood pressure was higher in individuals who had been exposed to a single course of prenatal glucocorticoids (Doyle *et al.*, 2000). However, follow-up data from the first and largest randomised controlled trial, the Auckland Steroid Trial, demonstrated that blood pressure not was different at age 6 or 30 years in individuals exposed to a single course of betamethasone (Dalziel *et al.*, 2004; Dalziel *et al.*, 2005). Currently, several multi-centred randomised controlled trials are underway to investigate the potential adverse effects of repeated doses of prenatal glucocorticoids in humans (IMPACT/PSANZ, 2001; MIRU, 2002; NICHD, 2005). The most recent reports from the randomised controlled trials have shown no difference in mean birth

weight or head circumference (Guinn *et al.*, 2001; Crowther *et al.*, 2006; Wapner *et al.*, 2006). However, at birth, the z-scores were lower for babies exposed to repeat corticosteroids than for those in the placebo group for weight-adjusted mean difference (Crowther *et al.*, 2006) and the repeat group had a reduction in multiples of the birth weight median by gestational age and more neonates weighing less than the 10^{th} percentile (Wapner *et al.*, 2006).

Impaired renal development is thought to be in involved in the programming of hypertension. In humans, low birth weight is associated with reduced nephron endowment (Brenner & Chertow, 1994; Mackenzie *et al.*, 1996; Manalich *et al.*, 2000; Hughson *et al.*, 2003; Amann *et al.*, 2004), an increased risk of developing hypertension (Barker *et al.*, 1990; Law *et al.*, 1993; Curhan *et al.*, 1996a; Curhan *et al.*, 1996b) and an increased susceptibility to early-onset end-stage renal disease in adults (Lackland *et al.*, 2000; Tulassay & Vasarhelyi, 2002). Furthermore, in animal models, alterations in the renal expression of glucocorticoid-sensitive genes (Whorwood *et al.*, 2001) and components of the RAS (Whorwood *et al.*, 2001; Moritz *et al.*, 2002b; Moritz *et al.*, 2005b) have been implicated in the programming of hypertension.

Experimental studies in sheep have been used to mimic late gestation antenatal glucocorticoid administration in humans. The aim of the present study was to assess the long-term effects of single or repeated doses of betamethasone administered to either the pregnant ewe or directly to the fetus on the adult kidney at age 3.5 years. Fetal injections were used to separate the effects of growth restriction and glucocorticoids, as maternal injections of betamethasone reduce fetal growth but direct fetal injections do not (Newnham *et al.*, 1999). Direct fetal injections are a possible route for antenatal glucocorticoid therapy as it has been shown to be as efficacious as maternal injections of betamethasone for causing pre-term lung maturation in sheep (Rebello *et al.*, 1997). However, because of the invasive nature of fetal injections, it is not currently employed in obstetric practice. Other data from the cohort of animals used in the present study have been published (Moss *et al.*, 2001; Sloboda *et al.*, 2002a; Sloboda *et al.*, 2003; Moss *et al.*, 2005). Single and repeated doses of maternal betamethasone reduced birth weight in the lambs; however, direct fetal administration had no effect on growth (Moss *et al.*, 2001). Basal mean arterial pressure (MAP) was not altered by prenatal treatment in these adult sheep (Moss *et al.*, 2005). By age 3 years, adult sheep exposed to repeated

doses of maternal betamethasone *in utero* had altered HPA activity with reduced basal cortisol levels (Sloboda *et al.*, 2003). These attenuated levels of endogenous glucocorticoid hormones may affect the expression of glucocorticoid-sensitive genes and proteins within tissues and thus alter local glucocorticoid hormone action. There have been no studies to assess the effects of late-gestation prenatal glucocorticoid administration on the adult kidney. The levels of GR and Na^+/K^+-ATPase-α_1 protein and expression of GR, Na^+/K^+-ATPase-α_1, MR, 11β-HSD-2, AT_1 receptor and A_o mRNA were measured in adult sheep kidneys to determine the normal expression and long-term impact of late-gestation prenatal betamethasone administration on the adult kidney.

5.2. EXPERIMENTAL PROCEDURES

5.2.1. Animals

Manuscripts describing the sheep used in this study have been published (Moss *et al.*, 2001; Sloboda *et al.*, 2002a; Sloboda *et al.*, 2003; Moss *et al.*, 2005). The prenatal treatment protocol is illustrated in Table 5.1. Pregnant ewes bearing single fetuses were injected with MPA at 100 d, prior to randomisation into treatment groups. Animals in the control groups (MS, FS) were administered normal saline at the injection time-points. Those in the treatment groups were exposed to single (M1, F1), or repeated (M4, F4), doses of betamethasone administered to the pregnant ewe (M-groups) or directly to the fetus (F-groups) on a weekly basis commencing at 104 d (70% of gestation; refer to section 2.2). Ewes in this study had a body weight of 49.5 ± 0.5 kg at 104 d, resulting in a total dose of approximately 25 mg of betamethasone in a single injection. Sheep offspring were born spontaneously in a field environment and tail docking, immunisation and castration of males was performed at approximately 2 months of age (Moss *et al.*, 2001). Two of the M4 lambs (#1 and #98) were raised by hand and supplemented with powdered milk (Divetalact®) as lactation was affected in some ewes in this group. All lambs were weaned at age 3 months. Adult offspring were euthanised at age 3.5 years for tissue collection (refer to section 2.3).

Table 5.1. Prenatal treatment protocol.

Pregnant ewes or fetuses were injected intra-muscularly with treatments as indicated below. The gestational timing of the injection is indicated on the left; term is 150 d.

	MATERNAL INJECTIONS			**DIRECT FETAL INJECTIONS**		
Gestational timing of injection	**MS**	**M1**	**M4**	**FS**	**F1**	**F4**
100 d	MPA	MPA	MPA	MPA	MPA	MPA
104 d	Saline	Beta	Beta	Saline	Beta	Beta
111 d	Saline	Saline	Beta	Saline	Saline	Beta
118 d	Saline	Saline	Beta	Saline	Saline	Beta
125 d	saline	Saline	Beta	Saline	Saline	Beta

MPA: medroxyprogesterone acetate, 150 mg. Saline: 5 mL 0.9% NaCl. Beta: Betamethasone (Celestone® Chronodose®), 0.5 mg/kg ewe body weight or estimated fetal body weight (section 2.2.3).

5.2.2. Molecular analyses

5.2.2.1. Histology and immunohistochemistry

Routine H&E staining was performed on 5 μm sections of paraffin-embedded adult sheep kidneys (section 2.4; Appendix 2). Where abnormal histology was evident, an additional section was stained with Masson's trichrome to assess the histopathology (section 2.4; Appendix 3, Figure 5.2). Immunohistochemistry was performed on 5 μm sections of left kidney to localise GR-immuno-reactive (IR) cells (section 2.5).

5.2.2.2. Western blot analysis

Western blot analysis was used to quantify renal GR and Na^+/K^+-ATPase-α_1 protein levels in total protein extracted from the right kidneys of offspring in this study (section 2.6). GR Western blots were probed with the primary antibody GR (M-20) (Santa Cruz Biotechnology, Inc.) at a concentration of 1 μg/mL (1:200) and the secondary antibody, ECL anti-rabbit IgG peroxidase-linked species-specific whole antibody (from donkey) (NA934; Amersham Biosciences UK Ltd), at a dilution of 1:25,000 (fetal administration blot) or 1:30,000 (maternal administration blot). Na^+/K^+-ATPase-α_1 blots were probed with the primary antibody MA3-929 (Affinity BioReagents) at a concentration of 0.19 μg/mL (1:40,000) and secondary antibody, anti-mouse IgG (whole molecule) peroxidase conjugate (A9044; Sigma-Aldrich, Inc.), at a dilution of 1:80,000.

5.2.2.3. Real-time RT-PCR

Total RNA was extracted from the right kidneys of adult offspring exposed to maternal or fetal administration of saline or betamethasone (section 2.7.1). Real-time RT-PCR was used to measure relative gene expression levels of GR, MR, 11β-HSD-2, Na^+/K^+-ATPase-α_1, A_o and AT_1 receptor mRNA in these samples (section 2.7.5-6). Gene expression levels were calculated relative to the saline control group. Some samples were excluded from analysis on the basis that they gave abnormal results as a consequence of contaminated or degraded RNA. The intra-assay co-efficient of variation for each gene was less than 10%.

5.2.3. Statistics

All data are presented as mean ± S.E.M. Linear regression analysis was used to determine the correlation between total kidney weight and birth weight. One-way ANOVA was used to compare Western blot and real-time RT-PCR data from animals according to injection route (maternal or fetal).

5.3. RESULTS

5.3.1. Body and kidney weight in adult sheep

Table 5.2 lists the mean ± S.E.M. of body weight, total kidney weight and the kidney-to-body weight ratio for each group. Values were similar between groups at age 3.5 years (Moss *et al.*, 2005).

There was a significant positive linear correlation between total kidney weight at age 3.5 years and birth weight in offspring whose mothers received prenatal treatment by maternal injection ($R^2 = 0.40$; $p = 0.02$; Figure 5.1). There was no correlation between birth weight and total adult kidney weight in offspring that were directly treated by fetal injection ($p = 0.28$).

5.3.2. Histology

Renal histopathology was diagnosed in a single kidney from five out of 29 adult sheep. A contingency table of normal and abnormal renal histology is below (Table 5.3).

5.3.2.1. Left kidney 3651 FS

The left kidney from ewe 3651 in the FS group weighed only 7.4 g; the right kidney weighed 89.3 g, indicating compensatory renal hypertrophy. Chronic reflux-associated pyelonephritis was diagnosed in the left kidney of 3651 FS (Figure 5.2 C). Radial scarring extended from the papilla to the cortex and was most evident in the upper and lower poles of the kidney. Scarred regions were associated with lymphocytic infiltration, interstitial fibrosis, tubular atrophy and thickening of arteriolar walls. Focal

Table 5.2. Body and kidney weights of adult sheep according to treatment group.

	BODY WEIGHT (kg)	TOTAL KIDNEY WEIGHT (g)	KIDNEY TO BODY WEIGHT RATIO (g/kg)
MS n = 5: 3 ♂, 2 ♀	61.8 ± 2.1	111.4 ± 6.2	1.80 ± 0.06
M1 n = 4: 2 ♂, 2 ♀	58.0 ± 4.5	102.4 ± 5.2	1.78 ± 0.09
M4 n = 5: 2 ♂, 3 ♀	59.0 ± 4.3	107.2 ± 10.0	1.81 ± 0.09
FS n = 4: 4 ♀	61.1 ± 1.3	110.4 ± 4.9	1.81 ± 0.10
F1 n = 7: 4 ♂, 3 ♀	57.4 ± 2.2	107.4 ± 3.5	1.89 ± 0.08
F4 n = 4: 2 ♂, 2 ♀	58.1 ± 1.8	110.3 ± 3.9	1.90 ± 0.09

Values are mean ± S.E.M. There was no significant difference between groups.

Data from Moss *et al.*, 2005.

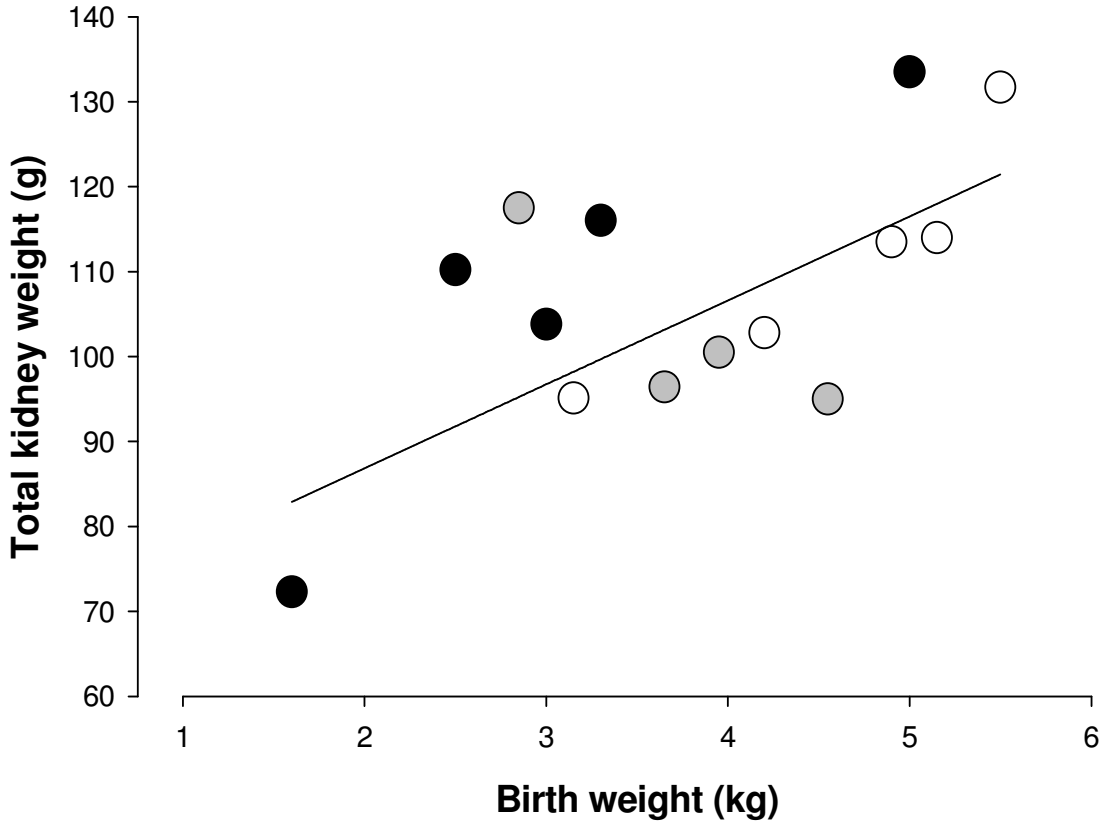


Figure 5.1. Relationship between total adult kidney weight and birth weight.

