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Prenatal Alcohol Exposure and the Effects on the Developing Kidney and Long-Term Adult Health Outcomes

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

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August 2009

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Abstract

It is well known that chronic fetal exposure to high levels of alcohol can result in developmental abnormalities. However, the effects of low concentrations or acute exposure to ethanol on fetal development have been less well studied, and this is particularly the case for the kidney. In recent years, a variety of environmental factors have been shown to result in a deficit in nephron endowment, and numerous studies have associated this nephron deficit with the development of adult hypertension and renal functional abnormalities. This thesis explores the effects of chronic and acute fetal ethanol exposure on kidney development in sheep and rat models. *In vitro* and *in vivo* studies were conducted.

To investigate the effects of chronic prenatal ethanol exposure on kidney development, pregnant ewes (Merino Border-Leicester) were intravenously infused with ethanol 0.75g/kg body weight per day, infused over 1hr from day 95 of gestation until day 134 of gestation (gestation in sheep is approximately 147 days). The fetal kidneys were then taken for unbiased stereological determination of total nephron number and quantitative real-time PCR analysis of gene expression. Ethanol-exposed fetal sheep kidneys contained 11% fewer nephrons than saline-exposed kidneys, but were similar in weight. Analysis of renal expression of 14 genes failed to identify any differences between ethanol-exposed and saline-exposed fetuses. This study has been published (Gray, SP, Kenna, K, Bertram, JF, Hoy, WE, Yan, EB, Bocking, AD, Brien, JF, Walker, DW, Harding, R & Moritz, KM: Repeated ethanol exposure during late gestation decreases nephron endowment in fetal sheep. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology,* 295**:** R568-R574, 2008).

To investigate the effects of acute prenatal ethanol exposure on kidney development and the long-term consequences for cardiovascular and renal function, pregnant Sprague-Dawley rats were administered ethanol (1g/kg) by gavage at embryonic days 13.5 and 14.5. Nephron number was determined in offspring at postnatal day 30, and cardiovascular and renal function were analysed at 6 months of age. Ethanol-exposed male and female offspring were growth

restricted in comparison to controls for the first 30 days of postnatal life, and at postnatal day 30 the kidneys contained approximately 20% fewer nephrons. At 6 months of age, ethanol-exposed male and female offspring had a 10mmHg elevation in mean arterial pressure without changes in heart rate. Male ethanol-exposed offspring had elevated glomerular filtration rate and renal blood flow, while female ethanol-exposed offspring had lower glomerular filtration rate and renal blood flow, with elevated renal vascular resistance.

Rat metanephric organ culture was employed to investigate the direct effects of ethanol on kidney development. In addition, retinoic acid, a promoter of kidney development was added to culture to determine whether any ethanol-induced alterations to kidney development could be prevented by co-culture with retinoic acid. Embryonic day 14 kidneys were cultured with ethanol (0.2%) and/or retinoic acid (10nM & 20nM), and ureteric branching morphogenesis was quantified after 48 hours. Kidneys cultured in the presence of ethanol contained 20% fewer ureteric branch points and tips than control kidneys. The ureteric trees in kidneys co-cultured with ethanol and retinoic acid (10nM) were similar to control kidneys, demonstrating retinoic acid could prevent the adverse effects of ethanol.

The results of the studies described in this thesis demonstrate that prenatal ethanol exposure in both the chronic and acute settings results in a permanent reduction in nephron number. Acute (2 days) prenatal ethanol exposure in rats resulted in elevated arterial pressure and sex-specific changes in renal function at 6 months. *In vitro* experiments demonstrated aberrant changes in ureteric branching following culture in the presence of ethanol, and these changes could be blocked by co-culture with retinoic acid. This suggests that the retinoic acid signalling pathway may, in part, contribute to the effects of ethanol on the developing kidney. The present findings of the adverse effects of low levels of alcohol exposure on kidney development suggest further studies in this area are warranted. Ultimately, information from studies such as these should be used to advise pregnant women, and women considering becoming pregnant.

General Declaration

In accordance with Monash University Regulation 17/ Doctor of Philosophy and Master of Philosophy (MPhil) regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in peer reviewed journals and two unpublished publications. The core theme of the thesis is the effects of alcohol exposure on the developing kidney. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working with the department of Anatomy & Developmental Biology, Monash University, under the supervision of Prof. John F Bertram and Dr. Karen M Moritz.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapters 2, 3 and 4 my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Publication State**	**Nature and extent of candidate's contribution**
2	Repeated Ethanol Exposure During Late Gestation Decreases Nephron Endowment in Fetal Sheep	Published; Gray *et al* 2008, *Am J Physiol Regul Integr Comp Physiol* . R568-R574	All tissue, data analysis and interpretation of data preformed by candidate. Publication written by candidate. 50% of animal work performed by candidate.
3	Acute Alcohol Exposure Early in Gestation Results in Long-Term Cardiovascular and Renal Functional Consequences	Prepared for Submission to *JASN*	Performed all experiments, complied and analysed all data and interpreted data and wrote the manuscript 80%
4	Retinoic Acid Rescues Ethanol-Induced Inhibition of Ureteric Branching Morphogenesis *In Vitro*.	Prepared for Submission to *Kidney International*	Performed all experiments, complied and analysed all data and interpreted data and wrote the manuscript 80%

Signed:

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Date:

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Acknowledgements

Firstly, I would like to thank the support of my supervisors, Professor John Bertram and Dr Karen Moritz for their continual support, patience, understanding and guidance throughout my PhD.

I would like to thank Associate Professor Kate Denton for her advice and guidance on the physiological aspects of the studies, I learnt a lot and am continually learning just how complex cardiovascular and renal physiology actually is.

Dr Luise Cullen-McEwen, a massive thank you for assisting in the *in vitro* studies. I can honestly say that the studies would never have gotten to the stage that they are at without your assistance.

I am grateful to all the research assistants that have helped me over the years, Ms Rebecca Douglas-Denton, Ms Debbie Arena, Ms Rebecca Flower and Mr Chaminda Premaratne your assistance made my studies progress much more smoothly than they otherwise would have. To all members of the Bertram lab, thank you for all of your assistance over the course of my PhD.

To my fellow PhD students, my second family. Reetu, Lina and Sakil, you guys made my PhD life more enjoyable from the start. Our regular “brainstorming” sessions provided a great support network to deal with a PhD. Ken, your friendship, support, advice and guidance over the years has been invaluable. The gang, Megan, Ryan, Sarah, Benjie, Jonathan, Kelly, Chantal and all the other students who have come and gone, you just being there made many days so much more enjoyable, and I don’t think that I would have enjoyed my time as much as I did without you all. In some way, shape and form you have all influenced me greatly and I hope that many of us will remain good friends for years to come. Thank you.

To the administration folk of the department, Megan and Chris, thanks for reminding me over the years when I had paperwork to fill out and when I had to have it done by. Thanks for just being there to take care of it all when clearly it was falling apart.

My Family, Mum, Dad, Sally, Nan, Pa, Gran, Gramps, Nanna (RIP) and the rest, thank you for your support and patience over the years. Specifically I would like to thank you all for smiling when I discussed my project, even though you may not have had the slightest bit of interest in what I was doing or even understood what I was going on about. Thank you for reminding me where I came from and how far I have come. Your contribution, no matter how small it may have been has meant more to me than you may think. Thank you

The housemate and great friend, Kim, thanks for putting up with my terrible moods, the random mental breakdowns, the flip outs and my disarray that was my mind towards the end. Thank you for the distractions that you provided and ensuring that I ate. Thanks.

Lastly, to all my friends, Justine, Phina, Mark, Kim, Ken, Megs, Megan and all the new ones that I have gained throughout my PhD, there are just so many of you. I don't really know where to begin. I would like to thank you all, as you have been there for me when I thought that it was all falling apart, you were there when I would complain for hours on end and you didn't stop me. You have been there throughout the entire process, your support, patience, guidance and advice over the years has meant the world to me and without it I doubt I would be writing this page. So thank you all so much and here is hoping that it all works out ok.

When all seems to fail, hold onto hope, things will improve

Publications & Abstracts

Peer Reviewed Papers:

Gray SP, Kenna K, Bertram JF, Hoy WE, Yan WB, Bocking AD, Brien JF, Walker DW, Harding R, Moritz KM, (2008). *"Repeated Ethanol Exposure During Late Gestation Decreases Nephron Endowment in Fetal Sheep".* American Journal of Physiology: Integrative, Comparative & Regulatory Physiology. 295: R568-R574.

Abstracts:

Gray SP, Cullen-McEwen LA, Moritz KM, Bertram JF, (2009) "*Retinoic Acid Ameliorates the Effects of Ethanol Exposure On Ureteric Branching Morphogenesis in Cultured Rat Metanephroi*". 45th Annual Australian & New Zealand Society of Nephrology Meeting, 2009.

Harding R, Kenna K, Stokes V, Parkington H, Tare M, **Gray SP**, Bocking A, Brien J, Sozo F, Walker D, (2009) "*Episodic Ethanol Exposure has Multiple Effects on the Fetus".* Annual Fetal and Neonatal Physiological Society Meeting, 2009.

Kenna K, Yan E, **Gray SP**, Bocking A, Brien J, Walker D, Harding R, (2009) "*Physiologic responses to daily alcohol infusion in the ovine fetus and ewe during late gestation".* Society for Gynaecological Investigation 2009 Annual Meeting.

Gray SP, Denton KM, Bertram JF, Moritz KM, (2008) *"Acute Prenatal Ethanol Exposure Results In Elevated Mean Arterial Pressure and Altered Renal Function in Offspring at Six Months of Age".* High Blood Pressure Research Council of Australia Annual Scientific Conference, 2008.

Gray SP, Cullen-McEwen LA, Bertram JF, Moritz KM, (2008) "*Ethanol Exposure and the Effects on the Developing Kidney".* 2nd NGED Early Career Researcher Scientific Meeting, A24. (Oral Presentation; Best PhD Student Presentation).

Gray SP, Cullen-McEwen LA, Moritz KM, Bertram JF, (2008) *"Ethanol Exposure Results in Fewer Ureteric Branch Points and Glomeruli in Cultured Metanephroi".* The Proceedings of the Australian Society for Biochemistry and Molecular Biology, **40**: A64. (Poster Presentation; Best Developmental Biology Poster Presentation).

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Kenna K, Yan E, **Gray SP**, Watson C, Bocking A, Brien J, Walker D, Haring R, (2007) *"Acute and Chronic Physiological Effects of Repeated Alcohol (Ethanol) Exposure in Maternal and Fetal Sheep"* Developmental Origins of Health and Adult Diseases, Satellite Conference: 62. (Poster Presentation).

Kenna K, Yan E, **Gray SP**, Bocking A, Brien J, Harding R, Walker D, (2007). *"Effects of ethanol exposure during late gestation on physiological status and growth in fetal sheep".* Fetal & Neonatal Workshop of Australia and New Zealand, 21st Annual Meeting: A23. (Oral Presentation).

Gray SP, Moritz KM, Bertram JF, (2007). *"The effects of alcohol exposure on the developing rat kidney".* Fetal & Neonatal Workshop of Australia and New Zealand, 21st Annual Meeting: A12. (Oral Presentation).

Kenna K, Yan E, **Gray SP**, Bocking A, Brien J, Harding R, Walker D, (2007). *"Physiological Effects of Repeated Maternal Ethanol Exposure During Late Gestation."* Journal of Paediatric Child Health **43** (Supplement 1): A121. (Poster Presentation).

List of Abbreviations

ADH	Alcohol Dehydrogenase
ALDH	Aldehyde Dehydrogenase
ARBD	Alcohol Related Birth Defects
ARND	Alcohol Related Neurodevelopmental Disorders
AT1	Angiotensin type 1
AT1b	Angiotensin II Receptor type 1b (rat only)
AT2	Angiotensin II Receptor type 2
AVP	Arganine Vasopressin
BAC	Blood Alcohol Concentration
BAX	Bcl-2 Associated X Protein
BEC	Blood Ethanol Concentration
BMP4	Bone Morphogenic Protein Type 4
BMP7	Bone Morphogenic Protein Type 7
CNS	Central Nervous System
CVD	Cardiovascular Disease
DGA	Days of Gestational Age
DOHaD	Developmental Origins of Health and Adult Diseases
ENaCα	Epithelial Sodium Channel type α
ENaCβ	Epithelial Sodium Channel type β
ENaCγ	Epithelial Sodium Channel type γ
FAS	Fetal Alcohol Syndrome
FASD	Fetal Alcohol Spectrum Disorders
FGF7	Fibroblast Growth Factor type 7
FF	Filtration Fraction

GDNF	Glial Cell Line Derived Neurotrophic Factor
GFRα1	Glial Cell Line Derived Neurotrophic Factor Family receptor α 1
GFR	Glomerular Filtration Rate
GN	Glomerular Number
HR	Heart Rate
IGF	Insulin Like Growth Factor
MAP	Mean Arterial Pressure
MEOS	Microsomal Ethanol Oxidising System
Na^+/K^+ ATPase (α)	Sodium-Potassium Adenosine Triphosphotase α Subunit
Na^+/K^+ ATPase (β)	Sodium-Potassium Adenosine Triphosphotase β Subunit
Na^+/K^+ ATPase (γ)	Sodium-Potassium Adenosine Triphosphotase γ Subunit
NH&MRC	National Health & Medical Research Council
NO	Nitric Oxide
Pax-2	Paired Box type 2
PN	Postnatal Age
PNA	Peanut Agglutinin
RA	Retinoic Acid
RARα	Retinoic Acid Receptor type α
RAS	Renin-angiotensin System
RBF	Renal Blood Flow
RPF	Renal Plasma Flow
RVR	Renal Vascular Resistance
RXRα	Retinoic X Receptor type α
SEM	Standard Error of the Mean
TGFβ1	Transforming Growth Factor β1
TGFβ2	Transforming Growth Factor β2

TGFβ3	Transforming Growth Factor β3
UBPs	Ureteric Branch Points
UTs	Ureteric Tips
WHO	World Health Organisation
Wnt4	Wingless type 4
Wnt11	Wingless type 11
WT-1	Wilms Tumour Gene

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Chapter 1

General Introduction

1.1. General Introduction

Alcohol is one of the most commonly consumed drugs, and the incidence of alcohol consumption by pregnant women is increasing in a number of countries. Furthermore, prenatal alcohol exposure is one of the most common fetal teratogenic exposures in the western world. The deleterious effects of chronic exposure to high levels of alcohol on fetal development are well established, however the effects of exposure to low or moderate levels, particularly acutely, are relatively unknown. This is of clinical importance, as there is some evidence to suggest that pregnant women are more likely to consume alcohol in short, acute episodes rather than in chronic volumes over a longer period of time.

A large amount of research has been conducted investigating the effects of prenatal alcohol exposure on the developing nervous and cardiovascular systems. However, surprisingly little research has focused on the effects of prenatal alcohol exposure on the developing kidney, although prenatal alcohol exposure has been reported to affect urine production and the ability of the kidney to regulate sodium and potassium homeostasis. This is important as the kidney plays a pivotal role in the long-term regulation of fluid and electrolyte balance, and arterial blood pressure. Furthermore, there is increasing evidence that suboptimal renal function, through a reduction in nephron endowment is associated with the development of adult disease, including hypertension.

Brenner and colleagues (1988) first proposed that the likelihood of developing hypertension in adulthood might be associated with having a lower nephron endowment. Subsequent studies in humans and in animal models have identified inverse correlations between nephron endowment and arterial pressure, with low nephron endowment associated with higher blood pressure and high nephron endowment associated with lower blood pressure. In addition, many studies have identified that exposure to a sub-optimal *in utero* environment can lead to a permanent reduction in nephron endowment and subsequent development of hypertension.

The suboptimal fetal environments evaluated have included maternal dietary protein restriction, elevation in maternal stress hormones, placental insufficiency and vitamin deficiencies. However, prior to the present study, the effects of fetal alcohol exposure on nephron endowment had not been investigated and the effects on long-term cardiovascular and renal function were not known.

This introductory chapter begins with a brief description of the structure, pharmacology and metabolism of alcohol, and then considers the epidemiology of alcohol consumption in the greater community, with a focus on pregnant women. The consequences of alcohol exposure *in utero* and the long-term health outcomes are then discussed in the context of fetal programming. Mammalian kidney development is then described as well as our current understanding of the effects of ethanol on kidney development. Finally, the hypothesis and aims of the experimental studies described in this Thesis are presented.

1.2. Alcohol

1.2.1 Chemical Structure

Alcohol (ethanol) is a simple 2-carbon alcohol-based carbohydrate forming a dipole molecule. Because of the few hydrogen bonds present in the molecule, alcohol is readily soluble in water, allowing for it to be readily absorbed by the body (Figure 1.1). Alcohol does not have an asymmetric carbon, allowing it to undergo chemical interactions with other biological substrates. The hydroxyl group provides a dipole that readily forms new hydrogen bonds with other substrates, such as proteins and other carbohydrates. These capabilities allow alcohol to permeate cells readily.

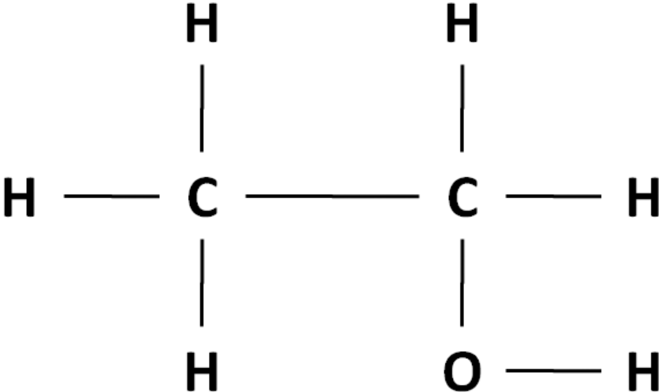

Figure 1.1 – The Chemical Structure of Ethanol

1.2.2 Pharmacology

Ethanol is a depressant drug, causing an anaesthetic type response in the central nervous system (CNS) [Eckardt *et al* 1998, Weiss and Porrino 2002]. The effects of alcohol are non-selective and therefore it can affect many cell functions and properties including: (1) membrane organisation, increasing the fluidity of the cellular membrane through recruitment of cholesterol to the membrane [Goldstein *et al* 1981]; (2) enzyme function, such that alcohol can bind to the active site of enzymes causing a conformational change and rendering then inactive [Tuma *et al* 2003]; and (3) ion channel activity, for example, the Na, K-ATPase channels increase in activity when alcohol is present, resulting in sodium leakage and thus altering sodium homeostasis [Blachley *et al* 1985].

However, the most studied effects of ethanol are those on the CNS, specifically the neurotransmitters gamma-aminobutyric acid (GABA), N-methyl-D-aspartate (NMDA) and serotonin pathways [Eckardt *et al* 1998]. Alcohol enhances the action of GABA, acting on the alpha receptor in a similar fashion to that of benzodiazepines, although the magnitude of the effect elicited by alcohol is smaller. More specifically, alcohol inhibits the release of the neurotransmitter by causing a depolarisation, inhibiting the opening of voltage-sensitive calcium channels in neurons. This causes hyperpolarisation of the neuronal membrane and mild sedation [Eckardt *et al* 1998]. The NMDA subtype of the glutamate receptor is considered to be the most sensitive to the actions of alcohol. The excitatory properties of NMDA are inhibited by the actions of alcohol causing a depressant phenotype. Other effects produced by alcohol include an enhancement of the excitatory effects through activation of the atypical clozapine (nAChRs) and 5-hydroxy tryptamine (5-HT_3) receptor subtypes of the serotonin receptor pathway [Eckardt *et al* 1998]. Overall, alcohol depresses the CNS, resulting in the typical behavioural phenotype observed in intoxicated individuals, which

includes slurred speech, motor incoordination, increased self-confidence and a sense of euphoria.

1.2.3 Alcohol Metabolism and the Effects on the Body

1.2.3.1 Alcohol Metabolism

Elimination of alcohol from the body occurs predominantly through metabolism, with small fractions of alcohol being excreted in the breath (0.7%), sweat (0.1%) and urine (0.3%) [Ramchandani *et al* 2001]. Alcohol metabolism occurs chiefly within the liver by hepatic oxidation and is governed by the catalytic enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). The rate of alcohol metabolism has a high degree of inter-individual and ethnic variability, which in part is due to allelic variants of the genes encoding ADH and ALDH which give rise to isoenzymes that metabolise alcohol at different rates [Ramchandani *et al* 2001]. However, the rate of alcohol clearance is relatively constant between individuals with 0.015g/dl of alcohol cleared per hour once alcohol consumption has stopped.

An important aspect of alcohol metabolism to note is that there is a large degree of variability between species in the rate at which alcohol is metabolised. While all mammalian species express the enzymes required to metabolise alcohol, the actual rate at which the enzymes function to clear alcohol from the system varies. Thus, rodents, including rats and mice, metabolise alcohol at a much faster rate than humans, while larger animal species such as sheep and non-human primates have similar rates of alcohol metabolism to humans [Matsumoto and Fukui 2002].

The process of alcohol metabolism can be broken down into three sequential steps, as shown in Figure 1.2. The first step involves the oxidation of alcohol to acetaldehyde, which is then

further oxidised to acetate. Finally, acetate is converted to carbon dioxide and water allowing for exhalation and excretion, respectively.

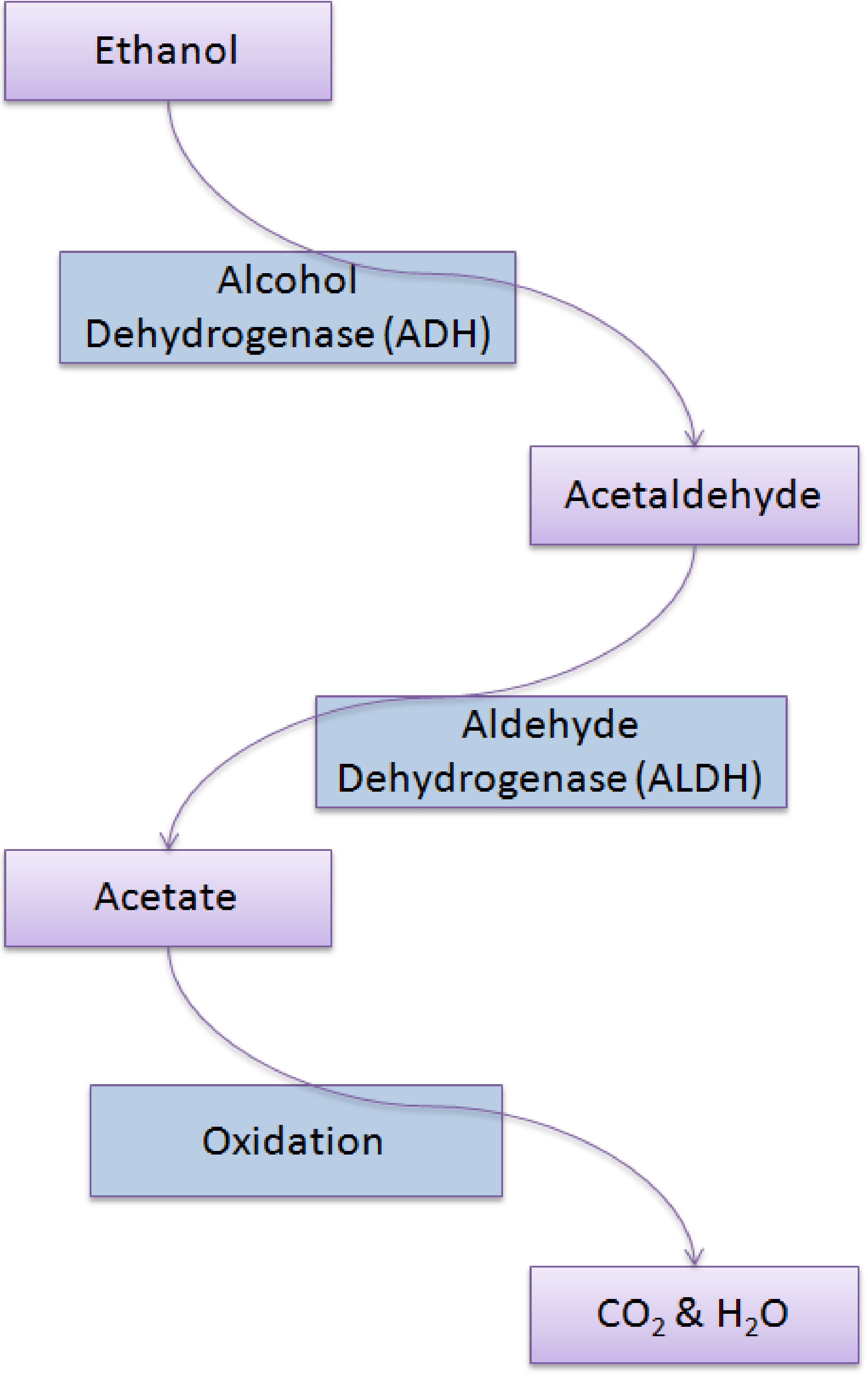

Figure 1.2 – Schematic Diagram of Ethanol Metabolism

1.2.3.1.1 Step 1: Ethanol Metabolism to Acetaldehyde

As previously stated, the first step involved in the metabolism of ethanol is the oxidation of ethanol to acetaldehyde. This process is principally governed by the enzyme ADH. However, this process can also occur through the activity of the microsomal ethanol oxidizing system (MEOS) [Suddendorf 1999].

1.2.3.1.1.1 Alcohol Dehydrogenase (ADH)

Enzymatic conversion of alcohol to acetaldehyde by ADH is the most common pathway utilised by the liver. ADH is a NAD^+ dependant cytosolic enzyme principally located in the liver but found to a lesser extent in the stomach, lungs and kidneys. ADH is a zinc-containing, dimeric molecule, which catalyses the oxidation of alcohol by the removal of hydrogen from the hydroxyl group forming an aldehyde complex, acetaldehyde [Suddendorf 1999, Lieber 2000].

$$CH_3CH_2OH + NAD^+ \rightarrow CH_3CHO + NADH + H^+$$

In the human and rodent there are five classes of ADH (ADHI-V) that have independent expression profiles that are time and tissue-specific [Suddendorf 1999]. While all of the ADH classes have the capacity to metabolise alcohol, it is predominantly ADHI and II that are ubiquitously expressed in the liver. ADHIII and IV are expressed in the mucosa of the gastrointestinal tract allowing for the initial metabolism of alcohol to occur in the wall of the gastrointestinal tract. The role of ADHV in alcohol metabolism has yet to be fully determined [Suddendorf 1999].

1.2.3.1.1.2 Microsomal Ethanol Oxidizing System (MEOS)

In the event that large amounts of alcohol have been consumed ADH becomes saturated and unable to metabolise alcohol to acetaldehyde fast enough. This results in the activation of an additional metabolic pathway, MEOS. MEOS is found within the smooth endoplasmic reticulum and is a NADPH-dependant system, using oxygen as a consumable.

$$CH_3CH_2OH + NADPH + H^+ + O_2 \rightarrow CH_3CHO + NADP^+ + 2H_2O$$

The key enzyme involved in this pathway is the alcohol inducible cytochrome P450E1 (CYP2E1), the levels of which are increased 4- to 10-fold following chronic alcohol administration [Lieber 2000].

1.2.3.1.2 Step 2: Metabolism of Acetaldehyde to Acetate

Acetaldehyde is toxic and needs to be quickly metabolised. ALDH metabolises acetaldehyde to acetate, via:

$$CH_3CHO + NAD^+ + H_2O \rightarrow CH_3CHOOH + NADH + H^+$$

While the enzymes involved in the oxidation of alcohol are primarily located within the liver, the two major isoforms of ALDH (ALDH1 and ALDH2) are found throughout the body [Lieber 2000]. This widespread distribution facilitates rapid metabolism of acetaldehyde to acetate before cellular damage occurs.

1.2.3.1.3 Step 3. Metabolism of Acetate to Carbon Dioxide (CO_2) and Water (H_2O)

Oxidation of acetate occurs relatively quickly in most organs producing CO_2 and H_2O [Lands 1991]. The clearance of acetate is dependent on age and sex, with the clearance rate slowing with age. Women metabolise acetate slower than men [Lands 1991].

1.2.3.2 Alcohol Consumption and Consequences on Nutrition

For many years it was believed that the poor nutritional status of alcoholics was due to their poor diet. However, evidence from experimental and clinical studies suggests that despite an adequate balanced diet, alcohol consumption has the capacity to alter one's nutritional status, leading to malnutrition and hepatotoxicity, compounding the systemic effects of alcoholic liver diseases, if present.

While the exact mechanism by which alcohol consumption causes malnutrition are unknown, it is believed to be the result of the multifactorial influence of poor diet, malabsorption, maldigestion and the interaction of alcohol with nutrients, inhibiting their use by the body.

1.2.3.2.1 Alcohol and Digestion (Maldigestion)

Alcohol in high concentrations increases the transit rate of food along the digestive tract, thereby limiting digestion. Alcohol decreases the number of type I (impeding) peristaltic waves of the jejunum, while increasing the type III (propulsive) waves in the ileum [Sullivan *et al* 1964, Marin *et al* 1973]. The reduction in number of type I waves results in the food progressing through the digestive tract faster than it would otherwise do, and with the enhanced propulsion waves in the ileum, the region of the gastrointestinal tract where most

digestion occurs, food fails to digest adequately before it is eliminated [Sullivan *et al* 1964, Marin *et al* 1973].

1.2.3.2.2 Alcohol and Nutrient Absorption (Malabsorption)

Excessive alcohol consumption or even a single dose of alcohol leads to disturbances in the intestinal absorption of nutrients. Chronic excessive alcohol consumption all but eliminates the absorption of sodium and water, resulting in diarrhoea and steatorrhea due to mucosal wall bleeding and duodenal erosion [Bode *et al* 2003]. One of the most sensitive vitamins to malabsorption is vitamin A, which is essential for normal vision. Vitamin A deficiencies can result in dark blindness. In acute episodes of alcohol consumption the levels of vitamin A do not change, however prolonged consumption of alcohol can lead to a deficiency in vitamin A in the liver [Lieber 2003]. Alcohol fed to rats daily for 4 to 6 weeks resulted in a 60% decrease in hepatic vitamin A levels and an additional 3 to 6 weeks of alcohol exposure led to a further 12% decline in vitamin A levels [Leo and Lieber 1999]. Even supplementing the diet with vitamin A could not elevate the level of vitamin A in the liver [Leo and Lieber 1999]. Despite the lower liver vitamin A levels, blood vitamin A levels were normal, indicating that alcohol could interrupt the conversion of β-carotene to vitamin A in the liver, but not the blood. Alcoholic patients often present with a reduced vitamin A level in their liver but their blood levels are normal. Similarly to the rat study, alcoholic patients typically have a normal blood concentration of β-carotene, suggesting that alcohol consumption alters the capacity of the liver to convert β-carotene to vitamin A [Lieber 2003]. Vitamins are not the only molecules that fail to be adequately absorbed following alcohol consumption, as essential amino acids have also been found to be not as adequately absorbed when animals are fed alcohol [Stanko *et al* 1979].

1.3 Epidemiology of Alcohol Consumption

The estimated worldwide per capita consumption of alcohol is 5.8 litres of pure (100%) per year [Anderson 2006, Popova *et al* 2007]. However, the per capita consumption of alcohol varies widely between countries. The highest rate of consumption occurs in European countries such as Germany, France and Russia, where approximately 12 litres of alcohol per year per adult is consumed [WHO 2004, Anderson 2006, Popova *et al* 2007]. The lowest volume of alcohol consumption occurs in Islamic countries of the Middle East and South Eastern Europe such as Saudi Arabia, the United Arab Emirates and Turkey, with an average of 0.6 - 1.3 liters per year per adult [WHO 2004, Anderson 2006, Popova *et al* 2007].

The worldwide per capita consumption of alcohol remained relatively stable for approximately 30 years, but in the last 5 to 10 years consumption has increased, particularly amongst the young and more specifically amongst young women [WHO 2004]. Most government agencies acknowledge that women, and more specifically young women, should not consume more than 1 standard drink per day, whereas the recommendations for men are generally no more than 2 standard drinks in one day [WHO 2004, NH&MRC 2009]. However, epidemiological studies have indicated that heavy drinking is more common in young individuals than older individuals, and it has been reported that women aged 18-39 years were significantly more likely to consume alcohol in episodic heavy drinking than those aged over 40 years [Nayak *et al* 2004, Strandberg-Larsen *et al* 2008]. Furthermore, it has been identified that approximately 7% of women in Britain of childbearing age consume alcohol at levels that are classified as risky [Nayak *et al* 2004]. With the increase in alcohol consumption amongst young women, it has been identified that there is also an increase in the percentage of women who fall pregnant while intoxicated and continue to engage in their normal drinking patterns until the pregnancy is confirmed [Nayak *et al* 2004, Chang *et al* 2006, Strandberg-Larsen *et al* 2008]. Usually,

pregnancy is not confirmed until approximately the 6th to 8th week of gestation, leaving 6 to 8 weeks of potential fetal alcohol exposure.

