H24/3242

MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON..... 2 August 2002 .....
.....[redacted].....
Sec. Research Graduate School Committee

Factors Affecting Structural Development of the Lung in Fetal Sheep

Rochelle Elizabeth Boland
B.Sc. (Hons)

Department of Physiology
Faculty of Medicine
Monash University

A thesis submitted in total fulfilment of the requirements
for the degree of Doctor of Philosophy

March 2002

In the middle of the journey of our life I came to myself
within a dark wood where the straight way was lost.
Ah, how hard a thing it is to tell what a wild,
and rough, and stubborn wood this was,
which in my thought renews the fear!

DANTE

The Divine Comedy

Table of Contents

Summary

The volume of liquid retained within the fetal lungs is of vital importance in stimulating adequate lung growth and maturation *in utero* and hence facilitating survival of the newborn at birth. The changes in the structural components of the lung that permit, or occur as a result of, alterations in lung expansion are the focus of investigation in this thesis.

Sustained periods of lung deflation essentially cause lung growth to cease, resulting in morphological deficits within the lung that compromise neonatal survival. Late in gestation, increased circulating cortisol concentrations are believed to mature many organ systems, including the lung. Exogenous corticosteroids have been used in the prevention of respiratory distress syndrome in premature infants, as they are thought to increase both biochemical and structural aspects of lung maturity. Cortisol also increases lung liquid secretion rates, lung volumes and lung growth. As increases in lung volume also increase lung growth and structural maturation, the effect of cortisol on lung growth and structure may be mediated by increases in lung compliance that allow greater increases in lung liquid volumes. I hypothesised that, in the absence of an increase in lung liquid volume, all of the effects of cortisol on lung growth and structure would be prevented. The experiments described in this thesis have shown that an increasing physiological dose of cortisol, delivered intravenously to the fetus over a 9-day period, accelerates lung maturation in both normal and hypoplastic lungs (Chapter 3). Cortisol improved morphological indices of lung maturation, including an increase in percentage luminal space, alveolar number and a thinning of inter-alveolar wall thickness. Furthermore, cortisol delivered at these physiological levels did not adversely affect fetal lung growth. This is an important finding, in the context of recent studies, which show detrimental effects of high-dose corticosteroids on lung growth. The alterations in morphological maturity of the lung following cortisol treatment occurred regardless of differing degrees of lung expansion. This suggests that cortisol acts *via* other mechanisms, independent of alterations in lung expansion, to stimulate structural changes in the lung and enhance lung maturity.

Structural changes in the lung are believed to play an important role in mediating the lung growth response to changes in lung expansion. Turnover of extracellular matrix

proteins, such as collagen, may play an important part in this morphological remodelling. Levels of matrix metalloproteinases, in particular, the gelatinases MMP-2 and MMP-9, may indicate that turnover of pulmonary collagen is occurring. In Chapter 4, I have shown that MMP-2 levels increased following a prolonged period of lung deflation, indicating that lung liquid drainage may decrease collagen content in the hypoplastic lung due to increased gelatinase activity and hence collagen turnover. In contrast, two days of increased lung expansion induced by tracheal obstruction, resulted in a transient decrease in MMP-2 levels, which was not sustained following longer periods of tracheal obstruction. Thus, increases in collagen content following tracheal obstruction are therefore likely to be due to increased *de novo* synthesis of collagen.

The increase in lung expansion induced by tracheal obstruction is a potent stimulus for fetal lung growth and net collagen accumulation. An increase in collagen content is believed to play an important role in allowing further growth to occur following tracheal obstruction. It was hypothesised that the prevention of collagen synthesis during a period of tracheal obstruction would reduce the accelerated rate of lung growth. Infusion of LACA, which prevents collagen synthesis, was not able to prevent the rapid increases in collagen accumulation, lung liquid volume or lung growth that occur following tracheal obstruction, at the doses investigated (Chapter 5). However, LACA infusion reduced the interstitial collagen fraction in normally expanded lungs. This finding suggested that collagen synthesis rates, following tracheal obstruction, were increased to levels that were not easily prevented by the dose of LACA administered.

In summary, this thesis has shown that structural remodelling of the lung is an important part in normal and altered lung growth. Lung structural maturity, but not lung growth, can be accelerated by treatment with cortisol. The pulmonary content of the structural protein collagen is decreased following a period of lung deflation and this is likely to be due to increases in collagen turnover. In contrast, the increase in collagen content following an increase in lung expansion is likely to be due to an increase in collagen synthesis.

Declaration

This thesis contains no material that has been accepted or submitted for the award of any other degree in any University or other institution. To the best of my knowledge, this thesis contains no material previously published or written by any other person, except where due reference is made in the text.

Rochelle Boland

March 2002

Publications

Abstracts and conference presentations:

Boland, R. E., Wallace, M. J., & Hooper, S. B. (2000). The role of collagen synthesis in the lung growth response to tracheal obstruction in fetal sheep. American Thoracic Society, Toronto, Canada, *American Journal of Respiratory & Critical Care Medicine* **161** (3), Abstract: A565.

Acknowledgments

First and foremost, I am greatly indebted to my supervisors Dr. Stuart Hooper and Dr. Megan Wallace. You have given freely of your time and have provided me with unending advice, assistance and friendship. It has been a pleasure to work with you both.

I would like to acknowledge the assistance of the Heads of Department, Professor Warwick Anderson and Professor Colin Gibbs, in allowing me to undertake my PhD. within the Department of Physiology. I am also grateful for the assistance provided to me by the University in the form of a Monash Graduate Scholarship.

To Alex Satragno, for all your assistance in surgery, and Alison Thiel for animal preparation, *post mortems*, and general lab assistance. Thanks also to all the girls who've helped in PMs over the years: Laura, Megan W, Andrea, Sharon, Megan P. Your help and your laughter made it an enjoyable experience! Big thanks to Laura, who taught me many of the techniques used in this thesis. Thanks to Dr. Heather Stanton, Dr. Mandy Fosang and Karena Last for your assistance with the zymography technique. Special thanks to Heather, for your particular effort and for guidance with Chapter 4. Thanks also to Saraid Billiards for teaching me the cortisol assay, to Ian Boundy for assistance with tissue processing and Gosia Zieba for assistance with the digital images.

To my friends in the Department, I thank you for your support and encouragement. I have many fond memories of time spent with you all: Laura, Andrea, Megan C, Megan W, Amanda, Amany, Brenda, Kelly, Nicole, Marty, Marcus D, Marcus N.

To my friends and family, I am grateful for all your interest and welcome distractions. To my mother, I dedicate this thesis to you.

And to Mark, you have not wavered in your support and love. It has been wonderful to share this time of my life with you. Thankyou.

Symbols and Abbreviations

Symbols

α	*alpha*
&	And
~	approximately
β	*beta*
=	equals
γ	*gamma*
<	less than
-	negative
/	Per
%	percentage
±	plus or minus
Σ	Sum of
X	times; cross

Abbreviations

Å	angstrom
ACF	aqueous counting fluid
ACTH	adrenocorticotrophic hormone
ADX	adrenalectomised
AEC	alveolar epithelial cell
ANOVA	analysis of variance
APMA	amino-phenyl-mercuric acid
ARDS	adult respiratory distress syndrome
ATPase	adenosine triphosphatase
AU	arbitrary units
BSA	bovine serum albumin
BW	body weight
[^{14}C]	radioactive isotope of carbon
°C	degree(s) Celsius
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
CDH	congenital diaphragmatic hernia
cf.	*confer* (compare)
CH_2Cl_2	dichloromethane
CHAPS	3[(3-cholamidopropyl)-dimethylammonio-1-propanesulfonate
Cl^-	chloride ion(s)
cm	centimetre(s)
cm^3	cubic centimetre(s)
cmH_2O	centimetre(s) of water
COPD	chronic obstructive pulmonary disease
cpm	count(s) per minute
d	day(s)
dH_2O	distilled water
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DSPC	disaturated phosphatidyl choline
DTT	dithiothreitol
e	base of natural logarithm ~ 2.718
e.g.	*exempli gratia* (for example)
ECM	extracellular matrix
EDTA	ethylenediaminetetra acetic acid
et al.	*et alii* (and others)
FBM	fetal breathing movement(s)
FCS	fetal calf serum
FGF	fibroblast growth factor
g	gram(s)
GA	gestational age
GR	glucocorticoid receptor
[^{3}H]	tritiated/radioactive isotope of hydrogen
H&E	Haemotoxylin and Eosin
H_2O	water
HCl	hydrochloric acid
HCO_3^-	bicarbonate ion(s)
Hct	hematocrit
HD	high dose
HEPES	N'[2-Hydroxyethyl]piparazine -N'-[2-ethanesulfonic acid]
hGR	human glucocorticoid receptor
hr	hour(s)
HSP	heat shock protein
HX	hypophysectomy
[^{125}I]	radioactive isotope of iodine
i.e.	*id est* (that is)
i.m.	intramuscular
i.v.	intravenous
ID	internal diameter

IGF	insulin-like growth factor
IL	interleukin
Inc	incorporated
IU	international units
K^+	potassium ion(s)
kb	kilobase(s)
kDa	Kilo Dalton(s)
kg	kilogram(s)
l	Litre(s)
LACA	L-Azetidine 2-carboxylic acid
LD	Low dose
ln	natural logarithm
LSD	least significant differences
Ltd	limited
LW	lung weight
M	molar
mA	milliamp(s)
µg	microgram(s)
mg	milligram(s)
min	minute(s)
µl	microlitre(s)
ml	millilitre(s)
µm	micrometre(s)
mm	millimetre(s)
mM	millimolar
mmH_2O	millimetre(s) of water
mmHg	millimetre(s) of mercury
mmol	millimole(s)
MMP	matrix metalloproteinase
M_r	relative molecular weight
mRNA	messenger ribonucleic acid
MT1-MMP	membrane type 1 MMP
mV	millivolt(s)
n	number
Na^+	sodium ion(s)
NaCl	sodium chloride
NaOH	sodium hydroxide
$NaPO_4$	sodium phosphate
ng	nanogram(s)
nm	nanometre(s)
NSB	Non-specific binding
O.D.	optical density
O_2	oxygen
OD	outer diameter
P	p value: level of significance
P	phosphorous
$PaCO_2$	arterial partial pressure of carbon dioxide
PAGE	polyacrylamide gel electrophoresis
PaO_2	arterial partial pressure of oxygen
PDGF	platelet-derived growth factor
PDGF-β-R	platelet-derived growth factor B receptor
PEG	polyethylene glycol
pH	pressure of hydrogen
PPNX	post pneumonectomy
Pty Ltd	proprietary limited
r	coefficient of correlation
RDS	respiratory distress syndrome
REM	rapid eye movement
RNA	ribonucleic acid
rpm	revolution(s) per minute
SaO_2	arterial saturation of oxygen
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	second(s)
SEM	standard error of the mean
SP	surfactant protein
t	time
T_3	triiodothyronine
T_4	thyroxine
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinases
TNF	tumour necrosis factor
TO	tracheal obstruction
TRH	thyroid releasing hormone
Tris	tris(hydroxymethyl)amino-methane
Tween20	polyoxyethylene-sorbitan monooleate
UK	United Kingdom
USA	United States of America
UV	ultraviolet
V	volt(s)
VEGF	very early growth factor
vs.	*versus*
v/v	volume per volume
w/v	weight per volume
Zn^{2+}	Zinc ion(s)

List of Figures

Chapter 4 The Role of Gelatinases in the Structural Remodelling of the Lung

Chapter 5 Role of Collagen Synthesis in the Lung Growth Response to Tracheal Obstruction

List of Tables

CHAPTER 1 Literature Review

1.1 General Introduction

The adequate development of the lung *in utero* is crucial for the survival of the newborn. Prior to birth, the lungs are liquid-filled and, unlike most other organs, do not perform the same function before birth as they do after birth. Immediately from the time of birth, the lungs must take on the role of gas exchange. Therefore, adequate development of the lungs during fetal life is essential for the transition from fetus to neonate to take place successfully. Given the critical nature of adequate lung development *in utero*, it is important to understand the factors that control, mediate and influence lung development.

The degree to which the lungs are expanded by lung liquid during fetal life has considerable effects on lung growth and maturation. Prolonged periods of lung deflation result in lung growth and structural deficits that severely compromise neonatal life. Conversely, increases in lung expansion increase lung growth and hence treatments that increase lung expansion *in utero* are being investigated as a means for reversing pre-existing fetal lung growth deficits. Late in gestation, increased circulating cortisol concentrations are believed to mature many organ systems including the lung, in preparation for birth. Exogenous corticosteroids have significant effects on lung structure, lung liquid volume and lung growth and have been used widely in the prevention of respiratory distress syndrome in prematurely born infants. Given the beneficial effects of corticosteroids, there is growing interest in using them to treat pulmonary hypoplasia. It is important however, to understand how corticosteroids affect biochemical and structural aspects of fetal lung maturity. In particular, corticosteroids may influence maturity of the lung by altering lung liquid volume.

Alterations in lung expansion affect structural components of the lung as well as parameters of lung growth. The extracellular matrix forms a network of fibres that holds the lungs together, connecting and signalling between many of the components of the lungs. In fact, the extracellular matrix may be important in mediating many of the structural changes that occur following alterations in lung expansion. Collagen is an extracellular matrix protein that is known to play an important role in lung development. However, little is known of how collagen is affected following alterations in lung expansion, or the effect that collagen may have upon such manipulations.

This thesis is directed at understanding the relationships between lung expansion, lung growth and remodelling of the extracellular matrix (ECM) and the influence that corticosteroids have on these relationships. As such, in this review of the literature, I shall discuss the development of the lungs during fetal life, the role of lung liquid in normal lung growth and the effect of alterations in lung expansion on lung growth. I shall place particular importance on reviewing the role of corticosteroids in lung growth and the importance of the extracellular matrix in lung growth and development in the fetus.

1.2 Stages of Fetal Lung Development

By convention, lung development in mammals has been divided into five stages: embryonic, pseudoglandular, canalicular, saccular and alveolar (Bryden *et al.*, 1973; Hodson, 1992). These stages of fetal lung development are largely defined by the appearance of the lungs at a microscopic level. However, lung development is, by nature, a continual process and each stage is arbitrarily defined to provide an approximate guide to understand the developmental processes of the lung at different gestational ages (Table 1.1).

The experiments in this thesis will focus on understanding mechanisms underlying the relationship between lung expansion and lung growth in the sheep fetus. The sheep fetus provides an ideal model for investigating fetal lung development in humans because, at birth, the fetal lungs are at a similar stage of development, although slightly more advanced in sheep than they are in humans. Although the sheep lung

progresses through saccular development, no specific gestational age window for this stage has been ascribed (Alcorn *et al.*, 1981).

Table 1.1 Stages of lung development in sheep, humans, rabbits and rats

Stages of lung development in sheep, humans, rabbits and rats where gestational length is ~147 days in sheep, 38 weeks in humans, 30 days in rabbits and 22 days in rats (Meyrick & Reid, 1977; Alcorn *et al.*, 1981; Burri, 1999). Note that the saccular phase of development in sheep has not been defined (Alcorn *et al.*, 1981).

Stage of Lung Development	Sheep (days)	Human (weeks)	Rabbit (days)	Rat (days)
Embryonic Stage	0 - 40	0 – 7	0 - 9	0 - 13
Pseudoglandular Stage	40 - 80	5 - 17	9 - 25	13 - 16
Canalicular Stage	80 - 120	16 - 26	23 - 28	16 - 18
Saccular Stage	-	24 - 35	27 - term	20 - term
Alveolar Stage	120 - after birth	32 weeks - 2 years	postnatal	postnatal

1.2.1 Embryonic Stage of Lung Development

The embryonic stage of lung development involves the laying down of all the basic components of the future airways, with the establishment of the lobar structure of the lungs (Alcorn *et al.*, 1981). On day 17 of gestation in the sheep fetus, a ventral outpouching of the foregut appears (Bryden *et al.*, 1973). This 'respiratory diverticulum' grows caudally to form the primitive trachea (DiFiore & Wilson, 1994) and divides to form the principal bronchi. These tubules increase in length and begin budding to form the lobar bronchi by day 22 of gestation (Bryden *et al.*, 1973). In the next few days, there are further dichotomous divisions to form the buds of the future segmental bronchi (Bryden *et al.*, 1973).

The developing lung is composed of epithelium derived from the endoderm that invades the splanchnic mesoderm. The mesoderm will later give rise to structures such as the connective tissue, pulmonary vessels and smooth muscle (Moore & Persaud, 1993). The epithelium and mesenchyme are separated by a basal lamina, containing type I collagen at the site of branching (Thurlbeck, 1992). The interaction that occurs between the epithelium and mesoderm is vital for growth to continue, as the

mesoderm appears to control the process of branching morphogenesis (Alescio & Cassini, 1962; Taderera, 1967; Spooner & Wessells, 1970; Wessells, 1970; Minoo & King, 1994). The vascular system also begins its development during this stage of lung growth. The pulmonary arteries derive from the sixth pair of aortic arches and the veins form from the left atrial portion of the heart (Burri, 1994). A number of vascular connections are made and the future airways become ensheathed in an arterial plexus (Boyden, 1977). By the end of this stage of development, the epithelium appears to be columnar and pseudostratified (Bryden *et al.*, 1973).

1.2.2 Pseudoglandular Stage of Lung Development

The lungs at this stage of development are so named due to their histologic appearance, which is gland-like. It is during this phase of lung development, approximately days 40 to 80 of gestation in sheep, that the majority of the divisions of the conducting portions of the future airways are completed, to the level of the terminal bronchioles (Hodson, 1992); this occurs in a centrifugal manner mediating out from the hilas (Alcorn *et al.*, 1981; Fukuda *et al.*, 1983). The appearance of ciliated epithelial cells, containing large quantities of glycogen (Hodson, 1992), suggests that the epithelium of the proximal airways has started to differentiate (Jeffery, 1998). As in the embryonic stage of fetal lung development, interactions between the epithelium and mesenchyme are necessary for normal airway morphogenesis to proceed (Spooner & Wessells, 1970; Deterding & Shannon, 1995). It is during this stage of development, that the volume of liquid within the future airways can first be measured; it has been measured at ~74 days of gestation in fetal sheep (Olver *et al.*, 1981a). Development of the pulmonary vascular tree continues to parallel that of the bronchial tree and this stage is also a period of cartilage formation around the large airways (Burri, 1984). Elastic fibres are found in the pulmonary vessels and in the primordia of the alveolar septa (Fukuda *et al.*, 1983; Fukuda *et al.*, 1984).

1.2.3 Canalicular Stage of Lung Development

This stage of lung development, which occurs from ~days 80 to 120 of gestation in fetal sheep, is highlighted by rapid growth of the future airways and pulmonary vasculature (Alcorn *et al.*, 1981). The respiratory portion of the future airways undergoes repeated divisions to form acinar units that will contain alveolar ducts, alveolar sacs and the future alveoli themselves (Hodson, 1992). As a result of the continuous division, there

is a large increase in the potential airspace and, therefore, in the lung luminal volume. The process of septation, which gives rise to the primary alveoli, begins late in this stage of lung development, continuing into the saccular and alveolar stages of lung development. Additionally, the respiratory epithelium starts to differentiate into type I and type II alveolar epithelial cells (type I and II AECs), by 95 and 110 days of gestation respectively (Alcorn *et al.*, 1981). The type I AECs provide the large surface area across which gas exchange takes place after birth, whereas the type II AECs synthesise, store and secrete surfactant from late in gestation. Surfactant is the surface-active material that reduces surface tension at the air-liquid interface in the air-filled lung. To facilitate gas exchange, the mesenchyme thins, as does the epithelial layer, allowing the capillaries and future airway epithelium to come closer and eventually allowing the basement membranes for each structure to fuse. These actions are accompanied by an enlargement of the airways (Alcorn *et al.*, 1981). Ultimately, this means that extrauterine life becomes possible towards the end of this phase of development.

1.2.4 Saccular Stage of Lung Development

This period of lung development is not usually designated as a separate stage in sheep (Alcorn *et al.*, 1981). However, in humans and rats, this stage of development is highlighted by increases in the future airspaces, as a result of continual thinning of the epithelium and interstitium, further differentiation of alveolar epithelial cells and the appearance of secondary crests within alveolar sacs (Burri, 1999).

1.2.5 Alveolar Stage of Lung Development

The alveolar stage of lung development (~day 120 - term and beyond in sheep), is signified by further alveolar epithelial cell differentiation (Mason & Shannon, 1997), an increase in the formation of alveoli and thus an increase in surface area and luminal volume and continued thinning of connective tissue and alveolar septa (Alcorn *et al.*, 1981).

Type I AECs form the majority of the gas exchange surface by the end of gestation (Alcorn *et al.*, 1981) and cover ~95% of the alveolar surface area in mature lungs (Crapo *et al.*, 1982; Zeltner *et al.*, 1987; Davies *et al.*, 1988), due to their elongated

cytoplasmic extensions (Alcorn *et al.*, 1981). Type II AECs, which are thought to be the progenitor of both type I and II AECs (Uhal, 1997), are generally cuboidal in shape, with microvilli on their apical surface. Type II AECs are typically located close to the base of alveolar septa extending into the luminal space and comprise ~5% of the alveolar surface area (Crapo *et al.*, 1982) in adult lungs. In fetal sheep by 128 days, type II AECs have been shown to represent ~30% of the total number of epithelial cells (Flecknoe *et al.*, 2000) and type I AECs represent ~65% of the total number. Type II AECs produce surfactant from late in gestation, as indicated by the presence of lamellar bodies within these cells (Alcorn *et al.*, 1981). In fact, surfactant is first detected in lung liquid between day 124 and day 135 of gestation in fetal sheep (Mescher *et al.*, 1975).

Alveolar crests start to extend deep into the terminal saccules during this stage of lung development, leading to the formation of alveolar septa (Alcorn *et al.*, 1981). Collagen fibrils and/or smooth muscles cells are found in the tips of the alveolar septa and elastin starts being deposited throughout the alveolar wall leading to an increase in pulmonary elastin late in gestation (Schellenberg & Liggins, 1987). Inter-alveolar wall thickness decreases from ~5.5 μm at 123 days of gestation to ~2 μm by 146 days of gestation (Crone *et al.*, 1983). The average number of alveoli in the terminal respiratory unit increases from 4.3 at 110 days of gestation to 12.5 by 141 days of gestation in fetal sheep (Alcorn *et al.*, 1981). Formation of new alveoli and the continual thinning of the alveolar septa during this stage also cause an increase in the percentage of lung volume occupied by luminal space from ~60% at 109 days of gestation (Alcorn *et al.*, 1977) to ~80% by term (deLorimier *et al.*, 1969). Therefore, at birth, there are a large number of alveoli, ~1000 - 2000 million present in the sheep lung (Davies *et al.*, 1988) and as in humans, the number and size of alveoli continue to increase postnatally in sheep (Davies *et al.*, 1988). In humans, there is a 10 to 30-fold increase in the number of alveoli following birth (Emery & Mithal, 1960; Davies & Reid, 1970; Thurlbeck, 1975). Rats on the other hand, are born during the saccular stage of lung development and don't have any alveoli at the time of birth (Burri, 1974).

Lung development is a continual process, beginning almost immediately post-conception and continuing into postnatal life. A number of factors influence the natural development of the lungs, but the most important is the retention of an appropriate volume of lung liquid within the future airways. Fetal lung liquid and its role in normal lung development will be discussed in the following two sections. Cortisol is believed

to play an important role in many of the maturational changes that occur late in gestation, including alterations in ECM properties, such as elastin and collagen, which may play a part in increasing lung compliance late in gestation. The role of corticosteroids in lung development will be discussed in further detail later in this chapter (Section 1.5).

1.3 Fetal Lung Liquid

Fetal lung liquid is a unique fluid produced by the fetal lung epithelium. Originally, this fluid was believed to be inhaled amniotic fluid, however, a number of studies have established that lung liquid is a product of the lung. In experiments in which the fetal neck or trachea was ligated, thus isolating the lungs from the amniotic sac, the lungs were found to be distended with a fluid that could not have been inhaled (Jost & Policard, 1948; Carmel *et al.*, 1965). Analysis of the liquid showed that its composition was not likely to be a derivative of plasma or inhaled amniotic fluid (Adams *et al.*, 1967; Adamson *et al.*, 1969; Adamson *et al.*, 1975). Measurements of ion fluxes and the movement of radio-labelled solutes then determined that fluid moves into the future airways as the result of an active process (Humphreys *et al.*, 1967; Normand *et al.*, 1971; Olver & Strang, 1974). Several studies over the last 30 years, primarily in sheep, have quantified lung liquid secretion rates and determined the volume of liquid within the lungs throughout gestation (Strang, 1991; Bland & Nielson, 1992; DiFiore & Wilson, 1994; Harding, 1994; Walters, 1994; Hooper & Harding, 1995). Importantly, the presence of an adequate volume of lung liquid has been shown to be vitally important in normal growth and development of the lung *in utero*.

1.3.1 Lung Liquid Composition

Fetal lung liquid is a colourless fluid, with a composition that is distinctly different from either plasma or amniotic fluid (Humphreys *et al.*, 1967; Boston *et al.*, 1968; Adamson *et al.*, 1969). The most notable difference between fetal lung liquid, plasma and amniotic fluid, is the high concentration of chloride ions (Cl^-) in fetal lung liquid, indicative of active chloride secretion into the lung lumen (Adamson *et al.*, 1969). In addition, the protein levels in fetal lung liquid are lower than those in plasma and amniotic fluid, probably due the small pore size of the pulmonary epithelium in the fetus (Normand *et al.*, 1971).

1.3.2 Lung Liquid Secretion

Evidence of lung liquid secretion in fetal sheep is well established (Adams *et al.*, 1963; Adams *et al.*, 1967; Olver & Strang, 1974; Olver, 1977; Vilos & Liggins, 1982). Using the impermeant tracer technique, lung liquid secretion has been measured in fetal sheep from mid-gestation: 1.6 ml/hr/kg body weight at day 74, increasing until term to around 3.5 ml/hr/kg body weight (Olver *et al.*, 1981a; Harding & Hooper, 1996). A number of studies have investigated the movement of radio-labelled solutes between the lung lumen, the lymphatics and the bloodstream to determine water and solute movement across the pulmonary epithelium (Humphreys *et al.*, 1967; Normand *et al.*, 1971; Olver & Strang, 1974). In summary, net movement of Cl^- towards the lung lumen generates an osmotic gradient that favours the large flow of liquid into the lung lumen (Strang, 1991). The postulated mechanism that drives this flow of liquid into the future airways is based on studies in the shark rectal gland (Silva *et al.*, 1977). These predict that Na^+-K^+-ATPase pump activity on the basolateral membrane provides the transmembranous concentration gradient for Na^+ to enter the cell, coupled to Cl^- *via* a Na^+-K^+-$2Cl^-$ cotransporter (Silva *et al.*, 1977; Gillie *et al.*, 2001). Cl^- then exits the cell across the apical surface *via* specific Cl^- channels which results in the net movement of Cl^- into the lung lumen (Olver *et al.*, 1981a; Schneeberger & McCarthy, 1986). The net movement of Cl^- into the lung lumen generates an osmotic gradient in the direction of the lung lumen and this osmotic gradient is believed to ultimately drive the mass movement of water (see Figure 1.1).

1.3.2.1 Control of Lung Liquid Secretion and Reabsorption

A number of hormonal factors have been shown to influence the rate of lung liquid secretion both *in vivo* and *in vitro*. Catecholamines, such as epinephrine, have potent effects on lung liquid secretion and act *via* β-adrenergic receptors to decrease lung liquid secretion and induce lung liquid reabsorption (Walters & Olver, 1978; Olver *et al.*, 1981b; Olver *et al.*, 1986). Vasopressin, although less potent than the catecholamines, also decreases lung liquid secretion (Olver *et al.*, 1981b; Perks & Cassin, 1982; Ross *et al.*, 1984; Hooper *et al.*, 1993a; Cummings *et al.*, 1995). Both epinephrine and vasopressin inhibit lung liquid secretion and induce lung liquid reabsorption by activating amiloride-inhibitable Na^+ channels on the luminal surface of epithelial cells (Olver *et al.*, 1986; Hooper *et al.*, 1993a). Cortisol and T_3 (triiodothyronine) interact with both epinephrine and vasopressin, especially towards term, to depress lung liquid secretion (Olver *et al.*, 1986; Cassin *et al.*, 1994) and increase lung liquid reabsorption

(Barker *et al.*, 1988; Barker *et al.*, 1991; Cassin *et al.*, 1994; Wallace *et al.*, 1995). *In vitro* studies showed that cortisol and aldosterone reduce lung liquid production close to term (Kindler *et al.*, 1993). However, cortisol infusions in late gestation fetal sheep have been shown to increase lung liquid secretion rates and to enhance lung liquid reabsorption in response to epinephrine (Wallace *et al.*, 1995). Conversely, adrenalectomy reduced or abolished the age-related increases in lung liquid secretion rates (Wallace *et al.*, 1996) and greatly diminished the capacity of the lung to reabsorb liquid.

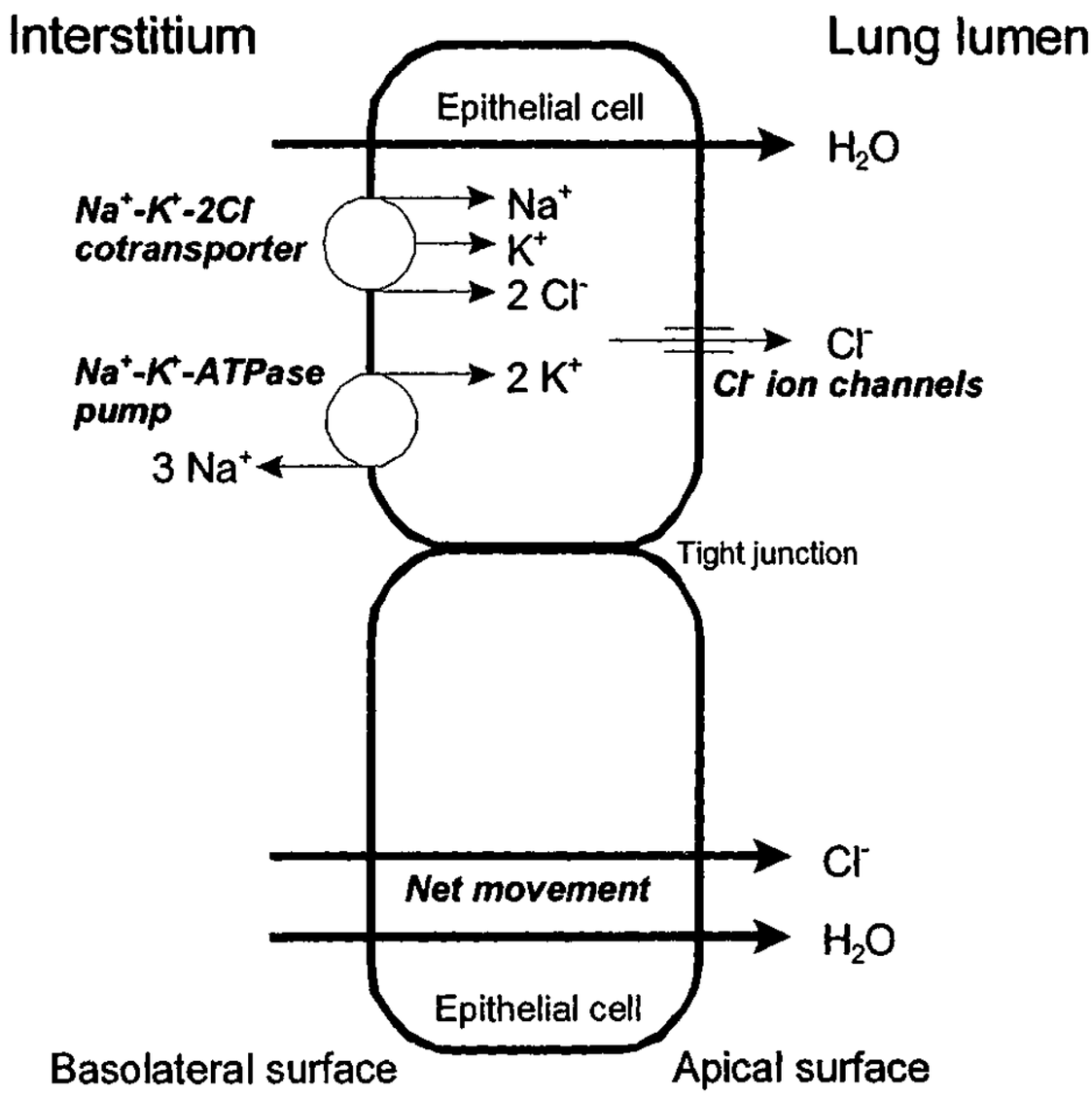

Figure 1.1 Mechanism of lung liquid secretion

This diagram represents the currently accepted model of lung liquid secretion. The secretion of lung liquid is believed to be driven by the Na^+-K^+-ATPase pump on the basolateral surface of the pulmonary epithelium, which generates a gradient for Na^+ entry. Na^+ enters the cell *via* a Na^+-K^+-2Cl^- cotransporter. The flow of Cl^- down an electrochemical gradient, through specific Cl^- channels into the lung lumen creates an osmotic gradient, which favours the bulk flow of water from the interstitium, into the lung lumen.

Alterations in lung liquid secretion and reabsorption rates were attributed to changes in the concentration or activity of some or all of the epithelial components thought to be involved in the secretion and reabsorption of lung liquid (Wallace *et al.*, 1995; Wallace *et al.*, 1996). At the time of birth, it is critical that the lung ceases lung liquid secretion and begins lung liquid reabsorption to clear the lungs of liquid in preparation for breathing air (Lines *et al.*, 1997). The effects of active labour and vaginal delivery enhance the clearance of liquid from the fetal lungs (Boon *et al.*, 1981; Brown *et al.*, 1983). In addition, it has been proposed that epinephrine and vasopressin may be largely responsible for the clearance of lung liquid at birth, as both their concentrations are elevated during labour (Brown *et al.*, 1983; Pochard & Lutz-Bucher, 1986) and amiloride can reduce the labour-induced clearance of lung liquid at birth (O'Brodovich *et al.*, 1990; Song *et al.*, 1992; Chapman *et al.*, 1994).

In addition to hormonal influences, physical factors also influence fetal lung liquid secretion rates. The relationship between lung expansion and lung liquid secretion is of particular interest. Generally, when the lung is expanded and the lung liquid volume is increased above normal levels, the rate of lung liquid secretion is reduced (Perks & Cassin, 1982; Nardo *et al.*, 1995; Nelson & Perks, 1996; Nardo *et al.*, 1998). Conversely, when the lung is deflated and lung liquid volume is decreased below normal levels, lung liquid secretion rate is increased (Harding *et al.*, 1993; Nardo *et al.*, 1995). Based on these findings, it was suggested that the changes in intraluminal pressure, which arise from changes in the level of lung expansion, influence lung liquid secretion rates. These findings also imply that the osmotic pressure that normally drives lung liquid secretion equates to a hydrostatic pressure of ~5 mmHg, because at this intraluminal pressure net lung liquid secretion rates cease (Nardo *et al.*, 1998). At this point, the intraluminal pressure is thought to counterbalance the osmotic pressure driving lung liquid secretion, causing net lung liquid production to cease (Hooper & Harding, 1995).

1.3.3 Lung Liquid Volume

Lung liquid is present in measurable amounts from mid-gestation in fetal sheep (Olver *et al.*, 1981a; Harding & Hooper, 1996). At 74 days of gestation (term ~147 days), the volume of lung liquid is ~1 ml (~3.0 - 4.0 ml/kg) (Olver *et al.*, 1981a; Harding & Hooper, 1996). The volume of lung liquid gradually increases throughout gestation (Humphreys *et al.*, 1967; Normand *et al.*, 1971; Olver *et al.*, 1981a) to ~35 - 40 ml/kg (Hooper &

Harding, 1995) and is maintained until or close to the onset of labour (Bland *et al.*, 1979; Kitterman *et al.*, 1979; Dickson *et al.*, 1986; Lines *et al.*, 1997; Berger *et al.*, 1998). The volume of liquid maintained within the future airspaces is determined by the balance between the rate of lung liquid secretion and the rate of efflux of liquid from the trachea.

The larynx is the major site of control of lung liquid volume (Harding *et al.*, 1980; Harding *et al.*, 1986a; Harding *et al.*, 1986b) as it regulates lung liquid efflux from the trachea. Sectioning of the recurrent laryngeal nerve, which innervates the larynx, leads to reductions in lung liquid volume (Harding *et al.*, 1984). Adductor activity of the larynx restricts lung liquid efflux and generates an intraluminal pressure of 0.75 - 1.0 mmHg from days 105 - 115 of gestation in sheep and 2.0 - 2.5 mmHg from day 115 - 130 (Dickson & Harding, 1991). Thereafter, an intraluminal pressure of ~2.0 mmHg is maintained until term (Vilos & Liggins, 1982; Dickson & Harding, 1991).

Fetal breathing movements (FBMs) have been measured from at least 40 days of gestation in fetal sheep (Thorburn & Schneider, 1972) and are largely associated with contractions of the diaphragm and relaxation of the laryngeal muscles, but can also involve the intercostal muscles (Harding, 1997). During FBMs, the lung's high intraluminal pressure, in relation to amniotic pressure, as well as a reduced upper airway resistance, favour the loss of liquid from the lung lumen (Harding *et al.*, 1986a). Abolishing diaphragmatic activity by synthetic nerve blockade (Miller *et al.*, 1993) or transection of the spinal cord above the level of phrenic outflow (Harding *et al.*, 1993) both result in a reduction in lung liquid volume and ultimately lung hypoplasia (Harding *et al.*, 1993). Therefore, the contraction of the diaphragm during FBMs is believed to oppose the loss of liquid and assist in maintaining an appropriate lung liquid volume and hence continued lung growth (Harding *et al.*, 1993).

1.4 Lung Expansion and Lung Growth

Tracheal obstruction is a technique by which the fetal trachea is obstructed, distal to the larynx, thus preventing the efflux of liquid from the lungs. Continued secretion of liquid into the lung lumen results in the accumulation of lung liquid and thus an increase

in the degree of lung expansion. In contrast, lung liquid drainage involves continuous gravimetrical drainage of lung liquid from the fetal lungs *via* a cannula, preventing the normal accumulation of lung liquid and promoting lung deflation. The pioneering study by Alcorn *et al.* (1977) examined the effects of prolonged periods of tracheal ligation and lung liquid drainage on fetal lung growth and clearly demonstrated the importance of an appropriate volume of lung liquid for normal lung growth and development. Lungs that had been drained of lung liquid for 21 - 28 days weighed less than their respective age-matched controls, had thicker inter-alveolar walls and had altered little from control lungs taken at the beginning of the experimental period. Tracheal ligation for 21 - 28 days resulted in lungs that were heavier than the lungs of age-matched controls and displayed a reduction in type II alveolar epithelial cell number and thinning of the alveolar walls. In this study, it was shown that alterations in lung liquid volume not only affected lung growth, but also affected structural maturation of the lungs. Following Alcorn *et al.*, Moessinger *et al.* (1990) simultaneously ligated the left mainstem bronchus and drained the right lung of liquid in fetal sheep for 25 days. This caused the left lung to become over-expanded and hyperplastic, as measured by DNA content, whereas the right lung was under-expanded and hypoplastic relative to age-matched controls. This remarkable finding indicated, for the first time, that altering the degree of lung expansion led to changes in lung growth primarily *via* local influences, rather than by systemic factors. Together, the studies by Alcorn *et al.* and Moessinger *et al.* showed that retention of an appropriate volume of lung liquid within the lung lumen is necessary for normal growth and development of the fetal lungs.

1.4.1 Decreased Lung Expansion and Lung Hypoplasia

Pulmonary hypopiasia in humans is usually defined clinically in terms of lung to body weight ratio (LW/BW) and is associated with a reduced DNA content compared to age-matched controls (Wigglesworth & Desai, 1981). Pulmonary hypoplasia can result in severe respiratory insufficiency and is potentially lethal (Nakayama *et al.*, 1985; Adzick *et al.*, 1989), further highlighting the necessity for the lungs to develop appropriately *in utero*. A decrease in the degree of lung expansion is believed to be central to most causes of lung hypoplasia (Hooper & Harding, 1995) and can result from reductions in amniotic fluid volume, reduced intrathoracic volume, musculo-skeletal disorders or experimental manipulations (Alcorn *et al.*, 1977; Fewell *et al*, 1981; Wigglesworth *et al.*, 1981; Harrison *et al.*, 1996).

1.4.1.1 Reductions in Amniotic Fluid Volume

Amniotic fluid is primarily composed of fetal urine, with a relatively smaller volume being contributed by fetal lung liquid (Brace *et al.*, 1994). Reduced amniotic fluid volume, or oligohydramnios, is caused by various conditions including urinary tract obstructions, renal agenesis or premature rupture of the fetal membranes (Moessinger *et al.*, 1989; Nicolini *et al.*, 1989). It is most likely that oligohydramnios reduces lung liquid volume and hence impairs lung growth (Wigglesworth *et al.*, 1981) due to increased compression of the fetus by the uterus (Dickson & Harding, 1989; Harding *et al.*, 1990; Savich *et al.*, 1992), resulting in increased flexion of the fetal spine and increasing abdominal, pleural and tracheal pressures, thus favouring loss of lung liquid (Harding *et al.*, 1990).

1.4.1.2 Musculo-Skeletal Disorders

As discussed previously (Section 1.3.3), fetal breathing movements (FBMs) play an important role in the regulation of lung liquid volume throughout gestation. Diaphragmatic contractions during FBMs are a requirement for maintenance of normal lung liquid volume and thus lung growth (Harding *et al.*, 1993; Miller *et al.*, 1993). Prevention of FBMs *via* phrenic nerve section results in reduced lung liquid volume and impaired lung growth (Alcorn *et al.*, 1980; Fewell *et al.*, 1981; Liggins *et al.*, 1981a). However, the reduction in lung volume is most probably due to the resultant atrophy of the diaphragm muscle (Liggins *et al.*, 1981a). The reductions in lung growth associated with the absence of FBMs are believed to be due to reduced lung expansion as a result of increased lung liquid efflux during laryngeal activation thus resulting in a decrease in lung liquid volume (Harding & Hooper, 1996).

1.4.1.3 Reductions in Intrathoracic Volumes

Space-occupying lesions in the thoracic cavity result in decreased intrathoracic volumes and lung hypoplasia. Such lesions include cysts, tumours, pleural effusions and herniation of abdominal contents through the diaphragm (Maeda *et al.*, 1989; Adzick *et al.*, 1993), with the latter condition being the most comprehensively studied, both clinically and using animal models. Congenital diaphragmatic hernia (CDH) is present in 1 in 2200 of all human births and is characterised by a diaphragm that does not close during embryonic development, allowing herniation of abdominal viscera into the thorax (Butler & Claireaux, 1962; Nathanson & Nadel, 1984). The invading

abdominal contents reduce the space within the thoracic cavity and therefore, limit the space the lungs can expand into and induce lung compression, thereby reducing or preventing lung growth. Severe pulmonary hypoplasia is the primary cause of death in these infants, particularly on the first day of birth (Nathanson & Nadel, 1984). Despite recent advances in perinatal surgical techniques, the outcome of the CDH patient remains poor (Harrison *et al.*, 1978; Adzick *et al.*, 1985; Harrison, 1996). The lungs of CDH patients are hypoplastic as well as immature, with retarded structural development, inadequate development of the surfactant system and they are incompliant (Glick *et al.*, 1992). A large body of work has focussed on understanding the mechanisms that lead to pulmonary hypoplasia associated with CDH. Consequently, two animal models of diaphragmatic hernia have been developed. The nitrofen-induced rat model involves the maternal injection of nitrofen early in development (Sluiter *et al.*, 1992; Alfonso *et al.*, 1993; Suen *et al.*, 1993; Losty *et al.*, 1996; Ijsselstijn *et al.*, 1997) which interferes with the formation of the diaphragmatic anlage (Kluth *et al.*, 1990). The resulting diaphragmatic hernia allows the penetration of abdominal contents into the thorax, limiting thoracic volume and thus lung growth. In larger animals, in particular rabbits (Tannuri *et al.*, 1998a; Tannuri *et al.*, 1998b) and sheep (deLorimier *et al.*, 1967; Glick *et al.*, 1992; Hill *et al.*, 1994; Hassett *et al.*, 1995; O'Toole *et al.*, 1996; Schnitzer *et al.*, 1996; Hedrick *et al.*, 1997; Lipsett *et al.*, 2000), surgically created hernias have been used to study many aspects of retarded lung development associated with CDH and its possible treatments. These lungs are biochemically immature as measured by disaturated phosphatidyl choline/DNA ((DSPC)/DNA), total DSPC, lung weights, lung weight/body weight, protein/DNA and total protein content (Suen *et al.*, 1993). Additionally, they have elevated collagen levels and are less compliant (Hassett *et al.*, 1995). The rat CDH model shows increased local expression of tropoelastin and type I procollagen in the lung (Taira *et al.*, 1999). Morphometric analysis of the effects of diaphragmatic hernia on morphological aspects of growth shows that there are reductions in lung volumes, gas-exchanging areas, parenchymal/nonparenchymal ratio and parenchymal airspace/tissue ratio (Lipsett *et al.*, 2000).

1.4.1.4 Gravitational Drainage or Removal of Lung Liquid

Gravitational drainage of lung liquid has been used to study the effects of decreased lung liquid volumes on lung growth (Alcorn *et al.*, 1977; Moessinger *et al.*, 1990; Nardo *et al.*, 1995; Davey *et al.*, 1999). In 1977, Alcorn *et al.* first studied the effects of lung liquid drainage over a sustained period (~24 days) in the sheep. It was shown that

lung deflation resulted in retarded lung growth and maturation as measured by lung weights and morphometric parameters such as alveolar wall thickness and altered alveolar epithelial cell proportions. Nardo *et al.* (1995) showed that lung growth deficits established *in utero* by the lung liquid drainage technique resulted in significantly reduced lung wet weights and lung DNA contents. Such lung growth deficits did not allow for survival at birth (Harding *et al.*, 2000a), but the growth deficit and consequent survival could be reversed by tracheal obstruction *in utero* (see Section 1.4.3) (Nardo *et al.*, 1995; Davey *et al.*, 1999).

1.4.2 Increased Lung Expansion and Lung Growth

A number of early studies indicated that obstruction of the fetal trachea leads to accumulation of lung liquid within the fetal lungs and ultimately lung hyperplasia (Jost & Policard, 1948; Carmel *et al.*, 1965; Lanman *et al.*, 1971). Not only did these studies suggest that the lungs were responsible for the production of liquid that expanded them, but they also indicated that it was the volume of liquid itself that was providing a stimulus for growth. Human laryngeal atresia occurs when the upper airway becomes closed by cartilaginous tissue early in gestation. This occludes the upper airways and prevents the natural efflux of lung liquid from the lungs (Smith & Bain, 1964). *Post mortem* examination of infants with laryngeal atresia shows that the condition increases lung growth (Wigglesworth *et al.*, 1987; Scurry *et al.*, 1989; Hedrick *et al.*, 1994; Harrison *et al.*, 1996) and advances alveolarisation and other markers of lung development (Silver *et al.*, 1988). The surface area of the lungs is increased, as is alveolar number and the alveolar walls are thinner (Wigglesworth *et al.*, 1987; Hedrick *et al.*, 1994). This acceleration in lung development is believed to be due to increased expansion of the lungs due to accumulation of lung liquid, because large volumes of liquid can be aspirated from the lungs of these infants (Wigglesworth *et al.*, 1987). When a complete laryngeal atresia occurs simultaneously with renal agenesis (Scurry *et al.*, 1989), the lungs are grossly enlarged and hyperplastic. In that condition, the obstruction of the upper airway is believed to prevent or reverse the lung hypoplasia normally associated with oligohydramnios induced by renal agenesis (see Section 1.4.1.1).

1.4.2.1 Tracheal Obstruction

The most commonly employed method of increasing lung expansion experimentally is to prevent the efflux of liquid from the lung lumen by either tracheal obstruction (TO) or ligating the fetal trachea. Alcorn *et al.* (1977) demonstrated that an increase in lung liquid volume, induced by tracheal ligation, not only increased lung weights, but also accelerated structural maturation of the lung. As mentioned previously, Moessinger *et al.* (1990) showed that local distension by lung liquid, rather than systemic factors, was the major determinant of fetal lung growth. It should be noted that replacing lung liquid with saline resulted in small decreases in lung liquid volumes and inhibition of hypertrophy in one study (Papadakis *et al.*, 1997), suggesting that humoral factors within the lung liquid may play a minor role in the increase in lung growth following tracheal obstruction. However, in another study, regular replacement of lung liquid with a mock lung liquid did not influence the lung growth response to 10 days of TO (Nardo *et al.*, 1998). Therefore, it is widely believed that the volume of liquid maintained within the lung is the most important factor determining the rate of lung growth in the fetus.

Increases in lung growth following TO have been shown to be time-dependent. Tracheal obstruction for a period as short as 7 days in fetal sheep results in an increase in DNA content and synthesis rates, protein content and lung dry weights, similar to those of lungs that have been obstructed for a 25 day period (Moessinger *et al.*, 1990; Hooper *et al.*, 1993b). As there is no difference in the level of expansion following 7 or 25 days of TO, the lungs have been described as being maximally expanded by 7 days following TO and further growth is probably restricted to levels similar to control fetuses due to the constraints imposed by the chest wall (Nardo *et al.*, 1998). This finding has important implications in studying the mechanisms that regulate lung growth following TO, because presumably these mechanisms are going to be most active within the first 7 days of TO.

A recent study (Nardo *et al.*, 1998) examined the time-course of the lung growth response to TO over 10 days in fetal sheep late in gestation. Lung liquid volume doubled within the first day but no further increases were recorded until after the second day of TO, after which time lung liquid volume increased linearly until day 7. Intratracheal pressure increased to maximal levels within one day and stayed at a similar level for the remainder of the 10 day period of TO. The increase in DNA content between day 2 and day 7 of TO closely paralleled the increase in lung liquid volume and was not correlated with the change in intratracheal pressure, indicating that lung

liquid volume is the stimulus for growth following TO. DNA synthesis rates were maximal (~800% greater than control) at 2 days of TO and then decreased from these very high levels to ~60% above control on days 4 and 7, returning to control levels by 10 days of TO. Similar changes in cell proliferation labelling index values have been reported in which it was shown that, at 2 days of TO, ~12% of lung cells divide over an 8 hour period (Nardo *et al.*, 2000). The initial elevation in DNA synthesis rates indicates that the cellular mechanisms which result in lung growth following TO are particularly active within the first 2 days of TO. The large increase in DNA synthesis rates in the first 2 days, in comparison to the levels on days 4 and 7, also indicates that there may be 2 different phases in the growth response following TO. We propose that there is an initial phase (0 - 2 days) in which lung liquid volumes increase rapidly, creating a stretch stimulus that results in large increases in DNA synthesis rates. In addition, as lung liquid volume does not change between 1 and 2 days of TO, the lung may have reached an initial structural limit after 1 day. Thus, a second growth phase (3 - 7 days) is hypothesised, in which further lung growth is dependent on structural remodelling of the lung. Collagen content, which is not elevated at 2 days, is elevated by 10 days of TO (Nardo *et al.*, 1998), suggesting that ECM remodelling occurs in parallel with the increase in DNA content and may be an important factor in facilitating the changes that allow increases in lung growth to occur between 3 and 7 days of TO in late gestation fetal sheep.

The effects of TO are also dependent on the gestational age of the fetus (Keramidaris *et al.*, 1996). Two days of TO late in gestation (125 - 127 days of gestation: alveolar stage of lung development) in fetal sheep induces a greater increase in DNA synthesis rates than earlier in gestation (112 - 114 days of gestation: canalicular stage of lung development) (Keramidaris *et al.*, 1996). This differential response to TO at varying gestational ages was attributed to age-related changes in lung compliance that would allow greater increases in lung liquid volume and therefore, greater increases in lung growth in more mature fetuses (Keramidaris *et al.*, 1996). Tracheal obstruction in rabbits has also been shown to be gestational age-dependent with lung growth occurring more quickly in older fetuses (De Paepe *et al.*, 1999). However, when 10 or 15 days of TO was performed in the early canalicular stage of lung development in fetal sheep (<90 days of gestation), the increase in DNA content was double that measured for the same period of TO during the alveolar stage of lung development (Hooper *et al.*, 1993b; Nardo *et al.*, 1998; Probyn *et al.*, 2000). Important differences in the type of growth that occurred in these younger fetuses were noted. Particularly, there was a large increase in the growth of the mesenchymal tissue, which is not observed in more

mature fetuses (Probyn *et al.*, 2000). The authors suggested that the large increase in mesenchyme would produce a lung structure incompatible with efficient gas exchange after birth (Probyn *et al.*, 2000).

A study by Boland *et al.* in 1997 further highlighted the vital relationship between lung compliance, lung liquid volume and lung growth. The rationale was that the increase in lung compliance induced by cortisol would allow a greater increase in lung liquid volume following TO (Boland *et al.*, 1997), due to structural alterations of the lung (Schellenberg *et al.*, 1987a). As a result of this increase in lung liquid volume, pretreatment with cortisol induced an increase in lung growth in comparison to fetuses with obstructed tracheas that did not receive the cortisol pretreatment. Importantly, this study suggested that alterations in lung expansion may be induced by structural maturation of the lung, which was accelerated in this instance by corticosteroid treatment. Together, these studies suggest that lung growth is profoundly influenced by mechanical distension and that the compliance of the lung regulates the type and degree of growth that occurs.

1.4.2.2 *In Vitro* Models of Stretch-Induced Lung Growth

A number of studies have focussed on stretching fetal lung cells *in vitro* to elucidate the relationship between increases in lung distension and lung growth (Skinner, 1989; Liu *et al.*, 1992; Bishop *et al.*, 1993; Scott *et al.*, 1993). It should be noted that these studies all utilise phasic stretch to simulate FBMs and are not designed to mimic the effects of sustained stretch that is induced by TO. Isolated fibroblasts or type II AECs are plated on three-dimensional matrices and phasically stretched and relaxed in a manner generally designed to replicate FBMs in terms of frequency and duration. However, *in utero*, individual FBMs occur in an isovolumetric manner and result in only minor changes in thoracic dimensions (Harding & Liggins, 1996). In addition, the composition of the cells in culture does not accurately mimic the cellular organisation of the lung *in vivo*. Nevertheless, these techniques have led to interesting results, including increases in cell number and increases in DNA synthesis rates (Liu *et al.*, 1992), in phasically stretched lung cell cultures when compared to unstretched lung cells. Such results support the belief that mechanical forces act directly on lung cells to stimulate lung growth and support a role for FBMs in normal lung growth (Liu *et al.*, 1992). Increases in growth promoting substances such as platelet-derived growth factor BB (PDGF-BB) and its receptor are thought to be important in mediating the

growth that occurs as a result of phasic stretch. The role of such growth factors and the response of lung cells to stretch will be discussed shortly (Section 1.4.4).

1.4.3 Effects of Altered Lung Expansion on Lung Development

Structural maturity of the lungs is also a feature of lungs exposed to periods of increased lung expansion. Hashim *et al.* (1995) showed that long-term TO increased airspace fraction as well as radial alveolar counts and alveolar number per body weight. In fact, Nardo *et al.* (2000) completed detailed morphometric analysis of lung structure and showed that increases in luminal volume, alveolar number and surface area also occur in a time-dependent manner following tracheal obstruction. Alterations in the level of lung expansion also affect AEC phenotype, such that a decrease in lung expansion promotes an increase in type II AECs while an increase in lung expansion decreases type II AECs (Alcorn *et al.*, 1977; Bin Saddiq *et al.*, 1997; Piedboeuf *et al.*, 1997; De Paepe *et al.*, 1998; Flageole *et al.*, 1998; Flecknoe *et al.*, 2000). Flecknoe *et al.* (2000) showed that there is a time-dependent change in the proportion of AECs with a decrease in the proportion of type II AECs within 2 days of TO and a delayed increase in the proportion of type I AECs. A transient increase in an intermediate cell type, displaying characteristics of both cells, also occurred suggesting type II AECs transformed into type I AECs (Flecknoe *et al.*, 2000). Not surprisingly, the expression and production of surfactant proteins and the number of surfactant-storing lamellar bodies are also reduced following TO (Piedboeuf *et al.*, 1997; Benachi *et al.*, 1999; Lines *et al.*, 1999). In addition, TO has been shown to reduce pulmonary compliance (O'Toole *et al.*, 1996) and to impair pulmonary blood flow (Papadakis *et al.*, 1998).

The generally positive influences of TO on lung growth and development have led to the suggestion that TO may be beneficial in accelerating lung growth in order to reverse pre-existing lung growth deficits. In instances of pre-existing pulmonary hypoplasia, TO has been utilised to return many indices of lung development to normal when merely correcting the underlying physiological problem had limited beneficial effects (Nardo *et al.*, 1995). Nardo *et al.* (1995) showed that lung hypoplasia, as a result of prolonged lung liquid drainage, may be reversed by as little as six days of TO. In fact, TO has been used in human fetuses as a means of correcting lung growth deficits *in utero* (Flake, 1996; Harrison *et al.*, 1996; Graf *et al.*, 1997). However, problems associated with human fetal surgery, existing complications with the fetus

and/or mother and differing rates of growth following TO in humans mean that this technique is being trialled with limited success (Harrison *et al.*, 1996). Therefore, despite marked increases in biochemical growth and morphological changes following TO, deleterious effects including reductions in type II AECs and surfactant production and limited success in the clinical environment mean that the therapeutic use of TO in humans requires further experimentation before it can be more successfully applied.

1.4.4 Mechanisms Mediating the Effects of Expansion on Lung Growth

It is now clear that alterations in lung liquid volume result in biochemical and morphological changes in the lung. The mechanisms by which these changes in lung liquid volumes are translated into a stimulus at the cellular level are unknown, although they are likely related to the ability of the cells themselves to sense changes in their environment. Changes in lung liquid volume and the resulting changes in cell shape and the matrix surrounding them may be due to a number of signalling systems that ultimately result in changes in cell proliferation, differentiation, protein synthesis and ECM remodelling. Such changes may be mediated by a number of mechanisms which include local production of growth factors (reviewed in: Tanswell *et al.*, 2000), ECM-mediated signalling, *i.e. via* integrins (discussed in detail later in this literature review: 1.6.4), or by stretch-sensitive ion channels.

Stretching lung cells *in vitro* and *in vivo* has been shown to have variable effects on the expression of growth factors such as platelet-derived growth factor (PDGF) (Liu *et al.*, 1992; Liu *et al.*, 1995a; Liu *et al.*, 1995b; Wallace *et al.*, 2002). *In vitro*, Liu *et al.* (1995c) showed that mechanical strain increased the expression and protein concentration of PDGF-B and its receptor (PDGF-β–R). The addition of PDGF-B antisense oligonucleotides or a receptor antagonist abolished the increase in DNA synthesis associated with mechanical strain (Liu *et al.*, 1995a). *In vivo*, however, PDGF-B mRNA expression was decreased following 2, 4 and 10 days of tracheal obstruction (Wallace *et al.*, 2002), suggesting that it is not involved in the increase in DNA synthesis associated with TO. Recently, calmodulin 2 gene expression was shown to be elevated after 2 days of TO, returning to control levels after 4 and 10 days of TO (Gillett *et al.*, 2002). As calmodulin is essential for cell proliferation, the authors suggested a role for calmodulin in the expansion-induced increases in DNA synthesis rates (Gillett *et al.*, 2002). In addition, other growth factors such as IGF-II (Hooper *et*

al., 1993b; Wallace *et al.*, 2002) and TGF-β (Liu *et al.*, 1995a; Wallace *et al.*, 2002), are expressed following alterations in lung stretch. IGF-II decreases *in vivo* with a decrease in lung expansion and conversely increases following an increase in lung expansion (Harding *et al.*, 1993; Hooper *et al.*, 1993b; Wallace *et al.*, 2002). However, the increase in IGF-II and TGF-β expression does not occur until 7 or 10 days of tracheal obstruction, when the majority of the increases in lung growth have already occurred (Hooper *et al.*, 1993b; Wallace *et al.*, 2002). Stretch-induced upregulation of VEGF gene expression and protein levels have been demonstrated *in vitro* using mixed pulmonary cells (Muratore *et al.*, 2000). *In vivo*, VEGF expression decreased following tracheal obstruction (Wallace *et al.*, 2002) and is, therefore, unlikely to play a role in stretch-mediated lung growth following TO. IGF-I gene expression is decreased in experimental diaphragmatic hernia, but is restored following tracheal ligation (Nobuhara *et al.*, 1998).

Evidence for the presence of stretch-sensitive ion channels in fetal lung cells has been provided (Liu *et al.*, 1994). Ion channels are transmembrane proteins that permit signalling of cells by controlling the movement of ions (Riley *et al.*, 1990). Such channels are believed to allow calcium fluxes, followed by downstream activation of the cyclic AMP-dependent protein kinase (Riley *et al.*, 1990). Indeed, Liu *et al.* (1995c) concluded that the effects of mechanical strain on fetal lung cells was mediated by phospholipase C, D and protein kinase C in fetal lung cells. Alternatively, a direct force may act upon a cell to redistribute tension-bearing cytoskeletal and contractile elements (see Section 1.6.4; Riley *et al.*, 1990). Thus, mechanical forces may be transmitted directly to the nucleus *via* changes in extracellular and intracellular structure. Although we are only beginning to understand the complex nature of cellular responses to stretch, the interplay between the ECM, growth factors, ion channels and secondary messengers seemingly provide a signalling system that is acutely sensitive to changes in mechanical forces and therefore, alterations in lung expansion.

1.5 Corticosteroids and Fetal Lung Development

Corticosteroids play a crucial role in preparing the fetal lung for life after birth. Liggins first noted that corticosteroids accelerated lung maturation in 1969 (Liggins, 1969) and

since then numerous studies have focussed attention on this important group of steroid hormones. Corticosteroids are used widely to reduce the severity of respiratory distress syndrome (RDS) suffered by prematurely born infants (Moya & Gross, 1988; Crowley *et al.*, 1990) and in combination with other hormones and treatments, particularly with postnatal surfactant treatment, have greatly improved neonatal outcomes (Farrell *et al.*, 1989; Jobe *et al.*, 1993). The impacts on lung development in premature infants are great, however, focus is now turning to short- and long-term effects of perinatal corticosteroid treatment.