Total kidney weight in adult sheep at age 3.5 years was significantly correlated (R^2 = 0.40, p = 0.02) with birth weight after prenatal exposure to maternal saline (MS:○), single betamethasone (M1:◍) or repeated doses of betamethasone (M4:●)

Table 5.3. Frequency of abnormal and normal renal histology in adult sheep.

	MS	**M1**	**M4**	**FS**	**F1**	**F4**
Abnormal histology	0	1	2	1	0	1
Normal histology	5	3	3	3	7	3
Total *n*	5	4	5	4	7	4

glomerular sclerosis and fibrosis were localised in the cortex. There was marked tubule loss and increased collagen deposition in the medulla.

5.3.2.2. Left kidney 121 F4

Cystic renal dysplasia was the clinical diagnosis of the left kidney from wether 121 in the F4 group (Figure 5.2 D). The cortex of this kidney was noticeably thinner compared to other kidneys. There was marked cystic dilatation of Bowman's space within glomeruli. There was also evidence of sclerotic and fibrotic degenerating glomeruli. Lymphocytic infiltration and arteriolar wall-thickening were evident in the cortex. Tubular calcification and atrophy were observed in the medulla.

5.3.2.3. Right kidney 3666 M1

The right kidney from ewe 3666 in the M1 group weighed only 10.4 g; the left kidney weighed 84.6 g, indicating compensatory hypertrophy of the contra-lateral kidney. The clinical diagnosis of this specimen was chronic reflux-associated pyelonephritis (Figure 5.2 E). Widespread lymphocytic infiltration was evident in the subcapsular space in the cortex. Vascular wall-thickening and collagen deposition surrounding the basement membrane of glomeruli was evident in the cortex. There was apparent glomerular sclerosis and fibrosis. Tubular atrophy and urinary casts composed of congealed Tamm-Horsfall mucoprotein were seen in the cortex and medulla.

5.3.2.4. Right kidney #98 M4

The right kidney from ewe #98 in the M4 group was diagnosed with focal reflux-associated nephritis (Figure 5.2 F). A single radial scar extended from the papilla to the cortical capsule. Within the cortical scarred region, there was marked tubular atrophy, lymphocytic infiltration, thickening of arteriolar walls and focal glomerular sclerosis and fibrosis. Tubular atrophy was also evident in the medulla and was associated with increased collagen deposition surrounding the necrotic tubules.

5.3.2.5. Right kidney #1 M4

The right kidney from ewe #1 in the M4 group weighed 20.7 g, with the contra-lateral kidney weighing 51.6 g. The histopathology of the right kidney was diagnosed as renal dysplasia (Figure 5.2 G & H). There was widespread lymphocytic infiltration in the cortex and medulla. Interstitial fibrosis and marked tubular atrophy were associated with intense collagen deposition (blue staining). Sclerotic, fibrotic and cystic glomeruli were evident. In the cortex, there was marked thickening of arteriolar walls. Tubular atrophy and urinary casts were evident in the cortex and medulla. The papilla of this kidney was fibrotic with few tubules.

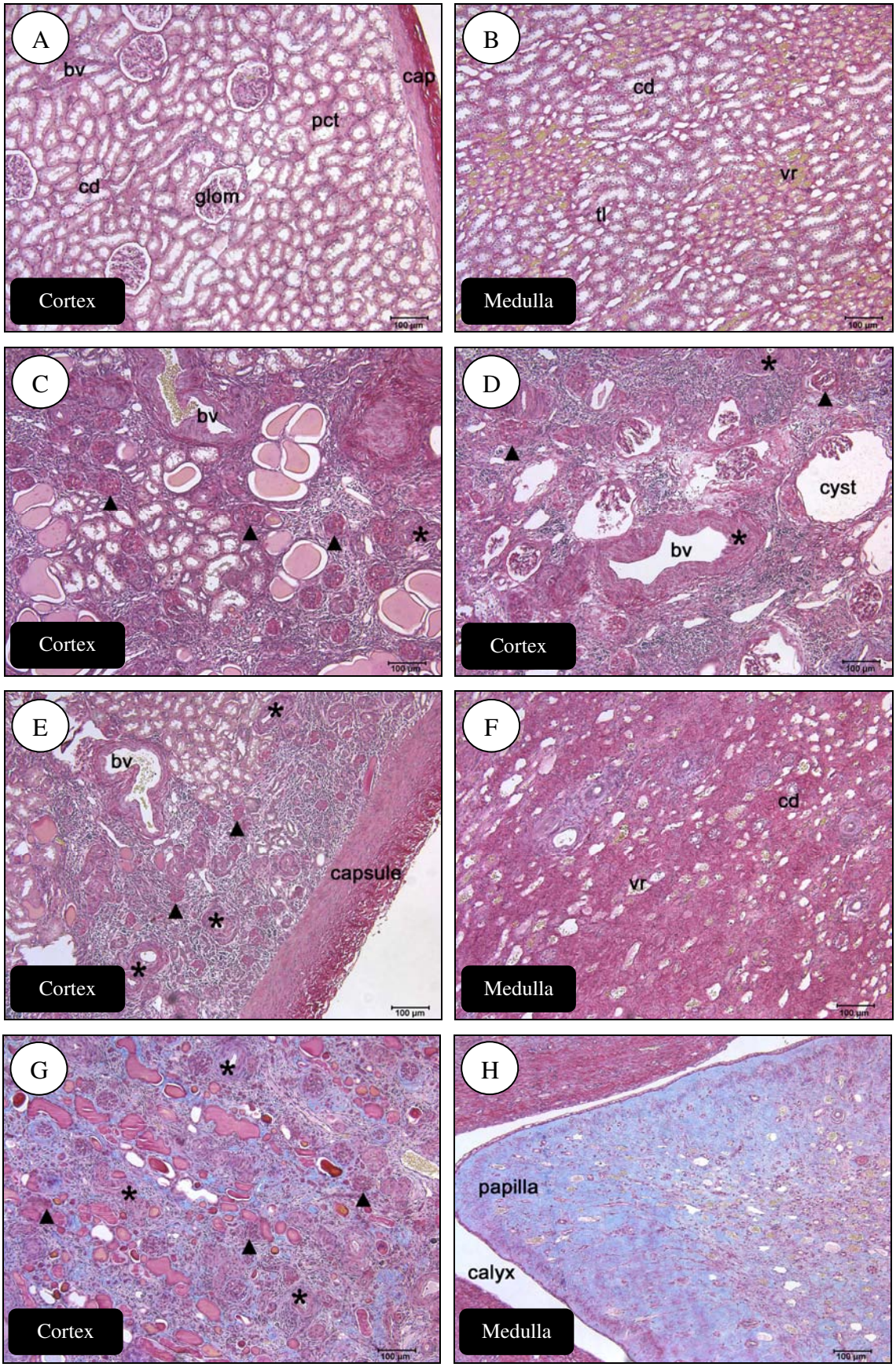
5.3.3. GR mRNA and protein expression

There was no difference in the relative expression of GR mRNA in the kidneys of adult sheep that had been exposed to maternal saline or betamethasone administration in late gestation ($p = 0.49$; Figure 5.3 A). Similarly, there was no effect of prenatal exposure to maternal betamethasone on renal GR protein levels ($p = 0.38$; Figure 5.3 B) or the localisation and intensity of GR immuno-staining (Figure 5.4). GR immuno-staining was localised to the nuclei and cytoplasm of cells along the entire nephron. Staining was also present in mesangial cells of the glomerular tuft and smooth muscle cells of the tunica media in blood vessels. The intensity of GR immuno-staining was greater in the cortex and outer medullar than in the inner medulla.

Renal GR mRNA expression levels were similar in adult animals that had been exposed to direct fetal i.m. injections of saline or betamethasone ($p = 0.38$; Figure 5.5 A). There was no effect of prenatal treatment on renal GR protein levels in the fetal-injection ($p = 0.38$; Figure 5.5 B) offspring at age 3.5 years. Consistent with these data, the localisation and intensity of GR immuno-staining was similar across the fetal-injection groups (Figure 5.6) and was not different to staining observed in the maternal-injection groups.

Figure 5.2. Histopathology of adult sheep kidneys.

Digital micrographs of sections from adult sheep kidneys stained with Masson's trichrome. Using this technique, nuclei stained black; muscle, fibrin and some cytoplasmic granules stained red; collagen stained blue and erythrocytes stained yellow. Panels **A** (cortex) and **B** (medulla) are from a normal adult sheep kidney that had not been exposed to any prenatal treatment. Panels **C** (ewe 3651 FS), **E** (ewe 3666 M1) and **F** (ewe #98 M4) are from kidneys diagnosed with *chronic or focal reflux-associated pyelonephritis*. Lymphocytic infiltration (black nuclei), tubular atrophy ("thyroidisation"), thickened arterioles (*) and sclerotic glomeruli (▲) were evident in the cortex (**C** and **E**). In the radial scar region in the medulla (**F**), there was a marked loss of tubules and interstitial fibrosis. Panel **D** (wether 121 F4) shows *cystic renal dysplasia* characterised by cystic dilatation of Bowman's capsule. Within the cortex, lymphocytic infiltration, thickened arteriolar walls (*) and sclerotic glomeruli (▲) were prevalent. Panels **G** (cortex) and **H** (medulla) are from a kidney diagnosed with *renal dysplasia* (ewe #1 M4). The blue stain in these sections demonstrates marked interstitial collagen deposition. Tubular atrophy, thickened arteriolar walls (*) and fibrotic and sclerotic glomeruli (▲) were evident in the cortex (**G**). Section **H** shows a hyaline fibrotic papilla with obvious tubule loss. All digital micrographs have a scale bar of 100 μm. bv = blood vessel; cap = renal capsule; cd = collecting duct; glom = glomerulus; pct = proximal convoluted tubule; tl = thin limb of Loop of Henle; vr = vasa rectae.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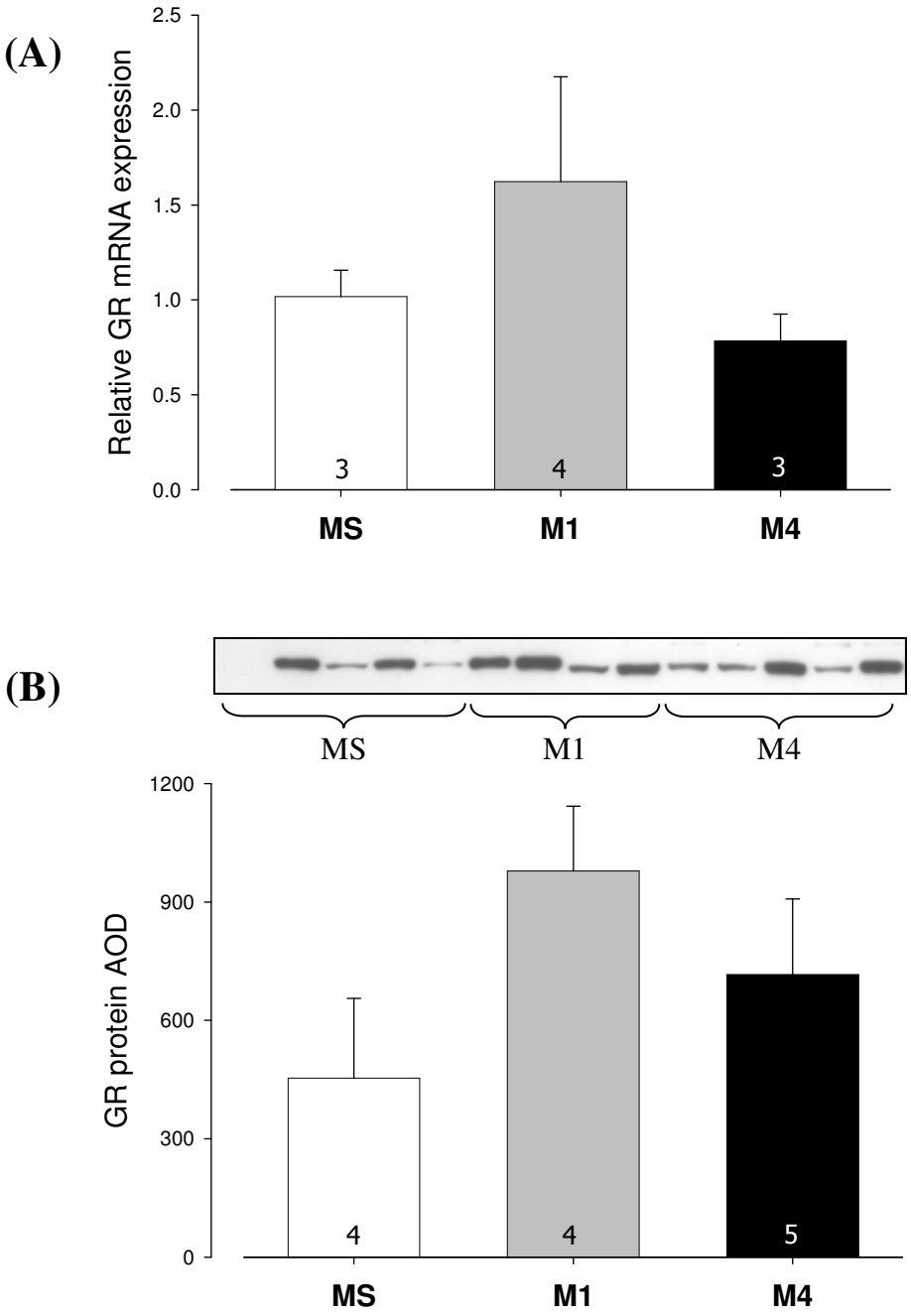
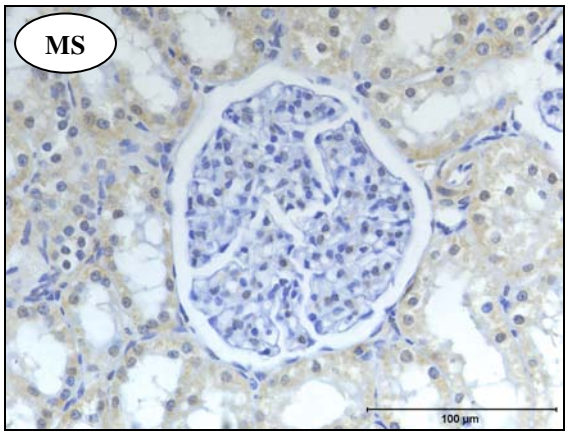
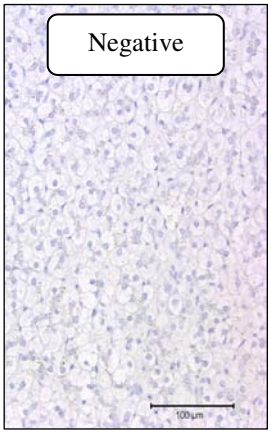
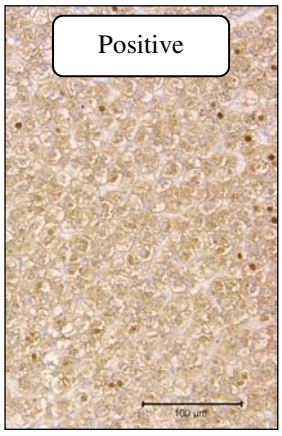