1.4 Alcohol Consumption during Pregnancy and the Consequences

1.4.1 International Guidelines on Alcohol Consumption During Pregnancy

The guidelines given to pregnant women advising the "safe" levels of alcohol consumption vary amongst western countries. While many of the guidelines issued by government health agencies in Western countries, such as Australia, USA, Britain and majority of mainland Europe, advise pregnant women to abstain from drinking while pregnant, most advise that women who choose to consume alcohol should not drink to intoxication, but should limit their intake to 2 to 3 standard drinks in the one sitting and never exceed 7 to 8 standard drinks in a week [WHO 2004]. In Australia the guidelines issued to pregnant women have recently been reviewed by the National Health & Medical Research Council (NH&MRC). The new guidelines recommend that 'not drinking is the safest option' [NH&MRC 2009]. Similar guidelines are advised by British and US health agencies with a consistent message of abstinence being recommended [O'Leary *et al* 2007]. However, the new Australian guidelines also state that women who choose to drink should never become intoxicated and should never consume more than 3 standard drinks in the one sitting or exceed 7 standard drinks per week [NH&MRC 2009]. These new Australian guidelines, while considered an improvement on what was previously recommended to pregnant women, have been suggested to not go far enough and have received criticism from the medical and scientific community [Whitehall 2007, Peadon *et al* 2007]. For example, it has been suggested that the guidelines should be similar to those of the USA and New Zealand where a consistent abstinence of alcohol consumption is recommended [O'Leary *et al* 2007]. O'Leary *et al* (2007) considers that a consistent message of abstinence avoids the confusion and inconsistency that exists in the current guidelines, and also points out that the different states of Australia have different additional recommendations on top of those issued by the NH&MRC. For example, the New

South Wales Department of Health recommends that abstinence is the safest option as even a small amount of alcohol may be harmful. However, the Western Australian Department of Health only advises that a reduction in alcohol consumption is recommended [Peadon *et al* 2007]. Employing a nationally consistent message of abstinence would avoid the confusion that currently exists between states.

The reported incidence of alcohol consumption by pregnant women also varies between western countries. While the majority of Western countries recommend that abstinence is the safest option, many studies report that while many women abstain from consuming alcohol while pregnant, a minority of women continue to consume alcohol [Caetano *et al* 2006, Chang *et al* 2006, Colvin *et al* 2007, Giglia *et al* 2007]. A recent Western Australian study reported that women generally reduced their consumption of alcohol and the number of standard drinks on a typical occasion as their pregnancy progressed. However, 58.7% of women still consumed alcohol in at least one trimester of their pregnancy [Colvin *et al* 2007]. In addition, it was found that 10 to 14% of pregnant women consumed alcohol above the recommended levels [Colvin *et al* 2007]. However, information pertaining to the number of standard drinks consumed in a typical drinking occasion was not obtained, making it difficult to estimate the level of dangerous drinking that was occurring. In a similar study, also undertaken in Western Australia, it was identified that 35% of pregnant women consumed alcohol during pregnancy, with 82% of these women consuming up to 2 standard drinks per week [Giglia *et al* 2007]. A small percentage (13%) of women consumed more than 2 standard drinks in the one week, while 7% consumed more than 7 standard drinks in the one week [Giglia *et al* 2007]. This pattern of alcohol consumption by pregnant women is not isolated to Australia, with US and British studies similarly reporting that many pregnant women consume alcohol while pregnant [Caetano *et al* 2006, Change *et al* 2006, Tsai *et al* 2007]. For example, Caetano *et al* (2006) reported that 44.3% of British women drank while pregnant, and that 1.2% of these women engaged in binge drinking. Tsai *et al* (2007) reported that 28.5% of American

pregnant women consumed 5 or more standard drinks on a typical drinking occasion [Tsai *et al* 2007]. In Russia, where 90% of non-pregnant women consume alcohol on a daily basis, the incidence of alcohol consumption by pregnant women is relatively common [Kristjanson *et al* 2007]. In a questionnaire survey of pregnant women in Russia it was identified that 60% continued to consume alcohol at the same levels as prior to becoming pregnant, while 7.4% consumed more than 5 standard drinks the last time they had a drinking session [Kristjanson *et al* 2007]. One major disadvantage of employing a questionnaire survey to obtain epidemiological data on alcohol consumption is that it allows for under-reporting, in this case to avoid the social stigma associated with alcohol consumption while pregnant. Nevertheless, more than 90% of the pregnant women surveyed acknowledged that alcohol consumption had detrimental effects on the developing fetus [Kristjanson *et al* 2007]. Several other studies have also reported that a large majority of pregnant women acknowledge that the alcohol they are consuming is detrimental to their developing fetus [Chang *et al* 2000, Giglia *et al* 2007].

1.4.2 Consequences of Alcohol Consumption During Pregnancy

The consequences of alcohol exposure on the developing fetus have been widely studied. The term Fetal Alcohol Spectrum Disorders (FASD) has been coined to describe all aspects of prenatal alcohol exposure, which includes the best characterised syndrome, Fetal Alcohol Syndrome (FAS). In addition, two less well characterised, but more common effects of alcohol exposure are the Alcohol Related Birth Defects (ARBD) and Alcohol Related Neurodevelopmental Disorders (ARND).

1.4.2.1 Fetal Alcohol Spectrum Disorders (FASD)

FASD refers to an umbrella of alcohol-related fetal developmental abnormalities including FAS, ARBD and ARND. It has been estimated that the incidence of FASD in the US, Australia and Europe is up to 1% [Harris *et al* 2003, Elliott *et al* 2004]. While it is generally thought that FASD is more strongly associated with higher levels of alcohol consumption, animal studies have suggested that even a single episode of alcohol consumption may contribute to the manifestation of a FASD syndrome.

1.4.2.1.1 Fetal Alcohol Syndrome (FAS)

FAS is the most severe congenital birth defect that is a consequence of alcohol exposure. The incidence of FAS varies from country to country and is more common in indigenous communities [Harris *et al* 2003]. In the USA, the incidence varies from 2.8 per 1,000 live births to as much as 9.8 per 1,000 live births in the Native American communities [Chiriboga 2003, Peadon *et al* 2007]. In Australia, the incidence of FAS is similar to that of the USA, although the incidence of FAS in indigenous Australians (4.7 per 1,000 live births) is not as high as in Native Americans [Peadon *et al* 2007]. The Cape Province communities of South Africa have the highest worldwide incidence of FAS, with an incidence of approximately 46 cases per 1,000 live births [Peadon *et al* 2007]. It is speculated that the incidence of FAS is actually a lot higher in many communities of Australia and the USA, but children remain undiagnosed, due to a lack of knowledge of the symptoms by health professionals [Peadon *et al* 2007].

FAS is a consequence of prolonged chronic alcohol exposure throughout most of gestation. Children born with FAS have characteristic facial features, which include flat nasal bridge, smooth philtrum, thin upper lip and upturned nose (Figure 1.3). In addition, FAS children

display pre- and/or post-natal growth failure, and structural and/or functional abnormalities of the CNS. These functional abnormalities of the CNS often result in children having difficulties with learning and memory, and often behavioural and emotional problems are evident [Chiriboga 2003, Sant'Anna *et al* 2006, Peadon *et al* 2007].

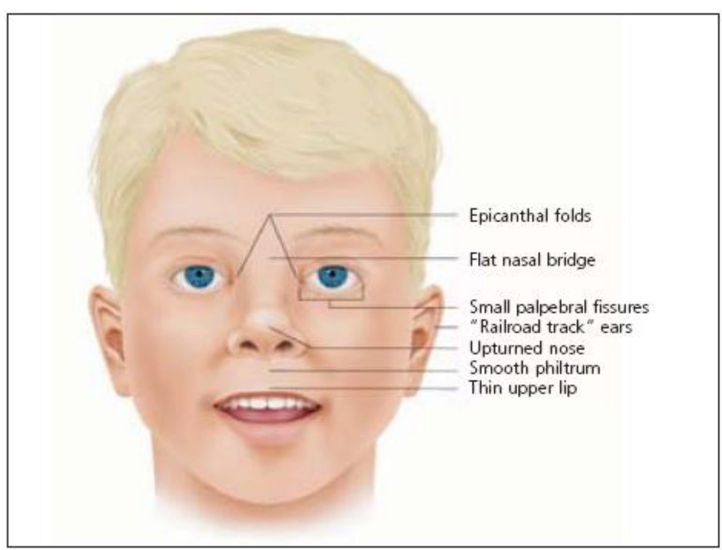

Figure 1.3 – Clinical Features of Fetal Alcohol Syndrome. Image from Wattendorf and Muenke. 2005.

1.4.2.1.2 Alcohol Related Birth Defects (ARBD)

A number of congenital birth defects have been attributed to alcohol exposure *in utero*, however these birth defects can occur without any diagnosis of FAS. The incidence of ARBD in Australia and other Western countries is not currently known as it is often difficult to make an adequate diagnosis. This is because the congenital defects can appear without confirmed knowledge of prenatal alcohol exposure, and because of a lack of knowledge amongst health professionals as to the causes of the birth defects [Peadon *et al* 2007]. Children diagnosed with ARBD often display many characteristic structural features which include cardiac, skeletal, renal and facial defects [Peadon *et al* 2007].

1.4.2.1.3 Alcohol Related Neurodevelopmental Disorders (ARND)

Children with confirmed exposure to alcohol may not display symptoms of FAS or ARBD but still have a range of cognitive, emotional and behavioural problems. These children are classified as having ARND. There is no data on the prevalence or incidence of ARND in Australia or other Western countries as this category of alcohol-related disorders has only been recently identified [Peadon *et al* 2007]. Children diagnosed with ARND have a range of neurodevelopmental disorders, which include behavioural and cognitive abnormalities, impaired performance of complex tasks and a higher level of language deficits [Peadon *et al* 2007].

1.4.2.2 Animal Models of Prenatal Alcohol Exposure

In order to study the effects of alcohol exposure on fetal development and subsequent postnatal health a range of animal models have been utilised. Most animal studies to date have been conducted using rats, mice and sheep. The sheep and rat are convenient in terms of alcohol administration, and they are appropriate for long-term analysis of growth and physiology. Furthermore, alcohol metabolism and clearance are somewhat comparable to the human [Suddendorf 1999, Lieber 2000]. However, the large body size, particularly of the sheep, makes studies expensive, and the relative lack of molecular biological tools makes it difficult to investigate the mechanisms of alcohol teratogenesis.

Recently, the zebrafish (*Danio rerio*) has been employed to investigate the more subtle developmental effects of alcohol teratogenesis that cannot be studied in a rodent. The zebrafish, with its small size, external development and ease of observation and manipulation, has been used to investigate the consequences of alcohol exposure on skeletogenesis and behaviour [Carvan *et al* 2004]. In these studies, alcohol at concentrations ranging up to

300mM was added to the tank water 4 hours after fertilisation and maintained for 6 days post-fertilisation, raising the alcohol concentration within the embryo [Carvan *et al* 2004]. Furthermore, with the extensive genetic and molecular knowledge on zebrafish development, further insights into the consequences of alcohol exposure will become evident.

While there is no single ideal animal model for investigating the effects of prenatal alcohol exposure on development, as each animal model has its own pros and cons, some studies have used alcohol concentrations that are very high and therefore unrealistic. For example, Mayock *et al* (2007) administered pregnant ewes 1.5g/kg of alcohol intravenously, which resulted in a BAC of 0.21g/dl immediately after infusion. Similarly, Maier *et al* (1998) identified that administration of alcohol to pregnant rat dams by gavage at a concentration of 2.25g/kg, 4.5g/kg and 6.5g/kg gave BAC's of 0.126g/dl, 0.273g/dl and 0.355g/dl respectively. The BAC's obtained in these studies are high, and are somewhat unrealistic in terms of what BAC a pregnant women would obtain while drinking. For example, in adult men a BAC greater than 0.3g/dl results in respiratory depression and sedation [Ramchandani *et al* 2001]. It is unlikely that a significant proportion of pregnant women would consume that much alcohol in one sitting to obtain a BAC within that range, as epidemiological studies have demonstrated that even if pregnant women choose to drink they are more likely to reduce their drinking [Chang *et al* 2000, Giglia *et al* 2007]. However, it cannot be ruled out that some women drink to this level, at least occasionally, during pregnancy.

Many studies have employed a liquid alcohol supplemented based diet for administering alcohol to pregnant rats and mice [Assadi *et al* 1991, Vavrousek-Jakuba *et al* 1991, Fuseler 1993, Snow *et al* 2007]. Typically, the liquid-based diet has a percentage of calories derived from alcohol, such as the Leiber-D'Carli's liquid diet in which 36% of the total calories is derived from alcohol, giving a BAC of approximately 0.15g/dl. This method of alcohol administration is advantageous as it allows chronic continual alcohol administration. Furthermore, as the animal feeds over a considerable timeframe, the peak BAC achieved is

lower than that achieved via gavage [Varousek-Jakuba *et al* 1991]. However, a major downfall with this model is that the peak BAC achieved varies from day to day, as it is dependent on how much liquid diet the animal consumes [Varousek-Jakuba *et al* 1991].

The alcohol concentration, duration of exposure and animal model utilised for studies of prenatal alcohol exposure is largely dependent on the scientific questions being asked, as one model may be appropriate for one organ system but be entirely inappropriate for another organ system. For example, studies investigating key time points of neurological development have administered alcohol at a time when the brain is most developmentally plastic, such as between postnatal days 4 and 12 in the rat when a large amount of brain maturation and neuronal proliferation is occurring [Miller *et al* 1996]. However, this time point would be inappropriate if, for example, the early stages of kidney development were the major focus, as at that time point in the rat kidney development is largely complete [Burrow 2000].

Overall, there is no single ideal animal model for studying the effects of prenatal alcohol exposure as each model has its own pros and cons. The most appropriate model to utilise is the one that will address the aims of the study designed, while at the same time provide a BAC that is relevant to that found in pregnant women.

1.4.2.3 The Effects of Alcohol on Fetal Development

The effects of alcohol on the developing fetus are widespread with each developing organ system being affected differently. While it is generally considered that the developing nervous system is one of the most vulnerable systems, it is becoming increasingly evident that the developing cardiovascular and respiratory systems are also very vulnerable to the effects of alcohol. It is also clear that the timing and dose of alcohol exposure are important parameters. Below are brief descriptions of the principal effects of alcohol on the developing nervous,

cardiovascular, musculoskeletal, respiratory and urogenital systems. These descriptions include evidence from both human and animal studies.

1.4.2.3.1 Development of the Nervous System

As mentioned above, the developing nervous system is considered to be one of the most vulnerable systems to the effects of alcohol. Because of this, the effects of alcohol on the developing nervous system have been well studied. Studies of children exposed to alcohol *in utero* have identified a multitude of effects ranging from neuroanatomical malformations [Clarren *et al* 1978, Peiffer *et al* 1979], to cognitive dysfunction [Coles *et al* 1991], to behavioural disorders [Coles *et al* 1985]. Animal studies have identified that different regions of the developing brain are differentially affected by alcohol in a time- and dose-dependent manner. Young *et al* (2006) found that raising the maternal BAC to 50mg/dl was enough to cause neuroapoptosis in the infant mouse brain without causing a reduction in brain weight. Maier *et al* (1998) found that a BAC of 0.2g/dl in the mouse resulted in growth differences in the different regions of the brain with the most significant reduction occurring within the cerebellum. In a follow-up study it was identified that the reduction in cerebellar weight was associated with reductions in Purkinje and granule cell numbers [Maier *et al* 2001]. Reductions in DNA and protein content have also been observed in those brain regions most affected by fetal alcohol exposure [Miller 1996].

1.4.2.3.2 Cardiovascular Development

Cardiovascular malformations are commonly observed in children exposed to high concentrations of alcohol during fetal development [Daft *et al* 1986, Adickes *et al* 1990]. The effects of alcohol on the developing cardiovascular system are dose and time (fetal stage)

dependant. For example, Webster *et al* (1984) found ventricular septal defects in the hearts of mice exposed to alcohol on days 8, 9 or 10 of gestation, but no defects were present in mice exposed to alcohol on day 7 of gestation. Turcotte *et al* (2002) found that prenatal alcohol exposure in the rat did not result in any gross heart defects, but defects in the contractile responses of the thoracic aorta were observed. There is also evidence that the influence of alcohol on heart development is dependent on genetic background. In chicks, Cavieres *et al* (2000) found that the more pure bred strains were more prone to the effects of alcohol exposure than cross-breeds.

1.4.2.3.3 Musculoskeletal Development

There is increasing evidence that prenatal alcohol exposure contributes to the incidence of fetal bone malformations. One primary diagnostic feature of FAS is short stature, indicating defects in bone development *in utero* [Spohr *et al* 1993]. Habbick *et al* (1998) and Day *et al* (2002) found that exposure to high levels of alcohol can result in permanent short stature and delayed mean bone age in children up to 14 years of age. Studies in rodents have identified reduced body length and length of individual bones, delayed ossification and decreased skeletal maturity in response to prenatal alcohol exposure [Lee *et al* 1983, Keiver *et al* 1996, 1997, 2004].

One area that has received very little research attention is the effects of alcohol on the development of muscle. Animal models of fetal alcohol exposure often report growth retardation in the form of reduced body weight, which could be a consequence of decreased muscular development. Rodent studies have shown reductions in weight and fibre content of the Tibialis Anterior and Extensor Digitorum Longus muscles of alcohol-exposed rats [David *et al* 2005].

1.4.2.3.4 Respiratory Tract Development

The effects of alcohol exposure on the developing respiratory tract have not been well characterised. Children diagnosed with FAS are often prone to respiratory infections. Studies have demonstrated that macrophage activity within the lung is compromised following prenatal alcohol exposure, with the macrophages demonstrating a defect in phagocytosis of bacteria and an increased rate of apoptosis [Ping *et al* 2007]. Wang *et al* (2007) found that lungs of alcohol-exposed mice weighed less than control lungs, and by histological examination identified that they were developmentally immature.

1.4.2.3.5 Urogenital Development

FAS patients often have small kidneys that have failed to rotate correctly. This causes kinks in the ureter, urinary flow problems and resultant hydroureter [Qazi *et al* 1979, Havers *et al* 1980, Taylor *et al* 1994]. In sheep studies, prenatal alcohol exposure results in a transient reduction in urine flow close to term [Clarke *et al* 1987a, Clarke *et al* 1987b]. Rats prenatally exposed to alcohol display a urinary concentrating defect when challenged with high sodium or potassium diets and that histological examination demonstrated mitochondrial atrophy and tubular hypertrophy [Assadi *et al* 1991, 1992]. Gallo *et al* (1986) reported a reduction in renal protein and DNA content in alcohol-exposed rat offspring. Furthermore, a link has been made between light-to-moderate alcohol exposure during development and an increase in the risk of renal anomalies among offspring [Moore *et al* 1997]. Despite these identified defects in kidney function in adulthood following fetal alcohol exposure, very little is known about the effects on kidney development.

1.4.2.4 Mechanisms through which Alcohol Disrupts Normal Development

The mechanisms through which alcohol acts to disrupt the development of the fetus has received little attention. However, with the advancement of molecular biology techniques, new insights into the molecular and developmental mechanisms of alcohol's action are becoming evident. Two mechanisms through which alcohol acts are discussed below.

1.4.2.4.1 Retinoic Acid

Retinoic acid (RA), the active metabolite of vitamin A synthesis, is an important regulator of embryonic development [Crabb *et al* 2001]. Interestingly, children born following vitamin A deficiencies display many of the developmental abnormalities observed in children diagnosed with FAS, including facial and skeletal abnormalities, and mental retardation [Zachman *et al* 1998, Crabb *et al* 2001, Radhika *et al* 2002]. The production of RA from vitamin A employs the same enzymes involved in alcohol metabolism (ADH & ALDH), and individuals who are intoxicated, even in acute situations, have lower circulating RA levels [Deltour *et al* 1996, Crabb *et al* 2001]. This has been confirmed in both human and animal studies, where acute alcohol exposure reduced the synthesis of RA in both the liver and intestine [Deltour *et al* 1996, Crabb *et al* 2001, Parlesak *et al* 2005].

The fetus is dependent on having sufficient levels of RA to ensure normal development. It has been proposed that the adverse effects of fetal alcohol exposure are due, in part at least to the fetus being exposed to low levels of RA *in utero* [Deltour *et al* 1996, Crabb *et al* 2001]. Studies have demonstrated that pregnant rats administered alcohol via a liquid-based diet have reduced circulating RA levels and reduced amounts of stored retinal esters [Parlesak *et al* 2005]. Furthermore, culture of whole E10.5 rat embryos in the presence of alcohol leads to underdevelopment of both the first and second branchial arches as well as growth restriction.

Chen *et al* (1996) found that tissue levels of RA in alcohol-cultured embryos were mildly reduced, again suggesting that the effects of alcohol are in part mediated by a reduction in available RA. Similarly, Deltour *et al* (1996) reported that mouse embryos cultured in the presence of 100mM alcohol had reduced levels of RA. In a *Xenopus* model of prenatal alcohol exposure, Yelin *et al* (2005) identified a reduced level of RA in Spemann's Organizer. Despite these findings in multiple models, there remains no definitive experimental evidence that links the effect of prenatal alcohol exposure with diminished RA levels. However, the accumulated evidence does suggest that the reduction in RA levels contributes to the developmental defects observed following prenatal alcohol exposure.

1.4.2.4.2 Oxidative Stress

Consumption of alcohol is associated with an increase in the abundance of oxygen free radicals, resulting in increased oxidative stress within the cell. This increase in oxidative stress contributes to increased cell death in free radical sensitive tissues, such as the brain and liver. Elimination of oxygen free radicals, and therefore a reduction in cellular oxidative stress, is achieved through the action of antioxidants, such as Vitamin E, Vitamin C, folic acid and β-carotene. The developing fetus is particularly susceptible to the effects of oxidative stress. This is because the fetus's ability to eliminate reactive oxygen species through the action of antioxidants is lower than the adult. Addolorato *et al* (1997) reported that offspring of rats fed low levels of alcohol during pregnancy had reduced growth and antioxidant activity in their livers while their mothers were unaffected. It has also been reported that rats exposed to alcohol during gestation and the lactation period had higher activity levels of oxidative damage in their livers and pancreas. Interestingly, concurrent administration of folic acid with alcohol mitigated the effects of alcohol on the oxidative state of the liver and pancreas [Cano *et al* 2001]. It has been proposed that an increased cellular oxidative state causes an increase

in DNA damage in highly proliferative embryonic tissue leading to programmed cell death, thereby reducing the number of cells within susceptible embryonic tissues. This proposal was supported by the findings of Mitchell *et al* (1998) who cultured rat hippocampal cells in the presence of alcohol. This resulted in increased neuronal apoptosis which was ameliorated by the addition of the antioxidant vitamin E or β-carotene [Mitchell *et al* 1998]. Based on these and similar findings it has been hypothesised that the reduction in brain weight and growth of children diagnosed with FAS, may in part be the result of increased neuronal apoptosis due to the highly oxidative state.

1.5 Long-Term Consequences of Prenatal Alcohol Exposure

Children exposed to alcohol *in utero* are known to have developmental disabilities that manifest in the form of delayed growth, deficits in memory and learning, and deficits in motor coordination. These disabilities have long-term consequences in the form of limiting education and influencing employment prospects. However, it is becoming more evident with the increased rate of diagnosis and documentation of children exposed to alcohol *in utero* that while there may not always be overt developmental deficits, there may be subtle developmental abnormalities that affect the long-term health of the individual.

There is increasing evidence that children who do not present with the symptomology to be diagnosed with FAS, but were still exposed to alcohol *in utero,* may have subtle genetic or structural abnormalities that affect their long-term health. For example, several studies have shown that chronic alcohol administration leads to a reduction in plasma levels of the hormone arginine vasopressin (AVP), which is thought to contribute to the higher incidence of diabetes insipidus in offspring [Collins *et al* 1992, Hirschl *et al* 1994]. This finding was confirmed in rat studies which showed that alcohol exposure results in a reduction in the number of AVP producing neurons in the supraoptic nucleus [Madeira *et al* 1993]. Furthermore, Castells *et al* (1981) found that children exposed to alcohol *in utero*, when challenged with an oral glucose test were more insulin resistant than normal children because they were hyperinsulinemic and hyperglycaemic. In addition, as previously stated, a link has been made between renal anomalies and moderate prenatal alcohol exposure, which could influence renal health in adulthood [Moore *et al* 1997].

The long-term consequences of prenatal alcohol exposure on adult health suggests that prenatal alcohol exposure contributes to the developmental origins of health and disease (DOHaD). The DOHaD hypothesis states that a fetus developing in a compromised environment, for example due to malnutrition or exposure to a teratogen, makes

developmental decisions in order to enhance survival in the short-term. While these changes may be beneficial in the short term, they may render the offspring more susceptible to adult-onset diseases, such as hypertension and diabetes. This has lead to studies of the long-term consequences of alcohol exposure *in utero*. These studies will be discussed in Section 1.9.

1.6 The Fetal Origins of Adult Disease Hypothesis

The fetal origins of adult disease hypothesis, or the developmental origins of health and diseases (DOHaD) as it is more commonly known, was first proposed by epidemiologist David Barker and colleagues in 1986, when they proposed that individuals exposed to a poor growth environment *in utero* were in some way predisposed to the development of cardiovascular disease (CVD) in adulthood. This hypothesis proposes that exposure to a sub-optimal *in utero* environment, such as poor nutrition, results in fetal adaptations to ensure immediate survival through changes in gene expression and structural changes; however, these same changes enhance the susceptibility to disease in adulthood. This hypothesis is often referred to as fetal or developmental "programming". The adult onset diseases that have been linked to fetal development in a sub-optimal *in utero* environment include CVD, metabolic disease, diabetes, renal disease, osteoporosis and some mental and behavioural disorders [Curhan *et al* 1996, Hoy *et al* 1999, Lackland *et al* 2000, Williams *et al* 2002, Huxley *et al* 2002, Hales *et al* 2003, Falkner *et al* 2004, Harvey *et al* 2004, Huxley *et al* 2004].

1.6.1 Cardiovascular Disease (CVD) and Hypertension

People who reside in western countries are at an increased risk of developing CVD compared with people who live in non-westernized communities. This increased incidence/prevalence of CVD has been largely attributed to environmental factors, including diet, sedentary lifestyle and reduced physical activity. However, there is increasing evidence that environmental and genetic factors do not fully explain the high rates of CVD. As Barker and colleagues originally proposed, a sub-optimal *in utero* environment may "programme" CVD in adult life. Barker *et al* (1993) were first to show an association between low birth weight and the subsequent development of CVD. While many subsequent studies have reported that

individuals born small have an increased risk of developing CVD-related diseases, there is also the argument that increased CVD risk is more due to the nutritional status of the mother than low birth weight [Huxley *et al* 2004, Falkner *et al* 2004]. Experimental studies have provided further insights into the association between a sub-optimal *in utero* environment and adult CVD.

Most animal studies of developmental programming have fed pregnant animals a low protein diet. Many of these studies have reported that offspring born of low protein mothers have low birth weights and subsequently go on to develop hypertension in adulthood [Langley-Evans *et al* 1999, Woods *et al* 2004]. One key study reported by Langley-Evans *et al* (1999), found that rat offspring exposed to a low protein diet throughout gestation had an 18mmHg increase in blood pressure compared to control animals. This finding has been confirmed in subsequent studies, which have also shown that offspring exposed to a low protein diet throughout gestation and those that were exposed in the second half (E15 to E21) of gestation had a higher blood pressure in comparison to control offspring [Woods *et al* 2004]. What is interesting is that the window of vulnerability in these animals coincides with the key period of kidney development. The kidneys of low protein offspring were found to contain fewer nephrons than control kidneys [Woods *et al* 2004]. The relationship between fetal programming and the kidney will be discussed further in Section 1.8.

1.7 Kidney Development

Mammalian kidney development involves the sequential formation of pronephroi, mesonephroi and metanephroi, the permanent kidneys. All three excretory structures arise from the intermediate mesoderm via a series of molecular and morphological events. Both the pronephroi and mesonephroi are transient structures and are necessary for development of the metanephric kidney. Metanephric kidney development in humans begins during week 5 of gestation [Vize *et al* 1997], in the sheep at 27 days gestation [Moritz and Wintour 1999] and at embryonic (E) 11.5 in the rat and mouse [Burrow 2000]. Completion of nephrogenesis varies across species, with both human and sheep completing nephrogenesis before birth, 36 weeks gestation in the human and 130 days gestation in the sheep [Moritz and Wintour 1999]. However, nephrogenesis in the mouse and the rat continues postnatally, finishing at approximately postnatal (PN) day 5 in the mouse and PN7 in the rat [Burrow 2000].

1.7.1 Pronephros

The pronephros is the most primitive excretory structure to develop in mammals. It forms at approximately 22 days gestation in the human and at E8 in the mouse and rat. The majority of the pronephric duct degenerates in mammals, but the caudal portion of the duct forms part of the Wolffian duct [Vize *et al* 1997].

1.7.2 Mesonephros

The mesonephric kidney develops once the pronephric duct reaches the presumptive mesenchyme and induces the development of mesonephric tubules [Saxen and Lehtonen 1987]. Mammalian mesonephric tubules have nephron-like structures that are functional and

excrete urine. Regression of these structures begins once metanephric kidney development commences, and full regression of the mesonephric kidney is complete by approximately 16 weeks of gestation in the human [Beck *et al* 1985]. Portions of the mesonephros give rise to components of the male and female reproductive tracts.

1.7.3 Metanephros

Metanephric kidney development begins with the budding of the ureteric duct from the Wolffian duct. The ureteric bud migrates towards the metanephric mesenchyme, a mass of undifferentiated mesenchymal cells of several lineages. Once the ureteric bud has grown into the metanephric mesenchyme, molecular signals secreted from the mesenchyme induce the ureteric bud to bifurcate. The process of ureteric branching morphogenesis has thus commenced, which ultimately gives rise to the collecting duct system and the renal calyces.

At the tip of each branch of the forming ureteric tree, metanephric mesenchymal cells condense, forming a mesenchymal condensate. The condensate cells proliferate and then epithelialize, giving rise to an epithelial vesicle, which eventually differentiates into a nephron. The epithelial vesicle first forms a comma-shaped body through an invagination of the caudal region of the vesicle. Invagination of the distal region of the comma-shaped body subsequently forms an S-shaped body. The lower limb of the S-shaped body gives rise to the renal corpuscle and glomerulus, with the remainder of the S-shaped body giving rise to the proximal tubule, the loop of Henle and the distal tubule. Nephron maturation is characterised by migration of capillaries into the lower limb of the S-shaped body forming the glomerular tuft of capillaries. Nephrogenesis occurs within the nephrogenic zone, a thin band of tissue at the periphery of the kidney (Figure 1.4).

1.7.3.1 Key Molecular Regulators of Kidney Development

The development of the metanephric kidney involves the interplay of multiple factors that regulate cell survival, proliferation, apoptosis, differentiation and motility. These factors must be expressed in a precise spatiotemporal pattern to coordinate the development of the kidney. These regulatory factors include growth factors, transcription factors, extracellular matrix molecules, hormones, oncogenes and proto-oncogenes. To date, more than 400 molecules have been implicated in metanephric development. A brief description of some of the key regulatory molecules involved in kidney development is presented below.

1.7.3.1.1 Ureteric Branching Morphogenesis

The initiation and maintenance of ureteric tree growth and development requires the coordinated activity of molecules involved in promoting branching morphogenesis, as well as factors that inhibit branching morphogenesis. Key regulators of ureteric branching morphogenesis are glial cell line derived neurotrophic factor (*GDNF*), wingless type 11 (*Wnt11*), bone morphogenetic protein 4 and 7 (*BMP4, 7*), paired box gene 2 (*Pax2*), retinoic acid receptor (*RAR*), transforming growth factor β1 (*TGFβ1*), fibroblast growth factor 7 (*FGF7*) and insulin growth factor (*IGF*).

GDNF is a distant member of the *TGFβ* superfamily of signalling molecules. *GDNF* is first expressed in the metanephric adjacent to the ureteric bud site, and later is required in the metanephric mesenchyme adjacent to ureteric branch tips [Arighi *et al* 2005, Costantini *et al* 2006]. *GDNF* signalling occurs through the tyrosine kinase receptor, *Ret*, which is located on tip epithelial cells [Pachnis *et al* 1993, Sainio *et al* 1997]. The key role of *GDNF* in kidney development is to regulate induction of the outgrowth of the ureteric bud from the Wolffian duct and to promote and maintain branching of the ureteric tree [Sainio *et al* 1997]. *GDNF*

homozygous null mutant (knockout) mice display bilateral renal agenesis [Moore *et al* 1996, Pichel *et al* 1996, Sanchez *et al* 1996] with heterozygous null mutants have reduced nephron number [Cullen-McEwen *et al* 2001].

Wnt11 is a member of the *Wnt* family of genes which encode glycoprotein growth factors and differentiation factors. *Wnt11* is first expressed in the nephric duct and later becomes restricted to the tips of the branching ureteric tree [Kispert *et al* 1996, Merkel *et al* 2007].

Transcripts of all BMPs have been identified in the developing kidney, and BMPs 4 and 7 have been shown to play key roles in kidney development [Dudley and Robertson 1997, Godin *et al* 1999]. *BMP4* is expressed in the stroma surrounding the Wolffian duct and ureteric bud, while its receptors are located in the ureteric epithelium [Dudley and Robertson 1997]. *BMP4* inhibits inappropriate budding from the Wolffian duct, inhibits ureteric branching and promotes elongation of ureteric branches [Obara-Ishihara *et al* 1999]. In addition, *BMP4* is required for formation of the nephric duct from intermediate mesoderm [Raatikainen-Ahokas *et al* 2000]. *BMP7* is the only member of the *BMP* family expressed in the metanephric mesenchyme [Piscione *et al* 1997, Gupta *et al* 1999]. *BMP7* is involved in maintaining the survival of the metanephric mesenchyme, but is also involved in the regulation of ureteric branching morphogenesis, with low concentrations promoting branching morphogenesis, and high concentrations inhibiting branching morphogenesis [Piscione *et al* 1997].

Paired box - 2 (*Pax-2*) is one of the earliest known markers of mesenchymal cell condensation and is also expressed in the induced epithelium of the renal vesicle [Dressler *et al* 1990, Dressler and Douglass 1992]. *Pax-2* regulates the expression of several other key kidney developmental genes, including *WT-1* and *GDNF*. *PAX-2* knockout mice display a range of renal abnormalities including renal hypoplasia, increased apoptosis and ureter abnormalities [Brophy *et al* 2001, Bouchard *et al* 2002].