Fetal production of cortisol increases exponentially during the last 12 days of gestation in the ovine fetus (Magyar *et al.*, 1980; Wintour *et al.*, 1980). It is this increase in fetal plasma cortisol concentrations that induces many maturational changes in the fetus, including maturation of the lung and it is also responsible for inducing labour in the sheep (reviewed in: Liggins, 1994). Indeed the increase in fetal plasma cortisol concentrations parallels the increase in lung compliance and the increase in fetal lung liquid volumes prior to birth (Schellenberg & Liggins, 1987).

1.5.1 Cortisol Production in the Fetus

Cortisol is produced by the fetal adrenal gland (Nathanielsz, 1976) in response to adrenocorticotrophic hormone (ACTH), which is produced in the anterior pituitary gland. From 90 - 120 days of gestation in sheep, fetal plasma cortisol concentrations are relatively low and a large proportion of the cortisol is of maternal origin (Beitins *et al.*, 1970). After 130 days of gestation, fetal plasma cortisol concentrations increase markedly from ~1.5 ng/ml at 130 days of gestation, to > 50.0 ng/ml at term (~147 days) (Bassett & Thorburn, 1969; Mescher *et al.*, 1975). In late gestation, fetal adrenalectomy prevents the increase in circulating cortisol concentrations, further demonstrating that the increase is the result of increased production by the fetal adrenal gland, rather than increased passage through the placenta (Wintour *et al.*, 1980; Liggins *et al.*, 1985).

1.5.2 Corticosteroids and Fetal Lung Development

Initially, maternal administration of corticosteroids was shown to cause premature delivery in fetal sheep, but was also associated with accelerated lung development in

the fetus so that these newborns were able to survive (Liggins, 1969). The survival of the newborn lambs was attributed to acceleration in the development of the surfactant system. Since this time, corticosteroids have been shown to have effects on a variety of developmental parameters such as lung volume, surfactant properties and ultrastructural changes that lead to increases in lung compliance.

1.5.2.1 Effects of Corticosteroids on Lung Liquid Volumes

Exogenous corticosteroids result in an increase in lung liquid volume in a number of animal models (Platzker *et al.*, 1975; Johnson *et al.*, 1978; Beck *et al.*, 1981; Seidner *et al.*, 1988; Kendall *et al.*, 1990; Ikegami *et al.*, 1995; Wallace *et al.*, 1995). On the other hand, in adrenalectomised fetal sheep, whereby the primary source of fetal cortisol is removed, there is a decrease in both the volume and secretion rate of fetal lung liquid (Wallace *et al.*, 1996). Following pretreatment with cortisol, TO resulted in a greater increase in lung liquid volume than occurred with TO alone and this greater increase occurred without any change in intraluminal pressure (Boland *et al.*, 1997) relative to non-cortisol treated fetuses. Therefore, the greater increase in lung liquid volume was attributed to an associated increase in fetal lung compliance. This hypothesis is supported by previous work that has shown that corticosteroids alter the mechanical properties of the lungs, leading to increased lung compliance (see Section 1.5.2.2) (Schellenberg *et al.*, 1987a). This increase in lung compliance also occurs in the liquid-filled lung indicating that it is partially independent of changes in surfactant synthesis.

1.5.2.2 Effects of Corticosteroids on Lung Compliance, Collagen and Elastin

Lung compliance is essentially the ability of the lungs to expand in response to increased pressure and is, therefore, a measure of distensibility, or the ability of the lungs to stretch. As there is no air-liquid interface in the fetal lung, increases in pulmonary compliance must be due to alterations in lung architecture, possibly *via* alterations in ECM proteins such as collagen and elastin. Several studies have found an increase in collagen content (Beck *et al.*, 1981; Schellenberg & Liggins, 1987; Campos *et al.*, 1992), elastin content (Schellenberg & Liggins, 1987; Campos *et al.*, 1992) and tropoelastin gene expression (Pierce *et al.*, 1995a) following corticosteroid treatment. However, these findings are contradictory to many studies *in vitro* that have shown that corticosteroids impair collagen synthesis (Kivirikko & Laitinen, 1965; Cutroneo *et al.*, 1981; Kehrer *et al.*, 1983).

1.5.2.3 Effects of Corticosteroids on Surfactant Proteins

A sudden increase in fetal plasma corticosteroids does not seem to be the primary stimulus for the appearance of surfactant, as surfactants are found in lung liquid in fetal sheep from up to 10 days before the increase in endogenous corticosteroids occurs (Mescher *et al.*, 1975). However, corticosteroids are believed to induce the rapid accumulation of surfactant that occurs prior to delivery (Mescher *et al.*, 1975). Exogenous corticosteroids accelerate most aspects of the surfactant synthesis pathway: by maturing type II AECs which produce surfactant (Kikkawa *et al.*, 1971; Stettner & Ledwozyw, 1995), by increasing synthesis of disaturated phosphatidyl choline (DSPC) (Brumley *et al.*, 1977) and by synthesis of the surfactant proteins themselves (Motoyama *et al.*, 1971). There are however, some discrepancies within the literature which raise questions as to the effects of increased corticosteroids prior to birth on surfactant. Removal of the pituitary gland (hypophysectomy (HX)) results in inadequate function of the adrenal gland and due to a lack of adrenocorticoid activity, surfactant synthesis is impeded (Liggins *et al.*, 1981b; Crone *et al.*, 1983). However, cortisol treatment of hypophysectomised fetal sheep did not increase the phospholipid content (Liggins *et al.*, 1981b). Recent observations from transgenic mice (review: Jobe & Ikegami, 2000) suggest that low, maternal levels of corticosteroids are sufficient to support some aspects of lung maturation (Muglia *et al.*, 1999), including development of the surfactant proteins. Furthermore, most mice that are glucocorticoid receptor deficient die soon after birth as a result of inadequate airway development, but these mice have normal amounts of surfactant proteins (Cole *et al.*, 1995).

1.5.2.4 Effects of Corticosteroids on Lung Morphometry

Exogenous corticosteroids accelerate many aspects of structural maturation of the lung (Kikkawa *et al.*, 1971), including increased air spaces, thinning of alveolar septa (Crone *et al.*, 1983) and invasion of capillaries into alveolar walls (Ballard, 1986). Corticosteroid treatment of fetuses also decreases the parenchymal/nonparenchymal ratio (Kendall *et al.*, 1990) in the lung and decreases interstitial thickness and septal wall thickness (Losty *et al.*, 1995). Conversely, fetal hypophysectomy, to eliminate endogenous corticosteroids, results in fetuses with thicker alveolar walls (Liggins *et al.*, 1981b; Crone *et al.*, 1983). Treatment of HX fetuses with ACTH or cortisol restores these values to near control levels (Liggins *et al.*, 1981b; Crone *et al.*, 1983). Recent studies using corticosteroids as a possible treatment *in utero*, for the correction of lung hypoplasia associated with experimental diaphragmatic hernia, have shown a number

of improvements in lung morphometry (Losty *et al.*, 1995; Losty *et al.*, 1996; Hedrick *et al.*, 1997). The lungs of the diaphragmatic hernia fetuses treated with corticosteroids show improvements in lung structure such that the lungs in these animals are similar to the lungs of normal age-matched controls (Losty *et al.*, 1995; Losty *et al.*, 1996; Hedrick *et al.*, 1997). However, high dose treatments of corticosteroids such as those performed by Hedrick *et al.* (1997) have been shown to have detrimental effects on lung and body growth (see Sections 1.5.2.5 and 1.5.2.6).

1.5.2.5 Effects of Corticosteroids on Lung Growth

The effects of corticosteroids on fetal lung growth are variable depending on the dose and age at treatment. Late in gestation exogenous corticosteroids have been shown to inhibit lung growth (DeLemos *et al.*, 1970; Kotas & Avery, 1971; Motoyama *et al.*, 1971; Hitchcock, 1980; Kotas & Avery, 1980; Stein *et al.*, 1993). Likewise, maternal delivery of cortisol analogues at high doses has also been shown to reduce fetal lung weights (Suen *et al.*, 1994; Ijsselstijn *et al.*, 1997). In contrast, fetal administration of cortisol at physiological levels does not reduce fetal lung weights (Wallace *et al.*, 1995; Boland *et al.*, 1997) and removal of the adrenal gland (adrenalectomy) has been shown to reduce lung weights indicating that cortisol is required for basal lung growth (Liggins *et al.*, 1985; Wallace *et al.*, 1996). It is becoming apparent that both route of delivery, number of doses (French *et al.*, 1999), timing and dose of the corticosteroids are important in determining whether growth restriction occurs. Given the nature of the effects of cortisol, it is important that lung weights be recorded to assess whether dose and route of delivery are having negative effects on lung growth. In one particular study, the dose of cortisol administered was 12 times the cumulative dose (Schnitzer *et al.*, 1996) used previously within our laboratory (Wallace *et al.*, 1996; Boland *et al.*, 1997). Although the authors found significant improvements in compliance (Schnitzer *et al.*, 1996) as well as in morphometric factors (Hedrick *et al.*, 1997), no data of the effects of the corticosteroid treatment on lung or body growth, or on total lung DNA or protein were presented. In light of the deleterious effects that such high doses of corticosteroids have on lung growth, failure to include data on lung weights is surprising.

1.5.2.6 Effects of Corticosteroids on Organ and Body Growth

Generally, corticosteroids produce beneficial effects on organ maturation, however the large doses of synthetic corticosteroids administered before prenatal delivery may also have serious side-effects (reviewed in: Nyirenda & Seckl, 1998; Seckl, 1998; Seckl, 2001), including a reduction in general body growth and impairment of neurodevelopment (Cotterrell *et al.*, 1972; Gumbinas *et al.*, 1973; Weischsel, 1974; Uno *et al.*, 1990; Uno *et al.*, 1994; Dunlop *et al.*, 1997; Smith *et al.*, 2000). Furthermore, an increasing body of evidence is accumulating indicating that elevated corticosteroids during fetal life are associated with an increased risk of cardiovascular problems from adolescence (Barker *et al.*, 1993; Doyle *et al.*, 2000) and increased glucose intolerance in adults (Nyirenda *et al.*, 1998). Direct fetal injection of betamethasone in sheep did not affect fetal body weights whereas the same dose directed maternally produced significant reductions in fetal body weight, placental weight and the weights of other major organs in the fetus (Newnham *et al.*, 1999). Repetitive doses of corticosteroids in sheep have also resulted in reductions in fetal body weights (Losty *et al.*, 1995; Ijsselstijn *et al.*, 1997; Ikegami *et al.*, 1997): by 15% after one dose, 19% after two doses and by 27% after three doses (Ikegami *et al.*, 1997). However, physiological doses of corticosteroids administered to fetal sheep, do not affect fetal body weights (Wallace *et al.*, 1995; Boland *et al.*, 1997). In these studies, increasing doses of cortisol were administered by an intravenous infusion into the fetus. The dose of cortisol was intended to mimic the preparturient rise in endogenous cortisol (Magyar *et al.*, 1980; Wintour *et al.*, 1980) and as such was considered to be a 'physiological dose'. Such doses were able to induce changes in lung maturational indices without altering fetal organ or body weights.

1.5.3 Corticosteroid Receptors and Signalling Pathways

The corticosteroid/glucocorticoid receptor (GR) has been localised to the cytoplasm in an inactive form when not bound by glucocorticoid (Nemoto *et al.*, 1990). Free GR forms complexes with the 90-kDa heat shock protein (HSP 90) and disruption of this complex yields a receptor incapable of binding glucocorticoid (Nemoto *et al.*, 1990). When glucocorticoids bind to the GR, the resulting complex is translocated to the nucleus where it binds to specific DNA elements known as glucocorticoid response elements to enhance or repress transcription of target genes (Nemoto *et al.*, 1990). The GR-hormone complex appears to modulate transcription by altering the formation of a productive transcription complex at the promoter, either by interaction with general

transcription factors (McEwan *et al.*, 1993) or by inducing changes in chromatin structure (Cordingley *et al.*, 1987). GR has been detected in adult and fetal lung of a variety of species (Beer *et al.*, 1983), and in many different cell types including type II AECs (Ballard & Ballard, 1974, Flint & Burton, 1984). The importance of GR in lung development has been highlighted by GR knockout mice which die of respiratory failure shortly after birth (Cole *et al.*, 1995). The lungs of these mice were severely atelectatic, with impairment of development from day 15 of gestation (term is 21 days) (Cole *et al.*, 1995). Recently, two isoforms of GR in humans were identified, hGRα and hGRβ, with hGRα protein detected in human lung epithelial cells (Pujols *et al.*, 2001). Both of these GR isoforms have been shown to be regulated by exogenous glucocorticoids (Pujols *et al.*, 2001). GR increases nearly sevenfold in ovine lung tissue during the second trimester and then declines somewhat during the third trimester (Flint & Burton, 1984). Therefore, the rise in GR concentration during mid-gestation may be a major mechanism by which corticosteroids can modulate lung maturation.

1.6 Lung Structure and the Extracellular Matrix

The ECM is a network of fibres that regulates a number of functions. Many of the components of the ECM interact with each other, resulting in a heterogeneous network that provides the lung with structural integrity. In the lung, the major component of the ECM is collagen, with elastin being the next most abundant protein. Collagen and elastin have been shown to be vital proteins in the normal function and development of the lung, whereas other ECM proteins, such as proteoglycans and glycoproteins may also play important roles. Additionally, the regulation of ECM protein turnover, by matrix metalloproteinases (MMPs) is of importance in ECM remodelling during lung development.

1.6.1 Collagen

Collagen is the major structural protein of the lung (Bradley *et al.*, 1974) and there are a number of different types of collagen found within the lung. Type I collagen provides tensile strength and is mainly found in bronchi and blood vessels with a small amount found in the interstitium (Seyer *et al.*, 1976; McLees *et al.*, 1977; Madri & Furthmayr,

1980; Davila *et al.*, 1995). Type II collagen is found in bronchial cartilage (Bradley *et al.*, 1974). Type III collagen is found mostly in the interstitium and alveolar septa, aggregated at the entrance rings of alveoli (Raghu *et al.*, 1985). Type III collagen provides the lung with flexibility and is quite pliable (Bradley *et al.*, 1974). Type IV and V collagens are found in the basement membrane (Kefalides, 1975) and are secreted by type II AECs (Sage *et al.*, 1983). Other collagens are also found in the lung at low levels (Bradley *et al.*, 1974). A number of growth factors influence the synthesis and secretion of collagen, including TGF-β and corticosteroids (Riley, 2000). TGF-β increases collagen production by fibroblasts (MacFarlane *et al.*, 1993), whereas corticosteroids have varied effects on collagen production and turnover (see Section 1.5.2.2).

The importance of collagen in branching morphogenesis during lung development is well established (Spooner & Faubion, 1980; Deterding & Shannon, 1995). In addition, studies in which collagen synthesis is prevented provide an insight into the importance of collagen during lung development (see Section 5.1). Lung collagen content increases towards the end of gestation (Schellenberg & Liggins, 1987), and may play a role in increasing lung compliance with gestational age. Collagen synthesis is high during development (Tolstoshev *et al.*, 1981; Jackson *et al.*, 1990), peaking during alveolarisation (Bradley *et al.*, 1974). Net collagen turnover is also highest during development (Mays *et al.*, 1989) and this is accompanied by elevated procollagen message levels (Larson, 1993). In fetal and postnatal rats, up to 40% of new collagen is rapidly degraded (Arden & Adamson, 1992; Arden *et al.*, 1993). Therefore remodelling of the lung interstitium by rapid turnover of lung collagen may facilitate rapid changes in lung structure during lung development. The role of collagen in alterations in lung expansion is not as well understood. Increases in lung expansion induced by TO result in elevated lung collagen content (Nardo *et al.*, 1998), which may be the result of alterations in the rates of collagen degradation and synthesis. Alterations in collagen turnover during TO may contribute to remodelling of the ECM and may be responsible for the second phase of the lung growth response to TO. Collagen content following a period of lung deflation is likely to be reduced, but the mechanisms that lead to these changes in collagen content in the lung following alterations in lung expansion are not known.

1.6.2 Matrix Metalloproteinases

Remodelling of the ECM plays an important role in the growth and structural development of many organs including the lung. One of the families of enzymes responsible for this remodelling is the matrix metalloproteinases (reviewed in: Senior & Shapiro, 1992; Ray & Stetler-Stevenson, 1994; Shapiro & Senior, 1999; Parks & Shapiro, 2001). MMPs are characterised as a family of at least 20 proteinases that degrade at least one component of the ECM, contain zinc at the catalytic site, are inhibited by their specific inhibitors (tissue inhibitors of metalloproteinases: TIMPs) and have similar amino acid sequences (Parsons *et al.*, 1997). MMPs are capable of degrading collagens, proteoglycans, fibronectin and laminin (Matrisian, 1990; Bode *et al.*, 1999) and in so doing play a role in facilitating cell migration, altering cellular behaviour, regulating three-dimensional structure and modulating the activity of biologically active molecules (Vu & Werb, 2000). MMPs are secreted from a variety of cell types in latent form and are activated by cleavage of the propeptide domain. The persistence of the propeptide domain in secreted MMPs is of extreme importance in the regulation of their activity. Therefore, turnover of the ECM is tightly controlled by the types of MMPs synthesised, how the activation takes place and the balance between net production of MMPs and their natural inhibitors, the TIMPs (Nagase, 1994).

Extracellular degradation of collagen is primarily due to the activity of the MMPs (Matrisian, 1990; Birkedal-Hansen *et al.*, 1993; Dollery *et al.*, 1995), and in particular by the gelatinases: MMP-2 (gelatinase A) and MMP-9 (gelatinase B). Gelatinases can degrade native collagens I, IV, V, VII and X to some extent but they are more active against the denatured form of collagen, gelatin (Murphy *et al.*, 1990; Corbel *et al.*, 2000). Thus, MMP-2 and -9 are thought to be important in the final degradation of fibrillar collagens after they have first been cleaved by collagenases and denatured (Kahari & Saarialho-Kere, 1999). There is a suggestion that the expression of proteases and their inhibitors is likely to be highly regulated during lung development when the lung is undergoing major structural changes (Hoidal & Hoidal, 2000), and increased levels of gelatinases may indicate that a tissue is going through active remodelling. Large decreases in parenchymal tissue late in gestation give rise to a greater airspace and this change is principally believed to be due to tissue remodelling. As substantial remodelling of the lung occurs following TO, it is highly likely that the gelatinases are playing an integral role in this process. *In vitro*, alterations in mechanical stretch of fetal lung cells has had variable effects on gelatinase expression

and levels (Tomasek *et al.*, 1997; Xu *et al.*, 1999). At present, no *in vivo* data exists on the effect of alterations in lung expansion on gelatinase levels; this was the subject of investigation in Chapter 4.

1.6.3 Elastin and Other Extracellular Matrix Proteins

Elastin gives a tissue compliance and in the case of the lung, the ability to recoil after transient stretch (Foster & Curtiss, 1990; Swee *et al.*, 1995). In neonates, pulmonary elastin mRNA (tropoelastin) is expressed in vascular smooth muscle cells and interstitial fibroblasts (Bruce, 1991). Elastin production increases during late fetal and early neonatal life and it ceases when the lungs are fully mature (Pierce *et al.*, 1995a). In particular, there is a steep rise in elastin accumulation during the canalicular period of lung development (Schellenberg & Liggins, 1987). Elastin deposits are greatest at the points from which the future alveolar septae, or secondary crests will protrude (McGowan, 1992), but it can also be found in the walls of bronchi, bronchioles, blood vessels and pleura (Fukuda *et al.*, 1984). The number of elastin fibres in the interalveolar walls increases until adult life (Quaglino *et al.*, 1993). The elastin promoter contains many potential binding sites for transcriptional regulatory factors including those for corticosteroids (Rosenbloom *et al.*, 1995) and maternal administration of corticosteroids increases elastin in the fetus (Pierce *et al.*, 1995b), as does dexamethasone treatment of organotypic lung cells in culture (Nakamura *et al.*, 2000).

Glycoproteins are a group of proteins that include fibronectin, tenascin, laminin and the family of receptors; the integrins. Fibronectin is a ubiquitous glycoprotein found in high levels in the developing lung, decreasing in late gestation and found only in low levels in the neonate (Sinkin *et al.*, 1995). It is a fibrillar component of the ECM and is primarily an adhesive glycoprotein that modifies matrix assembly. Fibronectin binds to integrins and modifies cell phenotype and may play a role in inducing MMPs (Werb *et al.*, 1989). Vitronectin interacts with cell surfaces and the ECM (Yamada *et al.*, 1985) and is a normal component of lung epithelial lining fluid (Roman *et al.*, 1990) Laminin is the most prevalent glycoprotein in basement membranes and is particularly important in cell adhesion and migration during lung development (Roman *et al.*, 1990). Tenascin and laminin both play roles in branching morphogenesis, promoting outgrowth and elongation of lung buds and influencing the structure of the basement membrane at the epithelial-mesenchymal interface (Abbott *et al.*, 1991; Thomas &

Dziadek, 1994). Integrins also belong to the glycoprotein family and are involved in ECM signalling (see Section 1.6.4).

Proteoglycans are hydrophilic molecules that form porous ground substance that attracts water, enabling tissue to withstand compressive forces (Roman & McDonald, 1992). Proteoglycans are a subset of glycoproteins, distinguished by glycosaminoglycan chains attached to a protein core. Proteoglycans are found in basement membranes and in the interstitium, where they bind and release growth factors and regulate cells involved in the synthesis of ECM proteins (Massague & Pandiella, 1993). This observation suggests that proteoglycans may mediate structural organisation and other facets of fetal lung development. Syndecan is a cell surface proteoglycan that binds to collagen, fibronectin, thrombospondin and growth factors (Bernfield & Sanderson, 1990), such as FGF and TGF-β (Ruoslahti & Yamaguchi, 1991). β-xyloside is an inhibitor of proteoglycans and when 16 day fetal mice were treated with β-xyloside, pseudoglandular lung epithelium did not form respiratory endings, contained fewer specialised cells and accumulated little additional surfactant (Smith *et al.*, 1990). This finding suggests that deposition of an ECM rich in proteoglycans is required to support maturation of the respiratory epithelium.

1.6.4 ECM Signalling Pathways

There is no doubt that the importance of ECM signalling, *via* cell-cell and cell-matrix communication has been the focus of much research recently. Cell surface receptors such as integrin receptors and clusters of intra- and ECM proteins known as focal adhesion points, help confer information that alters cellular activity and the environment around the cell. This section contains a broad overview of ECM signalling pathways with reference to lung-specific data (reviews: Liu & Post, 2000; Wirtz & Dobbs, 2000) where available.

Cytoskeletal components and intracellular molecules involved in signalling cascades aggregate at specialised focal adhesion points, which facilitate transfer of information into and out of cells (Spurzem, 1996). Integrins have been shown to bind to specific matrix proteins and can associate with clusters of receptors on the cell surface at these focal adhesion points (Spurzem, 1996). At these points are sites where cytoskeletal elements, including actin, interact with the cytoplasmic tails of the receptors, therefore

linking intra- and extracellular fibres (Spurzem, 1996). The theory that the assembly of the cytoskeleton regulates cell behaviour towards mechanical forces has been called 'tensegrity' (Ingber, 1991; Ingber *et al.*, 1993). Mechanical forces may be transmitted to the nucleus from the ECM, directly by intermediate filaments that bind to actin filaments, or by a number of second messenger pathways (Spurzem, 1996). Cells also communicate changes in structure directly to other neighbouring cells and examples of this have been shown in lung fibroblasts and human bronchial epithelial cells (Swartz *et al.*, 2001).

Integrins are transmembrane cell surface glycoproteins that play a vital role in cell-substrate adhesion and therefore extracellular signalling. Integrins are heterodimers composed of one of 15 α and one of 8 β subunits (Ruoslahti & Pierschbacher, 1987; McDonald, 1989; Akiyama *et al.*, 1990; Albelda & Buck, 1990), that bind extracellular components such as fibronectins, vitronectins and collagens (Gailit & Ruoslahti, 1988) and interact with the cytoskeleton of the cell (Buck & Horwitz, 1987; Burridge *et al.*, 1987, Roman *et al.*, 1989). β1 and β3 integrins are detected in developing lungs at very early stages of development in the fetus, with α5β1 being found in mesenchymal cells in murine lungs as early as 11 days of gestation (Roman & McDonald, 1992). The α3 subunit is expressed in epithelial cells of developing airways and αvβ3 receptors are present in epithelial and mesenchymal cells (Roman *et al.*, 1991). The α1, α2, α6 and α9 subunits are all present in the developing lung (Edelman *et al.*, 1994; Wang *et al.*, 1994) and the α1β1 and α2β1 heterodimers have been shown to function as collagen binding receptors in fetal lung fibroblasts (Caniggia *et al.*, 1995). Several reports have demonstrated regulation of gene expression by ligand binding to integrins (Werb *et al.*, 1989), and integrin signalling appears to mediate synthesis, degradation and organisation of the ECM (Riley, 2000). Integrins are involved in the signalling that mediates the secretion of MMPs and protease inhibitors (Werb *et al.*, 1989). This finding is of particular importance when we consider that when the lung is under- or over-distended, changes in physical forces may alter integrin signalling, resulting in a transformation in the surrounding ECM. Alterations in tension in the ECM may be transmitted to the nuclei of cells *via* the cytoskeleton. In addition, breakdown of the ECM may affect the signalling into a cell and alter production of extracellular proteins or ultimately cell division.

1.7 Summary and Aims

Increases and decreases in lung liquid volume alter the degree of lung expansion and have profound influences on lung growth. Cortisol also profoundly affects lung growth and is believed to be responsible for maturation of many fetal organs late in gestation. Exogenous corticosteroids have been shown to increase biochemical and structural aspects of lung maturity and there is growing interest in using them to treat pulmonary hypoplasia. Animal studies have shown that administration of cortisol to the fetus in physiological doses increases lung liquid secretion rates, lung volumes and lung growth. The effect of cortisol on lung growth in such instances may be mediated by its ability to increase lung compliance thereby allowing greater increases in lung liquid volumes. If lung liquid volume is reduced by lung liquid drainage, the effects of cortisol on lung structure and lung growth may be prevented. In addition, numerous recent studies have shown that high-dose corticosteroids induce lung maturation at the expense of lung, body and other organ growth. Therefore, it is important to understand the effects of physiological doses of cortisol on lung growth and structural maturation during lung deflation and lung hypoplasia.

AIM 1: To determine the effects of a corticosteroid treatment at physiological doses on fetal lung growth and maturation in a lung undergoing continuous lung liquid drainage (Chapter 3).

Increases in lung liquid volume are highly correlated with increases in lung growth. A number of recent studies have shown that the growth response to tracheal obstruction is time-dependent and that most of the growth in the fetal sheep lung is completed within seven days of tracheal obstruction. Additionally, within this seven-day timeframe, the lung growth appears to occur in different phases. For example, within the first day of tracheal obstruction, lung liquid volumes increase by 100% and then do not increase further until after two days of tracheal obstruction. In addition, DNA synthesis rates are highly elevated above control values within the first two days of tracheal obstruction. However, after two days of tracheal obstruction, these DNA synthesis rates, although still elevated above control values, are not markedly reduced compared with this initial large increase. In contrast, collagen appears to remain close to control values within the first two days of tracheal obstruction, before increasing above control values by seven days of tracheal obstruction. These observations suggest that there may be a biphasic growth response to an increase in lung expansion

induced by tracheal obstruction. An initial phase (0 - 2 days) may involve the rapid increase in lung liquid volumes, which by mechanosignalling would stimulate the large increases in DNA synthesis rates. After this time (3 - 7 days), structural changes must take place to allow for further increases in lung liquid volume and therefore, lung growth to occur. In particular, collagen remodelling by gelatinases (Aim 2), or *de novo* synthesis of collagen (Aim 3), may allow the luminal volume to increase. We suggest that the second growth phase following TO is dependent upon the synthesis of new collagen. Therefore, we have hypothesised that a collagen synthesis inhibitor will prevent the increase in lung growth that normally occurs following TO.

AIM 2: To determine the levels of the gelatinases MMP-2 and MMP-9, following periods of lung expansion, lung deflation and treatment with corticosteroids (Chapter 4).

AIM 3: To examine the effects of a collagen synthesis inhibitor, L-Azetidine 2-Carboxylic Acid (LACA), on lung growth following tracheal obstruction (Chapter 5).

CHAPTER 2 General Methods

2.1 Animal Welfare

Ewes were placed in metabolism cages for approximately one week prior to surgery to ensure that they had acclimatised and were feeding properly. Ewes were fed each day with lucerne chaff and were given free access to water. Night/day cycles were regulated by timers to provide 12 hours of light daily and the ambient temperature was maintained between 18 and 20 °C. 24 hours prior to surgery the ewes were denied access to food but were allowed unrestricted access to water. All experimental procedures on animals were approved by the Monash University Committee for Ethics in Animal Experimentation.

2.2 Fetal Surgery

Surgery was performed on pregnant Merino X Border-Leicester ewes and fetuses at various gestational ages depending on experimental protocol: refer to individual chapters for details. Anaesthesia of the ewe and fetus was induced using an intravenous injection of thiopentone sodium (20 ml Pentothal 50 mg/ml, Boehringer Ingelheim Pty Ltd, Australia), into the jugular vein of the ewe. Following the insertion of an endotracheal tube (Size 8, Portex Ltd, England) into the ewe's trachea, anaesthesia was maintained using 0.5 - 2.0% halothane (Fluothane, Zeneca, Australia) in O_2, regulated by a Midget 3 vaporiser anaesthetic machine (CIG, Australia). The ewe was ventilated during surgery using a mechanical ventilator (Campbell, ULCO Engineering, Australia).

The ewe was prepared for surgery by shaving the right flank, neck and abdominal area, which were then scrubbed with Hibicet antiseptic solution (Cetamide 15% w/v, chlorohexidine gluconate 1.5% w/v and isopropyl alcohol 4% v/v, ICI Pharmaceuticals, Australia), followed by Betadine surgical scrub (7.5% w/v povidone-iodine, Faulding Pharmaceuticals, Australia) and finally with Betadine aseptic solution (10% w/v povidone-iodine, Faulding Pharmaceuticals, Australia). As a final preparation, the ewe's abdomen was rinsed using alcoholic hibitane solution (Hibiclens in ethanol: chlorohexidine gluconate 4% w/v, isopropyl alcohol 4% w/v, ICI Pharmaceuticals, Australia).

Prior to surgery, all instruments, drapes and gowns were sterilised by autoclaving and the fetal and maternal catheters were sterilised by exposure to ethylene oxide. The surgeons prepared for surgery by scrubbing hands and arms with Hibiclens antiseptic solution (chlorohexidine gluconate 4% w/v, isopropyl alcohol 4% w/v, ICI Pharmaceuticals, Australia) and wore hats, masks, sterile gowns and gloves (Gammex, Ansell, Australia).

The ewe was placed in a supine position on the operating table and covered with sterile drapes leaving only the area surrounding the incision site exposed. A midline skin incision was made in the ewe's abdomen, extending from the outer margin of the udder, to the area of the umbilicus, taking care to avoid the large mammary veins. The subcutaneous and adipose tissues were cleared to expose the *linea alba* which was then incised to expose the uterus. The head of the fetus was located by palpation and delivered through the abdominal incision before a subsequent incision was made through the uterus and fetal membranes overlying the fetal head. A midline skin incision was made in the ventral, mid cervical region of the fetal neck below the level of the larynx. The trachea was then dissected free, taking care not to damage the recurrent laryngeal nerves and two silk threads were placed underneath it. Two large bore silicone catheters (Medical grade Silastic, length: 150 cm, ID: 3.175 mm, OD: 6.35 mm, Dow Corning, USA), filled with heparinised saline (0.9% NaCl, 1% heparin v/v 5,000 IU/ml, Baxter Healthcare Pty Ltd & Fisons Pty Ltd, Australia), were then inserted into the fetal trachea. One of these catheters was directed towards the fetal lung with the top located above the bifurcation of the trachea and the other was directed towards, but did not enter the larynx. Both catheters were secured using the silk threads placed around the fetal trachea. Polyvinyl catheters (Medical grade polyvinyl, ID: 0.86 mm, OD: 1.52 mm, Dural Plastics, Australia), filled with heparinised saline, were implanted

into a fetal carotid artery and jugular vein to collect blood samples and for delivering infusions. The incision made in the fetal neck was then sutured closed and the catheters secured to the skin of the fetus using silk thread. An extra saline-filled catheter (Medical grade polyvinyl, ID: 1.5 mm, OD: 2.7 mm, Dural Plastics, Australia), was sutured to the fetal skin to measure amniotic sac pressure. Antibiotics were administered to the fetus (2 ml i.m. Procaine Penicillin, 200 mg/ml and dihydrostreptomycin, 250 mg/ml, Intervet, Australia) before it was returned to the uterus. The uterus was then closed in two layers and particular care was taken to prevent amniotic fluid leakage around the catheters. The catheters were exteriorised through an incision in the right flank of the ewe and a sterile 3-way tap (Discofix, Braun, Germany) was attached to each catheter. The 3-way taps on the tracheal catheters were connected *via* a male-male adaptor (Device Technologies, Australia) to permit the normal flow of lung liquid into and out of the lungs. When connected, these tracheal catheters act as an exteriorised 'tracheal loop'. A polyvinyl catheter (Medical grade polyvinyl, ID: 1.5 mm, OD: 2.7 mm, Dural Plastics, Australia) was also implanted into the ewe's jugular vein to permit delivery of a lethal dose of anaesthetic at the end of the experimental period. The *linea alba* and skin incision in the ewe's abdomen were closed using an interrupted and continuous suture pattern respectively. Netting (Setonet, size 7, Seton Healthcare Group, Australia) was placed around the ewe's abdomen and thorax. All catheters were secured underneath this netting in clean plastic bags. The incision site was sprayed with iodine spray (50 g/l polyvinylpyrrolidone-iodine, Troy Laboratories Pty Ltd, Australia) and covered with a sterile pad.

Towards the end of surgery, the administration of halothane was reduced and finally stopped when all procedures were complete. Once the ewe had commenced spontaneous breathing, the ventilator was turned off and the endotracheal tube was finally removed when the ewe's swallowing reflex had returned. The ewe was returned to her metabolism cage and was given unrestricted access to food and water when she had fully recovered from the anaesthesia. The animals were allowed at least 5 days to recover before experiments began.

2.3 Monitoring of Maternal and Fetal Well Being

Ewes were monitored daily with attention paid to intake of food and water and to healing of incision sites. The arterial blood gas status of each fetus was measured every 1 - 2 days and catheters were flushed with heparinised saline to prevent blood clotting in the catheters. Fetal arterial blood samples of approximately 0.4 ml were taken to measure arterial pH, $PaCO_2$, PaO_2, SaO_2 and hematocrit (Hct) using an ABL510 Blood Gas and Acid Base Analyser (Radiometer, Denmark). The readings were adjusted to 39 °C, which approximated fetal body temperature (Walker, 1988).

2.4 Lung Liquid Secretion Rate and Volume Measurements

Fetal lung liquid secretion rates and volumes were measured using the dye dilution technique as previously described by Perks and Cassin (1985) and by Hooper *et al.* (1988). Dextran Blue 2000 (Molecular weight 2,000,000, Stokes radius 270 Å, radius of gyration 380 Å, Pharmacia Chemicals, Sweden) was used as an indicator. The molecular weight and radius of this dye ensures that the pulmonary epithelium is impermeable to the dye and therefore, the dye remains within the lung liquid. Before each experiment, 500 mg of this dye was sterilised using dry heat and then 10 ml of sterile heparinised saline was added to produce a final concentration of 50 mg/ml. At the beginning of the experiment, the tracheal catheter directed towards the lungs was connected to a sterile glass syringe, which acted as a reservoir into which the lung liquid was drained, by gravity. The top of this glass syringe was open to the atmosphere *via* a bacterial air filter (Midisart 2,000, Sartorius, Germany). A volume of dye solution (3 - 5 ml) was added to the drained lung liquid and then lung liquid and dye were returned to the lung by raising the syringe above the level of the ewe's abdomen. The dye was mixed by draining and replacing lung liquid over a 45 minute period. Thereafter, once every 15 minutes for a period of 90 minutes liquid was drained and mixed and a sample of lung liquid (1 - 1.5 ml) was removed before returning the remaining liquid to the lung.

A standard curve was generated using the standard dye concentrations of 0, 0.25, 0.50, 1.00, 1.50 and 2.00 mg/ml. 340 µl aliquots of the standards and samples were pipetted in duplicate into a multiwell Titertek plate and the absorbance of each sample was measured at 620 nm in a multichannel spectrophotometer (Titertek Multiskan MC, Flow Laboratories, UK). The concentrations of the unknown samples were calculated by interpolation of the standard curve (Figure 2.1), which was generated from average absorbances of the standard dye concentrations. The volume of liquid in the lungs at each sample time was then calculated using the equation identified below (Equation 1), which accounted for the mass of volume of dye removed in previous samples and the dead space volume of the catheter. In practice, lung liquid volumes did not increase with time as the sample volume removed was altered to approximate the volume of liquid secreted in the previous intervening period. However, the calculated volumes increased because the volumes removed in previous samples were added into the equation.

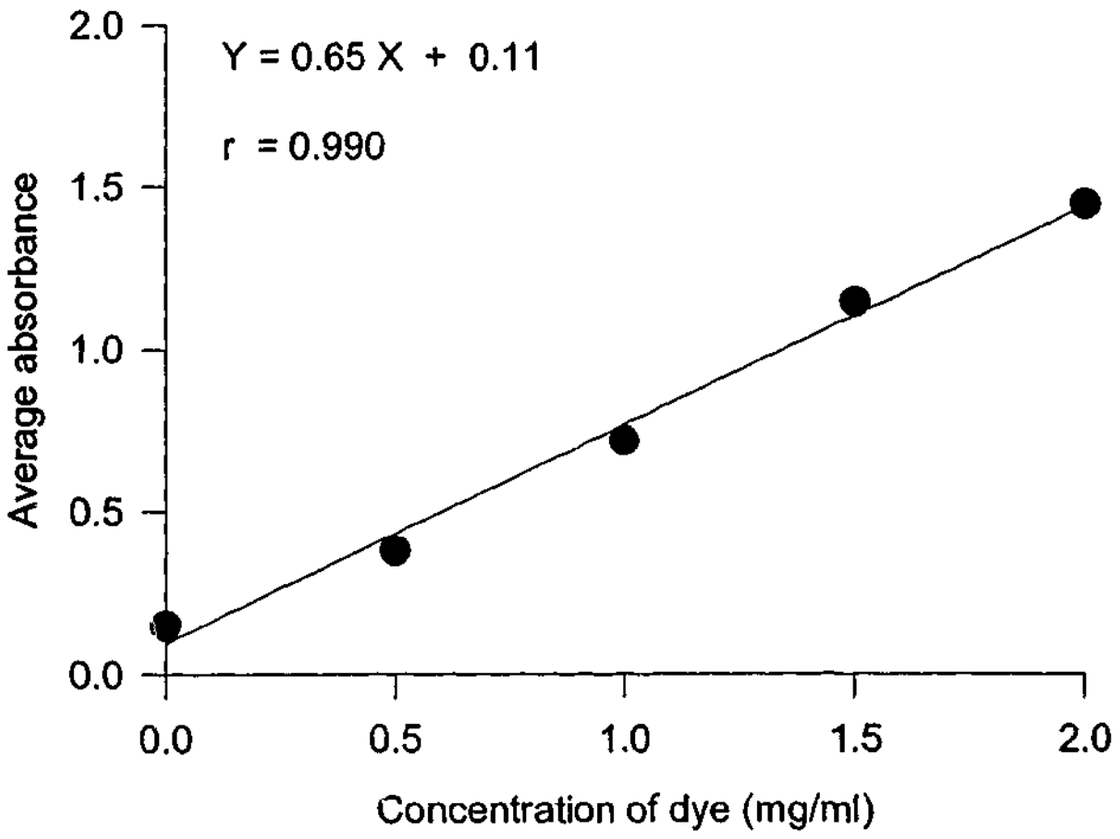

Figure 2.1 The standard curve used to calculate Dextran Blue dye concentration in samples of lung liquid

This graph is an example of the standard curve used to measure lung liquid volume and secretion rate. It describes the average absorbance (mean ± SEM) of Dextran Blue dye solution in duplicate samples, at known concentrations of 0.5, 1.0, 1 5 and 2.0 mg/ml. The standard curve is used to determine the known concentration of dye in samples of lung liquid collected during a lung liquid volume experiment.

Using linear regression analysis, the line of best fit was calculated and the rate of change in lung liquid volume over time determined the lung liquid secretion rate. Initial lung liquid volume was determined by extrapolating the line of best fit back to time zero, which was the time when the lung was initially isolated from the upper airway (Figure 2.2).

Equation 1

$$V_t = \left(\frac{X - \sum X}{C_t} \right) + \sum V - V_{CD}$$

where:

V_t	=	Volume of lung liquid at time t (ml)
X	=	Mass of dye added to lung liquid (mg)
ΣX	=	Sum of mass of dye removed in previous samples (mg)
C_t	=	Concentration of dye in sample at time t (mg/ml)
ΣV	=	Sum of the volume removed in previous samples (ml)
V_{CD}	=	Volume of catheter dead space (ml)

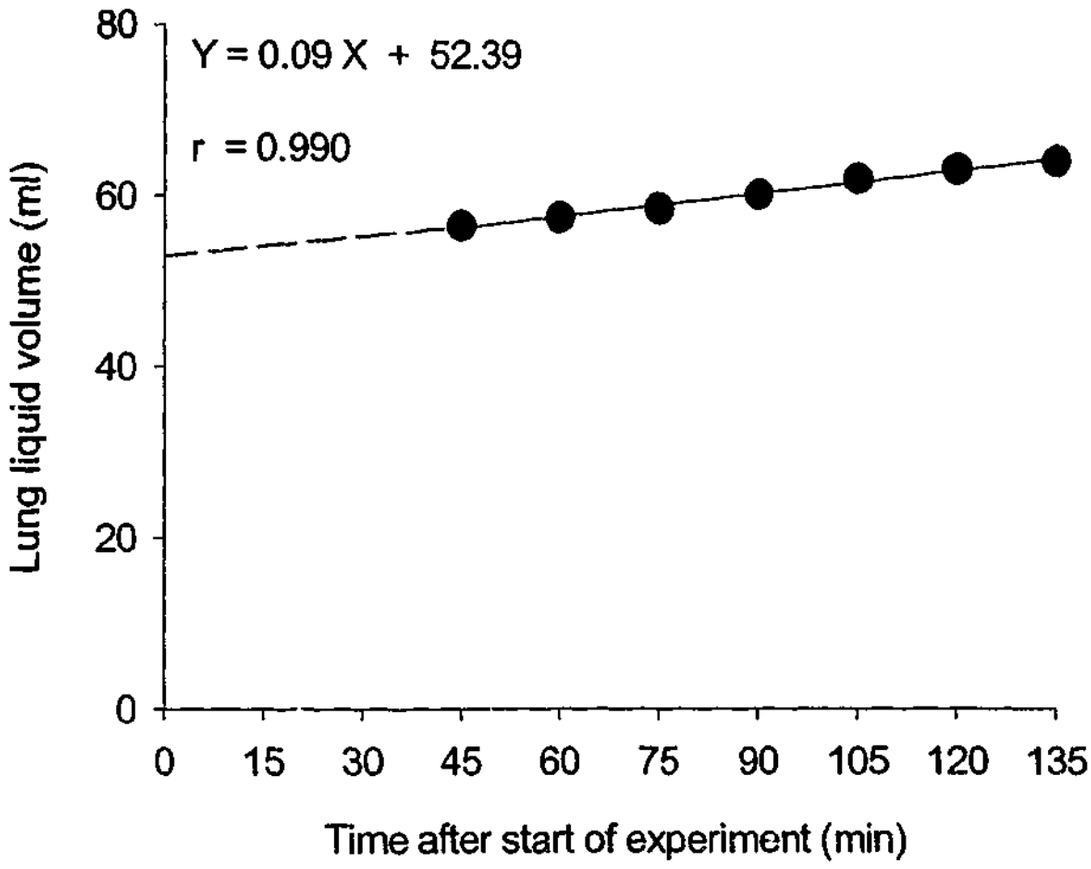

Figure 2.2 An example of the graph used to calculate fetal lung liquid secretion rate and initial lung liquid volume

Lung liquid secretion rate was calculated as the gradient of the regression line for the increase in lung liquid volume (ml) during the experimental period (min). Time 0 is the time when the lung was isolated from the upper airway and connected to the external reservoir. The dashed line represents the 45 min period in which the dye is mixed with lung liquid. Initial lung liquid volume is determined by extrapolating the regression line to intercept the y-axis.

The method used to measure lung liquid volume and secretion rates has been validated by the simultaneous injection of Dextran Blue 2000 and [^{125}I]-albumin (Perks & Cassin, 1985). Further data from our laboratory has shown that following the addition of Dextran Blue 2000 to fetal lung liquid *in vivo*, 98.2 ± 3.7% can be retrieved by repeatedly draining and replacing lung liquid over a period of ~2 hours (unpublished observations, n = 7). This finding supports previous work that has shown that Dextran Blue 2000 does not enter the fetal circulation (Hooper *et al.*, 1988).

2.5 *Post Mortem* and Tissue Collection

On the final day of the experiments, lung liquid was removed from the lungs by draining it into a cylinder by gravity and the volume was recorded. The ewe and fetus were then humanely killed by an overdose of pentobarbitone (325 mg/ml, Arnolds of Reading, Australia) administered intravenously to the ewe. The abdomen and uterus were opened to expose the fetus. The tracheal catheters were clamped and the fetus was weighed. The fetal lungs, kidneys, heart and liver were removed and weighed. The left mainstem bronchus was ligated and the left lung removed distal to the ligature and portions were frozen in liquid nitrogen and stored at -70 °C for subsequent biochemical analysis. The right lung was pressure fixed with 4% paraformaldehyde at 20 cmH_2O.

2.6 Estimation of Fetal Body Weights

Fetal body weights on the day of each experiment were calculated using the equation (Equation 2) reported by Lumbers *et al.* (1985).

Equation 2

$$BWE = \frac{e^{\left((\ln(GAE)5.09) - 16.88\right)}}{e^{\left((\ln(GAD)5.09) - 16.88\right)}} \times BWD$$

where:

BWE	=	Estimated body weight on the day of the experiment (kg)
GAE	=	Gestational age on the day of the experiment (days)
BWD	=	Body weight at death (kg)
GAD	=	Gestational age at death (days)

2.7 Biochemical Estimates of Lung Growth

2.7.1 DNA Assay

DNA assays were performed to measure DNA concentration and total DNA content of the fetal lungs using a fluorometric DNA assay (Labarca & Paigen, 1980). DNA standards (Calf Thymus DNA, Sigma, USA) were prepared by dissolving approximately 5 mg of DNA in sodium phosphate ($NaPO_4$) buffer (0.05 M $NaPO_4$ with 3 M NaCl, 0.002 M EDTA, pH 7.4), to achieve a concentration of approximately 200 μg/ml. This solution was then diluted to approximately 100 μg/ml and the concentration was determined by measuring the absorbance at 260 nm (UV/VIS 918, GBC Equipment Pty Ltd, Australia): an absorbance of 1 O.D. (optical density) at 260 nm is equivalent to 50 μg/ml of double stranded DNA. The DNA standards were dispensed into aliquots and stored at -20 °C. The fluorochrome (bisBenzimide H33258, Calbiochem, USA) was prepared prior to the day of the assay, at a concentration of 100 μg/ml in distilled H_2O and was stored in the dark at 4 °C. On the day of the assay, DNA standards were diluted to concentrations of 8.7, 26.0, 43.3, 69.2 and 86.5 μg/ml in $NaPO_4$ buffer. In addition, the fluorochrome was further diluted to 2.5 μg/ml with $NaPO_4$ buffer (0.125 M NaH_2PO_4, 3 M NaCl, pH 7.4). Approximately 700 mg of each tissue, which had been stored at -70 °C, was weighed and put in 50 ml tubes on ice. $NaPO_4$ buffer was then added to each sample to a final volume of 10 ml. The samples were then homogenised (Ultra-Turrax T25, Janke and Kunkel, IKA-Labortechnik, Germany) for 60 - 180 seconds and centrifuged (Beckman, Model J-6B Centrifuge, USA) at 2500 rpm for 5 minutes. 50 μl aliquots of the DNA standards and the supernatants of the homogenised samples (diluted 1:7 with $NaPO_4$ buffer) were added to plastic 2 ml cuvettes in triplicate. To each cuvette, 850 μl of 0.002 M EDTA was added followed by 600 μl of the diluted fluorochrome. The cuvettes were immediately covered in foil to avoid exposure to light and stored in the dark at room temperature for 15 - 180 minutes. The fluorescence of each standard and sample was measured using a fluorometer (F-2,000 Fluorescence Spectrophotometer, Hitachi, Japan), at an excitatory wavelength of 480 nm and an emission wavelength of 356 nm. The average emission value for each standard was used to create a standard curve and the line of best fit was calculated using linear regression (Figure 2.3).

The DNA concentration of each sample was calculated using the average emission value for each sample by interpolation of the standard curve. The following equations

(Equations 3 & 4), were used to calculate the DNA concentration and content of each tissue.

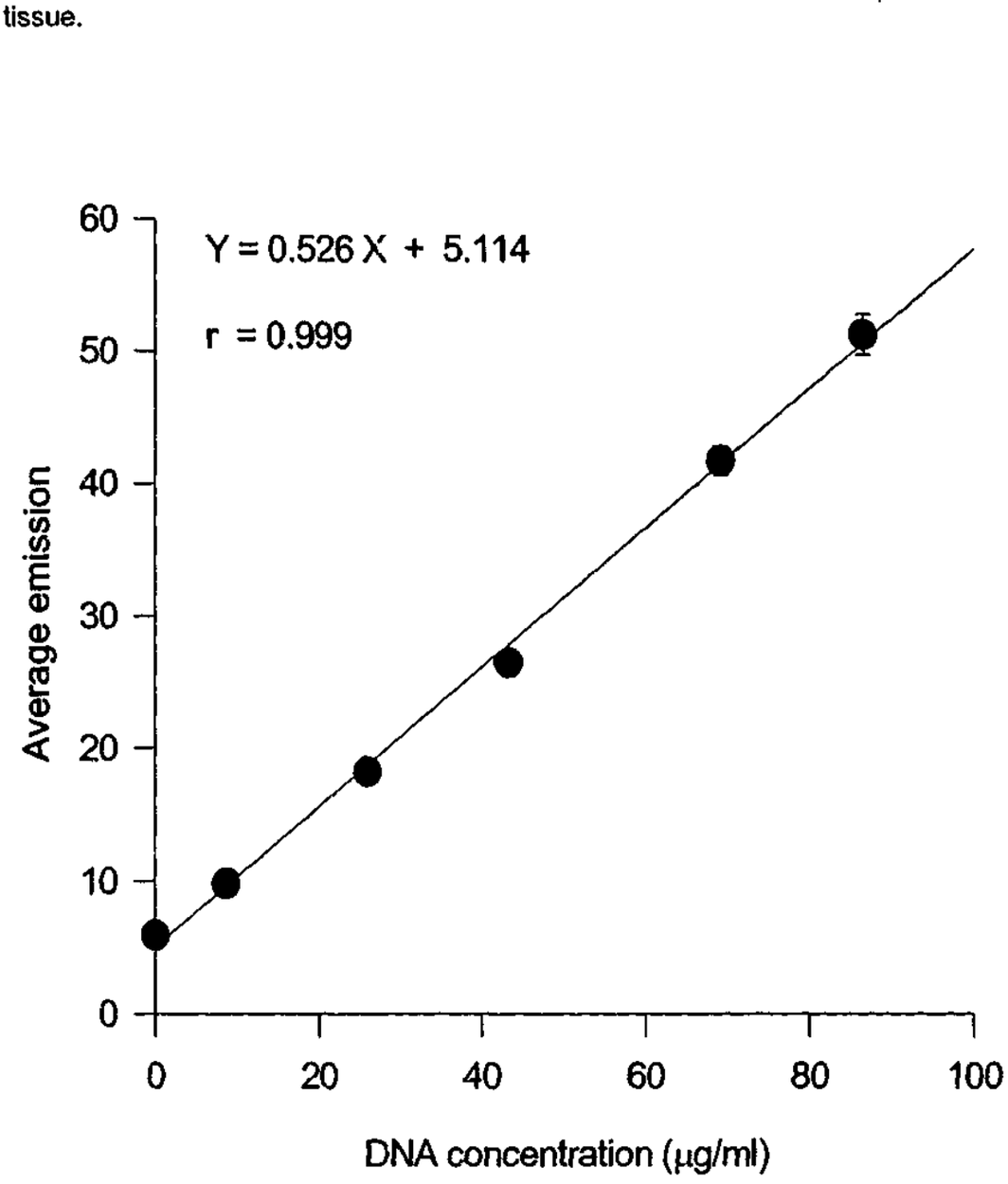

Figure 2.3 Standard curve used to calculate DNA concentration in fetal lung

The mean emission (480 nm) ± SEM of DNA extracted from calf thymus at concentrations of 8.7, 26.0, 43.3, 69.2 and 86.5 µg/ml: averaged from 8 DNA assays. The DNA concentration in lung tissue samples was determined by interpolation of the standard curve generated at the time the samples were analysed. Where error bars cannot be seen they fall within the symbol.

Equation 3

$$[DNA] = \frac{\text{Mean Emission} - A}{B} \times \frac{70}{W}$$

Equation 4

$$DNA_{content} = \frac{[DNA] \times O}{BWD}$$

where:

[DNA]	=	DNA concentration (mg/g sample)
$DNA_{content}$	=	DNA content of tissue (mg/kg body weight)
B	=	Gradient of DNA standard curve
A	=	Y-intercept of DNA standard curve
O	=	Organ weight (g)
W	=	Tissue (sample) weight (g)
BWD	=	Body weight at death (kg)

2.7.2 Protein Assay

Protein assays were performed to measure protein concentration and total protein content (Bradford, 1976) of the fetal lungs. Approximately 400 mg of tissue was weighed accurately and an appropriate volume of distilled water was added to produce a final concentration of 50 mg of tissue per ml of distilled water. The tissue was then homogenised (Ultra-Turrax T25, Janke and Kunkel, IKA-Labortechnik, Germany) at 20,000 rpm for 60 - 180 seconds. Protein standards were prepared using Bovine Serum Albumin (BSA: A-7888, Sigma Aldrich, Australia) at concentrations of 6.25, 12.5, 25, 50 and 100 μg/ml. 160 μl aliquots of these standards and the sample homogenates were added to multiwell Titertek plates in quadruplicate. Dye reagent (Bio-Rad Protein Assay, Bio-Rad Laboratories, USA) was added to each well, the plates were vortexed for 1 minute and allowed to stand for 10 minutes before the absorbance was measured in a multichannel spectrophotometer at 620 nm (Titertek Multiskan MC, Flow Laboratories, UK). The protein concentration of each sample was calculated using the average absorbance for each sample by interpolation of the standard curve (Figure 2.4). The protein content (mg/kg) of the lung was then calculated by correcting for lung weight and body weight.

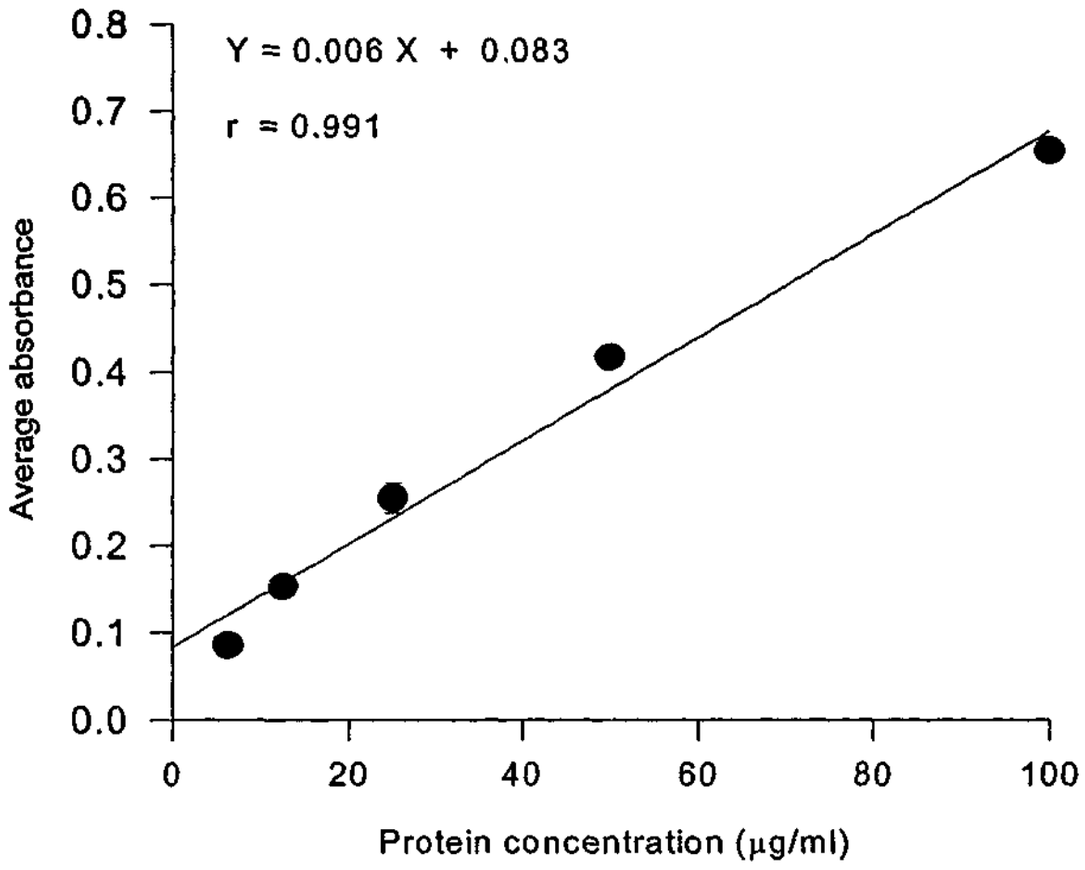

Figure 2.4 Standard curve used to calculate protein concentration in lung samples

The mean absorbance (620 nm) ± SEM of bovine serum albumin at concentrations from 6.25 to 100 µg/ml: averaged from 8 protein standard curves. The protein concentration of the lung tissue samples was determined by interpolation of the standard curve generated at the time the samples were analysed. Where error bars cannot be seen, they fall within the symbol.

2.7.3 Hydroxyproline Assay

Hydroxyproline is commonly used as a marker for collagen (Stegemann & Stalder, 1967), as this modified amino acid is present in high concentrations in collagen. To determine the hydroxyproline content of lung tissue (Stegemann & Stalder, 1967), approximately 0.5 g of frozen fetal tissue was accurately weighed and placed into Pyrex culture tubes (Corning 13 x 100 mm, Sigma Aldrich, Australia). To each culture tube, 1.0 ml of 6 M HCl was added for every 100 mg of tissue, the tissues were then hydrolysed for 16 h at 110 °C. The samples were then dried under vacuum overnight (Speedvac Concentrator, Savant, USA) at room temperature. The remaining tissue pellet was resuspended in 2.0 ml of assay buffer (10-fold dilution of stock buffer with dH_2O [stock buffer: 5% w/v citric acid, 1.2% v/v glacial acetic acid, 12% w/v sodium acetate.$3H_2O$, 3.4% w/v NaOH, 3 drops toluene]). The samples were then diluted 1:50, 1:100 and 1:200 with assay buffer to give a final volume of 2.0 ml.

Trans-4-hydroxyl-L-proline (Sigma Aldrich, Australia) was used as the hydroxyproline standard and was dissolved in dH_2O to give a final concentration of 10.0 µg/ml. This stock solution was then further diluted with assay buffer to give final concentrations of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 µg/ml. One ml of freshly prepared chloramine-T reagent (1.41% w/v chloramine-T, 26% v/v n-propanol and 53.3% v/v stock buffer) was added to each standard and tissue diluent and the samples were incubated at room temperature for 20 min. One ml of freshly prepared dimethylaminobenzaldehyde reagent (15% dimethylaminobenzaldehyde, 60% v/v n-propanol, 25% v/v 60% perchloric acid) was then added to each sample. All samples were mixed thoroughly and incubated at 60 °C for 15 min. Following the incubation period, the samples were cooled to room temperature using tap water and the absorbance of each sample and standard was then measured for 1 min at 550 nm using a spectrophotometer (UV/VIS 918, GBC Equipment Pty Ltd, Australia). The average absorbance values for the hydroxyproline standards were used to construct a standard curve (Figure 2.5) and the x- and y-axis intercepts were calculated by determining the line of best fit.

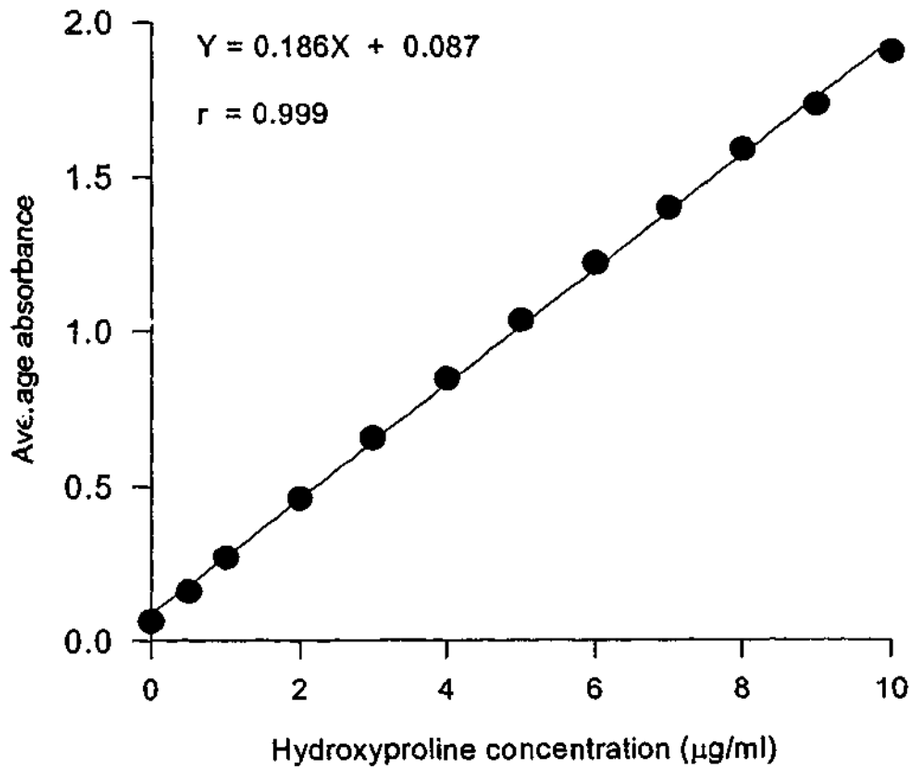

Figure 2.5 The standard curve used to calculate hydroxyproline concentration

The mean absorbance (550 nm) ± SEM, of hydroxyproline at concentrations of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 µg/ml: values were averaged from 8 hydroxyproline assays. The hydroxyproline concentration of the lung tissue samples was determined by interpolation of the standard curve generated at the time the samples were analysed. Where error bars cannot be seen, they fall within the symbol.