Figure 5.3. Renal GR mRNA and protein expression in adult offspring kidneys after prenatal maternal injections. Bars show mean ± S.E.M. of renal GR mRNA (A) and protein (B) expression in adult offspring after maternal saline (MS), single betamethasone (M1) or repeated betamethasone (M4) administration. Numbers of animals in each group are labelled within the bars. There was no statistical difference among groups in renal GR mRNA ($p = 0.49$) or protein expression ($p = 0.38$).

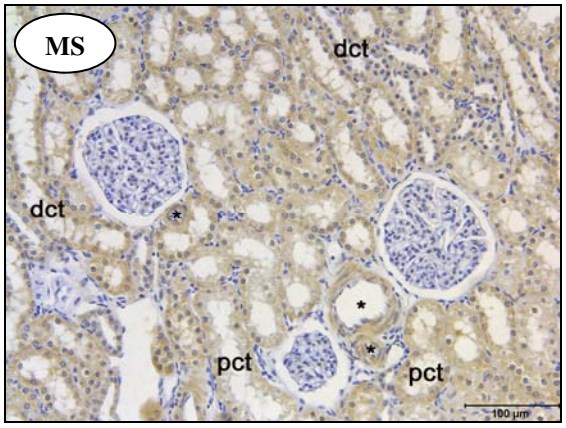
Figure 5.4. GR immuno-staining in adult sheep kidneys after prenatal maternal injections of saline or betamethasone. Representative digital micrographs of kidney sections from adult sheep exposed to prenatal maternal saline (MS), a single dose of betamethasone (M1) or four repeated doses of maternal betamethasone (M4). At the top left, a magnified digital micrograph of a glomerulus in a MS animal shows GR immuno-staining in mesangial cells within the glomerular tuft. Digital micrographs of the negative (primary antibody pre-absorbed with peptide) and positive controls (sheep liver) are shown top middle and right. Brown stain = GR immuno-stain. cd = collecting duct; dct = distal convoluted tubule; mtal = medullary thick ascending limb; pct = proximal convoluted tubule; pr = pars recta; tl = thin loop of Henle; vr = vasa recta. Black arrows show macula densae and asterisks denote blood vessels. Scale bar illustrates 100 μm.