RA and its receptors (*RARs*) are key regulators of fetal development, and in kidney development they play important roles in promotion of ureteric bud outgrowth from the

Wolffian duct and in ureteric branching morphogenesis [Moreau *et al* 1998]. The expression of *c-Ret* is regulated by *RAR*, as *in vitro* studies adding RA to cultured metanephroi show that the expression of *c-Ret* is significantly increased, resulting in enhanced ureteric branching morphogenesis [Mendelsohn *et al* 1999]. In addition, *RARs* are critically involved in the patterning of the stromal cell population, as double mutant *RAR* mice show defective stromal patterning, impaired ureteric bud outgrowth, as well as decreased *c-Ret* expression [Mendelsohn et al 1999].

TGFβ1 inhibits ureteric budding as well as ureteric branching [Rogers *et al* 1993, Ritvos *et al* 1995]. *TGFβ1* is also an important regulator of renal architecture through regulating the expression of matrix degrading enzymes.

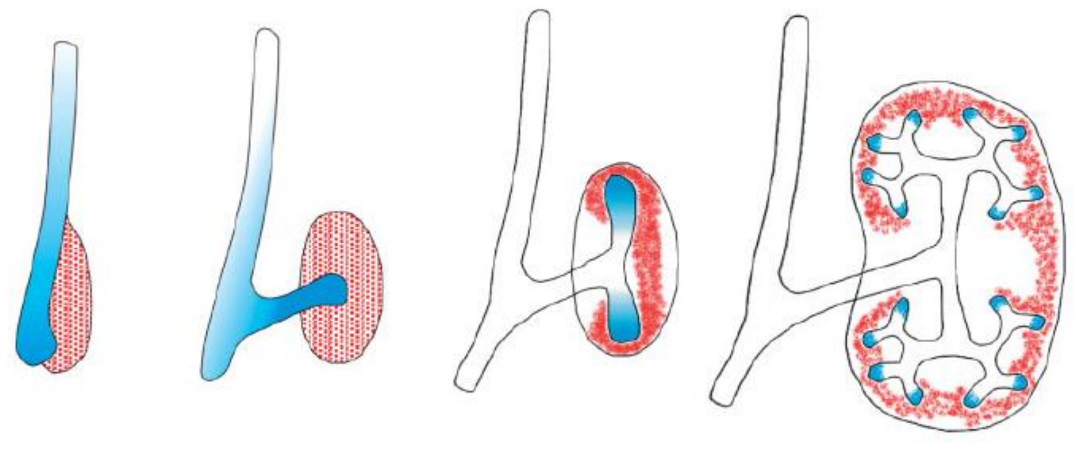

Figure 1.4 – Schematic representation of metanephric kidney development, showing ureteric bud invagination (Blue) into the metanephric mesenchyme (Red) and subsequent ureteric bud bifurcation and branching morphogenesis. From Costantini & Shakya, 2006.

1.7.3.1.2 Nephron Development and Maturation

The critical stages of nephron induction and maturation involve considerable and rapid changes in gene expression to ensure that the metanephric mesenchyme is induced to condense and then converts to epithelium (Figure 1.5). Key genes involved in nephrogenesis are the transcription factors *Pax-2*, *WT-1* and the growth factors *BMP7* and *Wnt-4*.

As previously mentioned, *Pax-2* is one of the earliest genes expressed in induced mesenchyme and mesenchymal condensates. Expression of *Pax-2* continues during the early stages of nephron development, however, it is down-regulated in S-shaped bodies [Dressler and Douglas 1992]. Disruption of *Pax-2 in vitro* disrupts nephron induction and maturation, indicating that it is critical for the induction of nephrogenesis [Rothenpieler and Dressler 1993].

WT-1 is initially expressed in the uninduced metanephric mesenchyme before ureteric bud formation [Kreidberg *et al* 1993]. Once the metanephric mesenchyme is induced, its expression is more localised to the developing podocytes of the glomerular aggregates [Armstrong *et al* 1993, Nishinakamura *et al* 2001]. *WT-1* is involved in mesenchyme to epithelial conversion and is expressed at its highest levels in the lower limb of the S-shaped body where it inhibits *Pax-2* expression. In *WT-1* transgenic mice in which *Pax-2* expression is also down-regulated, glomerular development fails, indicating that *WT-1* is required for the maintenance of *Pax-2* expression enabling glomerular development [Dressler *et al* 1993].

BMP7 promotes cells in the nephrogenic zone of the developing kidney to remain in a stem cell like state, inhibiting differentiation [Dudley *et al* 1999]. Furthermore, *BMP7* is critically involved in the maintenance of mesenchymal survival, which allows for nephrogenesis to be ongoing in the developing kidney [Miyazaki *et al* 2000].

Wnt-4 protein is secreted by the induced metanephric mesenchyme and the *Wnt-4* gene is expressed in the condensing mesenchyme soon after induction, remaining until the S-shaped

body fuses with the ureteric epithelium [Kispert *et al* 1996]. *Wnt-4* knockout mice have small kidneys that lack nephrons [Stark *et al* 1994]. These kidneys display significant branching suggesting *Wnt-4* is involved in the conversion of mesenchyme to epithelium [Stark *et al* 1994].

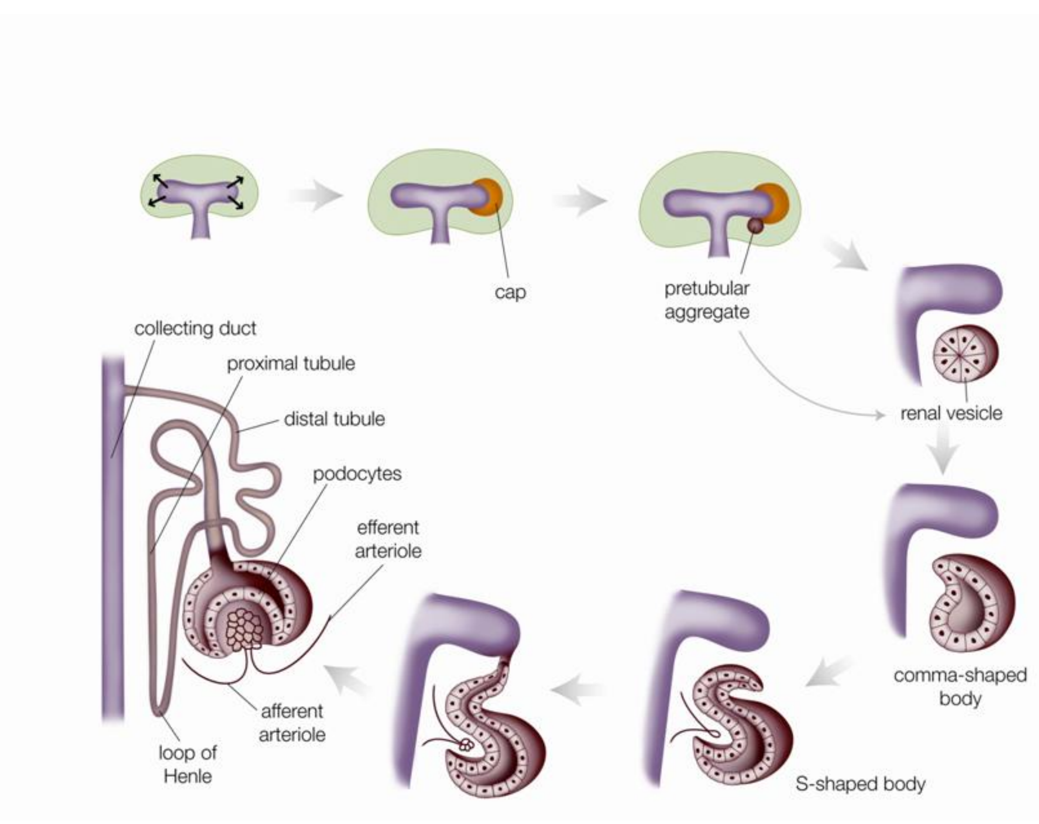

Figure 1.5 – Schematic representation of nephron development in the mammalian metanephros. From Moritz et al, 2008.

1.8 Fetal Programming of the Kidney

As briefly discussed earlier, the developing kidney appears to be particularly vulnerable to the adverse effects of developmental programming. As will be described in Sections 1.8.2, studies in a range of species have reported reduced nephron endowment in the kidneys of offspring exposed to a sub-optimal *in utero* environment [Langley-Evans *et al* 1999, Wintour *et al* 2003, Woods *et al* 2004, Singh *et al* 2007, Wlodek *et al* 2007, Zohdi *et al* 2007, Dickinson *et al* 2007, Moritz *et al* 2008].

The kidneys play an important role in the regulation of adult blood pressure through their ability to regulate the excretion of sodium and water, via the pressure-natriuresis mechanism [Vander 1997, Guyton *et al* 2000]. The pressure-natriuresis mechanism operates as a negative feedback system, whereby elevations in arterial pressure cause an increase in renal sodium and water excretion above that of sodium and water intake, until arterial pressure is reduced. This process is ongoing and the kidney has a large capacity to excrete sodium and water until arterial pressure is reduced to its equilibrium point, the point where sodium and water intake match sodium and water excretion, normalising blood pressure [Vander 1997, Guyton *et al* 2000]. There are two main events which can result in a permanent increase in arterial pressure: the first involves high level intake of sodium and water such that the kidney can no longer excrete it adequately (as may occur in pathological states), and the second involves a shift in the pressure-natriuresis curve such that the equilibrium point is higher than normal.

However, some individuals with normal sodium and water intake have elevated arterial pressure. Results from several recent studies suggest these individuals with hypertension, but no overt renal deficits, have a relatively low nephron number [Keller *et al* 2003, Hoy *et al* 2006, Hughson *et al* 2006]. Brenner and colleagues (1988) first proposed a role for low nephron number in the aetiology of hypertension. Specifically, Brenner *et al* (1988) hypothesised that a reduced glomerular filtration surface area resulting from a congenital or

acquired low nephron number would limit the body's ability to excrete sodium and water, ultimately increasing fluid volume. This would contribute to a rise in blood pressure through an expansion of extracellular fluid volume and a shift in the pressure-natriuresis curve. This increase in blood pressure, possibly coupled with postnatal "hits" to the kidney [Nenov *et al* 2000] may lead to glomerular damage through an increase in individual glomerular pressure, possibly leading to additional glomerular loss. A simplified schematic diagram of the Brenner *et al* (1988) hypothesis is shown in Figure 1.6.

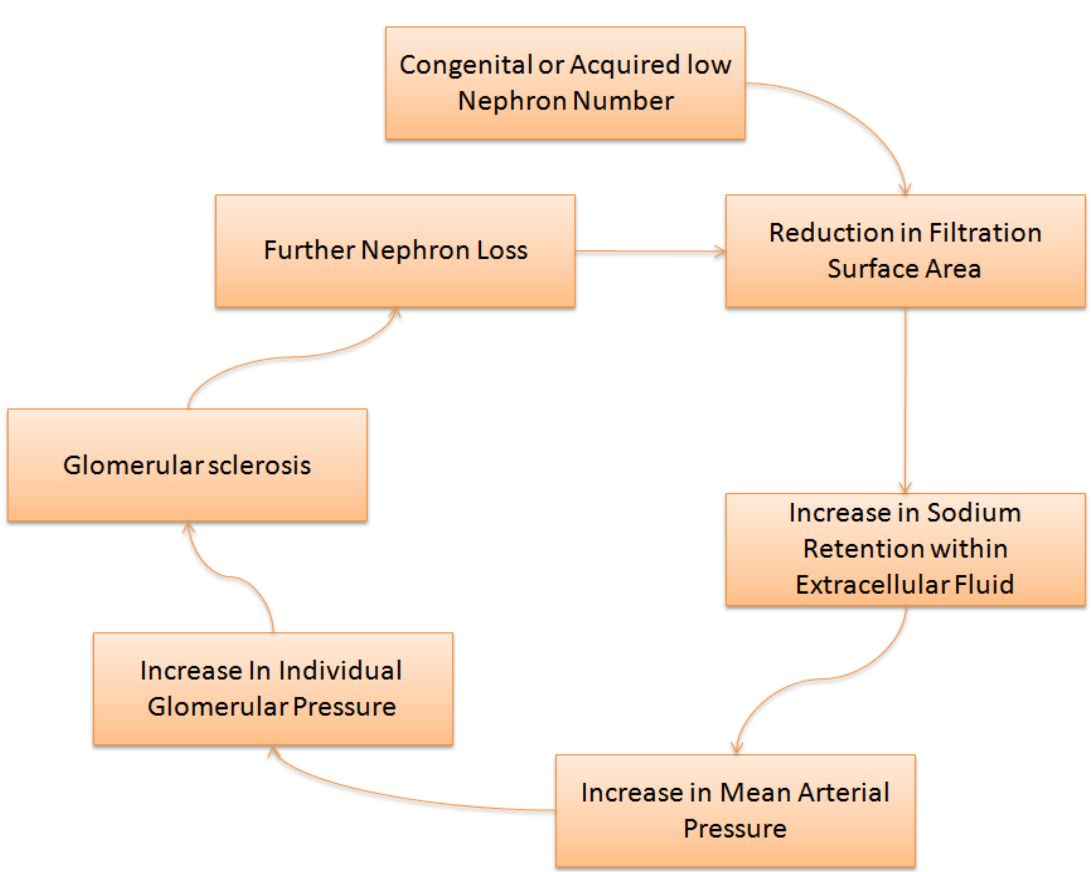

Figure 1.6 – Simplified schematic diagram of the Brenner Hypothesis, demonstrating the relationship between low nephron number and the elevation in mean arterial pressure.

1.8.1 Nephron Number and Hypertension

Total nephron number in the normal human kidney varies up to 10-fold, from 200,000 to more than 2,000,000 [Nyengaard 1999, Keller *et al* 2003, Hughson *et al* 2006, Hoy *et al* 2003, 2005, 2006, 2008, Douglas-Denton *et al* 2006]. As previously described, Brenner *et al* (1988) hypothesised individuals with a nephron number at the lower end of the scale have a predisposition to the development of hypertension (Figure 1.6). Keller *et al* (2003) were the first to report a significant inverse correlation between human nephron number and hypertension. Those subjects with low nephron number had high blood pressure whilst those with a high nephron number had lower blood pressure. Hoy *et al* (2006) reported that nephron number in Indigenous Australians without a history of hypertension was higher than those with a history of hypertension. Hughson *et al* (2006) reported that US whites with higher mean arterial pressure had lower nephron number than those with lower mean arterial pressure. However, this association was not observed in African Americans. Subsequent studies involving more subjects have failed to reproduce this finding in white Americans [Hughson *et al* 2008]. It is currently unknown if those individuals with a low nephron number and higher blood pressure were programmed during fetal development to have the low nephron number or if the low nephron number was acquired in postnatal life. Animal models of low nephron number are providing some of the answers to whether nephron number is programmed *in utero*.

1.8.2 Fetal Programming of Nephron Number and Hypertension: Animal Models

A large body of experimental evidence suggests that prenatal insults such as those induced by inadequate maternal diet, placental insufficiency or maternal hormonal status result in low nephron number and subsequent development of hypertension [Langley *et al* 1994, Merlet-

Benichou *et al* 1994, Celsi *et al* 1998, Langley-Evans *et al* 1999, Woods *et al* 2001, Bauer *et al* 2002, Wintour *et al* 2003, Woods *et al* 2004, Dickinson *et al* 2007, Hoppe *et al* 2007, Singh *et al* 2007a, Singh *et al* 2007b, Moritz *et al* 2008]. Having said that, it is becoming increasingly accepted that low nephron number does not always lead to increased blood pressure [Woods *et al* 2005, Poladia *et al* 2006, Haas 2006, Moritz *et al* 2009]. Nevertheless, many studies have demonstrated a fetal programming effect on both nephron number and blood pressure, and the key models employed are described below.

One of the most extensively studied models of fetal programming of the kidney involves the feeding of a low protein diet to pregnant rodents. In a key study, Langley-Evans *et al* (1999) fed a low protein diet to pregnant rat dams throughout their pregnancy and found that the offspring had a lower nephron number at 4 weeks of age than control (normal protein) offspring. Furthermore, the low protein offspring had a higher systolic blood pressure at 4 and 19 weeks of age. In a subsequent study, Woods *et al* (2004) demonstrated that rat offspring exposed to a low protein diet in the second half of gestation had a lower nephron endowment than offspring exposed to a low protein diet only in the first half of gestation and the controls, who were not exposed to a low protein environment. In the rat fetus, nephrogenesis only occurs in the second half of gestation. Of particular importance, Woods *et al* (2004) found that only those offspring with low nephron endowment developed hypertension.

Fetal exposure to elevated levels of glucocorticoids (natural and synthetic) has also been shown to result in offspring with a lower nephron number which develop hypertension in adulthood. Studies by Dodic *et al* (1998, 2002) in sheep found that administration of the synthetic glucocorticoid, dexamethasone, for a 48 hour period in early gestation (26-28 days) resulted in the development of hypertension in both male and female offspring coupled with a reduction in nephron endowment. In rodent studies, it was identified that if dexamethasone was administered to the pregnant dam on E15-E16 or E17-E18 of gestation, the offspring had a reduced nephron number and developed hypertension [Ortiz *et al* 2003]. However, when

dexamethasone was administered earlier (E11-E12) or later (E19-E20), nephron number and blood pressure were unaffected [Ortiz *et al* 2001, 2003]. In a later study utilising the natural glucocorticoid, corticosterone, Singh *et al* (2007a) found that exposure in the rat over a 48 hour period (E14-E15) resulted in offspring with low nephron number that went on to develop hypertension. In whole rat metanephric culture, Singh *et al* (2007b) found that culture in the presence of dexamethasone gave rise to kidneys with fewer ureteric branch points and glomeruli. This indicated a direct effect of corticosterone on the developing kidney and suggests that a low nephron number was due to reduced ureteric branching morphogenesis. Similarly, in the spiny mouse (*Acomys cahirinus*) Dickinson *et al* (2007) found that maternal dexamethasone administration between E20 and E23 (gestation is 40 days) resulted in offspring with reduced nephron number. Expression of genes known to inhibit ureteric branching (*BMP4, TGFβ1, Wnt4)* was shown to be significantly increased. These studies suggest that the early stages of kidney development are "critical windows" for the adverse effects of developmental programming.

What is important to appreciate is that while many studies have reported decreases in nephron number coupled with changes in blood pressure, some studies have used techniques that are not considered optimal or the "gold standard". For example, Ortiz *et al* (2001, 2003) used an acid-maceration technique to quantify nephron number. This requires the kidney to be chemically digested with acid leaving only the glomeruli, which are subsequently counted microscopically. The major limitation of this technique is that some glomeruli may be digested and therefore uncounted, generating an underestimate of nephron number. Langley-Evans *et al* (1999) employed a nephron counting technique in which glomeruli were counted in 3-5 longitudinal histological sections of paraffin-embedded kidneys. These counts were then mathematically extrapolated to provide an estimate for the whole kidney. As glomeruli are not evenly distributed within the cortex, and glomerular sampling with this technique is biased by glomerular size, this technique may generate inaccurate estimate. However, the

"gold standard" disector/fractionator unbiased stereological method has repeatedly been shown to provide accurate and precise estimates of total glomerular number [Bertram 2001].

Both Ortiz *et al* (2001, 2003) and Langley-Evans *et al* (1999) used a tail-cuff technique to measure blood pressure in rats. The technique requires the animal be restrained in a cylindrical tube for the duration of the measurement (up to 15 minutes). This is a stressful event for the animal, and it is commonly accepted that this technique is more a reflection of a stress test than an accurate analysis of blood pressure [Plehm *et al* 2006]. Alternative techniques for measuring conscious blood pressure in rodents are available and preferred, and include tail-artery catheterisation [Bergstrom *et al* 1998, Stevenson *et al* 2000, Kett *et al* 2004, Singh *et al*, 2007] and radio-telemetry [Denton and Barnett 2008, Sampson *et al* 2008].

1.9 Alcohol and Fetal Programming

It has only been in recent years that alcohol has been considered in the context of fetal programming. This awareness has arisen from epidemiological studies that have identified that children diagnosed with FAS have long-term health issues [Sphor *et al* 1993, Welch-Carre 2005, Zhang *et al* 2005]. It is the more subtle effects of alcohol exposure that are being considered in the context of fetal programming, and many of these may be the consequence of low to moderate alcohol exposure.

Some of the earliest studies of prenatal alcohol exposure studied long-term behavioural consequences. For example, rats exposed to alcohol *in utero* displayed increased activity when analysed in open field and in running wheels [Bond 1981, Meyer *et al* 1986]. This increased activity was found to be more pronounced in younger rats than older rats, indicating changes throughout life compensated in part for the deficits in neurological function that were present in the young rats [Abel *et al* 1986]. More recent studies have shown that prenatal alcohol exposure increases the incidence of seizures in a rat model of epilepsy, reduces thyroid function and leads to defects in the development and function of the hypothalamic-pituitary-adrenal axis [Wilcoxon *et al* 2004, Lan *et al* 2006, Glavas *et al* 2006, Russo *et al* 2008].

Prenatal alcohol exposure has also been shown to result in permanent changes in the regulation of insulin, arginine vasopressin release and serum triglyceride levels [Elton *et al* 2002, Pennington *et al* 2002, Knee *et al* 2004]. Measurement of insulin sensitivity in rats prenatally exposed to alcohol found that red muscle fibre glucose uptake was significantly reduced, however whole body insulin sensitivity was unaffected. Elton *et al* (2002) speculated that these animals, while not showing whole body insulin sensitivity, could be predisposed to the development of insulin sensitivity if the right environmental stimulus was administered, such as a high glucose diet. However, these findings are heavily debated, as similar studies have identified that alcohol-exposed offspring are insulin resistant [Chen 2004], fail to

regulate glucose homeostasis when given a glucose challenge [Villarroya *et al* 1985], and have hypoglycaemia/hyperinsulinemia throughout the neonatal period [Lopez-Tejero *et al* 1989]. Many of these apparent varied effects of prenatal alcohol exposure can be attributed to the model utilised, including the timing of alcohol exposure and the dose of alcohol.

Circulating triglyceride levels have been shown to be influenced by prenatal alcohol exposure in a sex-dependent manner. In a rat model of prenatal alcohol exposure, Pennington *et al* (2002) found that male offspring had a significant increase in serum triglycerides which was not present in female offspring. Interestingly, when male offspring were castrated and female offspring were administered testosterone, the results were reversed.

Little experimental evidence exists for fetal programming effects of alcohol on other organ systems, including the kidney. However, Assadi *et al* (1991) found that rats prenatally exposed to alcohol had a defect in their capacity to concentrate sodium and potassium when challenged with high sodium or potassium diets. However, the effect of prenatal alcohol exposure on adult blood pressure has not previously been investigated.

1.10 Hypothesis and Aims of Present Project

While the teratogenic effects of high level fetal alcohol exposure are well known, relatively little is known about the effects of alcohol on kidney development. Moreover, the long-term effects of fetal alcohol exposure on nephron endowment and adult renal and cardiovascular function have received little attention.

1.10.1 Scope of PhD Project

The overall goal of the experimental studies described in this thesis was to define the effects of chronic and acute fetal alcohol exposure on kidney development. Two animal models were employed. First, chronic studies were conducted in sheep, a species in which nephrogenesis is completed before birth. Second, studies of acute ethanol exposure were conducted in the rat. Both *in vitro* and *in vivo* studies were conducted in rats. In both sheep and rat studies, nephron number was used as an index of kidney development. This is because nephrons are the functional units of the kidney and because nephrogenesis is a complex multistage process. Therefore, any adverse affect of ethanol exposure on any stage or stages of nephrogenesis is likely to influence final nephron endowment. In addition, the effects of acute prenatal ethanol exposure in rats on adult blood pressure and renal function were assessed.

Specific Aims and Hypotheses

Hypothesis 1: Chronic ethanol exposure during the period of peak nephrogenesis in the fetal sheep results in fetal growth restriction and reduced kidney weight. This reduction in kidney weight is coupled with reduced nephron endowment.

- **Aim 1:** To determine the effects of chronic (GDA95-GDA134) prenatal alcohol exposure in sheep on:
    a. Total glomerular (nephron) number at 134 days of gestation.
    b. The levels of expression of genes involved in kidney development/function.

Hypothesis 2: Acute ethanol exposure in the fetal rat, at a time point when the kidney is in the early stages of development, results in growth restriction, reduced kidney weight at PN30 and reduced nephron endowment. In adulthood, offspring display altered cardiovascular and renal function.

- **Aim 2:** To determine the effects of acute (E13.5 and E14.5) prenatal alcohol exposure in rats on:
    a. Total glomerular (nephron) number in male and female offspring at PN30.
    b. Blood pressure and renal function in male and female offspring at 6 months of age.
    c. Renal morphology.
    d. The expression levels of key genes involved in kidney development.

- **Aim 3:** To use rat metanephric organ culture to explore the mechanisms through which ethanol exposure affects kidney development, and to specifically determine:
    a. The effects of culture in the presence of ethanol on ureteric branching morphogenesis and glomerular number.
    b. Whether exogenous RA can block the adverse effects of ethanol on kidney development.

Chapter 2

General Methods

2.1 Animal Groups

This section provides details of general animal maintenance and preparation such as surgical techniques, and the rationale behind specific procedures. All animal experiments were approved by an Animal Ethics Committee of Monash University (School of Biomedical Sciences Animal Ethics Committee A), and were conducted according to the National Health and Medical Research Council of Australia guidelines.

2.1.1 Sheep Study

Merino Border-Leicester sheep were mated by a commercial supplier and had an ultrasound examination performed to determine the number of fetuses present and confirm gestational age. Only ewes carrying single fetuses were used for these studies as it have previously been identified that presence of multiple fetuses can influence nephron endowment [Mitchell *et al* 2004]. Pregnant ewes remained on pasture until 80 days of gestation at which point they were transported to the Clayton campus of Monash University and housed in individual cages in a room containing a minimum of four ewes within the Department of Physiology. Ewes were allowed to acclimatize to the animal house facility for 10 days before surgery commenced.

2.1.1 Surgical Procedures

<u>Maternal surgery</u>:

At 90 days gestational age (DGA), 10 ewes carrying single fetuses were anesthetised with halothane (2%) and underwent surgery for implantation of arterial and venous catheters [Cock *et al* 1997]. Briefly, a small incision was made in the jugular vein, and a catheter (0.5 cm diameter) was inserted and tied off with sutures. Following this, blunt dissection was used to

isolate the carotid artery, where blood flow was slowed using clamps. An incision was made and a catheter (0.5 cm) inserted approximately 10cm into the artery and tied off with suture, and blood flow restored. Catheters were flushed daily with heparinised saline (5000IU Sodium Heparin, Troy Laboratories, Australia in 1 l of 0.9% sodium chloride, Baxter Healthcare Pty Ltd, Australia) in order to keep them patent. The exteriorised ends of the catheters were contained within a plastic bag attached to the back of the ewe to prevent chewing.

Feeding and alcohol administration:

After surgery ewes were allowed free access to water and a standard amount of "hard-feed" (~1kg, hay and lucerne chaff) daily. Food that was not eaten was collected, weighed, recorded and returned to the ewe, to try to ensure that food intake was standard for all ewes. Ewes were weighed once every 2 weeks for re-calculation of ethanol dose and to ensure normal weight gain. From 95 to 124 DGA, five randomly chosen ewes received a daily 1 hour intravenous infusion of 0.75g/kg ethanol diluted in saline commencing at 9:00am. The five control ewes received a 1 hour intravenous infusion of an equivalent volume of saline. As alcohol was a source of calories, approximately 120g (~250 calories) of additional food was provided to ewes in the saline cohort each day.

Fetal surgery:

At 126 DGA, sheep were anesthetised for fetal surgery [Cock *et al* 1997]. Briefly, general anaesthesia of the ewe and fetus was induced by injection of 1g/kg of maternal body weight of sodium thiopentone (Pentothal®, Abbott Laboratories, Illinois, USA) and then maintained by inhalation of 1-2% isofluorane in oxygen (1.5-2.5% in 70/30 O_2/N_2O). Ewes were intubated to assist with breathing and to maintain anaesthesia. Next, the ewe's abdomen was shaved and aseptically cleaned in preparation for surgery. A 10-15 cm incision was made in the

abdominal wall and the uterus exposed. The position of the fetus within the uterus was determined by gently manipulating the uterus. The fetal hindlimbs were identified and the uterus opened (~5cm incision). Care was taken to minimise loss of amniotic fluid. A catheter (2mm) was implanted into a fetal brachial artery for the collection of fetal blood and two catheters (0.5cm) were sutured onto the fetal skin to allow collection of amniotic fluid. The uterus was sutured tightly closed and catheters were exteriorised onto the ewe flank and surgical incisions were closed. Procaine penicillin (500mg total) and dihydrostreptomycin (500mg total) (Depomycin; Intervet, Australia) were injected intramuscularly into the fetus before the uterine incision was closed. Ewes received a 500mg intravenous injection of antibiotics to limit infection (Ampicillin, Douglas Pharmaceuticals Australia Ltd, Australia). After surgery ewes were returned to their home cages and monitored for surgical recovery and signs of pain (~3-6 hours). If the ewes were seen to be grinding their teeth or showed other signs of discomfort or pain (i.e. not standing up, not eating), they received an intramuscular injection of Xylazil (0.2mg in sterile water, Troy Laboratories, Australia). A total of 6 ewes were administered Xylazil (4 in the saline group and 2 in the alcohol group). Ewes were allowed an additional 24 hours recovery before ethanol administration commenced.

Fetal and maternal blood sampling:

Daily ethanol or saline infusions were resumed on 127 DGA and continued until necropsy at 134 DGA. From 131 to 133 DGA, fetal and maternal arterial blood samples (3ml) were obtained immediately before starting each daily ethanol or saline infusion, and then at +1, 2, 4, 8, 12 and 24 hours (i.e. immediately before the next daily dose). Blood alcohol concentration (BAC) was measured in these blood samples using an established method [Bonnichsen *et al* 1957]. In addition, amniotic fluid (1ml) was collected from one of the catheters in the amniotic fluid compartment 1 hour before the start of the infusions (-1 hour) and 1 hour after the infusions on 133 DGA. Maternal and fetal blood (2ml) samples were collected from the

vascular catheters and centrifuged to obtain plasma, which was stored at -20°C until analysis of osmolality and plasma ions.

2.1.2 Rat Studies

Breeding protocols:

Sprague-Dawley rats were purchased from Monash Animal Services. At 8-10 weeks of age virgin females were mated overnight and the presence of a seminal plug the following morning was designated embryonic day 0.5 (E0.5). Dams were randomly allocated to one of three experimental groups (Ethanol, SHAM, Control) and then housed individually. Standard rat chow and water were provided *ad libitum* with a 12 hour light/dark cycle. At 10.00am on E13.5, rats in the ethanol group were administered 1g of ethanol per kg of maternal body weight diluted in saline by oral gavage. This entailed restraining the rats by hand, inserting a tube via the mouth and oesophagus into the stomach, and slowly pushing the ethanol solution (1ml total volume) into the stomach. Once complete, the gavage tube was removed and animals were returned to their home cages. For the next 4 hours, animal movement and eating and drinking patterns were monitored. Animals that showed signs of distress (lack of movement, not eating or drinking) were further monitored for recovery. All animals recovered within 3 hours of administration and were fully recovered by 4 hours after administration. Rats were re-checked 10 hours after ethanol administration to ensure full recovery. Ethanol administration was repeated on E14.5.

The SHAM group received an equivalent volume of saline on E13.5 and E14.5 by gavage, while the Control group did not receive any treatment. The Control group served as a control for the effects of stress associated with the gavage procedure, as it has previously been established that enhanced maternal stress can be detrimental to fetal development, including kidney development [Moritz *et al* 2002, Wintour *et al* 2003, Singh *et al* 2007].

Pregnant dams were weighed at E12.5, E13.5, E14.5, E15.5, E16.5, E18.5 and E20.5. They were allowed to litter down naturally, and evidence of pup cannibalisation was monitored. Each pup was individually weighed every second day from PN2 until PN30. Pups were sexed, ear clipped for individual identification and weaned on PN21. After weaning, offspring of the same sex were housed with their siblings and checked daily by animal house staff and weekly by laboratory staff for general wellbeing and weight measurement, until postmortem or when blood pressure was measured.

Determination of BAC:

To determine maternal BAC following gavage, separate cohorts of rats were used (N=4 SHAM and N=4 ethanol). This was because the stress associated with blood sampling was considered possibly detrimental to fetal kidney development. Blood (500µl) was collected from the tail vein of pregnant dams 1 hour after ethanol or saline gavage on E13.5. In brief, rats were placed under a heat lamp for 20 minutes to promote vasodilatation and allowing for vessels to be visually identified. Then, the rats were restrained within a towel, the tail exposed, and a 21G needle was inserted into the tail vein at the base of the tail and blood was removed. Blood was spun in a centrifuge at 10,000rpm, plasma was removed and BAC was measured in plasma using the QuantiChrom™ Ethanol Assay Kit (BioAssay Systems, California, USA).

2.2 Amniotic Fluid, Urine and Plasma Sample Analysis

Sheep plasma, urine and amniotic samples (~500μl each) were analyzed for Na^+, K^+, Cl^-, urea and creatinine concentrations, and urinary protein concentration was also determined. All assays were performed using a Synchron CX-5 Clinical system (Beckman, Australia). Osmolality was measured using freezing point depression (Advanced Osmometers, Australia). Rat plasma and urine samples (~500μl each) were analysed for Na^+, K^+ and Cl^- concentrations, and osmolality as above, and urinary protein concentration was also determined.