The concentration of hydroxyproline was determined from the absorbance of the samples and by interpolation of the hydroxyproline standard curve. The total hydroxyproline content (mg/kg) of the lung was then determined by correcting for fetal lung and body weights.

2.8 Cortisol Assay

Fetal blood samples were collected every 2 - 3 days, centrifuged (BR 3.11 Jouan Inc, USA) at 2000 rpm for 10 minutes and the plasma collected and stored at -20 °C. The radioimmunoassay used to measure cortisol concentrations in plasma was based on the method used by Bocking *et al.* (1986). Cortisol was extracted from 100 µl aliquots of fetal plasma in duplicate using 2 ml of CH_2Cl_2 (Dichloromethane, Merck, Australia) and then the aqueous and solvent phases were allowed to separate. A 1 ml aliquot of the solvent phase was transferred to a plastic tube and the solvent evaporated off under a stream of air at 37 °C. A standard curve was constructed by diluting a stock solution of cortisol (Hydrocortisone 5 µg/ml, Sigma Aldrich, Australia), to generate the following concentrations: 0, 0.195, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25 and 50 ng/ml. Duplicate 100 µl aliquots of each standard were added to 1.0 ml CH_2Cl_2 in glass tubes, vortexed and evaporated under a stream of air at 37 °C. In tubes to assess the non-specific binding (NSB) of tritiated cortisol ([^{3}H]-cortisol), 100 µl of cortisol buffer, 100 µl of human γ-globulin and 100 µl of 70% ethanol were extracted with 1 ml of CH_2Cl_2 and dried under air at 37 °C. All samples, standards and NSB pellets were resuspended in cortisol buffer (0.05 M phosphate buffer, 0.1% w/v Na^+ azide, pH 7.4). The recovery of [^{3}H]-cortisol from plasma was determined by extracting 100 µl [^{3}H]-cortisol in 100 µl dH_2O, 2 ml CH_2Cl_2 and 100 µl fetal sheep plasma.

The antiserum used in the radioimmunoassay was kindly donated by Dr. R. I. Cox (CSIRO, Australia) and was raised in sheep against the antigen 4-Pregnen-11β, 17α, 21-triol-3, 20-dione-21-acetate 3 (CMO-BSA Steraloids, USA). The cross-reactivity of the cortisol antiserum with cortisone, corticosterone, 17-hydroxyprogesterone, progesterone, testosterone and 4-androstene-3,17 dione was 20, 1, 4, 1, < 1 and < 1% respectively. 100 µl of a cortisol antiserum, 100 µl of human γ-globulin (8 mg/ml, Commonwealth Serum Laboratories, Australia) and 100 µl [^{3}H]-cortisol (10,000

cpm/0.1 ml, Amersham, Australia) were added to each sample, standard and total tube. The tubes were vortexed, covered and stored at 4 °C overnight.

After overnight incubation, 1 ml of human γ-globulin and 1 ml of 22% polyethylene glycol (PEG) were added to all tubes to separate antibody-bound hormone from unbound hormone. The tubes were vortexed and then centrifuged at 3000 rpm (Model J-6B, Beckman Instruments, USA) for 15 minutes at 4 °C. The supernatant was discarded and the remaining pellet was resuspended in 200 µl cortisol assay buffer: 100 µl of assay buffer was also added to the recovery and total tubes. 1 ml of aqueous counting fluid (ACF, Amersham, Australia) was added to all tubes and vortexed. The amount of radioactivity present was measured using a liquid scintillation β-counter (model LS 5,000TA, Beckman Instruments, USA). The concentration of cortisol in each sample was determined by interpolation of the standard curve. The intra- and inter-assay coefficients of variation were 8% and 22% respectively at concentrations of 49.2 ± 5.1 and 6.6 ± 0.3 ng/ml. The recovery efficiency for the extraction of cortisol from plasma was 100%. The minimum detectable dose of cortisol in fetal plasma was < 0.03 ng/ml.

2.9 Histological Examination of the Fetal Lung

Following fixation of the right fetal lung (Section 2.5), the trachea was ligated and the lung was immersed in a container of fresh fixative and left for 24 hours at 4 °C. The lung was then sectioned transversely into 0.5 cm slices from a random beginning. These slices were laid, inferior cut surface up, for estimation of right lung volume by the Cavalieri method (see Section 2.9.1.1). The slices were then sectioned (~1 cm x 1 cm) in a random manner and a portion of these new slices were processed in an automated processor (Histokinette, Hendrey Relays and Electrical Equipment Ltd, UK). The processing of the tissue slices involved dehydration by sequential immersions in 70%, 80%, 90% and 100% alcohol. Alcohol was removed from the tissues by immersion in a clearing solution (Histosol, Interpath Services, Australia) and the tissues were then ultrafiltrated with two changes of molten paraffin wax. The tissues were then removed from the automated processor and a third wax change took place under vacuum to ensure complete penetration of the tissue with wax. The tissues were then embedded

into paraffin blocks and allowed to set. Tissue sections were cut from paraffin blocks at 5 μm (Jung Biocut 2035, Geprufte Sicherhiet, Germany), transferred to a water bath at 45 °C and floated onto coded glass microscope slides.

2.9.1 Morphometric Measurements

The use of stereology allows quantitative three-dimensional measurements to be made from two-dimensional images (Michel & Cruz-Orive, 1988; Bolender *et al.*, 1993). Tissue sections were stained with Haematoxylin and Eosin (H&E) and viewed using a projection microscope (Olympus BX50F4, Olympus Optical Company, Japan), with a X40 objective lens. The final magnification after projection was X720. For each animal, 3 blocks were sectioned and 2 views from each of 2 sections per block (*i.e.* 12 views per animal), were analysed as outlined below, avoiding any area which contained major airways or blood vessels. All measurements refer to the dimension of the fixed lung volume.

2.9.1.1 Cavalieri Method for Estimating Lung Volume

An estimate of fixed right lung volume was calculated for each fetal lung using the Cavalieri method as previously described (Michel & Cruz-Orive, 1988). A grid of evenly spaced lines (Figure 2.6a) was superimposed over the cut lung slices. The number of line intersections overlying the lung slices was counted. Fixed right lung volume was then determined from the following equation:

Equation 5 $$V = \sum p \times A \times k \times t$$

where:

V	=	fixed right lung volume
Σp	=	sum of all line intersections falling on the lung
A	=	area of 1 square on the grid
k	=	k^{th} slice selected
t	=	mean thickness of each slice

2.9.1.2 Percentage of Tissue and Luminal Space

The percentage of tissue and luminal space in each right fetal lung was determined using techniques similar to those described by Adkisson & Callas (1982) and Crone *et al.* (1983). A 21-line, 42-point grid (Figure 2.6b) was superimposed over the projected image of the lung and the number of endpoints overlying tissue or luminal space were counted. The percentage of tissue and luminal space was then determined by the following formulae:

Equation 6

$$\% \text{ tissue space} = \frac{\text{number of endpoints overlying tissue}}{\text{total number of endpoints counted}}$$

Equation 7

$$\% \text{ luminal space} = \frac{\text{number of endpoints overlying airspace}}{\text{total number of endpoints counted}}$$

The total volume of tissue and luminal space in the right lung was determined by multiplying the percentage of tissue or luminal space by the total volume of the lung.

2.9.1.3 Alveolar Diameter

The mean alveolar diameter was determined using the linear intercept technique described by Weibel (1963). A straight line of known length (Figure 2.6c) was superimposed over the projected image of the lung and the number of intercepts with septa counted. To account for septal tissue thickness, alveolar diameter was corrected for mean luminal fraction. The size of the alveoli was then determined by the following formula:

Equation 8

$$D_a = \left(\frac{N \times L}{\sum M_i}\right) \times F$$

where:

D_a	=	alveolar diameter
N	=	number of times the measurement was made
L	=	length of the line
ΣM_i	=	the sum of the number of intercepts
F	=	the percentage of luminal space

To correct for the magnification of the image, the length of the line was determined using a 0.1 mm graticule (Asep Minigrid, Southern Biological Services Pty Ltd, Australia) projected to the same magnification as the lung tissue.

2.9.1.4 Alveolar Number

The total number of alveoli within the right fetal lung was calculated according to the technique originally described by Weibel and Gomez (1962). In brief, a square test grid containing 25 equidistant points (Figure 2.6d) was placed over a random section of lung and the number of alveoli within a test area and the number of points within the alveolar space were determined. Alveolar space as a percentage of total grid area was calculated. Any alveolar profile falling on the lower or right side of the grid outline was not included in the analysis. By then determining the number of alveoli per unit area and fraction of alveolar space, it is possible to calculate total alveolar number according to the following formula:

Equation 9

$$N_T = \frac{1}{b} \times \frac{N_A^{3/2}}{V^{1/2}} \times RV$$

where:

N_T	=	total number of alveoli within the right lung
b	=	1.55, the assumed shape constant for alveoli as determined by Weibel & Gomez (1962)
N_A	=	number of alveoli per unit area
V	=	percentage of alveolar space
RV	=	right lung volume (cm^3)

2.9.1.5 Inter-alveolar Wall Thickness

The arithmetic thickness of the lung is the average thickness of the septal wall, which is the thickness of the tissue forming the air-blood barrier. The measurement of inter-alveolar wall thickness was adapted from the techniques described by Weibel (1963) to determine arithmetic thickness. A grid comprising of 15 lines of known length, evenly distributed in 3 planes, was superimposed over the projected image of the lung (Figure 2.6e). To correct for the magnification of the image, the length of the line was determined using a 0.1 mm graticule projected to the same magnification as the lung image. The number of intersections between the lines and tissue and the number of endpoints overlying the tissue were then counted (see Equation 10).

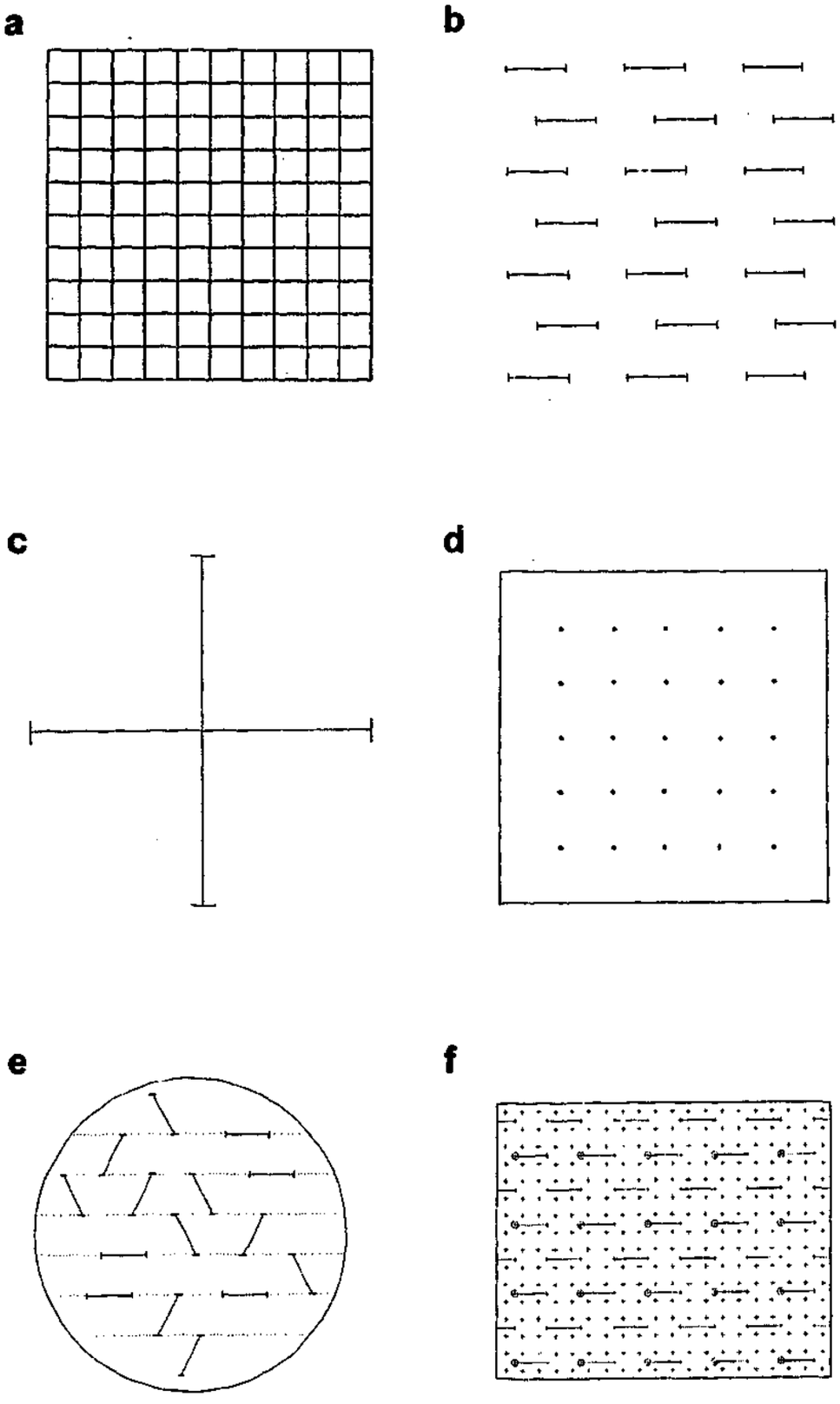

Figure 2.6 Morphometric grids

The grids used for determination of **a**) lung volume, **b**) percentage of tissue and luminal space, **c**) alveolar diameter, **d**) alveolar number, **e**) inter-alveolar wall thickness and **f**) collagen content. Where necessary, the length of the lines was determined by projecting a 0.1 mm graticule over the grid.

Equation 10

$$\text{Inter-alveolar wall thickness} = \frac{z \times p}{2n}$$

where:

z	=	length of the sampling line
p	=	number of end points of sampling lines overlying the tissue
n	=	number of times the line intercepts the tissues

2.9.2 Analysis of Lung Collagen Content

Tissue sections were stained with 0.01% w/v Picrosirius Red (Sirius Red F3BA, Verona Dyestuffs, USA) in saturated aqueous picric acid for 1 hour and differentiated in 0.01 M HCl for 60 seconds, as previously described (Junqueira *et al.*, 1979). This treatment stains collagen fibres pink/red while background tissue is stained yellow. The differential staining allowed the collagen fibres to be quantitated. Interstitial collagen fraction was determined by laying a grid (Figure 2.6f) over the projected image of the lung section. Points on the grid were defined as the intersections of the 'uncircled, free crosses' (*i.e.* +). The fraction of interstitial collagen in the right lung was determined using the following equation:

Equation 11

$$\% \text{ collagen} = \frac{\text{number of points overlying collagen}}{\text{total number of points}}$$

Interstitial collagen volume was determined by multiplying the interstitial collagen fraction by right lung volume.

2.10 Statistical Analysis

All values reported in the text are presented as the mean ± standard error of the mean (SEM). Statistical significance was set at $P < 0.05$. Appropriate statistical tests are described in detail at the end of the Methods section in each experimental chapter.

CHAPTER 3 Effect of Cortisol on Lung Development in Fetuses with Lung Hypoplasia

3.1 Introduction

Late in gestation, fetal cortisol concentrations rise markedly and this rise is believed to be responsible for the maturation of many fetal organs, particularly the lungs. Pioneering studies by Liggins in the 1960's revealed that the delivery of corticosteroids to the fetus in late gestation rapidly matured the lungs and reduced the severity of respiratory insufficiency suffered at birth by preterm infants (Liggins, 1969). As a result of this finding, exogenous corticosteroids have been used widely to reduce the risk of respiratory distress syndrome (RDS) suffered by prematurely born infants (Liggins & Howie, 1972; Crowley *et al.*, 1990). Undoubtedly, many of the benefits of prenatal corticosteroid treatment arise from the maturation of the surfactant system, however, corticosteroids have also been shown to enhance structural maturation of the lung (review: Liggins, 1994). Exogenous corticosteroids increase lung compliance (Mitzner *et al.*, 1979; Liggins *et al.*, 1985), most likely by directly altering lung structure, in particular, by remodelling of the extracellular matrix (ECM) proteins such as collagen and elastin (Schellenberg *et al.*, 1987a). Conversely, hypophysectomy, which removes the pituitary gland, eliminates the source of adrenocorticotrophic hormone (ACTH), leading to a reduction in endogenous cortisol production by the adrenal gland and impairing the structural development of the lung (Crone *et al.*, 1983).

Due to the many beneficial effects of treating premature infants with corticosteroids, there is growing interest in studying the effects of corticosteroids in infants with pulmonary hypoplasia. For example, in congenital diaphragmatic hernia (CDH), the lung is severely hypoplastic as well as immature, with retarded structural development, inadequate development of the surfactant system and reduced pulmonary compliance (Glick *et al.*, 1992). It is believed that corticosteroids may assist in reversing many of

these aspects of immaturity and thus improve postnatal survival. In fact a number of recent studies have examined the effects of corticosteroid treatment in animal models of CDH and treated fetuses show improvements in lung morphometry (Losty *et al.*, 1995; Losty *et al.*, 1996; Hedrick *et al.*, 1997). However, corticosteroids may mature lungs at the expense of lung growth. Early studies on corticosteroid treatment of experimental CDH have shown that high-dose corticosteroid injections enhance lung maturity at the expense of lung growth (Suen *et al.*, 1994; Ijsselstijn *et al.*, 1997) and/or fetal body growth (Losty *et al.*, 1995; Ijsselstijn *et al.*, 1997). In addition to the negative effects on organ and body growth, numerous human and animal studies have suggested that high-dose prenatal corticosteroid treatments may have serious side effects including impairment of neurodevelopment and increased risk of cardiovascular problems (reviewed in: Nyirenda & Seckl, 1998; Seckl, 1998; Seckl, 2001).

Cortisol infusion at physiological doses has previously been shown in our laboratory (Wallace *et al.*, 1995; Boland *et al.*, 1997) to elevate fetal plasma cortisol concentrations to levels similar to those that are seen prior to birth (Magyar *et al.*, 1980). These physiological levels of cortisol prematurely increase lung liquid volume and lung liquid secretion rate (Wallace *et al.*, 1995). Whereas, reductions in fetal cortisol levels, induced by adrenalectomy (Wallace *et al.*, 1996), prevent the age-related increases in lung liquid volume and secretion rate. When fetuses received a pretreatment of cortisol followed by a period of TO, lung liquid volume and lung growth were greatly accelerated when compared to the saline-infused tracheal obstructed fetuses (Boland *et al.*, 1997), a change attributed to cortisol-mediated alterations in lung compliance. Importantly, doses of cortisol used in studies from our laboratory have had no deleterious effects on lung or body weights. This finding is in contrast to other studies that have used high doses of corticosteroids, where lung maturity is increased at the expense of lung growth (Suen *et al.*, 1994; Ijsselstijn *et al.*, 1997) and/or fetal body growth (Losty *et al.*, 1995; Ijsselstijn *et al.*, 1997). In one particular study, the dose of cortisol administered was 12 times the cumulative dose (Schnitzer *et al.*, 1996) used previously within our laboratory (Wallace *et al.*, 1995; Boland *et al.*, 1997). The authors found significant improvements in dynamic compliance and an increase in the protein/DNA ratio (Schnitzer *et al.*, 1996) as well as improvements in morphometric factors (Hedrick *et al.*, 1997). However, no data of the effects of the corticosteroid treatment on lung or body growth, or on total lung DNA or protein were presented. The presentation of such data is especially important considering the known negative effects of large doses of cortisol on body and organ growth as described above.

The effect of cortisol on fetal lung development may be mediated *via* an increase in compliance, allowing an increase in fetal lung expansion which then induces maturational changes in lung structure. It is our hypothesis that preventing the lung from expanding during cortisol administration may prevent most of the effects of cortisol on lung structure and lung growth. Thus, the aim of this study was to determine the effect of cortisol on fetal lung growth and maturation in a lung undergoing continuous lung liquid drainage, which has been shown to induce pulmonary hypoplasia in fetal sheep (Alcorn *et al.*, 1977; Moessinger *et al.*, 1990; Nardo *et al.*, 1995; Davey *et al.*, 1999).

3.2 Methods

Surgery was performed on 20 pregnant ewes and their fetuses (Merino X Border-Leicester) at 109.4 ± 0.9 days of gestation (term is ~147 days), to implant fetal and maternal vascular catheters and fetal tracheal catheters as described in Section 2.2. Fetal well-being was monitored on a daily basis by measuring fetal PaO_2, $PaCO_2$, pH, SaO_2 and Hct (ABL30, Radiometer, Denmark). A minimum of 5 days was allowed for the ewe and fetus to recover from surgery before experiments began.

3.2.1 Experimental Protocol

To determine the biochemical and morphological effects of cortisol treatment on normal and hypoplastic lungs, fetuses were divided into one of four groups:

1) A *saline-only* group, in which saline was infused intravenously into the fetus for a period of 9 days (122 - 131 days GA, n = 5),
2) A *cortisol-only* group in which cortisol was infused intravenously (i.v.) into the fetus at increasing concentrations (see below) for a period of 9 days (122 - 131 days GA, n = 5),
3) A *saline & drain* group in which fetal lung liquid was drained gravimetrically into a sterile bag for a period of 20 days (111 - 131 days GA) and which also received a saline infusion as per group 1 (n = 5),
4) A *cortisol & drain* group in which lung liquid was drained gravimetrically into a sterile bag for a period of 20 days (111 - 131 days GA) and which also received a cortisol infusion in increasing concentrations as per group 2 (n = 5).

Groups 2 and 4 (*cortisol-only* and *cortisol & drain*) received 9 day infusions of cortisol (Hydrocortisone sodium succinate, Solu Cortef, Upjohn Pty Ltd, Australia) into the fetal jugular vein. Cortisol was administered in increasing doses over this 9 day period: 1.5 mg/day on 122 - 123 days GA, 2.5 mg/day on 124 - 125 days GA, 3.0 mg/day on 126 - 127 days GA, 3.5 mg/day on 128 - 129 days GA and 4.0 mg/day on 130 - 131 days GA. Cortisol was prepared daily in equal volumes of heparinised saline and was delivered at a rate of 1.2 ml/hr using an infusion pump (Perfusor VI, Braun, Germany). This regime was similar to that used by Boland *et al.* (1997), to prematurely induce a rise in fetal plasma cortisol concentrations similar to that observed in the last 10 days of

gestation before labour in sheep (Magyar *et al.*, 1980). However, this dose of cortisol is insufficient to induce labour in that species (Elsner *et al.*, 1980; Nathanielsz *et al.*, 1982; Mason *et al.*, 1989). The saline-infused groups (*saline-only* and *saline & drain*) were given equal volumes of heparinised saline which were delivered at the same rate (1.2 ml/hr) as the cortisol-infused fetuses. Fetal blood samples (~2 ml) were collected from all animals every 2 - 3 days for the measurement of fetal plasma cortisol concentrations (Section 2.8). All infusions were continued until the time of *post mortem* (131 days of gestation).

Lung liquid was drained from the lungs of fetuses in both the *saline & drain* and *cortisol & drain* groups for a period of 20 days from day 111 - 131 of gestation. The fetal tracheal loop was connected to a sterile bag (500 ml, Tuta, Australia) and lung liquid was drained by gravity continuously for this 20 day period. Collection bags were secured to the ewes' metabolism cages below the level of the flooring. Drainage pressures were previously determined in our laboratory to be ~15 cmH_2O whilst the ewes were lying down and ~40 cmH_2O whilst the ewes were standing up (unpublished data). Drainage bags were emptied daily and the volume of drained liquid was recorded so that daily lung liquid drainage rates and cumulative volumes of drained lung liquid could be determined. The lung liquid drainage rates were corrected for the estimated fetal body weight at each gestational age (Lumbers *et al.*, 1985; Section 2.6).

All experiments were terminated at 130.8 ± 0.1 days of gestation. The fetal lungs were drained of liquid *via* the tracheal catheter and the tracheal loop was blocked before the ewe and fetus were humanely killed by an overdose of sodium pentobarbitone administered to the ewe (130 mg/kg i.v.). The fetus was weighed and the fetal lungs, kidneys, heart and liver were removed and weighed. The left mainstem bronchus was ligated and the left lung removed distal to the ligature and portions were frozen in liquid nitrogen and stored at -70 °C for subsequent biochemical analysis. The right lung was pressure fixed with 4% paraformaldehyde at 20 cmH_2O (Section 2.5).

3.2.2 Biochemical and Histological Methods

Portions of frozen lung tissue were accurately weighed and used for biochemical analysis of DNA, protein and hydroxyproline content as described in the General Methods section (Sections 2.7.1 - 2.7.3).

Sections of the right lung were processed in paraffin, cut at 5 μm and stained with Haematoxylin and Eosin. Lung sections were viewed on a X40 microscope lens and projected onto a screen. The final magnification was determined by simultaneous projection of a 0.1 mm graticule and was X720. The right lung volume was determined using the Cavalieri method as described in Section 2.9.1.1. Stereological measurements of: right lung tissue fraction and volume (Section 2.9.1.2), luminal fraction and volume (Section 2.9.1.2), alveolar diameter (Section 2.9.1.3), alveolar number (Section 2.9.1.4) and inter-alveolar wall thickness (Section 2.9.1.5) were then made using grids shown in Figure 2.6.

3.2.3 Statistical Analysis

The results are presented as mean ± standard error of the mean (SEM). The accepted level of significance for all statistical analyses was $P < 0.05$. Statistical analyses were performed using the computerised statistical packages SigmaStat (Version 2.0, Jandel Corporation, USA) and SPSS (Version 10.0.5, SPSS Incorporated, USA). Differences in fetal body weights, lung and organ weights, DNA, protein and hydroxyproline contents and right lung volumes were analysed by a one-way analysis of variance (ANOVA). Significant differences between values were then identified with a Least Significant Difference (LSD) test. Differences in fetal arterial blood gas and acid-base indices, fetal plasma cortisol concentrations, lung liquid drainage rates and cumulative drainage volumes were analysed by a two-way ANOVA for repeated measures with treatment and gestational age as factors. If an interaction was identified, an LSD test isolated differences by doing pairwise comparisons of the data points. Additionally, the slope of the curves for cumulative volume of drained lung liquid for the *saline & drain* and *cortisol & drain* fetuses was assessed for parallelism by performing linear regression analysis on the data points after commencement of the cortisol infusion. Differences in morphometric measurements were analysed by nested one-way ANOVAs. When differences were found, they were identified by an LSD test.

3.3 Results

All fetuses were considered healthy according to their arterial blood gas and acid-base status for the duration of the experimental period. Furthermore, the experimental manipulations had no significant effect on fetal arterial blood gases and acid-base status (Table 3.1). Body weights were not different between any of the groups at the time of *post mortem* (Table 3.2).

Table 3.1 Fetal arterial blood gas and acid-base status

This table shows the mean ± SEM arterial blood gas and acid-base status of fetal sheep for *saline-only*, *cortisol-only*, *saline & drain* and *cortisol & drain* fetuses, over the duration of the experimental period. The experimental manipulations had no significant effect on the arterial blood gas and acid-base status of fetal sheep.

	saline-only (n = 5)	*cortisol-only* (n = 5)	*saline & drain* (n = 5)	*cortisol & drain* (n = 5)
pH	7.36 ± 0.01	7.37 ± 0.01	7.37 ± 0.01	7.37 ± 0.01
$PaCO_2$ (mmHg)	43.6 ± 1.5	42.6 ± 0.5	42.5 ± 1.3	40.3 ± 1.7
PaO_2 (mmHg)	22.6 ± 1.3	25.2 ± 1.5	23.9 ± 1.5	24.4 ± 1.3
SaO_2 (%)	65.9 ± 2.6	74.1 ± 2.8	70.8 ± 3.7	70.7 ± 3.2
Hct (%)	28.6 ± 2.3	27.9 ± 1.3	29.1 ± 1.1	29.0 ± 0.8

Table 3.2 Fetal body weight

Fetal body weight (kg) ± SEM at 130.8 ± 0.1 days of gestation in each of the experimental groups of fetuses used in this study. The experimental manipulations had no significant effect on fetal body weight.

	saline-only (n = 5)	*cortisol-only* (n = 5)	*saline & drain* (n = 5)	*cortisol & drain* (n = 5)
Body weight (kg)	3.4 ± 0.3	3.6 ± 0.1	3.0 ± 0.4	3.1 ± 0.2

3.3.1 Fetal Plasma Cortisol Concentrations

Prior to the infusion of cortisol (day 120.0 ± 0.3), the plasma cortisol concentrations were similar in all 4 groups of fetuses (*saline-only*: 2.3 ± 1.1 ng/ml; *cortisol-only*: 7.6 ± 3.3 ng/ml; *saline & drain*: 1.9 ± 0.4 ng/ml and *cortisol & drain*: 2.7 ± 0.5 ng/ml; Figure 3.1). Mean plasma cortisol concentrations for both of the cortisol-infused groups (*cortisol-only* and *cortisol & drain*) were significantly greater than the plasma cortisol concentrations of both the *saline-only* and *saline & drain* groups of fetuses throughout the infusion period (days 122 - 131). By day 131 of gestation, circulating cortisol concentrations in both cortisol-infused groups had risen to 33.4 ± 3.4 ng/ml. The mean plasma cortisol concentrations for the two saline-infused groups did not change during the experimental period and were not significantly different from each other.

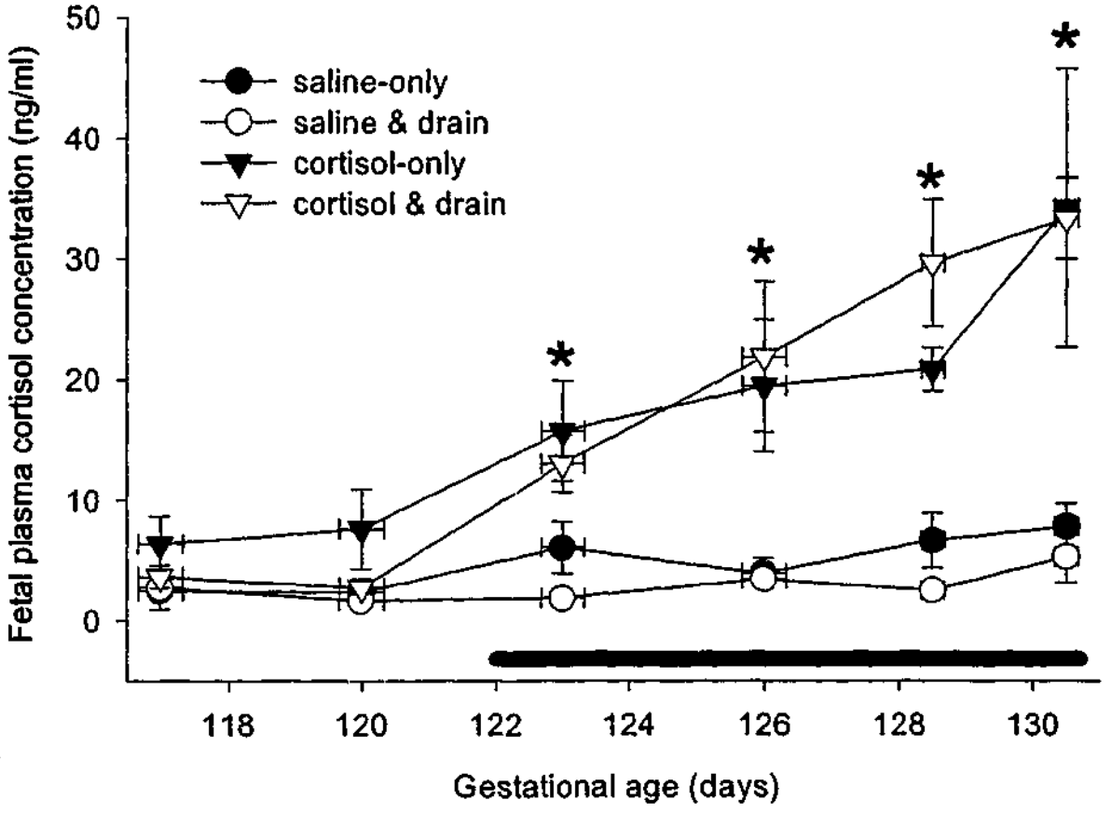

Figure 3.1 Fetal plasma cortisol concentrations

Cortisol concentrations (mean ± SEM) in fetal plasma of saline-infused (circles) and cortisol-infused (triangles) fetuses. Open symbols represent fetuses that have undergone a 20-day period of lung liquid drainage whereas the closed symbols represent fetuses not exposed to lung liquid drainage. The closed bar represents the period during which the cortisol and saline infusions were administered. The asterisks indicate mean cortisol concentrations in the cortisol-infused fetuses that are significantly different ($P < 0.05$) from both of the saline-infused groups at the corresponding gestational age.

3.3.2 Rate and Volume of Drained Lung Liquid

The cumulative volume of lung liquid drained by day 131 of gestation was not significantly different between the *saline & drain* and *cortisol & drain* groups (Figure 3.2). However, when linear regression analysis was performed on the cumulative lung volume data points during the infusion period (days 123 - 131 of gestation), it showed the treatment with cortisol caused a significant increase in the slope of the curve ($P < 0.05$: Figure 3.2). Lung liquid drainage rate (ml/hr) and drainage rate corrected for estimated fetal body weight (ml/hr/kg) were significantly different ($P < 0.05$) between the *cortisol & drain* and *saline & drain* groups on days 127, 128 and 129 of gestation (Figure 3.3.), but not on days 130 and 131 of gestation.

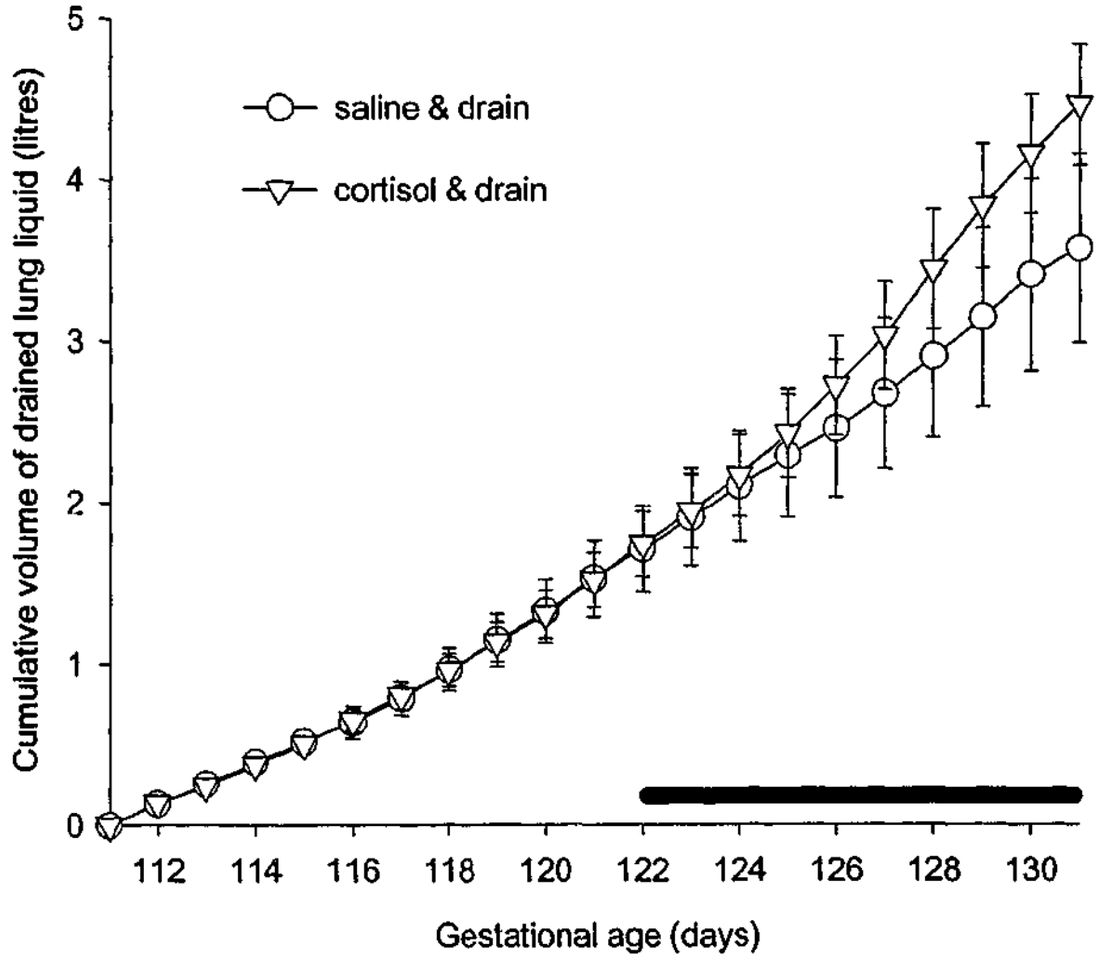

Figure 3.2 Cumulative volume of drained lung liquid throughout the drainage periods

Cumulative volume (litres) ± SEM of liquid drained from the lungs between 111 and 131 days of gestation, in the *cortisol & drain* fetuses (triangles) and in the *saline & drain* fetuses (circles). The black bar represents the period of saline or cortisol infusion.

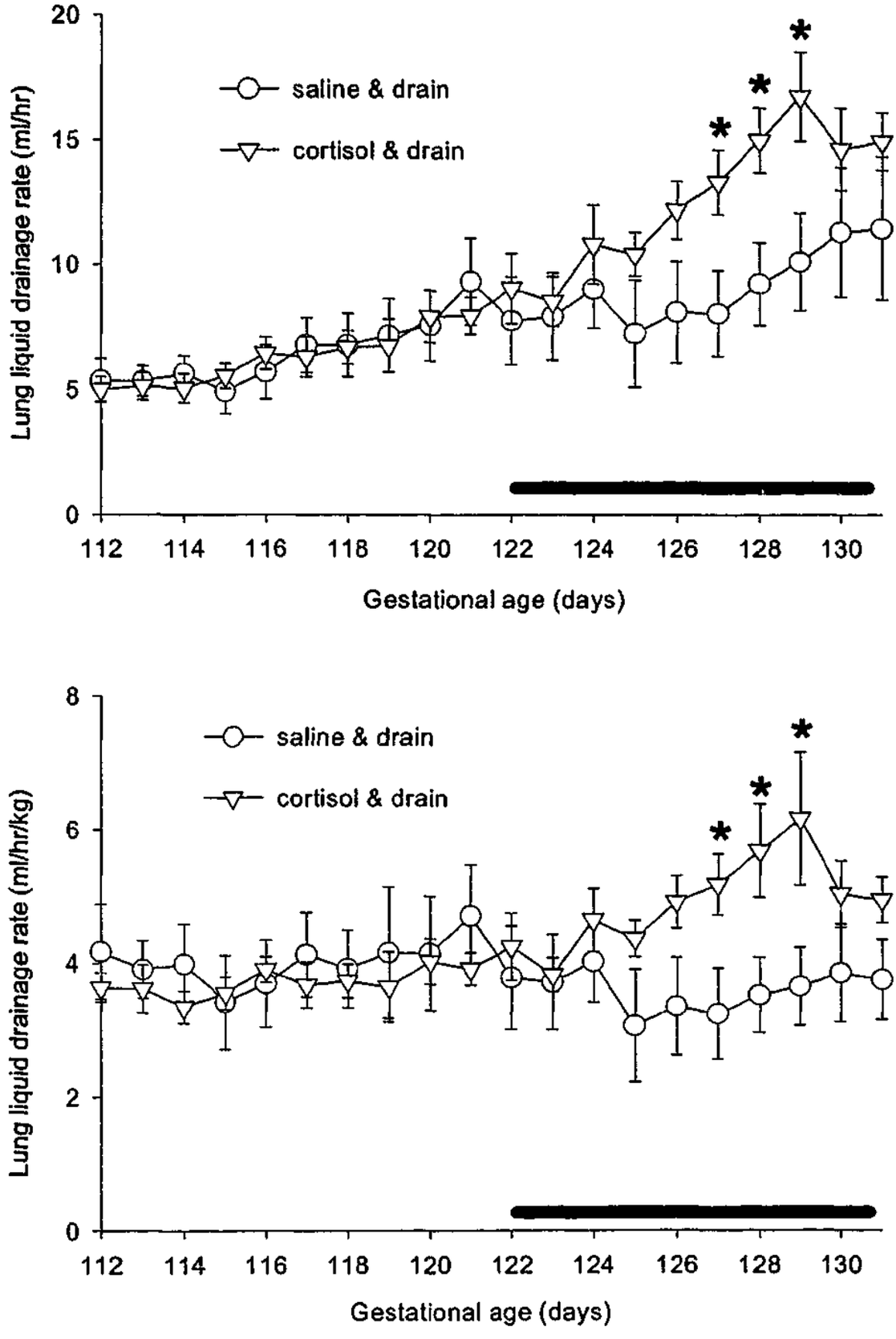

Figure 3.3 Lung liquid drainage rates

Upper panel: lung liquid drainage rates (ml/hr) ± SEM and ***lower panel***: drainage rates corrected for estimated fetal body weight (ml/hr/kg) ± SEM throughout the drainage period (111 - 131 days GA). Asterisks represent significant differences ($P < 0.05$) in lung liquid drainage rate between the *cortisol & drain* (triangles) and *saline & drain* (circles) groups of fetuses at the corresponding gestational age. The black bar represents the period of saline and cortisol infusion.

3.3.3 Fetal Lung and Organ Weights

The lung weights (g and g/kg body weight) of fetuses that had undergone a period of lung liquid drainage (*saline & drain:* 48.9 ± 7.7 g, 16.3 ± 1.0 g/kg; *cortisol & drain:* 50.3 ± 4.0 g, 16.5 ± 0.4 g/kg) were significantly reduced when compared to lung weights for *saline-only* (122.1 ± 12.7 g, 35.9 ± 0.7 g/kg) and *cortisol-only* (129.5 ± 9.5 g, 35.6 ± 2.0 g/kg) fetuses ($P < 0.05$; Figure 3.4). A period of lung liquid drainage resulted in a 54% reduction in lung weight corrected for body weight (g/kg) and a 60% reduction in lung weight (g) relative to fetuses that did not undergo a period of lung liquid drainage. Cortisol infusion did not alter lung weights.

There were no significant differences between any of the treatment groups in the wet weights (g) of fetal liver, heart or kidneys (Table 3.3). When corrected for fetal body weight (g/kg) there were no differences in the weights of the liver or kidney, however, there was a reduction ($P < 0.05$) in fetal heart weight when corrected for body weight (g/kg) in the *cortisol-only* group (6.9 ± 0.3 g/kg) when compared to heart weights in both *saline & drain* (8.4 ± 0.1 g/kg) and *cortisol & drain* (8.2 ± 0.3 g/kg) fetuses but not when compared to the *saline-only* (7.6 ± 0.4 g/kg) group of fetuses (Table 3.3).

3.3.4 DNA, Protein and Hydroxyproline Contents

3.3.4.1 DNA Concentration and Content

Lung liquid drainage resulted in a significantly ($P < 0.05$) higher DNA concentration (mg/g) in both drainage groups of fetuses (*saline & drain*: 7.4 ± 0.7 mg/g and *cortisol & drain*: 7.2 ± 0.5 mg/g) in comparison to non-drained fetuses (*saline-only*: 4.9 ± 0.2 mg/g and *cortisol-only:* 5.4 ± 0.1 mg/g; Figure 3.5). As lung liquid drainage reduced fetal lung weights by 54%, total lung DNA content (mg/kg body weight) was lower ($P < 0.05$) in both of the drained groups (*saline & drain*: 122.2 ± 13.8 mg/kg and *cortisol & drain*: 118.1 ± 8.4 mg/kg) in comparison to non-drained groups of fetuses (*saline*: 175.9 ± 6.6 mg/kg and *cortisol*: 192.5 ± 12.0 mg/kg). The DNA concentration (mg/g lung weight) and total DNA content (mg/kg body weight) of the fetal lung were not affected by cortisol infusion (*saline & drain vs. cortisol & drain* and *saline-only vs. cortisol-only*).

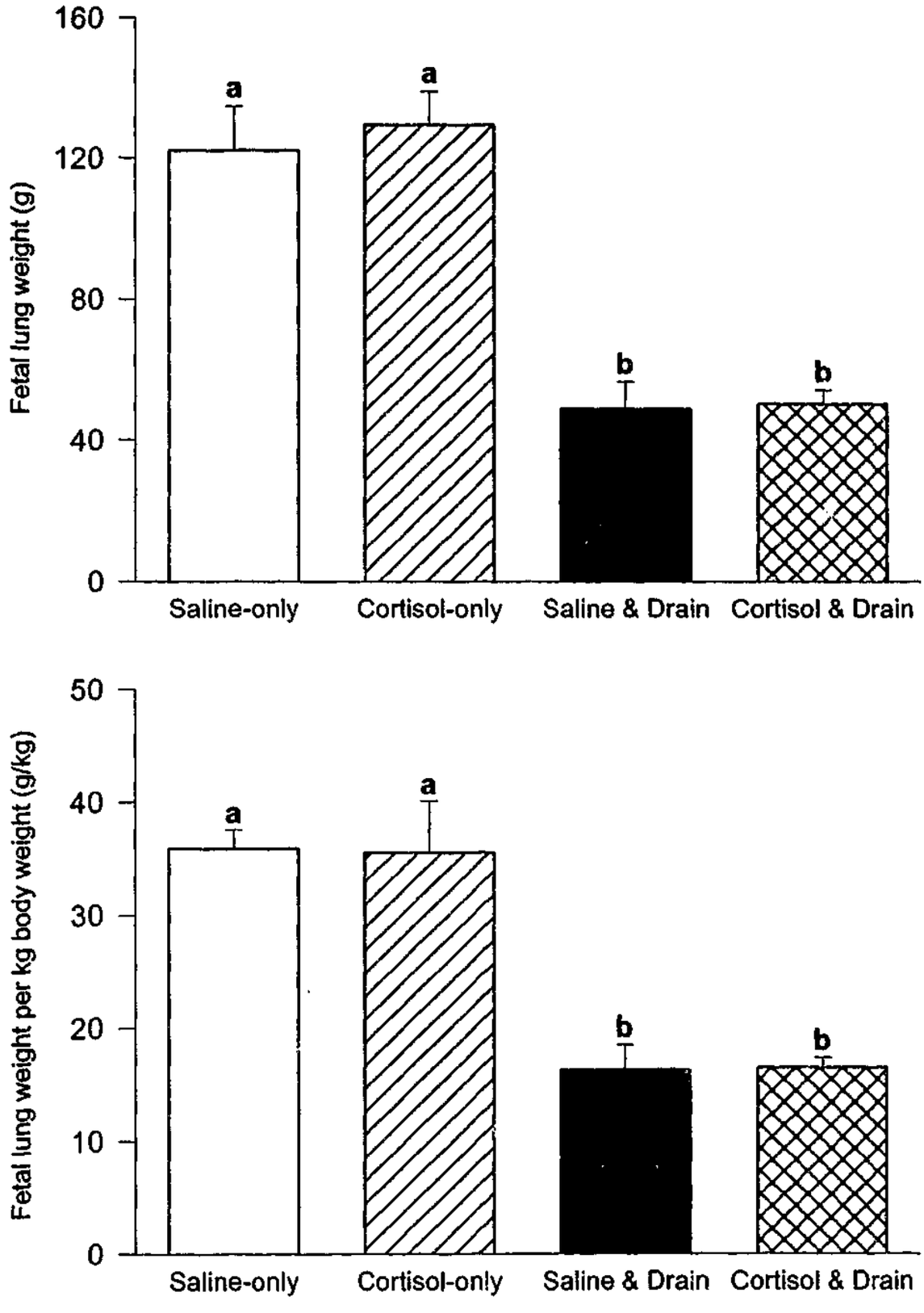

Figure 3.4 Fetal lung weights

Upper panel: mean wet lung weights ± SEM (g) and ***lower panel***: mean wet lung weights adjusted for fetal body weight ± SEM (g/kg), measured in *saline-only* (n = 5), *cortisol-only* (n = 5), *saline & drain* (n = 5) and *cortisol & drain* (n = 5) groups of fetuses at 130.8 ± 0.1 days of gestation. Within each graph, values that do not share a common letter are significantly different from each other ($P < 0.05$).

Table 3.3 Fetal organ weights

Mean fetal liver, kidney and heart weights ± SEM (g, g/kg), measured at 130.8 ± 0.1 days of gestation in the *saline-only*, *cortisol-only*, *saline & drain* and *cortisol & drain* groups of fetuses. Values that do not share a common letter are significantly different from each other ($P < 0.05$).

	saline-only (n = 5)	*cortisol-only* (n = 5)	*saline & drain* (n = 5)	*cortisol & drain* (n = 5)
Liver weights (g)	81.3 ± 7.2	104.2 ± 4.0	87.3 ± 15.5	88.7 ± 8.9
(g/kg)	24.2 ± 0.7	28.8 ± 1.0	29.3 ± 4.0	29.1 ± 1.6
Kidney weights (g)	20.8 ± 2.7	21.1 ± 1.0	19.4 ± 2.5	19.0 ± 2.5
(g/kg)	6.1 ± 0.2	5.8 ± 0.2	6.6 ± 0.4	6.2 ± 0.5
Heart weights (g)	25.7 ± 3.2	24.8 ± 1.0	24.6 ± 3.0	25.1 ± 2.3
(g/kg)	7.6 ± 0.4^{ab}	6.9 ± 0.3^{b}	8.4 ± 0.1^{a}	8.2 ± 0.3^{a}

3.3.4.2 Protein Concentration, Content and Protein-to-DNA Ratio

A period of lung liquid drainage resulted in a higher ($P < 0.05$) protein concentration (mg/g) of the left lung in both drained groups of fetuses (*saline & drain*: 76.6 ± 1.7 mg/g and *cortisol & drain:* 72.9 ± 3.3 mg/g) in comparison to non-drained groups of fetuses (*saline-only*: 53.7 ± 2.5 mg/g and *cortisol-only*: 59.1 ± 2.3 mg/g; Figure 3.6). However, as lung weight was reduced by 54% in drained fetuses, the total protein content (mg/kg) of the left lung was lower in both drained groups of fetuses (*saline & drain*: 1198.0 ± 73.1 mg/kg and *cortisol & drain* 1202.8 ± 54.0 mg/kg) when compared to both non-drained groups of fetuses (*saline-only*: 1927.6 ± 98.0 and *cortisol-only:* 2089.9 ± 95.1 mg/kg; Figure 3.6). A period of cortisol infusion did not alter the protein concentration (mg/g lung weight) or content (mg/kg body weight) of the left lung (*saline & drain vs. cortisol & drain* and *saline-only vs. cortisol-only*). Protein-to-DNA ratios were calculated and were not different between any of the treatment groups (Figure 3.7: upper panel).

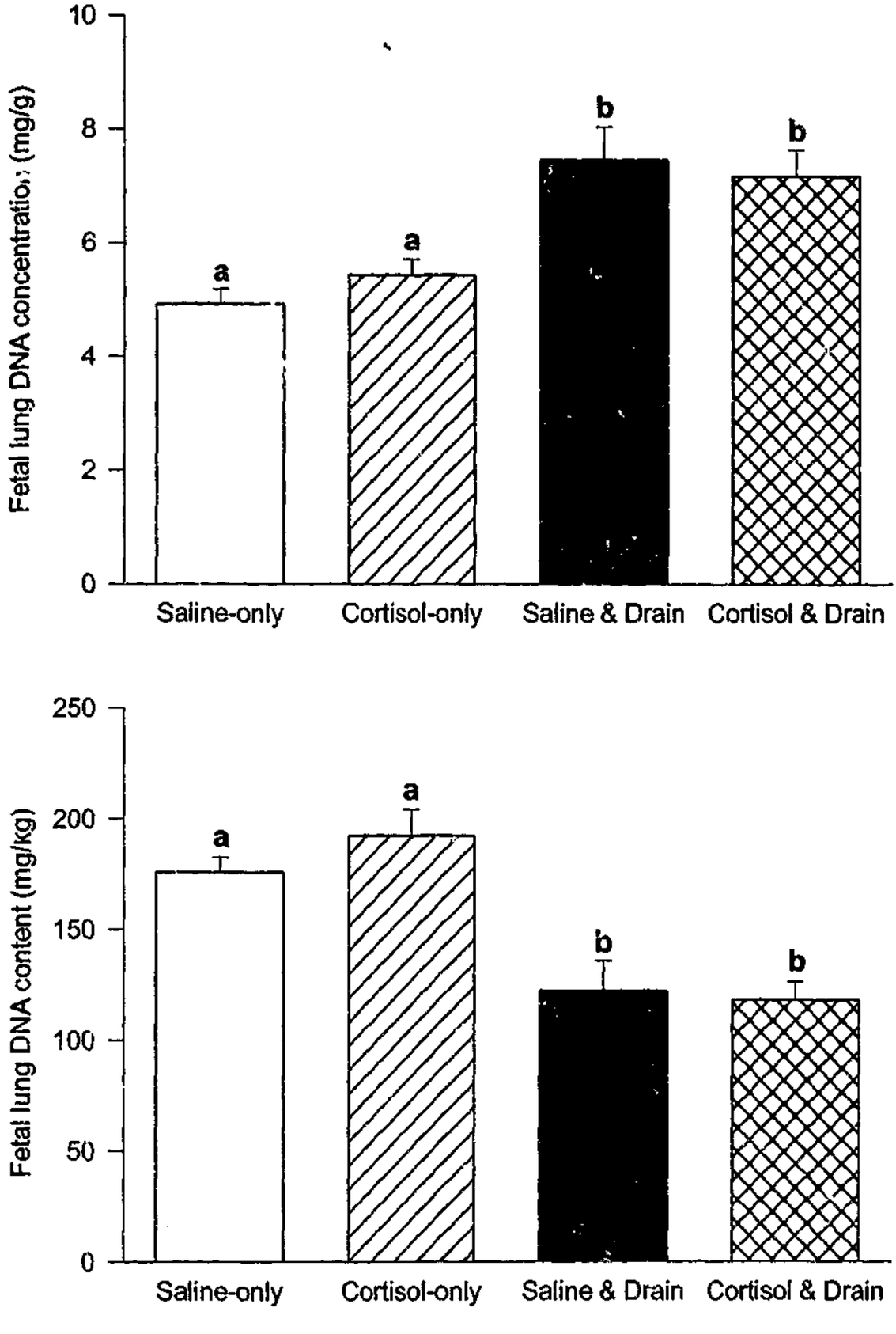

Figure 3.5 Fetal lung DNA concentration and content

Upper panel: fetal lung DNA concentration (mg/g lung tissue) ± SEM and ***lower panel***: fetal lung DNA content (mg/kg of fetal body weight) ± SEM measured in *saline-only* (n = 5), *cortisol-only* (n = 5), *saline & drain* (n = 5) and *cortisol & drain* (n = 5) groups of fetuses. Values that do not share a common letter are significantly different from each other ($P < 0.05$).

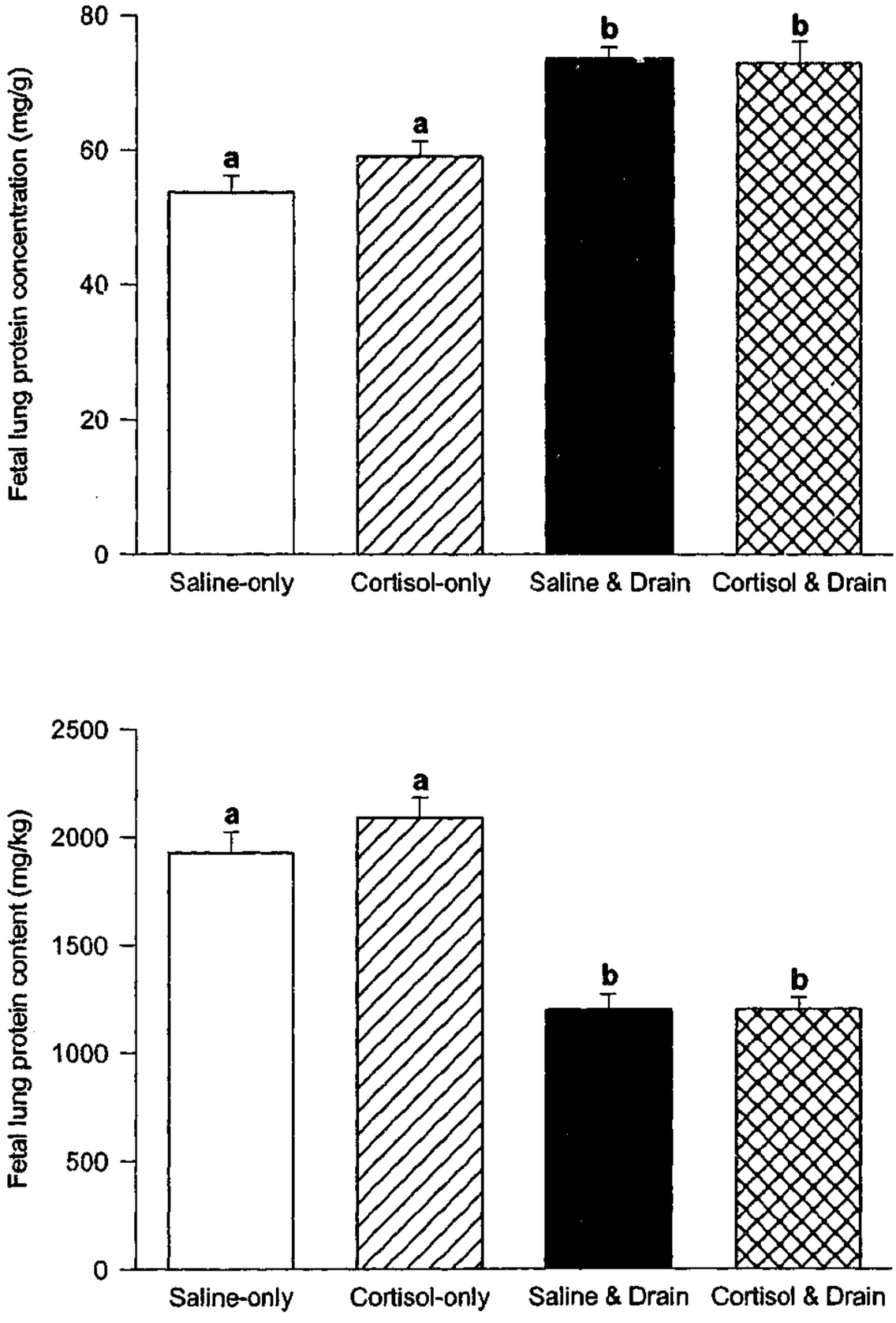

Figure 3.6 Fetal lung protein concentration and content

Upper panel: fetal lung protein concentration (mg/g of lung tissue) ± SEM and ***lower panel***: fetal lung protein content (mg/kg of fetal body weight) ± SEM measured in *saline-only* (n = 5), *cortisol-only* (n = 5), *saline & drain* (n = 5) and *cortisol & drain* (n = 5) groups of fetuses. Values that do not share a common letter are significantly different from each other ($P < 0.05$).

3.3.4.3 Hydroxyproline Concentration, Content and Hydroxyproline-to-Protein Ratio

The hydroxyproline concentration of the left lung was significantly greater ($P < 0.05$) in both drained groups of fetuses (*saline & drain*: 1.4 ± 0.1 mg/g and *cortisol & drain*: 1.5 ± 0.1 mg/g) when compared to non-drained lungs (*saline-only*: 0.9 ± 0.1 mg/g and *cortisol-only*: 0.8 ± 0.1 mg/g). Cortisol infusion did not influence hydroxyproline concentration (Figure 3.8: upper panel). Hydroxyproline content of the fetal lung for *saline & drain* (22.9 ± 1.1 mg/g) and *cortisol & drain* (24.2 ± 1.6 mg/kg) fetuses was significantly lower than that for *saline-only* (34.9 ± 1.9 mg/kg) fetuses but was not different to that in *cortisol-only* fetuses (29.2 ± 3.5 mg/kg; Figure 3.8: lower panel). The hydroxyproline-to-protein ratio of the fetal lung was found to be elevated in the *cortisol & drain* group (0.020 ± 0.001) in comparison to the *cortisol-only* group (0.014 ± 0.002; $P < 0.05$). There were no other significant differences in hydroxyproline-to-protein ratio (Figure 3.7: lower panel) between the different treatment groups.

3.3.5 Morphological Measurements

Representative lung tissue sections from each of the experimental groups can be seen in Figure 3.9.

3.3.5.1 Right Lung Volume, Tissue and Luminal Fractions and Tissue and Luminal Volumes

Cortisol infusion alone, without an associated lung liquid drainage period resulted in an increased luminal space (%), a decreased tissue space (%) and a decreased right lung volume (cm^3) and right lung volume adjusted for body weight (cm^3/kg) when compared to saline-infused fetuses (Table 3.4). There was no difference in total luminal volume adjusted for body weight between *saline-only* and *cortisol-only* groups whereas total tissue volume was significantly decreased in the *cortisol-only* group in comparison to the *saline-only* group, as was the tissue-to-luminal space ratio (Table 3.4).