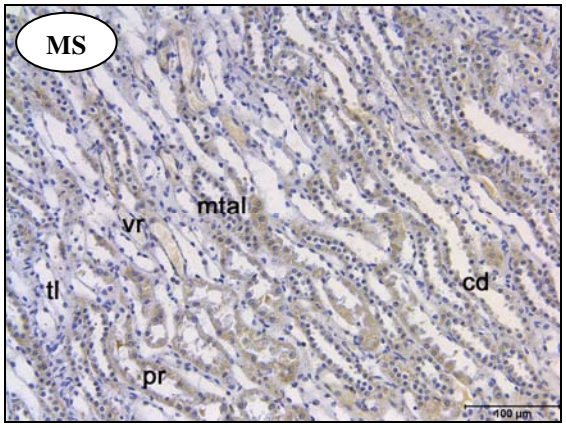


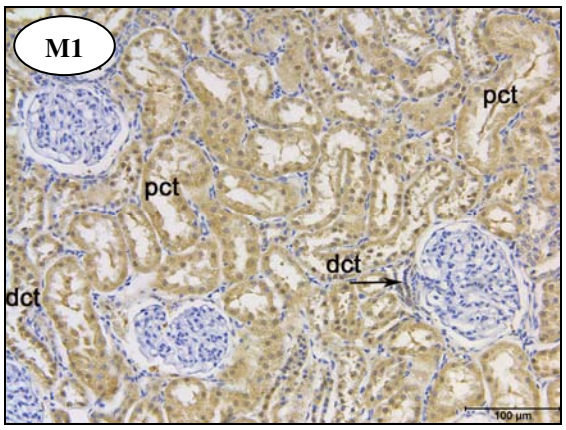


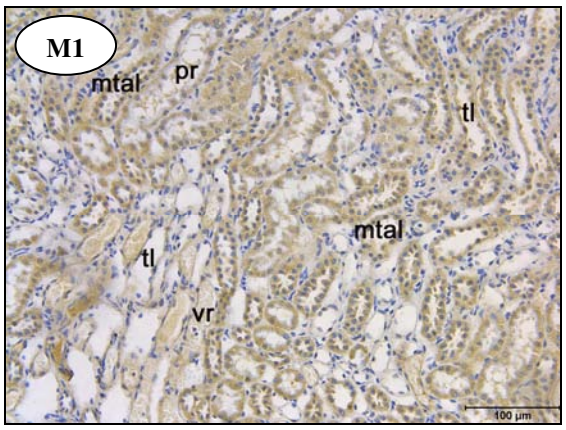


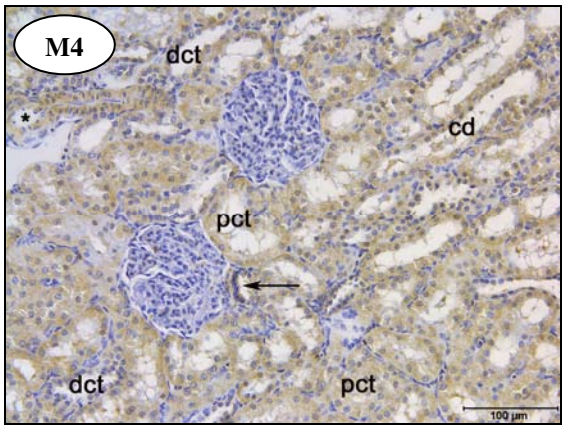
CORTEX **MEDULLA**

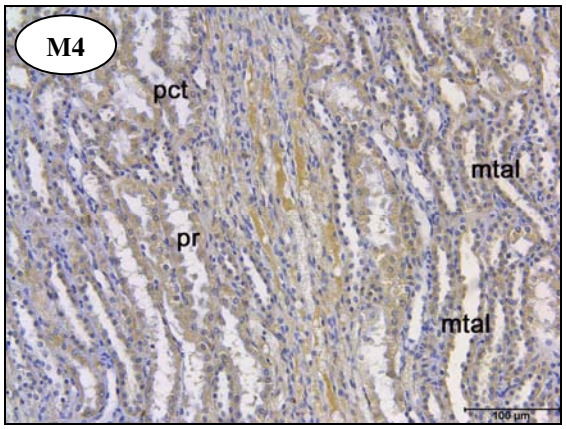












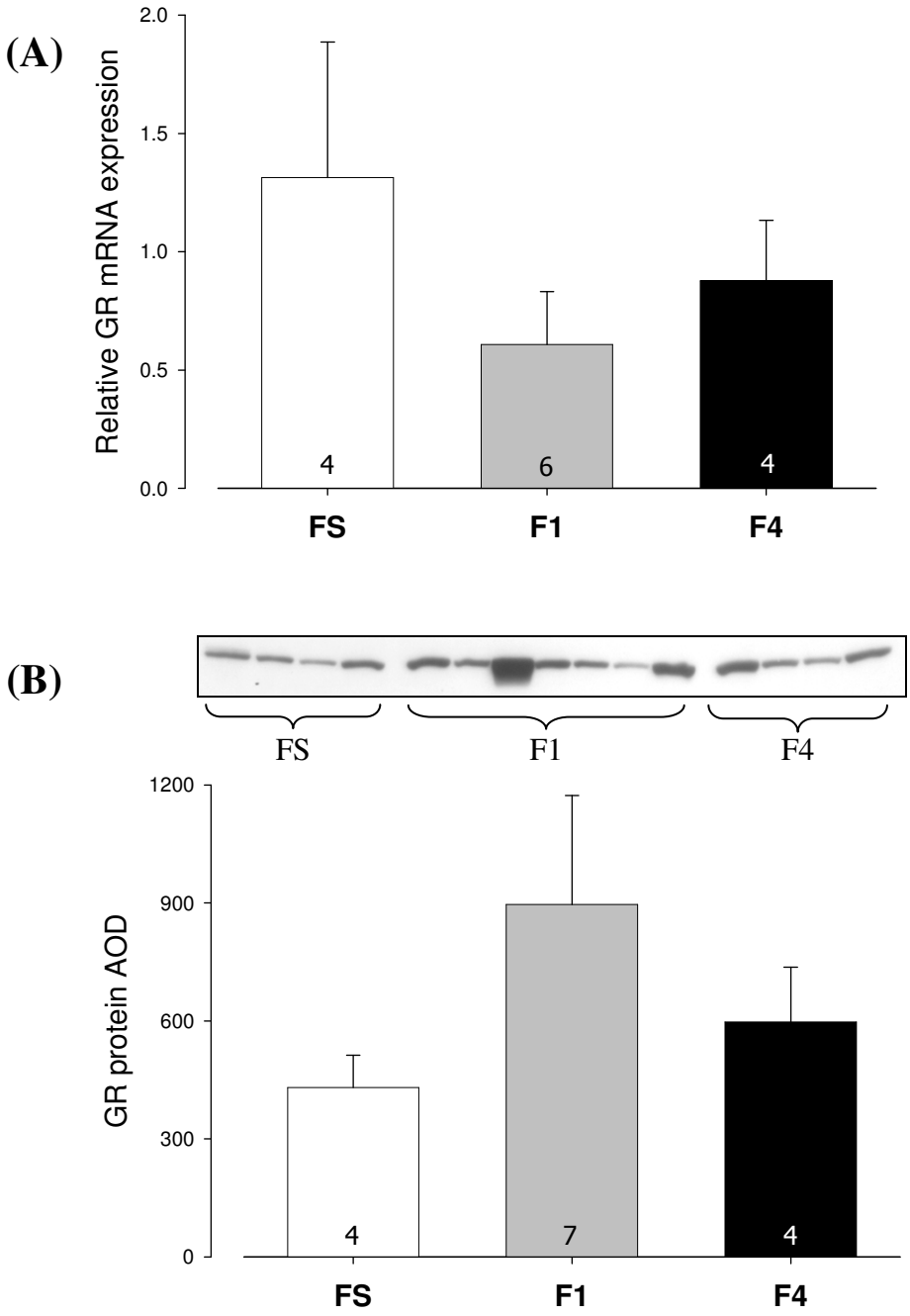
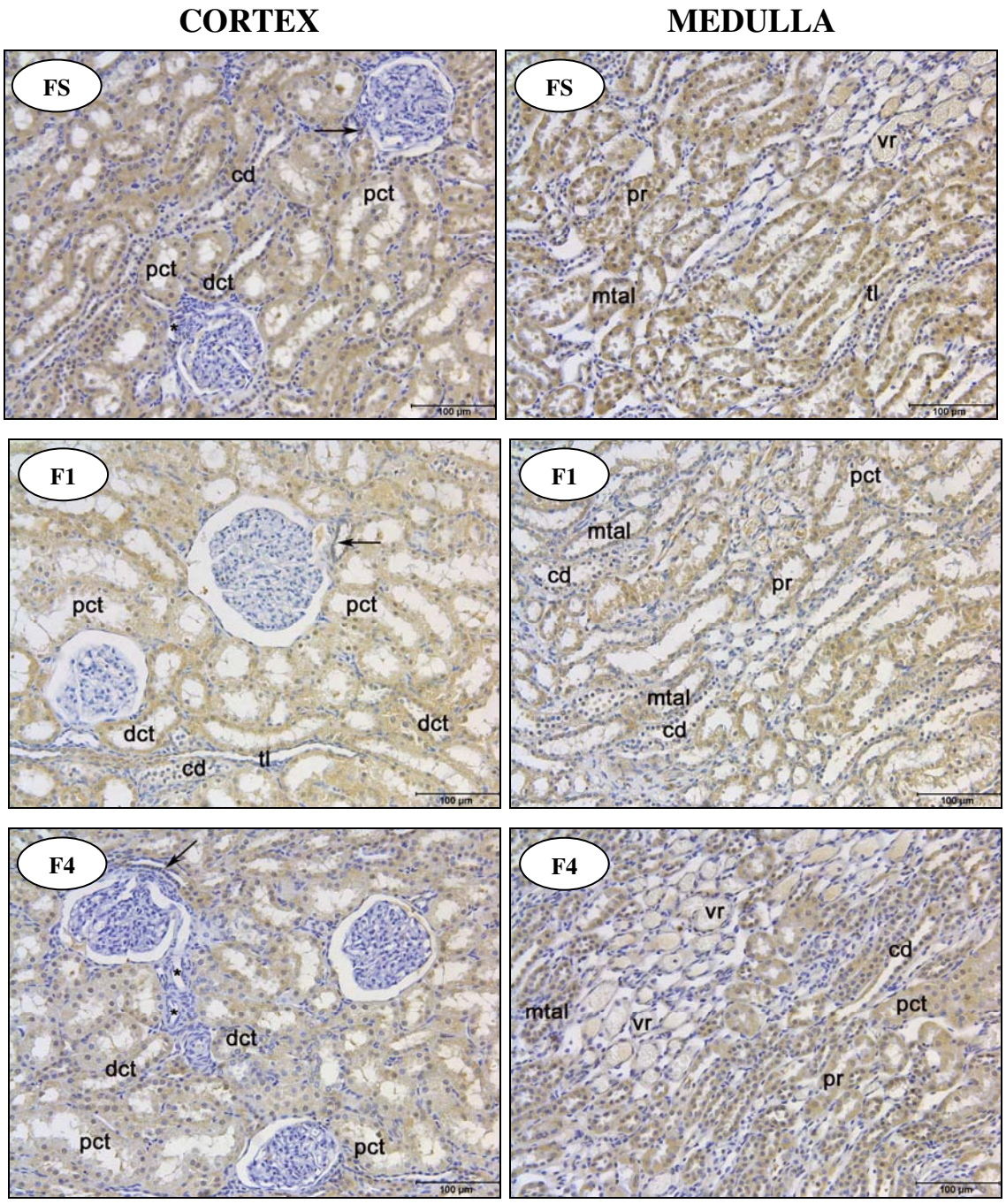


Figure 5.5. Renal GR mRNA and protein expression in adult sheep after prenatal fetal injections. Bars show group mean ± S.E.M. of renal GR mRNA (A) and protein (B) expression in adult sheep after saline (FS), single betamethasone (F1) or repeated betamethasone injections given directly to the fetus. Numbers of samples in each group are labelled within the bars. There was no statistical difference among groups in renal GR mRNA ($p = 0.38$) or protein ($p = 0.38$) expression.

Figure 5.6. GR immuno-staining in kidney sections from adult sheep after prenatal fetal injections. Digital micrographs of kidney sections from adult sheep after fetal injections of saline (FS), single betamethasone (F1) or repeated betamethasone (F4). cd = collecting duct; dct = distal convoluted tubule; mtal = medullary thick ascending limb; pct = proximal convoluted tubule; pr = pars recta; tl = thin loop of Henle; vr = vasa recta. Black arrows show macula densae and asterisks denote blood vessels. Digital micrographs were captured at 200x magnification; the scale bar illustrates 100 μm**.**


CORTEX
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100 µm
F4
*
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dct
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100 µm

5.3.4. Na^+/K^+-ATPase-α_1 mRNA and protein expression

There was no difference in the relative expression of Na^+/K^+-ATPase-α_1 mRNA ($p = 0.29$; Figure 5.7 A) or protein ($p = 0.45$; Figure 5.7 B) in kidneys of adult sheep that had been exposed to maternal saline or betamethasone administration.

Similarly, relative renal Na^+/K^+-ATPase-α_1 mRNA ($p = 0.21$; Figure 5.8 A) and protein ($p = 0.11$; Figure 5.8 B) levels in adult sheep were not affected by prenatal intra-fetal injections of saline or betamethasone.

5.3.5. Mineralocorticoid receptor mRNA expression

Prenatal exposure to maternal ($p = 0.57$; Figure 5.9 A) or fetal ($p = 0.99$; Figure 5.9 B) betamethasone administration did not alter the relative expression of renal MR mRNA in adult sheep.

5.3.6. 11β-HSD-2 mRNA expression

Prenatal maternal betamethasone tended to increase relative renal 11β-HSD-2 mRNA expression, but this difference did not reach statistical significance ($p = 0.07$; Figure 5.10 A). Relative renal 11β-HSD-2 mRNA expression in adult sheep was not affected by fetal injections of betamethasone ($p = 0.29$; Figure 5.10 B).

5.3.7. Angiotensinogen mRNA expression

There was no difference in the relative renal expression of A_o mRNA in adult sheep that had been exposed to maternal ($p = 0.31$; Figure 5.11 A) or fetal ($p = 0.99$; Figure 5.11 B) injections of betamethasone.

5.3.8. AT_1 receptor mRNA expression

Relative renal AT_1 receptor mRNA expression in adult sheep was not affected by prenatal exposure to maternal ($p = 0.33$; Figure 5.12 A) or fetal ($p = 0.26$; Figure 5.12 B) betamethasone injections.

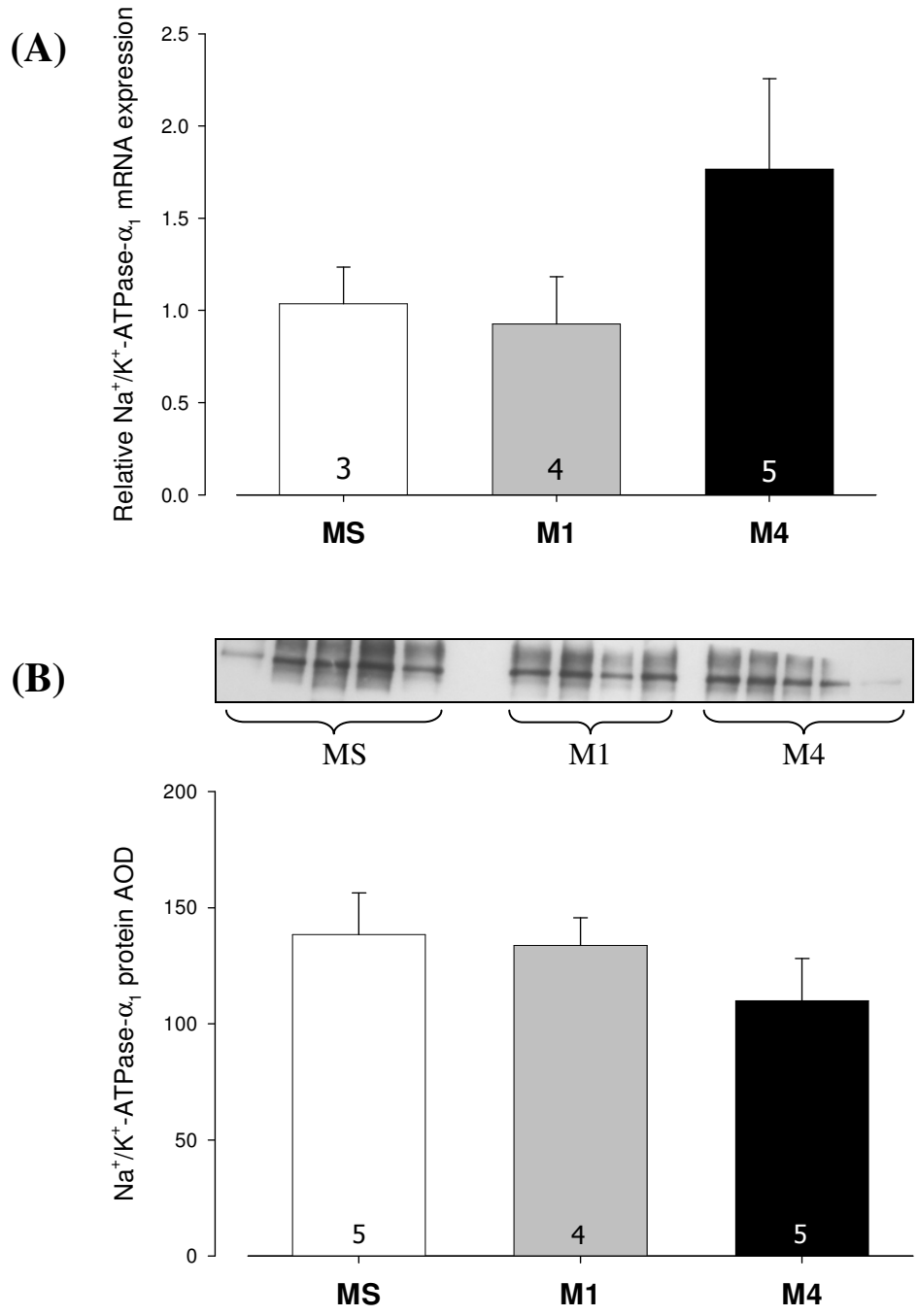

Figure 5.7. Relative renal Na^+/K^+-ATPase-α_1 mRNA and protein expression in adult offspring after prenatal maternal injections. Bars show group mean ± S.E.M. of renal Na^+/K^+-ATPase-α_1 mRNA (A) and protein (B) expression in adult offspring after maternal saline (MS), single betamethasone (M1) or repeated betamethasone (M4) administration. Numbers of samples in each group are labelled within the bars. There was no difference in Na^+/K^+-ATPase-α_1 mRNA ($p = 0.29$) or protein ($p = 0.45$) levels among groups.