2.3 Mean Arterial Pressure (MAP) and Heart Rate (HR) Determination in Rats

At 6 months of age, conscious MAP and HR of male and female ethanol, SHAM and Control rats was measured using an indwelling tail artery catheter. Anaesthesia was induced using gaseous anaesthetic isofluorane (5% v/v isofluorane plus oxygen; Rhodia Australia). A midline incision was made in the proximal end of the tail, the fascia was cut away and the tail artery exposed. The artery was cut and a catheter (1.1mm diameter) was inserted and secured with ligatures. Surgery always took less than 20 minutes. The rats were allowed to recover for at least 1 hour after completion of surgery. Then, they were placed back in their home cages in a quiet area and the catheters were connected to a pressure transducer. The Universal Acquisition Program (TracerDAQ™ Measurement Computing, MA, USA) was used to obtain MAP and HR measurements over a 1 hour period. Our laboratories have used this method extensively on previous occasions [Bergstrom *et al* 1998, Stevenson *et al* 2000, Kett *et al* 2004, Singh *et al*, 2007]. All data were checked carefully and values that were clearly abnormal (due to catheter blockage or unusual animal movement) were removed.

2.4 Renal Function Studies in Rats

At completion of all blood pressure recordings, rats were injected with long acting anaesthetic (Inactin; Thiobutabarbital sodium, Sigma-Aldrich) at a dose of 150mg/kg in saline for the renal function experiments. Once the rats were fully anaesthetized, they were placed on their back on a heat mat and a midline incision was made on the ventral aspect of the neck and blunt dissection was used to locate the trachea and jugular vein [Zimanyi *et al* 2006, Denton *et al* 2001]. Small incisions were made into the trachea and jugular vein to allow insertion of catheters (jugular 1.1mm, trachea 0.5cm) which were secured with ligatures. To replace and maintain fluids during surgery, 2% bovine serum albumin (BSA, Sigma-Aldrich, Australia) in saline (0.9% sodium chloride) was infused (1ml/hr/100g bodyweight) via the catheter in the jugular vein. Next, a small incision was made in the lower abdomen and blunt dissection used to locate the bladder. A small cut was made on the ventral surface of the bladder and a 1.1mm catheter was inserted and secured with ligatures.

To determine renal function, radiolabelled para-aminohippuric acid (0.5µCi/hr ^{14}C-PAH, Perkin Elmer, USA) and Inulin (1µCi/hr ^{3}H-inulin, Perkin Elmer, USA) were infused (1.5ml/hr) via the jugular vein for 75 minutes. The first 60 minutes of PAH/Inulin infusion was allowed for the animal to stabilize after surgery and for the radioisotopes to reach equilibration in the plasma. Clearance measurements commenced following the 1 hour PAH/Inulin infusion. Urine was collected from the bladder for 15 minutes. Following clearance measurements and urine collection, blood (2ml) was collected from the tail artery. In addition, blood was collected into heparinised hematocrit tubes (Drummond Scientific, USA) for determination of hematocrit (Hct). To determine urine and plasma levels of ^{14}C-PAH and ^{3}H-Inulin, 20µl aliquots of plasma were placed in vials with 2ml scintillation fluid in triplicate. Samples were placed in a scintillation counter (Beckman LS6000TA, Beckman Coulter, USA). Each sample was counted for 10 minutes and the disintegrations per minute

(DPM) from each triplicate were averaged. GFR was calculated as the clearance of ^{3}H-Inulin using:

$$\text{GFR (ml/min)} = \text{Urine Flow Rate (ml)}/[(\text{DPM (Urine)}/\text{DPM (Plasma)}]$$

Effective renal plasma flow (ERPF) was calculated as the clearance of ^{14}C-PAH using:

$$\text{ERPF (ml/min)} = \text{Urine Flow Rate (ml)}/[(\text{DPM (Urine)}/\text{DPM (Plasma)}]$$

Filtration fraction (FF), effective renal blood flow (ERBF) and renal vascular resistance (RVR) were calculated using:

$$\text{FF} = \text{GFR (ml/min)}/\text{ERPF (ml/min)}$$

$$\text{ERBF} = \text{ERPF (ml/min)}/(1\text{-Hct})$$

$$\text{RVR} = \text{MAP (mmHg)}/\text{ERBF(ml/min)}$$

Rats were killed at completion with an overdose of pentobarbital sodium (100mg/kg, i.p. Troy Laboratories, Australia) and organs (kidneys, heart, lung, liver) collected and weighed.

2.5 Stereology

Unbiased stereological methods were used to estimate kidney volume, total glomerular (and thereby nephron) number, mean glomerular and renal corpuscle volumes, and total renal glomerular and renal corpuscle volumes in both sheep and rats. The stereological methods used in this thesis are considered the gold-standard methods, and have been recommended by both *Kidney International* [Bertram 2001] and the *Journal of the American Society of Nephrology* [Nyengaard 1999, Madsen 1999]. These so-called design-based methods provide accurate (unbiased) and precise quantitative estimates of tissue morphology. Here, *unbiased* refers to the fact that no geometric assumptions are required to obtain the stereological estimates. The earlier model-based methods (see Weibel 1979) required knowledge of the geometry (size, size distribution, shape) of the object (kidney or glomerulus) under investigation. Often, this information was not available, and therefore geometric assumptions were required. These assumptions were often incorrect, being based on little or no evidence, and led to biased estimates.

The stereological methods used for each species were identical, apart from some slight differences for kidney volume and glomerular number estimation. The differences for the sheep study are described in Chapter 3. The following description of stereological methods therefore pertains to the rat studies undertaken in Chapter 4. The stereological analyses were conducted in a blinded fashion, and were performed by the candidate (SPG). Despite these techniques being theoretically unbiased, experience in our group has shown that stereological counting can change over time. In other words, the same person might subconsciously alter their counting approach, and thereby outcomes, over time. To ensure this did not confound the present study, total glomerular number and mean glomerular volume were re-estimated in the first kidney analysed in each group. In every case, the repeat estimates were within 10%

of the original estimate, indicating a satisfactory level of reproducibility in the stereological analysis.

2.5.1 Kidney Fixation and Sampling

The left kidneys of rats were decapsulated and immersion-fixed whole in 5ml of 10% formalin. After fixation for several days, kidneys were washed in 70% ethanol in preparation for sampling. The kidneys were then cut into quarters. Sampled blocks were embedded in glycolmethacrylate (Technovit 7100, Heraeus Kulzer Gmbh, Germany) and sectioned at 20μm thickness with a Leica DM2165 Supercut rotary microtome. The tenth and eleventh sections were collected for further analysis, with the first section chosen at random, and were stained with periodic acid-Schiff (PAS) reagent.

2.5.2 Estimation of Total Kidney Volume

Total kidney volume was estimated using the Cavalieri principle [Gundersen and Jensen 1987, Gundersen *et al* 1988]. In brief, every tenth section was viewed on a Fuji Minicopy reader with a superimposed orthogonal grid (3cm x 3cm) at a final magnification of 24.25X, and grid points that overlaid kidney tissue were counted. Kidney volume (V_{kid}) was estimated using:

$$V_{kid} = 10 \times P_s \times a(p) \times t,$$

where 10 refers to the fact that every tenth section was analysed, t is average section thickness, a(p) is the area associated with each grid point, and P_s is the total number of points hitting kidney tissue.

2.5.3 Estimation of Total Glomerular Number, Mean Glomerular and Renal Corpuscle Volumes, and Total Glomerular and Renal Corpuscle Volumes

Total glomerular (and thereby nephron) number was estimated using the physical disector/fractionator method [see Bertram 2001, Cullen-McEwen *et al* 2001, 2003]. Complete section pairs (i.e. with no artificial section edges and no missing tissue) were projected side-by-side at a final magnification of x298 using a pair of Olympus BH-2 light microscopes modified for projection. The images were projected onto orthogonal test grids (2cm x 2cm) as shown in Figure 2.5.2.1. One microscope was fitted with a standard manual stage that could be rotated, while the other was fitted with an electronic motorized stage (Autoscan EL300, Autoscan Systems P/L, Melbourne, Australia) controlled by a laptop computer running Trackscan (version 7.0, Autoscan Systems P/L, Melbourne, Australia). Using the motorized stage, every 10th section was randomly, uniformly and systematically sampled in the X and Y directions using a field step of 1600µm. Grid points overlying kidney tissue (P_{kid}) in the 10th section, as well as grid points overlying glomeruli (P_{glom}) and renal corpuscles (P_{corp}) were counted.

Next, the stage of the left microscope was manually rotated to align the field of view of the 11th section with that of the 10th section. Once aligned, those glomeruli present in the image of the 10th section and sampled by an unbiased counting frame [Gundersen *et al* 1977] but not present in the image of the 11th section (designated Q^-), and vice versa, were counted according to the disector principle of Sterio (1984). On average, approximately 150 glomeruli (Q^-) were counted in each rat kidney. This has been shown to provide an estimate with a coefficient of error of less than 10% [see Nyengaard 1999]. The following formula was used to estimate the total number of glomeruli in a kidney ($N_{glom,kid}$):

$$N_{glom,kid} = 10 \times P_s/P_f \times 1/(2fa) \times Q^-$$

where 10 is the inverse of the section sampling fraction, P_s is the total area of kidney sections, P_f is the area of sections used for counting glomeruli, 1/(2fa) is the fraction of the total section area used to count glomeruli, and Q^- is the actual number of glomeruli counted. The following formulae were used to estimate mean glomerular volume (V_{glom}), mean renal corpuscle volume (V_{corp}), and the total volumes of all glomeruli ($V_{glom,tot}$) and all renal corpuscles ($V_{corp,tot}$) in the kidney:

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

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

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

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

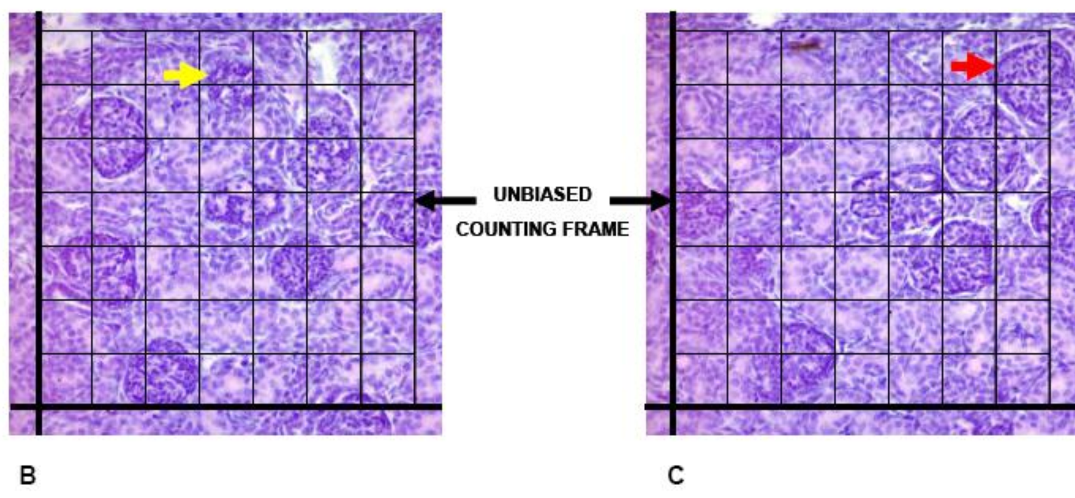

Figure 2.5.2.1. The physical disector/fractionator method used to estimate total glomerular (nephron) number. (A) Double microscopes projecting corresponding images from the 10th and 11th sections. Note that one microscope (on the right) is fitted with a motorised stage. (B and C) Images of the 10th and 11th sections seen at higher magnification with superimposed unbiased counting frames and stereological test grids. The yellow arrow on the 10th section (B) indicates a glomerulus that is not present in the 11th section (C). The red arrow on the 11th section (C) indicates a glomerulus that is not present in the 10th section (B). These two glomeruli are counted (Q^-). Only glomeruli sampled by the unbiased sampling frame are analysed. See Gundersen (1977) for a full description.

2.6 Whole Metanephric Organ Culture

Whole metanephric organ culture, originally established by Grobstein (1956), has been extensively used for more than 50 years to study the effects of exogenous factors on kidney development. It allows whole metanephroi to be cultured for up to 6 or more days in the presence of known concentrations of agents of interest, in this case, ethanol. Under control culture conditions, the metanephroi increase in size, and many of the stages and processes of metanephric development occur, including ureteric branching, nephron induction, and nephron (and glomerular) growth and differentiation. While metanephroi develop more quickly in the presence of serum, most laboratories utilise serum-free conditions so that the composition of the media is precisely known.

In the present studies, virgin Sprague-Dawley female rats were placed with a male rat for 4 hours. When a seminal plug was detected, this indicated time of mating and was designated as embryonic day 0.5 (E0.5). At E14.5, the dams were surgically anaesthetised with Pentobarbitone Sodium (150mg/kg of body weight administered i.p, Lethabard, Troy Laboratories, Australia), a midline abdominal incision was made, the uterus identified and embryos were quickly located, removed, weighed and decapitated. Whole metanephroi were isolated from embryos within a weight range of 130-150mg, in order to limit variability in kidney size and development. Embryos were pinned to a silicone plate, the abdomen was opened and liver, stomach and intestinal organs removed. Then the urogenital ridge was isolated and the gonads removed, leaving the kidney. Whole metanephroi were placed on 3.0µm pore polycarbonate transfilter membranes (Osmonics, Proetics, New South Wales, Australia) in 24-well tissue culture plates containing 350µl of serum-free culture media at 37°C and 5% CO_2. The culture medium consisted of DMEM:Ham's F12 liquid medium (Sigma-Aldrich) supplemented with 5µg/ml transferrin (Sigma-Aldrich, Australia), 12.9µl/ml L-Glutamine (Sigma-Aldrich, Australia), Penicillin (100U/ml, Sigma-Aldrich, Australia), and

Streptomycin (100μg/ml, Sigma-Aldrich, Australia). Metanephroi were allocated randomly into one of the following six groups: 0% ethanol (control), 0% ethanol plus 10nM retinoic acid (RA, all-*trans*-retinoic acid, Sigma-Aldrich, Australia), 0% ethanol plus 20nM RA, 0.2% ethanol, 0.2% ethanol plus 10nM RA or 0.2% ethanol plus 20nM RA. The concentrations of RA used were based on the study of Vilar *et al* (1996) who found that addition of RA at a concentration of 100nM, 10-fold higher than the concentration used in the current study, resulted in a 200% increase in nephron number after 6 days culture. Therefore, 10 and 20nM RA were selected as appropriate concentrations to use as it would be expected that this would provide an increase in glomerular number of approximately 20 to 30%. To investigate the effects of ethanol and/or RA on ureteric branching morphogenesis, metanephroi were cultured for 48 hours with media replaced after 24 hours. To determine the effects of ethanol and/or RA on glomerular number, metanephroi were cultured for 5 days, with media replaced after 24 hours and then left for the following 4 days. At the conclusion of the culture period, metanephroi were fixed with ice cold 100% methanol, sealed in parafilm and kept at 4°C until wholemount immunofluorescence staining was undertaken.

2.7 Quantitation of Ureteric Branching Morphogenesis in Whole Cultured Metanephroi

For visualisation and quantitation of the ureteric tree in cultured metanephroi, following fixation the metanephroi were washed briefly in 1% Tween 20 (PBST) and incubated with primary antibody (monoclonal mouse anti-pan cytokeratin (Sigma-Aldrich) at a dilution of 1:100 at 37°C for 2 hours. Metanephroi were then washed in PBST before addition of the secondary antibody, Alexa 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR) at a dilution of 1:100 at 37°C for 2 hours. Metanephroi were then washed briefly in PBST before mounting in PBS/glycerol mounting medium (Sigma-Aldrich). The metanephroi were then visualized using a Provis epifluorescence microscope (Olympus) and manually skeletonised. Ureteric branch points (defined as the intersection of three or more branches) and tips were identified and counted as shown in Figure 2.7.1.

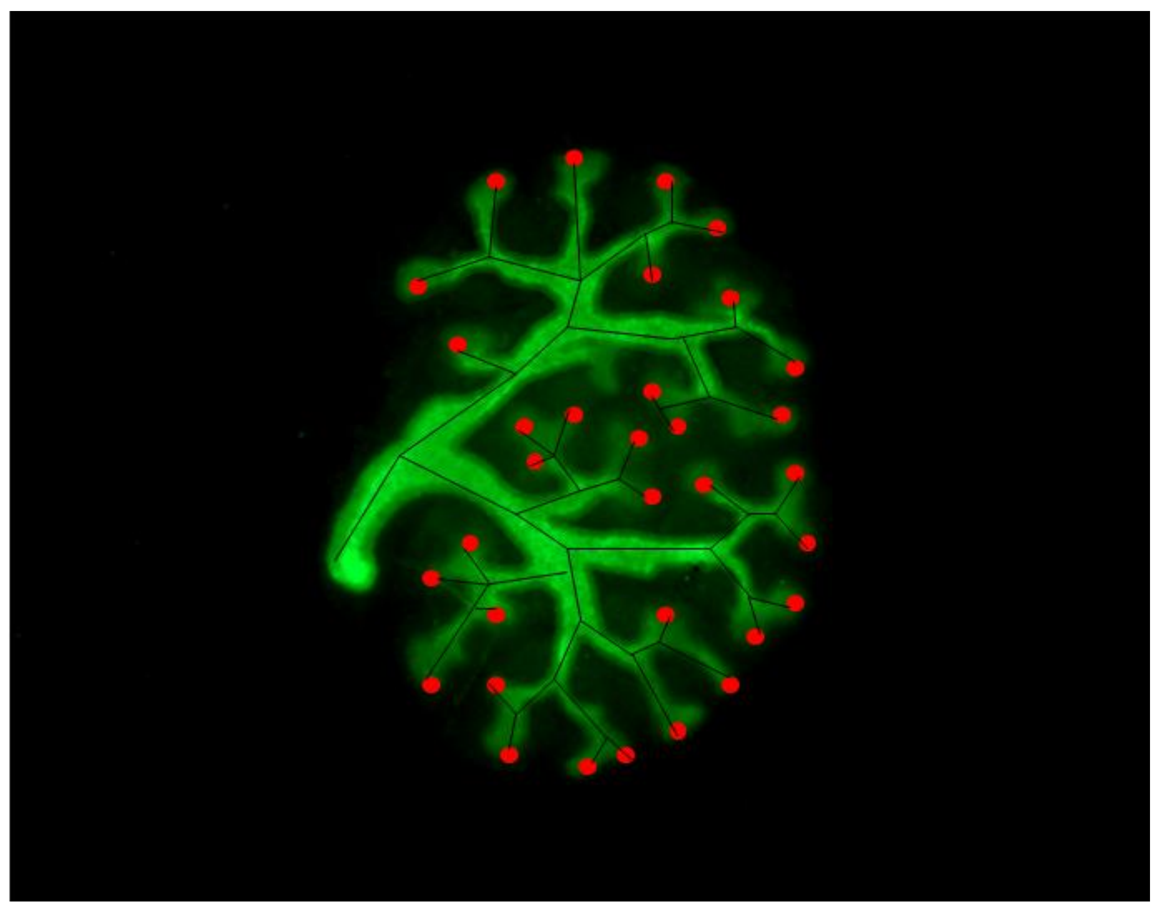

Figure 2.7.1: Quantitation of Ureteric Branch Points and Tips. The immunostained ureteric tree in a whole metanephros cultured for 48hrs is shown. Black lines represent manual skeletonisation of ureteric branches, with red dots representing ureteric tips. The kidney displayed in this image contains a total of 30 ureteric branch points and 33 ureteric tips.

2.8 Counting Glomeruli in Whole Cultured Metanephroi

Following fixation, those metanephroi to be prepared for glomerular number estimation were briefly washed in PBS. They were incubated in 50mM NH_4Cl at room temperature for 1 hour and then permeabilized with 0.1% saponin in PBS for 1 hour at room temperature. After digestion with 2% H_2O_2 in methanol at room temperature for 30 minutes, metanephroi were washed in 0.1% saponin in PBS for 30 minutes. Metanephroi were then incubated in 0.1U/ml of neuraminidase (Sigma-Aldrich) in 1% $CaCl_2$ in PBS for 2 hours at 37^oC, before being washed twice for 30 minutes in 0.2% saponin in PBS. Metanephroi were then incubated in peanut agglutinin (PNA) (50µg/ml; Sigma-Aldrich) in 0.3% Triton in PBS with 1:100 dilution of ions overnight at 4^oC. Metanephroi were then extensively washed in 0.1% saponin to remove excess PNA and then mounted on cavity slides with fluorescence preserving mounting media (Sigma-Aldrich). Metanephroi were then observed under the Provis epifluorescence microscope for direct counting of glomeruli (Figure 2.8.1).

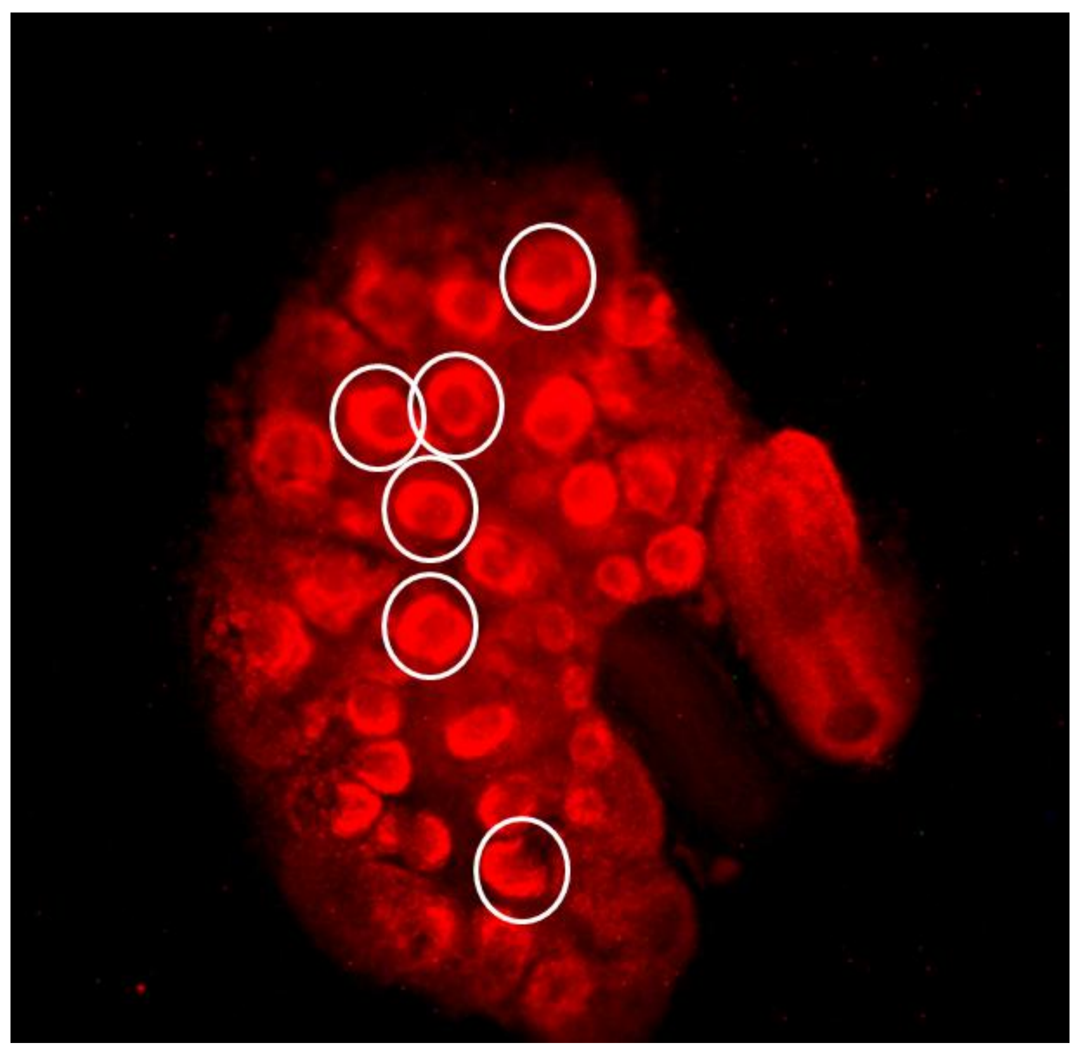

Figure 2.8.1: Glomerular Quantitation. This figure depicts the method of glomerular counting in whole cultured metanephroi. Within each white circle, a single developing glomerulus is seen and counted. The kidney displayed above contains a total of 32 glomeruli.

2.9 Gene Expression Studies

2.9.1 RNA Extraction and cDNA Construction

Total RNA was extracted from slices of sheep and rat kidneys containing cortex and medulla using RNeasy extraction kits (Qiagen, Australia). In brief, samples were placed in a sterile eppendorf tube and to this 350µl of a guanidine-isothiocyanate-containing (GITC) lysis buffer (RLT) (containing 3.5µl β-mercaptoethanol) was added. Samples were then lysed until completely homogenized. Lysate was then repeatedly centrifuged to purify RNA using wash buffer (RW1), with flow through again discarded. The RNeasy column was transferred to a new tube and washed with RPE buffer and centrifuged at 10,000 rpm for 15 seconds. The column was then transferred to a 1.5ml RNA collection tube and 50µl of DEPC-treated water was added to RNeasy membrane and centrifuged at 13,200rpm for 1 minute. The concentration of RNA was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., CA, USA). The Bioanalyzer provides information of both RNA quantity and quality. One microgram of each RNA sample was reverse transcribed as previously described [Singh *et al* 2007]. Briefly, RNA was reverse transcribed into cDNA. 1 µg of each RNA sample was reverse transcribed in a 30.75µl reaction containing 5µl of 10 x Taqman reverse transcriptase buffer (Invitrogen, Australia), 11µl of 25mM $MgCl_2$ (Invitrogen, Australia), 10µl of 100µM of 2'-deoxynucleoside 5'-triphosphate (Invitrogen, Australia), 2.5µl of 50mM random hexamers (Invitrogen, Australia), 1µl of RNase inhibitor (Invitrogen, Australia) and 1.25µl of Multiscribe™ reverse transcriptase (PE Applied Biosystems, Foster City, CA, USA). To control for genomic DNA contamination, negative reactions were prepared, where the 1.25µl of Multiscribe™ was replaced with sterile water. Reverse transcription reactions were run in a BIORAD i-cycler™ at 25°C for 10 minutes, 48°C for 30

minutes, 95°C for 5 minutes and then held at 4°C until used. These samples were stored at -20°C.

2.9.2 Real-Time PCR

Real-Time PCR reactions quantify mRNA by utilizing a fluorescence-based technology. The Eppendorf RealPlex Cycler real-time machine used in these studies can detect fluorescence between 500 nm and 660 nm (BUSTIN 2000). This system uses the Taqman® assay (Perkin-Elmer-Applied Biosystems, Foster City, CA, USA).

The sequences of genes analysed in the ovine study are presented in Table 2.9.2.1. Primer/probe sequences were obtained from www.ncbi.nlm.nih.gov/query and aligned using the ClustalW alignment program provided on www.ebi.ac.uk/clustalw. Primer and probe sequences were designed using Primer express software. Primer concentrations of 50nM, 300nM and 900nM of both reverse and forward primers were trialled to determine optimal concentrations. Gene expression analysis was performed for two angiotensin II receptors (AT1 and AT2), renin, members of the insulin-like growth factor family and their receptors (IGF1, IGF1R, IGF2, IGF2R), and six sodium ion channels (ENaCα, ENaCβ, ENaCγ, NaKα, NaKβ and NaKγ). Optimisation of primer/probe sets had been performed prior to commencement of this thesis and have been published (Dodic *et al* 2002)

The sequences of genes (Wnt4, AT1b, AT2, BMP-4, TGFβ1, GFRα1, TGFβ2, TGFβ3, FGF7, Wnt11, RARα and RXRα) analysed in the rat study are presented in Table 2.9.2.2 and were designed using similar techniques as described above. Other genes, including GDNF, BAX, Bcl-2 and ADH were analysed using an Assay-on-Demand from Applied Biosystems. Prior to use all primer/probe sets underwent optimisation and validation to ensure optimal results. Firstly, primer and probe concentrations were optimised. Then all genes were tested in a multiplex reaction to ensure they could be run in a single reaction with a house-keeping gene.

This is essential to ensure that both the gene of interest and the house-keeping gene amplify with equal efficiency and results are similar if run in a single or multiplex reaction. These experiments had revealed that all genes could be multiplexed with 18S, except for AT2 in the rat, as optimization experiments revealed the CT was altered if run in a multiplex reaction. Thus, the AT2 in the rat experiments was run as a singleplex reaction. The Assay-on Demand kits were run at the concentrations indicated by the manufacturer. However, the validation experiment to ensure they could be multiplexed with 18S was performed in the laboratory of Dr Karen Moritz.

Table 2.9.2.1. Primer and probe sequences for genes used for quantitative RT-RCR in sheep.

Gene	Forward Primer (5'-3') Reverse Primer (5'-3') Probe (5'-3')	Working Concentrations (nM)
ATI Receptor	GGGCTGTCTACACTGCTATGGAA CCGGAAGCGATCTTACATAGGTA ACCGCTGGCCCTTCGGCAA	900 900 150
ATII Receptor	TGTTCTGGCGTTCATCATTTG CCATCCAAGCTAGAGCATCCA TGGCTTCCCTTCCATGTTCTGACCTTC	300 300 75
Angiotensinogen	CTCTCCCACGCTCACTAGACTTG CTCTTCTAGTTGTCCAAGTACGTA CCACGGACCCAAATCTCGCTGC	300 300 75
Renin	TGAGCCAGGACCTGGTGACT TCCGTGACCTCGCCAAAG TCTGTGTGACTGTGATCCCGCCA	900 50 200
IGF1	CAGCGCCACACCGACAT CCTGCACTCCCTCTGCTTGT TCTTCAAATGTACTTCCTTCTGAGCCTTGGG	300 300 50
IGF1R	CGCCTCTACTTTGTCTTTGCA TCACTGGCCCAGGAATGTC CCTGCTCCTTCTGCAGGCATGGTT	300 300 50
IGF2	CGTGCTTCCGGACGACTT TGCTTCCAGGTGTCAGATTGG ACAGCATACCCCGTGGGCAAGTTCT	900 300 50
IGF2R	CCAGCGGATGAGTGTCATCA ACCTCCCCGGTGAACACA CCTCTGCCGTTGTTACCTGCTGTCTGA	900 900 50
ENaCα	CATCTTCACCTGCCGTTTCA CGGGTGGTGGAAGTGAGAGT CCAGGACTCCTGCAATGAGGCGAA	300 300 50
ENaCβ	GCAAAGTAGCCATGAGACTATGCA GGTGGCGCTGCTGAAGTT CAATGGAACCACATGCACCTTCCG	900 300 75
ENaCγ	TCAGGGAGCCAACTACTGCAA CCTGGTGCAGTTGGTAGTAGCA ACCAGCAGCACCCCAACTGGATGTA	300 300 75
Na/K ATPase (α subunit)	AACGGCTTCCTCCCTAATCAC GTCGTTGATCCAACGGTCATC TGCTGGGCATCCGAGTGACCTG	300 300 50
Na/K ATPase (β subunit)	GCCTGGCGGGCATCTT GTGGGCTTAAATTCACTGATGGT TCGGAACCATCCAAGTGATGCTGCT	300 300 50
Na/K ATPase (γ subunit)	GCCCTGGCCTTCATCGT TTGCCGGTGCTTCTTCTTG CTCGTCATCATCCTCAGCAAAAGATTCCG	50 300 75

Table 2.9.2.2. Primer and probe sequences for genes used for quantitative RT-RCR in rats.

Gene	Forward Primer (5'-3') Reverse Primer (5'-3') Probe (5'-3')	Working Concentration (nM)
GFRα1	CGTCTTAACTGCAATACAC AACCGCTACAATATCGAAAG AAATGTCACTGACTTGGGTTTGGGC	300 300 150
TGFβ1	TCGACATGGAGCTGGTGAAA GAGCCTTAGTTTGGACAGGATACTG AAGCGCATCGAAGCCTCCGTG	300 300 150
TGFβ2	CGACATGCCGTCCCACTTC CTGCCCACTGAGCCAGAG CCCTCCGAAACTGTCTGCCCAG	300 300 150
TGFβ3	TTCAGCCCAATGGAGACATAC CGGCCATGGTCATCTTCGT TGGAAAATGTTCACGAGGTGATGGAA	300 300 150
FGF7	GACAAACGAGGCAAAGTGAAAGG TGCCACAGTCCTGATTTCCA ACCCAGGAGATGAGGAACAGCTACA	300 300 150
Wnt4	AGGATGCTCGGACAACATCG CGCATGTGTGTCAAGATGGC AAACGACGGCCAGAGAATTA	300 300 75
Wnt11	TGCGTCTACACAACAGTGAAGTG GGAGCAGGAGCCAGATACC AGACAGGCTCTACGTGCCTCCC	300 300 150
RARα	CCGGACTCCGCTTTGGAATG GGGCTGGGCACTATCTCTTC TCAAACCACTCCATCGAGACCCA	300 300 150
RXRα	CGGAGCTGGTGTCGAAGATG CCCTTAGAGTCAGGGTTGAAGA TGACATGCAGATGGACAAGACGGA	300 300 150
AT1b	GGGCTGTCTACACTGCTATGGAA CCGGAAGCGATCTTACATAGGTA ACCGCTGGCCCTTCGGCAA	300 150 50
AT2	TGTTCTGGCGTTCATCATTTG CCATCCAAGCTAGAGCATCCA TGGCTTCCCTTCCATGTTCTGACCTTC	300 300 75
BMP4	CGAGCCATGCTAGTTTGATACCT CCGCGTGGCCCTGAA TCGGCGATTTTTCTTCCCGGTCT	300 300 100

2.9.3 Quantitation of Relative Gene Expression

A comparative cycle of threshold fluorescence (C_T) method was used to quantify relative levels of gene expression, using 18S as an internal control (housekeeping gene). For each individual sample, the C_T value for 18S was subtracted from the C_T value for the gene of interest to give a ΔC_T. The ΔC_T of the calibrator (the mean ΔC_T of their respective control groups) was subtracted from each sample to give a $\Delta\Delta C_T$ value. This was inserted into the equation $2^{-\Delta\Delta C_T}$ to give a final expression relative to the calibrator, using the following equations:

$$\Delta C_{T\text{ (gene of interest)}} = C_{T\text{ (gene of interes)}} - C_{T\text{ (18S)}}$$

$$\Delta\Delta C_{T\text{ (gene of interest)}} = \Delta C_{T\text{ (gene of interest)}} - C_{T\text{ (calibrator)}}$$

$$\text{Relative Expression} = 2^{-\Delta\Delta C_{T\text{(gene of interest)}}}$$

2.10 Statistics

Data are expressed as mean ± standard error of the mean (SEM) except where otherwise indicated, and were analysed using one-way analysis of variance (ANOVA), two-way ANOVA, two-tailed unpaired t-tests, and Tukey's post-hoc test as appropriate running on the SPSS (version 14) statistical software package. A probability (P) value < 0.05 was classified as being statistically significant. Further details relating to specific tests for each data set are provided in the respective experimental chapters.