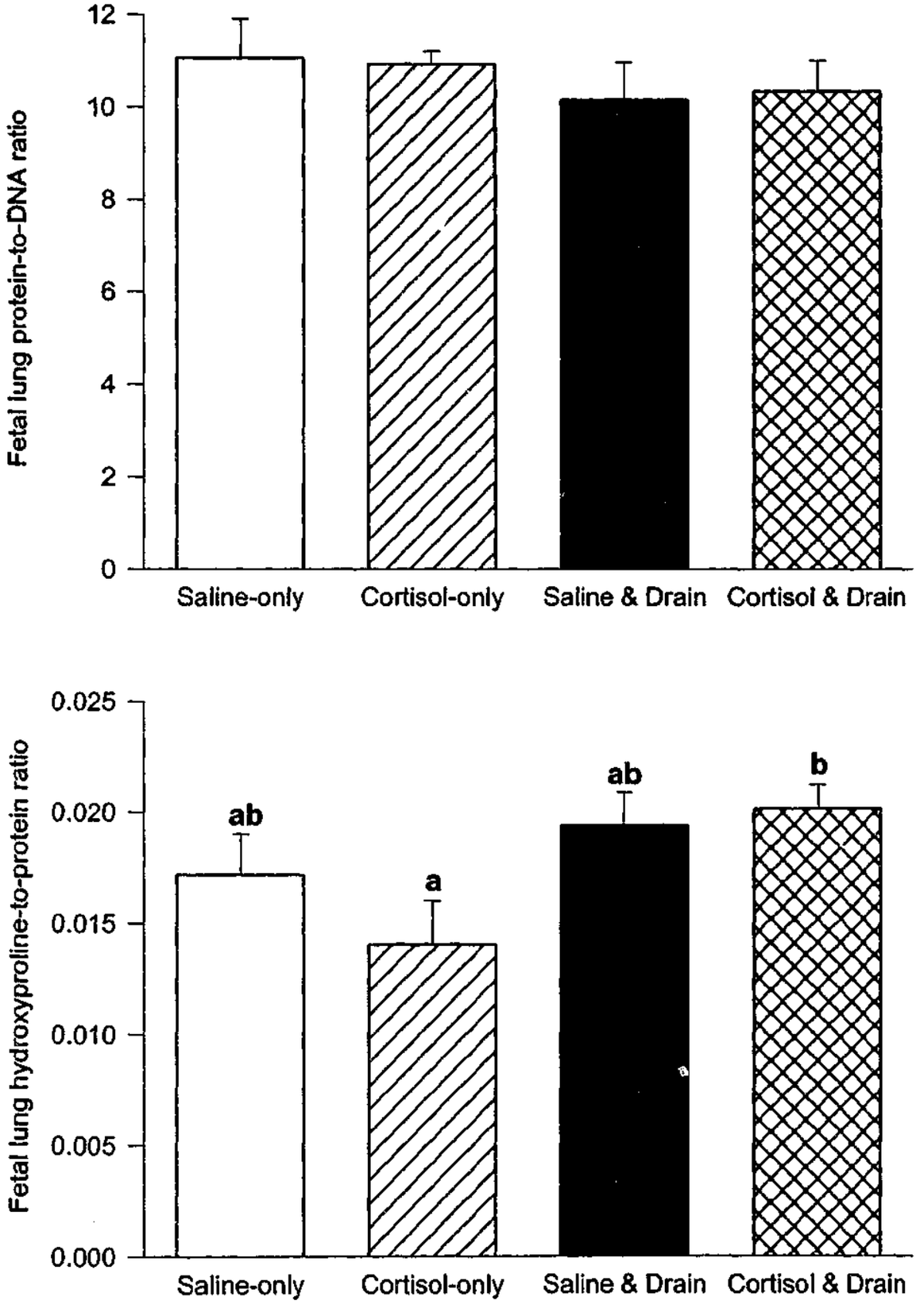

Figure 3.7 Fetal lung protein-to-DNA ratio and hydroxyproline-to-protein ratio

Upper panel: fetal lung protein-to-DNA ratio ± SEM and ***lower panel***: fetal lung hydroxyproline-to-protein ratio ± SEM, as measured in *saline-only* (n = 5), *cortisol-only* (n = 5), *saline & drain* (n = 5) and *cortisol & drain* (n = 5) groups of fetuses. No significant differences were found in the upper panel. In the lower panel, values that do not share a common letter are significantly different from each other ($P < 0.05$).

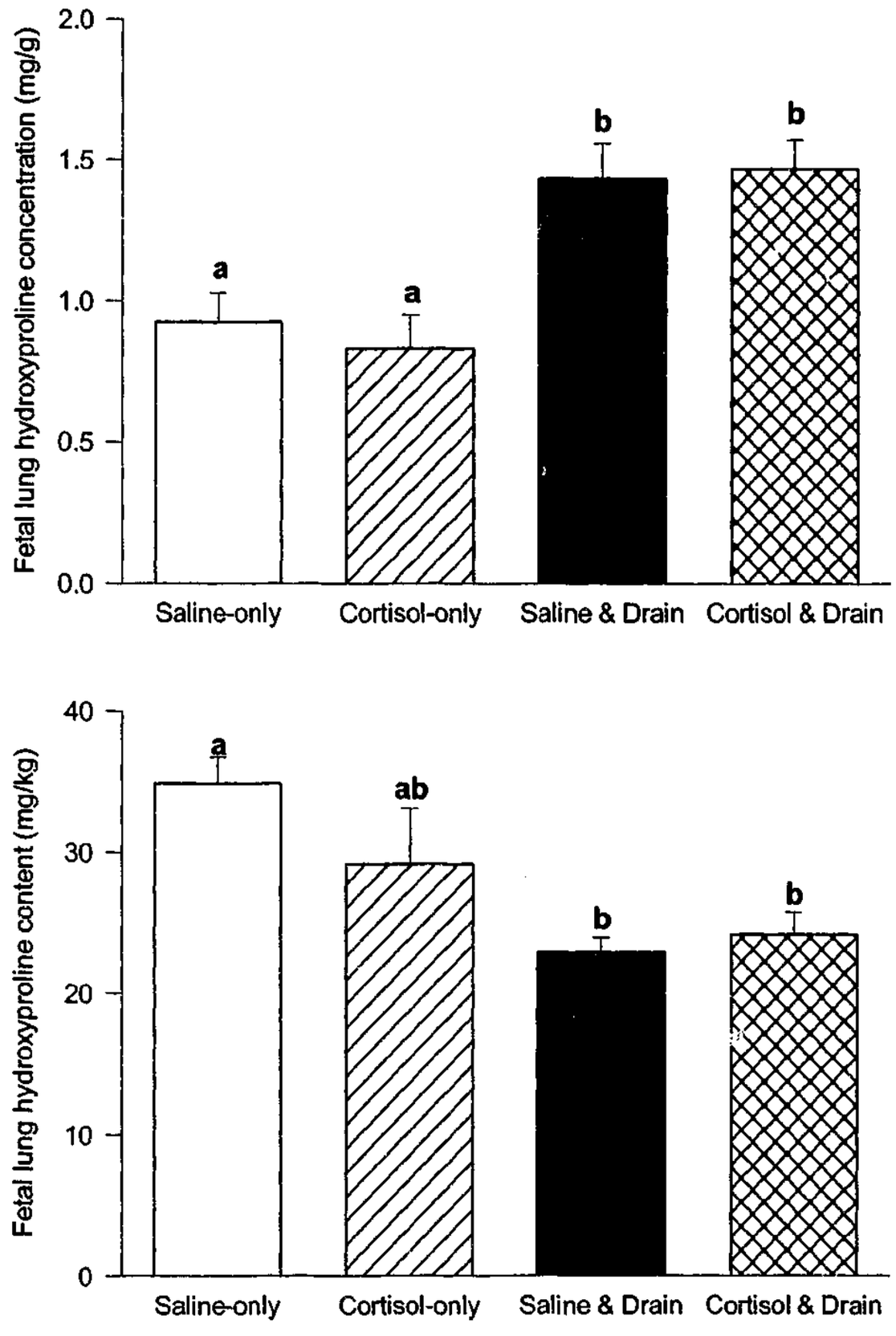

Figure 3.8 Fetal lung hydroxyproline concentration and content

Upper panel: fetal lung hydroxyproline concentration (mg/g lung weight) ± SEM and ***lower panel***: fetal lung hydroxyproline content (mg/kg body weight) ± SEM measured in *saline-only* (n = 5), *cortisol-only* (n = 5), *saline & drain* (n = 5) and *cortisol & drain* (n = 5) groups of fetuses. Values that do not share a common letter are significantly different from each other ($P < 0.05$).

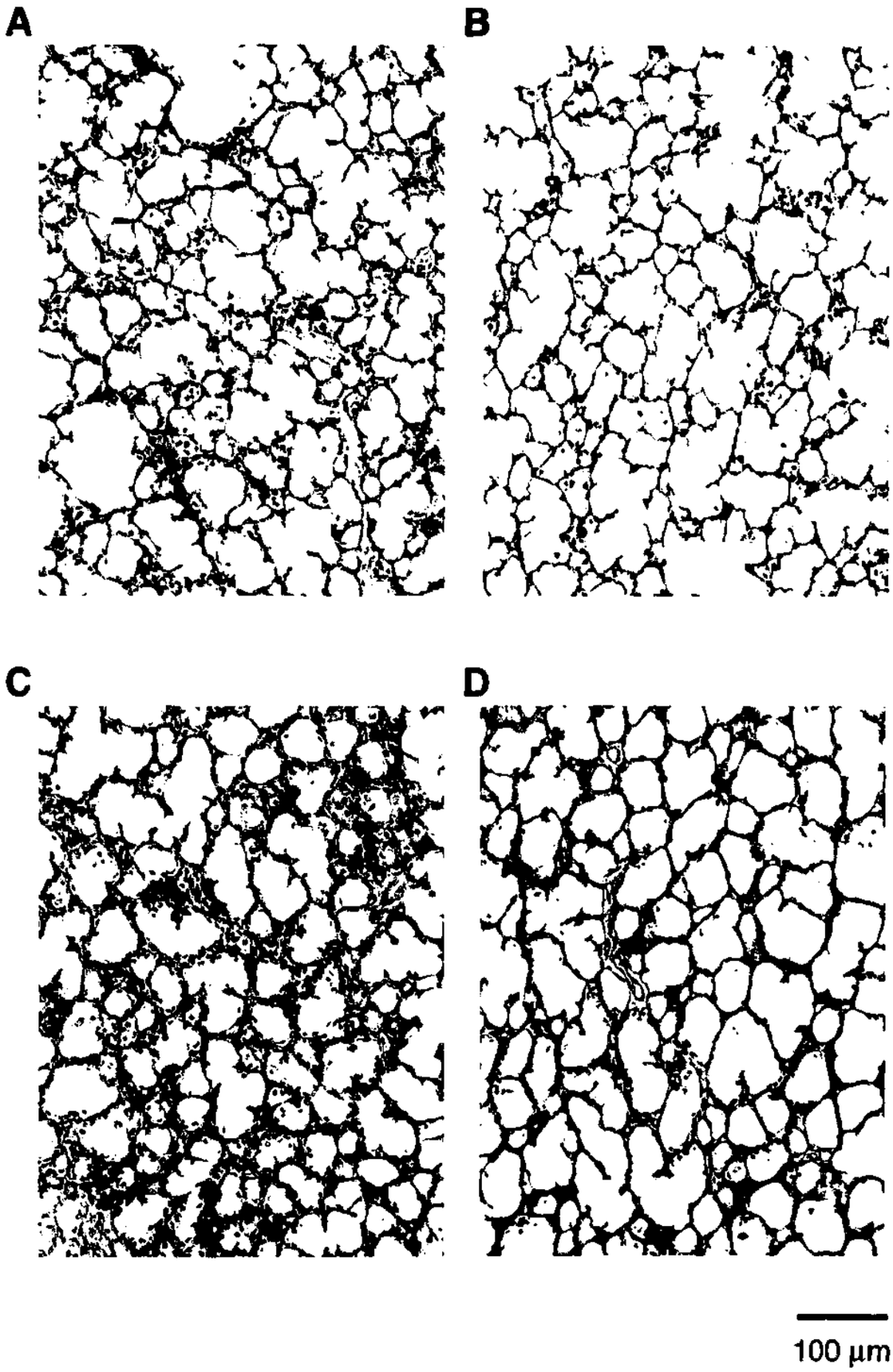

Figure 3.9 Histological appearance of the fetal lungs

Lung tissue sections as seen under the light microscope from ***A)*** *saline-only*, ***B)*** *cortisol-only*, ***C)*** *saline & drain* and ***D)*** *cortisol & drain* fetuses at 130.8 ± 0.1 days of gestation. The sections were 5 µm thick and were stained with Haematoxylin and Eosin. Sections were photographed at a final magnification of X150. The black bar indicates the scale of each picture.

Table 3.4 Fetal lung volume, tissue space and luminal space

The percentage of space within the ***right*** *lung occupied by tissue or lumen, lung volume (cm^3 and cm^3/kg), tissue volume (cm^3/kg), luminal volume (cm^3/kg) and tissue-to-luminal space ratio, in each of the four groups of fetuses. All values refer to the* ***right*** *lung at 130.8 ± 0.1 days of gestation. For each parameter, values that do not share a common letter are significantly different from each other ($P < 0.05$).*

	saline-only	cortisol-only	saline-then-cortisol	cortisol-then-cortisol
Percentage luminal space (%)	76.3 ± 3.3 [a]	82.7 ± 2.8 [b]	60.0 ± 3.6 [c]	70.7 ± 3.5 [d]
Percentage tissue space (%)	23.6 ± 3.3 [a]	17.3 ± 2.8 [b]	40.0 ± 3.6 [c]	29.3 ± 3.5 [d]
Right lung volume (cm^3)	81.6 ± 5.5 [a]	74.9 ± 2.5 [b]	28.9 ± 3.1 [c]	30.7 ± 1.9 [c]
Right lung volume adjusted for body weight (cm^3/kg)	24.4 ± 1.2 [a]	20.6 ± 0.4 [b]	9.9 ± 0.2 [c]	10.1 ± 0.2 [c]
Total luminal volume adjusted for body weight (cm^3/kg)	18.7 ± 1.2 [a]	17.1 ± 0.7 [a]	5.9 ± 0.4 [b]	7.2 ± 0.4 [b]
Total tissue volume adjusted for body weight (cm^3/kg)	5.8 ± 0.8 [a]	3.6 ± 0.6 [bc]	4.0 ± 0.4 [b]	3.0 ± 0.4 [c]
Tissue-to-luminal space ratio	0.31 ± 0.06 [a]	0.21 ± 0.04 [b]	0.67 ± 0.06 [c]	0.42 ± 0.07 [d]

The percentage luminal space was significantly lower in the lungs of fetuses drained of lung liquid when compared to *saline-only* fetuses. Similarly percentage tissue space was significantly higher in the lungs of fetuses drained of lung liquid when compared to those of *saline-only* fetuses. Right lung volume was significantly lower in *saline & drain* fetuses in comparison to *saline-only* fetuses, as was right lung volume adjusted for body weight. Total luminal volume (adjusted for body weight) was significantly lower in *saline & drain* fetuses when compared to *saline-only* fetuses, as was total tissue volume. Tissue-to-luminal space ratio was also significantly higher in *saline & drain* fetuses than in *saline-only* fetuses (Table 3.4).

Cortisol infusion increased percentage luminal space in *cortisol & drain* animals towards, but not reaching, *saline-only* levels and likewise reduced the percentage tissue space towards but not reaching *saline-only* levels. Right lung volume in lung liquid drained fetuses, before and after adjustment for fetal body weight, was not affected by cortisol infusion and remained significantly lower than the non-drained fetuses. Likewise, total luminal volume (adjusted for fetal body weight) tended to be increased by cortisol in the drained groups of fetuses (non-significant), but was significantly lower than both of the non-drained groups. Total tissue volume however, was significantly reduced in the *cortisol & drain* group when compared to the *saline & drain* and *saline-only* groups. Tissue-to-luminal space ratio was lower in the *cortisol & drain* group than in the *saline & drain* group. Tissue-to-luminal space ratio in the *cortisol & drain* group was reduced towards, but not reaching, values for *saline-only* and *cortisol-only* groups (Table 3.4).

3.3.5.2 Alveolar Diameter, Alveolar Number and Inter-alveolar Wall Thickness

Mean alveolar diameter was not different between the *saline-only* (31.4 ± 2.9 μm) and *cortisol-only* (36.7 ± 3.8 μm) or *saline & drain* (25.7 ± 2.5 μm) groups of fetuses (Figure 3.10). There was also no difference between the *saline-only* (31.4 ± 2.9 μm) and *cortisol & drain* (35.5 ± 5.2 μm) fetuses (Figure 3.10). However, there was a significant difference ($P < 0.05$) in alveolar diameter between the *cortisol-only* (36.7 ± 3.8 μm) and *saline & drain* (25.7 ± 2.5 μm) groups of fetuses. This reduction in alveolar diameter in the *saline & drain* group appeared to be ameliorated by the infusion of cortisol.

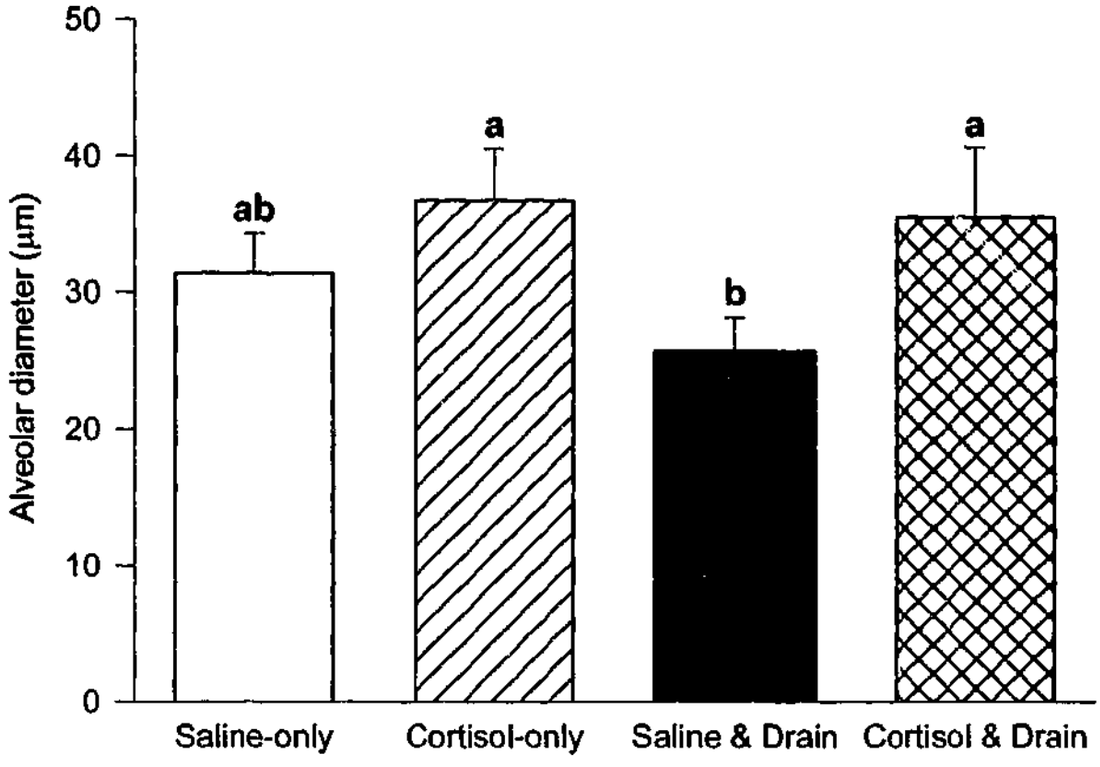

Figure 3.10 Alveolar diameter

Mean alveolar diameter (μm) ± SEM in the right lung of: *saline-only* (n = 5), *cortisol-only* (n = 5), *saline & drain* (n = 5) and *cortisol & drain* (n = 5) groups of fetuses. Values that do not share a common letter are significantly different from each other ($P < 0.05$).

Alveolar number was increased by cortisol infusion (*cortisol-only*: 522.5 ± 55.1 $X10^6$; *cortisol & drain*: 67.0 ± 7.6 $X10^6$) relative to the respective saline-infused groups of fetuses (*saline-only*: 380.8 ± 17.3 $X10^6$; *saline & drain*: 41.4 ± 7.3 $X10^6$; Figure 3.11). Cortisol infusion increased alveolar number by 37% in normal lungs and by 62% in hypoplastic lungs. A period of lung liquid drainage significantly reduced ($P < 0.05$) alveolar number in comparison to both non-drained groups. Alveolar number was increased in *cortisol & drain* fetuses in comparison to *saline & drain* fetuses but remained significantly less than *saline-only* levels (Figure 3.11: upper panel).

Cortisol infusion reversed the effect of lung liquid drainage (*cortisol & drain*: 16.0 ± 2.5 μm) on inter-alveolar wall thickness, returning it to *saline-only* levels (12.6 ± 2.1 μm; Figure 11: lower panel). Inter-alveolar wall thickness tended not to be different between the *saline-only* group (12.6 ± 2.1 μm) and the *cortisol-only* group (9.4 ± 1.4 μm), but both groups had significantly ($P < 0.05$) thinner inter-alveolar walls than *saline & drain* fetuses (21.5 ± 3.7 μm).

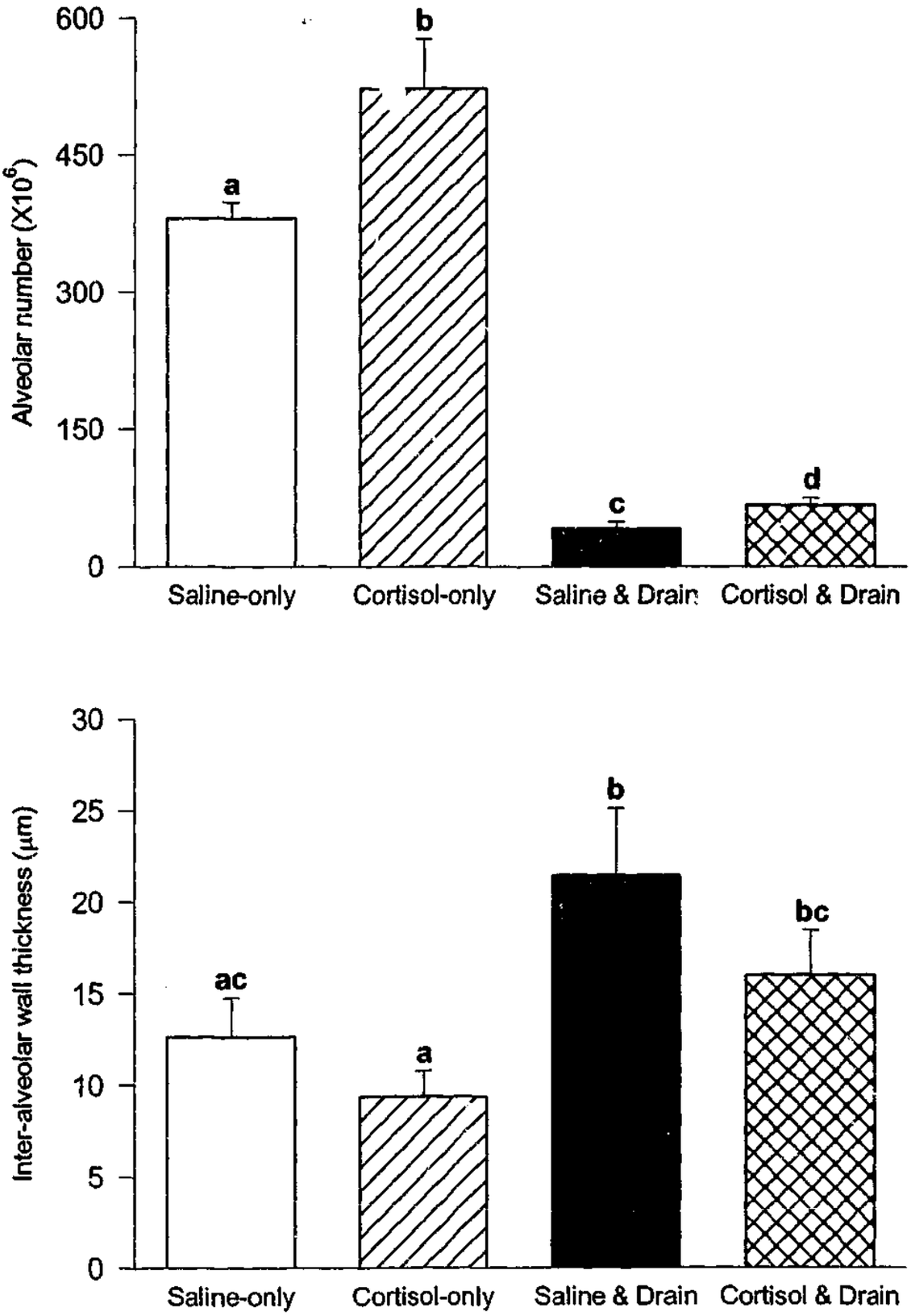

Figure 3.11 Alveolar number and inter-alveolar wall thickness

Upper panel: mean alveolar number ± SEM in the right lung and ***lower panel***: mean inter-alveolar wall thickness (μm) ± SEM in the right lung of: *saline-only* (n = 5), *cortisol-only* (n = 5), *saline & drain* (n = 5) and *cortisol & drain* (n = 5) groups of feluses. Values that do not share a common letter are significantly different from each other ($P < 0.05$).

3.4 Discussion

Cortisol accelerates many aspects of lung development, however, it also increases lung volume, most probably by increasing lung compliance. As an increase in lung volume, in the absence of an increase in cortisol, can also induce acceleration in lung growth and structural development, we hypothesised that structural alterations in the developing lung brought about by exogenous or endogenous cortisol may be due to alterations in lung expansion. Therefore, it was hypothesised that a prolonged period of lung deflation would inhibit the effects of cortisol on structural or morphometric aspects of lung maturity. The aim of this study therefore, was to examine the effect of cortisol on fetal lung growth and maturation in a lung undergoing continuous lung liquid drainage. Lung liquid drainage has proven to be a potent inhibitor of lung growth, as indicated by large reductions in lung weight and DNA, protein and hydroxyproline contents, ultimately resulting in lung hypoplasia. Importantly, this study has shown that an increasing dosage of cortisol delivered intravenously to the fetus over a 9 day period did not improve nor reduce lung growth as assessed by fetal lung weights, total DNA, protein and hydroxyproline contents and concentrations and protein-to-DNA ratio. However, there were considerable alterations in morphological indices of fetal lung maturation, with cortisol treatment decreasing the percentage right lung tissue space and increasing the percentage right lung luminal space in both normal and hypoplastic lungs. Thus, a reduction in lung expansion was countered by a period of cortisol infusion, despite the negative stimulus due to lung liquid drainage. In addition, advantageous alterations in morphological indices occurred even though the dose used in this study was much less than that used in other studies in which detrimental effects on fetal lung and body growth were observed. Cortisol delivered at these physiological levels did not affect fetal body weight or fetal liver or kidney weights. Therefore, physiological doses of cortisol were able to induce morphological maturation in both normal and hypoplastic lungs without affecting many of the biochemical indices of lung growth. Importantly, these alterations in structural maturation following cortisol treatment appear to occur despite changes in lung expansion.

3.4.1 Fetal Well Being and Fetal Plasma Cortisol Concentrations

All fetuses included in this study were considered healthy during the course of the experiments as assessed by fetal arterial blood gas and acid-base status. Periods of

saline and cortisol infusion and lung liquid drainage did not affect fetal arterial blood gas and acid-base values.

The cortisol infusion regime was identical to that which we have used previously (Boland *et al.*, 1997) and the plasma cortisol concentrations achieved were similar to those observed in that study. The plasma cortisol concentrations in the cortisol-infused fetuses rose gradually over the infusion period, but did not reach concentrations that are sufficient to induce parturition in fetal sheep (Elsner *et al.*, 1980; Nathanielsz *et al.*, 1982; Mason *et al.*, 1989). Previous studies have calculated that fetal plasma cortisol concentrations must be greater than 40 - 50 ng/ml for approximately 3 days to induce parturition in sheep (Elsner *et al.*, 1980; Nathanielsz *et al.*, 1982; Mason *et al.*, 1989). There was no increase in the fetal plasma cortisol concentrations measured in the saline-infused fetuses at any time in the experimental period. This is in accordance with previous studies (Magyar *et al.*, 1980), which have shown that the pre-parturient increase in cortisol concentrations in fetal sheep does not generally occur until 11.8 ± 1.0 days before labour, which is ~135 days of gestation in the breed of sheep used in the present study.

3.4.2 Fetal Lung Liquid Drainage Rates and Cumulative Volumes

A number of previous studies have used lung liquid drainage as a simple method to induce pulmonary hypoplasia in fetal sheep (Alcorn *et al.*, 1977; Moessinger *et al.*, 1990; Nardo *et al.*, 1995; Davey *et al.*, 1999). These studies have measured both the rate of lung liquid drained and the cumulative volume of lung liquid drained. Alcorn *et al.* (1977) found that in the fetuses that were drained of lung liquid for 21 - 25 days in late gestation, the mean drainage rates over the entire drainage period ranged from 7.3 to 14.3 ml/hr between different fetuses during the course of the drainage period. In the study by Moessinger *et al.* (1990) in which the right lung was drained of lung liquid whilst the left mainstem bronchus was simultaneously ligated, drainage rates from the right lung only, averaged 4.4 ml/hr (range being 1.9 - 6.6 ml/hr) over the course of the drainage period (105 - 129 days of gestation). Additionally, Nardo *et al.* (1995) showed that lung liquid drainage rate increased from 3.9 ± 0.2 ml/hr at 105 days to 14.4 ± 0.5 ml/hr at 134 days of gestation. When adjusted for fetal body weight, the rate of lung liquid drainage in that study did not change with increasing gestational age and was approximately 3.5 ml/hr/kg throughout the experimental period. Davey *et al.* (1999)

also demonstrated that there was an age-related increase in lung liquid drainage rates from 6.5 ± 0.5 ml/hr at 113 ± 0.3 days of gestation to 17.7 ± 1.3 ml/hr at 138.0 ± 0.2 days of gestation. Mean lung liquid drainage rates corrected for fetal body weight in that same study were 5.4 ± 0.1 ml/hr/kg (Davey *et al.*, 1999). Clearly, the values of lung liquid drainage rates from the saline-infused fetuses in the present study fall within the range of values shown in the above studies.

An intravenous cortisol infusion in fetal sheep at similar concentrations as used in this study has been previously shown to elevate lung liquid secretion rates (Wallace *et al.*, 1995) and as a result, increase lung liquid volumes. In the present study, cortisol-infused fetuses had a significantly greater lung liquid drainage rate at three timepoints during the infusion period. However, by the end of the infusion period, lung liquid drainage rates were not significantly different between the *saline & drain* and *cortisol & drain* groups of fetuses, although they appeared to be elevated. Additionally, the rate of accumulation of lung liquid drained was significantly different between *saline & drain* and *cortisol & drain* groups of fetuses after the cortisol infusion commenced. A number of mechanisms have been postulated for this increase in lung liquid secretion rate following administration of corticosteroids. An alteration in lung luminal surface area resulting in an increased secretory area (Crone *et al.*, 1983) is one possible explanation. Indeed, morphological responses to cortisol treatment including an increase in alveolar number and an increase in luminal space, suggest that surface area is increased following cortisol infusion which supports this hypothesis. However, because lung liquid drainage rates were relatively equal by the time of *post mortem*, it is not likely that an increase in surface area would account for the changes observed in lung liquid drainage rates with cortisol infusion. Such alterations in lung liquid secretion rates are more likely to be due to transitory changes in the concentration or activity of some or all of the epithelial components thought to be involved in the secretion of lung liquid, such as Na^+-K^+-ATPase, Na^+-K^+-$2Cl^-$ cotransporter and Cl^- channels, as suggested previously (Wallace *et al.*, 1995).

Expressed in relation to fetal body weight, lung liquid drainage rates of fetuses prior to the onset of either infusion, were within the normal range (2 - 5 ml/hr/kg) of lung liquid secretion rates (Mescher *et al.*, 1975; Olver *et al.*, 1981a; Harding & Hooper, 1996). Similar to the results presented in a previous study (Nardo *et al.*, 1995), a gestational age-related increase in drainage rates per kilogram of fetal body weight (Olver *et al.*, 1981a; Hooper & Harding, 1995) did not occur. Yet, when corrected for wet lung

weight at the time of *post mortem*, the drainage rates in these fetuses were much greater than the lung liquid secretion rates of control fetuses (Nardo *et al.*, 1995). This apparent stimulatory effect of drainage on lung liquid secretion was attributed to hydrostatic pressure changes within the lung lumen caused by this experimental technique (Nardo *et al.*, 1995). Lung liquid secretion is driven by an osmotic pressure gradient (Olver *et al.*, 1974; Olver *et al.*, 1986). This pressure is thought to be opposed by the hydrostatic pressure within the lung lumen (Carmel *et al.*, 1965; Miller *et al.*, 1993). When the lung is drained of liquid, the hydrostatic pressure is reduced, therefore increasing the osmotic pressure gradient that drives lung liquid secretion (Hooper & Harding, 1995).

3.4.3 Fetal Body Weights and Biochemical Indices of Lung Growth

Lung DNA and protein contents were markedly reduced as a result of a 20 day period of lung liquid drainage. These results support previous evidence that prolonged lung liquid drainage results in pulmonary hypoplasia, as determined by fetal lung weights and DNA and protein contents (Moessinger *et al.*, 1990; Nardo *et al.*, 1995; Davey *et al.*, 1999). Furthermore, this indicates that lung liquid drainage is a potent inhibitor of lung growth in fetal sheep. Indeed, lung liquid drainage for a similar period results in hypoplastic lungs that are unable to support life after birth (Davey *et al.*, 1999). Hydroxyproline content, a marker for collagen, was significantly decreased following lung liquid drainage. Certainly, collagen content appears to be affected in a similar manner as protein and DNA contents. Following an increase in lung expansion, DNA, protein and hydroxyproline contents increase (Nardo *et al.*, 1998). The results found in the present study, therefore further emphasise that changes in DNA, protein and hydroxyproline are regulated by the degree of lung expansion.

In the present study, a 20-day period of lung liquid drainage resulted in a 60% reduction in fetal wet lung weights (g), in both groups of drained fetuses. This reduction in wet lung weight following lung liquid drainage is similar to the 60% (Alcorn *et al.*, 1977), 58% (right lung only; Moessinger *et al.*, 1990) and 58% (Nardo *et al.*, 1995) reductions found in previous studies. In addition, the reductions in fetal lung weights following lung liquid drainage in the present study are similar to the reductions in lung weight produced by the nitrofen-induced model of CDH (Sluiter *et al.*, 1992; Alfonso *et al.*, 1993; Suen *et al.*, 1993; Suen *et al.*, 1994; Losty *et al.*, 1995; Losty *et al.*,

1996; Ijsselstijn *et al.*, 1997) and surgically created diaphragmatic hernias in rabbits (Tannuri *et al.*, 1998a; Tannuri *et al.*, 1998b) and sheep (deLorimier *et al.*, 1967; Glick *et al.*, 1992; Hill *et al.*, 1994; Hassett *et al.*, 1995; O'Toole *et al.*, 1996; Lipsett *et al.*, 2000) as well as human autopsy findings from fetuses (Beals *et al.*, 1992, Asabe *et al* 1997).

A period of cortisol infusion at physiological doses was unable to reverse the growth deficit in the hypoplastic lungs. In fact, the cortisol infusion did not affect lung weights, lung DNA or protein contents, or the protein-to-DNA ratio in normal or hypoplastic lungs. Likewise, cortisol had no effect on DNA and protein concentrations, hydroxyproline concentration or content, nor in the protein-to-DNA ratio following infusion with cortisol. In other studies, fetal or maternal corticosteroid infusions have had either no effect (Johnson *et al.*, 1978; Liggins *et al.*, 1988, Warburton *et al.*, 1988; Ikegami *et al.*, 1991) or have reduced some or all of the above lung growth parameters (Schellenberg *et al.*, 1987b, Adamson & King, 1988, Kudlacz *et al.*, 1989; Stein *et al.*, 1993; Suen *et al.* 1994; Ijsselstijn *et al.*, 1997). Importantly, in the present study, a premature increase in fetal cortisol concentrations did not affect fetal body weight either. This corresponds to other measurements made in our laboratory that show that delivery of corticosteroids at physiological doses to the fetus alter neither fetal body weight nor fetal lung weight (Wallace *et al.*, 1995; Boland *et al.*, 1997). However, other studies that have observed maturation of lung morphology following treatment with much greater doses of corticosteroids in animal models of CDH, results in decreases in lung weights (Suen *et al.* 1994; Ijsselstijn *et al.*, 1997) and/or fetal body weights (Losty *et al.*, 1995; Ijsselstijn *et al.*, 1997). Similar reductions in birth weight have also been found following antenatal administration of synthetic corticosteroids in non-CDH models (Reinisch *et al.*, 1978; Johnson *et al.*, 1981; Jobe *et al.*, 1998; Sloboda *et al.*, 2000). Administration of corticosteroids in other animal studies and indeed in human clinical trials, have shown reductions in somatic growth and neurologic function (reviewed: Ballard & Ballard, 1976; Seckl, 1998). It is becoming apparent that both route of delivery, number of doses (French *et al.*, 1999) and the timing and dose of delivery of the corticosteroid itself are important factors in determining whether growth restrictions occur.

A period of lung liquid drainage resulted in an increase in lung hydroxyproline concentration and a decrease in hydroxyproline content. Treatment with cortisol did not alter hydroxyproline concentration and had a slight tendency to reduce the

hydroxyproline content (not significant) in *cortisol-only* fetuses towards but not reaching that of drained fetuses. Cortisol has previously been shown to decrease the rate of collagen synthesis in other organs *in vitro* (Kivirikko & Laitinen, 1965, Kivirikko *et al.*, 1965). *In vivo* experiments have shown that cortisol treatment leads to an increase in pulmonary hydroxyproline concentration (Schellenberg *et al.*, 1987a). However, this increase was associated with a large decrease in lung dry weights (Schellenberg *et al.*, 1987a). Thus, it is likely that despite increases in lung hydroxyproline concentrations, lung collagen content is unchanged following treatment with cortisol in these fetuses. Similar results were observed by Anceschi *et al.* (1992). Importantly, the current study shows that alterations in lung expansion have a greater effect on lung collagen content than that of physiological doses of cortisol.

3.4.4 Morphological Indices of Lung Maturation

In the present study, lungs drained of liquid were morphologically similar to those described in younger fetuses. This is especially apparent when viewed in terms of tissue and luminal space. *Saline & drain* fetuses had 70% greater tissue space than *saline-only* fetuses and a similar observation was made between *cortisol & drain* and *cortisol-only* fetuses. Inter-alveolar wall thickness was greater in the hypoplastic lungs when compared to the normal lungs. As described previously, an adequate volume of lung liquid actively stretches the lung, stimulating lung growth (Moessinger *et al.*, 1990; Hooper & Harding, 1995). Thus, lung liquid drainage removes this stretch stimulus resulting in cessation of lung growth and impairment of morphometric indices of lung maturity (Alcorn *et al.*, 1977; Davey *et al.*, 1999). Lungs drained of liquid resembled those from fetuses of an earlier gestational age (Alcorn *et al.*, 1977; Nardo *et al.*, 1995). Hypoplastic lungs from CDH fetuses are characterised by having reduced radial alveolar counts, which are a measure of the complexity of the alveolar region of the lungs (Asabe *et al.*, 1997, Losty *et al.*, 1996), a reduction in surface area (Losty *et al.*, 1996), an increase in tissue space and a corresponding decrease in luminal space (Ijsselstijn *et al.*, 1997; Losty *et al.*, 1996). In the present study, alveolar diameter was not significantly different between *saline-only* and *saline & drain* fetuses, although it did appear to be slightly lower in the *saline & drain* fetuses. It is important to note that measurements of alveolar diameter in young or immature lungs may be affected because there are fewer true alveoli and may therefore reflect the diameter of airway canals rather than true alveoli. Alveolar number was markedly reduced in both drained groups of fetuses and again highlights the developmental immaturity of the lungs in these fetuses. In the present study, alveolar number in the *saline-only* fetuses at 130

days of gestation (when term is ~147 days of GA) was similar to those levels found in previous studies from our laboratory (Nardo *et al.*, 2000) and is in accordance with previously published data in sheep at term (Davies *et al.*, 1988).

Previous studies on corticosteroid treated fetal sheep have revealed that cortisol accelerates many morphological indices of lung maturation (Boshier *et al.*, 1989; Kendall *et al.*, 1990; Kendall *et al.*, 1991). Such alterations include a decrease in tissue space and an increase in luminal space, thinning of the alveolar epithelium and increases in lung volumes. In the present study, similar improvements in lung maturity were produced but with much lower, physiological doses of corticosteroids. In the study by Kendall *et al.* (1990), fetuses were infused with cortisol at 17 mg per day until the onset of labour (58 ± 3 hours after commencement of cortisol infusion). This dose was at least 4 times greater than the maximum daily dose that animals received in the present study. Such high doses of cortisol led to fetal plasma cortisol concentrations that are known to precipitate labour (Elsner *et al.*, 1980; Nathanielsz *et al.*, 1982; Mason *et al.*, 1989; Kendall *et al.*, 1990). Similarly, Schnitzer *et al.* (1996) and Hedrick *et al.* (1997) infused sheep CDH fetuses with high doses of cortisol, 50 mg per dose with a dose every 12 hours for a period of 3 days. These studies showed that cortisol treatment accelerated structural maturity of the lungs, as indicated by morphometric indices including radial alveolar count and inter-airspace wall distance. However, the studies by Schnitzer *et al.* (1996) and Hedrick *et al.* (1997) utilised a total quantity of 300 mg of cortisol in 60 hours but fetal plasma cortisol concentrations were not recorded. In contrast, the doses used in the present study were designed to mimic the normal preparturient rise in fetal plasma concentrations and used a meagre 25 mg of cortisol over 9 days. Therefore, the results of the current study suggest that much lower doses of cortisol than those used by other investigators can deliver the same beneficial effects on lung maturation in the absence of any detrimental effects on fetal lung or body growth. Such a finding is of particular interest given the growing concern of detrimental effects of repeated, high-dose corticosteroid treatment *in utero* (French *et al.*, 1999; Sloboda *et al.*, 2000).

Physiological doses of cortisol had substantial effects on tissue and luminal space fractions. Cortisol accelerated the progression towards a greater luminal space and a reduced tissue space fraction in both the normal and hypoplastic lungs. Cortisol acted to reduce inter-alveolar wall thickness and increased alveolar diameter in *cortisol & drain* fetuses when compared to *saline & drain* fetuses. Cortisol infusion accelerated

the development of alveoli and its effects can be observed in the light microscope images (Figure 3.9), which clearly show the acceleration in the appearance of alveolar septae between *cortisol & drain* and *saline & drain* fetuses. However, alveolar number was greatly affected by a change in lung expansion, with an ~900% difference in the alveolar number between *saline & drain* fetuses and *saline-only* animals, as opposed to only an ~60% improvement in right lung alveolar number by treatment of these drained lungs with cortisol (*cortisol & drain* fetuses). This highlights the important relationship between alveolar number and the degree of lung expansion and correlates with previous findings from our laboratory suggesting that an increase in lung expansion is a potent stimulus of alveolarisation (Nardo *et al.*, 2000). In that study, alveolar number was ~37% greater following 4 days of tracheal obstruction, staying at about these levels after 10 days of tracheal obstruction. The percentage differences in alveolar number between lung liquid drained, normal and tracheal obstructed lungs indicate that an adequate volume of lung liquid is essential for the formation of alveoli in sheep.

Cortisol is believed to play a major role in accelerating lung maturation immediately prior to birth. One of the ways that cortisol is believed to accelerate such changes is *via* alterations in the extracellular matrix. This study has shown that acceleration in lung maturation following treatment with corticosteroids occurs irrespective of the degree of lung expansion. With the exception of overall lung growth and alveolar number, cortisol was able to return many indices in hypoplastic lungs substantially towards those seen in control fetuses. Therefore, it is likely that cortisol acts directly on the extracellular matrix to bring about structural changes in the lung. Alterations in lung growth and structural maturation are tightly regulated by the degree of lung expansion and we hypothesised that the cortisol-induced increase in lung liquid volume may mediate many of the maturational effects of cortisol on lung development. However, cortisol appears to act *via* different mechanisms than lung expansion to accelerate aspects of lung maturity. Acting *via* intracellular cortisol receptors, cortisol may directly facilitate extracellular matrix remodelling to attenuate the interstitium. Previously, cortisol treatment has been shown to increase extracellular matrix proteins such as elastin and collagen (Schellenberg *et al.*, 1987a; Campos *et al.*, 1992), although surprisingly, there were no effects on lung collagen in this study. Changes in ECM components, such as elastin and collagen, are believed to be responsible for increases in lung distensibility and compliance that occur following treatment with cortisol (Beck *et al.*, 1981; Fiascone *et al.*, 1987) and are also of benefit to the air-breathing newborn. Thus, cortisol appears to act directly to facilitate ECM remodelling and acceleration in the structural maturity of the lungs.

3.4.5 Summary

This study has shown that an increasing dosage of cortisol delivered intravenously to the fetus over a 9 day period accelerates lung maturation in both normal and hypoplastic lungs. Cortisol delivered at these physiological levels did not have a deleterious effect on fetal lung or body growth. In the context of recent studies, showing detrimental effects of high-dose corticosteroids this is an interesting finding. Importantly, however, the alterations in structural maturity following cortisol treatment appeared to occur regardless of changes in lung expansion. This suggests that cortisol acts *via* other mechanisms, independent of alterations in lung expansion, to stimulate structural changes in the lung and to enhance lung maturity.

CHAPTER 4 Role of Gelatinases in Structural Remodelling of the Lung

4.1 Introduction

The collagens are a family of ECM proteins that provide the lung with structural integrity and are involved in important functions such as cell adhesion, cellular signalling and matrix assembly. Turnover of collagen is believed to play a fundamental role in tissue remodelling throughout life, permitting alterations in lung architecture. During lung development, both collagen synthesis (Tolstoshev *et al.*, 1981; Jackson *et al.*, 1990) and turnover (Mays *et al.*, 1989) are elevated, highlighting that tissue remodelling is particularly important in prenatal lung growth. In fact, collagen synthesis rates appear to be highest during the latter stages of *in utero* lung development (Bradley *et al.*, 1974), when the lung undergoes marked changes in morphology. As a consequence of these high collagen synthesis rates, lung collagen content increases towards the end of gestation (Schellenberg & Liggins, 1987). Increases in fetal lung expansion also lead to an increase in lung collagen content (Nardo *et al.*, 1998). Conversely, a decrease in lung expansion results in a decrease in collagen content (Chapter 3). As collagen provides the structural scaffolding of the lung, it follows that an acceleration of lung growth would require an extension to this structural framework requiring greater amounts of collagen. On the other hand, a slowing in lung growth should reduce growth of the structural framework thereby reducing collagen accumulation. It is unclear, however, if these changes in collagen content are a result of changes in collagen production or in collagen turnover.

Net collagen accumulation within the lung is a finely balanced relationship between *de novo* synthesis and degradation. Collagen is actively broken down by a number of enzymes, including the matrix metalloproteinases (MMPs). The MMPs are in turn regulated by the tissue inhibitors of metalloproteinases (TIMPs: Nagase, 1994; Parsons

et al., 1997). An increase in *de novo* synthesis of collagen with no alterations in collagen breakdown would cause an increase in collagen content. Likewise, no alterations in *de novo* synthesis of collagen but a decrease in MMP breakdown of collagen would also lead to an increase in collagen content. Therefore, methods that measure MMP levels and activity may provide some insight into how alterations in collagen content in the developing fetal lung are regulated.

The MMP family comprises over 20 proteinases that are capable of degrading collagens, proteoglycans, fibronectin and laminin (Matrisian, 1990; Bode *et al.*, 1999). Turnover of the ECM is tightly controlled by several mechanisms. Firstly, the types of MMPs synthesised will influence ECM degradation due to the substrate specificities of the enzymes. Secondly, enzyme latency is maintained by the propeptide, which is bound to and blocks the catalytic site. Activation of an MMP requires the stepwise proteolysis of this propeptide. Thirdly, turnover of the ECM is also regulated by the balance between the net production of MMPs and their natural inhibitors, the TIMPs (Nagase, 1994). The gelatinases (MMP-2 and MMP-9), can degrade native collagens I, IV, V, VII and X to some extent, but they are more active against the denatured form of collagen: gelatin (Murphy *et al.*, 1990; Corbel *et al.*, 2000). Thus, MMP-2 and -9 are thought to be important in the final degradation of fibrillar collagens after they have been cleaved by collagenases (Kahari & Saarialho-Kere, 1999). Several studies have documented changes in the levels and expression of gelatinases during fetal lung development. MMP-2 is present in the lung during development, particularly late in gestation, in a number of species (Reponen *et al.*, 1992; Arden *et al.*, 1993; Malicdem *et al.*, 1993; Tournier *et al*, 1994; Rolland *et al.*, 1995; Kinoh *et al.*, 1996; Fukuda *et al.*, 2000). Fukuda *et al.* (2000) found that the ratio of active to latent MMP-2 increases towards the end of gestation in fetal rabbit lungs. In that study, MMP-2 was localised to type II alveolar epithelial cells along with its activator membrane type 1-MMP (MT1-MMP: Fukuda *et al.*, 2000). Interestingly, Arden *et al.* (1993) measured MMP-2 levels in fetal rat lung and showed that MMP-2 levels increased in late gestation, concomitantly with an increase in collagen synthesis rates, but not with an increase in collagen content. In contrast, MMP-9 is not normally found in the fetal lung (Arden *et al.*, 1993; Dunsmore *et al.*, 1998; Fukuda *et al.*, 1998; Eickelberg *et al.*, 1999) until very late in gestation (Rolland *et al.*, 1995, Fukuda *et al.*, 2000). However, MMP-9 is elevated in newborn lung in comparison to both fetal and adult lungs (Delacourt *et al.*, 1995; Fukuda *et al.*, 2000) and is prominent in pathological conditions such as chronic obstructive pulmonary disease (COPD) and asthma (Shapiro, 1999; Segura-Valdez *et*

al., 2000), hyperoxia-induced lung injury (Radomski *et al.*, 1998), bronchiectasis (Sepper *et al.*, 1994) and lung tumours (Pei *et al.*, 1999).

Following tracheal obstruction the lung expands rapidly and over a period of 10 days collagen content increases (Nardo *et al.*, 1998). During this ten day period, the normal maturation of lung structure is accelerated. Conversely, a prolonged period of lung liquid drainage prevents the increase in collagen content (Chapter 3) when compared to that of normal fetal lungs. Thus, changes in collagen content parallel the alterations in lung expansion in the fetus. As ECM remodelling is most likely an important factor in allowing further lung growth to occur following increases in lung expansion (Nardo *et al.*, 1998), it seems likely that gelatinase levels are altered in the sheep models of lung hyperplasia and hypoplasia induced by changes in the degree of fetal lung expansion. No data is currently available regarding gelatinase levels or activity in fetal sheep lung, nor has the effect of decreased or increased lung expansion on gelatinase levels been documented in fetal sheep. As corticosteroids also accelerate structural maturation of the lung with significant ECM remodelling, it is also likely that the MMPs are involved. Corticosteroids are known to inhibit collagen synthesis *in vitro* (Kivirikko & Laitinen, 1965; Kivirikko *et al.*, 1965), but they also downregulate MMP transcription (Angel *et al.*, 1987; Schonthal *et al.*, 1988; Huhtala *et al.*, 1991). The results presented in Chapter 3 show clearly that even low levels of corticosteroids accelerate structural development of the lung, both in normal and hypoplastic lungs. Thus, cortisol may have direct effects on ECM remodelling and in particular, may alter collagen levels. Measuring gelatinase levels in the lungs of these animals may indicate ECM remodelling in cortisol treated animals. Hence, the effects of corticosteroid treatment on gelatinase levels during normal lung growth, as well as during alterations in lung growth following increases and decreases of lung expansion are important in order to understand ECM remodelling during lung development.

The aims of this study were to measure changes in MMP-2 and MMP-9 protein levels following periods of increased lung expansion, decreased lung expansion and treatment with corticosteroids. Gelatin zymography was chosen for gelatinase detection, as it is a very sensitive technique, capable of detecting MMP-2 and MMP-9 at nanogram levels. It also shows whether the MMPs present in the tissue samples are in the active or latent form.

4.2 Methods

The tissue samples analysed in this chapter were obtained from previous studies in our laboratory; Nardo *et al.* (1998), Boland *et al.* (1997) and from the study discussed in Chapter 3 of this thesis. In all experiments, surgery was performed on pregnant Merino X Border-Leicester ewes at 104 - 120 days GA to implant fetal vascular and tracheal catheters as described in Section 2.2. Ewes and fetuses were allowed at least five days to recover from surgery prior to the start of the experiments.

4.2.1 Experimental Protocol

Tissue samples were collected from three different studies as outlined below.

1) Lung hypoplasia was induced in two groups of fetuses by draining lung liquid into a sterile bag for a period of 20 days, between 111 and 131 days GA. The effect of cortisol on lung hypoplasia was examined by infusing cortisol (for nine days: as described in Chapter 3) to one of the groups of fetuses with lung hypoplasia. Cortisol was infused into a second group as a control.

a) *Saline-only* (n = 4): as described in Chapter 3,

b) *Cortisol-only* (n = 4): as described in Chapter 3,

c) *Saline & drain* (n = 4): as described in Chapter 3,

d) *Cortisol & drain* (n = 4): as described in Chapter 3.

The experiments ceased at 131 days of GA.

2) To increase the degree of fetal lung expansion and induce lung hyperplasia, the fetal trachea was obstructed (TO) by preventing the flow of liquid through the exteriorised tracheal loop, for either:

a) No days of obstruction: *control* (n = 4): as described in Nardo *et al.* (1998),

b) *2dTO* (n = 4): as described in Nardo *et al.* (1998),

c) *4dTO* (n = 4): as described in Nardo *et al.* (1998),

d) *10dTO* (n = 4): as described in Nardo *et al.* (1998).

In fetuses that underwent a period of tracheal obstruction, the final day of tracheal obstruction was 128 days GA, at which time the experiments ceased.

3) To establish the effects of exogenous cortisol on the lung growth response to tracheal obstruction, fetal corticosteroids were delivered to the fetus for a period of 9 days (as described in Boland *et al.*, 1997) prior to the commencement of a 3 day period of tracheal obstruction (128 to 131 days GA):

a) *Saline-control* (n = 4): as described in Boland *et al.* (1997),

b) *Saline & 3dTO* (n = 4): as described in Boland *et al.* (1997),

c) *Cortisol & 3dTO* (n = 4): as described in Boland *et al.* (1997).

The experiments ceased at 131 days of gestation.

At the end of the experimental period (128 or 131 days GA), all ewes and fetuses in the above studies were humanely killed with an intravenous injection of sodium pentobarbitone administered to the ewe. During the *post mortem* examination, fetal lung tissue was collected, frozen and stored at -70 °C, as described in Section 2.5.

4.2.2 Gelatin Zymography

Gelatin zymography is a technique that can be used to identify the presence of gelatinases in active or latent forms. Sodium dodecyl sulphate (SDS) gels containing a substrate were first described by Heussen and Dowdle (1980) and were later adapted to form the quantitative enzymatic assay, zymography (Beranger *et al.*, 1994; Kleiner & Stetler-Stevenson, 1994). Zymography employs the technique of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which allows separation of proteins based on their size. In the case of zymography, however, the preferred substrate of the enzyme is incorporated into the SDS gel. To examine the activity of the gelatinases MMP-2 and MMP-9, gelatin is incorporated into the gel as the gelatinases have a high affinity for this protein (Birkedal-Hansen *et al.*, 1993). Following electrophoresis, the gel is washed to remove the SDS and incubated overnight in buffer to allow the separated gelatinases to degrade the gelatin in the gel. The gel is then stained with a protein-reactive dye, which binds to proteins in the gel. In contrast, areas of enzymatic activity, where proteins have been degraded, appear as clear bands against a dark, stained background. It is important to note that

zymography detects both latent and active forms of the gelatinases, due to the action of SDS, which unfolds latent enzymes, exposing the catalytic site. Following removal of SDS, the enzymes only partially refold within the gel matrix, such that both latent and active MMPs are capable of degrading gelatin.

4.2.2.1 Tissue Extraction

To determine the optimal method for extracting gelatinases from lung tissue, multiple tissue samples from one fetus were homogenised in the following homogenisation buffers: 1) 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.002% w/v Tween-20 (Passi *et al.*, 1999), 2) 2.5% SDS (Pardo *et al.*, 1996), 3) 500 mM Tris-HCl (pH 7.6), 200 mM NaCl, 10 mM $CaCl_2$, 1% Triton X-100 (Frisdal *et al.*, 2000), or 4) 10 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 20 mM N'[2-hydroxyethyl]piparazine-N'-[2-ethanesulfonic acid] (HEPES), 150 mM NaCl (pH 7.5: Perez-Ramos *et al.*, 1999). For each extraction, 0.5 g of tissue was added to 1 ml of homogenisation buffer. Tissues were homogenised using an Ultra-Turrax homogeniser (Ultra-Turrax T25, Janke and Kunkel, IKA-Labortechnik, Germany). The protein concentration of each homogenate was determined by protein assay (Section 2.7.2) using the appropriate homogenisation buffer as a blank. To assess the best buffer for homogenisation, tissue homogenates were then analysed by gelatin zymography. Similar protein yields were noted for each extraction method and the gelatinase profile on the zymogram was similar, irrespective of the homogenisation buffer.

Therefore, all subsequent tissue samples (0.5 mg) were homogenised using the Ultra-Turrax homogeniser, in 1 ml of homogenisation buffer 1 (50 mM Tris-HCl, 150 mM NaCl, Tween-20). Tissues were homogenised for a period of 1 - 2 minutes, then centrifuged for 10 min at 2,500 rpm (Beckman, Model J-6B Centrifuge, USA), at 4 °C. All samples and homogenates were kept on ice at all times. Following centrifugation, the supernatant was aliquoted and stored at -20 °C for future protein assays and zymography (Section 4.2.2.3).

4.2.2.2 Preparation of Gelatin

A collagen stock (bovine skin, type I collagen, Sigma, Australia) was dissolved at 2 mg/ml in 0.2 M acetic acid at 4 °C. The collagen solution was dialysed in water overnight at 4 °C. Following dialysis, the collagen stock was preserved with 0.02%

sodium azide and stored at 4 °C. On the day of zymography, an aliquot of the collagen stock was heated to 60 °C for a period of 20 min to denature the collagen. The denatured collagen was cooled on ice and added to the gel media at a concentration of 0.5 mg/ml.

4.2.2.3 Gelatin Zymography

Gelatin zymography was performed on 7% SDS-PAGE gels (lower gel 0.4 M Tris-HCl, pH 8.8; upper gel 0.3 M Tris-HCl, pH 6.8) containing 0.5 mg/ml gelatin in the lower gel (Section 4.2.2.2). In order to standardise the quantity of protein loaded into each lane of a zymogram, a protein assay was performed on each tissue homogenate (Section 2.7.2). Tissue homogenates were diluted to a final concentration of 0.2 - 0.4 μg/μl with a 1:5 dilution of non-reducing Laemmli sample buffer (5X concentrated stock: 2% w/v SDS, 10 μg/ml bromophenol blue, 1% w/v glycerol in 62.5 mM Tris-HCl at pH 6.8: Laemmli, 1970). A molecular weight marker was prepared with reducing Laemmli sample buffer (non-reducing sample buffer with 0.4 M dithiothreitol (DTT) 4:1) and reduced by boiling. To aid identification of the gelatinases, culture medium from a human fibrosarcoma cell line (HT-1080: American Type Culture Collection, USA) containing latent, intermediate and active MMP-2 and latent MMP-9 was used on each gel as a positive control (Figure 4.1: Ginestra *et al.*, 1997; Stanton *et al.*, 1998). The intermediate form of MMP-2 in the HT-1080 cells contains a partially cleaved propeptide domain. HT-1080 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM), with 10% fetal calf serum (FCS). To collect conditioned media, cells were cultured for 48 hours in serum-free DMEM. Aliquots of culture medium were stored at -20 °C. 45 μl of prepared tissue homogenates, HT-1080 culture medium and the molecular weight marker were loaded into the gel and electrophoresed in Laemmli electrophoresis running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.3) until the bromophenol blue band reached the bottom of the gel. On each gel, 4 samples from each of two groups were analysed. The first 4 samples were prepared from 4 'control' fetuses and the second 4 samples from 'treated' fetuses. Each gel was performed at least twice using freshly prepared tissue homogenates.

After electrophoresis, the gels were washed twice in 2.5% v/v Triton X-100 for 30 minutes per wash to remove the SDS. Following washing with Triton X-100, gels were rinsed in water and incubated overnight at 37 °C, in zymography buffer (100 mM Tris-HCl, 30 mM $CaCl_2$, 0.04% w/v sodium azide and 0.02% v/v Brij, pH 7.4). Gelatin-

degrading activity was detected by staining with Coomassie blue R-250 (Sigma, Australia) for at least 30 minutes. The gel was then destained periodically with 30% v/v methanol and 1% v/v acetic acid, until clear bands of lysis were revealed. The gel was incubated in 10% v/v glycerol overnight and dried between sheets of cellophane. The molecular weights of the lysis bands were determined with reference to high molecular mass standards (29 - 205 kDa, Life Technologies, Australia).

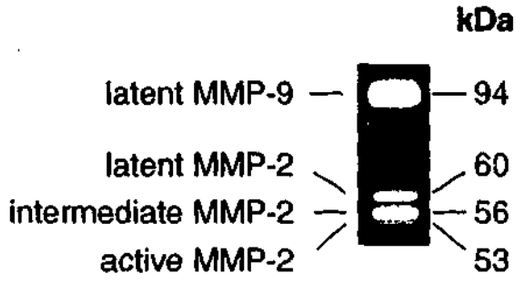

Figure 4.1 HT-1080 culture media run on a gelatin zymogram

HT-1080 culture media run on a gelatin zymogram. This tumour cell line expresses latent MMP-9 (94 kDa) and latent and intermediate MMP-2 (60 kDa and 56 kDa respectively) as well as a faint band of active MMP-2 (53 kDa).