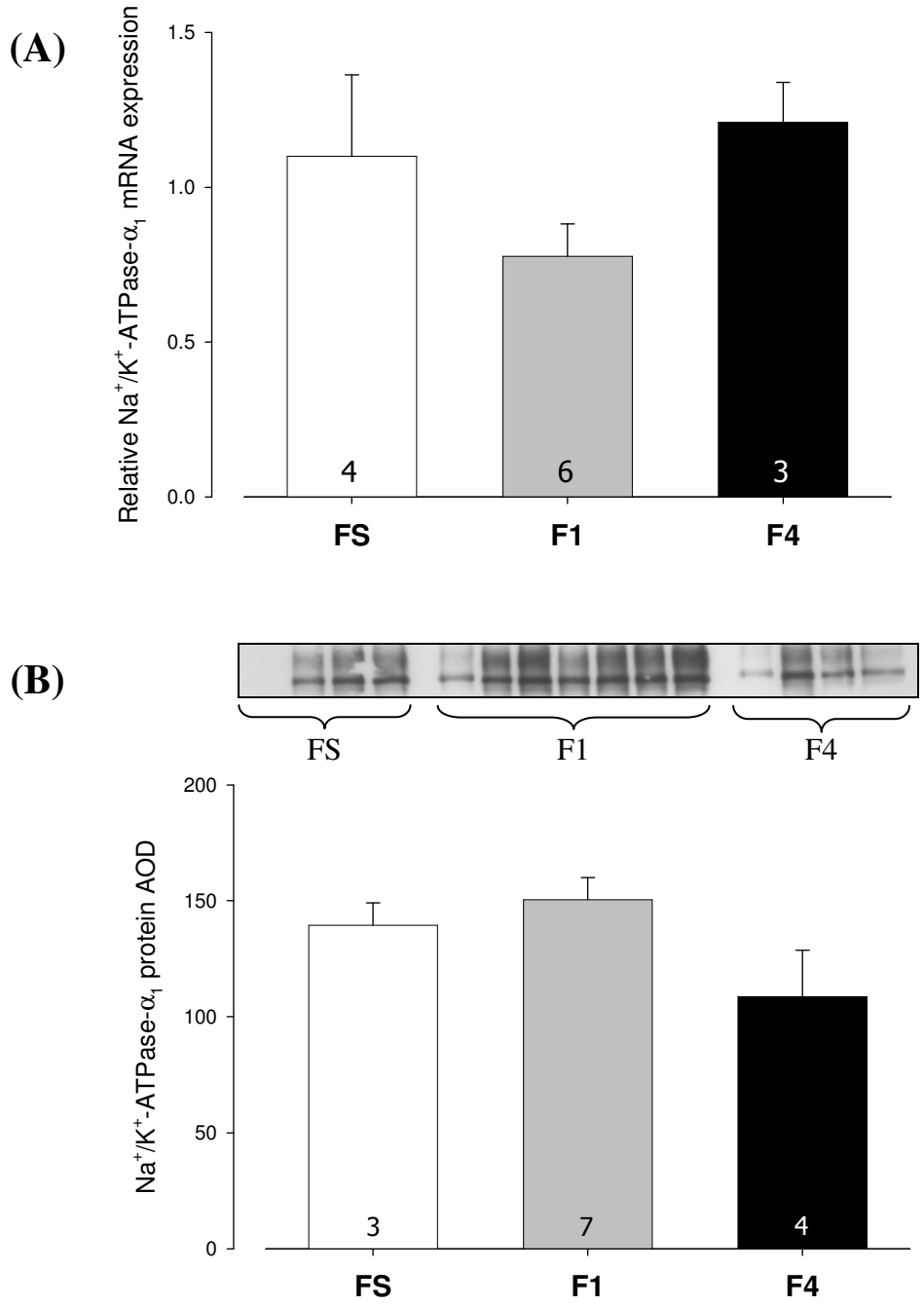

Figure 5.8. Relative renal Na^+/K^+-ATPase-α_1 mRNA and protein expression in adult offspring after fetal injections. Bars show group mean ± S.E.M. of renal Na^+/K^+-ATPase-α_1 mRNA (A) and protein (B) expression in adult offspring after fetal saline (FS), single betamethasone (F1) or repeated betamethasone (F4) administration. Numbers of samples in each group are labelled within the bars. There was no difference in Na^+/K^+-ATPase-α_1 mRNA ($p = 0.21$) or protein ($p = 0.11$) levels among groups.

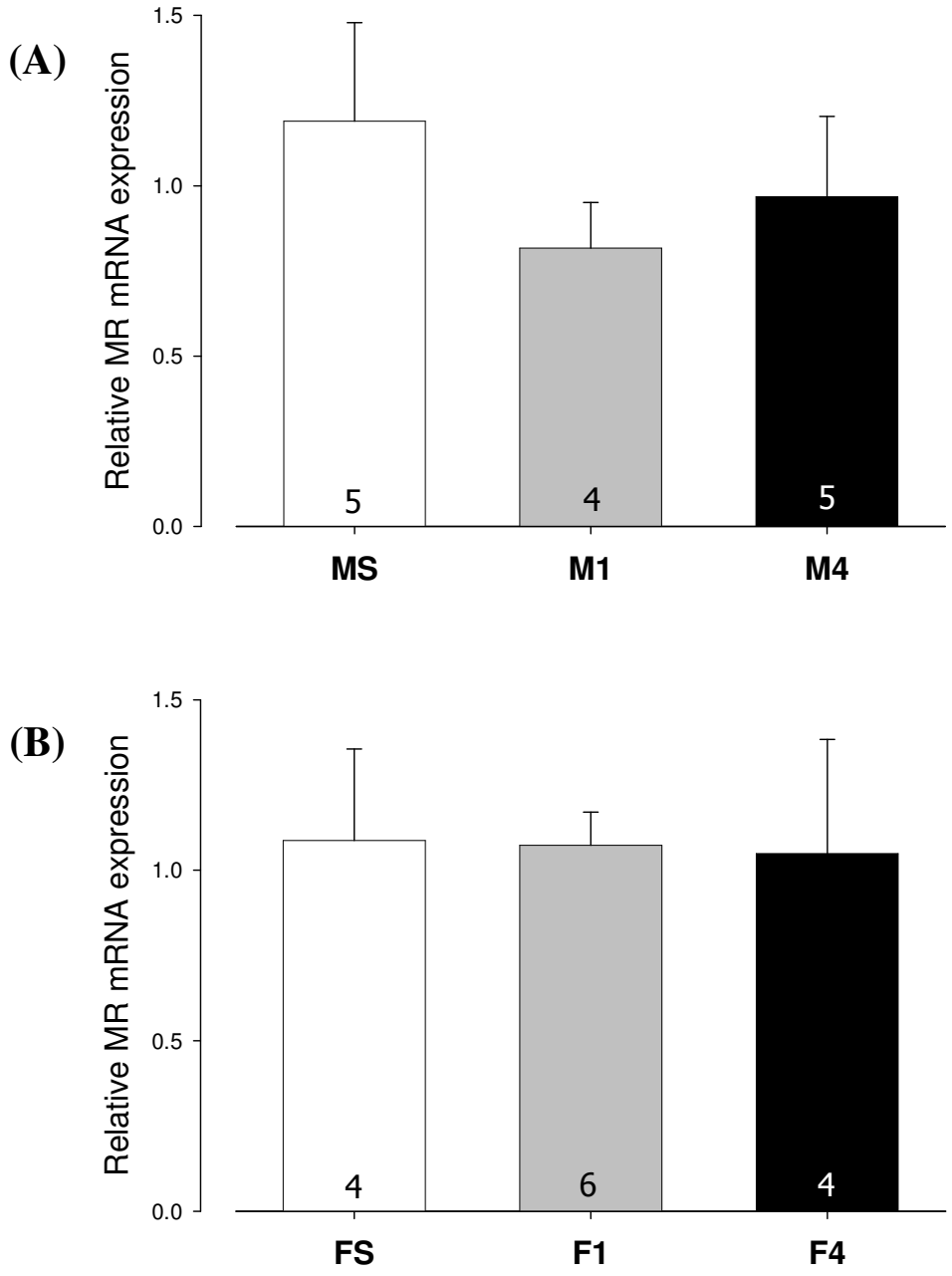

Figure 5.9. Relative renal MR mRNA expression.

Bars show group mean ± S.E.M. of relative MR mRNA levels in adult sheep kidneys after prenatal exposure to maternal (A) or fetal (B) injections of saline or betamethasone. Numbers of samples in each group are labelled within the bars. There was no effect of prenatal betamethasone exposure on relative renal MR mRNA levels in the maternal-injection ($p = 0.57$) or fetal-injection ($p = 0.99$) groups.

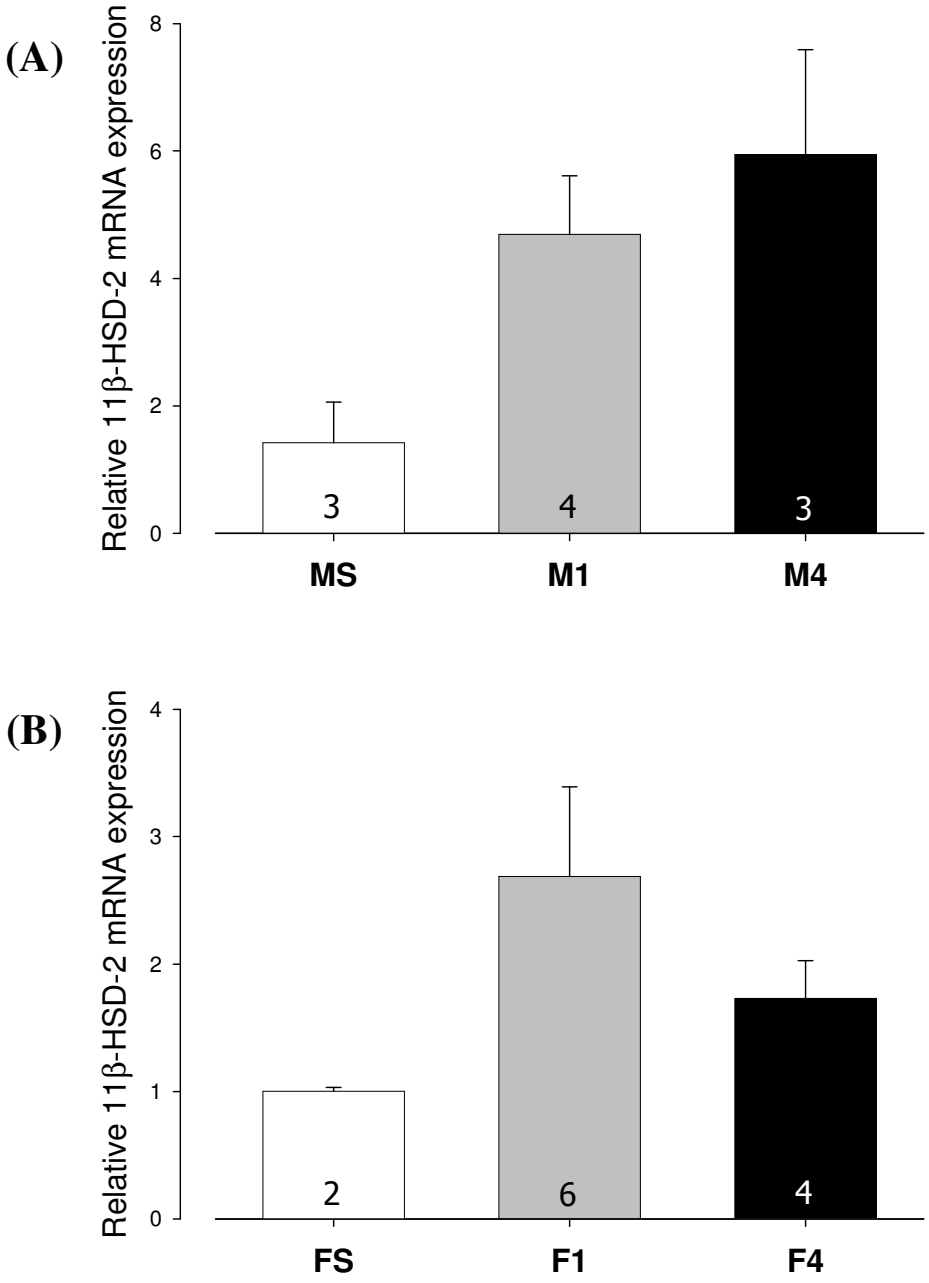

Figure 5.10. Relative renal 11β-HSD-2 mRNA expression.

Bars show mean ± S.E.M. of relative 11β-HSD-2 mRNA levels in adult sheep kidneys after prenatal exposure to maternal (A) or fetal (B) injections of saline or betamethasone. Numbers of samples in each group are labelled within the bars. There was no effect of prenatal betamethasone exposure on relative renal 11β-HSD-2 mRNA levels in the maternal-injection ($p = 0.07$) or fetal-injection ($p = 0.29$) groups.