Before commencement of each experiment, power calculations were performed to determine minimum sample sizes required to detect a defined difference between groups with statistical significance. However, this was not the case for the ovine study (Chapter 3) which was designed prior to commencement of my PhD. In the acute ethanol study in rats, power analysis indicated that 7 rats were required in each of the six experimental groups (control male and female, SHAM male and female, ethanol male and female) in order to detect a 20% difference in total nephron number between groups with a power of 0.8. The analysis assumed group standard deviations for nephron number estimates of 10%, based on earlier studies from our laboratory. Similar power analyses were used to determine the number of rats for the cardiovascular and renal function analysis.

As renal gene expression is known to be highly variable during development [Brunskill *et al* 2008; Challen *et al* 2005; Caruana *et al* 2006; Stuart *et al* 2003], the assumptions in the power analysis for the *in vivo* gene expression studies described in Chapter 5 were different from other studies within this thesis. In this case, the power analysis was designed to detect a 50% difference between group means, and assumed a standard deviation of 15% and a power of at least 0.8 for a total of two groups (ethanol-exposed kidneys and saline-exposed kidneys). From these calculations we achieved a group size of five rats.

Finally, when conducting studies on species such as the rat which have large litters of variable size, it is important to distinguish between an "individual pup" and "whole litter". In such circumstances, an appropriate control for "the litter effect" should be employed. The litter effect, which is the tendency of littermates to present a similar phenotype or response to one another than to animals from non littermates, can be controlled by drawing a single animal from the litter for any one comparison and is generally considered optimal for studies where the dam receives the experimental stimulus. When using multiparous species for developmental studies, the litter should be considered the experimental unit, and pups from the same litter and treatment group should not be considered independent measures. Failure to do so can result in type 1 statistical errors [Holson and Pearce, 1992]. The counter point to this is that for some experimental designs (such as culturing of embryonic kidneys) this approach contravenes the policies of reduction and refinement entrenched in guidelines for use of animals in scientific experiments. For example it would not be appropriate to kill a dam and discard all but one embryo in the litter.

A second method for controlling the litter effect involves using the same (multiple) number of animals in a litter for study and then returning a mean of these animals for the litter; the so called compound mean. This method minimises the litter effect, although the compression of many observations into a single mean results in a loss of data points. This reduces the number of degrees of freedom in the ANOVA, translating into a reduction in statistical power.

In this thesis, we incorporated a weight for litter in the ANOVA as suggested by Festing (2006). This method incorporates a least squared means regression analysis to the ANOVA and is weighted by litter. By using this model, the litter effect was reduced but the inherent variability of the data and higher degrees of freedom were retained. Because each litter contributed equally to the model this method allows us to draw differing numbers of animals per litter but retain the overall concept that data from each litter cannot be treated as an independent sample. This method has been used by many research groups in studies reported

in high impact papers [Elms *et al* 2007, Erhuma *et al* 2008, Yates *et al* 2009, Harrison *et al* 2009].

Chapter 3

Declaration for Thesis Chapter 3

Declaration by Candidate

In the following chapter, Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contribution (%)
All tissue collection, data analysis and interpretation of data preformed by candidate. First draft of publication written by candidate. 50% of animal work performed by candidate.	70%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of Contribution	Extent of Contribution (%) for student co-authors only
Kelly Kenna	Performed 50% of animal work	5%
John Bertram	Draft Revision/co-supervision of candidate, study design	
Wendy Hoy	Study Design	
Edwin Yan	Helped with animal work and logistics of study	
Alan Bocking	Study Design	
James Brien	Study Design	
David Walker	Draft Revision	
Richard Harding	Draft Revision	
Karen Moritz	Draft Revision/co-supervision of candidate, study design	

Candidate's Signature:

	Date:

Declaration by co-authors

The undersigned hereby certify that:

1) The above declaration correctly reflects the nature and extent of the candidate's contribution to this work and the nature of the contribution of each of the co-authors.

2) They meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise.

3) They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication.

4) There are no other authors of the publication according to these criteria.

5) Potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit.

6) The original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)

Department of Anatomy & Developmental Biology, and Department of Physiology, Monash University, Victoria, Australia.

	Signature:	**Date:**
Signature 1: Ms. Kelly Kenna		7/8/09
Signature 2: Prof. John Bertram		27/7/09
Signature 3: Prof. Wendy Hoy		28/7/09
Signature 4: Dr. Edwin Yan		5/8/09
Signature 5: Alan Bocking		10/6/09
Signature 6: James Brien		3/6/09
Signature 7: Dr. David Walker		28/7/09
Signature 8: Prof. Richard Harding		6/8/09
Signature 9: Dr. Karen Moritz		28/7/09

Repeated Ethanol Exposure During Late Gestation Decreases Nephron Endowment in Fetal Sheep

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Running title: Ethanol exposure decreases nephron number in fetal sheep

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Abstract

Maternal alcohol consumption during pregnancy can affect fetal development, but little is known about effects on the developing kidney. Our objectives were to determine the effects of repeated ethanol exposure during the latter half of gestation on glomerular (nephron) number and expression of key genes involved in renal development or function in the ovine fetal kidney. Pregnant ewes received daily intravenous infusion of ethanol (0.75g/kg, n=5) or saline (control, n=5) over 1 hour from 95-133 days of gestational age (DGA; term is approximately 147 DGA). Maternal and fetal arterial blood samples were taken before and after the start of the daily ethanol infusions for determination of blood ethanol concentration (BEC). Necropsy was performed at 134 DGA and fetal kidneys collected for determination of total glomerular number using the physical disector/fractionator technique; at this gestational age nephrogenesis is completed in sheep. Maximal maternal and fetal BECs of 0.12±0.01g/dL (mean±SEM) and 0.11±0.01g/dL, respectively, were reached 1 hour after starting maternal ethanol infusions. Ethanol exposure had no effect on fetal body weight, kidney weight or the gene expression of members of the renin-angiotensin system, insulin-like growth factors and sodium channels. However, fetal glomerular number was lower after ethanol exposure (377,585±8,325) than in controls (423,177±17,178, $P<0.001$). The data demonstrate that our regimen of fetal ethanol exposure during the latter half of gestation results in an 11% reduction in nephron endowment, without affecting the overall growth of the kidney or fetus or the expression of key genes involved in renal development or function. A reduced nephron endowment of this magnitude could have important implications for the cardiovascular health of offspring during postnatal life.

Key words: nephron number, kidney, fetus, ethanol exposure, sheep

Introduction

Alcohol (ethanol) consumption during pregnancy remains common in most developed countries, but recommended guidelines on safe levels of alcohol consumption vary significantly [42]. Excessive alcohol consumption during pregnancy is known to result in the fetal alcohol syndrome (FAS), with characteristic craniofacial dysmorphology, growth restriction, and intellectual dysfunction as the principal diagnostic features manifesting after birth [13, 30, 34, 48]. Even moderate levels of alcohol exposure are now known to adversely affect learning and behaviour in infants and children. While there is ample evidence that prenatal ethanol exposure can affect CNS development, few studies have investigated effects on other critical organs, such as the kidney. Studies of children diagnosed with FAS demonstrate that some have renal malformations [32, 37]; their kidneys are often small, misshapen and frequently fail to rotate correctly during development [22, 37]. However, the effects of less severe prenatal exposures to ethanol are relatively unexplored.

Animal studies suggest that prenatal ethanol exposure can affect the developing kidney. For example, rats that were prenatally exposed to ethanol had decreased renal function compared to controls at 90 days of postnatal age [2]. Although these findings suggest tubular dysfunction, there were no overt histological differences between the kidneys of the ethanol-exposed offspring and the kidneys of the controls [2]. Another study reported decreased renal protein and DNA content in 9 day old rats prenatally exposed to ethanol, but these effects were not evident in adulthood [18]. In instrumented near-term fetal sheep, maternal ethanol administration (1g/kg maternal body weight) during gestation led to a transient decrease in urinary output [10]. However, none of these previous studies have examined the effect of prenatal ethanol exposure on the number of glomeruli (nephrons) in the kidneys. This is important because nephrons are the functional units of the kidney, nephrogenesis in the human is complete before birth, and because a reduced nephron endowment is permanent and has

been linked to the development of adult-onset diseases, such as hypertension [7, 23]. Therefore, we have used an animal model, the sheep, in which kidney development is similar to that of humans to determine whether prenatal ethanol exposure has the capacity to decrease nephron endowment. As in the human, nephrons are formed only during fetal life in sheep and no new nephrons develop after birth [48].

In the present study, the effects of daily maternal ethanol administration during the period of peak nephrogenesis on glomerular number were examined in the near-term ovine fetus. Our primary objective was to test the hypothesis that repeated prenatal ethanol exposure, via maternal ethanol administration, results in smaller fetal kidneys with decreased nephron endowment. The kidneys were studied at 134 days of gestational age (DGA), as nephrogenesis is complete at 130 DGA (term is about 147 DGA) in the sheep fetus. Signalling-molecule members of the renin-angiotensin system (RAS) are involved in influencing the development and/or function of the fetal kidney and have been shown to have altered expression in the sheep [29] and rodents following developmental perturbations [35, 45]. The renal RAS is important for normal kidney development as well as the regulation of arterial blood pressure in the adult. Expression of other genes important for renal function, including sodium channels have also been shown to be altered following a prenatal perturbation [5, 43]. Thus, a second objective was to determine the effects of prenatal ethanol exposure on the expression of genes involved in renal development and/or function.

Materials & Methods

Animal groups: All experiments were approved by the Animal Ethics Committee of Monash University, and conducted according to the National Health and Medical Research Council of Australia guidelines. At 90 DGA, 10 Merino Border-Leicester ewes carrying single fetuses were anesthetised with halothane (2%) and underwent surgery for implantation of arterial and venous catheters [11]. After surgery ewes had free access to food and water. As the alcohol infused to ewes provides a source of calories, approximately 120g (~250 calories) of additional food was provided to ewes in the saline cohort to compensate for calories derived from ethanol.

From 95 to 124 DGA, five randomly chosen ewes received a daily one-hour intravenous infusion of 0.75g/kg of ethanol diluted in saline starting at 9:00am. The 5 control ewes received a one-hour intravenous infusion of an equivalent volume of saline. At 126 DGA, the animals were again anesthetised and underwent fetal surgery [11]. Briefly, general anesthesia of the ewe and fetus was induced by 1g sodium thiopentone (i.v.) and maintained by inhalation of 1-2% isofluorane in oxygen. The ewe's abdomen and uterus were incised to expose the fetus, avoiding loss of amniotic fluid. A catheter was implanted into a fetal brachial artery for the collection of fetal blood and two catheters were sutured to the fetal skin to allow collection of amniotic fluid. After closure of all incisions, catheters were exteriorised through an incision on the flank of the ewe. After recovery of the ewe, the daily ethanol infusions were resumed on 127 DGA and continued until necropsy at 134 DGA. From 131 to 133 DGA, fetal and maternal arterial blood samples (3ml) were obtained immediately before starting each daily ethanol or saline infusion, and then at +1, 2, 4, 8, 12 and 24 hours (i.e. immediately before the next daily dose). Blood ethanol concentration (BEC) was measured in these blood samples using an established method [6].

Amniotic fluid and plasma sample analysis: Amniotic fluid (1ml) was collected from one of the catheters in the amniotic fluid compartment 1 hour before the start of the infusions (-1 hour) and 1 hour after the infusions (2 hour) on 133 DGA. Maternal and fetal blood (2ml) samples were collected from the vascular catheters and centrifuged to obtain plasma. All samples were analyzed for Na^+, K^+, Cl^-, urea, creatinine and total protein concentrations using a Synchron CX-5 Clinical system (Beckman, Australia). Osmolality was measured using freezing point depression (Advanced Osmometers, Australia).

Post-mortem examination and fetal kidney collection: At 134 DGA, ewes and fetuses were euthanized with a maternal overdose of pentobarbital sodium (100mg/kg maternal body weight, i.v.). The fetus and its kidneys were removed and weighed. The right kidney was cut into 1-mm-thick slices containing samples of cortex and medulla, which were then snap frozen in liquid nitrogen. The left kidney was cut into approximately equal halves before immersion fixation in 300ml of 10% formalin for 24 hours. After fixation, the kidney halves were washed in 70% ethanol in preparation for further sampling.

Fetal kidney sampling: Each half of the left fetal kidney was cut into quarters, and each quarter was cut into 1.5mm slices. Every fifth slice was collected, with the first slice chosen at random; each collected slice was cut into blocks of tissue of approximately equal size. Blocks were arranged from smallest to largest, and every sixth block was sampled, with the first being chosen at random. Sampled blocks (10-12 per animal) were embedded in glycolmethacrylate (Technovit 7100, Heraeus Kulzer Gmbh, Germany) and sectioned at 20µm thickness with a Leica DM2165 Supercut rotary microtome. The tenth and eleventh sections were collected for further analysis, with the first section chosen at random, and were stained with periodic acid-Schiff (PAS) reagent.

Total kidney volume: Total kidney volume was estimated using the Cavalieri principle. In brief, every tenth section was viewed on a Fuji Minicopy reader with a superimposed orthogonal grid (3 cm x 3cm), and points that hit kidney tissue were counted. We used the formula:

$$V_{kid} = 5 \times 6 \times 10 \times P_s \times a(p) \times t,$$

where 5 is the inverse of the first sampling fraction, 6 is the inverse of the second sampling fraction, 10 accounts for the fact that every tenth pair of sections was analysed, t is average section thickness, a(p) is the area associated with each grid point, and P_s is the total number of points hitting kidney tissue.

Total glomerular number: Total glomerular number ($N_{glom,kid}$) was estimated using the physical disector-fractionator method [4]. This method is considered the gold standard methodology for determination of nephron endowment [3, 31]. Each kidney was randomly analysed "blind" by the same person (SPG). Slides with complete kidney sections (and their corresponding pair) were projected at a magnification of x298 with Olympus BH-2 light microscopes modified for projection. The fields were projected onto an orthogonal grid (6cm x 6cm). We used the formula:

$$N_{glom,kid} = 5 \times 6 \times 10 \times P_s/2P_f \times Q^-$$

where 5 is the inverse of the first sampling fraction, 6 is the inverse of the second sampling fraction, 10 is the inverse of the third sampling fraction, P_s is the total area of kidney sections, P_f is the area of sections used for counting glomeruli (2 refers to the fact that counting was performed in both directions to double counting efficiency), and Q^- is the actual number of glomeruli counted. Glomeruli were only counted if they were sampled with an unbiased counting frame and were not present in the adjacent projected section.

Glomerular tuft and renal corpuscle volumes: Grid points overlying glomerular tufts (P_{glom}) and renal corpuscles (P_{corp}) were counted to estimate mean and total glomerular and corpuscle volumes. The following formulae were used:

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

$$V_{glom.tot} = V_{glom} \text{ x } N_{glom,kid}$$

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

$$V_{corp.tot} = V_{corp} \text{ x } N_{glom,kid}$$

Fetal kidney morphology: Samples of the left kidney not taken for glomerular counting were embedded in paraffin and sectioned at 5μm thickness. Sections were stained with hematoxylin and eosin, PAS or Masson's trichrome, and then were analysed by light microscopy.

Real-time PCR quantification: Total RNA was extracted from samples containing both renal cortex and medulla using RNeasy extraction kits (Qiagen). A 1μg amount of RNA was reverse transcribed into cDNA [16]. Gene expression analysis was performed for angiotensin II receptors, AT1 and AT2 were measured and renin and members of the insulin-like growth factor family and their receptors (IGF1, IGF1R, IGF2, IGF2R). Six sodium ion channels (ENaCα, ENaCβ, ENaCγ, NaKα, NaKβ and NaKγ) were also analysed (Table 1). A comparative cycle of threshold fluorescence (C_T) method was used, with 18S as an internal control (housekeeping gene). For each individual sample, the C_T value for 18S was subtracted from the C_T value for the gene of interest to give a ΔC_T for each sample. The ΔC_T of the calibrator (in this case, the mean ΔC_T of the saline group) was subtracted from each sample to give a $\Delta\Delta C_T$ value. This was inserted into the equation $2^{-\Delta\Delta C_T}$ to give a final expression relative to the calibrator.

Data analysis: Data are expressed as mean ± standard error of the mean (SEM), except where otherwise indicated. Fetal plasma and amniotic fluid data were analysed using repeated-measures two-way ANOVA with treatment and time being factors. Comparison of data between the ethanol and saline treatment groups was performed using two-tailed unpaired t-tests. Statistical significance was accepted at $P<0.05$.

Results

Blood ethanol concentrations (BECs): Maximal maternal and fetal BECs, between 131 and 134 DGA, were 0.12±0.01g/dL and 0.11±0.01g/dL, respectively, occurring at the end of the one-hour maternal ethanol infusion. Eight hours after starting the ethanol infusion, fetal and maternal BECs were non-detectable (Figure 1).

Amniotic fluid composition: There were no differences between the ethanol-exposed and control groups in amniotic fluid concentrations of Na^+, K^+, Cl^-, urea, creatinine and total protein at 133 DGA (Table 2). In addition, there was no difference between groups in the amniotic fluid concentrations of these substances after the 1-hour maternal infusion compared with levels before the infusion.

Maternal and fetal plasma analysis: Data on maternal and fetal plasma composition at 133 DGA are given in Table 3. Before the start of the infusions, maternal plasma osmolality was similar in the two groups; it increased in both groups after the infusions, with the elevation tending to be greater in the ethanol-infused ewes ($P_{treatment}$=0.005, P_{time}=0.001, $P_{treatment*time}$=0.164). Maternal plasma concentrations of Na^+, K^+, urea, creatinine and total protein were similar in the saline and ethanol treatment groups, both before starting and after completing the 1-hour maternal infusion.

Fetal plasma osmolality was not different between the two treatment groups before the start of the infusions. In both groups fetal plasma osmolality increased after the infusions; the increase was greater in the ethanol fetuses compared to controls ($P_{treatment}$=0.011, P_{time}<0.001, $P_{treatment*time}$=0.044). Fetal plasma concentrations of Na^+, K^+, urea, creatinine and total protein were similar in the saline and ethanol treatment groups.

Maternal and fetal body weights, and fetal kidney weight: At necropsy on 134 DGA, there was no difference in maternal body weight between the ethanol and control groups (57±3kg and 59±4kg, respectively). Similarly, there were no differences in fetal body weight or fetal kidney weight (Table 4).

Fetal total nephron number: Data for fetal kidney volume, total nephron number, and mean and total glomerular volumes at 134 DGA are shown in Figure 2. Fetal mean and total renal corpuscle volumes are shown in Table 4. Fetal total nephron number was 11% lower in the ethanol treatment group (377,585±8,325) than the saline group (423,177±17,178) ($p<0.0001$). Values for all other parameters were similar in the two treatment groups.

Real-time PCR analysis: The relative mRNA levels of all genes examined were similar in the fetal kidneys of the saline and ethanol treatment groups at 134 DGA (Table 5).

Fetal kidney morphology: There was no evidence of fetal kidney pathology or mal-development at 134 DGA in either of the treatment groups. Glomeruli showed no signs of hypercellularity, sclerosis or enlargement. There was no evidence of renal interstitial fibrosis or cellular infiltration, and renal blood vessels appeared to be normal. In addition, there was no evidence of a nephrogenic zone in either group, indicating that the process of nephrogenesis was complete in all animals (Figure 3).

Table 1:

Gene	Forward (5'-3') Reverse (5'-3')	Probe (5'-3')
ATI Receptor	GGGCTGTCTACACTGCTATGGAA	ACCGCTGGCCCTTCGGCAA
	CCGGAAGCGATCTTACATAGGTA	
ATII Receptor	TGTTCTGGCGTTCATCATTTG	TGGCTTCCCTTCCATGTTCTGACCTTC
	CCATCCAAGCTAGAGCATCCA	
	CTCTCCCACGCTCACTAGACTTG	CCACGGACCCAAATCTCGCTGC
Angiotensogen	CTCTTCTAGTTGTCCAAGTACGTA	
	TGAGCCAGGACCTGGTGACT	TCTGTGTGACTGTGATCCCGCCA
Renin	TCCGTGACCTCGCCAAAG	
IGF1	CAGCGCCACACCGACAT	TCTTCAAATGTACTTCCTTCTGAGCCTTGGG
	CCTGCACTCCCTCTGCTTGT	
IGF1R	CGCCTCTACTTTGTCTTTGCA	CCTGCTCCTTCTGCAGGCATGGTT
	TCACTGGCCCAGGAATGTC	
IGF2	CGTGCTTCCGGACGACTT	ACAGCATACCCCGTGGGCAAGTTCT
	TGCTTCCAGGTGTCAGATTGG	
IGF2R	CCAGCGGATGAGTGTCATCA	CCTCTGCCGTTGTTACCTGCTGTCTGA
	ACCTCCCCGGTGAACACA	
ENaCα	CATCTTCACCTGCCGTTTCA	CCAGGACTCCTGCAATGAGGCGAA
	CGGGTGGTGGAAGTGAGAGT	
ENaCβ	GCAAAGTAGCCATGAGACTATGCA	CAATGGAACCACATGCACCTTCCG
	GGTGGCGCTGCTGAAGTT	
ENaCγ	TCAGGGAGCCAACTACTGCAA	ACCAGCAGCACCCCAACTGGATGTA
	CCTGGTGCAGTTGGTAGTAGCA	
Na/K ATPase (α subunit)	AACGGCTTCCTCCCTAATCAC	TGCTGGGCATCCGAGTGACCTG
	GTCGTTGATCCAACGGTCATC	
Na/K ATPase (β subunit)	GCCTGGCGGGCATCTT	TCGGAACCATCCAAGTGATGCTGCT
	GTGGGCTTAAATTCACTGATGGT	
Na/K ATPase (γ subunit)	GCCCTGGCCTTCATCGT	CTCGTCATCATCCTCAGCAAAAGATTCCG
	TTGCCGGTGCTTCTTCTG	

Table 2: Ovine amniotic fluid composition on 133 DGA at -1 hour and 2 hour after maternal saline or ethanol infusion; N=5 pregnant sheep in each treatment group.

	Saline		**Ethanol**	
	-1 Hour	**2 Hour**	**-1 Hour**	**2 Hour**
Na^+ (mmol/l)	132±7	143±24	134±10	127±9
K^+ (mmol/l)	5.0±1.2	5.6±1.8	5.2±1.5	5.4±1.2
Cl^- (mmol/l)	106±17	129±20	100±9	105±8
Creatinine (mmol/l)	0.1±0.1	0.2±0.1	0.7±0.2	0.6±0.2
Total Protein (g/l)	0.2±0.1	0.3±0.07	0.5±0.1	0.5±0.2
Uric Acid (mmol/l)	0.05±0.01	0.05±0.01	0.04±0.007	0.04±0.002
Urea (mmol/l)	10.3±1.8	10.7±2.4	8.9±0.9	10.7±3.1

Table 3: Ovine maternal and fetal plasma electrolyte and protein concentrations on 133 DGA at -1 hour and 2 hour after maternal saline or ethanol infusion; N=5 ewes and fetuses in each treatment group. Maternal and fetal data were analysed using a repeated measures two-way ANOVA with treatment and time being factors. *P<0.05 for the effect of treatment; #P<0.05 for the effect of time; and ^P<0.05 for the effect of treatment-time interaction.

	Maternal Plasma			
	Saline		**Ethanol**	
	-1 Hour	**2 Hour**	**-1 Hour**	**2 Hour**
Na^+ (mmol/l)	143±1	145±1	144±1	146±1
K^+ (mmol/l)	3.9±0.1	4.3±0.1	4.0±0.06	4.3±0.1
Creatinine (µmol/l)	65±6	75±5	63±2	61±4
Urea (mmol/l)	7.6±0.6	7.5±0.5	6.4±0.3	6.8±0.1
Total Protein (g/l)	59.4±1.8	58.2±0.4	60.6±2.3	59.0±2.1
Osmolality (mOsmol/KgH_2O)	290±1*#	301±2*#	298±4*#	317±2*#
	Fetal Plasma			
	Saline		**Ethanol**	
	-1 Hour	**2 Hour**	**-1 Hour**	**2 Hour**
Na^+ (mmol/l)	141±1	145±2	141±1	143±1
K^+ (mmol/l)	4.0±0.02	3.9±0.1	3.8±0.06	3.6±0.07
Creatinine (µmol/l)	162±11	169±9	146±17	147±19
Urea (mmol/l)	8.5±0.6	8.8±0.5	7.1±0.1	7.1±0.2
Total Protein (g/l)	34.4±1.1	34.4±1.0	33.8±1.1	34.5±1.6
Osmolality (mOsmol/KgH_2O)	282±3*#	293±2*#^	284±3*#	310±1*#^

Table 4: Effect of maternal infusion of saline or ethanol during 95-133 DGA on body weight, kidney weight, and mean and total renal corpuscle volume of fetal sheep at 134 DGA; N=5 in each treatment group.

	Saline	**Ethanol**
Body Weight (kg)	4.12±0.21	3.81±0.25
Left Kidney Weight (g)	13.17±1.21	12.50±1.08
Right Kidney Weight (g)	12.99±1.19	12.08±0.95
Total Kidney Weight/Body Weight (g/kg)	3.201±0.283	3.276±0.171
Mean Renal Corpuscle Volume ($x10^{-3}$ cm^3)	0.625±0.056	0.669±0.034
Total Renal Corpuscle Volume (cm^3)	0.265±0.025	0.253±0.014

Table 5: Relative mRNA levels in the kidney of ATIR, ATIIR, Angiotensinogen, renin, IGF1, IGF1R, IGF2, IGF2R, ENaCα, ENaCβ, ENaCγ, Na/KATPase (α, β, γ subunits) in ethanol- and saline-exposed sheep fetuses. Values are Mean±SEM, N=5 in each group.

	Saline	**Ethanol**
ATI Receptor	1.15±0.31	1.99±1.29
ATII Receptor	1.12±0.25	0.77±0.17
Angiotensinogen	1.01±0.08	0.76±0.19
Renin	1.57±0.60	1.41±0.51
IGF1	3.14±1.85	1.07±0.35
IGF1R	1.25±0.25	0.97±0.17
IGF2	1.16±0.32	1.21±0.48
IGF2R	1.45±1.12	1.11±0.74
ENaCα	1.21±0.29	1.80±0.89
ENaCβ	1.52±0.70	1.31±0.33
ENaCγ	1.89±0.75	1.60±0.67
Na/K ATPase (α subunit)	1.04±0.16	0.73±0.14
Na/K ATPase (β subunit)	1.22±0.32	1.30±0.42
Na/K ATPase (γ subunit)	1.58±0.72	1.71±0.57

Figure 1: Maternal (closed circles) and fetal (open circles) blood ethanol concentrations (BEC) measured prior to (-1 hour and immediately prior to infusion) and at +1, 2, 4, 8, 12 and 24 hours after commencement of the 1 hour of ethanol infusion. All data are mean ± SEM, N=5 in each group. Values for BEC in saline infused ewes and fetuses were zero (data not shown).

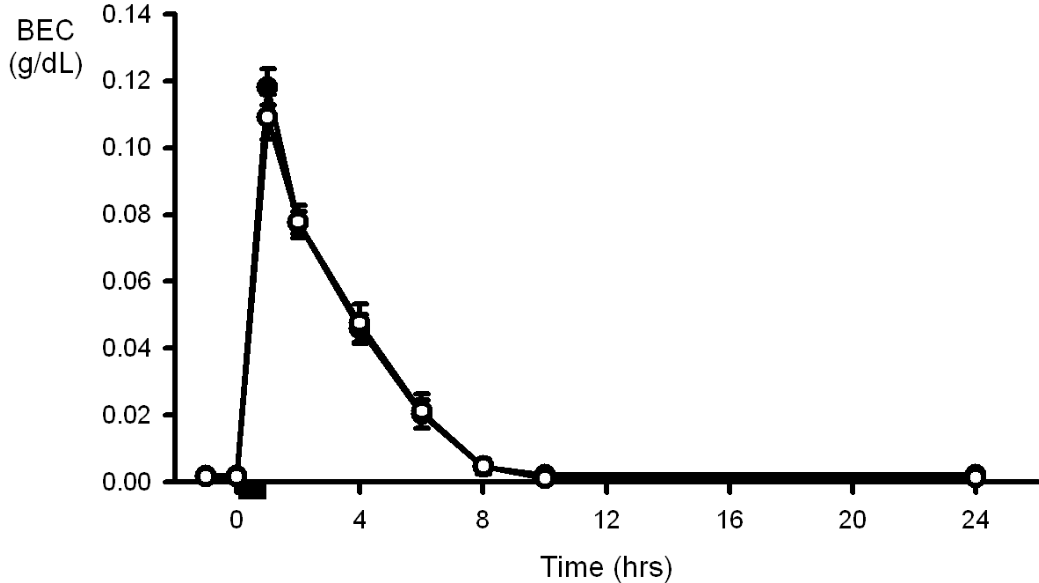


Figure 2: (A) Kidney volume, (B) total nephron number, (C) mean glomerular volume, and (D) total glomerular number in fetal sheep at 134 DGA following maternal infusion of saline or ethanol during 95-133 DGA; N = 5 in each treatment group; ***P<0.001.

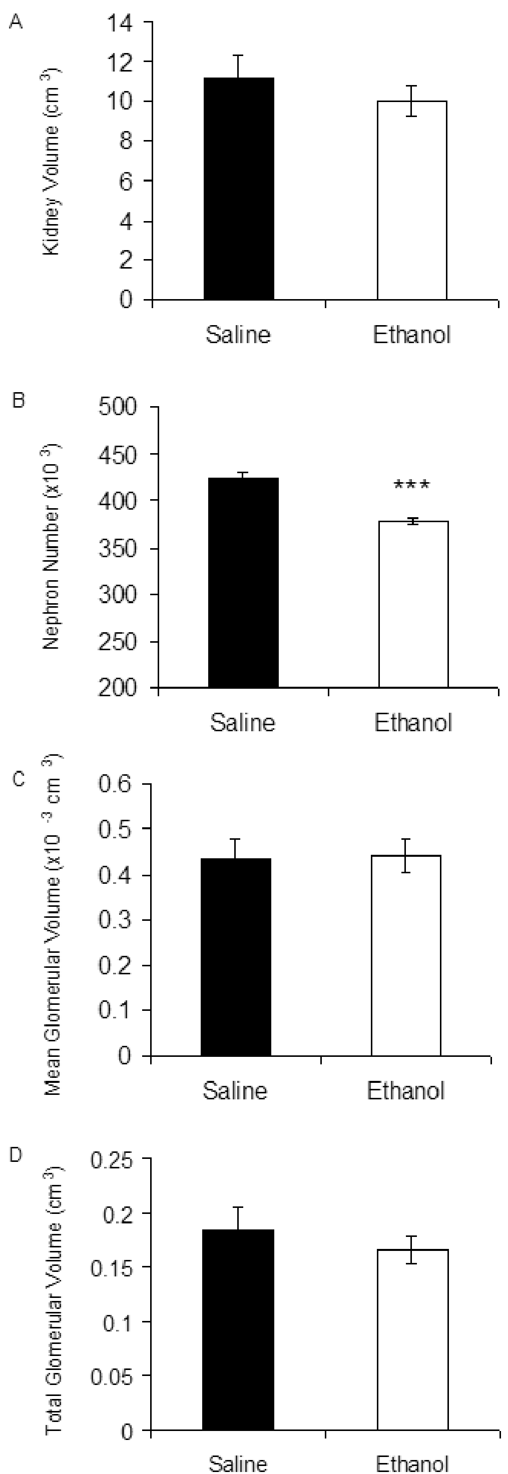
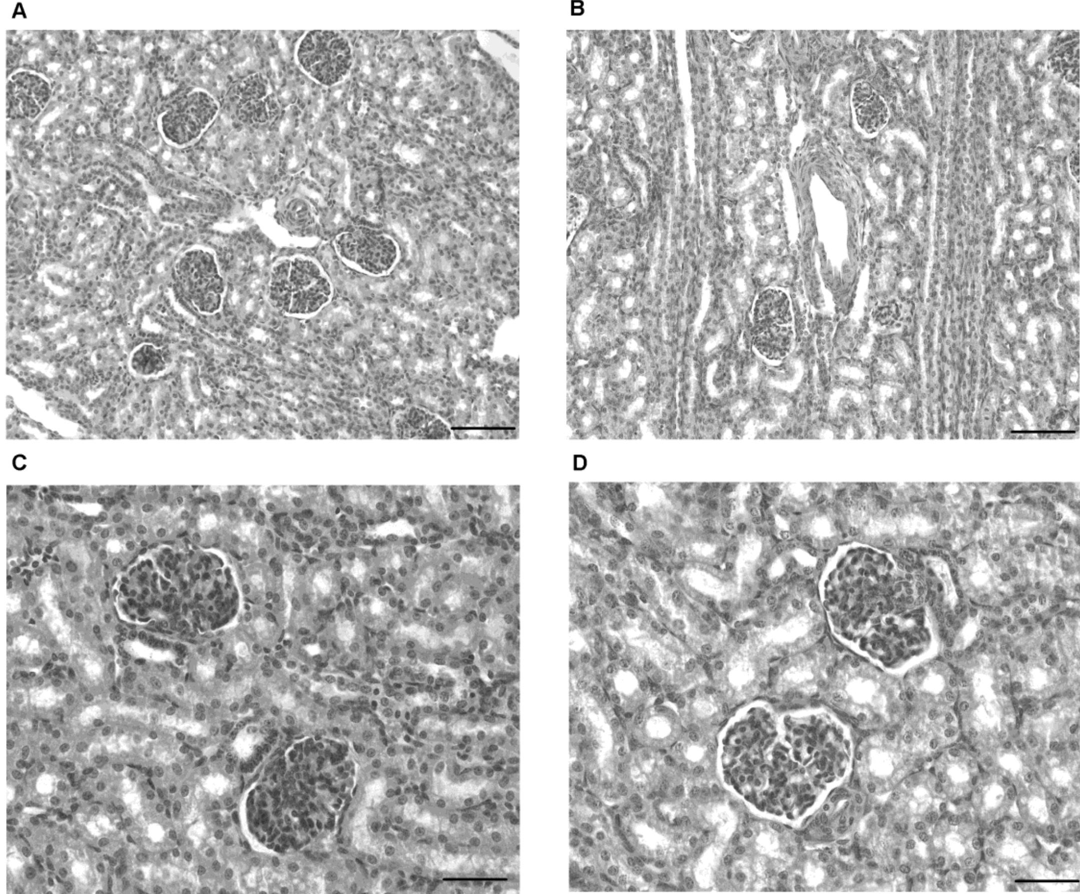


Figure 3: Photomicrographs of representative sections of ovine fetal kidney at 134 DGA following maternal infusion of saline (A & C) or ethanol (B & D) during 95-133 DGA. No evidence of renal pathology was observed in either treatment group. A and B, scale bar = 100μm; C and D, scale bar = 50μm.