4.2.2.4 EDTA and APMA treatments

Zymography has the potential to detect other gelatinases besides the metalloproteinases. To confirm that the lysis bands were indeed due to metalloproteinase activity, a gel was incubated overnight in zymography buffer containing 60 mM ethylenediaminetetra acetic acid (EDTA). EDTA chelates the Zn^{2+} ion in the active site of metalloproteinases, thus inhibiting MMP activity. Duplicate samples of a tissue homogenate and HT-1080 conditioned media were run on either side of a gel. Following electrophoresis, the gel was cut in half and each half was incubated separately overnight. One half was incubated in zymography buffer containing EDTA and the other in normal zymography buffer. Absence of lysis bands in the half of the gel incubated with EDTA positively identifies the clear bands in the control half as metalloproteinases.

To confirm the presence of MMP-2 in tissue samples and to identify latent and active forms, the samples were incubated with the organomercurial compound amino phenyl mercuric acetate (APMA), prior to zymography. APMA disrupts the bonds in latent

MMPs that link the propeptide to the Zn^{2+} in the active site, promoting enzyme unfolding followed by autocatalytic cleavage of the propeptide to give the active form. Tissue homogenates were diluted to 0.3 μg/μl in 20 mM Tris-HCl, 10 mM $CaCl_2$, 0.02% Na^+ azide and 0.2% Brij (pH 7.5). APMA was added to achieve a final concentration of 2 mM. Samples were incubated for periods of 0, 24, 48 and 72 hours at 37 °C before being placed at -20 °C. Samples were then assessed by gelatin zymography to determine whether APMA treatment caused a shift in enzyme molecular mass.

4.2.3 Quantitation of Zymograms

4.2.3.1 Image Analysis and Concentration Curve

Pixel density of lysis zones on gelatin zymograms can be used to quantitate changes in gelatinase levels. When the gels were dry, they were scanned and then converted to grey-scale images in Adobe Photoshop (Adobe Photoshop LE, Adobe Systems, USA). Using ImageQuaNT image analysis software (Molecular Dynamics, USA), the pixel densities for the lysis zones were quantitated and corrected for background staining in each lane of the zymogram. Where necessary, images were enlarged to enable accurate measurement of the pixel densities.

To determine that the pixel density of the zones of lysis was proportional to the concentration of protein loaded per lane, tissue homogenates were prepared using serial dilutions, at protein concentrations of 6.72, 3.36, 1.68, 0.84, 0.42, 0.21, 0.105 and 0.0525 μg/μl. These samples were then loaded, run, incubated and dried as described previously (Section 4.2.2.3). The concentration curve gel was analysed (see below) and pixel densities for each protein concentration were plotted. Linear regression analysis of the data was performed.

4.2.3.2 Statistical Analysis

The results are presented as mean pixel density (arbitrary units: AU) ± SEM. Statistical analyses were performed using the computerised statistical package SigmaStat (Version 2.0, Jandel Corporation, USA). As stated previously, on each gel, 4 tissue homogenates from 'control' fetuses and 4 tissue homogenates from 'treated' fetuses were compared. Each lane contained lung tissue homogenate from a different fetus

and each gel was performed at least twice. Mean pixel densities of active and latent forms of the gelatinases for 'control' and 'treated' fetuses on a particular gel were then calculated. A student's unpaired t-test was used to determine whether there were statistically significant differences between treated and control fetuses. To determine if there were any differences in the gelatinase levels between all of the experimental groups within a particular study, data for each fetus were calculated as a fraction of the mean density of the 'control' group run on the same gel. A one-way ANOVA was then performed and significant differences between groups were identified by performing an LSD test. Total MMP pixel density (latent plus active MMP) and percentage of active MMP relative to total MMP were analysed and are presented in tabular form. A student's unpaired t-test was used to determine whether there were statistically significant differences between 'treated' and 'control' fetuses. The accepted level of significance for all statistical analyses was $P < 0.05$.

4.3 Results

4.3.1 Gelatinases Present in Fetal Sheep Lung

Fetal sheep lung contained a major band of gelatinase activity at 60 kDa that co-migrated with latent human MMP-2 in HT-1080 conditioned medium (Figure 4.2). A second gelatinase band at 53 kDa co-migrated with active human MMP-2.

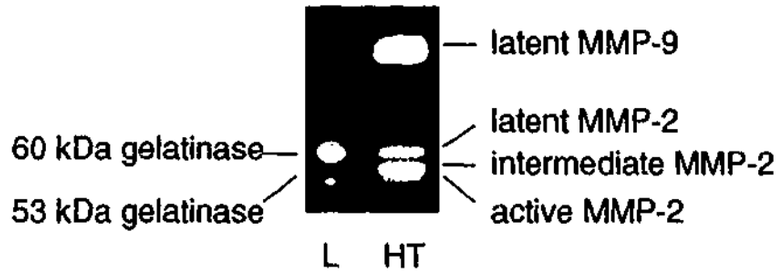

Figure 4.2 Gelatinases present in fetal sheep lung

A fetal sheep lung homogenate (L) and HT-1080 fibrosarcoma cell conditioned medium (HT) were electrophoresed on 7% SDS-PAGE gels containing denatured collagen and analysed for gelatin-degrading activity by zymography. Gelatinase bands at 60 kDa and 53 kDa in the fetal sheep lung homogenate co-migrated with human latent and active MMP-2 present in HT-1080 conditioned medium.

To confirm that these bands were generated by metalloproteinase activity, EDTA was added to the zymogram incubation buffer to inhibit metalloproteinases (refer to section 4.2.2.4). The gelatin-degrading activities in sheep lung tissue and in HT-1080 conditioned medium were completely inhibited by EDTA (Figure 4.3 A), identifying the 60 and 53 kDa gelatinases as metalloproteinases. It was considered most likely that the 60 kDa and 53 kDa metalloproteinases corresponded to sheep latent and active MMP-2 respectively. The identity of the MMPs was confirmed by treating homogenised lung tissue from control fetuses with APMA. The APMA treatment promotes MMP activation and the autocatalytic loss of the propeptide, which can be detected on SDS-PAGE as a reduction in the molecular mass of approximately 10 kDa (Nagase, 1994). Figure 4.3 B shows that continued incubation of lung tissue homogenates with APMA caused a loss of the 60 kDa band and a rise in the levels of the 53 kDa band, indicative of MMP activation. Hence the 60 kDa band was positively identified as latent sheep MMP-2 and the 53 kDa band as active sheep MMP-2.

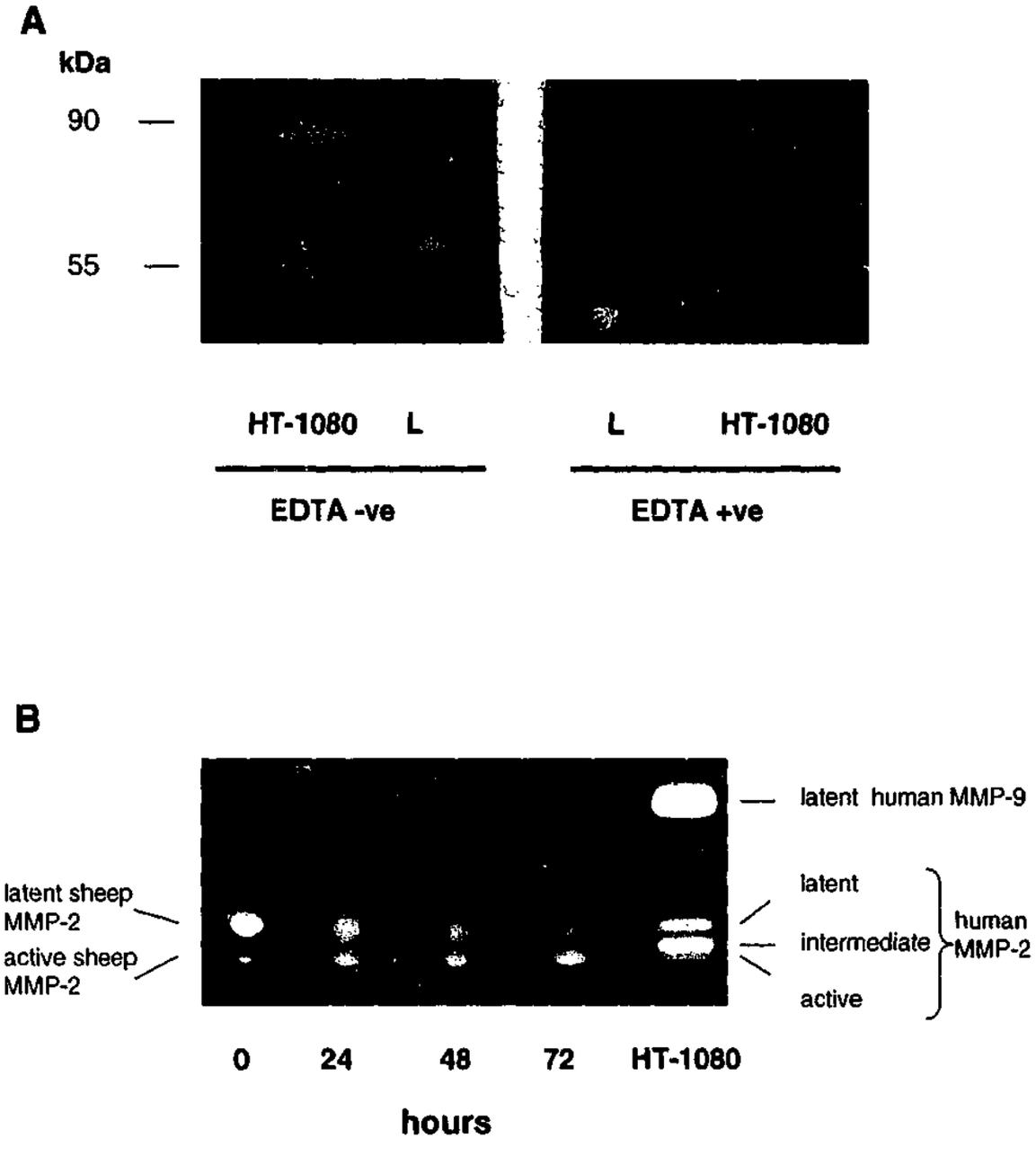

Figure 4.3 EDTA and APMA treatment

A) EDTA treatment: gelatin zymogram run with two identical halves, each loaded with the same lung tissue sample (L) and HT-1080 conditioned medium. Following electrophoresis, the gel was cut in half and one half was incubated in normal zymography buffer, whilst the other was incubated in zymography buffer with EDTA prior to staining for proteins. EDTA treatment prevented gelatinase activity.

B) APMA treatment: gelatin zymogram of HT-1080 conditioned medium and a lung tissue homogenate treated with APMA for 0, 24, 48 or 72 hours. APMA treatment promotes conversion of latent MMP-2 to active MMP-2 by removal of the propeptide domain. Activation of MMP-2 occurs in a time-dependent manner.

A higher molecular weight gelatinolytic band was detected in a few fetal lung tissue samples from various treatment groups. This higher molecular weight band most likely corresponds to ovine latent MMP-9. As this band was only seen rarely and did not appear to be affected by treatment, it has not been included in the discussion of the results. All graphs, figures and data will refer to the latent and active forms of MMP-2.

4.3.2 Concentration Curve

Serial dilutions of a fetal lung sample of known protein concentration were loaded on a gelatin zymogram. Each dilution produced zones of lysis corresponding to latent and active MMP-2 (Figure 4.4 A), which were quantitated by densitometry. Pixel densities for latent and active MMP-2 were summed and plotted as a function of the log of protein concentration (Figure 4.4 B). The two samples with the highest concentration of protein (3.34 and 6.68 μg/μl) were excluded from the analysis, due to streaking of lysis bands by non-MMP proteins present in the sample (Figure 4.4 A). Figure 4.4 B shows that there is a strong linear relationship between the protein concentration of the lung tissue sample and the volume of the lysis band generated. The correlation coefficient of this curve was 0.999. For subsequent gels, the volume of samples loaded was adjusted to achieve a protein concentration that fell within the central area of this curve: ranging from 0.2 – 0.4 μg/μl. However, in any one gel, the final protein concentration of tissue homogenates loaded in each lane was the same.

A

0.0525 0.105 0.21 0.42 0.84 1.68 3.34 6.68 µg/µl

B

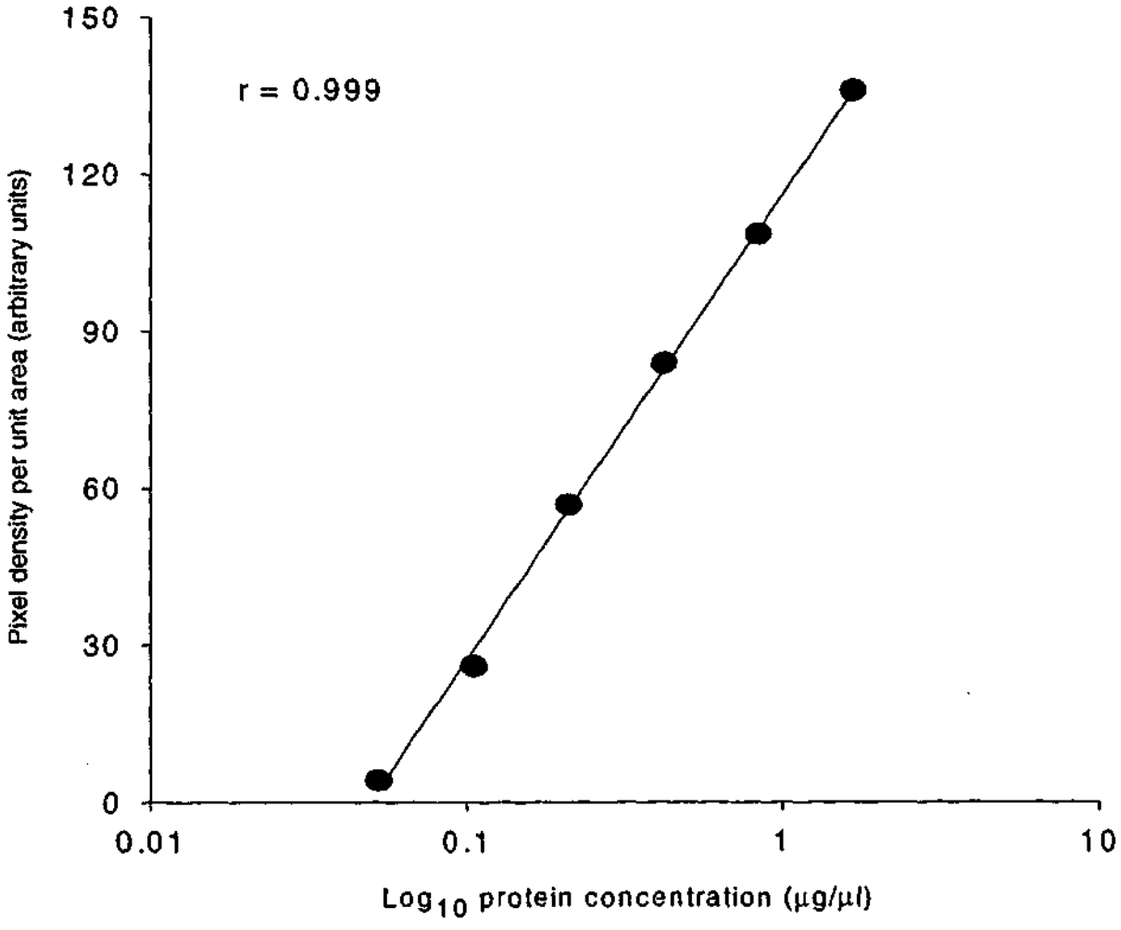

Figure 4.4 Gelatinase concentration curve

A) Serial dilutions of one lung tissue sample were loaded into a gelatin zymogram to determine if concentration of protein was related to size of lysis zone. Values below each lane indicate the final protein concentration of the sample loaded in that lane.
B) Pixel density (number of pixels per unit area) for each zone of lysis shown in Figure 4.4 A, was measured by image analysis and plotted against protein concentration. Linear regression analysis was performed on all points, with the exception of those concentrations where large protein streaks were deemed to interfere with the size of the lysis zone (3.34 & 6.68 µg/µl). The correlation of coefficient for the regression was 0.999. Protein loading for all subsequent zymograms was performed at concentrations that fell within the linear portion of the curve (0.2 – 0.4 µg/µl).

4.3.3 Gelatin Zymography of Tissues

4.3.3.1 Cortisol Treatment of Normal and Hypoplastic Fetal Lungs

Levels of active and latent MMP-2 were similar in the *saline-only* (active: 26.3 ± 4.2 AU (arbitrary units); latent: 79.2 ± 5.3 AU) and *cortisol-only* (active: 19.8 ± 2.6 AU; latent: 71.2 ± 2.0 AU) groups (Figure 4.5 (i)). However, following a period of lung liquid drainage, levels of latent MMP-2 were 29% higher in *saline & drain* fetuses when compared to *saline-only* fetuses (68.5 ± 3.1 AU *cf.* 54.0 ± 4.5 AU). Similarly, levels of latent MMP-2 were 31% higher in *cortisol & drain* fetuses when compared to *saline-only* fetuses (76.0 ± 3.8 AU *cf.* 57.9 ± 5.9 AU; Figure 4.5 (i) and (ii)). Notably, active MMP-2 levels were 74% greater in *saline & drain* fetuses in comparison with *saline-only* fetuses (35.1 ± 3.4 AU *cf.* 20.2 ± 4.9 AU), however levels were not increased in *cortisol & drain* fetuses in comparison with *saline-only* fetuses (25.4 ± 3.5 AU *cf.* 23.5 ± 4.9 AU; Figure 4.5 ii, iii).

The average pixel densities of latent and active MMP-2 for *cortisol-only*, *saline & drain* and *cortisol & drain* fetuses were then expressed as a percentage of the average pixel density of *saline-only* fetuses (Figure 4.6). Active MMP-2 levels were greater in the *saline & drain* fetuses (173.0 ± 10.5%) than in any other treatment group (Figure 4.6: upper panel). The active MMP-2 levels in *cortisol & drain* fetuses (128.3 ± 12.3%) were significantly elevated in comparison to that in the *cortisol-only* (87.7 ± 10.1%) fetuses, but were not significantly different from that in the *saline-only* fetuses (100.0 ± 12.3%: Figure 4.6: upper panel). Latent MMP-2 levels were elevated in *saline & drain* (136.2 ± 5.0%) and *cortisol & drain* fetuses (146.1 ± 6.7%) to a similar degree above the levels in both *saline-only* (100.0 ± 4.4%) and *cortisol-only* fetuses (98.5 ± 4.2%; Figure 4.6: lower panel).

Total MMP-2 levels were calculated as the sum of latent and active MMP-2 pixel densities for each treatment group and compared to *saline-only* fetuses. Total MMP-2 was elevated in the *saline & drain* fetuses above that of *saline-only* fetuses (Table 4.1). There were no other significant differences in total MMP-2 levels between 'treated' and 'control' groups. However, the total MMP-2 levels tended to be higher in the *cortisol & drain* group of fetuses when compared to the *saline-only* group of fetuses ($P = 0.08$).

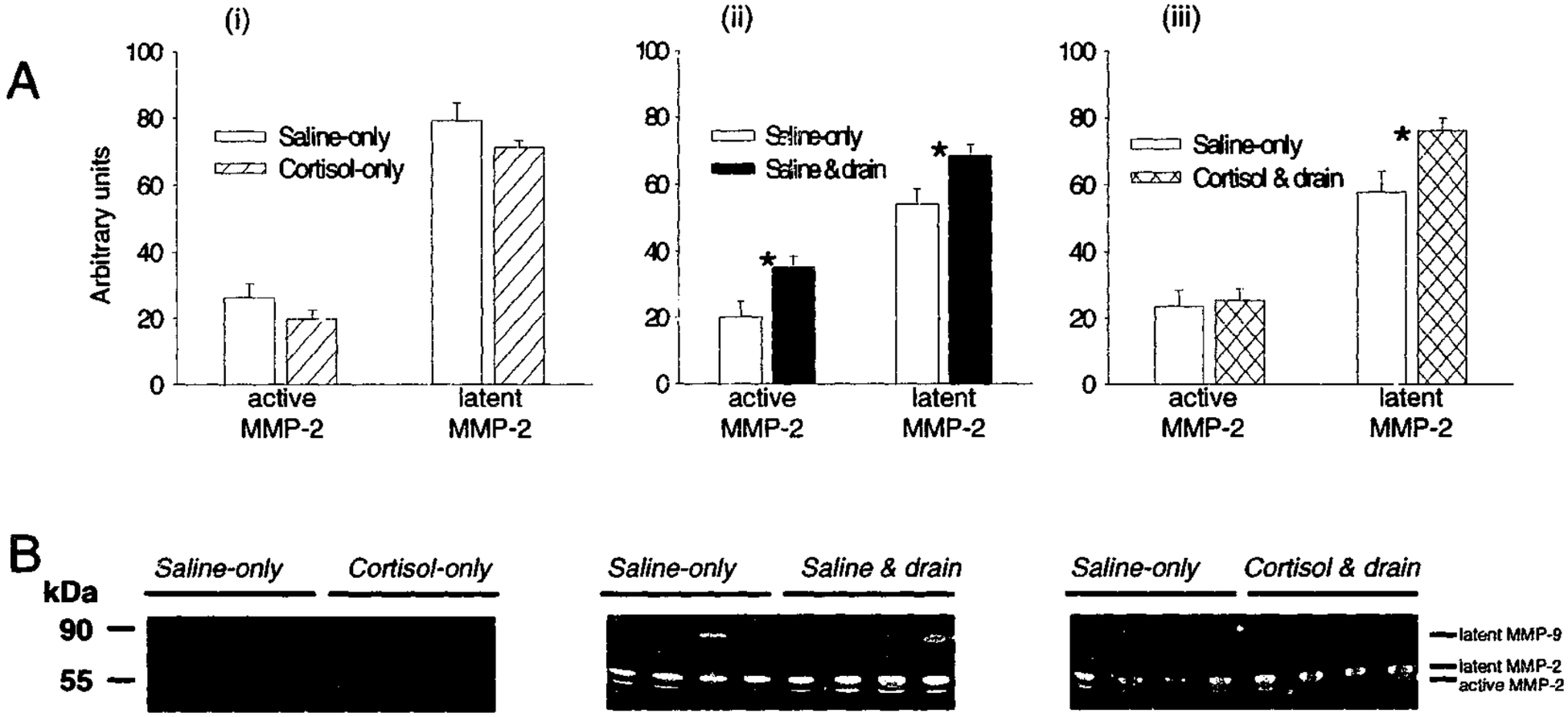

Figure 4.5 MMP-2 levels following cortisol treatment of normal and hypoplastic fetal lungs

A) Graphs representing levels of active and latent MMP-2 (number of pixels per unit area ± SEM) in fetal lung tissue of: (i) *saline-only* versus *cortisol-only*, (ii) *saline-only* versus *saline & drain* and (iii) *saline-only* versus *cortisol & drain* groups. Significant differences ($P < 0.05$) between groups run on the same gel are indicated by an asterisk.

B) Examples of gelatin zymograms for: (i) *saline-only* versus *cortisol-only*, (ii) *saline-only* versus *saline & drain* and (iii) *saline-only* versus *cortisol & drain* groups. Numbers on the left hand side of the first gel indicate sizes of molecular mass markers. The identity of each MMP is indicated on the right hand side.

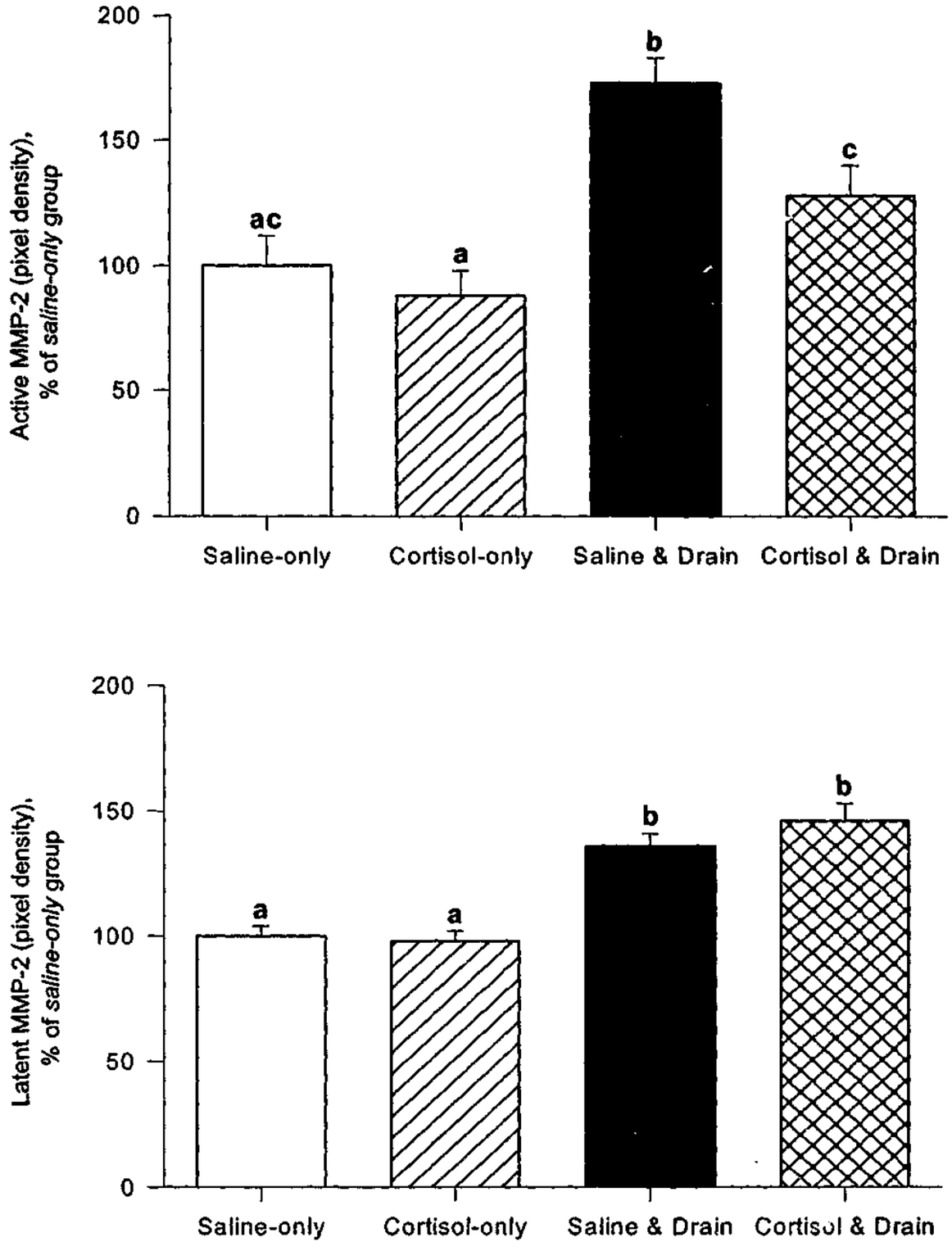

Figure 4.6 Summary of changes in MMP-2 levels following cortisol treatment of normal and hypoplastic fetal lungs

Upper panel: Active MMP-2 levels (mean ± SEM) in normal and hypoplastic fetal lung tissue following treatment of the fetuses with cortisol. ***Lower panel:*** Latent MMP-2 levels (mean ± SEM) in normal and hypoplastic fetal lung tissue following treatment with cortisol. These graphs were generated from the combined data from 2 zymogram gels for each comparison. Active and latent MMP-2 levels for each treatment group were calculated as a percentage of the mean active or latent MMP-2 levels for the *saline-only* group run on the same gel. Values that do not share a common letter are significantly different from each other ($P < 0.05$).

The proportion of active MMP-2 as a percentage of total MMP-2 was elevated in the *saline & drain* fetuses in comparison with the *saline-only* fetuses (Table 4.1).

Table 4.1 Total MMP-2 and the proportion of active MMP-2 levels following cortisol treatment of normal and hypoplastic fetal lungs

Total MMP-2 levels for each treatment group were calculated and expressed as mean total pixel density (arbitrary units) of active plus latent MMP-2 ± SEM for each group of fetuses. Active MMP-2 was then calculated as a percentage of total MMP-2 (%) (mean ± SEM). Significant differences between a treatment group and the *saline-only* group are marked with an asterisk ($P < 0.05$).

	Total MMP-2	Active MMP-2 / Total MMP-2
saline-only	76.2 ± 12.1	22.0 ± 2.4%
cortisol-only	70.2 ± 8.1	20.3 ± 1.7%
saline-only	96.2 ± 10.5	27.8 ± 2.2%
saline & drain	142.4 ± 15.5*	33.3 ± 0.9%*
saline-only	103.9 ± 15.5	26.3 ± 2.5%
cortisol & drain	150.5 ± 19.1	24.4 ± 1.3%

4.3.3.2 Tracheal Obstruction for 2 - 10 days

Levels of active MMP-2 were statistically similar in lung tissue from *control* fetuses and fetuses subjected to 2 (*control*: 17.5 ± 4.8 AU; *2dTO*: 10.3 ± 5.3 AU), 4 (*control*: 23.3 ± 5.8 AU; *4dTO*: 19.7 ± 4.7 AU) and 10 (*control*: 39.7 ± 10.7 AU; *10dTO*: 56.5 ± 10.5 AU) days of tracheal obstruction (Figure 4.7). Levels of latent MMP-2 were statistically similar in lung tissue from *control* fetuses and fetuses subjected to 2 (*control*: 68.8 ± 9.1 AU; *2dTO*: 68.6 ± 10.3 AU), 4 (*control*: 89.8 ± 8.4 AU; *4dTO*: 90.2 ± 4.6 AU) and 10 (*control*: 115.0 ± 9.1 AU; *10dTO*: 145.1 ± 12.6 AU ($P = 0.10$)) days of tracheal obstruction (Figure 4.7). The average pixel densities of latent and active MMP-2 for *2dTO*, *4dTO* and *10dTO* groups were then expressed as a percentage of the average values for the *control* fetuses (Figure 4.8). Active MMP-2 levels were not statistically different in any of the groups of fetuses (*control*: 100.0 ± 11.1%; *2dTO*: 62.3 ± 14.7%; *4dTO*: 91.1 ± 10.2% and *10dTO*: 113.8 ± 16.5%; Figure 4.8: upper panel). However, it would appear that there was a trend for a decrease in active MMP-2 levels after 2 days of tracheal obstruction ($P = 0.06$), with active MMP-2 levels returning to similar levels

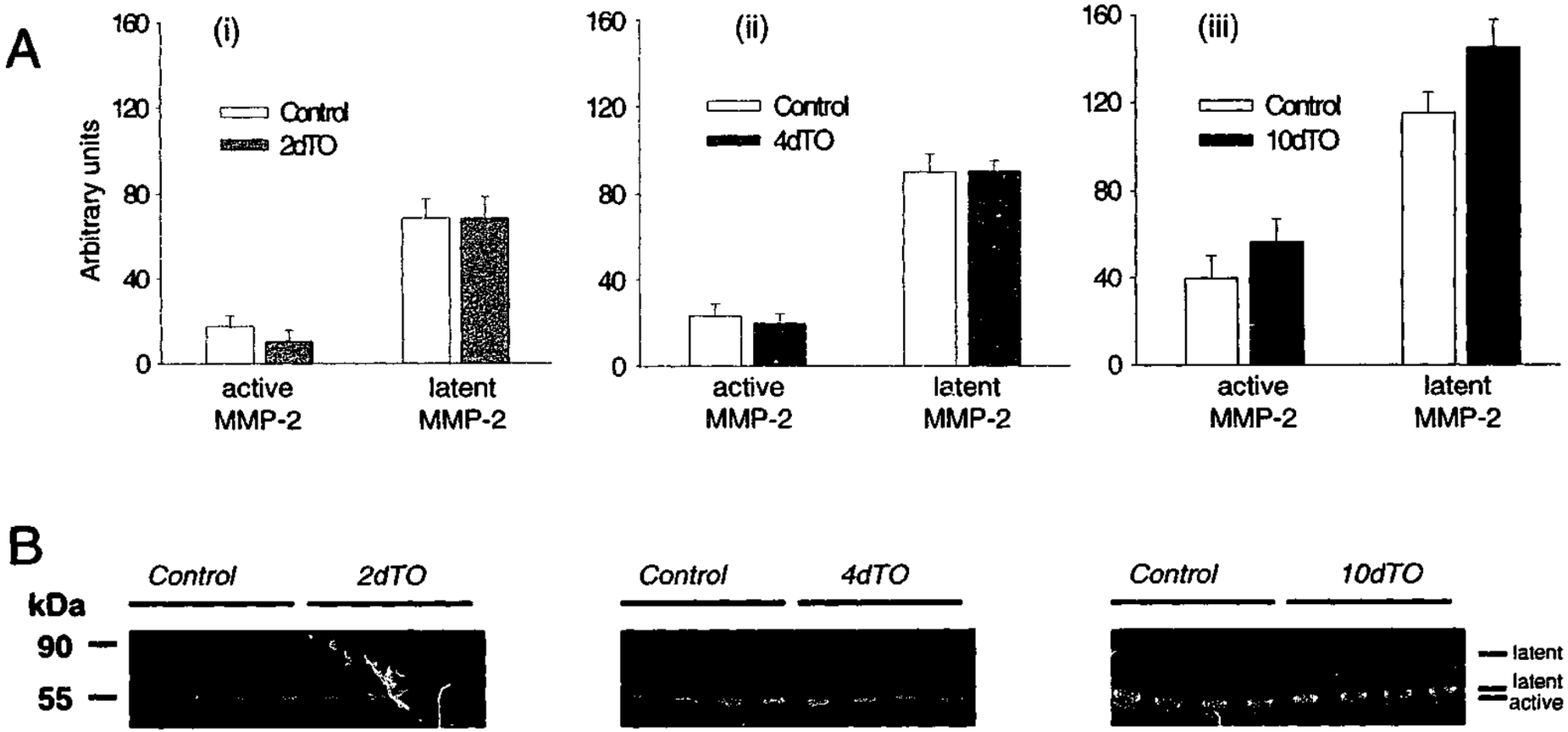

Figure 4.7 MMP-2 levels in fetal lung following tracheal obstruction

A) Graphs representing levels of active and latent MMP-2 (number of pixels per unit area ± SEM) in fetal lung tissue of: (i) *control* versus *2dTO*, (ii) *control* versus *4dTO* and (iii) *control* versus *10dTO* groups. There were no significant differences.

B) Examples of gelatin zymograms for: (i) *control* versus *2dTO*, (ii) *control* versus *4dTO* and (iii) *control* versus *10dTO* groups. Numbers on the left hand side of the first gel indicate the size of molecular mass markers. The identity of each MMP is indicated on the right hand side.

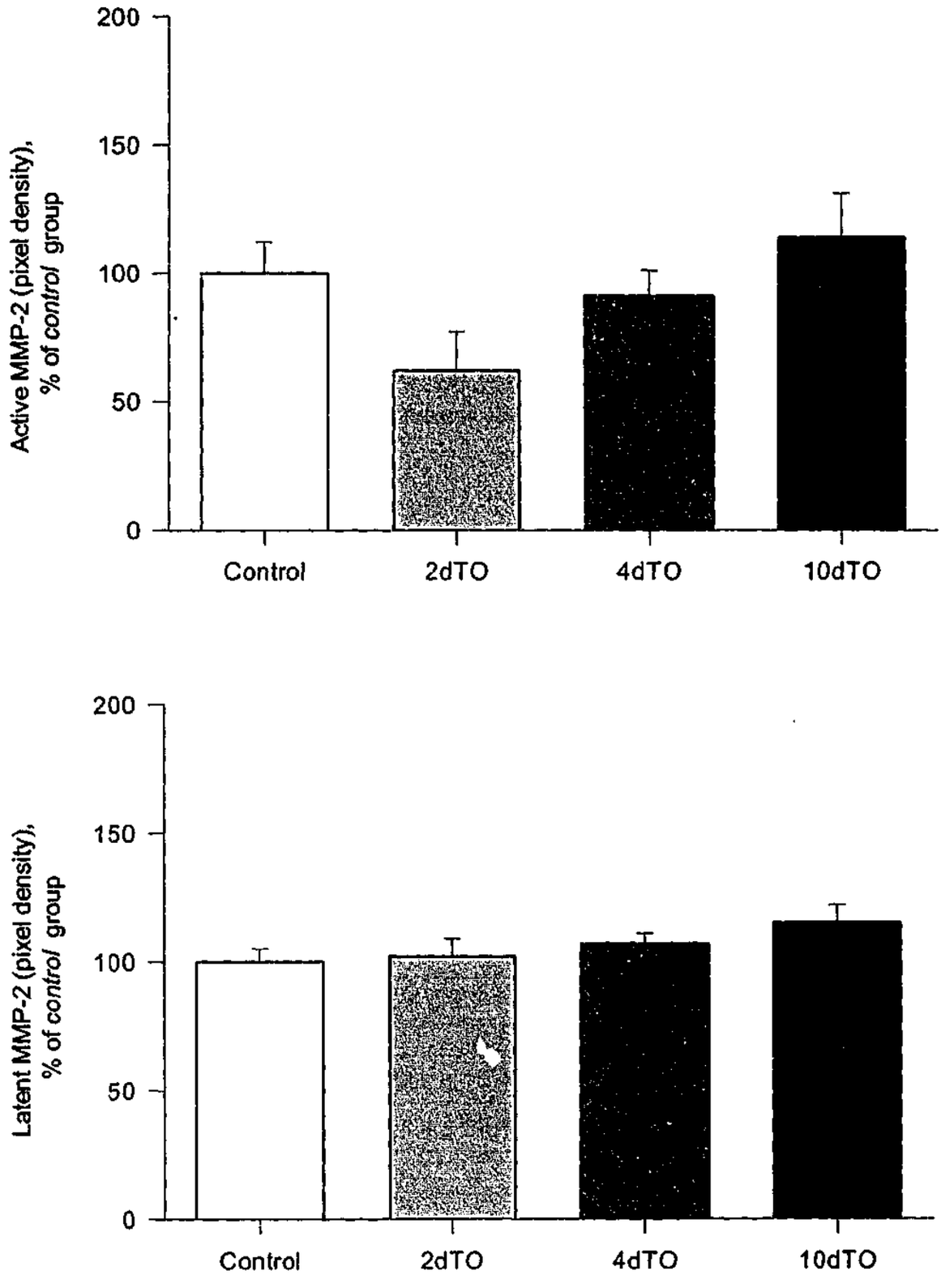

Figure 4.8 Summary of changes in MMP-2 levels in fetal lung tissue following tracheal obstruction

Upper panel: Active MMP-2 levels (mean ± SEM) in fetal lung tissue following tracheal obstruction. ***Lower panel***: Latent MMP-2 levels (mean ± SEM) in fetal lung tissue following 2, 4 and 10 days of tracheal obstruction. These graphs were generated from the combined data from 2 zymogram gels for each comparison. Active and latent MMP-2 levels for each treatment group were calculated as a percentage of the mean active or latent MMP-2 levels for the *control* group run on the same gel. There were no significant differences between groups.

as *control* fetuses by 4 and 10 days of tracheal obstruction. Latent MMP-2 levels were not altered significantly by a period of tracheal obstruction (*control*: 100.0 ± 4.6%; *2dTO*: 101.6 ± 7.0%; *4dTO*: 107.5 ± 4.2% and *10dTO*: 114.8 ± 6.7%; Figure 4.8: lower panel).

Total MMP-2 levels were calculated as the sum of latent and active MMP-2 pixel densities for each treatment group and compared to *control* fetuses. Total MMP-2 was not different between the treatment groups and *control* fetuses (Table 4.2). The proportion of active MMP-2 as a percentage of total MMP-2 was reduced in the *2dTO* fetuses in comparison with the *control* fetuses (Table 4.2).

Table 4.2 Total MMP-2 and the proportion of active MMP-2 levels following 2, 4 and 10 days of tracheal obstruction

Total MMP-2 levels for each treatment group were calculated and expressed as mean total pixel density (arbitrary units) of active plus latent MMP-2 ± SEM for each group of fetuses. Active MMP-2 was then calculated as a percentage of total MMP-2 (%) (mean ± SEM). Significant differences between a treatment group and the *control* group are marked with an asterisk ($P < 0.05$).

	Total MMP-2	Active MMP-2 / Total MMP-2
Control	77.7 ± 7.3	21.4 ± 1.7%
2dTO	64.4 ± 11.7	13.5 ± 2.4%*
Control	120.5 ± 8.2	26.8 ± 3.2%
4dTO	124.5 ± 7.1	24.1 ± 3.0%
control	122.2 ± 15.4	29.4 ± 2.7%
10dTO	144.4 ± 24.1	28.8 ± 1.3%

4.3.3.3 Cortisol Pretreatment and Tracheal Obstruction

Levels of active MMP-2 were similar between the *saline-control* (20.9 ± 2.3 AU) and *saline & 3dTO* (21.1 ± 3.5 AU) and between the *saline-control* (24.7 ± 3.3 AU) and *cortisol & 3dTO* (23.1 ± 2.1) groups (Figure 4.9). Levels of latent MMP-2 were similar between the *saline-control* (85.4 ± 3.4 AU) and *saline & 3dTO* (82.8 ± 4.6 AU) and between the *saline-control* (97.1 ± 9.5 AU) and *cortisol & 3dTO* (99.2 ± 5.0 AU) groups

(Figure 4.9). The average pixel densities of latent and active MMP-2 for *saline & 3dTO* and *cortisol & 3dTO* groups were expressed as a percentage of the *saline-control* fetuses (Figure 4.10). Active MMP-2 levels were similar in all of the groups of fetuses (*saline-control*: 100.0 ± 6.8%; *saline & 3dTO*: 96.7 ± 9.5% and *cortisol & 3dTO*: 86.0 ± 6.6%; Figure 4.10: upper panel). Similarly, latent MMP-2 levels in treated groups were similar to *saline-control* values (*saline-control*: 100.0 ± 2.1%; *saline & 3dTO*: 118.6 ± 9.2% and *cortisol & 3dTO*: 112.4 ± 5.0%; Figure 4.10: lower panel).

Total MMP-2 levels were calculated as the sum of latent and active MMP-2 pixel densities for each treatment group and compared to *saline-control* fetuses from the same gel. Total MMP-2 levels were not different in any of the treatment groups (Table 4.3) in comparison to the control groups. The proportion of active MMP-2 as a percentage of total MMP-2 was reduced in the *cortisol & 3dTO* fetuses in comparison with the *saline-control* fetuses ($P < 0.05$: Table 4.3).

Table 4.3 Total MMP-2 and the proportion of active MMP-2 levels following tracheal obstruction and pretreatment with cortisol.

Total MMP-2 levels for each treatment group were calculated and expressed as mean total pixel density (arbitrary units) of active plus latent MMP-2 ± SEM for each group of fetuses. Active MMP-2 was then calculated as a percentage of total MMP-2 (%) (mean ± SEM). Significant differences between a treatment group and the *saline-control* group are marked with an asterisk ($P < 0.05$).

	Total MMP-2	Active MMP-2 / Total MMP-2
saline-control	104.9 ± 7.0	26.1 ± 1.7%
saline & 3dTO	116.2 ± 5.6	22.7 ± 2.2%
saline-control	88.6 ± 13.8	21.8 ± 1.1%
cortisol & 3dTO	92.3 ± 11.9	17.6 ± 0.9%*

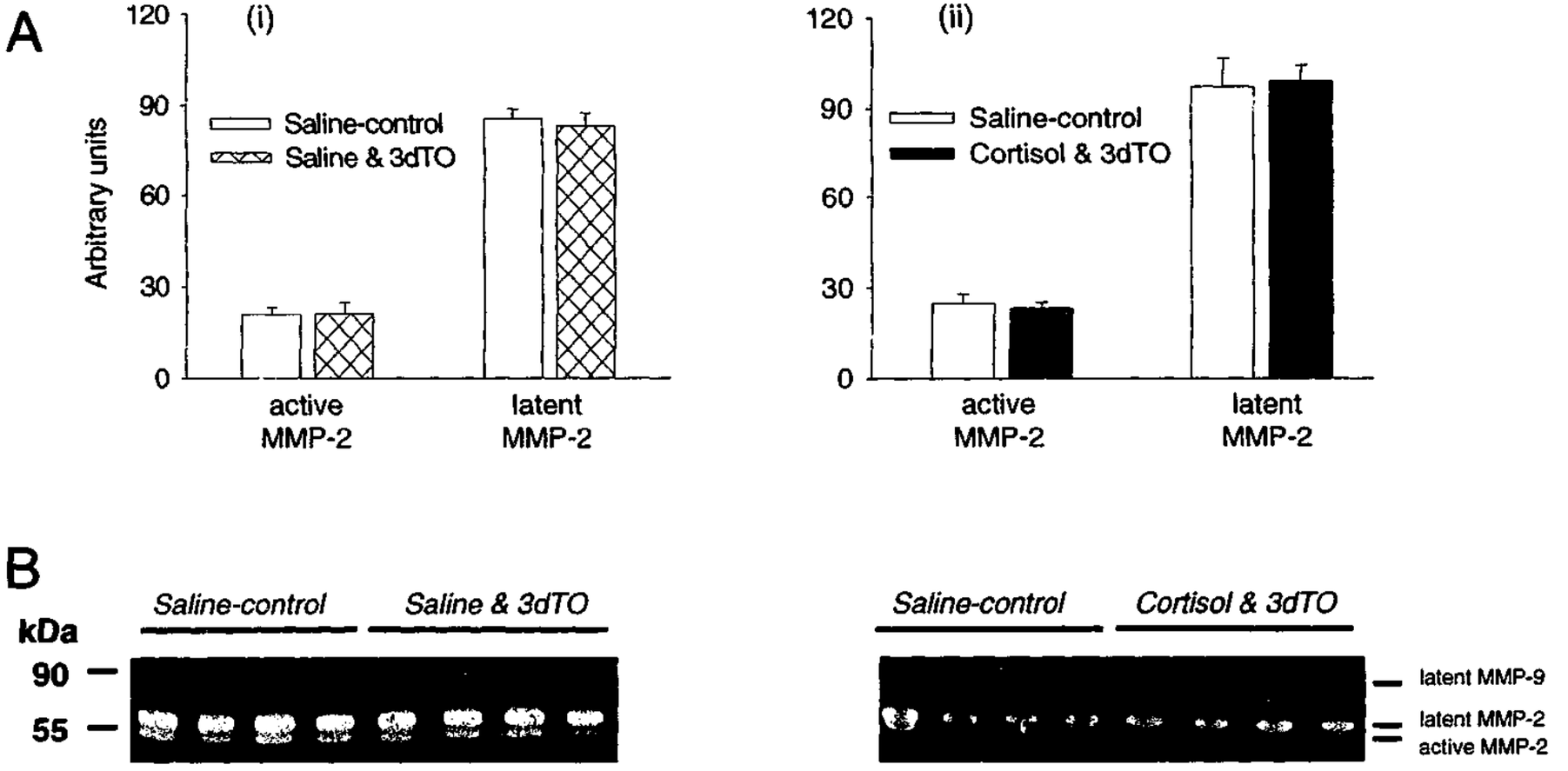

Figure 4.9 MMP-2 levels in fetal lung tissue following cortisol pretreatment and tracheal obstruction

A) Graphs representing levels of active and latent MMP-2 (number of pixels per unit area ± SEM) in fetal lung tissue of: (i) *saline-control* versus *saline & 3d TO* and (ii) *saline-control* versus *cortisol & 3d TO* groups. There were no significant differences.

B) Examples of gelatin zymograms for: (i) *saline-control* versus *saline & 3dTO* and (ii) *saline-control* versus *cortisol & 3dTO* groups. Numbers on the left hand side of the first gel indicate the size of molecular mass markers. The identity of each MMP is indicated on the right hand side.

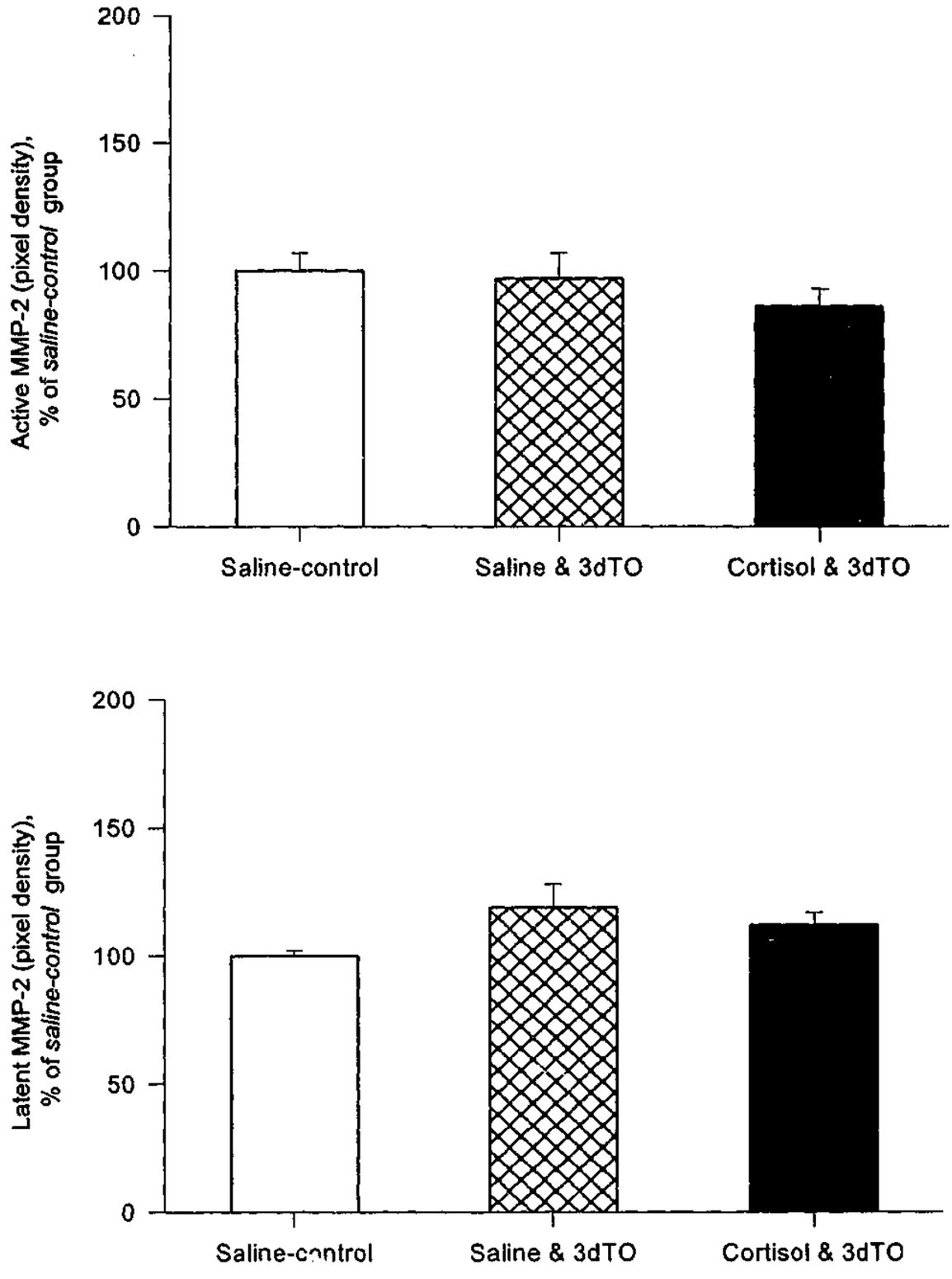

Figure 4.10 Summary of changes in MMP-2 levels following cortisol pretreatment and tracheal obstruction

Upper panel: Active MMP-2 levels (mean ± SEM) in fetal lung tissue following cortisol pretreatment and tracheal obstruction. ***Lower panel***: Latent MMP-2 levels (mean ± SEM) in fetal lung tissue following cortisol pretreatment and tracheal obstruction. These graphs were generated from combined data from 2 zymogram gels per comparison. Active and latent MMP-2 levels for each treatment group were calculated as a percentage of the mean active or latent MMP-2 levels for the *saline-control* group run on the same gel. There were no significant differences between groups.

4.4 Discussion

Alterations in gelatinase levels were examined following changes in fetal lung expansion and treatment with corticosteroids. Following alterations in lung expansion, ECM remodelling is believed to facilitate the structural changes in the lung. If this is the case, changes in the levels of enzymes that degrade structural proteins of the lung, such as the gelatinases, may indicate that remodelling of the ECM is occurring. Periods of lung deflation were found to significantly increase the amounts of active, latent and total MMP-2 in fetal sheep lung. A period of cortisol infusion during lung deflation had no effect on the increased levels of latent MMP-2, but returned active and total MMP-2 levels to control values. Biochemical analysis of hypoplastic lungs, induced by lung deflation, in the previous chapter showed that collagen content was decreased in hypoplastic lungs in comparison to control lungs. Results from gelatin zymography suggest that such alterations in collagen accumulation following decreases in lung expansion may be due in part to an increase in extracellular breakdown of collagen. Conversely, increases in lung expansion reduced the proportion of total MMP-2 that is active MMP-2 and tended to reduce the overall level of active MMP-2. Following tracheal obstruction, collagen content increases. This increase in collagen may be due to both an increase in collagen synthesis as well as a small decrease in collagen breakdown. Pretreatment with physiological doses of corticosteroids did not alter gelatinase levels in normally grown lungs, but reduced the percentage of active MMP-2 in hyperplastic lungs, which is in keeping with the greater ECM remodelling we suggest occurs in these lungs.

In summary, the zymography results show that active and latent MMP-2 are present in fetal sheep lung late in gestation (days 128 - 131). Decreases in lung expansion resulted in significant increases in the levels of active, latent and total MMP-2, whereas increases in lung expansion caused a transient decrease in active MMP-2 levels. MMP-9 was only noted in a few lung samples in very low quantities and did not appear to be affected by any of the treatments studied in this chapter and have not been quantified.

4.4.1 Gelatinase Levels in Fetal Sheep Lung

Gelatin zymography proved to be a suitable method for detecting sheep gelatinases. Gelatinases detected by this technique were inhibited by EDTA (Birkedal-Hansen *et al.*,

1993), as expected for metalloproteinases and had similar electrophoretic mobility to human MMP-2 and MMP-9. Results showed that latent and active MMP-2 were easily detected in fetal sheep lung, but that MMP-9 was rarely detectable, in accordance with studies of fetal lung development in other species (Dunsmore *et al.*, 1998; Fukuda *et al.*, 1998; Eickelberg *et al.*, 1999).

4.4.2 MMP-2 Levels Following Decreases in Lung Expansion

One of the most interesting findings of this study was that draining the fetal lung of liquid caused an increase in MMP-2 levels. Total MMP-2, latent MMP-2 and active MMP-2 were all increased in *saline & drain* fetuses in comparison to *saline-only* fetuses. This increase in gelatinase levels occurred in fetuses with hypoplastic lungs, which contain a lower collagen content than those of control fetuses (Chapter 3). It is highly likely therefore, that these increased gelatinase levels may actively reduce the collagen content, as gelatinases are implicated in the breakdown of extracellular collagens.

In most cell types, MMP-2 is constitutively expressed and does not respond to stimulation by growth factors or cytokines (Alexander & Werb, 1991). It was interesting therefore, that active and latent MMP-2 levels were increased in *saline & drain* fetuses and this increase may be due to changes in alveolar epithelial cell (AEC) phenotype. Alterations in lung expansion have been shown to affect alveolar epithelial cell phenotype (Alcorn *et al.*, 1977; Flecknoe *et al.*, 2000). Following a period of lung liquid drainage, the proportion of AECs which were type II AECs increased (Alcorn *et al.*, 1977). In fact, type II AEC proportion was increased in both groups of fetuses drained of lung liquid in Chapter 3 (S. Flecknoe: unpublished data). MMP-2 secretion has been localised to type II AECs (Pardo *et al.*, 1998; Fukuda *et al.*, 2000), as has MT1-MMP levels (Fukuda *et al.*, 2000), in fetal rat and rabbit lungs. It is possible, therefore, that changes in mechanical stress upon lung tissue following alterations in lung expansion may impact upon MMP-2 levels *via* alterations in the numbers of type II AECs or by direct effects on lung fibroblasts. Mechanical stress applied to human fetal lung fibroblasts resulted in a decreased activation of MMP-2 (Tomasek *et al.*, 1997), and decreased MMP-2 and MT1-MMP mRNA levels. However, in a 'mechanically relaxed' environment, MMP-2 activation was promoted (Tomasek *et al.*, 1997; Yan *et al.*, 2000). In addition, agents that disrupt the cellular cytoskeleton (cytochalasins) have been

shown to increase the levels of MMP-2 (Aggeler *et al.*, 1984; Werb *et al.*, 1986; Gervasi *et al.*, 1996; Tomasek *et al.*, 1997; Upadhya & Strasberg, 1999). Thus, decreases in lung liquid volume, which promote an increase in the proportion of type II AECs and a decrease in tissue stretch, may act to increase MMP-2 secretion.

Treatment of the fetus with cortisol during lung liquid drainage had no effect on the levels of latent MMP-2, but prevented the increase in active MMP-2 levels and thus a significant increase in total MMP-2 levels, associated with the drainage of lung liquid. It should be noted however, that total MMP-2 levels for the *cortisol & drain* group of fetuses tended to be higher than that of the *saline-only* fetuses. MMP-2 is activated *via* a unique pathway in comparison with other MMPs. MMP-2 activation involves active MT1-MMP, a membrane-bound MMP, which initiates cleavage of the propeptide domain producing an intermediate form of MMP-2 (Murphy *et al.*, 1999). Full activation of MMP-2 then involves autoproteolysis (Murphy *et al.*, 1999) to remove the remainder of the propeptide domain. Paradoxically, TIMP-2, originally thought to inhibit MMP-2, is also involved in the activation of MMP-2 (Murphy *et al.*, 1999). TIMP-2 binds to the active site of MT1-MMP and together they act as a 'receptor' for latent MMP-2 (Murphy *et al.*, 1999). Once latent MMP-2 is bound to this receptor, adjacent TIMP-free and active MT1-MMPs on the cell surface can commence the cleavage of the propeptide domain of MMP-2 (Murphy *et al.*, 1999). However, if the TIMP-2 concentration increases so that all the MT1-MMP molecules become complexed with TIMP-2, there remains no free MT1-MMP to initiate the proteolysis (Murphy *et al.*, 1999). Hence, the activation of latent MMP-2 is particularly sensitive to TIMP-2 concentrations as well as MT1-MMP concentrations. Although both latent and active MMP-2 increased following a period of lung liquid drainage, total MMP-2 increases are most likely due to a large increase in active MMP-2, as indicated by an increase in the percentage of active MMP-2 levels. Following treatment with cortisol, the percentage of active MMP-2 in relation to total MMP-2 returned to control levels. Cortisol may be acting to reduce MMP-2 activation *via* effects on MT1-MMP or TIMP-2. In fact, other studies have suggested that MT1-MMP is regulated by corticosteroids as well as by a number of cytokines and growth factors (Lohi *et al.*, 1996). The effect of corticosteroids to reduce the levels of active gelatinases in hypoplastic lungs may ultimately reduce the turnover of lung collagen and allow lung collagen content to increase. However, collagen content was not different between *saline & drain* and *cortisol & drain* fetuses (Chapter 3) and a decrease in active MMP-2 may be counterbalanced by decreases in the levels of collagen synthesis.

It should be noted that although zymography can detect whether MMPs are present in the active form or not, it cannot predict MMP activity. MMP in the active form is a target for inhibition by the TIMPs. During zymography, active MMP-2/TIMP complexes are disrupted by the SDS in the gel, allowing MMP-2 to migrate free of TIMP and degrade the gelatin. Hence, further studies employing activity assays (Cawston *et al.*, 2001) would be required to address whether MMP-2 in lung tissue samples is free of TIMP and hence potentially active *in vivo*. Techniques such as reverse zymography, *in situ* hybridisation, immunolocalisation, Northern and Western blot analyses would also be useful to indicate how TIMP expression is affected by changes in lung volume.

4.4.3 MMP-2 Levels Following Increases in Lung Expansion

Previous studies have shown that prolonged obstruction of the fetal trachea leads to accelerated lung growth and includes an increase in lung collagen content (Nardo *et al.*, 1998). This increase in collagen content is believed to be an important factor in allowing lung growth to continue during the course of the tracheal obstruction period. Structural remodelling of the extracellular matrix of the lung and in particular of collagen, would allow increases in lung volume to occur. Gelatinase levels were not statistically altered following tracheal obstruction for periods of 4 or 10 days. However, following 2 days of tracheal obstruction, active MMP-2 levels tended to be lower than those of *control* fetuses and the percentage of active MMP-2 levels were lower than that of the *control* fetuses. The high degree of variability within the active MMP-2 levels for the *2dTO* fetuses may have contributed to this fall in active MMP-2 being non-significant. However, a low number of animals used in the study is more likely to have contributed to this being a non-significant difference. Thus, a larger group size and running a greater number of lanes on a gel may clarify whether a significant difference in active MMP-2 levels occurs after 2 days of tracheal obstruction, in comparison to control fetuses. Results from the cortisol infusion and tracheal obstruction study, in which a group of fetuses was obstructed for 3 days and received an infusion of saline showed that active MMP-2 levels were similar to control values by 3 days of TO. Therefore, if a decrease in active MMP-2 levels does occur following an increase in lung expansion, it is likely that this would occur very early following TO. If this decrease in active MMP-2 occurs, it could signify an alteration in collagen turnover in the initial period following lung expansion when the lung expands to an initial limit (Nardo *et al.*, 1998). To reiterate, within a day of tracheal obstruction, the lung liquid volume doubles, but does not increase further until after 2 days of TO. The lung is

thought to reach an initial structural limit within the first day of tracheal obstruction and further increases in lung liquid volume are likely to require structural remodelling of the lung (Nardo *et al.*, 1998). In the early phase of lung growth following tracheal obstruction, collagen turnover may well be reduced by a decrease in gelatinase levels and an increase in collagen synthesis may not yet be stimulated. In the second phase, a higher collagen synthesis rate, and normal levels of collagen degradation would allow ECM turnover with collagen accumulation. The stimulus for a potential change in active MMP-2 levels is unknown but may be due to decreases in the proportion of type II AECs after 2 days of TO (Flecknoe *et al.*, 2000), or mechanical stress leading to a decrease in MMP-2 activation (Tomasek *et al.*, 1997). It is unlikely that the primary reason for the decrease in MMP-2 levels at two days of tracheal obstruction is due to a decrease in type II AECs since by 10 days of TO, < 2% of all AECs were type II AECs (Flecknoe *et al.*, 2000), yet total MMP-2 levels were not different from control levels. As the alterations in active MMP-2 levels appear to occur shortly after the experiment has commenced, it is more likely they involve a response due to physical changes in the lung. One could speculate that the rapid doubling of lung liquid volume, accompanied by a sharp increase in intraluminal pressure (Nardo *et al.*, 1998), may provide an enormous mechanical stimulus, thereby altering MMP-2 activation.