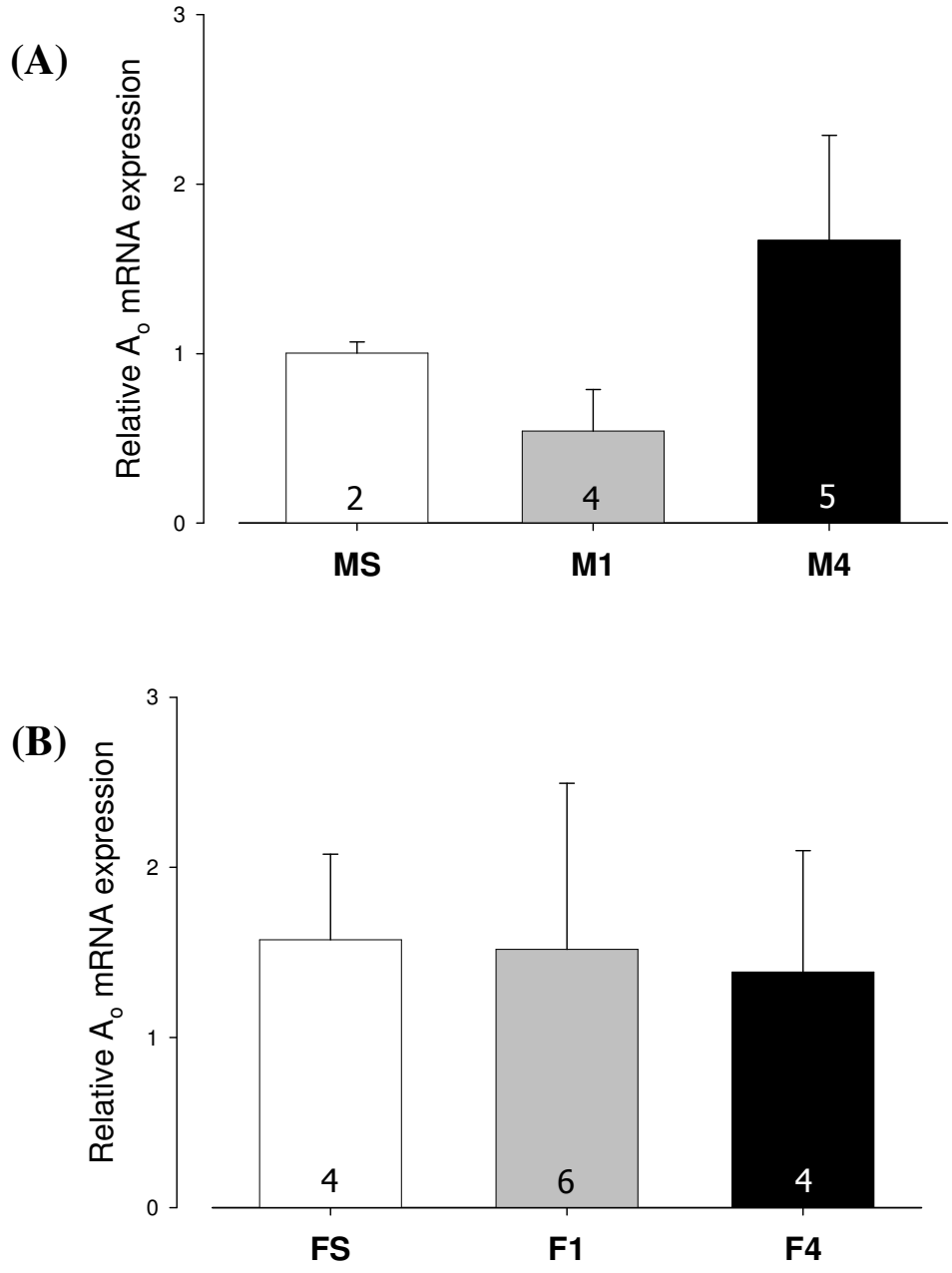

Figure 5.11. Relative renal A_o mRNA expression.

Bars show mean ± S.E.M. of relative A_o mRNA levels in adult sheep kidneys after prenatal exposure to maternal (A) or fetal (B) injections of saline or betamethasone. Numbers of samples in each group are labelled within the bars. There was no effect of prenatal betamethasone exposure on relative renal A_o mRNA levels in the maternal-injection ($p = 0.31$) or fetal-injection ($p = 0.99$) groups.

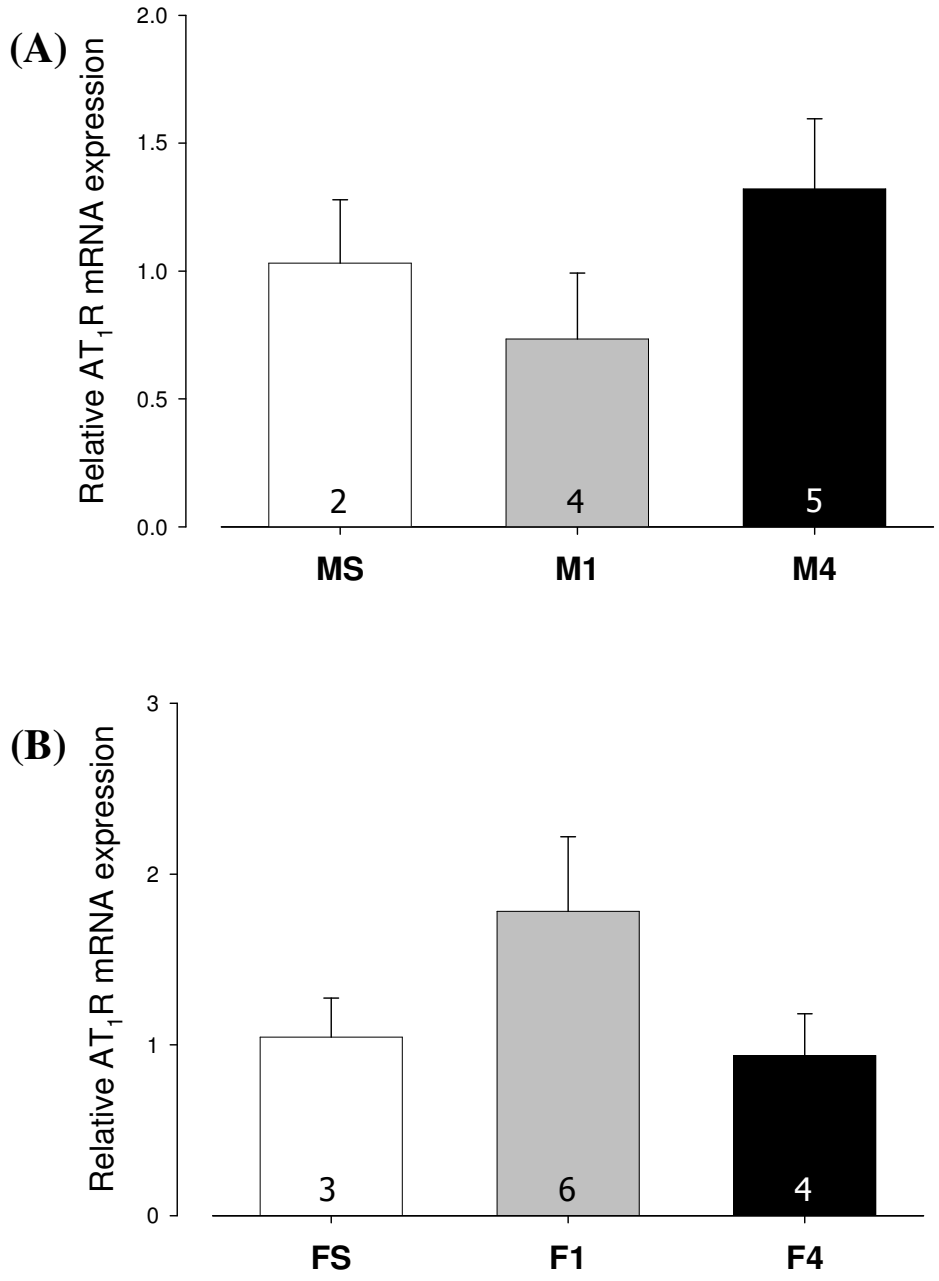

Figure 5.12. Relative AT_1 receptor mRNA expression.

Bars show mean ± S.E.M. of relative AT_1 receptor mRNA levels in adult sheep kidneys after prenatal exposure to maternal (A) or fetal (B) injections of saline or betamethasone. Numbers of samples in each group are labelled within the bars. There was no effect of prenatal betamethasone exposure on relative renal AT_1 receptor mRNA levels in the maternal-injection ($p = 0.33$) or fetal-injection ($p = 0.26$) groups.

5.4. DISCUSSION

This is the first study to assess the long-term outcomes of late-gestation prenatal glucocorticoid administration on the adult ovine kidney. Kidneys were analysed from adult offspring at age 3.5 years that had received i.m. injections of saline or betamethasone as fetuses or animals whose mothers were injected. Prenatal glucocorticoid administration did not alter total adult kidney weight; however, total adult kidney weight was related to birth weight in the maternal-injection group. There was no difference in the renal expression of GR, MR, 11β-HSD-2, Na^+/K^+-ATPase-α_1, A_o or AT_1 receptor mRNA in offspring exposed to prenatal betamethasone by maternal or fetal injection. These findings are consistent with the preservation of normal basal blood pressure in these adult sheep after prenatal betamethasone administration (Moss *et al.*, 2005).

Body weight, total kidney weight and the kidney-to-body weight ratio were similar between the groups at age 3.5 years. M1 and M4 offspring were smaller at birth than those in the MS group (Moss *et al.*, 2001); therefore, these animals must have undergone a degree of catch-up growth during the first few years of life. Total kidney weight at age 3.5 years was significantly correlated with birth weight in the maternal-injection groups, but not in offspring in the fetal-injection groups. The reason why total adult kidney weight was related to birth weight in the maternal, but not fetal, group is unknown. Maternal and fetal betamethasone administration are known to have different effects on growth (Newnham *et al.*, 1999; Moss *et al.*, 2001).

The normal prevalence of renal dysplasia and pyelonephritis in adult sheep has not been reported. Renal histopathology was evident in five animals at age 3.5 years and did not appear to be related to prenatal treatment. Abnormal histology was not evident in fetal kidneys (Chapter 4). Three out of five cases were reflux-associated pyelonephritis; the other two were renal dysplasia. Urine reflux is the most common cause of reflux-associated pyelonephritis: a bacterial infection that leads to focal scars and loss of functioning renal parenchyma (Clapp, 1992). Reflux is caused by incompetent vesico-ureteric valves, urinary tract obstruction or abnormal renal papillae (Clapp, 1992). Bacteria tend to infect the upper and lower poles of the kidney, because that is where the renal papillae are compounded and flattened, and therefore receptive to infection by

bacteria (Clapp, 1992). In all cases of abnormal histopathology, arteriolar wall thickening and sclerotic and fibrotic glomeruli were evident and may have contributed to the increased blood pressure observed at age 3 years in four of the five animals with renal histopathology (3666 M1, #98 M4, 121 F4 and #1 M4). The presence of degenerating glomeruli suggests that nephron number was reduced in these kidneys; however, it was unable to be determined in the adult kidneys using the physical disector/fractionator method as they were not collected appropriately. Previous studies in sheep have demonstrated that increased MAP is associated with reduced nephron endowment (Moritz *et al.*, 2002c; Wintour *et al.*, 2003; Gilbert *et al.*, 2005). Therefore, increased basal blood pressure in four out of five adult sheep with renal histopathology may be due to a reduce nephron endowment in the affected kidneys.

In this adult cohort study, basal MAP was not related to antenatal treatment (Moss *et al.*, 2005). This finding is consistent with the recent follow-up data from the Auckland Steroid Trial that reported that blood pressure was not different in subjects at age 30 years, who had been exposed to a single course of betamethasone *in utero* (Dalziel *et al.*, 2005). In contrast, an earlier study reported that mean systolic and diastolic blood pressure was increased in pre-term very low birth weight (VLBW, < 1501 g) adolescents (14-years-old) exposed to a single course of maternal glucocorticoids, compared to controls (Doyle *et al.*, 2000). However, some studies suggest that the gestational age at delivery strongly influences blood pressure (Siewert-Delle & Ljungman, 1998; Hack *et al.*, 2005). Systolic blood pressure was increased in 20-year-old pre-term VLBW individuals who were not exposed to prenatal glucocorticoids (Hack *et al.*, 2005). Hence, the gestational age at delivery may have a greater impact on postnatal basal blood pressure than exposure to antenatal glucocorticoids.

Although basal blood pressure may not be overtly altered in subjects exposed to prenatal glucocorticoids, blood pressure responses to physiological challenges (postnatal diet, disease, aging and stress) may differ to those of controls. A previous study in rats demonstrated that blood pressure was lower in offspring exposed to prenatal dexamethasone when measured by radio-telemetry; however, these animals exhibited stress-induced hypertension (Seckl, 2005). More recently it has been shown that there is an important interaction between prenatal glucocorticoid exposure and postnatal diet in rats; dexamethasone-induced hypertension in adult rats was prevented

by a postnatal diet high in omega-3 fatty acids (Wyrwoll *et al.*, 2006). The effect of stress or diet on blood pressure in adult sheep exposed to late-gestation prenatal glucocorticoids is unknown.

This is the first study to measure renal GR protein levels and localise GR-IR protein in adult sheep kidneys after prenatal betamethasone exposure. Prenatal treatment did not alter renal GR mRNA, protein levels or GR-IR protein localisation. The localisation of GR immuno-staining was consistent with studies in rabbit (Farman *et al.*, 1991) and human (Yan *et al.*, 1999) kidneys. Similar to results seen in the fetal kidney (Chapter 4), a novel finding of this study was GR immuno-staining in the tunica media of blood vessels (including the afferent and efferent arterioles) and in mesangial cells of glomerular tufts within the adult kidney. Mesangial cells are specialised smooth muscle cells that can alter glomerular surface area and hence affect GFR (Best & Taylor, 1985). These findings suggest that glucocorticoids can act directly on renal vasculature to alter renal blood flow and GFR.

Na^+/K^+-ATPase-α_1 mRNA and protein levels in adult kidneys were unaffected by prenatal maternal or fetal betamethasone administration. This finding suggests that there is likely to be no long-term effect of prenatal betamethasone exposure on basal sodium reabsorption in the kidneys of adult sheep. Altered renal sodium handling may predispose animals to hypertension; therefore, the lack of effect on Na^+/K^+-ATPase-α_1 mRNA and protein levels is consistent with the preservation of normal basal blood pressure in these animals.