Discussion

This study in sheep has shown that repeated fetal ethanol exposure during late gestation, at a time when nephrogenesis is occurring, results in an 11% lower nephron endowment in the fetus. However, our hypothesis that the ethanol exposure would result in decreased fetal body and kidney weights was not supported, nor did we find changes in gene expression for the renal RAS, IGFs or sodium channels in ethanol exposed fetuses. As nephrogenesis was complete at the time of nephron number analysis, the low nephron number identified in the ethanol-exposed fetuses is permanent and would persist into postnatal life. At present it is unclear whether an 11% reduction of nephron number would affect renal or cardiovascular function in postnatal life. However, it is known that a decrease in nephron number of 25-30% in the sheep results in hypertensive offspring [44]. Most studies in rodents that have observed compromised nephrogenesis following development in a suboptimal fetal environment also report nephron deficits of 25-30% and some of these studies demonstrate increased blood pressure in adult life [25, 35, 45]. Of particular relevance to the present study, Langley-Evans et al [25] reported a deficit in nephron number of 13% in rats exposed to a low-protein diet *in utero*. These rats subsequently developed elevated mean arterial blood pressure. Future studies to assess renal function and blood pressure in adult sheep repeatedly exposed to ethanol during fetal life, especially during the critical period of nephrogenesis, are required to determine if the observed reduction in nephron endowment is of long term physiological importance.

Despite the reduced nephron endowment, this study, found no difference in total glomerular volume between the two groups. Although not a direct measurement of total renal filtration surface area, this finding suggests there is unlikely to be any major alteration in total renal filtration surface area at 134 days gestation in the ethanol-exposed fetuses. This finding suggests that at least *in utero*, there is unlikely to be any substantial deficits in renal function.

While we cannot rule out the possibility of postnatal changes in total renal filtration surface area in the ethanol-exposed offspring, it was not possible to conduct these experiments in the current cohort. Given we have now identified a small, but statistically significant nephron deficit following prenatal ethanol exposure, future studies designed to determine total renal filtration surface area as well as renal function after birth are warranted. These studies would be necessary to provide future insights into the Brenner hypothesis in this model.

Epidemiological studies in humans affected by FAS show that fetal ethanol exposure over prolonged periods can lead to fetal growth restriction and low birth weight [1]. Low birth weight is often associated with reduced kidney weight and a deficit in nephron number. In such studies it is often assumed that the lower nephron number is a result of reduced kidney growth. For example, a maternal low-protein diet throughout pregnancy in rats results in lower offspring body and kidney weights, together with a nephron deficit of approximately 30% [25, 47]. However, as in the present study, decreases in nephron endowment can occur in the absence of changes in fetal body weight or kidney weight [14, 35, 44]. Whilst the mechanisms causing the reduction in nephron number following ethanol exposure observed in the present study need further investigation, we have preliminary data using rat metanephric organ culture that show ethanol exposure reduces ureteric branching morphogenesis [20]. Other models of reduced nephron number have shown changes in expression levels of genes regulating branching morphogenesis [14, 35] suggesting this may be a key mechanism underlying a nephron deficit.

A recent study of ethanol exposure in pregnant sheep, involving administration of the same daily maternal ethanol dosing regimen as in the present study from 116 to 118 DGA, found a 19% decrease in fetal body weight associated with transient suppression of maternal plasma IGF1 concentration (35%) and fetal plasma IGF2 concentration (28%) [19]. The authors

attributed the decreased fetal body weight to the suppression of maternal and fetal IGF concentrations. In the present study, although the period of alcohol exposure was much longer, we did not find any reduction in fetal body weight, fetal kidney weight, or fetal renal expression of IGF1 or 2. Whilst we have not measured maternal and fetal plasma IGFs, a comparison of the present study with that of Gatford *et al*. [19] suggests that fetal exposure to ethanol during the third-trimester equivalent over a longer gestational period may have different effects on the IGF system than does short-term exposure. The effects on fetal growth are also likely to be dependent on the fetal blood ethanol concentration and the stage of gestation during which exposure occurs [1].

Several studies have suggested that exposure to a compromised intrauterine environment results in alterations in the expression of certain genes, including those involved in kidney development and/or fluid and electrolyte balance. Such alterations in gene expression may contribute to the nephron deficit or may alter renal function and thus predispose the offspring to the development of hypertension in postnatal life. The best studied system is the RAS. In rats exposed to a low protein diet throughout pregnancy, the RAS is suppressed in newborn offspring [46, 39], a time at which nephrogenesis is continuing in this species. Suppression of the RAS may directly contribute to the nephron deficit as inhibition of the RAS with angiotensin converting enzyme inhibitors (ACE-I) can impair renal development [17]. However, by 4 weeks after birth, when nephrogenesis is complete, most studies in rats have found either no change or an upregulation of the RAS in offspring exposed to low protein in utero [28, 33] suggesting there may be a compensatory increase in the RAS following completion of nephron formation. A similar finding has been made in fetal sheep, in which an increase in AT1 receptor expression was found during late gestation or the early neonatal period following either glucocorticoid exposure [29] or maternal undernutrition [45]. In both of these sheep studies, gene expression was analysed, after the completion of nephrogenesis.

In another recent study we have shown no change in mRNA expression for components of the RAS in the fetal kidney at 130 days following growth restriction due to placental embolisation even though nephron endowment was reduced [50]. Another study has shown changes in the expression of fetal kidney sodium channels following maternal undernutrition [43]. However, in the present study there was no altered gene expression in the ovine fetal kidney after repeated ethanol exposure at 134 days of gestation. However, it is important to recognise that these findings do not exclude the possibility that changes in the protein levels did occur. However, performing protein analysis in the sheep is challenging, as many of the common antibodies used for protein analysis fail to provide adequate results in the sheep. Further studies examining gene and protein expression during the period of nephrogenesis as well as in the adult kidney would be of interest to determine if there is altered expression produced by repeated fetal ethanol exposure during development or in mature offspring.

Analysis of amniotic fluid composition can provide an indication of renal function in the fetus. The amniotic fluid composition in the present study suggests that fetal renal function was unlikely to be compromised by ethanol exposure. In a previous study in catheterised fetal sheep, acute ethanol exposure led to a transient decrease in fetal urine production [10]. In the rat, prenatal exposure to ethanol has been shown to produce postnatal defects in urine concentration, renal sodium conservation and potassium handling [2]. These findings raise the possibility that the ovine offspring in the present study, if studied as adults, may have compromised renal function, especially if challenged with a high sodium diet or dehydration.

Analysis of plasma samples from the ewe and fetus demonstrated that maternal and fetal osmolalities were higher in both groups after the 1-hour treatment; however, the increase in osmolality was greater in the ethanol-infused animals. Ethanol has a low molecular weight and would be expected to have a marked osmotic effect in blood. Changes in maternal and

fetal osmolality occurred without any changes in the concentration of sodium (or other electrolytes), indicating that the osmolality changes observed may have resulted directly from the osmotic contribution of ethanol itself. However, the effects on the saline treated ewes were surprising. It is possible that ewes were eating dry feed during the infusion, causing water to move from blood into the rumen to aid in digestion [15, 40]. Therefore, the increased plasma osmolality in the saline infused ewes may have been due to loss of water from blood; hence the greater increase in plasma osmolality in the ethanol-infused ewes may be due to this effect in combination with the osmotic contribution of ethanol itself. However, the effects on plasma osmolality were not persistent, as both the ewe and the fetus had apparently normal values before the ethanol or saline infusion on 133 DGA, after more than 30 days of maternal treatment.

Our findings on the fetal renal effects of ethanol exposure during late gestation have relevance to the human in terms of timing of fetal ethanol exposure and maternal ethanol intake. Recent data suggest that women who consume alcohol during pregnancy are more likely to drink during the third trimester than throughout gestation [12]. In another study, 13.5% of pregnant women reported that they consumed alcohol regularly during pregnancy, with 6.8% being more likely to consume alcohol in the last trimester [27]. In light of these data and the public misperception that alcohol consumption is less harmful to the fetus in the third trimester than in the first trimester [9, 26], knowledge of the effects of late gestational ethanol exposure on organ development is important. Our study clearly shows that alcohol exposure during nephrogenesis, which occurs largely during the third trimester in humans, can result in a reduced nephron endowment.

In relation to the maternal ethanol dose used in the present study, a mean maximal maternal BEC of 0.12g/dL was achieved, which would require a woman of average weight to consume

about three standard drinks in 1 hour [8]. Although this may seem to be a substantial amount of alcohol to consume, it has been shown that approximately 15% of pregnant women in the USA consume more than three standard drinks in one sitting more than three times per week [8]. In addition, 28.5% of pregnant women in the USA report engaging in a binge-drinking session at some point during their pregnancy, defined as consuming a minimum of four standard drinks within 3 hours [38]. This may be an under-estimate [41] as many do not recall accurately how much alcohol they consumed [24, 36].

Perspectives & Significance: Repeated fetal ethanol exposure during the period of nephrogenesis results in an 11% reduction in nephron endowment, without causing any overt growth restriction of the fetus or its kidney. This nephron deficit occurred in the absence of altered fetal renal gene expression of components of the RAS, IGFs or sodium channels. Further studies will be required to examine postnatal renal and cardiovascular outcomes following prenatal ethanol exposure via maternal ethanol administration. Our findings have important implications for pregnant women who drink alcohol during the latter half of pregnancy as they indicate that the developing kidney is susceptible to ethanol teratogenicity, that may result in persistent dysmorphology.

Acknowledgments

The authors acknowledge the expert technical assistance of Natasha Blasch, Alex Satrango, Rebecca Douglas-Denton, Debbie Arena, Andrew Jefferies and Sue Connell.

Grants

This work was supported by a Canadian Institutes of Health Research – New Emerging Team grant (NET-54014).

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Chapter 4

Declaration for Thesis Chapter 4

Declaration by Candidate

In the following chapter, Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contribution (%)
All tissue collection, data analysis and interpretation of data preformed by candidate. Manuscript of publication written by candidate.	80%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of Contribution	Extent of Contribution (%) for student co-authors only
Kate Denton	Draft Revision	
John Bertram	Draft Revision/co-supervision of candidate, study design	
Karen Moritz	Draft Revision/co-supervision of candidate, study design	

Candidate's Signature:

	Date:

Declaration by co-authors

The undersigned hereby certify that:

7) The above declaration correctly reflects the nature and extent of the candidate's contribution to this work and the nature of the contribution of each of the co-authors.

8) They meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise.

9) They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication.

10) There are no other authors of the publication according to these criteria.

11) Potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit.

12) The original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)

Department of Anatomy & Developmental Biology, Monash University, Victoria, Australia.

	Signature:	Date:
Signature 1: Assoc Prof. Kate Denton		28/7/09
Signature 2: Prof. John Bertram		27/7/09.
Signature 3: Dr. Karen Moritz		28/7/09

Acute Alcohol Exposure Early in Gestation Results in Long-Term Cardiovascular and Renal Functional Consequences

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Abstract

Knowledge of the effect of acute ethanol exposure on the developing kidney and its long-term consequences is limited. Our objective was to determine the effects of acute ethanol exposure during the early stages of kidney development in the rat on total nephron (glomerular) number at postnatal day (PN) 30 and cardiovascular and renal function at 6 months of age. Pregnant dams received ethanol (1g/kg body weight) via oral gavage on embryonic day 13.5 (E13.5) and E14.5, with a SHAM group receiving an equivalent volume of saline and a second control group not receiving the gavage treatment. Offspring exposed to ethanol were growth restricted during early postnatal life. At PN30 total nephron number was lower in ethanol-exposed male and female offspring (Males; 20,210±1,006 v 25,000±744 in SHAM and 26,061±1,288 in controls, P<0.001. Females; 21,239±926 v 24,144±350 in SHAM and 22,751±273 in controls, P<0.01). At 6 months of age, body weights were similar in all groups but mean arterial pressure was 10mmHg higher in ethanol-exposed offspring (Males; 116±2mmHg v 109±1mmHg in SHAM and 106±3mmHg in controls; P<0.01. Females; 119±2mmHg v 108±3mmHg in SHAM and 107±5mmHg in controls, P<0.01). Glomerular filtration rate was elevated in ethanol-exposed males and reduced in ethanol-exposed female offspring (Males; 0.801±0.157ml/min/gkw v 0.461±0.062ml/min/gkw in SHAM and 0.524±0.079ml/min/gkw in controls, P<0.01. Females; 0.601±0.080ml/min/gkw v 0.819±0.086ml/min/gkw in SHAM and 0.905±0.168ml/min/gkw in controls, P<0.01). These data demonstrate that acute ethanol-exposure during pregnancy in the rat results in a lower nephron number in offspring, long-term elevations in mean arterial pressure, and sex-specific changes in renal function. These findings have important ramifications for pregnant women who consume alcohol in binge doses during early pregnancy.

Introduction

In western communities, alcohol (ethanol) consumption by pregnant women is a relatively common occurrence [WHO 2004]. Some women, whilst reducing their daily ethanol consumption, do partake in episodes of acute (binge) drinking whilst pregnant [Caetano *et al* 2006]. While international guidelines generally recommend that pregnant women abstain from ethanol consumption, many still consume ethanol in the early stages of their pregnancy. This is of clinical importance as many pregnancies are unconfirmed until the end of the first trimester [Nayak *et al* 2004]. Furthermore, it is in the first trimester that the kidney begins to develop and the early stages of kidney development appear to be most developmentally plastic [Zoetis *et al* 2003, Burrow 2000, Moritz and Wintour 1999].

The knowledge of the teratogenic effects of acute ethanol-exposure on the fetus are limited. Animal studies have demonstrated that acute ethanol-exposure has the capacity to cause neuroapoptosis, neuronal cell loss, reduced brain growth and alveolar dysfunction [Wang *et al* 2007, Young *et al* 2005, Maier *et al* 1999, Maier *et al* 2001]. However, the number of studies investigating the effects of ethanol exposure during pregnancy on kidney development and long-term renal and cardiovascular function are limited.

Animal studies have shown that rats chronically exposed to ethanol through a liquid based ethanol (35% calories) supplemented diet throughout pregnancy had a reduced ability to concentrate urine at 90 days of age and the kidneys had reduced total DNA and protein content at 7 days of age [Assadi *et al* 1991, Gallo *et al* 1986]. Recently, we have shown that repeated maternal ethanol administration during the peak period of nephrogenesis in the fetal sheep results in an 11% lower nephron endowment [Gray *et al* 2008]. Furthermore, it has been shown that chronic prenatal ethanol exposure can result in an elevated mean arterial pressure (MAP) and altered vascular contractile response [Turcotte *et al* 2002]. However, no previous

study has investigated the effects of acute ethanol exposure on nephron endowment. This is important as a reduced nephron endowment is permanent and has been linked to the development of adult-onset diseases, such as hypertension [Brenner *et al* 1988, Hoy *et al* 2006]. In light of the identified decrease in renal function, reduced nephron number and an elevation in MAP in chronically ethanol-exposed offspring, it is important to investigate the consequences of acute ethanol exposure on nephron number and adult health given that many women drink before they are aware of their pregnancy.

In the present study, we hypothesised that acute prenatal ethanol exposure in the rat in the early stages of kidney development will result in a reduced nephron endowment at postnatal (PN) day 30 and alterations in adult cardiovascular and renal function. Our primary aim was to determine whether acute prenatal ethanol exposure results in smaller kidneys with a decreased nephron endowment at PN30, since nephrons are the functional units of the kidney. As low nephron number has been linked to the development of adult-onset diseases, our second objective was to investigate if MAP and renal function were different in ethanol-exposed offspring at 6 months of age.

Materials & Methods

Animal Groups: All experiments were approved by the Animal Ethics Committee of Monash University, and conducted according to the National Health and Medical Research Council of Australia guidelines. At 8 weeks of age 18 virgin female rats were time-mated overnight and the presence of a seminal plug the following morning was termed embryonic day 0.5 (E0.5). Dams were allocated to one of three experimental groups (Ethanol, SHAM, Control) and then housed individually. Food and water were provided *ad libitum* with a 12 hour light/dark cycle. At E13.5 the ethanol group were given 1g of ethanol per kg of maternal body weight diluted in saline by oral gavage at 10 am. Animals were then returned to their cages. The ethanol administration was repeated on E14.5. The SHAM group received an equivalent volume of saline on E13.5 and E14.5 by gavage, with the control group not receiving any treatment (to control for the effects of stress on fetal development). Pregnant dams were weighed at E12.5, E13.5, E14.5, E15.5, E16.5, E18.5 and E20.5. Pregnant dams were allowed to litter down naturally, pup viability was assessed with each pup being individually weighed every second day from PN2 until PN30. Pups were weaned on PN21.

Blood Alcohol Concentration (BAC): To determine the BAC achieved in the dam, a separate cohort of animals were used (N=4 SHAM and N=4 ethanol). Blood was collected from the tail vein (500μl) of pregnant dams 1 hour after gavage administration on E13.5. BAC was measured using QuantiChrom™ Ethanol Assay Kit (BioAssay Systems, California, USA).

Post-mortem examination at PN30: Two males and two females from each litter in all three groups (control, SHAM & ethanol) were culled at PN30 by an overdose of pentobarbital sodium (100mg/kg, i.p.). The brain, liver, heart, lungs and left and right kidneys were removed and weighed. The left kidneys were fixed in 10% formalin and prepared for stereological analysis.

Stereological Analysis: Total glomerular number and mean glomerular volume were determined using unbiased stereological methods as previously described [Cullen-McEwen *et al* 2001, 2003, Bertram 2001].

Mean Arterial Pressure (MAP) and Heart Rate (HR): At 6 months of age conscious MAP and HR were analysed from the tail artery as previously described [Bergstrom *et al* 1998, Stevenson *et al* 2000, Kett *et al* 2004, Singh *et al*, 2007]. At completion of all recordings, animals were anaesthetized (150mg/kg i.v. Inactin; Thiobutabarbital sodium, Sigma-Aldrich) for the renal function experiments.

Renal Function: Renal function was analysed using radiolabelled para-aminohippuric acid (0.5µCi/hr ^{14}C-PAH, Perkin Elmer, USA) and Inulin (1µCi/hr ^{3}H-inulin, Perkin Elmer, USA) infusion (1.5ml/hr) as described previously [Zimanyi *et al* 2006, Denton *et al* 2001].

Post-mortem examination at 6 months of age: After measuring arterial pressure and renal function, animals were culled with an overdose of pentobarbital sodium (100mg/kg, i.v.). The heart, lung, liver and left and right kidneys were removed and weighed. The left kidneys were fixed in 10% formalin for histological analysis.

Urine and plasma sample analysis: Urine and plasma samples (~500µl) were analyzed for Na^+, K^+ and Cl^- concentrations. Urinary protein concentration was assessed using a Synchron CX-5 Clinical system (Beckman, Australia). Osmolality was measured using freezing point depression (Advanced Osmometers, Australia).

Renal Morphology: Samples of the left kidneys removed from 6 month old animals were embedded in paraffin and sectioned at 5μm thickness. Sections were stained with hematoxylin and eosin, PAS or Masson's trichrome and analysed by light microscopy.

Data Analysis: Data are expressed as mean ± standard error of the mean (SEM), except where otherwise indicated. Males and females were analysed separately, with offspring weight gain being analysed using a one-way ANOVA weighted for litter testing for the effect of treatment at each time point. All other data were analysed using a one-way ANOVA weighted for litter testing for the effect of treatment. Followed by, Tukey's post-hoc test. A $P < 0.05$ was classed as being statistically significant.

Results

Gestational Weight Gain & Pup Viability: Ethanol administration did not affect gestational weight gain, with dams from all three groups having similar weight gain from E12.5 to E20.5 (Ethanol, 52±4g; SHAM, 53±6g; Controls 47±3 g). Similarly, ethanol administration did not affect pup viability, with pup numbers being comparable between groups at PN2 (Male; Ethanol, 7±1; SHAM, 5±1; Control 5±2. Female; Ethanol, 6±1; SHAM, 6±1; Control, 7±2).

Blood Alcohol Concentration (BAC): BAC measured 1 hour after gavage was 0.107±0.001g/dl in ethanol-treated dams and 0.000g/dl in SHAM dams.

Pup Growth until PN30: Male and female ethanol-exposed offspring weighed approximately 18% less than sex-matched SHAM and control offspring at PN 2 (Males; Ethanol, 4.1±0.1; SHAM, 5.1±0.1; Control, 4.9±0.1, $P<0.001$, ethanol v SHAM and control. Females; Ethanol, 4.5±0.1; SHAM, 5.0±0.1; Control, 5.3±0.1, $P<0.01$, ethanol v SHAM and control), and at all further time points examined (Figure 1). Fractional weight gain between time points was approximately 2 to 4g for all groups. There was no difference at any time point in the body weight of SHAM and control offspring.

Body & Organ Weights at PN30: Both male and female ethanol exposed offspring were significantly lighter than their sex-matched SHAM and control offspring at PN30 (Table 1). Male ethanol-exposed offspring had significantly lighter kidneys than SHAM and control offspring, while control offspring had smaller kidneys than SHAM offspring. The brain of male ethanol-exposed offspring was significantly lighter than SHAM and control offspring. However, once corrected for body weight, the brain was significantly heavier in SHAM and control offspring than ethanol-exposed offspring, whilst the relative weights of all other organs were comparable between the three groups (Table 1).

Female ethanol-exposed offspring had significantly lighter hearts than SHAM and control offspring. When organ weight was corrected for body weight, female ethanol-exposed offspring had significantly larger brains than SHAM and control offspring. Also, the kidneys of ethanol-exposed females were significantly larger in proportion to body weight than SHAM and control female offspring, whereas the relative weights of all other organs were comparable between groups (Table 1).

Nephron Number at PN30: Male ethanol-exposed offspring had significantly lower nephron number than male SHAM and control offspring, while female ethanol-exposed offspring had a significantly lower nephron number than SHAM offspring (Figure 2 (Males; Ethanol, 20,210±1,006; SHAM, 25,000±744; Control, 26,061±1,288, $P<0.001$, ethanol v SHAM and controls. Females; Ethanol, 21,239±926; SHAM, 24,144±350; Control, 22,751±273, $P<0.01$, ethanol v SHAM). Kidney volume was significantly smaller in ethanol-exposed males than SHAM and control male offspring. In contrast, female ethanol-exposed offspring had a similar kidney volume to SHAM and control female offspring (Figure 2B). In both male and female ethanol-exposed offspring, mean glomerular volume was significantly increased compared to sex-matched SHAM and control offspring (Males, $P<0.01$; Females, $P<0.05$). Mean renal corpuscle volume, and total glomerular and total renal corpuscle volumes were similar in the three groups.

Body & Organ Weights at 6 months of age: Both male and female ethanol-exposed offspring had similar body weights and organ weights to their sex-matched SHAM and control offspring at 6 months of age (Table 2).

Mean Arterial Pressure (MAP) and Heart Rate (HR) at 6 months of age: Both male and female ethanol-exposed offspring had significantly higher MAP than sex-matched SHAM and

control offspring at 6 months of age (Figure 3). There was no significant difference in heart rate of the ethanol-exposed male and female offspring in comparison to their sex-matched SHAM and control offspring.

Renal Function at 6 months of age: At 6 months of age, male ethanol-exposed offspring had a significantly higher glomerular filtration rate (GFR) and effective renal blood flow (ERBF) whilst female ethanol-exposed offspring had a significantly lower GFR and ERBF in comparison to their sex-matched SHAM and control offspring (Figure 4). Renal vascular resistance (RVR) was significantly elevated in ethanol-exposed female offspring compared with female SHAM and control offspring. However, male ethanol-exposed offspring had a similar RVR to SHAM and control offspring. Both male and female ethanol-exposed offspring had a similar filtration fraction to their sex-matched controls.

Urine and Plasma Analysis at 6 months of age: Male ethanol-exposed offspring had higher concentrations of urinary protein in comparison to sex-matched controls, while female ethanol-exposed offspring had similar urinary protein levels to SHAM and controls. Urine osmolalor and excretion rates of sodium, potassium and chloride were similar in ethanol-exposed male and female offspring in comparison to their sex-matched controls (Table 3). Plasma osmolality and the sodium, potassium and chloride concentrations of ethanol-exposed male and female offspring were similar to their sex-matched controls (data not shown).

Renal Morphology at 6 months of age: There was no evidence of kidney pathology at 6 months of age in any of the treatment groups. Glomeruli showed no signs of hypercellularity, sclerosis or enlargement. There was no evidence of renal interstitial fibrosis or cellular infiltration, and vessels appeared to be normal (data not shown).

Table 1:

Male and female absolute and corrected organ weights at PN30. Male and female data were analysed separately using a one-way ANOVA with a Tukey's post-hoc test. Data are mean ± SEM, N=8 per group, derived from 6 litters per treatment. Values that do not share a common letter/symbol are significantly different from each other ($P<0.05$).

	Absolute Weight (g)						
	Male				**Female**		
	Control	**SHAM**	**Ethanol**		**Control**	**SHAM**	**Ethanol**
Body Weight	$75\pm2^{\#}$	$80\pm2^{\#}$	$66\pm1^{*}$		71 ± 2^{A}	72 ± 2^{A}	60 ± 1^{B}
Left Kidney	$0.431\pm0.01^{\#}$	$0.473\pm0.01^{\wedge}$	$0.401\pm0.01^{*}$		0.401 ± 0.01^{A}	0.404 ± 0.01^{A}	0.395 ± 0.01^{A}
Right Kidney	$0.434\pm0.01^{\#}$	$0.485\pm0.01^{\wedge}$	$0.418\pm0.01^{*}$		0.407 ± 0.01^{A}	0.417 ± 0.01^{A}	0.397 ± 0.01^{A}
Brain	$1.557\pm0.01^{\#}$	$1.616\pm0.02^{\#}$	$1.531\pm0.01^{*}$		1.493 ± 0.02^{A}	1.566 ± 0.02^{A}	1.495 ± 0.02^{A}
Liver	$3.680\pm0.11^{\#}$	$4.075\pm0.25^{\#}$	$3.630\pm0.12^{\#}$		3.547 ± 0.11^{A}	3.371 ± 0.21^{A}	3.359 ± 0.08^{A}
Lung	$0.818\pm0.09^{\#}$	$0.804\pm0.03^{\#}$	$0.748\pm0.05^{\#}$		0.812 ± 0.10^{A}	0.807 ± 0.06^{A}	0.643 ± 0.01^{A}
Heart	$0.442\pm0.02^{\#}$	$0.464\pm0.01^{\#}$	$0.448\pm0.01^{\#}$		0.418 ± 0.02^{A}	0.425 ± 0.01^{A}	0.382 ± 0.01^{B}

	Organ Weight:Body Weight Ratio (mg/g)					
	Male			**Female**		
	Control	**SHAM**	**Ethanol**	**Control**	**SHAM**	**Ethanol**
Left Kidney	5.79±0.22$^{\#}$	5.94±0.20$^{\#}$	6.06±0.20$^{\#}$	5.94±0.21^{A}	5.61±0.21^{A}	6.53±0.22^{B}
Right Kidney	5.83±0.18$^{\#}$	6.09±0.25$^{\#}$	6.32±0.24$^{\#}$	5.79±0.25^{A}	5.80±0.22^{A}	6.55±0.22^{B}
Brain	20.91±0.60$^{\#}$	20.29±0.56$^{\#}$	23.01±0.40*	21.14±0.62^{A}	21.75±0.62^{A}	24.68±0.68^{B}
Liver	49.18±1.34$^{\#}$	51.28±3.54$^{\#}$	54.61±2.05$^{\#}$	49.85±1.08^{A}	46.67±3.03^{A}	55.49±2.01^{A}
Lung	10.90±1.14$^{\#}$	10.08±0.44$^{\#}$	11.20±0.77$^{\#}$	11.51±1.47^{A}	11.20±1.0^{A}	10.61±0.30^{A}
Heart	6.04±0.55$^{\#}$	5.83±0.21$^{\#}$	6.74±0.27$^{\#}$	5.74±0.37^{A}	5.89±0.22^{A}	6.32±0.22^{A}

Table 2:

Male and female absolute and corrected organ weights at 6 months of age. Male and female data analysed separately using a one-way ANOVA with a Tukey's post-hoc test. Data are mean ± SEM, N=8 per group, derived from 6 litters per treatment. There were no statistical differences for any parameter.

	Absolute Weight (g)						
	Male				**Female**		
	Control	**SHAM**	**Ethanol**		**Control**	**SHAM**	**Ethanol**
Body Weight	503±11	549±20	507±13		275±13	320±16	284±12
Left Kidney	1.68±0.04	1.84±0.08	1.74±0.07		0.99±0.03	1.08±0.04	0.96±0.04
Right Kidney	1.71±0.04	1.85±0.08	1.72±0.06		1.03±0.04	1.11±0.04	1.01±0.06
Liver	17.35±0.69	17.82±0.64	16.14±0.78		9.66±0.39	10.85±0.52	9.51±0.33
Lung	2.29±0.05	2.35±0.11	2.46±0.25		1.87±0.11	1.95±0.10	1.73±0.09
Heart	1.53±0.05	1.55±0.082	1.51±0.04		0.98±0.05	1.00±0.03	0.92±0.04

	Corrected Organ:Body Weight Ratio (g/kg)						
	Male				**Female**		
	Control	**SHAM**	**Ethanol**		**Control**	**SHAM**	**Ethanol**
Left Kidney	3.67±0.11	3.38±0.18	3.44±0.13		3.80±0.15	3.14±0.18	3.43±0.14
Right Kidney	3.42±0.13	3.39±0.20	3.39±0.10		3.80±0.15	3.51±0.19	3.57±0.17
Liver	34.50±1.18	32.54±0.96	31.68±1.03		35.46±1.39	33.93±0.76	33.68±1.00
Lung	4.57±0.13	4.31±0.19	4.84±0.44		6.98±0.64	6.19±34	6.15±0.35
Heart	3.06±0.08	2.82±0.11	3.01±0.15		3.34±0.06	3.18±0.18	3.28±0.15

Table 3:

Male and female urine excretion analysis performed at 6 months of age. Male and female data analysed separately using a one-way ANOVA with a Tukey's post-hoc test. Data are mean ± SEM, N=8 per group, derived from 6 litters per treatment. Values that do not share a common letter/symbol are significantly different from each other ($P<0.05$).

	Male				**Female**		
	Control	**SHAM**	**Ethanol**		**Control**	**SHAM**	**Ethanol**
Protein (g/l)	0.646±0.117*	0.774±0.135*	1.690±0.365^		0.398±0.117	0.646±0.117	0.134±0.037
Osmolality Excreion (mOsmol/in)	21.33±3.80	17.08±3.23	13.19±6.17		19.20±1.74	18.83±7.40	19.44±4.60
Na^+ Excretion (mmol/min)	1.67±0.94	1.55±0.36	1.24±0.69		2.46±0.10	3.87±2.27	2.46±0.90
K^+ Excretion (mmol/min)	1.13±0.26	0.45±0.20	0.58±0.21		1.54±0.13	1.03±0.35	1.11±0.24
Cl^- Excretion (mmol/min)	3.15±0.82	2.98±0.45	2.13±0.91		2.21±0.19	4.73±2.30	3.87±1.06

Figure 1: Male (A) and female (B) postnatal growth from PN2 to PN22 for the three groups of rats. Data are mean ± SEM. Male and female data analysed separately using a one-way ANOVA with a Tukey's post-hoc test at each time point. N>20 per group, derived from 6 litters per treatment, Tukey's P values, ***P<0.001.