Longer periods of tracheal obstruction, *i.e.* 3, 4 and 10 days of TO, had no effect on active and latent MMP-2 levels, as values were not different from those in control fetuses. However, long-term lung deflation led to marked increases in active and latent MMP-2 levels. These findings are in contrast to numerous studies that have shown that many components of the fetal lung are differentially regulated by the degree of lung expansion. Most notably, lung growth as measured by lung DNA content, protein content, collagen content and lung weights is differentially regulated by the degree of lung expansion (Moessinger *et al.*, 1990; Hooper *et al.*, 1993b; Nardo *et al.*, 1998; Chapter 3). Alveolar epithelial cell phenotype (Alcorn *et al.*, 1979) and surfactant gene expression (Lines *et al.*, 1999) are also differentially regulated in response to changes in lung expansion. In addition, expression of growth factors such as TGF-β and IGF-II increases following long-term tracheal obstruction (Hooper *et al.*, 1993b; Wallace *et al.*, 2002) and decreases following lung deflation (Hooper *et al.*, 1993b).

The results presented in this study are in contrast with *in vitro* studies in which periods of mechanical lung stretch did not alter gelatinase activities (Xu *et al.*, 1999). It should be noted that the study by Xu *et al.* (1999) utilised three-dimensional organotypic fetal

lung cell cultures on gelfoam sponges and compared ECM proteins on stretched and non-stretched lattices. This result is in contrast to the work by Tomasek *et al.* (1997), in which the degree of MMP-2 activation and MMP-2 and MT1-MMP mRNA levels were determined in free-floating lattices and plated lattices. In the latter study, free-floating lattices were deemed to be 'mechanically relaxed', whereas plated lattices were 'mechanically stressed', as indicated by intracellular stress fibres. Therefore, in relation to the Tomasek study, one could presume that Xu *et al.* commenced with a lattice already mechanically stressed. Thus, further stretching these cells did not induce MMP-2 production. In fact, mechanical stretch of the cells in the Xu study increased levels of procollagen mRNA for collagen-α1(IV) and collagen-α2(IV) (Xu *et al.*, 1999), further emphasising that the principal factor involved in the increase in collagen content following tracheal obstruction is likely to be an increase in the *de novo* synthesis of collagen.

4.4.4 Conclusions

In conclusion, gelatinase levels are increased following a prolonged period of lung deflation. As gelatinases degrade collagens, this increase in gelatinase levels may be associated with the net loss of collagen that occurs in the hypoplastic lungs of these fetuses. Increases in gelatinase levels may directly result from morphological changes in the lung following lung deflation such as the increase in type II alveolar epithelial cells and/or changes in the shape of cells such as fibroblasts. Given that levels of active MMP-2 were higher in *saline & drain* versus *cortisol & drain* groups of fetuses, it is surprising that collagen content is similar in both groups (Chapter 3). This similarity in collagen content may be a result of balancing factors, such as cortisol-mediated increases in collagen synthesis rates (Kivirikko & Laitinen, 1965; Kivirikko *et al.*, 1965; Schellenberg *et al.*, 1987b), or increases in the inhibitory TIMP. It may also be a reflection that the collagen assay is not sensitive enough to detect small changes in collagen content.

In summary, increases in gelatinase levels following lung liquid drainage may indicate that decreases in collagen content in the hypoplastic lung are as a result of increased gelatinase activity. Short-term tracheal obstruction tended to decrease active MMP-2 levels and could signify an alteration in collagen turnover in the initial period following lung expansion. Conversely, following longer-term tracheal obstruction, no significant differences in comparison to control fetuses were observed in gelatinase levels.

Therefore, increases in collagen content following tracheal obstruction are most likely due to increased *de novo* synthesis of collagen. The importance of collagen synthesis in the lung growth response to tracheal obstruction will be further investigated in the following chapter.

CHAPTER 5 Role of Collagen Synthesis in the Lung Growth Response to Tracheal Obstruction

5.1 Introduction

An increase in lung expansion induced by obstruction of the fetal trachea is a potent stimulus for fetal lung growth (Hooper & Harding, 1995; Nardo *et al.*, 1998). The lung growth response occurs in a time-dependent manner and in late gestation sheep, most of the growth is completed within seven days of obstructing the fetal trachea (Hooper *et al.*, 1993b; Nardo *et al.*, 1998). Within this seven day timeframe, the lung growth appears to be in two distinct phases. The initial phase (0 - 2 days of tracheal obstruction) is characterised by a very large increase in DNA synthesis rates (~800% above control levels: see Figure 5.1; Nardo *et al.*, 1998), whereas the second phase (3 - 7 days of tracheal obstruction) is characterised by much lower DNA synthesis rates, although they are still elevated above control levels (~70% above control; Nardo *et al.*, 1998). During this second phase of lung growth, increases in collagen content are observed (Nardo *et al.*, 1998), possibly due to increases in collagen synthesis rates (Chapter 4). We hypothesise that during this second phase of lung growth, ECM remodelling and in particular increases in collagen content play an important role in allowing further increases in lung growth to occur.

Within one day of tracheal obstruction, lung liquid volume doubles, yet there is no further increase in lung liquid volume between one and two days of tracheal obstruction (Keramidaris *et al.*, 1996; Nardo *et al.*, 1998). However, after 2 days of tracheal obstruction, there is a linear increase in lung liquid volume, reaching maximal values by 7 days of tracheal obstruction (Nardo *et al.*, 1998). The increases in lung growth between 2 and 10 days of tracheal obstruction are highly correlated to the increases in lung liquid volume ($r = 0.999$: Nardo *et al.*, 1998). As lung liquid volumes do not change between one and two days of tracheal obstruction, it has been suggested that

a limit to further increases in lung volume may have been reached at this time (Nardo *et al.*, 1998). In particular, a structural limit may be imposed by the lung tissue and further expansion after 2 days of tracheal obstruction may require remodelling of this structural framework. Thus, changes in the ECM at this time, particularly to components such as collagen, may provide a limit to growth during the second phase following tracheal obstruction by restricting the rate of lung growth to the rate of structural remodelling. Collagen content is significantly increased by 10 days of tracheal obstruction (Nardo *et al.*, 1998) indicating that collagen remodelling does occur following tracheal obstruction. If this hypothesis is correct and collagen remodelling plays a significant regulatory role in this second phase of the lung growth response to tracheal obstruction, then the inhibition of collagen synthesis should prevent or reduce the normal increase in growth in the fetal lung following tracheal obstruction.

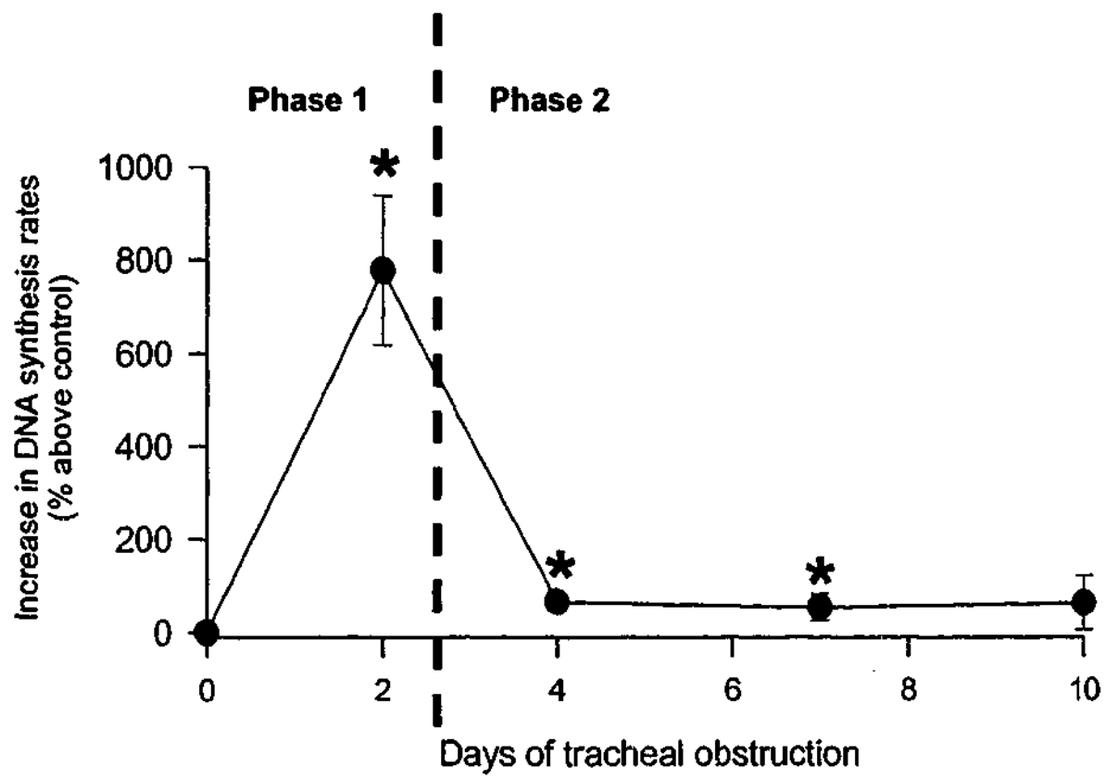

Figure 5.1 Lung DNA synthesis rates following tracheal obstruction

DNA synthesis rates following 2, 4, 7 and 10 days of tracheal obstruction in fetal sheep, expressed as a percentage of control values at each timepoint. Asterisks indicate values that are significantly different from control values ($P < 0.05$). The dashed bar through the graph separates the postulated two phases of lung growth following tracheal obstruction: phase 1: 0 - 2 days of tracheal obstruction; phase 2: >3 days of tracheal obstruction. Adapted from Nardo *et al.* (1998).

Proline analogues such as L-Azetidine 2-Carboxylic Acid (LACA) have been used to study the role that collagen synthesis plays in several developmental systems. LACA is a proline analogue that competes with proline for insertion in the procollagen polypeptide chain, disrupting the native collagen helix and inhibiting hydroxylation (Uitto & Prockop, 1974). Ultimately LACA inhibits the secretion of collagen from the cell (Uitto & Prockop, 1974), thereby preventing extracellular collagen accumulation (Lane *et al.*, 1971; Switzer & Summer, 1973). Studies in which lung explants have been incubated with LACA *in vitro* have shown that a lack of collagen synthesis results in a cessation of lung development (Alescio, 1973; Spooner & Faubion, 1980). Adamson and King (1987 & 1988) have administered LACA to pregnant rats (i.p.) and examined lung development in fetal and newborn offspring. They showed that interruptions in collagen synthesis result in decreased lung growth as measured by lung weights, lung DNA content and hydroxyproline (a marker of collagen) content. Disruption in collagen synthesis in fetal rats also delayed type II alveolar epithelial cell development and surfactant production (Adamson & King, 1987).

The aim of the current study was to observe the effects of continuous intravenous infusion of LACA on lung growth in fetal sheep during a period of tracheal obstruction. It was important that a time-period for tracheal obstruction be chosen in which increases in both lung growth and collagen content are occurring, therefore, 5 days of tracheal obstruction was selected. Based on the doses used in previous studies on the *in vivo* effects of LACA on lung development (Adamson & King, 1987; Adamson & King, 1988), two doses of LACA were chosen for infusion (i.v.) into separate groups of fetal sheep undergoing tracheal obstruction: a ***low*** dose (50 mg/day) and a ***high*** dose (250 mg/day; see Section 5.2.1).

5.2 Methods

Surgery was performed on 25 pregnant ewes and their fetuses (Merino X Border-Leicester) at 117.8 ± 0.4 days of gestation (term is ~147 days) to implant fetal and maternal vascular catheters and fetal tracheal catheters as described in Section 2.2. Fetal well-being was monitored on a daily basis by measuring fetal pH, PaO_2, $PaCO_2$ and SaO_2 (ABL30, Radiometer, Denmark). A minimum of 5 days was allowed for the ewe and fetus to recover from surgery before experiments began.

5.2.1 Experimental Protocol

To determine the effects of LACA on the lung growth response to tracheal obstruction, fetuses were divided into one of five groups:

1) A *Vehicle-only* group, in which vehicle was infused intravenously (i.v.) into the fetus for 5 days (125 - 130 days GA, n = 5),
2) A *Vehicle & 5dTO* group, in which the fetal trachea was obstructed for 5 days (125 - 130 days GA) and the fetus received an i.v. infusion of vehicle for the same 5 days,
3) A *LD LACA & 5dTO* group, in which the fetal trachea was obstructed for 5 days (125 - 130 days GA) and a low dose of LACA (50 mg/day i.v.) was infused into the fetus for 5 days (125 - 130 days GA, n = 5),
4) A *HD LACA & 5dTO* group, in which the fetal trachea was obstructed for 5 days (125 - 130 days GA) and a high dose of LACA (250 mg/day i.v.) was infused into the fetus for 5 days (125 - 130 days GA, n = 5),
5) A *HD LACA-only* group, in which a high dose of LACA (250 mg/day i.v.) was infused into the fetus for 5 days (125 - 130 days GA, n = 5).

LACA (50 or 250 mg: Auspep Pty Ltd, Australia) was mixed with 0.5 ml 70% acetic acid (as per product specification sheet: Auspep Pty Ltd, Australia) then made up to 30 ml with heparinised saline and infused into the fetal jugular vein at 1.2 ml/hr (Perfusion VI, Braun, Germany). The LACA was prepared fresh daily and administered at ***low*** (group 3: 50 mg/day) or ***high*** (groups 4 and 5: 250 mg/day) doses from day 125 until the time of *post mortem* on day 130 of gestation. The vehicle infusion consisted of 0.5 ml 70% acetic acid diluted to 30 ml with heparinised saline and infused at 1.2 ml/hr (i.v.). The

vehicle-infused and LACA-infused groups of fetuses each received a small bolus (~2 ml) immediately prior to the commencement of the infusion and each day thereafter. All infusions were continued until the time of *post mortem*.

The doses of LACA used in this study were chosen by extrapolating the doses used previously in the studies by Adamson and King (Adamson & King, 1987; Adamson & King, 1988). Many assumptions have been made in comparing the two very different modes of administration, including: route of administration, transfer of LACA across the placenta, metabolism of LACA, and distribution of LACA between maternal and fetal compartments. Adamson and King injected 200 mg/kg maternal body weight of LACA intraperitoneally twice per day over two days. Therefore, 400 mg/kg/day was delivered to each maternal rat (Sprague-Dawley). As the LACA was administered intraperitoneally and not directly into the fetus, we must consider that an appropriate proportion would have transferred across the placenta to the rat fetuses. At birth, rat pup body weights were approximately 3 - 5 g and thus, prior to birth, rat pup weights would have been significantly less than this (data interpreted from Adamson & King, 1988). Maternal rat weights (Sprague-Dawley) are approximately 300 - 350 g at the time of birth (Anderson *et al.*, 1980; Gargosky *et al.*, 1991) and it has been assumed that rat pup weights were approximately 1% of maternal weight at the time of treatment. This equates to ~4.0 mg/kg/day of LACA per fetal rat over the experimental period. Fetal sheep weights during the experimental period were estimated at being 3 - 4 kg. Thus, a ***high*** dose of LACA at 250 mg per day is equivalent to 60 - 80 mg/kg/day, a dose that is 15 - 20 X greater than that used in the Adamson and King studies. A ***low*** dose of LACA at 50 mg per day is equivalent to 12 - 16 mg/kg/day a dose 3 – 4 X greater than that used by Adamson and King. In addition, LACA was infused intravenously into the jugular vein which preferentially streams into the right ventricle/atrium providing the drug directly to the lungs.

Lung liquid volume measurements were made on the final day of experimentation, immediately prior to *post mortem*. Lung liquid volume measurements were made in accordance with the methods described in Section 2.4.

All experiments were terminated at 130.2 ± 0.1 days of gestation. The fetal lungs were drained of liquid *via* the tracheal catheter and the tracheal loop was blocked before the ewe and fetus were humanely killed by an overdose of sodium pentobarbitone administered to the ewe (130 mg/kg i.v.). The fetus was weighed and the fetal lungs,

kidneys, heart and liver were removed and weighed. The left mainstem bronchus was ligated and the left lung removed distal to the ligature. Portions of the left lung were frozen in liquid nitrogen and stored at -70 °C for subsequent biochemical analysis. The right lung was pressure fixed with 4% paraformaldehyde at 20 cmH_2O (Section 2.5).

5.2.2 Biochemical and Histological Methods

Frozen lung tissue samples were accurately weighed and used for biochemical analysis of DNA, protein and hydroxyproline concentrations and contents as described in the General Methods (Sections 2.7.1 - 2.7.3).

Sections of the right lung were processed in paraffin, cut at 5 μm and stained with Picrosirius Red which stains collagen fibres pink/red (Section 2.9.2). For each animal, 3 blocks were sectioned and 2 views from each of 2 sections per block (*i.e.* 12 views per animal), were analysed, avoiding any area which contained major airways or blood vessels. Stereological measurement of right lung collagen fraction (Section 2.9.2) was made using the grid shown in Figure 2.6 (f). Lung sections were viewed on a X20 microscope lens with the final projected magnification being X360 as determined with a 0.1 mm graticule.

5.2.3 Statistical Analysis

The results are presented as mean ± standard error of the mean (SEM). The accepted level of significance for all statistical analyses was $P < 0.05$. Statistical analyses were performed using the computerised statistical packages SigmaStat (Version 2.0, Jandel Corporation, USA) and SPSS (Version 10.0.5, SPSS Incorporated, USA). Fetal body weights, lung and organ weights, lung liquid volume and secretion rates, DNA, protein and hydroxyproline contents and concentrations were analysed by a one-way analysis of variance (ANOVA). Significant differences between values were then identified with a Least Significant Difference (LSD) test. Fetal arterial blood gas and acid-base indices were analysed by a two-way ANOVA for repeated measures with treatment and gestational age as factors. If an interaction was identified, an LSD test isolated differences by doing pairwise comparisons of the data points. Collagen fraction and volume were analysed by nested one-way ANOVAs. When significant differences were found, they were identified by an LSD test. The relationship between the

percentage increase in fetal lung hydroxyproline content and the percentage increase in fetal lung DNA content was calculated by linear regression analysis.

5.3 Results

All fetuses were considered healthy according to their arterial blood gas and acid-base status for the duration of the experimental period. Furthermore, the experimental manipulations had no significant effect on fetal arterial blood gases and acid-base status (Table 5.1). Body weights were not different between any of the groups at the time of *post mortem* (Table 5.2).

Table 5.1 Fetal arterial blood gas and acid-base status

This table shows the mean ± SEM of arterial blood gases and pH of fetal sheep throughout the duration of the experiment for *Vehicle-only*, *Vehicle & 5dTO*, *LD LACA & 5dTO*, *HD LACA & 5dTO* and *HD LACA-only* fetuses. The experimental manipulations had no significant effect on the arterial blood gas and acid base status of fetal sheep.

	Vehicle-only (n = 5)	*Vehicle & 5dTO* (n = 5)	*LD LACA & 5dTO* (n = 5)	*HD LACA & 5dTO* (n = 5)	*HD LACA-only* (n = 5)
pH	7.370 ± 0.004	7.374 ± 0.005	7.365 ± 0.002	7.368 ± 0.003	7.375 ± 0.002
$PaCO_2$ (mmHg)	47.2 ± 2.8	39.3 ± 1.2	42.6 ± 1.5	42.5 ± 1.3	39.8 ± 1.0
PaO_2 (mmHg)	22.3 ± 1.7	24.2 ± 1.2	23.2 ± 1.8	21.6 ± 1.1	25.6 ± 1.0
SaO_2 (%)	62.4 ± 3.6	71.3 ± 4.0	64.8 ± 3.9	66.0 ± 3.0	73.3 ± 2.4

Table 5.2 Fetal body weight

Mean fetal body weight ± SEM (kg) as measured at 130.2 ± 0.1 days of gestation in fetuses for each of the experimental groups used in this study. The experimental manipulations had no significant effect on fetal body weight.

	Vehicle-only (n = 5)	*Vehicle & 5dTO* (n = 5)	*LD LACA & 5dTO* (n = 5)	*HD LACA & 5dTO* (n = 5)	*HD LACA-only* (n = 5)
Fetal body weight (kg)	3.4 ± 0.5	4.0 ± 0.3	3.4 ± 0.3	3.6 ± 0.4	3.3 ± 0.2

5.3.1 Fetal Lung Liquid Volume

Lung liquid volumes (ml and ml/kg) on the final day of the experiment were not different between the fetuses without tracheal obstruction (*Vehicle-only*: 114.8 ± 12.4 ml; 33.0 ± 2.6 ml/kg versus *HD LACA-only*: 103.6 ± 8.2 ml; 32.8 ± 3.5 ml/kg; Figure 5.2). When corrected for fetal body weight, there were no apparent differences in lung liquid volume (ml/kg) between all the animals that underwent tracheal obstruction (*Vehicle & 5dTO*: 70.1 ± 4.6 ml/kg, *LD LACA & 5dTO*: 64.1 ± 6.0 ml/kg and *HD LACA & 5dTO*: 74.6 ± 5.3 ml/kg respectively; Figure 5.2: lower panel). All three tracheal obstruction groups, however, had significantly greater lung liquid volumes than the two groups that did not have obstructed tracheas. When lung liquid volume (ml) was not corrected for body weight, it was significantly greater in the tracheal-obstructed groups of fetuses (*Vehicle & 5dTO*: 279.1 ± 18.3 ml and *HD LACA & 5dTO*: 262.8 ± 37.8 ml) than in the fetuses without tracheal obstruction (*Vehicle-only*: 114.8 ± 12.4 ml and *HD LACA-only*: 103.6 ± 8.2 ml; $P < 0.05$). However, the *LD LACA & 5dTO* group (194.0 ± 4.0 ml) had a significantly lower lung liquid volume (ml) than that of the *Vehicle & 5dTO* group (279.1 ± 18.3 ml).

5.3.2 Fetal Lung and Organ Weights

The lung weights (g and g/kg) in all groups of fetuses that had been exposed to tracheal obstruction were significantly greater than lung weights from fetuses without tracheal obstruction ($P < 0.05$; Figure 5.3: upper panel). However, the lung weights (g) for the *LD LACA & 5dTO* fetuses (184.6 ± 20.6 g) were significantly lower than those of the *Vehicle & 5dTO* fetuses (236.6 ± 13.6 g). When corrected for fetal body weight (g/kg), lung weight did not differ between any of the groups of fetuses with tracheal obstruction (Figure 5.3: lower panel). There were no significant differences in the lung weights (g and g/kg) between the *Vehicle-only* and *HD LACA-only* groups.

There were no significant differences between any of the treatment groups in the wet weights of fetal liver, heart and kidneys in absolute values (g) or when corrected for fetal body weight (g/kg; Table 5.3).

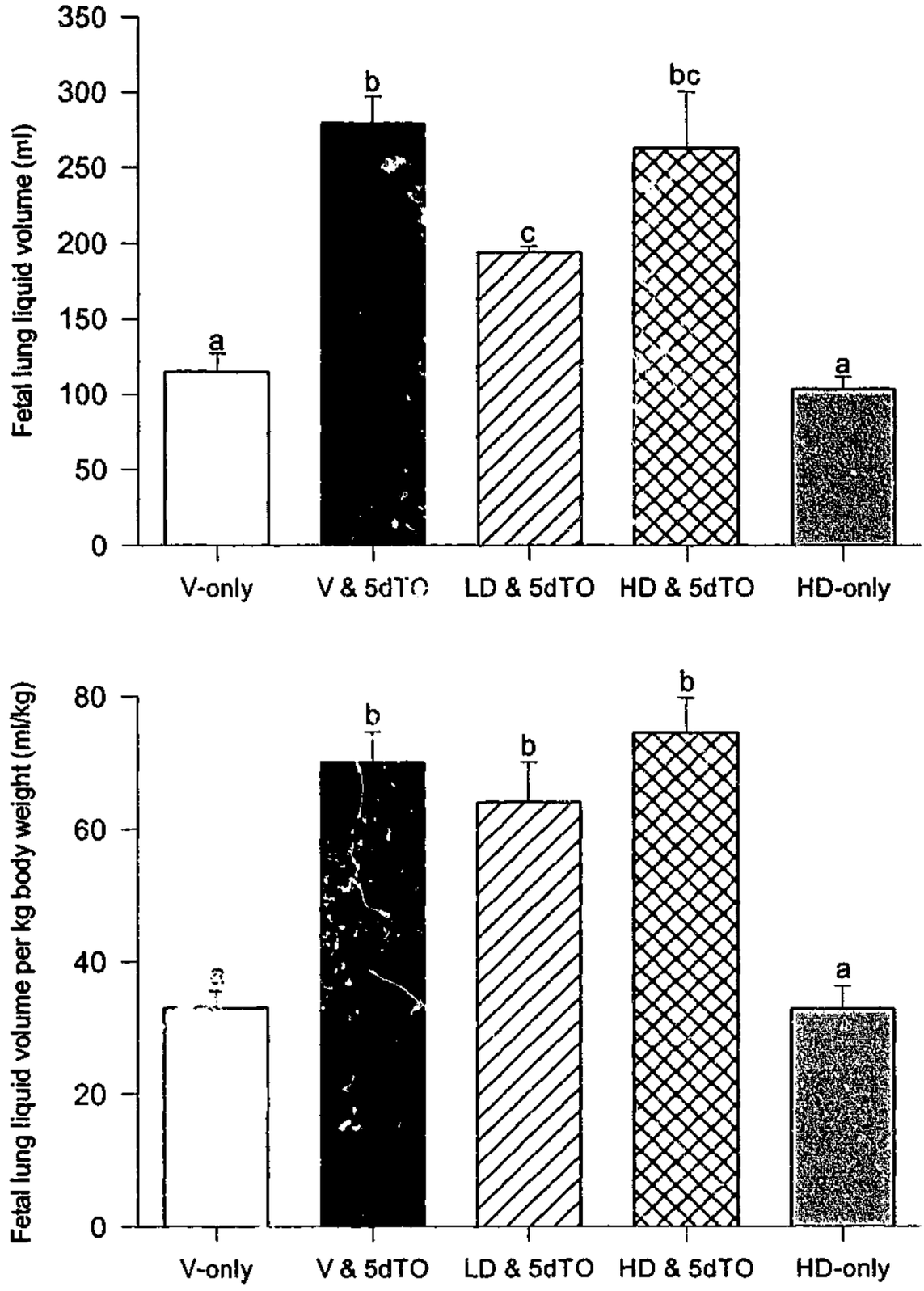

Figure 5.2 Fetal lung liquid volume

Upper panel: mean fetal lung liquid volume ± SEM (ml) and ***lower panel***: mean fetal lung liquid volume per kilogram of fetal body weight ± SEM (ml/kg) in *Vehicle (V)-only* (n = 5), *Vehicle & 5dTO* (n = 5), *LD LACA & 5dTO* (n = 5), *HD LACA & 5dTO* (n = 5) and *HD LACA-only* (n = 5) fetuses. Lung liquid volume was measured on the last day of each experiment, just prior to *post mortem* (130.2 ± 0.1 days GA). Values that do not share the same letter are significantly different from each other ($P < 0.05$).

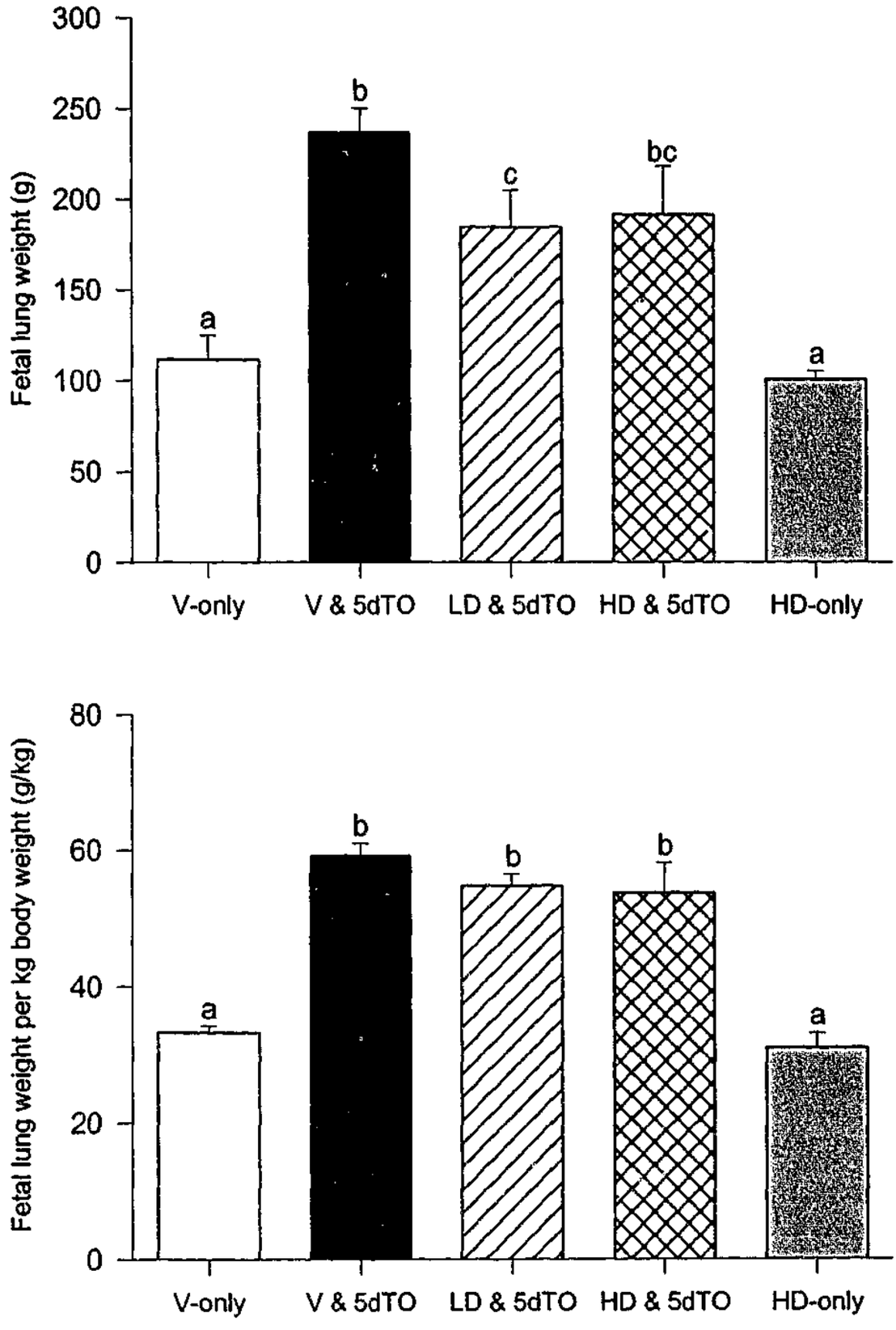

Figure 5.3 Fetal lung weights

Upper panel: mean wet lung weight ± SEM (g) and ***lower panel***: mean wet lung weight adjusted for fetal body weight ± SEM (g/kg) measured in *Vehicle-only* (n = 5), *Vehicle & 5dTO* (n = 5), *LD LACA & 5dTO* (n = 5), *HD LACA & 5dTO* (n = 5) and *HD LACA-only* (n = 5) fetuses at 130.2 ± 0.1 days of gestation. Values that do not share a common letter are significantly different from each other ($P < 0.05$).

Table 5.3 Fetal organ weights

Mean fetal liver, kidney and heart weights ± SEM (g and g/kg), measured at *post mortem* examination (130.2 ± 0.1 days GA) in the *Vehicle-only*, *Vehicle & 5dTO*, *LD LACA & 5dTO*, *HD LACA & 5dTO* and *HD LACA-only* fetuses. No significant differences between groups were identified.

	Vehicle-only (n = 5)	*Vehicle & 5dTO* (n = 5)	*LD LACA & 5dTO* (n = 5)	*HD LACA & 5dTO* (n = 5)	*HD LACA-only* (n = 5)
Liver weight (g)	85.2 ± 8.2	121.8 ± 12.3	94.8 ± 12.8	102.6 ± 12.3	86.5 ± 7.8
(g/kg)	25.7 ± 1.5	30.1 ± 1.5	27.9 ± 1.2	30.6 ± 2.0	26.4 ± 2.4
Kidney weight (g)	18.7 ± 1.1	24.2 ± 2.6	21.1 ± 1.5	21.3 ± 1.9	19.5 ± 1.2
(g/kg)	5.8 ± 0.5	6.0 ± 0.5	6.4 ± 0.4	6.4 ± 0.4	6.0 ± 0.5
Heart weight (g)	22.3 ± 1.7	26.1 ± 1.7	23.1 ± 2.9	24.7 ± 2.5	21.8 ± 1.9
(g/kg)	6.8 ± 0.7	6.5 ± 0.3	6.9 ± 0.5	7.5 ± 0.6	6.6 ± 0.4

5.3.3 DNA Concentration and Content

Lung DNA concentration (mg/g) in *Vehicle & 5dTO* fetuses (2.8 ± 0.1 mg/g) was significantly less than in both groups of fetuses without tracheal obstruction (*Vehicle-only*: 3.3 ± 0.1 mg/g and *HD LACA-only:* 3.7 ± 0.1 mg/g) as well as the *HD LACA & 5dTO* fetuses (3.2 ± 0.1 mg/g; Figure 5.4: upper panel). The lung DNA concentration (mg/g) of the *HD LACA-only* group (3.7 ± 0.1 mg/g) was significantly greater ($P < 0.05$) than the lung DNA concentration for all other groups. When corrected for total organ weight and body size, however, the total lung DNA content (mg/kg) was elevated in all groups of fetuses with tracheal obstruction (*Vehicle & 5dTO*: 163.3 ± 11.6 mg/kg, *LD LACA & 5dTO*: 158.6 ± 2.4 mg/kg and *HD LACA & 5dTO*: 165.0 ± 13.0 mg/kg) in comparison to fetuses without tracheal obstruction (*Vehicle-only*: 108.7 ± 3.4 mg/kg and *HD LACA-only*: 119.5 ± 8.2 mg/kg).

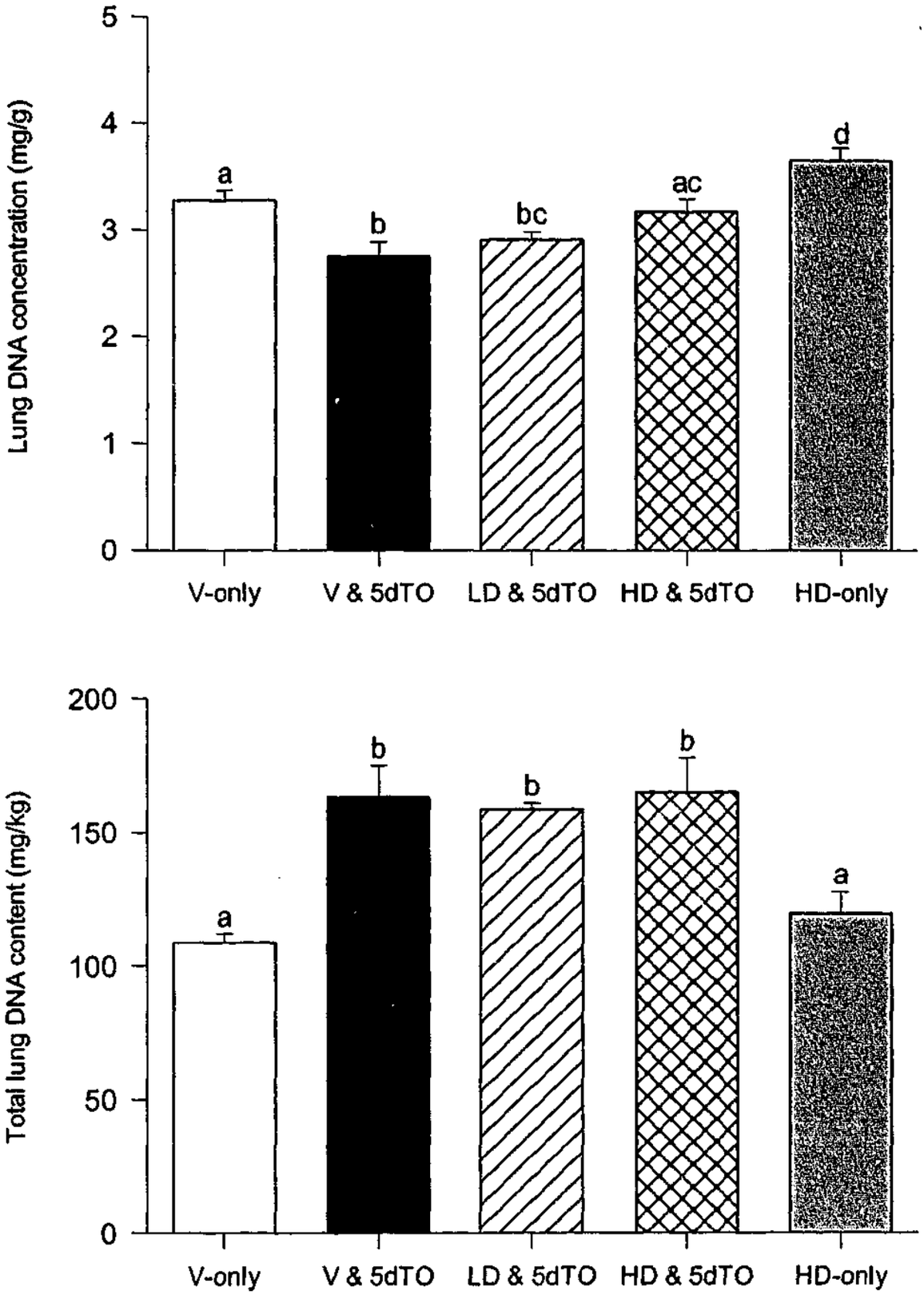

Figure 5.4 Fetal lung DNA concentration and content

Upper panel: mean DNA concentration ± SEM (mg/g lung tissue) and ***lower panel***: mean DNA content ± SEM (mg/kg of fetal body weight) of the lungs in *Vehicle-only* (n = 5), *Vehicle & 5dTO* (n = 5), *LD LACA & 5dTO* (n = 5), *HD LACA & 5dTO* and *HD LACA-only* (n = 5) fetuses (130.2 ± 0.1 days GA). Values that do not share a common letter are significantly different from each other ($P < 0.05$).

Although tracheal obstruction increased DNA content of the lung, the addition of LACA did not alter DNA content relative to the *Vehicle & 5dTO* group. Similarly, when a high dose of LACA was given alone, it did not cause an alteration in DNA content relative to that in the *Vehicle-only* group.

5.3.4 Protein Concentration, Content and Protein-to-DNA ratio

The protein concentration (mg/g) of the fetal lungs was not altered by any of the treatments (tracheal obstruction or treatment with LACA; Figure 5.5: upper panel). However, fetal lung protein content was elevated in all groups of fetuses exposed to a period of tracheal obstruction regardless of dosage of LACA received (*Vehicle & 5dTO*: 3780.0 ± 331.5 mg/kg, *LD LACA & 5dTO*: 4014.2 ± 320.9 mg/kg and *HD LACA & 5dTO*: 3538.5 ± 380.7 mg/kg) in comparison to fetuses without tracheal obstruction (*Vehicle-only*: 1998.6 ± 141.7 mg/kg and *HD LACA-only*: 2297.0 ± 140.8 mg/kg; Figure 5.5: lower panel). The protein-to-DNA ratio was significantly ($P < 0.05$) elevated in the *LD LACA & 5dTO* group (25.4 ± 1.8) in comparison to the *Vehicle-only* (18.5 ± 1.5), *HD LACA & 5dTO* (20.9 ± 1.4) and *HD LACA-only* (20.5 ± 0.9) groups. The *Vehicle & 5dTO* (23.1 ± 1.1) group had a protein-to-DNA ratio that was elevated only above that of the *Vehicle-only* (18.5 ± 1.5) group (Figure 5.6: upper panel).

5.3.5 Hydroxyproline Concentration, Content and Hydroxyproline-to-Protein Ratio

The hydroxyproline concentration of the fetal lung (mg/g of lung weight) was not different between any of the experimental groups (Figure 5.7: upper panel). However, the total lung hydroxyproline content (mg/kg) was significantly ($P < 0.05$) elevated in all groups of fetuses exposed to a period of tracheal obstruction (*Vehicle & 5dTO*: 45.1 ± 4.4 mg/kg, *LD LACA & 5dTO*: 45.4 ± 3.1 mg/kg and *HD LACA & 5dTO*: 44.1 ± 4.3 mg/kg) when compared to the fetuses without tracheal obstruction (*Vehicle-only*: 28.9 ± 1.2 mg/kg and *HD LACA-only*: 25.1 ± 1.5 mg/kg; Figure 5.7: lower panel). The dosage of LACA received by the fetus did not result in any further alterations in lung hydroxyproline concentration or content (Figure 5.7) and the hydroxyproline-to-protein ratio was not different between any of the experimental groups (Figure 5.6: lower panel).

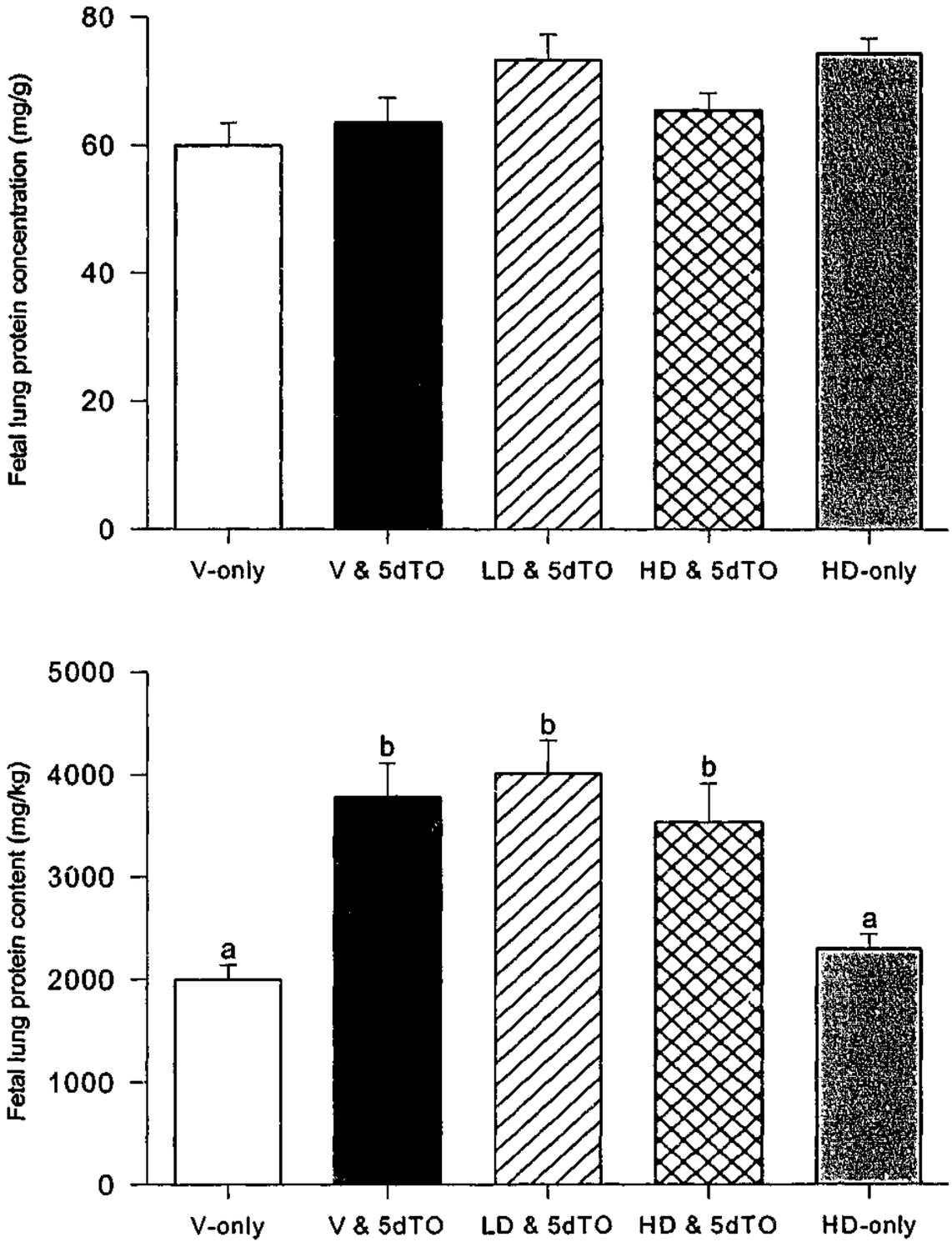

Figure 5.5 Fetal lung protein concentration and content

Upper panel: mean protein concentration ± SEM (mg/g of lung tissue) and ***lower panel***: mean protein content ± SEM (mg/kg of fetal body weight) of the fetal lung in *Vehicle-only* (n = 5), *Vehicle & 5dTO* (n = 5), *LD LACA & 5dTO* (n = 5), *HD LACA & 5dTO* (n = 5) and *HD LACA-only* (n = 5) fetuses (130.2 ± 0.1 days GA). No significant differences in protein concentration were identified (upper panel). In the lower panel, values that do not share a common letter are significantly different from each other ($P < 0.05$).

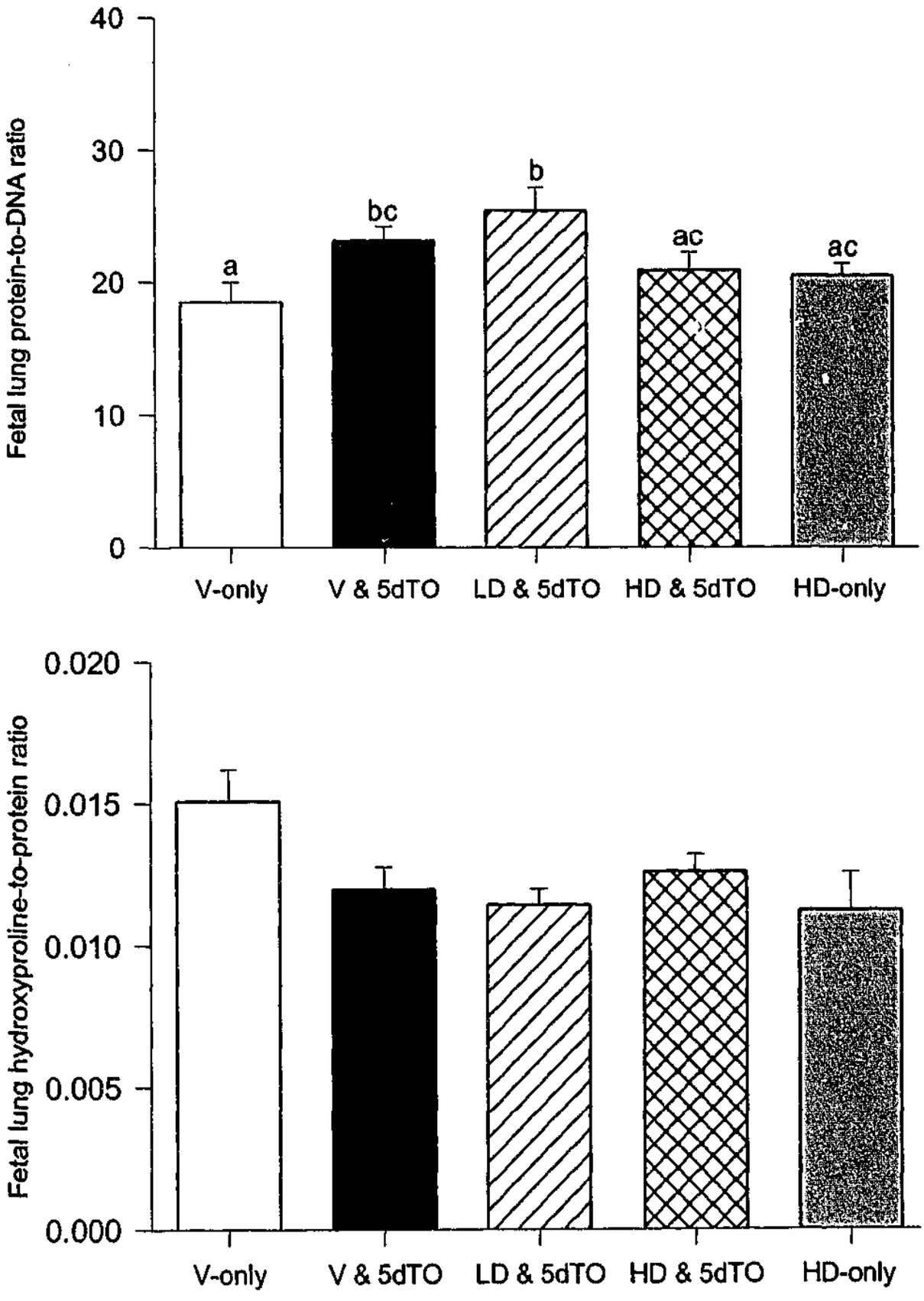

Figure 5.6 Fetal lung protein-to-DNA and hydroxyproline-to-protein ratio

Upper panel: mean fetal lung protein-to-DNA ratio ± SEM and ***lower panel***: mean fetal lung hydroxyproline-to-protein ratio ± SEM, as measured in *Vehicle-only* (n = 5), *Vehicle & 5dTO* (n = 5), *LD LACA & 5dTO* (n = 5), *HD LACA & 5dTO* (n = 5) and *HD LACA-only* (n = 5) fetuses (130.2 ± 0.1 days GA). In the upper panel, values that do not share a common letter are significantly different from each other ($P < 0.05$). No significant differences were identified between groups in the lower panel.

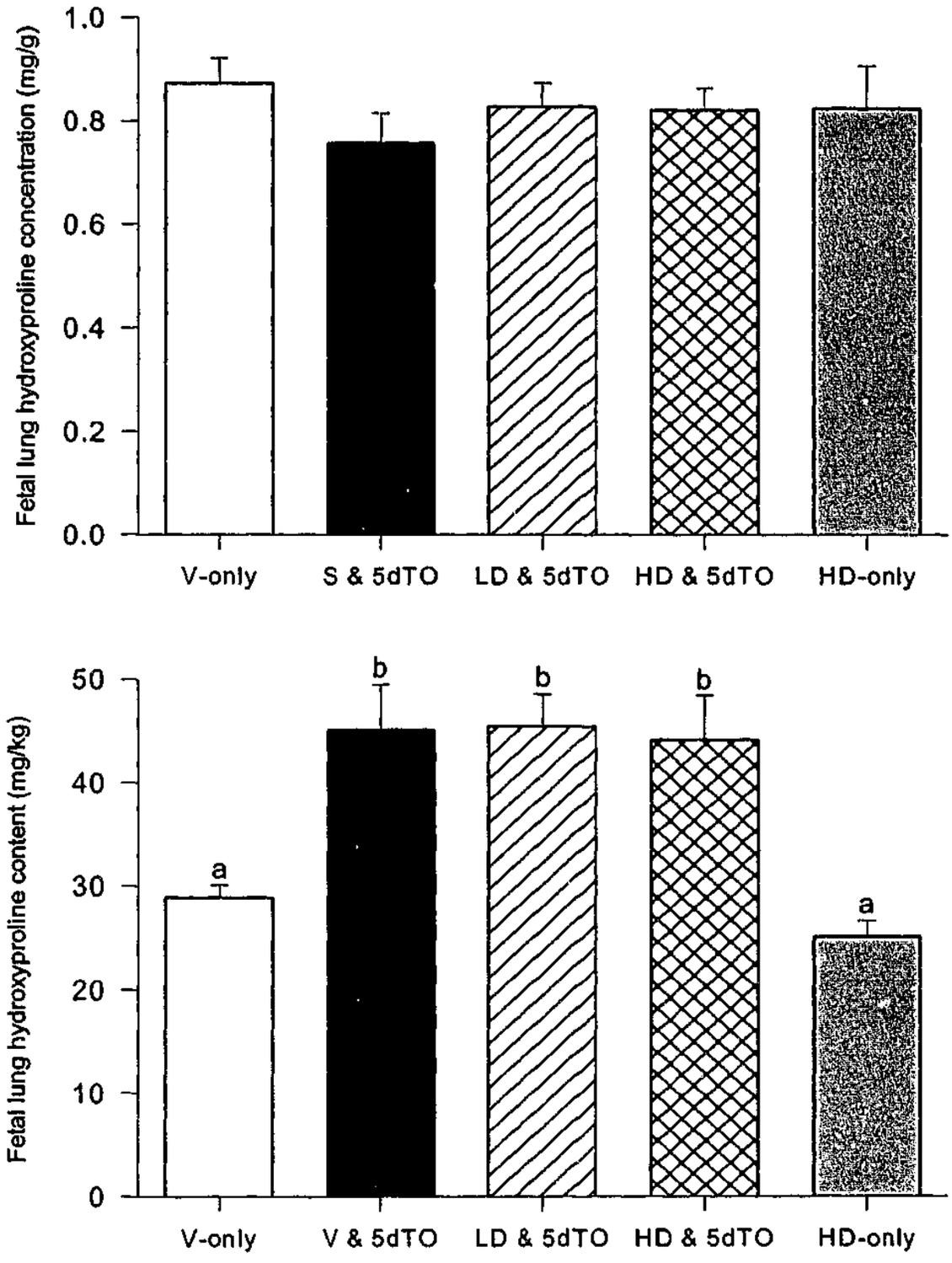

Figure 5.7 Fetal lung hydroxyproline concentration and content

Upper panel: mean fetal lung hydroxyproline concentration ± SEM (mg/g lung weight) and ***lower panel***: mean fetal lung hydroxyproline content ± SEM (mg/kg body weight) as measured in *Vehicle-only* (n = 5), *Vehicle & 5dTO* (n = 5), *LD LACA & 5dTO* (n = 5), *HD LACA & 5dTO* (n = 5) and *HD LACA-only* (n = 5) fetuses (130.2 ± 0.1 days GA). No significant differences were found between groups in the upper panel. In the lower panel, values that do not share a common letter are significantly different from each other ($P < 0.05$).

5.3.6 Interstitial Collagen Fraction

Examples of Picrosirius Red stain for collagen in *Vehicle & 5dTO* and *HD LACA & 5dTO* animals can be seen in Figure 5.8.

Interstitial collagen fraction (%): the fraction of the lung that is occupied by interstitial collagen, was decreased significantly ($P < 0.05$) in the *HD LACA-only* group of fetuses (1.5 ± 0.1%) when compared to all other groups of fetuses (*Vehicle-only*: 2.0 ± 0.2%; *Vehicle & 5dTO*: 2.2 ± 0.1%; *LD LACA & 5dTO*: 2.3 ± 0.1% and *HD LACA & 5dTO*: 2.0 ± 0.1%; Figure 5.9: upper panel). When corrected for right lung volume, interstitial collagen volume (cm^3) was significantly increased in all groups of fetuses that underwent tracheal obstruction (*Vehicle & 5dTO*: 4.3 ± 0.5 cm^3; *LD LACA & 5dTO*: 3.7 ± 0.4 cm^3 and *HD LACA & 5dTO*: 3.5 ± 0.3 cm^3) in comparison to fetuses without tracheal obstruction (*Vehicle-only*: 2.0 ± 0.3 and *HD LACA-only*: 1.4 ± 0.1 cm^3; Figure 5.9: lower panel). When corrected for right lung volume, the interstitial collagen volume for *HD LACA-only* fetuses was not different from that of the *Vehicle-only* group. As expected, the increases in interstitial collagen volume were similar to the increases observed in total lung collagen (hydroxyproline) content (Figure 5.7: lower panel).

5.3.7 Percentage Change in Hydroxyproline Content Following Tracheal Obstruction

Using the data on hydroxyproline content obtained in this study for *Vehicle-only* and *Vehicle & 5dTO* fetuses, in combination with data previously obtained from our laboratory (Keramidaris *et al.*, 1996; Nardo *et al.*, 1998), the time course of the percentage increase in hydroxyproline content was determined over 2, 4, 5 and 10 days following tracheal obstruction (Figure 5.10: upper panel). A significant increase in hydroxyproline content, above control values, occurs after 5 days of tracheal obstruction.

The percentage increase in collagen was then correlated to the increase in DNA content, again using data from this study and with data previously obtained from our laboratory (Keramidaris *et al.*, 1996; Nardo *et al.*, 1998). Linear regression analysis showed that the percentage increase in hydroxyproline content following tracheal obstruction is significantly related to the percentage increase in lung DNA content ($r = 0.955$, $n = 4$, $P < 0.05$; Figure 5.10: lower panel).

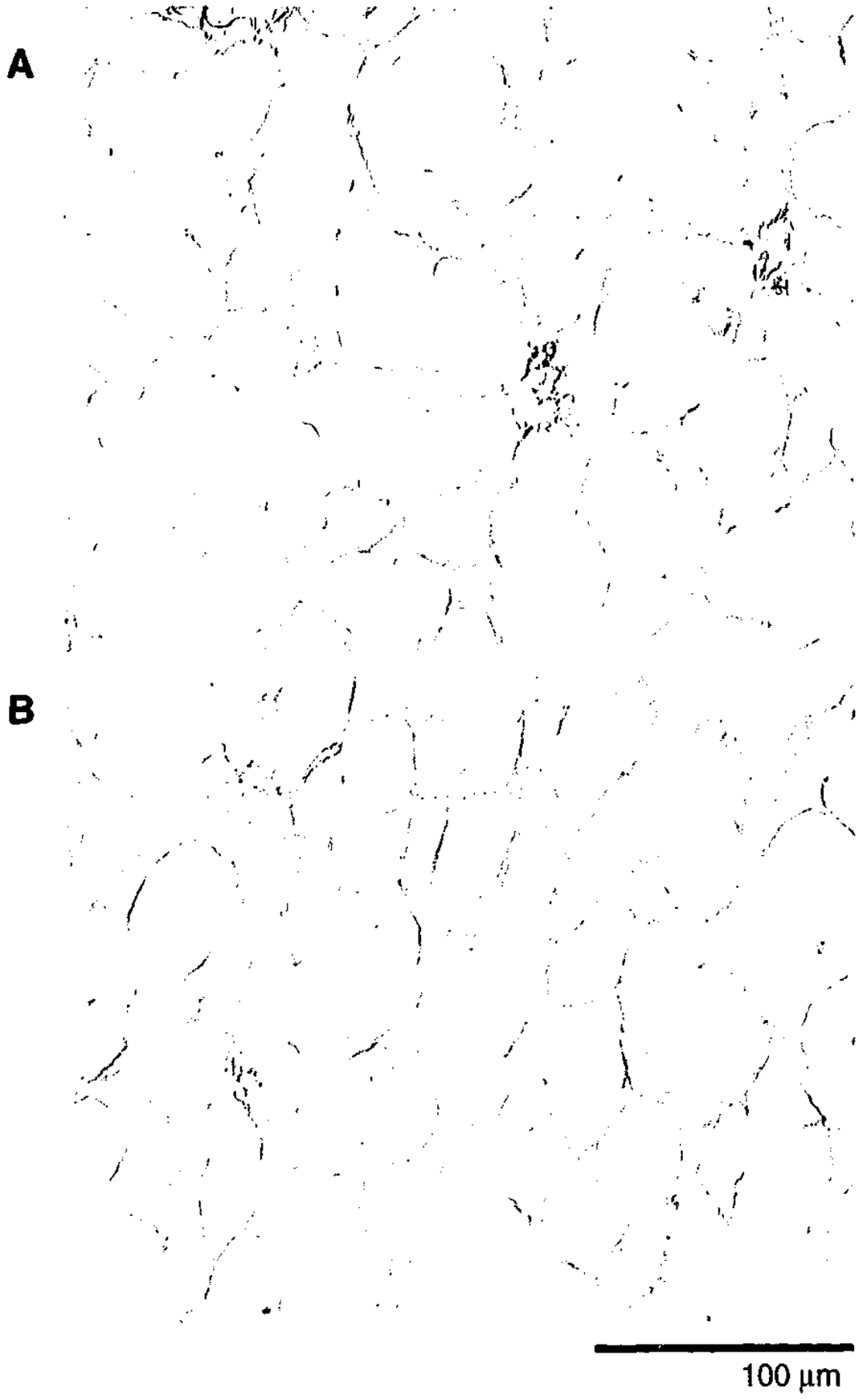

Figure 5.8 Histological appearance of Picrosirius Red stain in fetal lung

Tissue sections as seen under the light microscope from a ***A)*** *Vehicle & 5dTO* and a ***B)*** *HD LACA & 5dTO* fetus at 130.2 ± 0.1 days of gestation. The sections are 5 µm thick and have been stained with Picrosirius Red. Collagen fibres are stained pink/red. The sections are viewed at a final magnification of X420. The black bar indicates the scale of both pictures.