In the kidney, 11β-HSD-2 is a autocrine protector of the MR, preventing endogenous glucocorticoids from binding to the receptor (Edwards *et al.*, 1988; Stewart *et al.*, 1991). Prenatal exposure to fetal or maternal, single or repeated, betamethasone administration did not alter renal MR or 11β-HSD-2 mRNA in adult sheep. However, prenatal maternal betamethasone administration tended to increase renal 11β-HSD-2 mRNA, though this did not reach significance. Increased 11β-HSD-2 activity in the kidney would reduce glucocorticoid action but may augment mineralocorticoid activity as aldosterone would have greater access to the MR. Future studies could determine the protein levels of MR and 11β-HSD-2 and 11β-HSD-2 activity in the adult sheep kidney

after prenatal betamethasone administration. At present, there are no commercially available ovine-specific antibodies for MR and 11β-HSD-2.

Renal A_o and AT_1 receptor mRNA expression in adult sheep was unaltered by prenatal betamethasone exposure. This finding suggests that at least at the transcriptional level, the intra-renal RAS in adult sheep may not be affected by betamethasone exposure in late gestation. Normal intra-renal RAS expression is consistent with the observed lack of hypertension in these animals.

There are no published studies that have investigated the effects of prenatal glucocorticoid exposure on gene or protein expression in the adult sheep kidney. In this study I have demonstrated that prenatal betamethasone exposure, by maternal or fetal administration, does not have persistent effects on the adult ovine kidney. Renal gene expression of various factors involved in glucocorticoid action and the intra-renal RAS were unaffected by prenatal treatment. These findings are consistent with the preservation of normal basal blood pressure in sheep (Moss *et al.*, 2005) and humans (Dessens *et al.*, 2000; Dalziel *et al.*, 2004; Dalziel *et al.*, 2005) after prenatal glucocorticoid exposure in late gestation.

6. General Discussion

Antenatal glucocorticoid therapy is administered to pregnant women at risk of pre-term delivery between 24 and 34 weeks of gestation to induce fetal organ maturation. Increased fetal exposure to synthetic glucocorticoids during this period, when the fetal kidney is growing rapidly and nephrogenesis is occurring, may have adverse effects on the developing kidney. The overall aim of the studies comprising this thesis was to determine the effects of late-gestation betamethasone exposure on the fetal and adult kidney. The central hypothesis of this thesis was that exposure of the fetal kidney to synthetic glucocorticoids would change renal structure and cause long-term alterations in the expression of glucocorticoid-sensitive genes and proteins. In the studies comprising this thesis I have examined renal histology, the expression of GR, MR, 11β-HSD-2, Na^+/K^+-ATPase-α_1, A_o, renin, AT_1 and AT_2 receptor mRNA, Na^+/K^+-ATPase-α_1 protein levels, GR protein levels and the localisation of GR immuno-staining in fetal and adult sheep kidneys after betamethasone exposure in late gestation.

The origins of fetal growth restriction induced by late-gestation maternal betamethasone administration are likely to be multi-factorial. In sheep, maternal dexamethasone administration in mid-gestation reduced placental 11β-HSD-2 mRNA expression and restricted fetal growth (Kerzner *et al.*, 2002). Most recently, it has been demonstrated that placental insulin-like growth factor 2 (*Igf2*) and *Igf2*-receptor (*Igf2r*) gene expression in sheep is reduced after repeated doses of maternal betamethasone in late gestation (Roberts *et al.*, 2005). The *Igf2* gene has been proposed to control the placental supply of maternal nutrients to the fetus (Constancia *et al.*, 2002). Deletion of a specific *Igf2* transcript (P0) expressed in the labyrinthine trophoblast of the murine placenta reduced placental growth, decreased passive permeability of nutrients through the placenta and ultimately induced fetal growth restriction (Constancia *et al.*, 2002). Therefore, repeated maternal betamethasone administration in sheep may restrict fetal growth by increasing fetal glucocorticoid exposure and reducing trans-placental passage of essential nutrients to the fetus.

Betamethasone has variable effects

In this thesis I have demonstrated that prenatal betamethasone exposure has variable effects on fetal growth. Growth restriction was most evident in 121 d fetuses exposed to three weekly-doses of maternal betamethasone from 104 to 118 d: three out of four fetuses were growth-restricted at 121 d (Chapter 4). Growth restriction at 146 d was observed in four out of six fetuses that had been exposed to repeated maternal betamethasone administration; however, renal growth was restricted in only three out of six of these fetuses. The reason why betamethasone does not consistently affect fetal growth in sheep, or why some fetuses are more susceptible or resistant to its effects, is unclear.

Such variability in glucocorticoid effectiveness has been known, from clinical use of antenatal corticosteroids, for decades. Glucocorticoids are effective for preventing RDS in only 50% of treated infants (Crowley, 1995). Recent studies have suggested that the inter-individual variation in glucocorticoid-sensitivity can be partly explained by polymorphisms in the GR gene; the ER22/23EK and N363S polymorphisms, reduce and increase glucocorticoid-sensitivity, respectively (van Rossum & Lamberts, 2004; Russcher *et al.*, 2005). A previous study in sheep has demonstrated that fetal lung maturation induced by maternal betamethasone administration was augmented by the addition of intra-amniotic endotoxin (Newnham *et al.*, 2001). The authors proposed that the effectiveness of maternal betamethasone administration was greater in the presence of concurrent cytokine expression and release in the setting of intra-uterine inflammation (Newnham *et al.*, 2003). Therefore, GR gene polymorphisms and *in utero* cytokine expression are two factors that may modulate betamethasone action in the fetus and thus account for the variability of response.

Repetitive antenatal corticosteroid therapy in humans may have adverse effects on birth weight. Non-randomised cohort studies have reported reductions in birth weight of pre-term infants exposed to repeated doses of prenatal glucocorticoids (Reinisch *et al.*, 1978; Banks *et al.*, 1999; French *et al.*, 1999; Bloom *et al.*, 2001; Thorp *et al.*, 2002); however, data from randomised controlled trials reported that there was no effect of

repetitive antenatal glucocorticoids on mean birth weight (Guinn *et al.*, 2001; McEvoy *et al.*, 2002; Crowther *et al.*, 2006; Wapner *et al.*, 2006). Although, mean birth weight was not reduced, the Z-scores were lower for babies exposed to repeat corticosteroids (Crowther *et al.*, 2006), the repeat group had a reduction in multiples of the birth weight median by gestational age and there were significantly more babies with birth weights below the 10^{th} and 5^{th} percentiles for gestational age (Wapner *et al.*, 2006). Further investigation of repetitive antenatal corticosteroid therapy in humans is currently underway, with several large multi-centred randomised controlled trials.

Numerous epidemiological studies have demonstrated that low birth weight is associated with the development of adult-onset diseases such as hypertension and cardiovascular disease (Barker *et al.*, 1990; Law *et al.*, 1993; Barker, 1996). Furthermore, low birth weight is associated with a congenital nephron deficit in humans and experimental animals (Hinchliffe *et al.*, 1992; Merlet-Benichou *et al.*, 1994; Bassan *et al.*, 2000; Bauer *et al.*, 2002). Brenner and Chertow (1994) were the first to propose that congenital nephron endowment is a significant factor in the pathogenesis of hypertension and chronic renal disease: low birth weight individuals have a reduced nephron endowment and are more susceptible to developing renal disease and hypertension (Tulassay & Vasarhelyi, 2002; Lackland *et al.*, 2003). The exact mechanism of the reduced nephron endowment in low birth weight subjects is unknown; however, changes in DNA methylation, increased apoptosis in the developing kidney, alterations in the intra-renal RAS or increased fetal glucocorticoid exposure may play a role (Zandi-Nejad *et al.*, 2006).

Nephron endowment is reduced in fetuses with renal growth restriction

This is the first study to examine the effect of late-gestation maternal betamethasone administration on nephron endowment and glomerular size in term fetal sheep. Overall, total nephron number was not different between the control and betamethasone groups. However, nephron endowment and total glomerular volume were reduced in those betamethasone-exposed fetuses that exhibited renal growth restriction. This finding is consistent with previous studies where IUGR was associated with reduced nephron

endowment in humans (Hinchliffe *et al.*, 1992), rats (Merlet-Benichou *et al.*, 1994), rabbits (Bassan *et al.*, 2000) and pigs (Bauer *et al.*, 2002). Future studies could investigate the mechanism by which nephron number is reduced in betamethasone-exposed fetuses with renal growth restriction. Possible mechanisms may include altered expression of renal growth factors, increased apoptosis and/or reduced proliferation in the developing kidney.

No effect of sex on nephron endowment

This is the first study to examine the effect of sex on nephron endowment in term fetal sheep. In this thesis I have provided evidence which suggests that there is no difference in total nephron number between male and female fetal sheep. This finding is consistent with studies of nephron number in humans (Nyengaard & Bendtsen, 1992; Manalich *et al.*, 2000; Hoy *et al.*, 2003) and rats (Solomon, 1977; Kavlock & Gray, 1982; Munger & Baylis, 1988; Remuzzi *et al.*, 1988).

Maternal betamethasone administration did not cause persistent alterations in gene expression in the fetal kidney

I measured GR, MR, 11β-HSD-2, Na^+/K^+-ATPase-α_1, A_o, renin, AT_1 and AT_2 receptor mRNA and GR and Na^+/K^+-ATPase-α_1 protein levels in fetal sheep kidneys after exposure to maternal betamethasone administration in late gestation. Maternal betamethasone administration did not cause persistent alterations in renal gene expression in late-gestation fetal sheep. Only renin mRNA was transiently up-regulated in betamethasone-exposed fetal sheep at 116 and 121 d. Future studies could measure renal and plasma renin concentrations in betamethasone-exposed fetuses to examine whether the up-regulation in mRNA expression is translated to protein. Also, measurement of AngII levels in these animals may be informative because this growth factor is important for renal tubular growth and differentiation (Wolf, 2002). Plasma AngII levels in 127 d pre-term sheep were reduced after maternal betamethasone administration 24 hours before delivery (Berry *et al.*, 1997). There was no effect of prenatal maternal betamethasone administration on renal GR or Na^+/K^+-ATPase-α_1 protein levels. A limitation of this study was that protein levels could not be measured

for all the genes studied, due to the lack of commercially available antibodies with ovine immuno-reactivity.

The timing of prenatal glucocorticoid exposure appears to be the critical factor in determining the effect on renal gene expression. The effects of late-gestation maternal betamethasone administration I observed on fetal renal gene expression are in contrast to those of early-gestation maternal dexamethasone administration in sheep. In late-gestation fetal sheep, renal GR, MR, A_o, AT_1 receptor, AT_2 receptor and Na^+/K^+-ATPase-α_1 mRNA expression is increased after early-gestation maternal dexamethasone administration (Hantzis *et al.*, 2002; Moritz *et al.*, 2002b; Moritz *et al.*, 2005c). At the time of the early-gestation maternal dexamethasone administration (~ 27 d), the fetal ovine kidney is comprised mainly of metanephric mesenchyme, and the ureteric bud had branched only once (Moritz *et al.*, 2003). These findings suggest that increased fetal glucocorticoid exposure in early gestation programs the ovine fetal kidney, whereas exposure in late gestation does not. The early-gestation kidney may be more susceptible to the programming effects of glucocorticoids due to the vulnerability of the metanephric mesenchyme during early nephrogenesis.

Prenatal betamethasone exposure did not affect gene expression in the adult kidney

Consistent with my studies on fetal kidneys, I demonstrated that prenatal betamethasone exposure did not alter the expression of GR, MR, 11β-HSD-2, Na^+/K^+-ATPase-α_1, A_o and AT_1 receptor mRNA in adult sheep kidneys. Similarly, renal GR and Na^+/K^+-ATPase-α_1 protein levels were unaltered by prenatal treatment. The effect of early-gestation maternal dexamethasone on renal gene expression in the adult kidney has not been reported. Nephron number does not increase after birth; therefore, stereological studies were not performed on adult sheep kidneys. The rate of glomerular degeneration in adult sheep is unknown; however, the degree of glomerulosclerosis in hypertensive adult sheep exposed to early-gestation dexamethasone was similar to controls (Wintour *et al.*, 2003). Therefore, nephron numbers in adulthood should correlate closely with nephron endowment at birth (Chapter 3). The limitations of my study of adult sheep were the low number of samples in some groups for Western blot and real-time RT-

PCR experiments, a lack of ovine immuno-reactive antibodies available for protein analysis and the inability to study renal function. Animals in the adult cohort were part of another series of investigations (Moss *et al.*, 2001; Sloboda *et al.*, 2002a; Sloboda *et al.*, 2003; Moss *et al.*, 2005; Sloboda *et al.*, 2005) and were unavailable for renal function studies.

Adult sheep exposed to dexamethasone in early gestation have reduced nephron endowment (Wintour *et al.*, 2003) and are hypertensive from age 3 to 4 months (Dodic *et al.*, 1998); however, basal renal function was similar to controls (Moritz *et al.*, 2005a). MAP was measured in the adult sheep used for my studies; blood pressure was not related to prenatal treatment (Moss *et al.*, 2005) and it is unlikely that basal renal function was perturbed in these animals. These findings are consistent with data from the Auckland Steroid Trial that has demonstrated the preservation of normal basal blood pressure in adults exposed to maternal betamethasone *in utero* (Dalziel *et al.*, 2005).