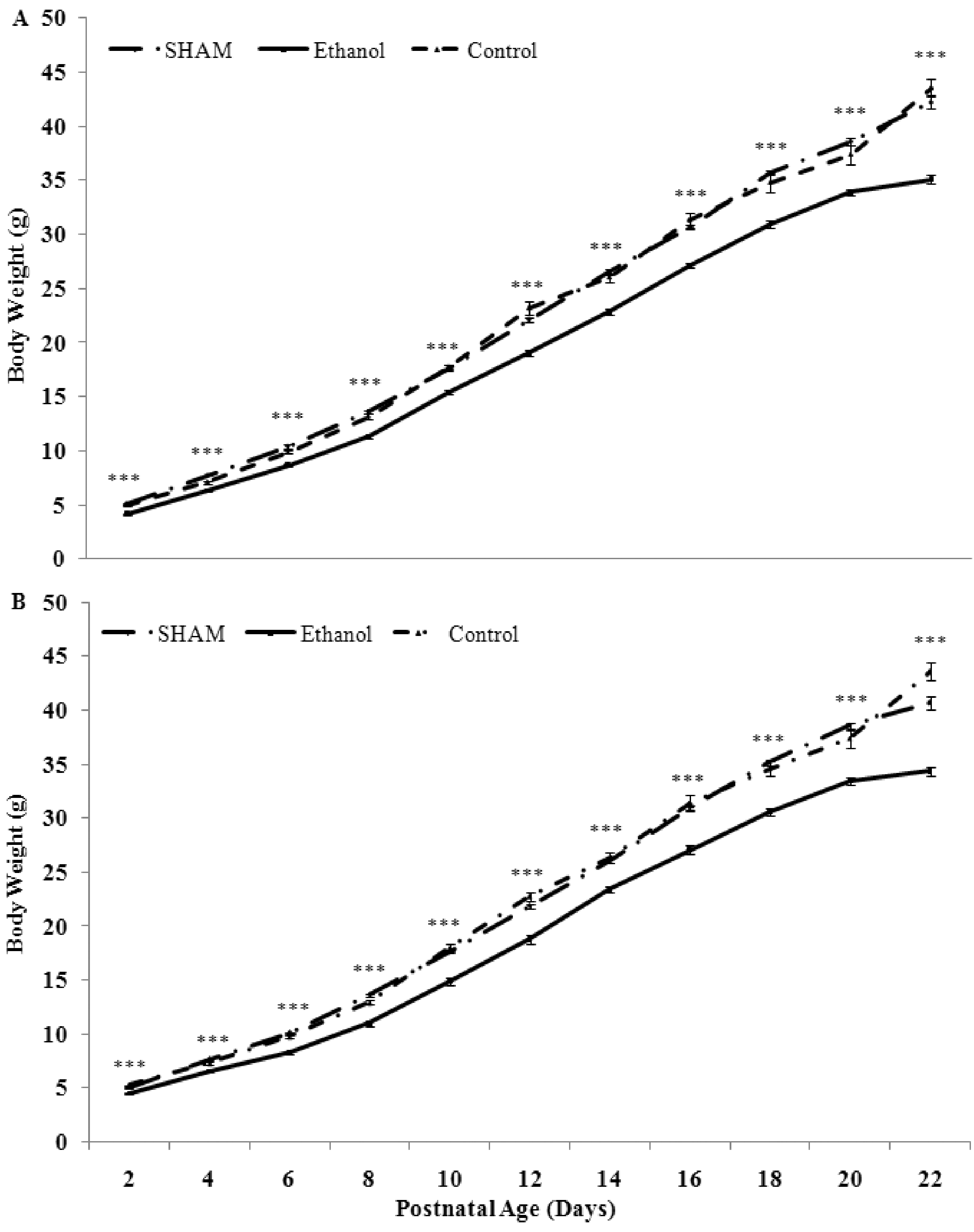


Figure 2: Male and female nephron number (A & E), kidney volume (B & F), mean glomerular volume (C & G) and total glomerular volume (D & H) at PN30 in the three groups of rats. Data are mean ± SEM. Male and female data were analysed separately using a one-way ANOVA with a Tukey's post-hoc test. N=8 per group, derived from 6 litters per treatment, Tukey's P values *P<0.05, **P<0.01, ***P<0.001.

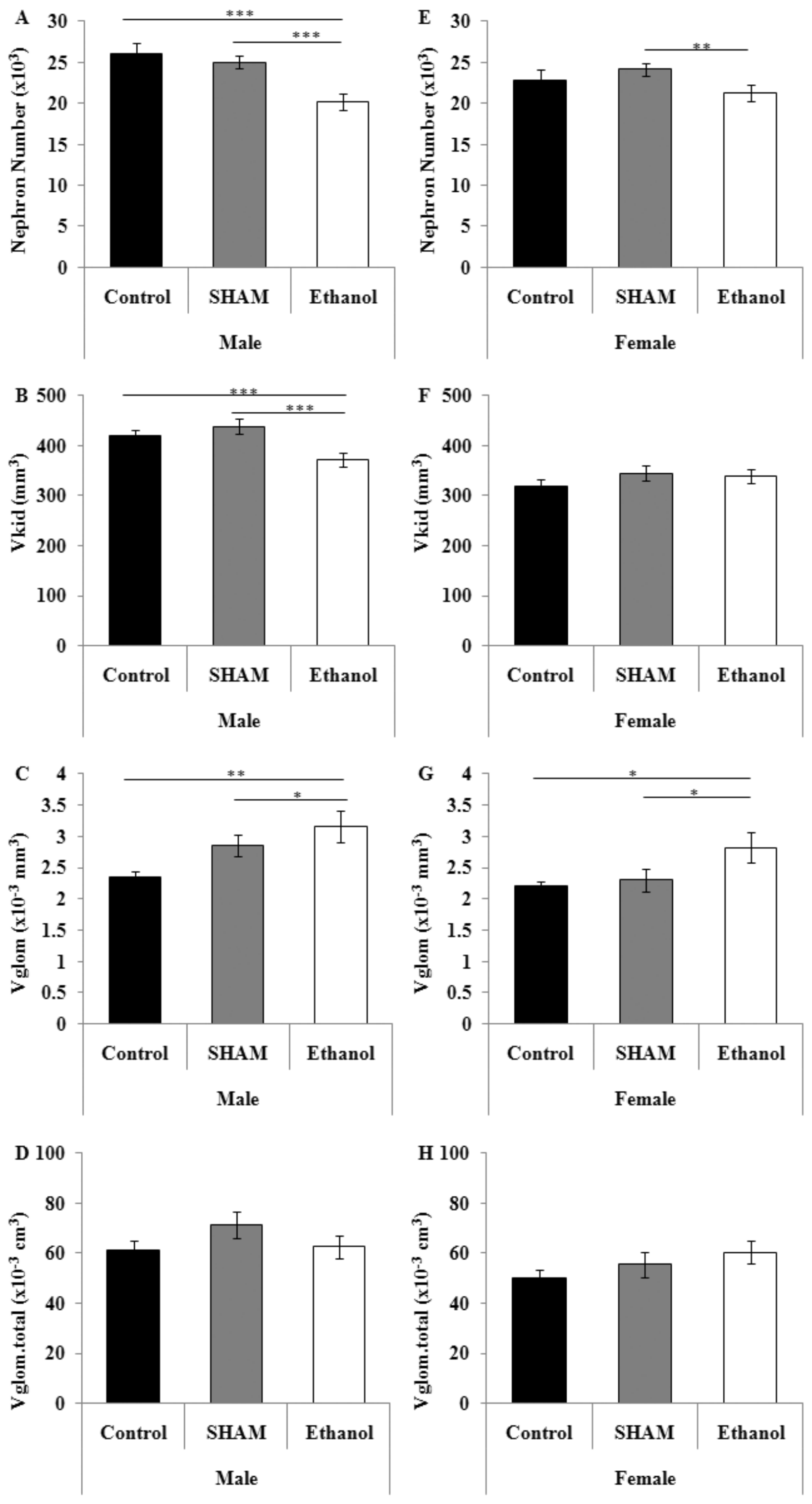


Figure 3: Male and female mean arterial pressure (A & C) and heart rate (B & D) at 6 months of age in the three groups of rats. Data are mean ± SEM. Male and female data were analysed separately using a one-way ANOVA with a Tukey's post-hoc test. N=8 per group, derived from 6 litters per treatment, Tukey's P values ***P<0.001.

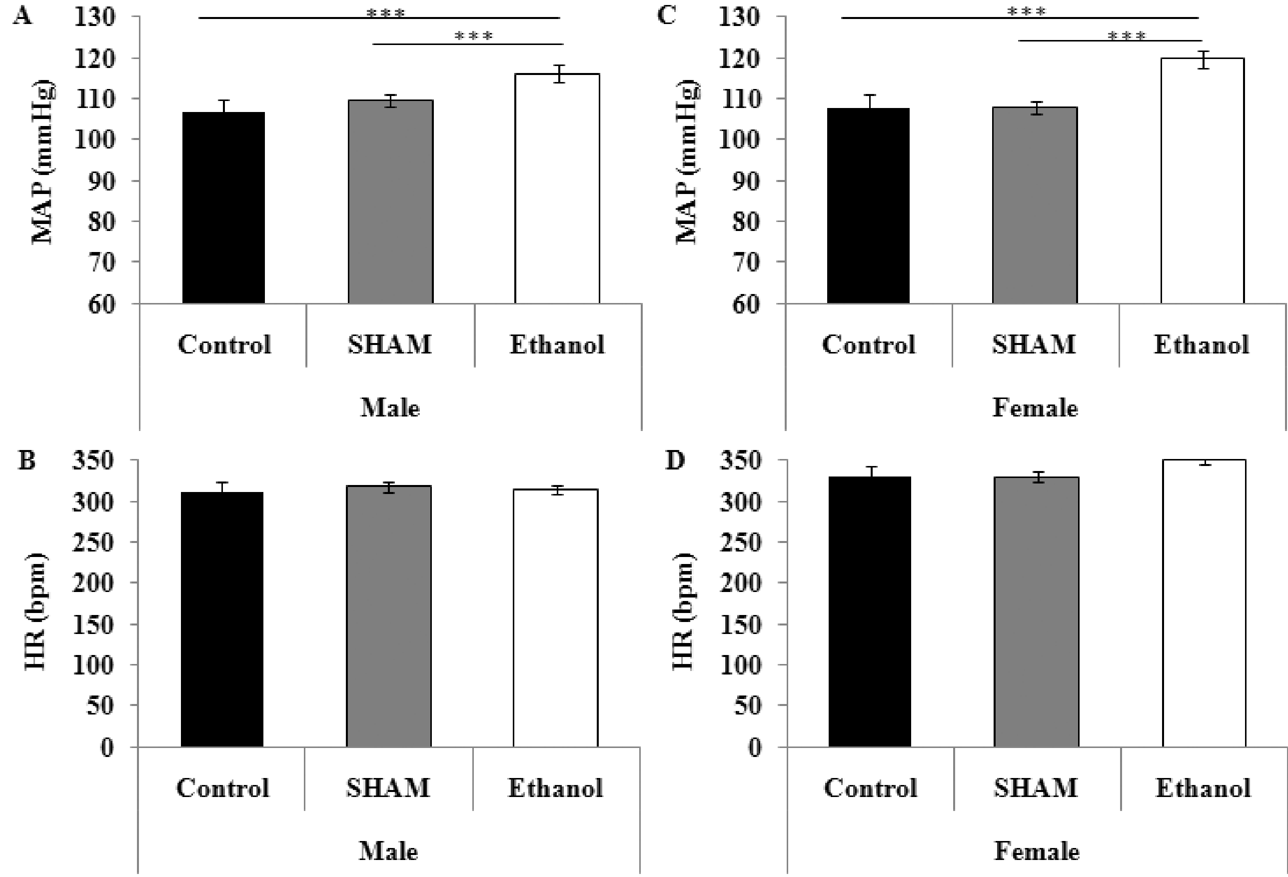
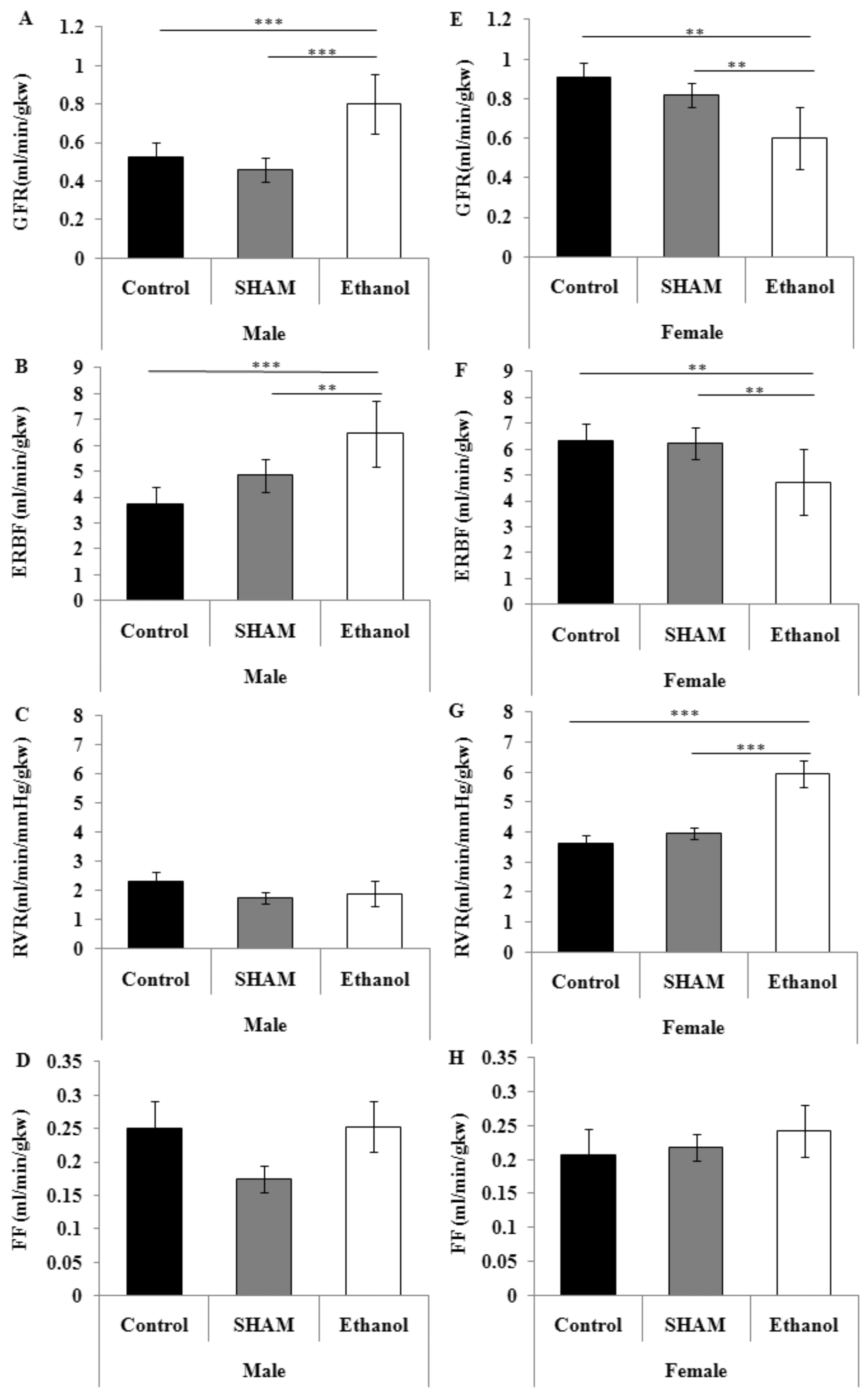


Figure 4: Male and female glomerular filtration rate (A & E), renal blood flow (B &F), renal vascular resistance (C & G) and filtration fraction (D & H) at 6 months of age in the three groups of rats. Data are mean ± SEM. Male and female data were analysed separately using a one-way ANOVA with a Tukey's post-hoc test. N=8 per group, derived from 6 litters per treatment, Tukey's P values **P<0.01, ***P<0.001.



Discussion

This study has shown for the first time that acute ethanol exposure at a time when the kidney is in its early stages of development causes early postnatal growth restriction and a permanent 10-20% reduction in nephron number at PN30. Furthermore, these findings support our hypothesis that acute ethanol exposure has long-term consequences on adult health, causing a 10mmHg elevation in MAP in ethanol-exposed offspring and sex-specific changes in renal function at 6 months of age. Together, the present findings suggest that prenatal ethanol exposure at an early stage of renal development results in long-term renal and cardiovascular deficits.

Our rat model of acute prenatal ethanol exposure has significant relevance to the human in terms of timing of exposure and the blood alcohol content obtained. Epidemiological studies report that pregnant women are more likely to consume alcohol in acute doses rather than in a chronic capacity [Ethen *et al* 2009, Tsai *et al* 2007]. Approximately 28.5% of pregnant women in the USA report engaging in binge drinking episodes at some point in their pregnancy, defined as consuming more than four standard drinks in the one sitting [Caetano *et al* 2006]. In the present study, maternal BAC measured 1 hour after ethanol administration was ~0.1g/dl. Similar models of maternal ethanol exposure find that maximal BAC ranges from 0.13-0.2g/dl, when alcohol is presented to the animals in their drinking water or administered by gavage [Knee *et al* 2004, McGivern *et al* 1982, Livy *et al* 2008, Varousek-Jakuba *et al* 1991]. The timing of ethanol exposure in the current study relates to the early stages of kidney development, when the ureteric bud has just started to invade the metanephric mesenchyme. This corresponds to ~7-8th week of human pregnancy [Zoetis *et al* 2003, Burrow 2000, Moritz and Wintour 1999]. This is a critical time-point in kidney development, as previous studies using maternal glucocorticoids have identified that perturbations at this time-point contribute to the programming of a reduced nephron endowment [Singh *et al* 2007,

Wintour *et al* 2003]. Our data further adds to the concept that early kidney development is a "critical window" in which *in utero* perturbations can elicit long term cardiovascular effects. Reductions in birth weight are common in animal models of fetal programming, such as low protein diet, placental insufficiency and ethanol models [Langley-Evans *et al* 1999, Woods *et al* 2004, Wlodek *et al*, 2007, Gatford *et al* 2007]. While birth weight was not recorded in the present study, data showed growth restriction in ethanol-exposed offspring, most probably occurring *in utero*. Furthermore, as we did not identify any differences in gestational weight gain in response to the ethanol administration, we propose that the observed growth restriction is a result of the ethanol-exposure and not undernutrition during development. Importantly, during lactation, the offspring exposed to ethanol did not become further growth restricted nor did they undergo significant 'catch-up' growth. Catch-up in body weight occurred post-weaning in these animals as evident by the fact that ethanol-exposed animals had similar body weights to control groups at 6 months of age. Fetal growth restriction has previously been demonstrated in animal models of prenatal ethanol exposure and in human cases of fetal alcohol syndrome (FAS) [Lan *et al* 2006, Spohr *et al* 1993, Vavrousek-Jakuba *et al* 1991]. In a human study of FAS children, body weights were smaller in infancy and were still smaller at 3 years of age [Spohr *et al* 1993].

In many models of prenatal programming, a reduction in nephron number is coupled with a reduction in kidney weight. Previous work investigating the effects of prenatal low protein exposure found that animals had smaller kidneys and a 30% reduction in nephron number [Langley-Evans *et al* 1999]. However, this is not always the case. For example, short-term prenatal glucocorticoid exposure can result in a reduced nephron endowment without changes in kidney weight [Moritz *et al* 2009]. Recently, we found no change in kidney weight but an 11% reduction in nephron number in response to ethanol-exposure during the latter half of gestation in an ovine model [Gray *et al* 2008]. In the present study, we identified a reduction

in kidney weight in male ethanol-exposed offspring, but no reduction in kidney weight in ethanol-exposed female offspring, even though there was a reduction in nephron number. The degree of nephron deficit was less in female ethanol-exposed offspring compared to male ethanol-exposed offspring, being approximately 10% in females and 20% in males. The exact cause of this difference between sexes is unknown, however, it has been established that sex hormones play a role in kidney development [Walker *et al* 2008]. This suggests that the kidneys of male fetuses may be more susceptible than female kidneys to the teratogenic properties of ethanol, causing not only a reduction in nephron number, but kidney weight as well. The observed reduction in nephron number is permanent, as at the time of analysis (PN30), nephrogenesis is complete in the rat. Interestingly, both male and female ethanol-exposed offspring displayed an increase in mean glomerular volume which is not a common finding in studies of reduced nephron number at such an early age [Gray *et al* 2008, Singh *et al* 2007, Wlodek *et al* 2007]. This increase in mean glomerular volume resulted in a normalisation of total glomerular volume in the kidney. While not conclusive, this suggests there is unlikely to be any alteration in total renal filtration surface area at PN30 in male and female-ethanol exposed offspring. We can speculate that the increase in mean glomerular volume may become even more pronounced as the ethanol-exposed rats age, possibly leading to an increase in filtration surface area, contributing to the hyperfiltration identified at 6 months of age. In addition, it is entirely possible that the difference in renal function between the sexes of ethanol-exposed offspring at 6 months of age could in part be due to differential degrees of glomerular hypertrophy and thereby sex differences in filtration surface area. In females, where renal function in ethanol-exposed animals has declined compared to male ethanol-exposed offspring, glomerular sclerosis or even glomerular loss contribute to an overall reduction in filtration surface area. Future studies in which total renal filtration surface area is estimated in ethanol-exposed and control offspring are warranted. Such studies would

provide further insights into the relevance of the Brenner hypothesis in male and female offspring in this model.

Many studies have demonstrated the relationship between a reduction in nephron number and the development of an elevation in MAP in both animal models and human studies [Wintour *et al* 2003, Langley-Evans *et al* 1999, Singh *et al* 2007, Wlodek *et al* 2007, Brenner 1988, Hoy *et al* 2006]. In the present study we have demonstrated that as a result of acute ethanol exposure there was an elevation in MAP of approximately 10mmHg at 6 months of age in offspring of both sexes. Similar elevations have been observed following short-term prenatal glucocoticoid exposure [Singh *et al* 2007, Wintour *et al* 2003]. A 12mmHg increase in MAP was found in rats that had been prenatally exposed to an acute elevation in the glucocoticoid, corticosterone, these rats also had a decrease in nephron number (20%) that was similar to the current study [Singh *et al* 2007]. Similarly, dexamethasone exposure in the early stages of kidney development in fetal sheep resulted in a nephron deficit and a 10mmHg increase in MAP in offspring at 7 years of age [Wintour *et al* 2003]. What is interesting is that both of these studies administered the exogenous glucocorticoids at a similar stage of kidney development (in the early stages of ureteric branching) as the ethanol in this current study and for a similar duration (2 days). This strongly suggests the early stages of metanephric development are particularly sensitive to perturbation, and that the effects of ethanol on the kidney may be influenced by the actions of maternal glucocorticoids, as similar findings were observed in both the current study and those investigating the effects of glucocorticoid exposure. Furthermore, as the actions of alcohol influence the activity of the hypothalamic-pituitary-adrenal axis, the site of glucocorticoid excretion; it is plausible to suggest that the findings observed within this current study may be mediated by the actions of glucocorticoids [Weinberg *et al* 1987, 2008].

Investigations to identify if the reduction in nephron number had any effect on renal function identified sex-specific changes in GFR, ERBF and RVR, with no changes in excretion rate of sodium, potassium and chloride. The elevations in GFR and ERBF in male ethanol-exposed offspring at 6 months of age could be due to an increase in glomerular volume (which was observed at PN30) and/or an increase in glomerular pressure contributing to the hyperfiltration. Hyperfiltration is associated with the early stages of renal insufficiency, such as in the early stages of diabetes [Vallon 2003]. Furthermore, ethanol-exposed male offspring had an elevation in urinary protein concentration, indicating that these animals were in the early stages of developing kidney disease. However, there were no overt signs of kidney damage by light microscopy. Surprisingly, in female ethanol-exposed offspring there was an elevation in RVR coupled with a reduction in GFR and ERBF. It is well recognised that there are sex differences in the role that nitric oxide (NO) play's in the kidney, with NO playing a greater role in the female kidneys, which could suggest a dysfunction in intrarenal NO production by endothelial cells [Baylis *et al* 1990]. Previous studies investigating NO blockade have reported changes in renal blood flow and glomerular filtration rate similar to those identified in the present study [Baylis *et al* 1990, Denton and Anderson 1994, Johnson *et al* 1992, Majid *et al* 1993]. Furthermore, ethanol-exposure prenatally or in the adult is known to affect vascular function [Turcotte *et al* 2002, Criscione *et al* 1989, Hatake *et al* 1991, Hatake *et al* 1994]. For example, Turcotte and colleagues (2002) identified that ethanol exposure throughout gestation results in alterations to endothelium dependent and independent vascular response. Similarly, in the adult rat ethanol consumption results in endothelial dysfunction resulting in defects in vessel contraction and relaxation [Criscione *et al* 1989, Hatake *et al* 1991, Hatake *et al* 1994]. As it is well known that ethanol can directly move from the maternal vasculature into the amniotic fluid [Brien *et al* 1987], it is reasonable to assume that ethanol would be having a direct effect on fetal vasculature development. As NO plays a differing role in the female compared to the male and that its actions are influenced by

estrogen, the effects of alcohol on NO could be resulting in endothelial dysfunction, resulting in the changes identified. Further studies are warranted to analyse the concentration of urinary nitrates and the effects of administering exogenous NO to the ethanol-exposed females to better identify if there is a dysfunction in NO production.

Conclusion

Acute prenatal ethanol exposure at a time when the kidney is in its earliest stages of development results in postnatal growth restriction and a permanent reduction in nephron number in rat offspring. Male and female ethanol-exposed offspring had significant elevations in MAP at 6 months of age and sex-specific changes in renal function, with male offspring having an elevation in GFR and ERBF, while female offspring had an elevation in RVR with a decrease in GFR and ERBF. Further studies are warranted to fully evaluate these sex-specific changes in renal function. The findings from the present study have important implications for pregnant women who drink alcohol in binge episodes early in gestation, as they indicate that the effects of alcohol are long-lasting and may influence adult health.

Acknowledgements

The authors acknowledge the expert technical assistance of Prof. John Dowling, Ms Rebecca Flower, Ms Rebecca Douglas-Denton, Ms Debbie Arena, Ms Sue Connell, Ms Julie Hickey and Mr Chaminda Premaratne.

Grants

This work was supported by a National Health & Medical Research Council grant (Grant ID 511162).

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Chapter 5

Declaration for Thesis Chapter 5

Declaration by Candidate

In the following chapter, Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contribution (%)
All tissue collection, data analysis and interpretation of data preformed by candidate. Manuscript of publication written by candidate.	80%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of Contribution	Extent of Contribution (%) for student co-authors only
Luise Cullen-McEwen	Draft Revision and assistance on experiments	
Karen Moritz	Draft Revision/co-supervision of candidate, study design	
John Bertram	Draft Revision/co-supervision of candidate, study design	

Candidate's Signature:

	Date:

Declaration by co-authors

The undersigned hereby certify that:

13) The above declaration correctly reflects the nature and extent of the candidate's contribution to this work and the nature of the contribution of each of the co-authors.

14) They meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise.

15) They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication.

16) There are no other authors of the publication according to these criteria.

17) Potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit.

18) The original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)

Department of Anatomy & Developmental Biology, Monash University, Victoria, Australia.

	Signature:	**Date:**
Signature 1: Dr. Luise Cullen-McEwen		28/7/09
Signature 2: Dr. Karen Moritz		28/6/2009
Signature 3: Prof. John Bertram		27/7/09.

Retinoic Acid Prevents Ethanol-Induced Inhibition of Ureteric Branching Morphogenesis *In Vitro*

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Abstract

The mechanism through which ethanol acts to disrupt kidney development is largely unknown. Retinoic acid (RA) is an important regulator of kidney development, and it is known that RA synthesis and ethanol metabolism share a common enzymatic pathway. Our objective was to determine the effects of ethanol exposure with/without exogenous RA on ureteric branching morphogenesis, glomerular development and expression of key kidney developmental genes in cultured rat metanephroi. The effects of *in vivo* fetal alcohol exposure on renal gene expression were also assessed. Metanephroi from embryonic day E14 Sprague-Dawley rats were cultured in DMEM/Ham's F12 media supplemented with either 0% ethanol, 0% ethanol & 10nM RA, 0% ethanol & 20nM RA, 0.2% ethanol, 0.2% ethanol & 10nM RA, or 0.2% ethanol & 20nM RA. Metanephroi were cultured for either 48 hours after which they were whole-mount fluorescently immunostained with monoclonal anti-pan cytokeratin antibody for visualisation and quantification of ureteric branch points and tips, or for 5 days after which they were whole-mount stained with rhodamine-labelled peanut agglutinin for visualisation and quantification of glomerular number. Metanephroi cultured with 0.2% ethanol had approximately 20% fewer branch points, branch tips and glomeruli than metanephroi cultured in the absence of ethanol. Exogenous RA significantly increased the numbers of branch points, branch tips and glomeruli, by approximately 25% in each case. Metanephroi cultured with both 0.2% ethanol & 10nM RA had a similar number of branch points, tips and glomeruli as control (0% ethanol) metanephroi. *In vivo* fetal ethanol exposure reduced the metanephric levels of GDNF, FGF7, Wnt11, TGFβ2 and TGFβ3 mRNAs. This study demonstrates that ethanol at a concentration of 0.2% inhibits ureteric branching morphogenesis in the rat kidney and that addition of RA can prevent this effect. These results suggest a possible pathway through which ethanol acts to disrupt normal kidney development.

Introduction

Alcohol (ethanol) is commonly consumed by pregnant women in western society [WHO 2004]. Alcohol is a known teratogen and the effects of chronic ethanol exposure on the developing fetus have been well established [Abel *et al* 1984, Day *et al* 1991]. However, the effects of acute ethanol exposure on development is not well characterised. It has been demonstrated that acute ethanol exposure in animal studies results in neuroapoptosis [Maier *et al* 1999, Maier *et al* 2001], reduced brain growth [Young *et al* 2005] and pulmonary alveolar dysfunction [Wang *et al* 2007]. Furthermore, it has been reported that culture in the presence of ethanol increases apoptosis in cultured skin cells [Cameron *et al* 2002], mitochondrial defects in myocardial cells [Mashimo *et al* 2003], and dendritic branching of neurons [Bingham *et al* 2004].

However, the number of studies investigating the effects of ethanol on the developing kidney is limited. In chronic studies of ethanol-exposed animals, adverse affects on kidney development, through a reduction in DNA and protein content [Gallo *et al* 1986] and defects in renal function [Assadi *et al* 1991] were observed. Recently, we have shown that repeated chronic ethanol administration to pregnant ewes results in an 11% reduction in nephron number in offspring [Gray *et al* 2008]. This is important as a reduced nephron endowment is permanent and has been linked to the development of adult-onset diseases, such as hypertension [Brenner *et al* 1988, Hoy *et al* 2006]. Mammalian kidney development involves complex molecular reciprocal interactions between the ureteric tree and the surrounding metanephric mesenchyme [Burrow 2000, Dressler 2006]. Branching of the ureteric tree is a critical process and plays a significant role in determining nephron number, as new nephrons only form adjacent to ureteric tips. The reduced nephron number observed following fetal ethanol exposure may be due to direct or indirect effects on ureteric branching.

Retinoic acid (RA), a Vitamin A derivative, and its receptors play critical roles in kidney development [Moreau *et al* 1998, Mendelsohn *et al* 1999]. In an early study, Wilson and colleagues (1953) showed that maternal vitamin A deficiency resulted in renal hypoplasia in rats which could be prevented by vitamin A administration to pregnant animals. Ethanol metabolism and RA synthesis share a common enzymatic pathway, which includes alcohol dehydrogenase and aldehyde dehydrogenase. It has been demonstrated in animal models that following ethanol exposure there is a transient reduction in the concentration of plasma RA [Deltour *et al* 1996, Crabb *et al* 2001]. In addition, it has been shown that the number of glomeruli in whole cultured rat metanephroi is increased after exogenous RA administration [Villar *et al* 1996]. It has been proposed that a transient reduction in RA levels is a contributing factor to the developmental presentation of fetal alcohol syndrome (FAS) [Deltour *et al* 1996, Goodyer *et al* 2007]. In the present study, we hypothesised that ethanol-induced inhibition of ureteric branching morphogenesis in rat metanephric culture would be ameliorated by co-culture with exogenous RA.

We also investigated the effects of *in vivo* fetal ethanol exposure on the expression levels of some key metanephric development genes. Glial cell-line derived neurotrophic factor (GDNF), bone morphogenetic proteins (BMPs), transforming growth factor βs (TGFβs), fibroblast growth factors (FGFs) and wingless type (Wnt) signalling molecules are important promoters/inhibitors of ureteric branching morphogenesis [Mendelsohn *et al* 1994, 1999, Merlet-Benichou *et al* 1997, Moreau *et al* 1998, Dressler 2006]. In the present study, the expression levels of GDNF, GFRα1 (a GDNF receptor), TGFβs 1, 2 and 3, FGF7, BMP4 and Wnts 4 and 11 were determined. Additionally, apoptosis plays a pivotal role in the maturation of the developing kidney, as do members of the renin angiotensin system (RAS) pathway [Saikumar *et al* 2003, Chen *et al* 2004]. Hence, we also investigated the effects of co-culture with ethanol and RA on the expression of Bcl-2 Associated X Protein (BAX), Bcl-2, and RAS

members angiotensin type 1b (AT1b) and angiotensin type 2 (AT2). Finally, the expression levels of RA receptors (RARα and RXRα) and alcohol dehydrogenase (ADH) were analysed.