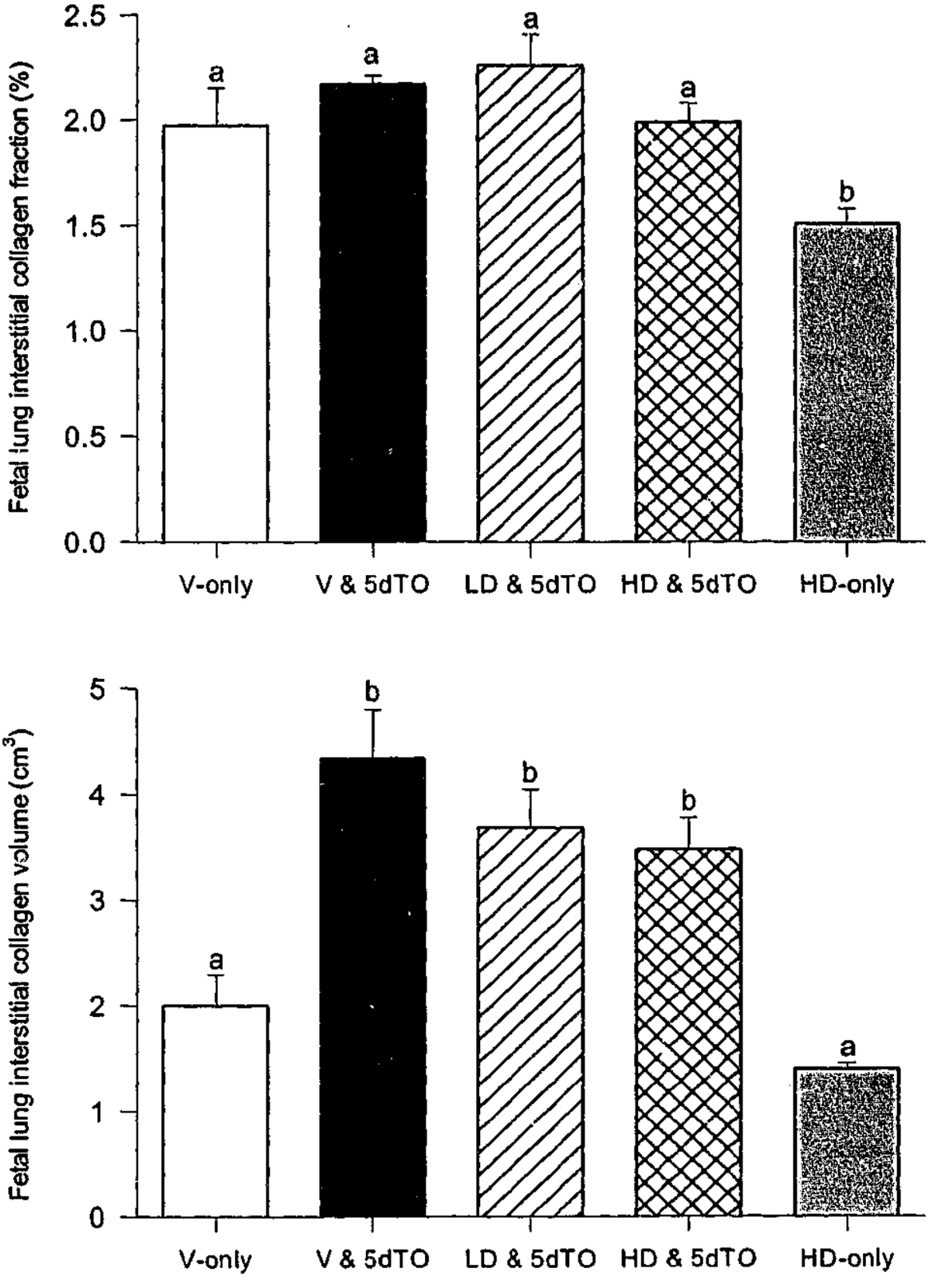

Figure 5.9 Fetal lung interstitial collagen fraction and interstitial collagen volume

Upper panel: mean fetal lung interstitial collagen fraction ± SEM (%) and ***lower panel***: mean fetal lung interstitial collagen volume ± SEM (cm^3) for *Vehicle-only* (n = 5), *Vehicle & 5dTO* (n = 5), *LD LACA & 5dTO* (n = 5), *HD LACA & 5dTO* (n = 5) and *HD LACA-only* (n = 5) fetuses (130.2 ± 0.1 days GA). Values that do not share a common letter are significantly different from each other ($P < 0.05$).

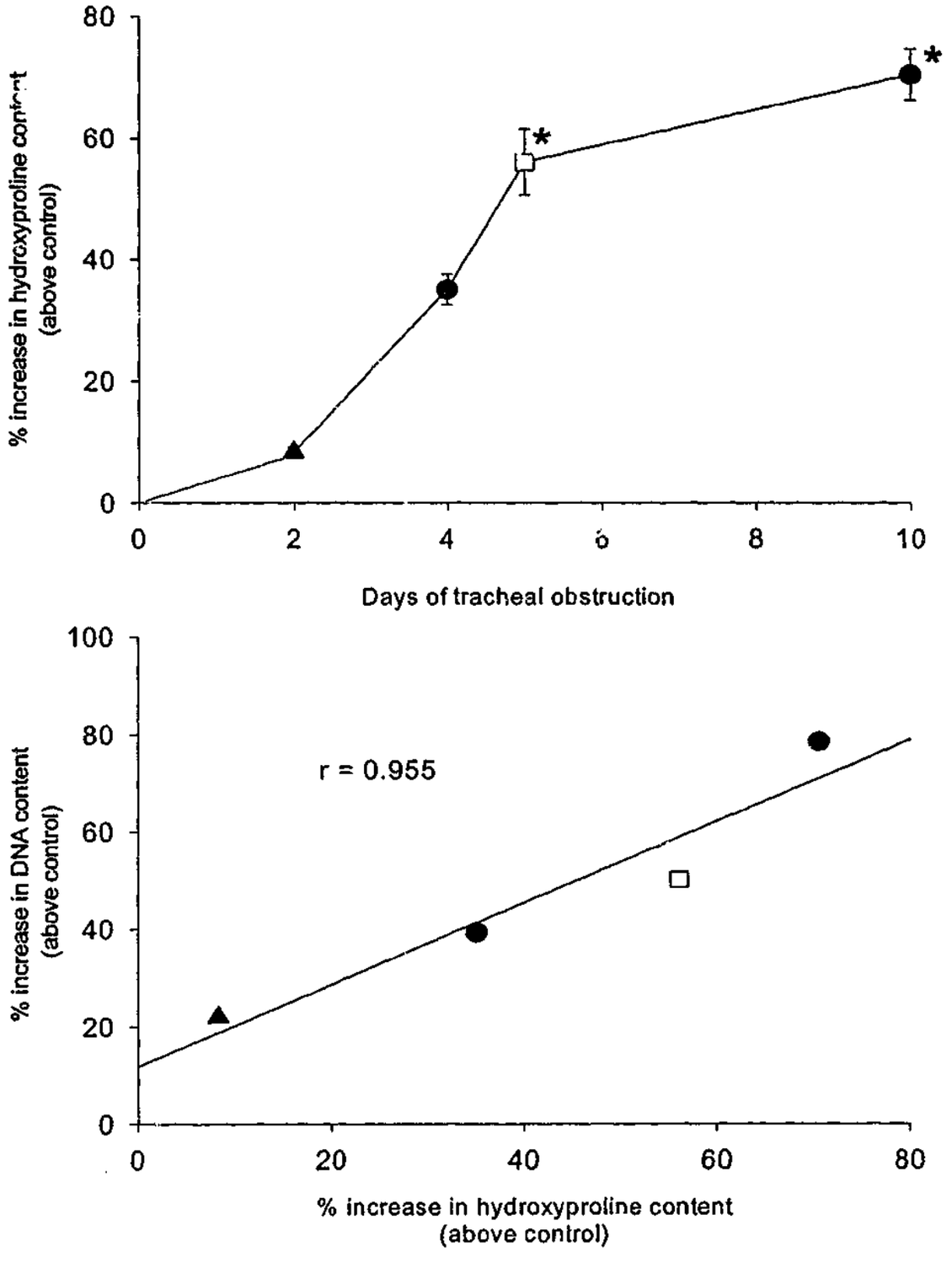

Figure 5.10 Percentage increase in fetal lung hydroxyproline content and relationship between fetal lung hydroxyproline and DNA contents following tracheal obstruction.

Upper panel: fetal lung hydroxyproline content as a percentage increase above control values after 2, 4, 5 and 10 days of tracheal obstruction. Where the increase in hydroxyproline content of the fetal lung is significantly greater ($P < 0.05$) than control values it is marked with an asterisk. ***Lower panel:*** the relationship between the percentage increase in DNA content and hydroxyproline content of the fetal lung following 2, 4, 5 and 10 days of tracheal obstruction. In both panels, data obtained from previous experiments in our laboratory is indicated by black circles (Nardo *et al.*, 1998) and black triangles (Keramidaris *et al.*, 1996) and data obtained from this study is indicated by a white square. The increase in DNA content is correlated to the increase in hydroxyproline content ($r = 0.955$, $P < 0.05$).

5.4 Discussion

The aim of the present study was to examine the role of collagen in the lung growth response to tracheal obstruction by inhibiting collagen synthesis with LACA during tracheal obstruction. Collagen content increases in response to an increase in fetal lung expansion, induced by tracheal obstruction and we hypothesised that inhibiting this increase in collagen would prevent or reduce the normal increase in fetal lung growth that occurs following tracheal obstruction. In this study, I have shown that the increase in collagen content following tracheal obstruction occurs in parallel with the increase in DNA content. Furthermore, the percentage increase in fetal lung collagen content is most rapid between 2 and 5 days of tracheal obstruction, when extracellular remodelling was hypothesised to play an important role in allowing further increases in lung growth to occur. Five days of tracheal obstruction induced a significant increase in collagen content in the fetal lung, in the presence or absence of the proline analogue, LACA. We initially tried a low dose of LACA, but preliminary analysis showed no effect, so the dose was increased 5-fold. Surprisingly, the infusion of LACA, at both doses, did not prevent or reduce the rapid accumulation of collagen following tracheal obstruction in fetal sheep, as measured by total hydroxyproline content and histological analysis of interstitial collagen in the lung.

5.4.1 LACA Treatment and Fetal Wellbeing

A dose of LACA thought likely to prevent collagen synthesis in the fetal sheep lung was chosen, based on previous *in vivo* studies in which LACA was used to study the effects of disrupting collagen synthesis on lung development in rats (Adamson & King, 1987; Adamson & King, 1988). This ***low*** dose of LACA (50 mg/day) and a dose 5-times higher, were given intravenously to fetal sheep for a period of 5 days. These doses of LACA were not detrimental to the health of the fetuses as determined by blood gas and acid-base status. Likewise, fetal body weight and organ weights were not adversely affected by the treatment. However, in one of the studies by Adamson and King, the dose of LACA injected was chosen as it only caused "limited deaths" of maternal rats (Adamson & King, 1988). Thus, it is likely that there were significant maternal health problems associated with high dose maternal treatments with LACA, however, these were not documented (Adamson & King, 1987; Adamson & King, 1988).

5.4.2 Lung Liquid Volume

Lung liquid volume, which determines the degree of fetal lung expansion, plays an important role in normal growth and development of the fetal lungs. In response to tracheal obstruction, lung liquid volume increases with time, expanding the lungs and stimulating lung growth. Following 1 day of tracheal obstruction, lung liquid volume doubles. Between 1 and 2 days of tracheal obstruction, lung liquid volume is unchanged, but increases linearly between 2 and 7 days of tracheal obstruction. We hypothesised that initially, the lung expands to a limit imposed by its own structural framework and that the increases in lung liquid volumes that occur between 2 and 7 days of tracheal obstruction are due to structural remodelling of the lung, and in particular, by remodelling of collagen. In the period between 2 and 7 days of tracheal obstruction in late gestation fetal sheep, the increase in DNA content of the lung is highly correlated to this increase in lung liquid volume (Nardo *et al.*, 1998). Thus, we hypothesised that inhibiting an increase in lung collagen following tracheal obstruction would prevent this increase in lung liquid volume and, therefore, lung growth. In the present study, lung liquid volumes were more than doubled after 5 days of tracheal obstruction in fetuses, relative to fetuses without an obstructed trachea. This increase in lung liquid volume closely paralleled the increase in DNA content and further confirms the very strong relationship between lung liquid volume and lung growth. It also highlights that lung liquid volume can be used to predict the degree of lung growth that occurs. However, this increase in lung liquid volume following tracheal obstruction was not altered by infusion of LACA. Although, the *LD LACA & 5dTO* group of fetuses had a lower lung liquid volume than that of the *Vehicle & 5dTO* group of fetuses in absolute terms, when corrected for fetal body weight it was not different from the *Vehicle & 5dTO* group of fetuses. It is likely, therefore, that the reduction in lung liquid volume in these fetuses was due to a lower fetal body weight, although not significant, rather than due to the effects of treatment with LACA.

5.4.3 Biochemical and Morphological Estimates of Collagen Content

Collagen content was assessed by measuring the hydroxyproline content of the lung tissue and by morphological assessment of tissue sections, with a dye that specifically stains collagen fibres. In the present study, 5 days of tracheal obstruction increased the total collagen of the lungs by ~55% above levels in control fetuses. Other studies from our laboratory have shown that fetal lung collagen content is increased by ~8% at

2 days of TO (not significant), by ~35% at 4 days of TO (not significant) and by ~70% by 10 days of tracheal obstruction (Keramidaris *et al.*, 1996; Nardo *et al.*, 1998). Together, these results indicate that there is a time-dependent increase in collagen content in the lung following tracheal obstruction and I have shown that this increase occurs in parallel with the increase in DNA content. The greatest increase in hydroxyproline content occurred between 2 and 5 days of tracheal obstruction when we believe ECM remodelling plays an important role in allowing further increases in lung growth to occur. The increase in collagen content may be due to a decrease in collagen breakdown or to an increase in collagen synthesis. However, results from the previous chapter indicate that gelatinase levels tended to be reduced following 2 days of tracheal obstruction only and were similar to that of control fetuses at 4 and 10 days of tracheal obstruction. Therefore, the large increases in collagen content that we have seen following TO are not likely to be the result of alterations in collagen turnover. An increase in collagen synthesis following tracheal obstruction may reflect an increasing number of collagen-producing cells or an increase in collagen synthesis rates per cell. Recently, a study by Nardo *et al.* (2000) has shown that fibroblasts, as well as type II AECs are among the cells that undergo division following tracheal obstruction. Given that both fibroblasts (Laurent *et al.*, 1988) and type II AECs (Simon *et al.*, 1993) secrete collagen, the increase in collagen content following tracheal obstruction may be, in part, due to an increase in the number of collagen-producing cells. *In vitro* studies have shown that stretch induces increases in collagen expression in organotypic fetal lung cell cultures (Xu *et al.*, 1999) and in fetal lung fibroblasts (Breen, 2000). Increased expression of tissue growth factors such as TGF-β (Lines *et al.*, 2000; Wallace *et al.*, 2002) and IGF-II (Hooper *et al.*, 1993b; Wallace *et al.*, 2002) occurs following periods of tracheal obstruction. This finding is important as TGF-β is known to increase the expression of procollagen (Raghu *et al.*, 1989). IGF-II is a potent mitogen and is elevated following 7 and 10 days of tracheal obstruction and is believed to play a role in remodelling of the lung ECM (Hooper *et al.*, 1993b; Wallace *et al.*, 2002). Thus, the increase in collagen content in the lung following tracheal obstruction may be due to a number of factors; tissue stretch may act to increase division of collagen producing cells, act directly to increase collagen synthesis per cell, or it may increase the synthesis of growth factors that specifically affect collagen production.

By preventing secretion of collagen from the cell, LACA should prevent the formation of new collagen fibrils (Lane *et al.*, 1971; Switzer & Summer, 1973). Given the high turnover of collagen in the lung during late fetal life, (Bradley *et al.*, 1974), an infusion of LACA should have rapid effects in altering the ECM. Indeed, Adamson and King

(1987 & 1988) have shown that lung growth parameters as well as lung collagen content are reduced within 2 days of treatment with LACA in fetal rats. Interstitial collagen fraction was reduced in *HD LACA-only* fetuses when compared to all other groups of fetuses, although lung collagen content was not altered from control levels in these fetuses. The latter finding is not surprising because it would take some time before a reduction in total lung collagen content, as a result of a reduction in collagen synthesis, could be detected due to the relatively high level of collagen pre-existing in the lung. We also found no difference in the interstitial collagen fraction in the tracheal obstruction groups of fetuses, regardless of saline or LACA infusion. Similarly, there was no difference in lung collagen content between the groups of fetuses that had their tracheas obstructed. These results indicate that collagen synthesis in fetuses with normally expanded lungs was inhibited by LACA, but that LACA was not capable of inhibiting collagen synthesis in the lung following a period of tracheal obstruction. This finding implies that tracheal obstruction is a potent stimulus for collagen synthesis and is not easily inhibited. The hydroxyproline assay measures all hydroxylated proline residues present in a sample and thus may be detecting other proteins such as elastin, which is also elevated by tracheal obstruction (Joyce *et al.*, unpublished observation). In addition, collagen is found in many parts of the lung, including areas that do not play a major role in the structural framework of the lung that limits its expansion. Thus, we measured the interstitial collagen fraction and volume by morphometric means, which was considered a more direct measurement of these structural components compared with total lung hydroxyproline content. Thus, the change in interstitial collagen fraction observed in the *HD LACA-only* group of fetuses, is considered a more sensitive measure of the change in lung collagen associated with the structural framework of the lung.

The observed increase in collagen content is likely to result from an increase in collagen synthesis rates following tracheal obstruction. In addition, an increase in collagen synthesis rates would require a greater dose of LACA for synthesis inhibition to occur. The increase in collagen synthesis rates may be due to an increase in collagen-producing cells following TO, thus leading to a total collagen synthesis rate that is far greater than the doses of LACA used can prevent. Given the nature of these results, it is interesting to postulate future directions of research to enhance our understanding of the role of collagen in the growth response to tracheal obstruction. Administering a higher dose of LACA would appear to be an obvious choice, however, the cost of such an exercise is prohibitive, as quantities of LACA required even for *in vitro* research are high. Similarly, measuring collagen synthesis rates *in vivo* is highly

expensive. Consequently, other alternatives must be considered. LACA could be administered directly into the pulmonary circulation or into the lung liquid of the fetus, which would allow smaller doses to be utilised. However, as previous studies have shown, results can differ between substances delivered in this manner and systemically (Cassin & Perks, 1982; Lumbers *et al.*, 1985). The precise involvement of collagen synthesis in the lung growth response to tracheal obstruction, therefore, remains undetermined and is very difficult to assess. However, it is likely that the increase in collagen content following tracheal obstruction is due to an increase above control levels in collagen synthesis rates.

5.4.4 Biochemical Estimates of Lung Growth

Previously, Nardo *et al.* (1998) have shown that the increase in lung DNA content following a period of tracheal obstruction is highly correlated to the increase in lung liquid volume ($r = 0.999$). In the present study, fetal lung weight, lung DNA and protein concentrations and contents were measured to assess the effects of LACA treatment on the lung growth response to tracheal obstruction. Lung weight, lung DNA and protein contents all increased following tracheal obstruction, regardless of whether LACA or saline was infused. The data obtained in the present study confirms the previous results of Nardo *et al.* (1998). That is, that increases in lung liquid volume are believed to be the principal determinant of the lung growth response to tracheal obstruction. As a result, the growth rate may be altered by changing the degree of lung expansion with lung liquid (Moessinger *et al.*, 1990; Boland *et al.*, 1997; Nardo *et al.*, 1998). Similar to the lack of effect of LACA on lung growth in tracheal obstructed fetuses, LACA infusion in fetuses without tracheal obstruction also had no effects on lung growth relative to the vehicle controls.

Previously, fetal exposure *via* maternal administration of LACA has been shown to reduce lung growth in fetal and postnatal rats (Adamson & King, 1987; Adamson & King, 1988). In those studies, 4 groups of rats were treated with LACA over a period of 2 days (from day 17, day 18, day 19 and day 20 of gestation, where term in rats is 22 days of gestation) followed by one day without treatment before *post mortem*. When compared to control rats, LACA treated fetal rats of all ages showed reduced fetal lung weights, lung DNA content and lung collagen content. Blockade of collagen synthesis by LACA was, therefore, shown to have marked effects on a number of biochemical estimators of lung growth, although it is possible that at least some of these effects

may have been due to the effect of LACA on the mother. Although the experiments by Adamson and King were similar to the current study in terms of dose received and the fact that the animals were administered LACA *in vivo*, there are many differences between the studies. For example, route of administration of LACA by Adamson and King was *via* maternal intraperitoneal injection. The ability of LACA to cross the placenta is little understood. LACA may have direct effects on the mother and in particular on the placenta, thus the effects on lung growth could be secondary effects as a result of effects on placental growth or function or effects of LACA on other maternal organs (such as adrenal gland function). Indeed, intra-uterine growth retardation by placental embolisation has been shown to alter lung DNA concentration and to increase the thickness of the air-blood barrier (Harding *et al.*, 2000b). The dose used by Adamson and King was chosen as it only caused "limited deaths" of maternal rats (Adamson & King, 1988). This suggests that higher doses than this may have severe effects on maternal health and may indicate that the intraperitoneal dose may exert its effects on the fetus *via* effects on the mother first. It is surprising then that maternal health was not described in that study (Adamson & King, 1987 &1988). As LACA is incorporated into other proline-containing molecules, a dose of LACA may have affected other proteins in the mother or fetus as it reduces general protein synthesis (Alescio, 1973; Spooner & Faubion, 1980). Another significant difference is the time period in which LACA was administered. In my study LACA was administered for 5 days, covering only a portion of the early alveolar stage of lung development (Table 1.1). In contrast, a 2 day treatment with LACA in rats spans entire stages of lung development (canalicular or saccular stage depending on time of injections; Adamson & King, 1987).

5.4.5 Conclusions

Tracheal obstruction is a potent stimulus for fetal lung growth and net collagen accumulation. The increase in collagen content following tracheal obstruction was greatest between 2 and 5 days when structural remodelling of the lung is believed to allow further increases in lung growth to occur. Furthermore, the increase in collagen content was correlated to the increase in DNA content. The increase in lung liquid volume following tracheal obstruction appeared to parallel this increase in DNA content and again highlights the importance of the relationship between these two factors. Also, it emphasises that an increase in lung liquid volume may be used to predict lung growth following tracheal obstruction. LACA infusion at the doses investigated was not able to prevent the rapid increases in collagen accumulation, lung liquid volume or lung

growth that occur following tracheal obstruction. However, a period of LACA infusion reduced the interstitial collagen fraction in normal lungs. Thus, collagen synthesis rates in normal fetal sheep lung in late gestation may be inhibited by LACA infusion, implying that collagen synthesis rates during a period of tracheal obstruction are greatly elevated. Collagen accumulation following tracheal obstruction was not easily prevented in fetal sheep at this gestational age and the underlying mechanism behind this collagen accumulation and its role in the lung growth response to tracheal obstruction remains are yet to be determined.

CHAPTER 6 General Discussion

The adequate growth and development of the lung *in utero* is critical for the survival of the neonate at birth and is dependent upon many factors, the most important being the presence of an appropriate volume of lung liquid. Alterations in lung liquid volume greatly affect both the growth and the structural maturation of the lung. Structural maturation of the lungs involves the development of the branches of the lungs to the level of alveoli and an appropriate number of alveoli, preparation of a thin blood-air barrier, development of a capillary network and differentiated alveolar epithelial cells. Structural maturation also entails the development of elastin and collagen fibrils, which give the lungs their compliant properties and which also form the architectural 'backbone' of the lungs. This thesis was primarily concerned with the effects of lung expansion and corticosteroids on lung structure, particularly in relation to the control of the ECM protein collagen.

Fetal lung hypoplasia in humans can result in severe respiratory insufficiency and is potentially lethal (Nakayama *et al.*, 1985; Adzick *et al.*, 1989), highlighting the need for the lungs to develop appropriately *in utero* and the importance of studying the mechanisms that cause and potentially reverse the underlying growth deficit. A number of studies have examined treatments to correct instances of pre-existing pulmonary hypoplasia both in human fetuses and in animal models (Nardo *et al.*, 1995; Flake, 1996; Harrison *et al.*, 1996; Graf *et al.*, 1997; Davey *et al.*, 1999). Research from our laboratory has shown that TO is a potent stimulus for lung growth and structural maturation of the lungs, reversing pulmonary hypoplasia and improving neonatal survival (Nardo *et al.*, 1995; Davey *et al.*, 1999). However, TO has been shown to have detrimental effects on the lung, altering the proportions of AECs (Alcorn *et al.*, 1977; Bin Saddiq *et al.*, 1997; Piedboeuf *et al.*, 1997; De Paepe *et al.*, 1998; Flageole *et al.*, 1998; Flecknoe *et al.*, 2000) and retarding the production of surfactant proteins (Piedboeuf *et al.*, 1997; Benachi *et al.*, 1999; Lines *et al.*, 1999). The therapeutic use of TO in human infants is being trialled with limited success due to

complications associated with *in utero* surgery (Harrison *et al.*, 1996). Coupled with the deleterious effects of TO outlined above, the search for less invasive and aggressive treatments is underway.

Two studies using a model of lung hypoplasia have compared the effects of cortisol treatment to both tracheal ligation and intra-amniotic surfactant treatment (Tannuri *et al.*, 1998a; Tannuri *et al.*, 1998b). Whilst tracheal ligation rapidly reversed the lung growth deficit associated with experimental diaphragmatic hernia in rabbits, surfactant and cortisol treatments were unable to reverse this growth deficit (Tannuri *et al.*, 1998a; Tannuri *et al.*, 1998b). Despite this finding, it was suggested that fetal corticosteroids and/or surfactant treatment may act as a substitute for tracheal ligation (Tannuri *et al.*, 1998a; Tannuri *et al.*, 1998b). The experiments in this thesis have shown that doses of cortisol that do not adversely affect fetal growth or health, advance structural maturity of the lung, but do not alter lung growth in normal or hypoplastic fetal lungs. Thus, it is unlikely that cortisol treatment alone would reverse the growth deficit associated with pulmonary hypoplasia in CDH fetuses. Although there are significant structural changes in the hypoplastic lungs treated with cortisol, the size and surface area of these lungs is probably still too small to support life after birth. Therefore, the benefit of using corticosteroids to treat pulmonary hypoplasia is more likely to arise from their use in combination with a treatment such as TO, where cortisol and an increase in lung expansion together can accelerate the increase in lung liquid volume and thus stimulate greater lung growth (Boland *et al.*, 1997). Recent studies have examined the effect of a period of tracheal obstruction, followed by release of tracheal obstruction and corticosteroids on reversing lung growth deficits (Bratu *et al.*, 2001a; Bratu *et al.*, 2001b). This regime improved lung morphology, however, the surfactant profile of these fetuses remained below the levels of control fetuses (Bratu *et al.*, 2001b). In another study the re-expression of the surfactant proteins following the release of tracheal obstruction depended upon the state of lung inflation which was very variable between fetuses (Lines *et al.*, 2001). In normal lungs, a period of tracheal obstruction, followed by release of tracheal obstruction and treatment with corticosteroids have additive effects, returning type II AEC density to near control levels (Kay *et al.*, 2001). Further research is required in this area and care needs to be taken to administer doses of cortisol that will not have detrimental effects on lung and body growth

A study by Boland *et al.* (1997) showed that lung liquid volume following tracheal obstruction was increased by a period of cortisol pretreatment. The authors suggested

that structural maturation of the lung, induced by corticosteroids, resulted in a more compliant lung that was able to expand more easily, allowing a greater volume of lung liquid to occupy the lung lumen and thus inducing greater lung growth (Boland *et al.*, 1997). As an increase in lung volume on its own can also induce acceleration in fetal lung growth and structural development, I hypothesised that structural alterations in the developing lung, brought about by exogenous or endogenous cortisol, may be due to cortisol-induced alterations in lung expansion. The effects of cortisol treatment on lung growth and structural maturation in both normal and hypoplastic lungs were examined. The results presented in this thesis (Chapter 3) show that cortisol improves many aspects of structural maturity regardless of the degree of lung expansion, but that lung growth is not affected by cortisol. The lack of any increase in lung growth, regardless of the improvements in lung maturation, may mean that these lungs are still unable to support life after birth. Thus, further experiments are needed to assess whether this protocol would be sufficient to prevent respiratory insufficiency at birth, or indeed prevent neonatal death. Future studies could include the following of a group of fetuses with hypoplastic lungs and treated with corticosteroids through to birth and assessing neonatal health, survival and respiratory parameters in the first weeks of life. Such a study may give us a clearer understanding of whether the size of the lungs or the maturity of the lungs are more important in determining survival at birth following pulmonary hypoplasia *in utero*.

Previous experiments (Nardo *et al.*, 1995) and results from Chapter 3 have shown that lung collagen content is reduced, when compared to control levels, following a period of lung liquid drainage. On the other hand, lung collagen content is increased following a period of lung expansion (Nardo *et al.*, 1998). To assess the degree of collagen turnover in these alterations in lung collagen content, the presence of the gelatinases, MMP-2 and MMP-9, was quantitated. Importantly, alterations in gelatinase levels may indicate changes in collagen turnover and, therefore, ECM remodelling. MMP-2 levels were increased following a period of lung deflation. A transient decrease in MMP-2 levels was observed following 2 days of TO, but no other changes were found in MMP-2 levels following longer periods of lung expansion. I have shown that the levels of the gelatinases are altered following changes in lung expansion, however much remains unknown about the role of ECM remodelling in the lung growth response to changes in lung expansion. Thus, there are many avenues for future research into lung collagen synthesis and turnover and their involvement in the growth response to changes in lung expansion. Other MMPs and enzymes are involved in collagen and ECM turnover and, in fact, in the regulation of the gelatinases themselves. Techniques exist to measure

these other MMPs and enzymes involved in collagen turnover. Collagen synthesis rates could be measured directly and investigation of the role of other ECM proteins that are likely to mediate changes in lung stretch and lung growth, such as elastin and the proteoglycans could be conducted.

Collagen content is differentially altered following changes in fetal lung expansion and is related to the degree of change in lung growth that occurs (Chapter 3, Chapter 5). Specifically, an increase in lung liquid volume accelerates lung growth and increases the lung collagen content in late gestation in fetal sheep. Conversely, lung liquid drainage retards the natural, age-related increase in lung collagen. I hypothesised that the increase in collagen content during tracheal obstruction played an important role in the lung growth response and that inhibition of synthesis of new collagen would prevent the increase in lung collagen content and have profound effects on lung growth. LACA, a proline analogue that prevents collagen secretion from the cell, did not affect lung collagen content in the tracheal obstructed animals, but did reduce the interstitial collagen fraction in normal lungs. I have shown that, at the doses delivered, LACA could not prevent the marked increase in collagen synthesis rates following TO. Thr , there is still a need to determine the mechanisms that are responsible for the increase in lung collagen content following TO, and the precise role this increase in pulmonary collagen plays in the lung growth response to an increase in lung expansion. Undoubtedly, collagen synthesis is a dynamic part of lung growth in the fetus, with high levels of *de novo* synthesis and turnover of collagen towards the end of gestation (Tolstoshev *et al.*, 1981; Mays *et al.*, 1989; Jackson *et al.*, 1990). Measurement of collagen synthesis rates needs to be conducted to ascertain the effects of changes in lung expansion on this ECM protein. Additional studies could be conducted that study the effects of disrupting existing collagen fibrils. However, as simply disrupting the existing collagen fibres may induce an increase in collagen synthesis to replace these fibres, the protocol could be conducted in conjunction with a modified LACA treatment (*i.e.* delivery *via* the lung lumen), to both disrupt existing collagen and prevent the secretion of new collagen. In this manner, we would be able to more accurately assess the role of collagen in normal lung development *in utero* and in response to changes in lung expansion.

The experiments in this thesis have shown that lung structural maturity can be advanced by treatment with physiological doses of cortisol, regardless of the degree of lung expansion. These improvements in lung structure occur without any alterations in

lung growth. Thus, cortisol treatment alone enhances structural maturity, but it is unlikely to reverse pre-existing lung growth deficits *in utero*. Structural maturity of the lung is also indicated by the amount of collagen contained within the lung, with lung collagen content being differentially regulated by changes in lung expansion. Lung collagen content increases following a period of lung expansion and decreases following a period of lung deflation. The results from this thesis suggest that the mechanisms that lead to these alterations in collagen content are likely to be different. An increase in the rate of *de novo* synthesis of collagen is believed to accelerate collagen accumulation following tracheal obstruction, whereas an increase in collagen turnover is likely to result in the reduced collagen content following a period of lung deflation. Thus, the experiments performed in this thesis, in conjunction with the postulated future experiments, will contribute to our understanding of the role that the ECM plays in the structural maturation of the lung, during retarded, normal and expanded lung development.

Reference List

Abbott, L. A., Lester, S. M., & Erickson, C. A. (1991). Changes in mesenchymal cell-shape, matrix collagen and tenascin accompany bud formation in the early chick lung. *Anatomy and Embryology* **183**, 299-311.

Adams, F. H., Deislets, D. T., & Towers, B. (1967). Control of flow of fetal lung liquid at the laryngeal outlet. *Respiration Physiology* **2**, 302-309.

Adams, F. H., Moss, A. J., & Fagan, L. (1963). The tracheal fluid in the fetal lamb. *Biology of the Neonate* **5**, 151-158.

Adamson, I. Y. R. & King, G. M. (1987). L-Azetidine-2-carboxylic acid retards lung growth and surfactant synthesis in fetal rats. *Laboratory Investigation* **57**, 439-445.

Adamson, I. Y. R. & King, G. M. (1988). Postnatal development of rat lung following retarded fetal lung growth. *Pediatric Pulmonology* **4**, 230-236.

Adamson, T. M., Boyd, R. D. H., Platt, H. S., & Strang, L. B. (1969). Composition of alveolar liquid in the foetal lamb. *Journal of Physiology* **204**, 159-168.

Adamson, T. M., Brodecky, V., Lambert, T. F., Maloney, J. E., Ritchie, B. C., & Walker, A. M. (1975). Lung liquid production and composition in the "*in utero*" foetal lamb. *Australian Journal of Experimental Biology and Medical Science* **53**, 65-75.

Adkisson, V. T. & Callas, G. (1982). Morphometric changes in the lungs of rats following adrenalectomy supplemented by desiccated thyroid. *Anatomy and Embryology* **203**, 147-156.

Adzick, N. S., Harrison, M. R., Flake, A. W., Howell, L. J., Golbus, M. S., & Filly, R. A. (1993). Fetal surgery for cystic adenomatoid malformation of the lung. *Journal of Pediatric Surgery* **28**, 806-812.

Adzick, N. S., Harrison, M. R., Glick, P. L., Nakayama, D. K., Manning, F. A., & deLorimier, A. A. (1985). Diaphragmatic hernia in the fetus: prenatal diagnosis and outcome in 94 cases. *Journal of Pediatric Surgery* **20**, 357-361.

Adzick, N. S., Vacanti, J. P., Lillehei, C. W., O'Rourke, P. P., Crone, R. K., & Wilson, J. M. (1989). Fetal diaphragmatic hernia: Ultrasound diagnosis and clinical outcome in 38 cases. *Journal of Pediatric Surgery* **24**, 654-658.

Aggeler, J., Frisch, S. M., & Werb, Z. (1984). Changes in cell shape correlate with collagenase gene expression in rabbit synovial fibroblasts. *Journal of Cell Biology* **98**, 1662-1671.

Akiyama, S. K., Nagata, K., & Yamada, K. M. (1990). Cell surface receptors for extracellular matrix components. *Biochimica et Biophysica Acta* **1031**, 91-110.

Albelda, S. M. & Buck, C. A. (1990). Integrins and other cell adhesion molecules. *FASEB Journal* **4**, 2868-2880.

Alcorn, D. G., Adamson, T. M., Lambert, T. F., Maloney, J. E., Ritchie, B. C., & Robinson, P. M. (1977). Morphological effects of chronic tracheal ligation and drainage in the fetal lamb lung. *Journal of Anatomy* **123**, 649-660.

Alcorn, D. G., Adamson, T. M., Maloney, J. E., & Robinson, P. M. (1980). Morphological effects of chronic bilateral phrenectomy or vagotomy in the fetal lamb lung. *Journal of Anatomy* **130**, 683-695.

Alcorn, D. G., Adamson, T. M., Maloney, J. E., & Robinson, P. M. (1981). A morphologic and morphometric analysis of fetal lung development in the sheep. *The Anatomical Record* **201**, 655-667.

Alescio, T. (1973). Effect of proline analogue, azetidine-2-carboxylic acid, on the morphogenesis *in vitro* of mouse embryonic lung. *Journal of Embryology and Experimental Morphology* **29**, 439-451.

Alescio, T. & Cassini, A. (1962). Induction *in vitro* of tracheal buds by pulmonary mesenchyme grafted on tracheal epithelium. *Journal of Experimental Zoology* **150**, 83-94.

Alexander, C. M., & Werb, Z. (1991). Extracellular matrix degradation. In *Cell Biology of the Extracellular Matrix*, ed. Hay, E. D., pp. 255-302.. Plenum Press, New York.

Alfonso, L. F., Vilanova, J., Aldazabal, P., Lopez, d. T., & Tovar, J. A. (1993). Lung growth and maturation in the rat model of experimentally induced congenital diaphragmatic hernia. *European Journal of Pediatric Surgery* **3**, 6-11.

Anceschi, M. M., Palmerini, C. A., Codini, M., Luzi, P., & Cosmi, E. V. (1992). Collagen and elastin in rabbit fetal lung: ontogeny and effects of steroids. *Journal of Developmental Physiology* **18**, 233-236.

Anderson, G. D., Ahokas, R. A, Lipshitz, J. & Dilts, P. V. (1980). Effect of maternal dietary restriction during pregnancy on maternal weight gain and fetal birth rate in the rat. *Journal of Nutrition* **110**, 883-890.

Angel, R. C., Botta, J. A., & Farias, R. N. (1987). Modification of L-triiodothyronine binding sites from rat erythrocyte membrane by heating and by proteinase treatments. *Biochimica et Biophysica Acta* **897**, 488-494.

Arden, M. G. & Adamson, I. Y. R. (1992). Collagen degradation during postnatal lung growth in rats. *Pediatric Pulmonology* **14**, 95-101.

Arden, M. G., Spearman, M. A., & Adamson, I. Y. R. (1993). Degradation of type IV collagen during the development of fetal rat lung. *American Journal of Respiratory Cell and Molecular Biology* **9**, 99-105.

Asabe, K., Tsuji, K., Handa, N., Kurosaka, N., & Kajiwara, M. (1997). Immunohistochemical distribution of surfactant apoprotein-A in congenital diaphragmatic hernia. *Journal of Pediatric Surgery* **32**, 667-672.

Ballard, P. L. (1986). Hormones and lung maturation. *Monographs on Endocrinology* **28**, 1-354.

Ballard, P. L. & Ballard, R. A. (1974). Cytoplasmic receptor for glucocorticoids in lung of the human fetus and neonate. *Journal of Clinical Investigation* **53**, 477-486.

Ballard, R. A. & Ballard, P. L. (1976). Use of prenatal glucocorticoid therapy to prevent respiratory distress syndrome. A supporting view. *American Journal of Diseases of Children* **130**, 982-987.

Barker, D. J., Osmond, C., Simmonds, S. J., & Wield, G. A. (1993). The relation of small head circumference and thinness at birth to death from cardiovascular disease in adult life. *British Medical Journal* **306**, 422-426.

Barker, P. M., Brown, M. J., Ramsden, C. A., Strang, L. B., & Walters, D. V. (1988). The effect of thyroidectomy in the fetal sheep on lung liquid reabsorption induced by adrenaline or cyclic AMP. *Journal of Physiology* **407**, 373-383.

Barker, P. M., Walters, D. V., Markiewicz, M., & Strang, L. B. (1991). Development of the lung liquid reabsorptive mechanism in fetal sheep: synergism of triiodothyronine and hydrocortisone. *Journal of Physiology* **433**, 435-449.

Bassett, J. M. & Thorburn, G. D. (1969). Foetal plasma corticosteroids and the initiation of parturition in sheep. *Journal of Endocrinology* **44**, 285-286.

Beals, D. A., Schloo, B. L., Vacanti, J. P., Reid, L. M., & Wilson, J. M. (1992). Pulmonary growth and remodeling in infants with high-risk congenital diaphragmatic hernia. *Journal of Pediatric Surgery* **27**, 997-1001.

Beck, J. C., Mitzner, W., Johnson, J. W. C., Hutchins, G. M., Foidart, J.-M., London, W. T., Palmer, A. E., & Scott, R. (1981). Betamethasone and the rhesus fetus: effect on lung morphometry and connective tissue. *Pediatric Research* **15**, 235-240.

Beer, D. G., Cunha, G. R., & Malkinson, A. M. (1983). Methods in laboratory investigation. Autoradiographic demonstration of the specific binding and nuclear localization of 3H-dexamethasone in adult mouse lung. *Laboratory Investigation* **49**, 725-734.

Beitins, I. Z., Kowarski, A., Shermeta, D. W., De Lemos, R. A., & Migeon, C. J. (1970). Fetal and maternal secretion rate of cortisol in sheep: diffusion resistance of the placenta. *Pediatric Research* **4**, 129-134.

Benachi, A., Delezoide, A. L., Chailley-Heu, B., Preece, M., Bourbon, J. R., & Ryder, T. (1999). Ultrastructural evaluation of lung maturation in a sheep model of diaphragmatic hernia and tracheal occlusion. *American Journal of Respiratory Cell and Molecular Biology* **20**, 805-812.

Beranger, J. Y., Godeau, G., Frances, C., Robert, L., & Hornebeck, W. (1994). Presence of gelatinase A and metalloelastase type protease at the plasma membrane of human skin fibroblasts. Influence of cytokines and growth factors on cell-associated metalloendopeptidase levels. *Cell Biology International* **18**, 715-722.

Berger, P. J., Kyriakides, M. A., Smolich, J. J., Ramsden, C. A., & Walker, A. M. (1998). Massive decline in lung liquid before vaginal delivery at term in the fetal lamb. *American Journal of Obstetrics and Gynecology* **178**, 223-227.

Bernfield, M. & Sanderson, R. D. (1990). Syndecan, a developmentally regulated cell surface proteoglycan that binds extracellular matrix and growth factors. *Philosophical Transactions of the Royal Society (London Britain) for Biological Sciences* **327**, 171-186.

Bin Saddiq, W., Piedboeuf, B., Laberge, J. M., Gamache, M., Petrov, P., Hashim, E., Manika, A., Chen, M. F., Belanger, S., & Piuze, G. (1997). The effects of tracheal occlusion and release on type II pneumocytes in fetal lambs. *Journal of Pediatric Surgery* **32**, 834-838.

Birkedal-Hansen, H., Moore, W. G., Bodden, M. K., Windsor, L. J., Birkedal-Hansen, B., DeCarlo, A., & Engler, J. A. (1993). Matrix metalloproteinases: a review. *Critical Reviews in Oral Biology and Medicine* **4**, 197-250.

Bishop, J. E., Mitchell, J. J., Absher, P. M., Baldor, L., Geller, H. A., Woodcock-Mitchell, J., Hamblin, M. J., Vacek, P., & Low, R. B. (1993). Cyclic mechanical deformation stimulates human lung fibroblast proliferation and autocrine growth factor activity. *American Journal of Respiratory Cell and Molecular Biology* **9**, 126-133.

Bland, R. D., Bressack, M. A., & McMillan, D. D. (1979). Labor decreases the lung water content of newborn rabbits. *American Journal of Obstetrics and Gynecology* **135**, 364-367.

Bland, R. D. & Nielson, D. W. (1992). Developmental changes in lung epithelial ion transport and liquid movement. *Annual Review of Physiology* **54**, 373-394.

Bocking, A. D., McMillen, I. C., Harding, R., & Thorburn, G. D. (1986). Effect of reduced uterine blood flow on fetal and maternal cortisol. *Journal of Developmental Physiology* **8**, 237-245.

Bode, W., Fernandez-Catalan, C., Grams, F., Gomis-Ruth, F. X., Nagase, H., Tschesche, H., & Maskos, K. (1999). Insights into MMP-TIMP interactions. *Annals of the New York Academy of Sciences* **878**, 73-91.

Boland, R. E., Nardo, L., & Hooper, S. B. (1997). Cortisol pretreatment enhances the lung growth response to tracheal obstruction in fetal sheep. *American Journal of Physiology* **273**, L1126-L1131.

Bolender, R. P., Hyde, D. M., & Dehoff, R. T. (1993). Lung morphometry: a new generation of tools and experiments for organ, tissue, cell, and molecular biology. *American Journal of Physiology* **265**, L521-L548.

Boon, A. W., Milner, A. D., & Hopkin, I. E. (1981). Lung volumes and lung mechanics in babies born vaginally and by elective and emergency lower segmental cesarean section. *Journal of Pediatrics* **98**, 812-815.

Boshier, D. P., Holloway, H., Liggins, G. C., & Marshall, R. J. (1989). Morphometric analyses of the effects of thyrotrophin releasing hormone and cortisol on the lungs of fetal sheep. *Journal of Developmental Physiology* **12**, 49-54.

Boston, R. W., Humphreys, P. W., Normand, I. C. S., Reynolds, E. O. R., & Strang, L. B. (1968). Formation of lung liquid in the lungs of the foetal lamb. *Biology of the Neonate* **12**, 306-335.

Boyden, E. A. (1977). Development and growth of the airways. In *Development of the Lung*, ed. Hodson, W. A., pp. 3-35. Marcel Dekker, New York.

Brace, R. A., Wlodek, M. E., Cock, M. L., & Harding, R. (1994). Swallowing of lung liquid and amniotic fluid by the ovine fetus under normoxic and hypoxic conditions. *American Journal of Obstetrics and Gynecology* **171**, 764-770.

Bradley, K. H., McConnell, S. D., & Crystal, R. G. (1974). Lung collagen composition and synthesis. *The Journal of Biological Chemistry* **249**, 2674-2683.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.

Bratu, I., Flageole, H., Laberge, J. M., Chen, M. F., & Piedboeuf, B. (2001a). Pulmonary structural maturation and pulmonary artery remodeling after reversible fetal ovine tracheal occlusion in diaphragmatic hernia. *Journal of Pediatric Surgery* **36** (5), 739-744.

Bratu, I., Flageole, H., Laberge, J. M., Possmayer, F., Harbottle, R., Kay, S., Khalife, S. & Piedboeuf, B. (2001b). Surfactant levels after reversible tracheal occlusion and prenatal steroids in experimental diaphragmatic hernia. *Journal of Pediatric Surgery* **36**(1), 122-127.

Breen, E. C. (2000). Mechanical strain increases type I collagen expression in pulmonary fibroblasts *in vitro*. *Journal of Applied Physiology* 88, 203-209.

Brown, M. J., Olver, R. E., Ramsden, C. A., Strang, L. B., & Walters, D. V. (1983). Effects of adrenaline and of spontaneous labour on the secretion and absorption of lung liquid in the fetal lamb. *Journal of Physiology* **344**, 137-152.

Bruce, M. C. (1991). Developmental changes in tropoelastin mRNA levels in rat lung: evaluation by *in situ* hybridization. *American Journal of Respiratory Cell and Molecular Biology* **5**, 344-350.

Brumley, G. W., Knelson, J. H., Schomberg, D. W., & Crenshaw, C. J. (1977). Whole and disaturated lung phosphatidylcholine in cortisol-treated, intrauterine growth-retarded and twin control lambs at different gestational ages. *Biology of the Neonate* **31**, 155-166.

Bryden, M. M., Evans, H., & Binns, W. (1973). Embryology of the sheep. III. The respiratory system, mesenteries and celom in the fourteen to thirty-four day embryo. *The Anatomical Record* **175**, 725-736.

Buck, C. A. & Horwitz, A. F. (1987). Cell surface receptors for extracellular matrix molecules. *Annual Review of Cell Biology* **3**, 179-205.

Burri, P. H. (1974). The postnatal growth of the rat lung III. Morphology. *The Anatomical Record* **180**, 77-98.

Burri, P. H. (1984). Fetal and postnatal development of the lung. *Annual Review of Physiology* **46**, 617-628.

Burri, P. H. (1994). Structural development of the lung in the fetus and neonate. In *Fetus and Neonate. Physiology and Clinical Implications.*, eds. Hanson, M. A., Spencer, J. A. D., Rodeck, L. H., & Walters, D., pp. 3-19. Cambridge University Press, United Kingdom.

Burri, P. H. (1999). Lung development and pulmonary angiogenesis. In *Lung Development*, eds. Gaultier, C., Bourbon, J. R., & Post, M., pp. 122-151. Oxford University Press, New York.

Burridge, K., Molony, L., & Kelly, T. (1987). Adhesion plaques: sites of transmembrane interaction between the extracellular matrix and the actin cytoskeleton. *Journal of Cell Science Supplements* **8**, 211-229.

Butler, N. & Claireaux, A. E. (1962). Congenital diaphragmatic hernia as a cause of perinatal mortality. *Lancet* 659-663.

Campos, G. A., Guerra, F. A., Brummer, E., Munoz, M. L., & Vega, I. A. (1992). Synergistic effect of cortisol and thyrotropin releasing hormone on lung maturation in the fetal sheep entails connective tissue maturation. *Biological Research* **25**, 95-100.

Caniggia, I., Han, R., Liu, J., Wang, J., Tanswell, A. K., & Post, M. (1995). Differential expression of collagen-binding receptors in fetal rat lung cells. *American Journal of Physiology* **268**, L136-L143.

Carmel, J. A., Friedman, F., & Adams, F. H. (1965). Fetal tracheal ligation and lung development. *American Journal of Diseases of Children* **109**, 452-456.

Cassin, S., DeMarco, V., Perks, A. M., Kuck, H., & Ellis, T. M. (1994). Regulation of lung liquid secretion in immature fetal sheep: hormonal interaction. *Journal of Applied Physiology* **77**, 1445-1450.

Cassin, S. & Perks, A. M. (1982). Studies of factors which stimulate lung fluid secretion in fetal goats. *Journal of Developmental Physiology* **4**, 311-325.

Cawston, T., Carrere, S., Catterall, J., Duggleby, R., Elliott, S., Shingleton, B., & Rowan, A. (2001). Matrix metalloproteinases and TIMPs: properties and implications for the treatment of chronic obstructive pulmonary disease. *Novartis Foundation Symposium* **234**, 205-28.

Chapman, D. L., Carlton, D. P., Nielson, D. W., Cummings, J. J., Poulain, F. R. & Bland, R. D. (1994). Changes in lung liquid during spontaneous labour in fetal sheep. *Journal of Applied Physiology* **76**, 523-530.

Cole, T. J., Blendy, J. A., Monaghan, A. P., Krieglstein, K., Schmid, W., Aguzzi, A., Fantuzzi, G., Hummler, E., Unsicker, K., & Schutz, G. (1995). Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung development. *Genes and Development* **9**, 1608-1621.

Corbel, M., Boichot, E., & Lagente, V. (2000). Role of gelatinases MMP-2 and MMP-9 in tissue remodeling following acute lung injury. *Brazilian Journal of Medical and Biological Research.* **33**, 749-754.

Cordingley, M. G., Riegel, A. T., & Hager, G. L. (1987). Steroid-dependent interaction of transcription factors with the inducible promoter of mouse mammary tumor virus *in vivo*. *Cell* **48**, 261-270.

Cotterrell, M., Balazs, R., & Johnson, A. L. (1972). Effects of corticosteroids on the biochemical maturation of rat brain: postnatal cell formation. *Journal of Neurochemistry* **19**, 2151-2167.

Crapo, J. D., Barry, B. E., Gehr, P., Bachofen, M., & Weibel, E. R. (1982). Cell number and cell characteristics of the normal human lung. *American Review of Respiratory Disease* **126**, 332-337.

Crone, R. K., Davies, P., Liggins, G. C., & Reid, L. (1983). The effects of hypophysectomy, thyroidectomy, and postoperative infusion of cortisol or adrenocorticotrophin on the structure of the ovine fetal lung. *Journal of Developmental Physiology* **5**, 281-288.

Crowley, P., Chalmers, I., & Keirse, M. J. N. C. (1990). The effects of corticosteroid administration before preterm delivery: an overview of the evidence from controlled trials. *British Journal of Obstetrics and Gynaecology* **97**, 11-25.

Cummings, J. J., Carlton, D. P., Poulain, F. R., Fike, C. D., Keil, L. C., & Bland, R. D. (1995). Vasopressin effects on lung liquid volume in fetal sheep. *Pediatric Research* **38**, 30-35.

Cutroneo, K. R., Rokowski, R., & Counts, D. F. (1981). Glucocorticoids and collagen synthesis: comparison of *in vivo* and cell culture studies. *Collagen and Related Research* **1**, 557-568.

Davey, M. G., Hooper, S. B., Tester, M. L., Johns, D. P., & Harding, R. (1999). Respiratory function in lambs after *in utero* treatment of lung hypoplasia by tracheal obstruction. *Journal of Applied Physiology* **87**, 2296-2304.

Davies, G. & Reid, L. (1970). Growth of the alveoli and pulmonary arteries in childhood. *Thorax* **25**, 669-681.

Davies, P., Reid, L., Lister, G., & Pitt, B. (1988). Postnatal growth of the sheep lung: a morphometric study. *The Anatomical Record* **220**, 281-286.

Davila, R. M., deMello, D. E., & Crouch, E. C. (1995). Ontogeny of type I procollagen expression during human fetal lung development. *American Journal of Physiology* **268**, L309-L320.

De Paepe, M. E., Johnson, B. D., Papadakis, K., & Luks, F. I. (1999). Lung growth response after tracheal occlusion in fetal rabbits is gestational age-dependent. *American Journal of Respiratory Cell and Molecular Biology* **21**, 65-76.

De Paepe, M. E., Johnson, B. D., Papadakis, K., Sueishi, K., & Luks, F. I. (1998). Temporal pattern of accelerated lung growth after tracheal occlusion in the fetal rabbit. *American Journal of Pathology* **152**, 179-190.

Delacourt, C., D'Ortho, M. P., Macquin-Mavier, I., Pezet, S., Harf, A., & Lafuma, C. (1995). Increased 92 kD gelatinase activity from alveolar macrophages in newborn rats. *American Journal of Respiratory and Critical Care Medicine* **151**, 1939-1945.

deLorimier, A. A., Manus, A. G., & Tyler, W. S. (1969). Morphometry of normal and hypoplastic lungs in newborn lambs. *Current Topics in Surgical Research* **1**, 431-442.

deLorimier, A. A., Tierney, D. F., & Parker, H. R. (1967). Hypoplastic lungs in fetal lambs with surgically produced congenital diaphragmatic hernia. *Surgery* **62**, 12-17.

DeLemos, R. A., Shermeta, D. W., Knelson, J. H., Kotas, R., & Avery, M. E. (1970). Acceleration of appearance of pulmonary surfactant in the fetal lamb by administration of corticosteroids. *American Review of Respiratory Disease* **102**, 459-461.

Deterding, R. R. & Shannon, J. M. (1995). Proliferation and differentiation of fetal rat pulmonary epithelium in the absence of mesenchyme. *Journal of Clinical Investigation* **95**, 2963-2972.

Dickson, K. A. & Harding, R. (1989). Decline in lung liquid volume and secretion rate during oligohydramnios in fetal sheep. *Journal of Applied Physiology* **67**, 2401-2407.

Dickson, K. A. & Harding, R. (1991). Fetal breathing and pressures in the trachea and amniotic sac during oligohydramnios in sheep. *Journal of Applied Physiology* **70**, 293-299.

Dickson, K. A., Maloney, J. E., & Berger, P. J. (1986). Decline in lung liquid volume before labor in fetal lambs. *Journal of Applied Physiology* **61**, 2266-2272.

DiFiore, J. W. & Wilson, J. M. (1994). Lung development. *Seminars in Pediatric Surgery* **3**, 221-232.

Dollery, C. M., McEwan, J. R., & Henney, A. M. (1995). Matrix metalloproteinases and cardiovascular disease. *Circulation Research* **77**, 863-868.

Doyle, L. W., Ford, G. W., Rickards, A. L., Kelly, E. A., Davis, N. M., Callanan, C., & Olinsky, A. (2000). Antenatal corticosteroids and outcome at 14 years of age in children with birth weight less than 1501 grams. *Pediatrics* **106**, E2.

Dunlop, S. A., Archer, M. A., Quinlivan, J. A., Beazley, L. D., & Newnham, J. P. (1997). Repeated prenatal corticosteroids delay myelination in the ovine central nervous system. *Journal of Maternal and Fetal Medicine*. **6**, 309-313.

Dunsmore, S. E., Saarialho-Kere, U. K., Roby, J. D., Wilson, C. L., Matrisian, L. M., Welgus, H. G., & Parks, W. C. (1998). Matrilysin expression and function in airway epithelium. *Journal of Clinical Investigation* **102**, 1321-1331.

Edelman, J. M., Chan, B. M., Uniyal, S., Onodera, H., Wang, D. Z., St John, N. F., Damjanovich, L., Latzer, D. B., Finberg, R. W., & Bergelson, J. M. (1994). The mouse VLA-2 homologue supports collagen and laminin adhesion but not virus binding. *Cell Adhesion Communications* **2**, 131-143.

Eickelberg, O., Kohler, E., Reichenberger, F., Bertschin, S., Woodtli, T., Erne, P., Perruchoud, A. P., & Roth, M. (1999). Extracellular matrix deposition by primary human lung fibroblasts in response to TGF-beta1 and TGF-beta3. *American Journal of Physiology* **276**, L814-L824.

Elsner, C. W., Magyar, D. M., Fridshal, D., Eliot, J., Klein, A., Glatz, T., Nathanielsz, P. W., & Buster, J. E. (1980). Time-trend analysis of plasma C-21 steroids in fetal and maternal sheep during the last 18 days of gestation. *Endocrinology* **107**, 801-808.

Emery, J. L. & Mithal, A. (1960). The number of alveoli in the terminal respiratory unit of man during late intrauterine life and childhood. *Archives of Disease in Childhood* **35**, 544-547.

Farrell, E. E., Silver, R. K., Kimberlin, R. N., Wolf, E. S., & Dusik, J. M (1989). Impact of antenatal dexamethasone administration on respiratory distress syndrome in surfactant-treated infants. *American Journal of Obstetrics and Gynecology* **161**, 628-633.

Fewell, J. E., Lee, C. C., & Kitterman, J. A. (1981). Effects of phrenic nerve section on the respiratory system of fetal lambs. *Journal of Applied Physiology* **51**, 293-297.

Fiascone, J. M., Jacobs, H. C., Moya, F. R., Mercurio, M. R., & Lima, D. M. (1987). Betamethasone increases pulmonary compliance in part by surfactant-independent mechanisms in preterm rabbits. *Pediatric Research* **22**, 730-735.

Flageole, H., Evrard, V. A., Piedboeuf, B., Laberge, J. M., Lerut, T. E., & Deprest, J. A. (1998). The plug-unplug sequence: an important step to achieve type II pneumocyte maturation in the fetal lamb model. *Journal of Pediatric Surgery* **33**, 299-303.

Flake, A. W. (1996). Fetal surgery for congenital diaphragmatic hernia. *Seminars in Pediatric Surgery* **5**, 266-274.

Flecknoe, S., Harding, R., Maritz, G., & Hooper, S. B. (2000). Increased lung expansion alters the proportions of type I and type II alveolar epithelial cells in fetal sheep. *American Journal of Physiology* **278**, L1180-L1185.

Flint, A. P. & Burton, R. D. (1984). Properties and ontogeny of the glucocorticoid receptor in the placenta and fetal lung of the sheep. *Journal of Endocrinology* **103**, 31-42.

Foster, J. A. & Curtiss, S. W. (1990). The regulation of lung elastin synthesis. *American Journal of Physiology* **259**, L13-L23.

French, N. P., Hagan, R., Evans, S. F., Godfrey, M., & Newnham, J. P. (1999). Repeated antenatal corticosteroids: size at birth and subsequent development. *American Journal of Obstetrics and Gynecology* **180**, 114-121.

Frisdal, E., Teiger, E., Lefaucheur, J. P., Adnot, S., Planus, E., Lafuma, C., & D'Ortho, M. P. (2000). Increased expression of gelatinases and alteration of basement membrane in rat soleus muscle following femoral artery ligation. *Neuropathology and Applied Neurobiology* **26**, 11-21.

Fukuda, Y., Ferrans, V. J., & Crystal, R. G. (1983). The development of alveolar septa in fetal sheep lung. An ultrastructural and immunohistochemical study. *The American Journal of Anatomy* **167**, 405-439.

Fukuda, Y., Ferrans, V. J., & Crystal, R. G. (1984). Development of elastic fibres of nuchal ligament, aorta, and lung of fetal and postnatal sheep: an ultrastructural and electron microscopic immunohistochemical study. *The American Journal of Anatomy* **170**, 597-607.

Fukuda, Y., Ishizaki, M., Kudoh, S., Kitaichi, M., & Yamanaka, N. (1998). Localization of matrix metalloproteinases-1, -2, and -9 and tissue inhibitor of metalloproteinase-2 in interstitial lung diseases. *Laboratory Investigation* **78**, 687-698.

Fukuda, Y., Ishizaki, M., Okada, Y., Seiki, M., & Yamanaka, N. (2000). Matrix metalloproteinases and tissue inhibitor of metalloproteinase-2 in fetal rabbit lung. *American Journal of Physiology* **279**, L555-L561.

Gailit, J. & Ruoslahti, E. (1988). Regulation of the fibronectin receptor affinity by divalent cations. *The Journal of Biological Chemistry* **263**, 12927-12932.

Gargosky, S. E., Owens, J. A., Walton, P. E., Owens, P. C., Wallace, J. C., & Ballard, F. J. (1991). Administration of insulin-like growth factor-I, but not growth hormone, increases maternal weight gain in late pregnancy without affecting fetal or placental growth. *Journal of Endocrinology* **130**, 395-400.

Gervasi, D. C., Raz, A., Dehem, M., Yang, M., Kurkinen, M., & Fridman, R. (1996). Carbohydrate-mediated regulation of matrix metalloproteinase-2 activation in normal human fibroblasts and fibrosarcoma cells. *Biochemical and Biophysical Research Communications* **228**, 530-538.

Gillett, A. M., Wallace, M. J., Gillespie, M. T., & Hooper, S. B. (2002). Lung expansion increases calmodulin mRNA levels within the fetal lung. *American Journal of Physiology* **282**, 440-447.

Gillie, D.J., Pace, J., Coakley, R.J., Koller, B.H., & Barker, P.M. (2001). Liquid and ion transport by fetal airway and lung epithelia of mice deficient in sodium-potassium-2-chloride transporter. *American Journal of Respiratory and Cell Molecular Biology* **25**(1), 14-20.