In summary, in my thesis I have presented original studies that have furthered our understanding of the impact of fetal glucocorticoid exposure on the ovine kidney. I have demonstrated that although prenatal maternal betamethasone administration does not appear to consistently alter nephron number or glomerular size in term fetal sheep, it may indirectly alter nephron endowment through effects on renal growth. Total nephron endowment was reduced in betamethasone-exposed fetal sheep with renal growth restriction. I have also provided evidence that suggests that late-gestation prenatal betamethasone exposure does not program permanent alterations in the renal expression of genes or proteins involved in glucocorticoid hormone action or the renin-angiotensin system in fetal or adult sheep. In conclusion, exposure of the developing fetal kidney to maternal betamethasone in late gestation may alter renal structure if renal growth is perturbed; however, there were no persistent alterations in the expression of various glucocorticoid-sensitive genes or proteins. These findings are consistent with the preservation of normal basal blood pressure in sheep and human studies of late-gestation maternal glucocorticoid administration, and are reassuring, given the use of antenatal corticosteroids in obstetric practice.

7. References

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8. APPENDICES

8.1. APPENDIX 1A: SHANDON PATHCENTRE® TISSUE PROCESSOR PROGRAM

Table A.1. Shandon Pathcentre® Tissue Processor program.

Adult sheep kidneys were processed using a Shandon Pathcentre® Tissue Processor (Life Sciences International (Europe), Cheshire, UK). The solutions and duration of immersion for the tissues is shown below.

SOLUTION	DURATION
100% Ethanol	33 min
100% Ethanol	37 min
100% Ethanol	56 min
100% Ethanol	1 h 8 min
100% Ethanol	1 h 18 min
Xylene	35 min
Xylene	59 min
Xylene	1 h 18 min
Paraffin wax	35 min
Paraffin wax	48 min
Paraffin wax	1 h 9 min
Paraffin wax	1 h 18 min

8.2. APPENDIX 1B: SHANDON CITADEL™ 1000 TISSUE PROCESSOR PROGRAM

Table A.2. Shandon Citadel™ 1000 Tissue Processor program.

Fetal kidneys were processed using a Shandon Citadel™ 1000 Tissue Processor.

SOLUTION	DURATION
70% Ethanol with 1% detergent	2 h
90% Ethanol with 1% detergent	2 h
100% Ethanol with 1% detergent	3 x 2 h
Toluene	2 x 1 h
Wax	2 x 1 h

8.3. APPENDIX 2: HAEMATOXYLIN & EOSIN STAIN

1. Deparaffinise and hydrate sections to tap water.
2. Stain in Haematoxylin for 6 min.
3. Wash sections in tap water.
4. Differentiate in acid alcohol (5 mL Hydrochloric Acid (36%), 1 L 70% Ethanol) by dipping once.
5. Blue in blueing solution (3 mL ammonia (25%), 400 mL tap water) for 15 s.
6. Wash in tap water.
7. Rinse in 70% alcohol.
8. Stain in eosin for 30 s.
9. Dehydrate, clear and mount.

Results:

- Nuclei stain dark blue/black.
- Cytoplasm stains pink.
- Erythrocytes stain red.

8.4. APPENDIX 3: MASSON'S TRICHROME STAIN

1. Deparaffinise and hydrate to tap water.
2. Stain sections for 3 min in Celestine Blue solution.
3. Wash in tap water.
4. Stain sections for 3 min in Haematoxylin.
5. Wash in tap water.
6. Blue in blueing solution for 10 s.
7. Wash in tap water.
8. Flood slides for 3 min with Picro Orange (2 g Orange G, 1 L 80% Ethanol saturated with Picric Acid).
9. Quickly dip in clean water twice.
10. Immerse sections in Biebrich Scarlet Acid Fuchsin (9 g Biebrich scarlet, 1 g acid fuchsin, 10 mL acetic acid, 1 L dH_2O) for 4 min, drain.
11. Incubate sections in 5% w/v Phosphotungtic Acid for 1 min.
12. Dip sections quickly six times in 1% v/v acetic acid.
13. Stain for 3-5 min in Aniline Blue (5 g aniline blue, 500 mL 1% v/v acetic acid).
14. Dip sections quickly six times in 1% v/v acetic acid.
15. Dehydrate, clear and mount with Ultramount (Scot Scientific Pty Ltd, Welshpool, WA, Australia).

Results:

- Nuclei stain black.
- Muscle, fibrin and some cytoplasmic granules stain red.
- Collagen, some reticulin, amyloid and mucin stain blue.
- Erythrocytes stain yellow.

8.5. APPENDIX 4: MOLECULAR WEIGHT MARKERS FOR WESTERN BLOTTING

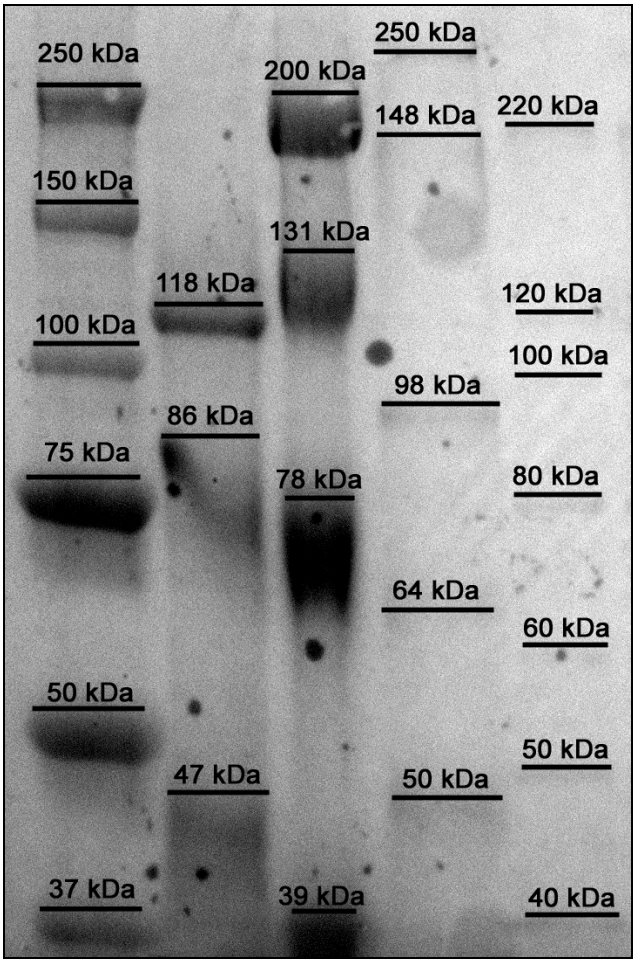

Figure A.1. Comparison of molecular weight markers for Western blotting.

Lane 1: Precision Plus Protein™ Standards All Blue (#161-0373; Bio-Rad Laboratories); Lane 2: Fermentas Prestained Protein Molecular Weight Marker (#SM0441; Fermentas); Lane 3: Kaleidoscope Prestained Standards (#161-0324; Bio-Rad Laboratories); Lane 4: SeeBlue® Plus 2 (Invitrogen); Lane 5: MagicMark™XP (Invitrogen).

8.6. APPENDIX 5: 0.1% v/v DEPC-DH_2O

1. Thoroughly clean glassware and bake overnight at 200 ºC.
2. Add 2 mL diethyl pyrocarbonate (Sigma-Aldrich, Inc.) to 2 L dH_2O, in an oven-baked 2 L bottle, in a fume hood.
3. Tighten cap, mix, then loosen cap and incubate overnight at 37 ºC.
4. Autoclave for 30 min at 121 ºC.

8.7. APPENDIX 6: EMBEDDING KIDNEY PIECES IN METHACRYLATE RESIN

(Protocol courtesy of Mrs Sue Connell at the Department of Anatomy and Cell Biology at Monash University, Melbourne, VIC, Australia)

1. Rinse fixed kidney pieces in 70% ethanol overnight.
2. Dehydrate tissue:
3. 70% ethanol: 1 x 1 h.
4. 100% ethanol: 3 x 1 h.
5. 100% butanol: 1 x 1 h, overnight.
6. Infiltrate tissue with *infiltration solution* overnight. *Infiltration solution*: Using Technovit 7100 Kit (Kulzer Histo-Technik; Heraeus Kulzer, Gmbh & Co. KG, Wehrheim, Germany), dissolve 1 g Hardener I in 100 mL of Technovit 7100 solution. Mix for approximately 30 min with wooden stick until dissolved. This solution can be stored at 4 ℃ for 4 weeks.
7. Place tissue in plastic embedding moulds with face to be cut facing the bottom of the mould.
8. Fill mold up with *embedding solution* and let it set at room temperature overnight or until set. Add label to mould after approximately 20 min.
9. Embedding solution: Using above *infiltration solution*, add 1 mL of Hardener II to 15 mL of *infiltration solution* and mix thoroughly with a wooden stick. Make up in batches of 30 mL or less as it may set quickly (within 5 to 7 min).
10. Adhere backing block to methacrylate block using Technovit 3040 resin, using a 2:1 ratio of powder to liquid (i.e. 2 g powder plus 1 mL liquid). Stir quickly with wooden stick and put a couple of drops onto the back of the methacrylate block, then quickly place backing block on top and allow to set. This resin sets very quickly so do not make up more than 6 g of powder and 3 mL of liquid at one time. This resin is also used to make up the backing blocks in the embedding moulds.

8.8. APPENDIX 7: PERIODIC ACID-SCHIFF STAINING OF METHACRYLATE SECTIONS

(Protocol courtesy of Mrs Rebecca Douglas-Denton at the Department of Anatomy and Cell Biology at Monash University, Melbourne, VIC, Australia)

1. Place sections in 1% periodic acid for 30 min.
2. Wash sections briefly in distilled water.
3. Place sections in Schiff's reagent for 30 min.
4. Wash sections under running tap water for 3 to 4 min (until the water turns bright pink in colour).
5. Place sections in haematoxylin for 30 min.
6. Wash sections under running tap water for 3 to 4 min (until the water runs clear).
7. Place sections in Scott's tap water for 3 to 4 min.
8. Wash sections briefly in tap water.
9. Allow sections to dry before clearing in xylene and cover-slipping.

8.9. APPENDIX 8: SEX-TYPING OF FETAL SHEEP

(Protocol courtesy of Mr Shaofu Li at the School of Women's and Infants' Health, The University of Western Australia, Perth, WA, Australia)

1. Using ultrasound, withdraw 10 mL of amniotic fluid from around the fetus using sterile techniques.

2. Place amniotic fluid into a sterile Falcon tube and centrifuge at 1,200 r.p.m. for 10 min at room temperature.

3. Discard supernatant and re-suspend cell pellet.

4. Add 500 µL of Red Cell Lysis Buffer into tube and transfer contents to a 1.5 mL microcentrifuge tube.

5. Rinse out Falcon tube with another 500 µL aliquot of buffer and add to 1.5 mL tube.

6. If the supernatant is red, the sample needs to be rinsed until it is clear.

7. Centrifuge the tube at 13,000 r.p.m. for 30 sec at room temperature.

8. Discard supernatant and re-suspend cell pellet.

9. Add 75 µL of PCR-grade water and 25 µL of Chelex solution and vortex well.

10. Heat tube at 100°C for 10 min in a heating block. Cool to room temperature and store at -20°C.

Table A.3. PCR master mix for sex-typing fetal sheep

COMPONENTS	x1 (µL)
10 x PCR reaction buffer (with 20 mM $MgCl_2$)	2.5
100 x dNTPs mix (dATP, dCTP, dGTP, dTTP: 100µM each)	0.2
SCUcd043.fwd (1 µM)	2.5
SCUcd043.rev (1 µM)	2.5
P1-5 EZ (1 µM)	2.5
P2-3 EZ (1µM)	2.5
FastStart Taq DNA Polymerase (5U/µL)	0.1
PCR water	0.7
Total	13.5

Components from Roche Diagnostic Australia Pty Ltd, Castle Hill, NSW, Australia.

Table A.4. Primer sequences for sex-typing fetal sheep

PRIMER	SEQUENCE
SCUcd043.fwd	5'-GTGACATGCCCATGACAGAAAG-3'
SCUcd043.rec	5'-GTGACATGCCTGGCTTAGTGTA-3'
P1-5 EZ	5'-ATAATCACATGGAGAGCCACAAGCT-3'
P2-3 EZ	5'-GCACTTCTTTGGTATCTGAGAAAGT-3'

- Run PCR for 4 min at 95°C, 1 min at 94°C, 40 cycles of 1 min at 61°C, 1.5 min at 72°C, 5 min at 72°C and hold at 4°C.

- Primer SCUcd043 produces a 1079 bp fragment which is the SRY gene product found only in males.

- Primer P1-5 EZ (P2-2 EZ) produces a 445 bp fragment which is the ZFX/ZFY gene product found in both males and females.