Ginestra, A., Monea, S., Seghezzi, G., Dolo, V., Nagase, H., Mignatti, P., & Vittorelli, M. L. (1997). Urokinase plasminogen activator and gelatinases are associated with membrane vesicles shed by human HT1080 fibrosarcoma cells. *The Journal of Biological Chemistry* **272**, 17216-17222.

Glick, P. L., Stannard, V. A., Leach, C. L., Rossman, J., Hosada, Y., Morin, F. C., Cooney, D. R., Allen, J. E., & Holm, B. (1992). Pathophysiology of congenital diaphragmatic hernia II: the fetal lamb CDH model is surfactant deficient. *Journal of Pediatric Surgery* **27**, 382-387.

Graf, J. L., Gibbs, D. L., Adzick, N. S., & Harrison, M. R. (1997). Fetal hydrops after *in utero* tracheal occlusion. *Journal of Pediatric Surgery* **32**, 214-216.

Gumbinas, M., Oda, M., & Huttenlocher, P. (1973). The effects of corticosteroids on myelination of the developing rat brain. *Biology of the Neonate* **22**, 355-366.

Harding, R. (1994). Development of the respiratory system. In *Textbook of Fetal Physiology*, eds. Harding, R. & Thorburn, G. D., pp. 140-167. Oxford University Press, Oxford.

Harding, R. (1997). Fetal breathing movements. In *The Lung: Scientific Foundations*, eds. Crystal, R. G., West, J. B., Weibel, E. R., & Barnes, P. J., pp. 1655-1663. Raven Lippincott, New York.

Harding, R., Bocking, A. D., & Sigger, J. N. (1986a). Influence of upper respiratory tract on liquid flow to and from fetal lungs. *Journal of Applied Physiology* **61**, 68-74.

Harding, R., Bocking, A. D., & Sigger, J. N. (1986b). Upper airway resistances in fetal sheep: the influence of breathing activity. *Journal of Applied Physiology* **60**, 160-165.

Harding, R. & Hooper, S. B. (1996). Regulation of lung expansion and lung growth before birth. *Journal of Applied Physiology* **81**, 209-224.

Harding, R., Hooper, S. B., & Dickson, K. A. (1990). A mechanism leading to reduced lung expansion and lung hypoplasia in fetal sheep during oligohydramnios. *American Journal of Obstetrics and Gynecology* **163**, 1904-1913.

Harding, R., Hooper, S. B., & Han, V. K. M. (1993). Abolition of fetal breathing movements by spinal cord transection leads to reductions in fetal lung liquid volume, lung growth and IGF-II gene expression. *Pediatric Research* **34**, 148-153.

Harding, R., Johnson, P., & McClelland, M. E. (1980). Respiratory function of the larynx in developing sheep and the influence of sleep state. *Respiration Physiology* **40**, 165-179.

Harding, R. & Liggins, G. C. (1996). Changes in Thoracic dimensions induced by breathing movements in fetal sheep. *Reproduction Fertility and Development* **8**, 117-124.

Harding, R., Sigger, J. N., Wickham, P. J. D., & Bocking, A. D. (1984). The regulation of flow of pulmonary fluid in fetal sheep. *Respiration Physiology* **57**, 47-59.

Harding, R., Tester, M. L., Moss, T. J., Davey, M. G., Louey, S., Joyce, B., Hooper, S. B., & Maritz, G. (2000a). Effects of intra-uterine growth restriction on the control of breathing and lung development after birth. *Clinical Experimental Pharmacology and Physiology* **27**, 114-119.

Harding, R., Cock, M. L., Louey, S., Joyce, B. J., Davey, M. G., Albuquerque, C. A., Hooper, S. B., & Maritz, G.S. (2000b) The compromised intra-uterine environment: implications for future lung health. *Clinical and Experimental Pharmacology and Physiology*. **27(12)**, 965-74.

Harrison, M. R. (1996). Fetal surgery. *American Journal of Obstetrics and Gynecology* **174**, 1255-1264.

Harrison, M. R., Adzick, N. S., Flake, A. W., Vanderwall, K. J., Bealer, J. F., Howell, L. J., Farrell, J. A., Rosen, M. A., Sola, A., & Goldberg, J. D. (1996). Correction of congenital diaphragmatic hernia *in utero* VIII: Response of the hypoplastic lung to tracheal obstruction. *Journal of Pediatric Surgery* **31**, 1339-1348.

Harrison, M. R., Bjordal, R. I., Langmark, F., & Knutrud, O. (1978). Congenital diaphragmatic hernia: the hidden mortality. *Journal of Pediatric Surgery* **13**, 227-230.

Hashim, E., Laberge, J.-M., Chen, M.-F., & Quillen, E. W. J. (1995). Reversible tracheal obstruction in the fetal sheep: effects on tracheal fluid pressure and lung growth. *Journal of Pediatric Surgery* **30**, 1172-1177.

Hassett, M. J., Glick, P. L., Karamanouklan, H. L., Rossman, J. E., Wilcox, D. T., & Azizkhan, R. G. (1995). Pathophysiology of congenital diaphragmatic hernia XVI: Elevated pulmonary collagen in the lamb model of congenital diaphragmatic hernia. *Journal of Pediatric Surgery* **30**, 1191-1194.

Hedrick, H. L., Kaban, J. M., Pacheco, B. A., Losty, P. D., Doody, D. P., Ryan, D. P., Manganaro, T. F., Donahoe, P. K., & Schnitzer, J. J. (1997). Prenatal glucocorticoids improve pulmonary morphometrics in fetal sheep with congenital diaphragmatic hernia. *Journal of Pediatric Surgery* **32**, 217-221.

Hedrick, M. H., Martinez Ferro, M., Filly, R. A., Flake, A. W., Harrison, M. R., & Adzick, N. S. (1994). Congenital high airway obstruction syndrome (CHAOS): a potential for perinatal intervention. *Journal of Pediatric Surgery* **29**, 271-274.

Heussen, C. & Dowdle, E. B. (1980). Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Analytical Biochemistry* **102**, 196-202.

Hill, A. C., Adzick, N. S., Stevens, M. B., Mori, H., Husseni, W., & Heymann, M. A. (1994). Fetal lamb pulmonary hypoplasia: pulmonary vascular and myocardial abnormalities. *Annals of Thoracic Surgery* **57**, 946-951.

Hitchcock, K. R. (1980). Lung development and the pulmonary surfactant system: Hormonal influences. *The Anatomical Record* **198**, 13-34.

Hodson, W. A. (1992). Normal and abnormal structural development of the lung. In *Fetal and Neonatal Physiology*, eds. Polin, R. A. & Fox, W. W., pp. 771-782. W.B. Saunders Company, Philadelphia.

Hoidal, J. R. & Hoidal, M. K. (2000). Proteolytic enzymes and their inhibitors in lung health and disease. In *Chronic Lung Disease in Early Infancy*, eds. Bland, R. D. & Coalson, J. J., pp. 859-883. Marcel Dekker, New York.

Hooper, S. B., Dickson, K. A., & Harding, R. (1988). Lung liquid secretion, flow and volume in response to moderate asphyxia in fetal sheep. *Journal of Developmental Physiology* **10**, 473-485.

Hooper, S. B., Han, V. K. M., & Harding, R. (1993b). Changes in lung expansion alter pulmonary DNA synthesis and IGF-II gene expression in fetal sheep. *American Journal of Physiology* **265**, L403-L409.

Hooper, S. B. & Harding, R. (1995). Fetal lung liquid: a major determinant of the growth and functional development of the fetal lung. *Clinical and Experimental Pharmacology and Physiology* **22**, 235-247.

Hooper, S. B., Wallace, M. J., & Harding, R. (1993a). Amiloride blocks the inhibition of fetal lung liquid secretion caused by AVP but not by asphyxia. *Journal of Applied Physiology* **74**, 111-115.

Huhtala, P., Tuuttila, A., Chow, L. T., Lohi, J., Keski-Oja, J., & Tryggvason, K. (1991). Complete structure of the human gene for 92-kDa type IV collagenase. Divergent regulation of expression for the 92- and 72-kilodalton enzyme genes in HT-1080 cells. *The Journal of Biological Chemistry* **266**, 16485-16490.

Humphreys, P. W., Normand, I. C. S., Reynolds, E. O. R., & Strang, L. B. (1967). Pulmonary lymph flow and the uptake of liquid from the lungs of the lamb at the start of breathing. *Journal of Physiology* **193**, 1-29.

Ijsselstijn, H., Pacheco, B. A., Albert, A., Sluiter, W., Donahoe, P. K., de Jongste, J. C., Schnitzer, J. J., & Tibboel, D. (1997). Prenatal hormones alter antioxidant enzymes and lung histology in rats with congenital diaphragmatic hernia. *American Journal of Physiology* **272**, L1059-L1065.

Ikegami, M., Jobe, A. H., Newnham, J., Polk, D. H., Willet, K. E., & Sly, P. (1997). Repetitive prenatal glucocorticoids improve lung function and decrease growth in preterm lambs. *American Journal of Respiratory and Critical Care Medicine* **156**, 178-184.

Ikegami, M., Polk, D. H., Jobe, A. H., Newnham, J., Sly, P., Kohen, R., & Kelly, R. (1995). Postnatal lung function in lambs after fetal hormone treatment. *American Journal of Respiratory and Critical Care Medicine* **152**, 1256-1261.

Ikegami, M., Polk, D., Tabor, B., Lewis, J., Yamada, T., & Jobe, A. (1991). Corticosteroid and thyrotropin-releasing hormone effects on preterm sheep lung function. *Journal of Applied Physiology* **70**, 2268-2278.

Ingber, D. E. (1991). Control of capillary growth and differentiation by extracellular matrix. Use of a tensegrity (tensional integrity) mechanism for signal processing. *Chest* **99**, 34S-40S.

Ingber, D. E., Karp, S., Plopper, G., Hansen, L., & Moore, G. J. (1993). Mechanochemical transduction across extracellular matrix and through the cytoskeleton. In *Physical Forces and the Mammalian Cell*, ed. Fransas, J. A., pp. 61-79. Academic Press, San Diego.

Jackson, J. C., Clark, J. G., Standaert, T. A., Truog, W. E., Murphy, J. H., Juul, S. E., Chi, E. Y., & Hodson, W. A. (1990). Collagen synthesis during lung development and during hyaline membrane disease in the nonhuman primate. *American Review of Respiratory Disease* **141**, 846-853.

Jeffery, P. K. (1998). The development of large and small airways. *American Journal of Respiratory and Critical Care Medicine* **157**, S174-S180.

Jobe, A. H. & Ikegami, M. (2000). Lung development and function in preterm infants in the surfactant treatment era. *Annual Review of Physiology* **62**, 825-846.

Jobe, A. H., Mitchell, B. R., & Gunkel, J. H. (1993). Beneficial effects of the combined use of prenatal corticosteroids and postnatal surfactant on preterm infants. *American Journal of Obstetrics and Gynecology* **148**, 509-513.

Jobe, A. H., Newnham, J., Willet, K., Sly, P., & Ikegami, M. (1998). Fetal versus maternal and gestational age effects of repetitive antenatal glucocorticoids. *Pediatrics* **102**, 1116-1125.

Johnson, J. W., Mitzner, W., Beck, J. C., London, W. T., Sly, D. L., Lee, P. A., Khouzami, V. A., & Cavalieri, R. L. (1981). Long-term effects of betamethasone on fetal development. *American Journal of Obstetrics and Gynecology* **141**, 1053-1064.

Johnson, J. W., Mitzner, W., London, W. T., Palmer, A. E., Scott, R., & Kearney, K. (1978). Glucocorticoids and the rhesus fetal lung. *American Journal of Obstetrics and Gynecology* **130**, 905-916.

Jost, A. & Policard, A. (1948). Contribution expérimentale a l'étude du développement prénatal du poumon chez le lapin. *Archives D'Anatomie Microscopique* **37**, 323-332.

Junqueira, L. C., Junqueira, L. C., & Brentani, R. R. (1979). A simple and sensitive method for the quantitative estimation of collagen. *Analytical Biochemistry* **94**, 96-99.

Kahari, V. M. & Saarialho-Kere, U. (1999). Matrix metalloproteinases and their inhibitors in tumour growth and invasion. *Annals of Medicine* **31**, 34-45.

Kay, S., Laberge, J. M., Flageole, H., Richardson, S., Belanger, S., & Piedboeuf, B. (2001) Use of antenatal steroids to counteract the negative effects of tracheal occlusion in the fetal lamb model. *Pediatric Research* **50** (4), 495-501.

Kefalides, N. A. (1975). Basement membranes; structural and biosynthetic considerations. *The Journal of Investigative Dermatology* **65**, 85-92.

Kehrer, J. P., Pearlman, R., & Smith, M. A. (1983). Direct effect of corticosteroids on *in vitro* collagen production by normal and damaged lung tissue. *Toxicology* **28**, 235-246.

Kendall, J. Z., Lakritz, J., Plopper, C. G., Richards, G. E., Randall, G. C. B., Nagamani, M., & Weir, A. J. (1990). The effects of hydrocortisone on lung structure in fetal lambs. *Journal of Developmental Physiology* **13**, 165-172.

Kendall, J. Z., Rivera-Alcina, M. E., Plopper, C. G., Weir, A. J., Randall, G. C., & Saldana-Gautier, L. (1991). A morphometric study of lung maturation in the adrenocorticotrophin- infused fetal lamb. *Biology of the Neonate* **60**, 258-272.

Keramidaris, E., Hooper, S. B., & Harding, R. (1996). Effect of gestational age on the increase in fetal lung growth following tracheal obstruction. *Experimental Lung Research* **22**, 283-298.

Kikkawa, Y., Kaibara, M., Motoyama, E. K., Orzalesi, M. M., & Cook, C. D. (1971). Morphologic development of fetal rabbit lung and its acceleration with cortisol. *American Journal of Pathology* **64**, 423-442.

Kindler, P. M., Chuang, D. C., & Perks, A. M. (1993). Fluid production by *in vitro* lungs from near-term fetal guinea pigs: effects of cortisol and aldosterone. *Acta Endocrinologica* **129**, 169-177.

Kinoh, H., Sato, H., Tsunezuka, Y., Takino, T., Kawashima, A., Okada, Y., & Seiki, M. (1996). MT-MMP, the cell surface activator of proMMP-2 (pro-gelatinase A), is expressed with its substrate in mouse tissue during embryogenesis. *Journal of Cell Science* **109** (5), 953-959.

Kitterman, J. A., Ballard, P. L., Clements, J. A., Mescher, E. J., & Tooley, W. H. (1979). Tracheal fluid in fetal lambs: spontaneous decrease prior to birth. *Journal of Applied Physiology* **47**, 985-989.

Kivirikko, K. I. & Laitinen, O. (1965). Effect of cortisone on the hydroxyproline in the serum and urine of young rats. *Acta Physiologica Scandinavica* **64**, 356-360.

Kivirikko, K. I., Laitinen, O., Aer, J., & Halme, J. (1965). Studies with 14C-proline on the action of cortisone on the metabolism of collagen in the rat. *Biochemical Pharmacology* **14**, 1445-1451.

Kleiner, D. E. & Stetler-Stevenson, W. G. (1994). Quantitative zymography: detection of picogram quantities of gelatinases. *Analytical Biochemistry* **218**, 325-329.

Kluth, D., Kangah, R., Reich, P., Tenbrinck, R., Tibboel, D., & Lambrecht, W. (1990). Nitrofen-induced diaphragmatic hernias in rats: an animals model. *Journal of Pediatric Surgery* **25**, 850-854.

Kotas, R. V. & Avery, M. E. (1971). Accelerated appearance of pulmonary surfactant in the fetal rabbit. *Journal of Applied Physiology* **30**, 358-361.

Kotas, R. V. & Avery, M. E. (1980). The influence of sex on fetal rabbit lung maturation and on the response to glucocortoid. *American Review of Respiratory Disease* **121**, 377-380.

Kudlacz, E. M., Navarro, H. A., & Slotkin, T. A. (1989). Phosphatidic acid phosphatase in neonatal rat lung: effects of prenatal dexamethasone or terbutaline treatment on basal activity and on responsiveness to *beta* adrenergic stimulation. *The Journal of Pharmacology and Experimental Therapeutics* **250**, 236-240.

Labarca, C. & Paigen, K. (1980). A simple, rapid, and sensitive DNA assay procedure. *Analytical Biochemistry* **102**, 344-352.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lane, J. M., Parkes, L. J., & Prockop, D. J. (1971). Effect of the proline analogue azetidine-2-carboxylic acid on collagen synthesis *in vivo*. II. Morphological and physical appearance of collagen containing the analogue. *Biochimica et Biophysica Acta* **236**, 528-541.

Lanman, J. T., Schaffer, A., Herod, L., Ogawa, Y., & Castellanos, R. (1971). Distensibility of the fetal lung with fluid in sheep. *Pediatric Research* **5**, 586-590.

Larson, J. E. (1993). Developmental control of collagen gene expression in the rat lung: confirmations of early observations of lung growth. *Pediatric Pulmonology* **15**, 205-208.

Laurent, G. J., Harrison, N. K., & McAnulty, R. J. (1988). The regulation of collagen production in normal lung and during interstitial lung disease. *Postgraduate Medical Journal* **64**, 26-34.

Liggins, G. C. (1969). Premature delivery of foetal lambs infused with glucocorticoids. *Journal of Endocrinology* **45**, 515-523.

Liggins, G. C. (1994). Fetal lung maturation. *Australian and New Zealand Journal of Obstetrics and Gynecology* **34**, 247-250.

Liggins, G. C. & Howie, R. N. (1972). A controlled trial of antepartum glucocorticoid treatment for prevention of the respiratory distress syndrome in premature infants. *Pediatrics* **50**, 515-525.

Liggins, G. C., Kitterman, J. A., Campos, G. A., Clements, J. A., Forster, C. S., Lee, C. H., & Creasy, R. K. (1981b). Pulmonary maturation in the hypophysectomised ovine fetus. Differential responses to adrenocorticotrophin and cortisol. *Journal of Developmental Physiology* **3**, 1-14.

Liggins, G. C., Schellenberg, J.-C., Finberg, K., Kitterman, J. A., & Lee, C. H. (1985). The effects of ACTH1-24 or cortisol on pulmonary maturation in the adrenalectomized ovine fetus. *Journal of Developmental Physiology* **7**, 105-111.

Liggins, G. C., Schellenberg, J.-C., Manzai, M., Kitterman, J. A., & Lee, C. C. H. (1988). Synergism of cortisol and thyrotropin-releasing hormone in lung maturation in fetal sheep. *Journal of Applied Physiology* **65**, 1880-1884.

Liggins, G. C., Vilos, G. A., Campos, G. A., Kitterman, J. A., & Lee, C. H. (1981a). The effect of spinal cord transection on lung development in fetal sheep. *Journal of Developmental Physiology* **3**, 267-274.

Lines, A. (2000). The effect of increased lung expansion on TGF-β1 and IGF-II mRNA levels in fetal sheep. *PhD Thesis; The regulation of surfactant proteins by alterations in lung expansion in fetal and newborn sheep*. Monash University, Victoria.

Lines, A., Gillett, A. M, Phillips, I. D., Wallace, M. J., & Hooper, S. B. (2001). Re-expression of pulmonary surfactant proteins following tracheal obstruction in fetal sheep. *Experimental Physiology* **86** (1), 55-63.

Lines, A., Hooper, S. B., & Harding, R. (1997). Lung liquid production rates and volumes do not decrease before labor in healthy fetal sheep. *Journal of Applied Physiology* **82**, 927-932.

Lines, A., Nardo, L., Phillips, I. D., Possmayer, F., & Hooper, S. B. (1999). Alterations in lung expansion affect surfactant protein A, B and C mRNA levels in fetal sheep. *American Journal of Physiology* **276**, L239-L245.

Lipsett, J., Cool, J. C., Runciman, S. I., Ford, W. D., Kennedy, J. D., Martin, A. J., & Parsons, D. W. (2000). Morphometric analysis of preterm fetal pulmonary development in the sheep model of congenital diaphragmatic hernia. *Pediatric and Developmental Pathology.* **3**, 17-28.

Liu, M., Liu, J., Buch, S., Tanswell, A. K., & Post, M. (1995a). Antisense oligonucleotides against PDGF-B and its receptor inhibit mechanical strain-induced fetal lung cell growth. *American Journal of Physiology* **269**, L178-L184.

Liu, M. & Post, M. (2000). Invited review: mechanochemical signal transduction in the fetal lung *Journal of Applied Physiology* **89**, 2078-2084.

Liu, M., Skinner, S. J., Xu, J., Han, R. N., Tanswell, A. K., & Post, M. (1992). Stimulation of fetal rat lung cell proliferation *in vitro* by mechanical stretch. *American Journal of Physiology* **263**, L376-L383.

Liu, M., Xu, J., Liu, J., Kraw, M. E., Tanswell, A. K., & Post, M. (1995c). Mechanical strain-enhanced fetal lung cell proliferation is mediated by phospholipases C and D and protein kinase C. *American Journal of Physiology* **268**, L729-L738.

Liu, M., Xu, J., Souza, P., Tanswell, B., Tanswell, A. K., & Post, M. (1995b). The effect of mechanical strain on fetal rat lung cell proliferation: comparison of two- and three-dimensional culture systems. *In vitro Cell Development and Biology of Animals* **31**, 858-866.

Liu, M., Xu, J., Tanswell, A. K., & Post, M. (1994). Inhibition of mechanical strain-induced fetal rat lung cell proliferation by gadolinium, a stretch-activated channel blocker. *Journal of Cell Physiology* **161**, 501-507.

Lohi, J., Lehti, K., Westermarck, J., Kahari, V. M., & Keski-Oja, J. (1996). Regulation of membrane-type matrix metalloproteinase-1 expression by growth factors and phorbol 12-myristate 13-acetate. *European Journal of Biochemistry* **239**, 239-247.

Losty, P. D., Pacheco, B. A., Manganaro, T. F., Donahoe, P. K., Jones, R. C., & Schnitzer, J. J. (1996). Prenatal hormonal therapy improves pulmonary morphology in rats with congenital diaphragmatic hernia. *Journal of Surgical Research* **65**, 42-52.

Losty, P. D., Suen, H. C., Manganaro, T. F., Donahoe, P. K., & Schnitzer, J. J. (1995). Prenatal hormonal therapy improves pulmonary compliance in the nitrofen-induced CDH rat model. *Journal of Pediatric Surgery* **30**, 420-426.

Lumbers, E. R., Smith, F. G., & Stevens, A. D. (1985). Measurement of net transplacental transfer of fluid to the fetal sheep. *Journal of Physiology* **364**, 289-299.

MacFarlane, D. J., O'Connor, C. M., & Fitzgerald, M. X. (1993). Collagen production in human lung fibroblasts in response to cytokines. *Biochemical Society Transactions* **22**, 49S.

Madri, J. A. & Furthmayr, H. (1980). Collagen polymorphism in the lung. An immunochemical study of pulmonary fibrosis. *Human Pathology* **11**, 353-366.

Maeda, H., Shirmokawa, H., Yamaguchi, Y., Sueishi, K., & Nakano, H. (1989). The influence of pleural effusions on pulmonary growth in the human fetus. *Journal of Perinatal Medicine* **17**, 231-236.

Magyar, D. M., Fridshal, D., Elsner, C. W., Glatz, T., Eliot, J., Klein, A. H., Lowe, K. C., Buster, J. E., & Nathanielsz, P. W. (1980). Time-trend analysis of plasma cortisol concentrations in the fetal sheep in relation to parturition. *Endocrinology* **107**, 155-159.

Malicdem, M., Taylor, W., Goerke, M., & Devaskar, U. (1993). Ontogeny of rat lung type IV collagenase mRNA expression and collagenolytic activity during the perinatal period. *Biology of the Neonate* **64**, 376-381.

Mason, J. I., France, J. T., Magness, R. R., Murry, B. A., & Rosenfeld, C. R. (1989). Ovine placental steroid 17α-hydroxylase/C-17,20-lyase, aromatase and sulphatase in dexamethasone-induced and natural parturition. *Journal of Endocrinology* **122**, 351-359.

Mason, R. J. & Shannon, J. M. (1997). Alveolar type II cells. In *The Lung: Scientific Foundations*, eds. Crystal, R. G., West, J. B., Weibel, E. R., & Barnes, P. J., pp. 543-555. Lippincott-Raven, New York.

Massague, J. & Pandiella, A. (1993). Membrane-anchored growth factors. *Annual Review of Biochemistry* **62**, 515-541.

Matrisian, L. M. (1990). Metalloproteinases and their inhibitors in matrix remodeling. *Trends in Genetics* **6**, 121-125.

Mays, P. K., McAnulty, R. J., & Laurent, G. J. (1989). Age-related changes in lung collagen metabolism. A role for degradation in regulation lung collagen production. *American Review of Respiratory Disease* **140**, 410-416.

McDonald, J. A. (1989). Receptors for extracellular matrix components. *American Journal of Physiology* **257**, L331-L337.

McEwan, I. J., Wright, A. P., Dahlman-Wright, K., Carlstedt-Duke, J., & Gustafsson, J. A. (1993). Direct interaction of the tau 1 transactivation domain of the human glucocorticoid receptor with the basal transcriptional machinery. *Molecular and Cellular Biology* **13**, 399-407.

McGowan, S. E. (1992). Extracellular matrix and the regulation of lung development and repair. *FASEB Journal* **6**, 2895-2904.

McLees, B. D., Schleiter, G., & Pinnell, S. R. (1977). Isolation of type III collagen from human adult parenchymal lung tissue. *Biochemistry* **16**, 185-190.

Mescher, E. J., Platzker, A. C. G., Ballard, P. L., Kitterman, J. A., Clements, J. A., & Tooley, W. H. (1975). Ontogeny of tracheal fluid, pulmonary surfactant, and plasma corticoids in the fetal lamb. *Journal of Applied Physiology* **39**, 1017-1021.

Meyrick, B. & Reid, L. M. (1977). Ultrastructure of alveolar lining and its development In *Development of the Lung*, ed. Hodson, W. A., p135. Marcel Dekker Inc., New York.

Michel, R. P. & Cruz-Orive, L. M. (1988). Application of the cavalieri principle and vertical sections method to lung: estimation of volume and pleural surface area. *Journal of Microscopy* **150**, 117-136.

Miller, A. A., Hooper, S. B., & Harding, R. (1993). Role of fetal breathing movements in control of fetal lung distension. *Journal of Applied Physiology* **75**, 2711-2717.

Minoo, P. & King, R. J. (1994). Epithelial-mesenchymal interactions in lung development. *Annual Review of Physiology* **56**, 13-45.

Mitzner, W., Johnson, J. W., Scott, R., London, W. T., & Palmer, A. E. (1979). Effect of betamethasone on pressure-volume relationship of fetal rhesus monkey lung. *Journal of Applied Physiology* **47**, 377-382.

Moessinger, A. C., Harding, R., Adamson, T. M., Singh, M., & Kiu, G. T. (1990). Role of lung fluid volume in growth and maturation of the fetal sheep lung. *Journal of Clinical Investigation* **86**, 1270-1277.

Moessinger, A. C., Santiago, A., Paneth, N. S., Rey, H. R., Blanc, W. A., & Driscoll, J. M., Jr. (1989). Time trends in necropsy prevalence and birth prevalence of lung hypoplasia. *Paediatric and Perinatal Epidemiology* **3**, 421-431.

Moore, K. L., & Persaud, T. V. (1993). *The Developing Human: Clinically Oriented Embryology*. W.B. Saunders Company, Philadelphia.

Motoyama, E. K., Orzalesi, M. M., Kikkawa, Y., Kaibara, M., Wu, B., Zigas, C. J., & Cook, C. D. (1971). Effect of cortisol on the maturation of fetal rabbit lungs. *Pediatrics* **48**, 547-555.

Moya, F. R. & Gross, I. (1988). Prevention of Respiratory Distress Syndrome. *Seminars in Perinatology* **12**, 348-358.

Muglia, L. J., Bae, D. S., Brown, T. T., Vogt, S. K., Alvarez, J. G., Sunday, M. E., & Majzoub, J. A. (1999). Proliferation and differentiation defects during lung development in corticotropin-releasing hormone-deficient mice. *American Journal of Respiratory Cell and Molecular Biology* **20**, 181-188.

Muratore, C. S., Nguyen, H. T., Ziegler, M. M., & Wilson, J. M. (2000). Stretch-induced upregulation of VEGF gene expression in murine pulmonary culture: a role for angiogenesis in lung development. *Journal of Pediatric Surgery* **35**, 906-912.

Murphy, G., Hembry, R. M., Hughes, C. E., Fosang, A. J., & Hardingham, T. E. (1990). Role and regulation of metalloproteinases in connective tissue turnover. *Biochemistry Society Transactions*. **18**, 812-815.

Murphy, G., Stanton, H., Cowell, S., Butler, G., Knauper, V., Atkinson, S., & Gavrilovic, J. (1999). Mechanisms for pro matrix metalloproteinase activation. *APMIS* **107**, 38-44.

Nagase, H. (1994). Matrix metalloproteinases. A mini-review. *Contributions to Nephrology* **107**, 85-93.

Nakamura, T., Liu, M., Mourgeon, E., Slutsky, A., & Post, M. (2000). Mechanical strain and dexamethasone selectively increase surfactant protein C and tropoelastin gene expression. *American Journal of Physiology* **278**, L974-L980.

Nakayama, D. K., Harrison, M. R., Chinn, D. H., Callen, P. W., Filly, R. A., Golbus, M. S., & deLorimier, A. A. (1985). Prenatal diagnosis and natural history of the fetus with a congenital diaphragmatic hernia: Initial clinical experience. *Journal of Pediatric Surgery* **20**, 118-124.

Nardo, L., Hooper, S. B., & Harding, R. (1995). Lung hypoplasia can be reversed by short-term obstruction of the trachea in fetal sheep. *Pediatric Research* **38**, 690-696.

Nardo, L., Hooper, S. B., & Harding, R. (1998). Stimulation of lung growth by tracheal obstruction in fetal sheep: relation to luminal pressure and lung liquid volume. *Pediatric Research* **43**, 184-190.

Nardo, L., Maritz, G., Harding, R., & Hooper, S. B. (2000). Changes in lung structure and cellular division induced by tracheal obstruction in fetal sheep. *Experimental Lung Research* **26**, 105-119.

Nathanielsz, P. W. (1976). The production and role of glucocorticoids from the fetal adrenal gland. In *Fetal Endocrinology. An Experimental Approach*, ed. Nathanielsz, P. W., pp. 151-175. North-Holland Publishing Company, Amsterdam.

Nathanielsz, P. W., Elsner, C., Magyar, D., Fridshal, D., Freeman, A., & Buster, J. E. (1982). Time trend analysis of plasma unconjugated and sulfoconjugated estrone and 3β-

delta^5-steroids in fetal and maternal sheep plasma in relation to spontaneous parturition at term. *Endocrinology* **110**, 1402-1407.

Nathanson, I. & Nadel, J. A. (1984). Movements of electrolytes and fluid across airways. *Lung* **162(3)**, 125-137.

Nelson, P. G. & Perks, A. M. (1996). Effects of lung expansion on lung liquid production *in vitro* by lungs from fetal guinea-pigs. I. Basic studies and the effects of amiloride and propranolol. *Reproduction Fertility and Development* **8**, 335-346.

Nemoto, T., Ohara-Nemoto, Y., Denis, M., & Gustafsson, J. A. (1990). The transformed glucocorticoid receptor has a lower steroid-binding affinity than the nontransformed receptor. *Biochemistry* **29**, 1880-1886.

Newnham, J. P., Evans, S. F., Godfrey, M., Huang, W., Ikegami, M., & Jobe, A. (1999). Maternal, but not fetal, administration of corticosteroids restricts fetal growth. *Journal of Maternal and Fetal Medicine*. **8**, 81-87.

Nicolini, U., Fisk, N. M., Rodeck, C. H., Talbert, D. G., & Wigglesworth, J. S. (1989). Low amniotic fluid pressure in oligohydramnios: is this the cause of pulmonary hypoplasia? *American Journal of Obstetrics and Gynecology* **161**, 1098-1101.

Nobuhara, K. K., DiFiore, J. W., Ibla, J. C., Siddiqui, A. M., Ferretti, M. L., Fauza, D. O., Schnitzer, J. J., & Wilson, J. M. (1998). Insulin-like growth factor-I gene expression in three models of accelerated lung growth. *Journal of Pediatric Surgery* **33**, 1057-1060.

Normand, I. C. S., Olver, R. E., Reynolds, E. O. R., & Strang, L. B. (1971). Permeability of lung capillaries and alveoli to non-electrolytes in the foetal lamb. *Journal of Physiology* **219**, 303-330.

Nyirenda, M. J., Lindsay, R. S., Kenyon, C. J., Burchell, A., & Seckl, J. R. (1998). Glucocorticoid exposure in late gestation permanently programs rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. *Journal of Clinical Investigation* **101**, 2174-2181.

Nyirenda, M. J., & Seckl, J. R. (1998). Intrauterine events and the programming of adulthood disease: The role of fetal glucocorticoid exposure. *International Journal of Molecular Medicine* **2**, 607-614.

O'Brodovich, H., Hannam, V., Seear, M. & Mullen, J. B. M. (1990). Amiloride impairs lung water clearance in newborn guinea pigs. *Journal of Applied Physiology* **68**, 1758-1762.

O'Toole, S. J., Sharma, A., Karamanoukian, H. L., Holm, B. A., Azizkhan, R. G., & Glick, P. L. (1996). Tracheal ligation does not correct the surfactant deficiency associated with congenital diaphragmatic hernia. *Journal of Pediatric Surgery* **31**, 546-550.

Olver, R. E. (1977). Fetal lung liquids. *Federation Proceedings* **36**, 2669-2675.

Olver, R. E., Ramsden, C. A., & Strang, L. B. (1981b). Adrenaline-induced changes in net lung liquid volume flow across the pulmonary epithelium of the fetal lamb: evidence for active sodium transport. *Journal of Physiology* **318**, 38P-39P.

Olver, R. E., Ramsden, C. A., Strang, L. B., & Walters, D. V. (1986). The role of amiloride-blockable sodium transport in adrenaline-induced lung liquid reabsorption in the fetal lamb. *Journal of Physiology* **376**, 321-340.

Olver, R. E., Schneeberger, E. E., & Walters, D. V. (1981a). Epithelial solute permeability, ion transport and tight junction morphology in the developing lung of the fetal lamb. *Journal of Physiology* **315**, 395-412.

Olver, R. E. & Strang, L. B. (1974). Ion fluxes across the pulmonary epithelium and the secretion of lung liquid in the foetal lamb. *Journal of Physiology* **241**, 327-357.

Papadakis, K., De Paepe, M. E., Tackett, L. D., Piasecki, G. J., & Luks, F. I. (1998). Temporary tracheal occlusion causes catch-up lung maturation in a fetal model of diaphragmatic hernia. *Journal of Pediatric Surgery* **33**, 1030-1037.

Papadakis, K., Luks, F. I., De Paepe, M. E., Piasecki, G. J., & Wesselhoeft Jr, C. W. (1997). Fetal lung growth after tracheal ligation is not solely a pressure phenomenon. *Journal of Pediatric Surgery* **32**, 347-351.

Pardo, A., Barrios, R., Maldonado, V., Melendez, J., Perez, J., Ruiz, V., Segura-Valdez, L., Sznajder, J. I., & Selman, M. (1998). Gelatinases A and B are up-regulated in rat lungs by subacute hyperoxia: pathogenetic implications. *American Journal of Pathology* **153**, 833-844.

Pardo, A., Selman, M., Ridge, K., Barrios, R., & Sznajder, J. I. (1996). Increased expression of gelatinases and collagenase in rat lungs exposed to 100% oxygen. *American Journal of Respiratory and Critical Care Medicine* **154**, 1067-1075.

Parks, W. C., & Shapiro, S. D. (2001). Review: Matrix metalloproteinases in lung biology. *Respiratory Research* **2**, 10-19.

Parsons, S. L., Watson, S. A., Brown, P. D., Collins, H. M., & Steele, R. J. (1997). Matrix metalloproteinases. *British Journal of Surgery* **84**, 160-166.

Passi, A., Negrini, D., Albertini, R., Miserocchi, G., & De Luca, G. (1999). The sensitivity of versican from rabbit lung to gelatinase A (MMP-2) and B (MMP-9) and its involvement in the development of hydraulic lung edema. *FEBS Letters* **456**, 93-96.

Pei, X. H., Nakanishi, Y., Takayama, K., Bai, F., & Hara, N. (1999). Granulocyte, granulocyte-macrophage, and macrophage colony-stimulating factors can stimulate the invasive capacity of human lung cancer cells. *British Journal of Cancer* **79**, 40-46.

Perez-Ramos, J., Lourdes Segura-Valdez, M., Vanda, B., Selman, M., & Pardo, A. (1999). Matrix metalloproteinases 2, 9, and 13, and tissue inhibitors of metalloproteinases

1 and 2 in experimental lung silicosis. *American Journal of Respiratory and Critical Care Medicine* **160**, 1274-1282.

Perks, A. M. & Cassin, S. (1982). The effects of arginine vasopressin and other factors on the production of lung fluid in fetal goats. *Chest* **81S**, 63S-65S.

Perks, A. M. & Cassin, S. (1985). The rate of production of lung liquid in fetal goats, and the effect of expansion of the lungs. *Journal of Developmental Physiology* **7**, 149-160.

Piedboeuf, B., Laberge, J.-M., Ghitulescu, G., Gamache, M., Petrov, P., Belanger, S., Chen, M.-F., Hashim, E., & Possmayer, F. (1997). Deleterious effect of tracheal obstruction on type 2 pneumocytes in fetal sheep. *Pediatric Research* **41**, 473-479.

Pierce, R. A., Mariani, T. J., & Senior, R. M. (1995a). Elastin in lung development and disease. *Ciba Foundation Symposium* **192**, 199-214.

Pierce, R. A., Mariencheck, W. I., Sandefur, S., Crouch, E. C., & Parks, W. C. (1995b). Glucocorticoids upregulate tropoelastin expression during late stages of fetal lung development. *American Journal of Physiology* **268**, L491-L500.

Platzker, A. C. G., Kitterman, J. A., Mescher, E. J., Clements, J. A., & Tooley, W. H. (1975). Surfactant in the lung and tracheal fluid of the fetal lamb and acceleration of its appearance by dexamethasone. *Pediatrics* **56(4)**, 554-561.

Pochard, J. L., & Lutz-Bucher, B. (1986). Vasopressin and oxytocin levels in human neonates. *Acta Paediatrica Scandinavica* **75**, 774-778.

Probyn, M. E., Wallace, M. J., & Hooper, S. B. (2000). Effect of increased lung expansion on lung growth and development near midgestation in fetal sheep. *Pediatric Research* **47**, 806-812.

Pujols, L., Mullol, J., Perez, M., Roca-Ferrer, J., Juan, M., Xaubet, A., Cidlowski, J. A., & Picado, C. (2001). Expression of the human glucocorticoid receptor alpha and beta isoforms in human respiratory epithelial cells and their regulation by dexamethasone. *American Journal of Cellular and Molecular Biology* **24**, 49-57.

Quaglino, D., Fornieri, C., Nanney, L. B., & Davidson, J. M. (1993). Extracellular matrix modifications in rat tissues of different ages. *Matrix* **13**, 481-490.

Radomski, A., Sawicki, G., Olson, D. M., & Radomski, M. W. (1998). The role of nitric oxide and metalloproteinases in the pathogenesis of hyperoxia-induced lung injury in newborn rats. *British Journal of Pharmacology* **125**, 1455-1462.

Raghu, G., Masta, S., Meyers, D., & Narayanan, A. S. (1989). Collagen synthesis by normal and fibrotic human lung fibroblasts and the effect of transforming growth factor-beta. *American Review of Respiratory Disease* **140**, 95-100.

Raghu, G., Striker, L. J., Hudson, L. D., & Striker, G. E. (1985). Extracellular matrix in normal and fibrotic human lungs. *American Review of Respiratory Disease* **131**, 281-289.

Ray, J. M. & Stetler-Stevenson, W. G. (1994). The role of matrix metalloproteases and their inhibitors in tumour invasion, metastasis and angiogenesis. *European Respiratory Journal* **7**, 2062-2072.

Reinisch, J. M., Simon, N. G., Karow, W. G., & Gandelman, R. (1978). Prenatal exposure to prednisone in humans and animals retards intrauterine growth. *Science* **202**, 436-438.

Reponen, P., Sahlberg, C., Huhtala, P., Hurskainen, T., Thesleff, I., & Tryggvason, K. (1992). Molecular cloning of murine 72-kDa type IV collagenase and its expression during mouse development. *The Journal of Biological Chemistry* **267**, 7856-7862.

Riley, D. J. (2000). Connective tissues in lung development and diseases in early infancy. In *Chronic Lung Disease in Early Infancy*, eds. Bland, R. D. & Coalson, J. J., pp. 669-710. Marcel Dekker, New York.

Riley, D. J., Rannels, D. E., Low, R. B., Jensen, L., & Jacobs, T. P. (1990). Effect of physical forces on lung structure, function and metabolism: NHLBI Workshop summary. *American Review of Respiratory Disease* **142**, 910-914.

Rolland, G., Xu, J., Dupret, J. M., & Post, M. (1995). Expression and characterization of type IV collagenases in rat lung cells during development. *Experimental Cell Research.* **218**, 346-350.

Roman, J., LaChance, R. M., Broekelmann, T. J., Kennedy, C. J., Wayner, E. A., Carter, W. G., & McDonald, J. A. (1989). The fibronectin receptor is organized by extracellular matrix fibronectin: implications for oncogenic transformation and for cell recognition of fibronectin matrices. *Journal of Cell Biology* **108**, 2529-2543.

Roman, J., Limper, A. H., & McDonald, J. A. (1990). Lung extracellular matrix: physiology and pathophysiology. *Hospital Practice (Office Edition)* **25**(11), 125-8, 131-5, 139-40.

Roman, J., Little, C. W., & McDonald, J. A. (1991). Potential role of RGD-binding integrins in mammalian lung branching morphogenesis. *Development* **112**, 551-558.

Roman, J. & McDonald, J. A. (1992). Expression of fibronectin, the integrin alpha 5, and alpha-smooth muscle actin in heart and lung development. *American Journal of Respiratory Cell and Molecular Biology* **6**, 472-480.

Rosenbloom, J., Abrams, W. R., Indik, Z., Yeh, H., Ornstein-Goldstein, N., & Bashir, M. M. (1995). Structure of the elastin gene. *Ciba Foundation Symposium* **192**, 59-74.

Ross, M. G., Ervin, G., Leake, R. D., Fu, P., & Fisher, D. A. (1984). Fetal lung liquid regulation by neuropeptides. *American Journal of Obstetrics and Gynecology* **150**, 421-425.

Ruoslahti, E. & Pierschbacher, M. D. (1987). New perspectives in cell adhesion: RGD and integrins. *Science* **238**, 491-497.

Ruoslahti, E. & Yamaguchi, Y. (1991). Proteoglycans as Modulators of Growth Factor Activities. *Cell* **64**, 867-869.

Sage, H., Farin, F. M., Striker, G. E., & Fisher, A. B. (1983). Granular pneumocytes in primary culture secrete several major components of the extracellular matrix. *Biochemistry* **22**, 2148-2155.

Savich, R. D., Guerra, F. A., Lee, C.-C. H., Padbury, J. F., & Kitterman, J. A. (1992). Effects of acute oligohydramnios on respiratory system of fetal sheep. *Journal of Applied Physiology* **73**, 610-617.

Schellenberg, J.-C. & Liggins, G. C. (1987). Elastin and collagen in the fetal sheep lung. I. Ontogenesis. *Pediatric Research* **22** , 335-338.

Schellenberg, J.-C., Liggins, G. C., Kitterman, J. A., & Lee, C.-C. H. (1987a). Elastin and collagen in the fetal sheep lung. II. Relationship to mechanical properties of the lung. *Pediatric Research* **22**, 339-343.

Schellenberg, J.-C., Liggins, G. C., & Stewart, A. W. (1987b). Growth, elastin concentration, and collagen concentration of perinatal rat lung: effects of dexamethasone. *Pediatric Research* **21**, 603-607.

Schneeberger, E. E. & McCarthy, K. M. (1986). Cytochemical localization of Na^+-K^+-ATPase in rat type II pneumocytes. *Journal of Applied Physiology* **60**, 1584-1589.

Schnitzer, J. J., Hedrick, H. L., Pacheco, B. A., Losty, P. D., Ryan, D. P., Doody, D. P., & Donahoe, P. K. (1996). Prenatal glucocorticoid therapy reverses pulmonary immaturity in congenital diaphragmatic hernia in fetal sheep. *Annals of Surgery* **224**, 430-437.

Schonthal, A., Herrlich, P., Rahmsdorf, H. J., & Ponta, H. (1988). Requirement for fos gene expression in the transcriptional activation of collagenase by other oncogenes and phorbol esters. *Cell* **54**, 325-334.

Scott, J. E., Yang, S.-Y., Stanik, E., & Anderson, J. E. (1993). Influence of strain on [^{3}H] thymidine incorporation, surfactant-related phospholipid synthesis, and cAMP levels in fetal type II alveolar cells. *American Journal of Respiratory Cell and Molecular Biology* **8**, 258-265.

Scurry, J. P., Adamson, T. M., & Cussen, L. J. (1989). Fetal lung growth in laryngeal atresia and tracheal agenesis. *Australian Paediatric Journal* **25**, 47-51.

Seckl, J. R. (1998). Physiologic programming of the fetus. *Clinical Perinatology* **25**, 939-62.

Seckl, J. R. (2001). Glucocorticoid programming of the fetus; adult phenotypes and molecular mechanisms. *Molecular and Cellular Endocrinology* **185**, 61-71.

Segura-Valdez, L., Pardo, A., Gaxiola, M., Uhal, B. D., Becerril, C., & Selman, M. (2000). Upregulation of gelatinases A and B, collagenases 1 and 2, and increased parenchymal cell death in COPD. *Chest* **117**, 684-694.

Seidner, S., Pettenazzo, A., Ikegami, M., & Jobe, A. (1988). Corticosteroid potentiation of surfactant dose response in preterm rabbits. *Journal of Applied Physiology* **64**, 2366-2371.

Senior, R. M. & Shapiro, S. D. (1992). Introduction: the matrix metalloproteinase family. *American Journal of Respiratory Cell and Molecular Biology* **7**, 119.

Sepper, R., Konttinen, Y. T., Sorsa, T., & Koski, H. (1994). Gelatinolytic and type IV collagenolytic activity in bronchiectasis. *Chest* **106**, 1129-1133.

Seyer, J. M., Hutcheson, E. T., & Kang, A. H. (1976). Collagen polymorphism in idiopathic chronic pulmonary fibrosis. *Journal of Clinical Investigation* **57**, 1498-1507.

Shapiro, S. D. (1999). The macrophage in chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine* **160**, S29-S32.

Shapiro, S. D. & Senior, R. M. (1999). Matrix metalloproteinases. Matrix degradation and more. *American Journal of Respiratory Cell and Molecular Biology* **20**, 1100-1102.

Silva, P., Stoff, J., Field, M., Fine, L., Forrest, J. N., & Epstein, F. H. (1977). Mechanism of active chloride secretion by shark rectal gland: role of Na-K-ATPase in chloride transport. *American Journal of Physiology* **233**, F298-F306.

Silver, M. M., Thurston, W. A., & Patrick, J. E. (1988). Perinatal pulmonary hyperplasia due to laryngeal atresia. *Human Pathology* **19**, 110-113.

Simon, R. H., Scott, M. J., Reza, M. M., & Killen, P. D. (1993). Type IV collagen production by rat pulmonary alveolar epithelial cells. *American Journal of Respiratory Cell and Molecular Biology* **8**, 640-646.

Sinkin, R. A., Sanders, R. S., Horowitz, S., Finkelstein, J. N., & Lomonaco, M. B. (1995). Cell-specific expression of fibronectin in adult and developing rabbit lung. *Pediatric Research* **37**, 189-195.

Skinner, S. J. M. (1989). Fetal breathing movements: a mechanical stimulus for fetal lung cell growth and differentiation. In *Advances in Fetal Physiology.*, eds. Gluckman, P. D., Johnston, B. M., & Nathanielsz, P. W., pp. 133-151. Perinatology Press, New York.

Sloboda, D. M., Newnham, J. P., & Challis, J. R. (2000). Effects of repeated maternal betamethasone administration on growth and hypothalamic-pituitary-adrenal function of the ovine fetus at term. *Journal of Endocrinology* **165**, 79-91.

Sluiter, W., Bos, A. P., Silveri, F., Tenbrinck, R., Kraakslee, R., Tibboel, D., Koster, J. F., & Molenaar, J. C. (1992). Nitrofen-induced diaphragmatic hernias in rats: pulmonary antioxidant enzyme activities. *Pediatric Research* **32**, 394-398.

Smith, C. I., Hilfer, S. R., Searls, R. L., Nathanson, M. A., & Allodoli, M. D. (1990). Effects of β-D-xyloside on differentiation of the respiratory epithelium in the fetal mouse lung. *Developmental Biology* **138**, 42-52.

Smith, G. N., Kingdom, J. C., Penning, D. H., & Matthews, S. G. (2000). Antenatal corticosteroids: is more better? *Lancet* **355**, 251-252.

Smith, I. I. & Bain, A. D. (1964). Congenital atresia of the larynx. *Annotology, Rhinology and Laryngology* **74**, 338-349.

Song, G., Sun, B., Curstedt, T., Grossman, G. & Robertson, B. (1992). Effect of amiloride and surfactant on lung liquid clearance in newborn rabbits. *Respiration Physiology* **88**, 233-246.

Spooner, B. S. & Faubion, J. M. (1980). Collagen involvement in branching morphogenesis of embryonic lung and salivary gland. *Developmental Biology* **77**, 84-102.

Spooner, B. S. & Wessells, N. K. (1970). Mammalian lung development: interactions in primordium formation and bronchial morphogenesis. *Journal of Experimental Zoology* **175**, 445-454.

Spurzem, J. R. (1996). Function at the junction: dynamic interactions between lung cells and extracellular matrix. *Thorax* **51**, 956-958.

Stanton, H., Gavrilovic, J., Atkinson, S. J., D'Ortho, M. P., Yamada, K. M., Zardi, L., & Murphy, G. (1998). The activation of ProMMP-2 (gelatinase A) by HT1080 fibrosarcoma cells is promoted by culture on a fibronectin substrate and is concomitant with an increase in processing of MT1-MMP (MMP-14) to a 45 kDa form. *Journal of Cell Science* **111**, 2789-2798.

Stegemann, H. & Stalder, K. (1967). Determination of hydroxyproline. *Clinica Chimica Acta* **18**, 267-273.

Stein, H. M., Oyama, K., Martinez, A., Chappell, B. A., Buhl, E., Blount, L., & Padbury, J. F. (1993). Effects of corticosteroids in preterm sheep on adaptation and sympathoadrenal mechanisms at birth. *American Journal of Physiology* **264**, E763-E769.

Stettner, S. & Ledwozyw, A. (1995). Prenatal starvation, betamethasone and lung development in newborn rats. *Acta Veterinaria Hungaria* **43**, 303-309.

Strang, L. B. (1991). Fetal lung liquid: secretion and reabsorption. *Physiological Reviews* **71**, 991-1016.

Suen, H. C., Catlin, E. A., Ryan, D. P., Wain, J. C., & Donahoe, P. K. (1993). Biochemical immaturity of lungs in congenital diaphragmatic hernia. *Journal of Pediatric Surgery* **28**, 471-475.

Suen, H. C., Losty, P., Donahoe, P. K., & Schnitzer, J. J. (1994). Combined antenatal thyrotropin-releasing hormone and low-dose glucocorticoid therapy improves the pulmonary biochemical immaturity in congenital diaphragmatic hernia. *Journal of Pediatric Surgery* **29**, 359-363.

Swartz, M. A., Tschumperlin, D. J., Kamm, R. D., & Drazen, J. M. (2001). Mechanical stress is communicated between different cell types to elicit matrix remodeling. *Proceedings of the National Academy of Sciences, U.S.A* **98**, 6180-6185.

Swee, M. H., Parks, W. C., & Pierce, R. A. (1995). Developmental regulation of elastin production. *The Journal of Biological Chemistry* **270**, 14899-14906.

Switzer, B. R. & Summer, G. K. (1973). Inhibition of collagen synthesis by α,α'-dipyridyl in human skin fibroblasts in culture. *In Vitro* **9**, 160-166.

Taderera, J. V. (1967). Control of lung differentiation *in vitro*. *Developmental Biology* **16**, 489-512.

Taira, Y., Oue, T., Shima, H., Miyazaki, E., & Puri, P. (1999). Increased tropoelastin and procollagen expression in the lung of nitrofen-induced diaphragmatic hernia in rats. *Journal of Pediatric Surgery* **34**, 715-719.

Takeuchi, T. & Prockop, D. J. (1969). Biosynthesis of abnormal collagens with amino acid analogues. I. Incorporation of L-azetidine-2-carboxylic acid and cis-4-fluoro-L-proline into procollagen and collagen. *Biochimica et Biophysica Acta* **175**, 142-155.

Tannuri, U., Maksoud-Filho, J. G., Santos, M. M., Tannuri, A. C., Rodrigues, C. J., & Rodrigues, A. J. (1998b). The effects of prenatal intraamniotic surfactant or dexamethasone administration on lung development are comparable to changes induced by tracheal ligation in an animal model of congenital diaphragmatic hernia. *Journal of Pediatric Surgery* **33**, 1198-1205.

Tannuri, U., Rodrigues, C. J., Maksoud-Filho, J. G., Santos, M. M., Tannuri, A. C., & Rodrigues, A. J. (1998a). The effects of prenatal intraamniotic surfactant or dexamethasone administration on lung development are comparable to changes induced by tracheal ligation in an animal model of congenital diaphragmatic hernia: studies of lung glycogen content, elastic fiber density, and collagen content. *Journal of Pediatric Surgery* **33**, 1776-1783.

Tanswell, A. K., Buch, S., Liu, M. & Post, M. (2000). Factors mediating cell growth in lung injury. In *Chronic Lung Disease in Early Infancy*, eds. Bland, R. D. & Coalson, J. J., pp. 493-534. Marcel Dekker, New York.

Thomas, T. & Dziadek, M. (1994). Expression of collagen alpha1(IV), laminin and nidogen genes in the embryonic mouse lung; implications for branching morhogenesis. *Mechanisms of development* **45**, 193-201.

Thorburn, G. D. & Schneider, W. (1972). The progesterone concentration in the plasma of the goat during the oestrous cycle and pregnancy. *Journal of Endocrinology* **52**, 23-36.

Thurlbeck, W. M. (1975). Postnatal growth and development of the lung. *American Review of Respiratory Disease* **111**, 803-844.

Thurlbeck, W. M. (1992). Prematurity and the developing lung. *Clinics in Perinatology* **19**, 497-519.

Tolstoshev, P., Haber, R., Trapnell, B. C., & Crystal, R. G. (1981). Procollagen messenger RNA levels and activity and collagen synthesis during the fetal development of sheep lung, tendon, and skin. *The Journal of Biological Chemistry* **256**, 9672-9679.

Tomasek, J. J., Halliday, N. L., Updike, D. L., Ahern-Moore, J. S., Vu, T. K., Liu, R. W., & Howard, E. W. (1997). Gelatinase A activation is regulated by the organization of the polymerized actin cytoskeleton. *Journal of Biological Chemistry* **272**, 7482-7487.

Tournier, J. M., Polette, M., Hinnrasky, J., Beck, J., Werb, Z., & Basbaum, C. (1994). Expression of gelatinase A, a mediator of extracellular matrix remodeling, by tracheal gland serous cells in culture and *in vivo*. *The Journal of Biological Chemistry* **269**, 25454-25464.

Uhal, B. D. (1997). Cell cycle kinetics in the alveolar epithelium. *American Journal of Physiology* **272**, L1031-L1045.

Uitto, J. & Prockop, D. J. (1974). Incorporation of proline analogues into collagen for peptides. Effects on the production of extracellular procollagen and on the stability of the triple-helical structure of this molecule. *Biochimica et Biophysica Acta* **336**, 234-251.

Uno, H., Eisele, S., Sakai, A., Shelton, S., Baker, E., DeJesus, O., & Holden, J. (1994). Neurotoxicity of glucocorticoids in the primate brain. *Hormones and Behaviour* **28**, 336-348.

Uno, H., Lohmiller, L., Thieme, C., Kemnitz, J. W., Engle, M. J., Roecker, E. B., & Farrell, P. M. (1990). Brain damage induced by prenatal exposure to dexamethasone in fetal rhesus macaques. I. Hippocampus. *Brain Research: Developmental Brain Research* **53**, 157-167.

Upadhya, G. A. & Strasberg, S. M. (1999). Evidence that actin disassembly is a requirement for matrix metalloproteinase secretion by sinusoidal endothelial cells during cold preservation in the rat. *Hepatology* **30**, 169-176.

Van Wart, H. E. & Birkedal-Hansen, H. (1990). The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proceedings of the National Academy of Sciences, U.S.A* **87**, 5578-5582.

Vilos, G. A. & Liggins, G. C. (1982). Intrathoracic pressures in fetal sheep. *Journal of Developmental Physiology* **4**, 247-256.

Vu, T. H. & Werb, Z. (2000). Matrix metalloproteinases: effectors of development and normal physiology. *Genes and Development* **14**, 2123-2133.

Walker, D. W. (1988). Effects of increased core temperature on breathing movements and electrocortical activity in fetal sheep. *Journal of Developmental Physiology* **10**, 513-523.

Wallace, M. J., Hooper, S. B., & Harding, R. (1995). Effects of elevated fetal cortisol concentrations on the volume, secretion and reabsorption of lung liquid. *American Journal of Physiology* **269**, R881-R887.

Wallace, M. J., Hooper, S. B., & Harding, R. (1996). Role of the adrenal glands in the maturation of lung liquid secretory mechanisms in fetal sheep. *American Journal of Physiology* **270**, R1-R8.

Wallace, M. J., Lines, A. M., Polglase, G. P., & Hooper, S. B. (2002). IGF-II, PDGF-B, TGFβ1 and VEGF mRNA levels during increased lung expansion in fetal sheep. *American Journal of Physiology* (submitted).

Walters, D. V. (1994). Fetal lung liquid: secretion and absorption. In *Fetus and Neonate. Physiology and Clinical Implications.*, ed. Walters, D. V., pp. 42-62. Cambridge University Press., United Kingdom.

Walters, D. V. & Olver, R. E. (1978). The role of catecholamines in lung liquid absorption at birth. *Pediatric Research* **12**, 239-242.

Wang, A., Fan, M. Y., & Templeton, D. M. (1994). Growth modulation and proteoglycan turnover in cultured mesangial cells. *Journal of Cell Physiology* **159**, 295-310.

Warburton, D., Parton, L., Buckley, S., Cosico, L., Enns, G., & Saluna, T. (1988). Combined effects of corticosteroid, thyroid hormones, and β-agonist on surfactant, pulmonary mechanics, and β-receptor binding in fetal lamb lung. *Pediatric Research* **24**, 166-170.

Weibel, E. R. (1963). *Morphometry of the Human Lung.* Springer-Verlag, Berlin.

Weibel, E. R. & Gomez, D. M. (1962). A principle for counting tissue structures on random sections. *Journal of Applied Physiology* **17**, 343-348.

Weichsel, M. E. (1974). Glucocorticoid effect upon thymidine kinase in the developing cerebellum. *Pediatric Research* **8**, 843-847.

Werb, Z., Hembry, R. M., Murphy, G., & Aggeler, J. (1986). Commitment to expression of the metalloendopeptidases, collagenase and stromelysin: relationship of inducing events to changes in cytoskeletal architecture. *Journal of Cell Biology* **102**, 697-702.

Werb, Z., Tremble, P. M., Behrendtsen, O., Crowley, E., & Damsky, C. H. (1989). Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. *Journal of Cell Biology* **109**, 877-889.

Wessells, N. K. (1970). Mammalian lung development: interactions in formation and morphogenesis of tracheal buds. *Journal of Experimental Zoology* **175**, 455-466.

Wigglesworth, J. S. & Desai, R. (1981). Use of DNA estimation for growth assessment in normal and hypoplastic lungs. *Archives of Disease in Childhood* **56**, 601-605.

Wigglesworth, J. S., Desai, R., & Guerrini, P. (1981). Fetal lung hypoplasia: biochemical and structural variations and their possible significance. *Archives of Disease in Childhood* **56** , 606-615.

Wigglesworth, J. S., Desai, R., & Hislop, A. A. (1987). Fetal lung growth in congenital laryngeal atresia. *Pediatric Pathology* **7**, 515-525.

Wintour, E. M., Coghlan, J. P., Hardy, K. H., Hennessy, D. P., Lingwood, B. E., & Scoggins, B. A. (1980). Adrenal corticosteroids and immunoreactive ACTH in chronically cannulated ovine foetuses with bilateral adrenalectomy. *Acta Endocrinologica* **95**, 546-552.

Wirtz, H. R. & Dobbs, L. G. (2000). The effects of mechanical forces on lung functions. *Respiration Physiology* **119**, 1-17.

Xu, J., Liu, M., & Post, M. (1999). Differential regulation of extracellular matrix molecules by mechanical strain of fetal lung cells. *American Journal of Physiology* **276**, L728-L735.

Yamada, K. M., Akiyama, S. K., Hasegawa, T., Hasegawa, E., Humphries, M. J., Kennedy, D. W., Nagata, K., Urushihara, H., Olden, K., & Chen, W. T. (1985). Recent advances in research on fibronectin and other cell attachment proteins. *Journal of Cell Biochemistry* **28**, 79-97.

Yan, L., Moses, M. A., Huang, S., & Ingber, D. E. (2000). Adhesion-dependent control of matrix metalloproteinase-2 activation in human capillary endothelial cells. *Journal of Cell Science* **113** (22), 3979-3987.

Zeltner, T. B., Caduff, J. H., Gehr, P., Pfenninger, J., & Burri, P. H. (1987). The postnatal development and growth of the human lung. I. Morphometry. *Respiration Physiology* **67**, 247-267.