Methods

Animals: All animal experiments were approved by the Animal Ethics Committee of Monash University, and conducted in accordance with the guidelines of the National Health and Medical Research Council of Australia, *ad libitum* access to standard rat chow and water with a 12 hour light/dark cycle.

Whole Metanephric Organ Culture: Sprague-Dawley female rats were time-mated for 4 hours where presence of a vaginal plug indicated time of mating, designated as embryonic day 0.5 (E0.5). At E14.5, the dams were surgically anaesthetised with Pentobarbitone Sodium, and their embryos removed and weighed. Whole metanephroi were isolated from embryos within a weight range of 130-150mg and placed on 3.0µm pore polycarbonate transfilter membranes (Osmonics, Poretics, New South Wales, Australia) in 24-well tissue culture plates with wells containing 350µl of serum-free culture media at 37°C and 5% CO_2. The culture medium consisted of DMEM:Ham's F12 liquid medium (Sigma-Aldrich) supplemented with 5µg/ml transferrin (Sigma-Aldrich), 12.9µl/ml L-Glutamine (Sigma-Aldrich), Penicillin (100U/ml), and Streptomycin (100µg/ml). Metanephroi were allocated randomly into one of the following six groups: 0% ethanol (control), 0% ethanol plus 10nM RA, 0% ethanol plus 20nM RA, 0.2% ethanol, 0.2% ethanol plus 10nM RA or 0.2% ethanol plus 20nM RA. To investigate the effects of ethanol and/or RA on ureteric branching morphogenesis, metanephroi were cultured for 48 hours with media replaced after 24 hours. To determine the effects of ethanol and/or RA on glomerular number, metanephroi were cultured for 5 days, with media replaced after 24 hours and then left for four days. At the conclusion of the culture period, metanephroi were fixed for examination using whole-mount immunofluorescence microscopy.

Immunofluorescence Staining: At the end of the culture period, metanephroi were fixed in methanol at -20°C for a minimum of 15min. After fixation, for visualisation of the ureteric tree, metanephroi were washed briefly in 1% Tween 20 (PBST) and incubated with primary antibody (monoclonal mouse anti-pan cytokeratin (Sigma-Aldrich)) at a dilution of 1:100 at 37°C for 2 hours. Metanephroi were then washed in PBST before addition of the secondary antibody; Alexa 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR) at a dilution of 1:100 at 37°C for 2 hours. Metanephroi were then washed briefly in PBST before mounting in PBS/glycerol mounting media (Sigma-Aldrich).

Quantification of Ureteric Branching Morphogenesis: Following immunostaining, metanephroi were visualized using an epifluorescence microscope (Olympus) and manually skeletonised. Ureteric branch points and tips were counted. Branch points were defined as the intersection of three or more branches (lines on the skeletonised image).

Lectin Histochemistry: Following fixation, those metanephroi to be prepared for glomerular number estimation were briefly washed in PBS. They were incubated in 50mM NH_4Cl at room temperature for 1 hour following which the tissue was permeabilized with 0.1% saponin in PBS for 1 hour at room temperature. After digestion with 2% H_2O_2 in methanol at room temperature for 30 minutes, metanephroi were washed in 0.1% saponin in PBS for 30 minutes. Metanephroi were then incubated in 0.1U/ml of neuraminidase (Sigma-Aldrich) in 1% $CaCl_2$ in PBS for 2 hours at 37°C, before being washed twice for 30 minutes in 0.2% saponin in PBS. Metanephroi were then incubated in peanut agglutinin (PNA) (50µg/ml; Sigma-Aldrich) in 0.3% Triton in PBS with 1:100 dilution of ions overnight at 4°C. Metanephroi were then extensively washed in 0.1% saponin to remove excess PNA and then mounted on cavity slides with fluorescence preserving mounting media (Sigma-Aldrich).

Quantification of Glomerular Number: Following lectin PNA histochemistry, metanephroi cultured for 5 days were observed under an epiflourescence microscope for direct counting of glomeruli.

In Vivo Studies of Gene Expression: To investigate whether maternal ethanol consumption alters fetal metanephric gene expression, *in vivo*, pregnant Sprague-Dawley rats were administered 1g/kg of ethanol via gavage on E13.5 and E14.5 of gestation, with a control group receiving a similar volume of saline. At E15.5, dams were surgically anaesthetised with Pentobarbitone Sodium and embryos were removed, cleaned, weighed and paired kidneys were collected and frozen for later extraction of RNA. Total RNA was extracted using RNeasy extraction kits (Qiagen). One microgram of each RNA sample was reverse transcribed as previously described [Singh *et al* 2007].

Relative Gene expression In Vivo: Levels of mRNA expression were determined via real-time PCR using an Applied Biosystems One step Machine. Sequences and optimal concentrations for Wnt4, AT1b, AT2, BMP-4 and TGFβ1 have previously been reported [Singh *et al* 2007, Dickinson *et al* 2007], while primer and probe sequences for GFRα1, TGFβ2, TGFβ3, FGF7, Wnt11, RARα and RXRα are presented in Table 1. GDNF, BAX, Bcl-2 and ADH were analysed using an Assay-on-Demand from Applied Biosystems. A comparative cycle of threshold fluorescence (CT) method was used with 18S as an internal control. All genes were multiplexed with 18S, except for AT2 as optimization experiments revealed the CT was altered if run in a multiplex reaction. Cycle threshold fluorescence for 18S was examined for each gene to ensure that there were no differences between ethanol and control metanephroi.

Calculation of Relative Gene Expression: The CT values for 18S were subtracted from the CT value for the gene of interest to give a ΔCT for each sample. The ΔCT of the calibrator (the

mean ΔCT for saline-exposed kidneys) was then subtracted from each sample to give a ΔΔCT value. This was then inserted into the equation 2-ΔΔCT to give a final relative expression relative to the calibrator.

Statistics: Data are presented as means ± SEM except where otherwise indicated. One-way ANOVA was utilised for analysis of ureteric branching morphogenesis and glomerular number. Tukey's post-hoc test was used to identify differences between groups. Two-tailed unpaired t-tests were used for analysis of gene expression. Statistical significance was accepted at $P<0.05$.

Results

Ethanol Reduces Ureteric Branching Morphogenesis In Vitro: Culture of whole rat metanephroi in the presence of ethanol did not influence the qualitative appearance of the ureteric tree (Figure 1). However, quantitative analysis of the ureteric tree after 2 days of culture showed in the presence of ethanol (0.2%) found the kidney contained 20% fewer branch points (0%, 40.63±1.82; 0.2%, 33.90±1.35, $P<0.001$) and tips (0%, 43.26±1.84; 0.2%, 37.15±1.30, $P<0.001$) than control metanephroi (Figure 2a and b).

Ethanol Inhibits Glomerulogenesis In Vitro: Culture in the presence of ethanol also adversely affected glomerular development. Metanephroi cultured for 5 days in the presence of 0.2% ethanol contained 25% fewer glomeruli than metanephroi cultured in control media (0%, 55.58±2.90; 0.2%, 42.66±1.79, $P<0.001$ (Figure 2c).

Exogenous Retinoic Acid Rescues Ureteric Branching Morphogenesis in the Presence of Ethanol: Metanephroi cultured in the presence of RA (10nM, 20nM) without ethanol and metanephroi cultured in the presence of both 0.2% ethanol and 20nM RA appeared larger than metanephroi cultured under other conditions (Figure 1). Quantitative analysis revealed that addition of RA to both 0% ethanol and 0.2% ethanol cultures significantly increased the number of ureteric branch points in comparison to metanephroi cultured in 0% or 0.2% ethanol without RA (Figure 3a). A similar effect was observed for ureteric tips, namely co-culture with 0.2% ethanol and 10nM RA resulted in a ureteric tip number similar to that of control, while culture in both 0.2% ethanol and 20nM RA increased ureteric tip number above that of control (Figure 3b).

Exogenous Retinoic Acid Rescues Glomerular Number in Presence of Ethanol: The addition of exogenous RA to both control cultures (0% ethanol) and 0.2% ethanol cultures significantly

increased the number of glomeruli at 5 days in comparison to metanephroi cultured in 0% ethanol or 0.2% ethanol. Significantly, co-culture with 0.2% ethanol and 10nM RA resulted in a similar glomerular number to that observed in control cultures (0% ethanol) (Figure 3c).

Gene Expression In Vivo: CT values for 18S were similar for all genes between control and ethanol exposed metanephroi (cycle 9.5 in control v cycle 9 in ethanol). The relative metanephric mRNA levels of GDNF, FGF7, Wnt11, TGFβ2 and TGFβ3 in E15.5 fetuses exposed to ethanol at E13.5 and E14.5 were significantly reduced compared to saline-treated fetuses. mRNA levels for all other genes examined were similar in control and ethanol-treated fetuses (Table 2).

Table 1:

Primer and probe sequences for genes used for quantitative RT-RCR

Gene	Forward (5'-3') Reverse (5'-3')	Probe (5'-3')
GFRα1	CGTCTTAACTGCAATACAC AACCGCTACAATATCGAAAG	AAATGTCACTGACTTGGGTTTGGGC
TGFβ2	CGACATGCCGTCCCACTTC CTGCCCACTGAGCCAGAG	CCCTCCGAAACTGTCTGCCCAG
TGFβ3	TTCAGCCCAATGGAGACATAC CGGCCATGGTCATCTTCGT	TGGAAAATGTTCACGAGGTGATGGAA
FGF7	GACAAACGAGGCAAAGTGAAAGG TGCCACAGTCCTGATTTCCA	ACCCAGGAGATGAGGAACAGCTACA
Wnt11	TGCGTCTACACAACAGTGAAGTG GGAGCAGGAGCCAGATACC	AGACAGGCTCTACGTGCCTCCC
RARα	CCGGACTCCGCTTTGGAATG GGGCTGGGCACTATCTCTTC	TCAAACCACTCCATCGAGACCCA
RXRα	CGGAGCTGGTGTCGAAGATG CCCTTAGAGTCAGGGTTGAAGA	TGACATGCAGATGGACAAGACGGA

Table 2:

Real-Time PCR analysis performed on E15.5 fetal kidneys following maternal exposure to saline or ethanol (1g/kg) at E13.5 and E14.5. Data are mean ± SEM and were analysed using a two-sampled unpaired t-test, N=5 kidney pairs per group. *P<0.05.

	Saline	**Ethanol**
GFRα1	1.19±0.319	1.37±0.555
GDNF	1.00±0.045	0.66±0.133*
TGFβ1	1.06±0.212	0.63±0.066
TGFβ2	1.03±0.144	0.44±0.199*
TGFβ3	1.01±0.079	0.62±0.094*
FGF7	1.00±0.045	0.50±0.158*
Wnt4	1.00±0.065	1.23±0.508
Wnt11	1.06±0.169	0.53±0.142*
BMP4	1.10±0.218	0.97±0.125
Bcl-2	1.03±0.138	0.95±0.327
BAX	1.00±0.067	1.17±0.118
AT1b	1.08±0.190	2.09±0.551
AT2	1.08±0.194	1.20±0.394
ADH	1.12±0.291	1.08±0.317
RARα	2.57±1.782	0.65±0.200
RXRα	1.13±0.281	1.04±0.568

Figure 1: Whole-mount immunofluorescence images of the ureteric tree of rat metanephroi after 48 hours culture in 0% ethanol (A), 0% ethanol and 10nM RA (B), 0% ethanol and 20nM RA (C), 0.2% ethanol (D), 0.2% ethanol and 10nM RA (E), or 0.2% ethanol and 20nM RA (F). Scale bar = 500µm.

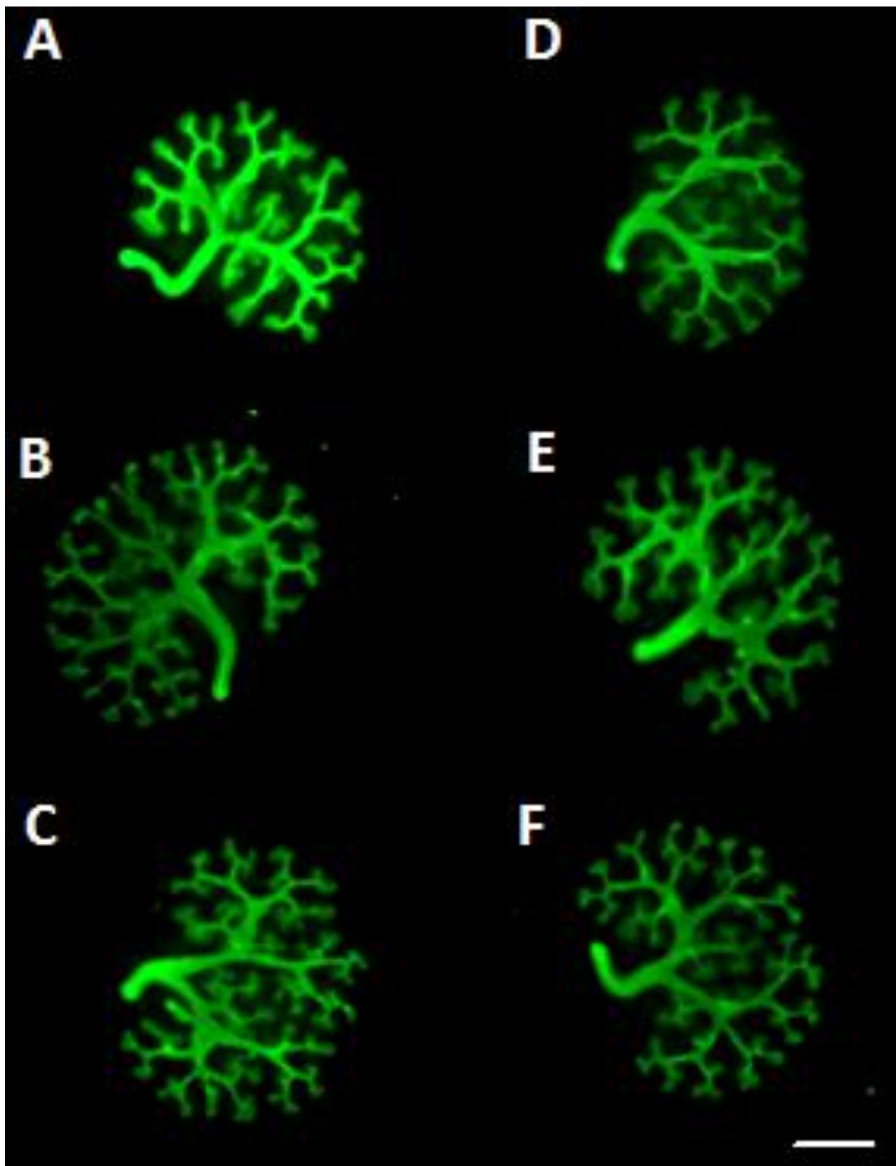


Figure 2: The number of ureteric branch points (A) and tips (B) after 48 hours culture, and glomerular number (C) after 5 days culture in 0% ethanol or 0.2% ethanol. Data are mean ± SEM and were analysed using a two-sampled unpaired t-test; ***P<0.001. N=10-15 metanephroi per group.

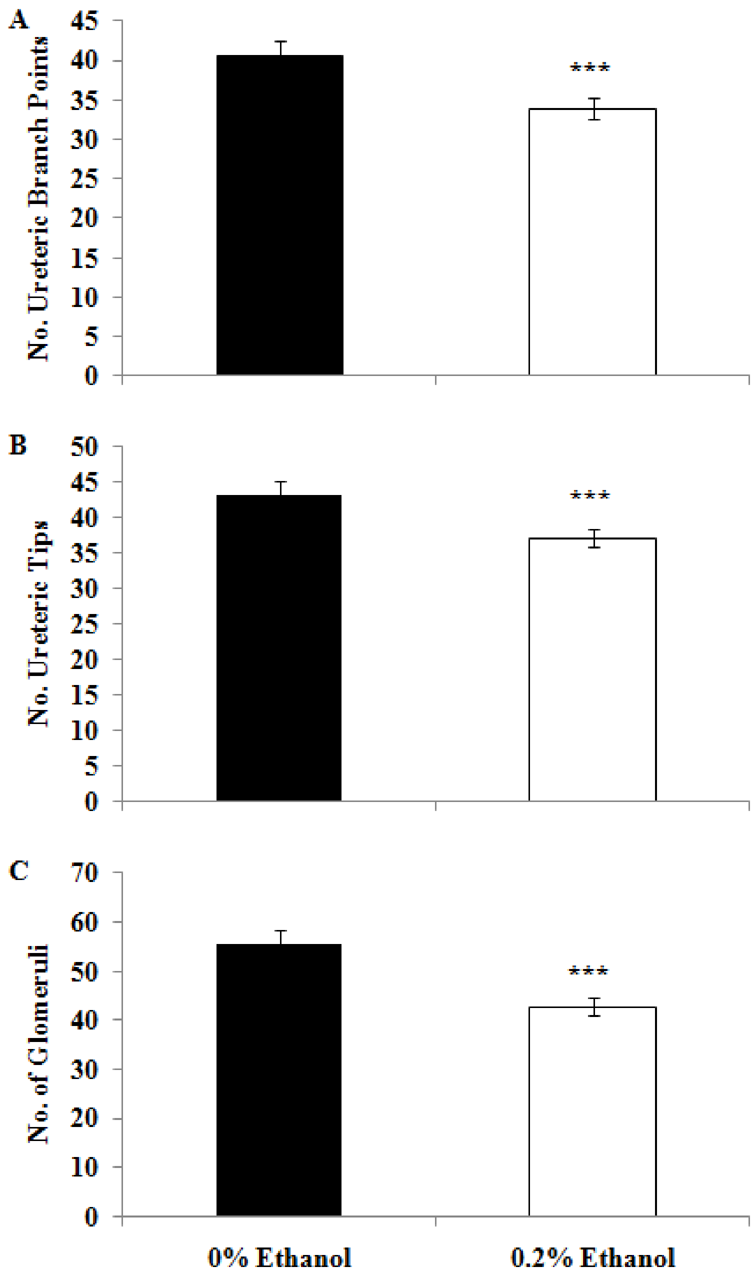
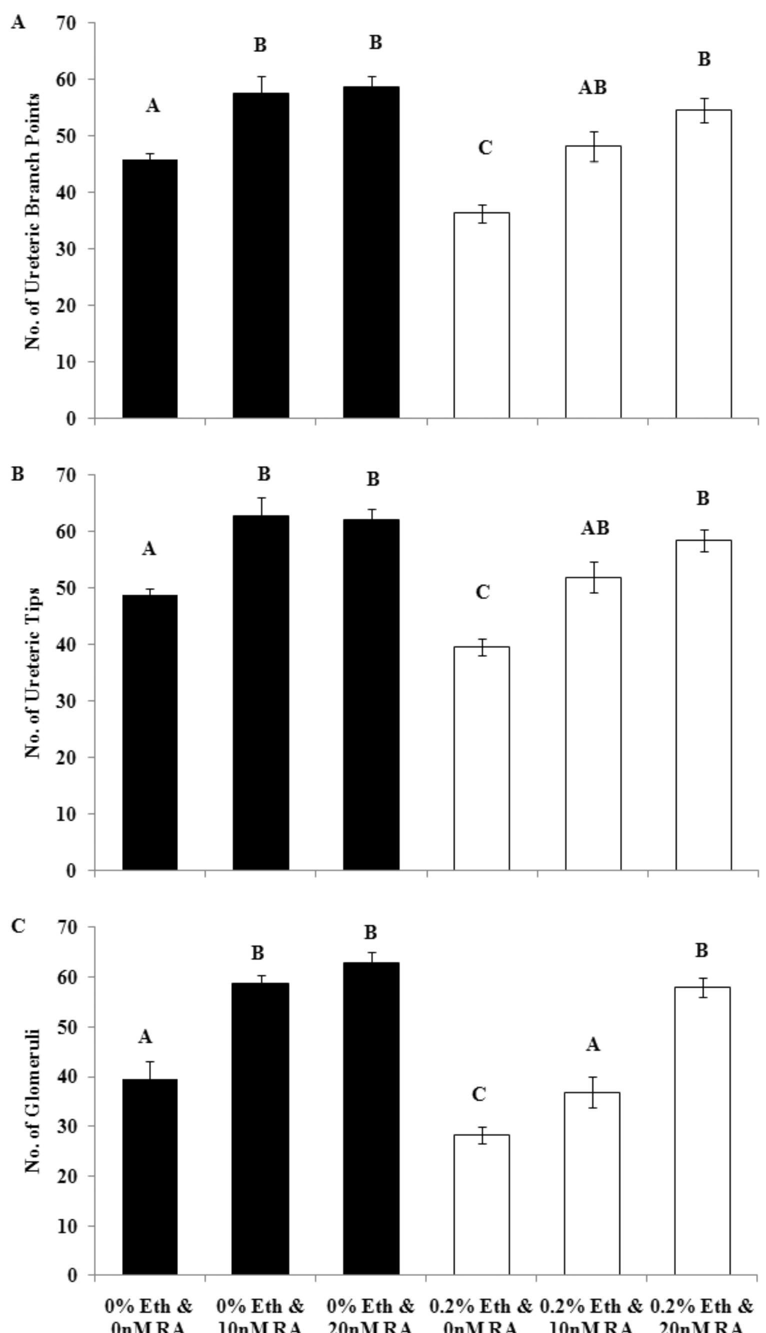


Figure 3: The number of ureteric branch points (A) and tips (B) after 48 hours and glomerular number (C) after 5 days culture with 0% or 0.2% ethanol with/without exogenous RA (10nM or 20nM RA). Data are mean ± SEM and were analysed using a one-way ANOVA with a Tukey's post-hoc test. Groups that do not share a common letter are significantly different from each other, $P<0.05$. N=10-15 metanephroi per group.



Discussion

This study has clearly demonstrated that ethanol inhibits rat kidney development *in vitro*. Utilising whole metanephric organ culture, we have shown that exposure to ethanol at a concentration of 0.2% reduces by approximately 20% the number of ureteric branch points, ureteric tips and glomeruli that are formed. Furthermore, this study has demonstrated that addition of RA, a promoter of kidney development, at a concentration of 10nM is able to rescue kidney development in ethanol-exposed metanephroi so that there was no longer an adverse effect of ethanol on kidney development. We also found that the expression levels of genes involved in ureteric branching morphogenesis and nephrogenesis were reduced in the kidneys of ethanol-exposed fetuses. These findings suggest that the reduction in nephron number identified *in vitro* is a result of a slowing/inhibition of ureteric branching morphogenesis, and that the RA synthesis pathway could play a potential role in the phenotype.

The concentration of ethanol used in the current study (0.2%) equates to a blood alcohol concentration (BAC) of approximately 0.157g/dl. A BAC of this level is equivalent to that achieved following consumption of approximately four standard drinks in one sitting. This level of drinking has been reported in some pregnant women [Ethen *et al* 2003, Nayak *et al* 2004, Colvin *et al* 2007]. Similar concentrations of ethanol have been used in previous studies investigating the teratogenic properties of ethanol in cultured cells/tissue [Cameron *et al* 2002, Mashimo *et al* 2003, Bingham *et al* 2004]. Furthermore, we have previously reported that repeated prenatal ethanol exposure and a BAC of 0.12g/dl results in a permanent nephron deficit in fetal sheep [Gray *et al* 2008].

The mechanisms through which alcohol acts to disrupt kidney development, resulting in a deficit in nephron number, are largely unknown. The present findings suggest that a reduction

in ureteric branching morphogenesis may contribute to the observed reduction in nephron number. Previous studies have also suggested a link between reduced branching morphogenesis and reduced nephron number in fetal programming. For example, exposure to dexamethasone for 2 days during gestation (E15-E16) in rats resulted in a significant nephron deficit [Ortiz *et al* 2001, 2003]. It was later identified through the use of metanephric organ culture that exposure to dexamethasone at an equivalent time-point to this current study as well as the studies of Ortiz and colleagues (2001, 2003) resulted in a reduction in ureteric branching morphogenesis and the number of glomeruli formed [Singh *et al* 2007]. This finding suggests that the observed reduction in nephron number induced *in vivo* by dexamethasone exposure could be mediated in part, directly or indirectly, by a reduction in ureteric branching morphogenesis, subsequently leading to a permanent reduction in the number of glomeruli formed.

RA, the active derivative of vitamin A synthesis, is a promoter of kidney development *in vivo* and *in vitro* [Vilar *et al* 1996, Lelievre-Pegorier *et al* 1998, Batourina *et al* 2001, Gilbert 2002]. As both vitamin A synthesis and ethanol metabolism employ a common enzymatic pathway, it has been proposed that diminished RA concentrations may contribute to FAS [Deltour *et al* 1996, Goodyer *et al* 2007]. Therefore, to further investigate the role of this pathway in the ethanol-induced phenotypic changes observed in the present study, metanephroi were co-cultured with ethanol and RA. Our results showed that addition of 10nM RA was able to stimulate kidney development, restoring the number of ureteric branch points, ureteric tips and glomeruli to levels observed in control cultured kidneys.

The effects of exogenous RA on metanephric development *in vitro* has been reported in several studies, occasionally with conflicting outcomes. Most studies have reported that culture in the presence of RA significantly increases the number of glomeruli found [Weller *et*

al 1991, Humes *et al* 1992, Vilar *et al* 1996]. For example, Vilar and colleagues (1996) found that addition of RA at a concentration of 100nM, 10-fold higher than the concentration used in the current study, resulted in a 200% increase in nephron number after 6 days in culture. However, Weller and colleagues (1991) found that RA failed to induce additional glomerular development in isolated mouse metanephroi after 72 hours in culture.

Significant changes in gene expression levels were found in kidneys of rats exposed to ethanol *in utero*. It is important in studies such as this to consider the expression of the housekeeping gene, in this case 18S . 18S expression is known to be influenced by gestational age [Tanic *et al* 2007], sex [Verma *et al* 2006, Derks *et al* 2008] and treatment [Schmittgen *et al* 2000. Derks *et al* 2008]. In the present study, 18S expression did not vary with either gestational age or ethanol exposure. However, we cannot rule out the effects of sex on 18S expression, as kidneys from both sexes were pooled for all groups. The expression of TGFβ2, TGFβ3, GDNF, FGF7 and Wnt11, which are all involved in the regulation of ureteric branching morphogenesis and nephrogenesis, were significantly down-regulated in ethanol-exposed kidneys. GDNF has a direct role in kidney development by promoting ureteric branching through its receptor c-ret [Burrow 2000]. GDNF heterozygous null mutant mice have reduced nephron endowment compared to wild-type littermates [Cullen-McEwen *et al* 2001], an observation that correlates with the reduced renal GDNF expression identified in ethanol-exposed fetuses and the reduced nephron number at PN30 observed in ethanol-exposed fetuses [Gray *et al* unpublished data]. Several members of the TGFβ superfamily are known to inhibit ureteric branching morphogenesis and promote branch lengthening [Oxburgh *et al* 2004]. The lengthening of ureteric branches ensures that branch tips are kept within the nephrogenic zone, the site of critical reciprocal signalling between the mesenchyme and ureteric tips [Santos and Nigam 1993, Nigam 1995, Bush *et al* 2004]. The down-regulation of TGFβ2 and TGFβ3 expression observed in the present study *in vivo*, suggests that the reduced nephron number

observed in ethanol-exposed offspring at PN30, and in kidneys cultured in the presence of ethanol may be due, at least in part, to dysregulated ureteric branching morphogenesis possibly involving inappropriate branch lengthening.

Mesenchyme survival and nephron induction are also critically important events in metanephric development, and defects in these processes can also result in reduced nephron number [Stark *et al* 1994, Guo *et al* 1996, Qiao *et al* 1999, Majumbar *et al* 2003]. Members of the FGF and Wnt signalling pathways are crucially involved in the induction process. The current study identified reduced expression of both Wnt11 and FGF7 in kidneys of fetuses exposed to ethanol. Both Wnt11 and FGF7 are expressed in the mesenchymal aggregates adjacent to the ureteric tips [Guo *et al* 1996, Qiao *et al* 1999, Majumbar *et al* 2003]. The kidneys of FGF7 homozygous null embryos are smaller than wildtype kidneys, and contain 30% less nephrons than wildtype kidneys in adulthood [Guo *et al* 1996, Qiao *et al* 1999]. The reduction in both Wnt11 and FGF7 expression observed in the present study suggests that the reduced nephron number identified *in vivo* [Gray *et al* unpublished data] and *in vitro* could be in part a product of failure of nephron induction. While changes in gene expression do not always translate to changes in protein levels, analysis of protein in embryonic rat kidneys would require many hundreds of kidneys, making the experiments unrealistic in this study. However, these studies do provide indications of the ‘best’ candidates for future analysis of protein expression. Taken together, the present *in vivo* findings of metanephric gene expression suggest that the phenotypic effects elicited by ethanol involve an interplay of repressive signals on ureteric branching morphogenesis which in turn influence nephron induction, resulting in a reduction in nephron endowment

Conclusions

The results from the present study suggest a potential mechanistic pathway through which ethanol acts to inhibit metanephric development. Fetal rat kidneys cultured in the presence of ethanol demonstrated a significant reduction in ureteric branching morphogenesis and glomerular number. This phenotype was completely blocked by co-culture with 10nM RA, a known promoter of kidney development. *In vivo* studies identified ethanol-induced changes in the expression levels of genes known to regulate ureteric branching and nephrogenesis. The present findings suggest that the identified reduction in nephron number *in vivo* [Gray *et al* unpublished data] could be in part a consequence of a direct or indirect reduction in ureteric branching morphogenesis.

Acknowledgements

The authors acknowledge Assoc Prof. Mary Wlodek for supply of primer and probe sets.

Grants

This work was supported by a National Health & Medical Research Council grant (Grant ID 511162).

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Chapter 6

General Discussion

6.1 Overall Summary

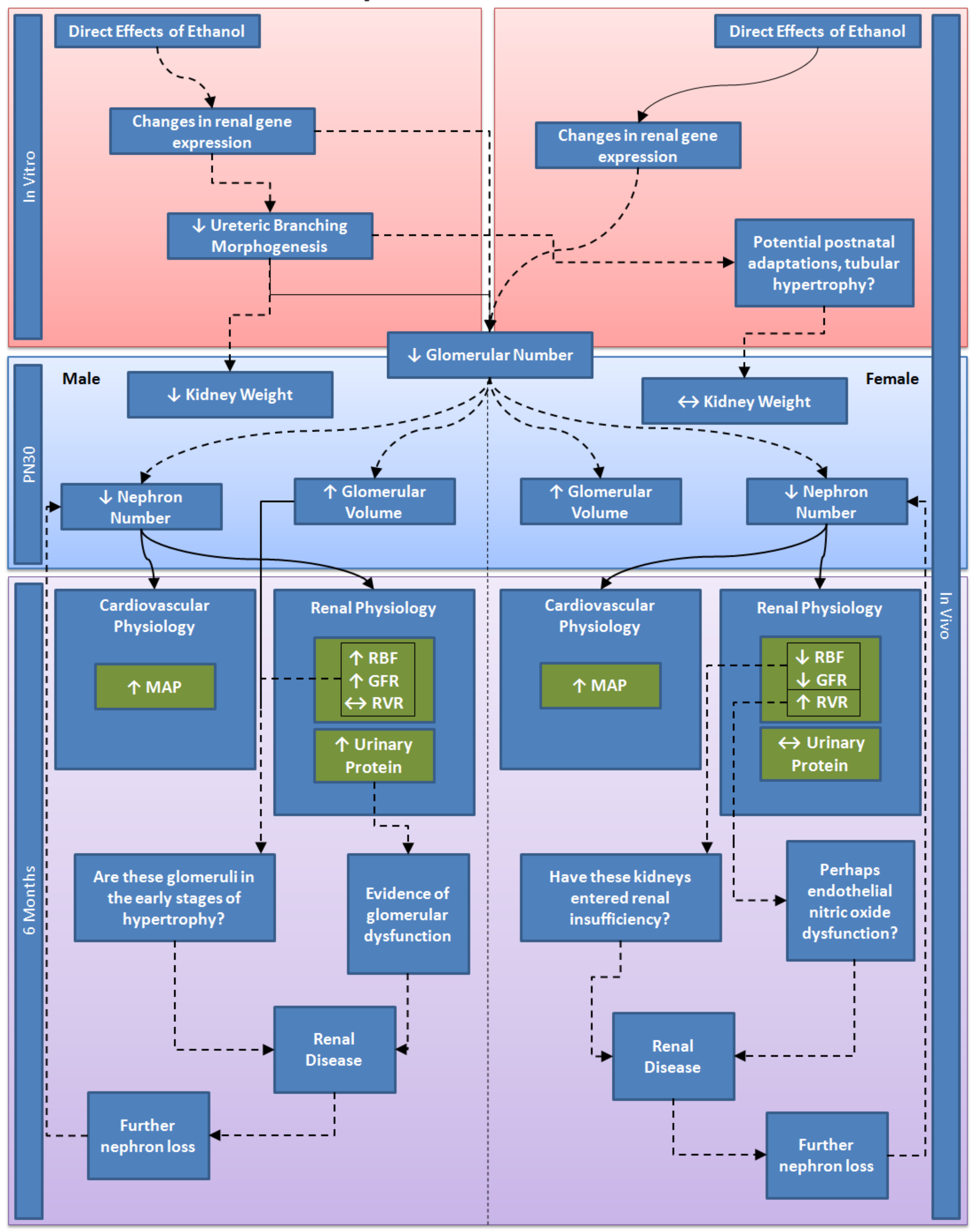
The findings of the studies described in this thesis indicate that both acute and chronic prenatal alcohol exposure result in a permanent reduction in the number of nephrons within the kidney. Chronic prenatal alcohol exposure in sheep resulted in an 11% nephron deficit in late gestation foetuses, at a time when nephrongenesis is complete. Acute prenatal ethanol exposure in rats resulted in a reduction in nephron endowment and was associated with long-term physiological consequences, with elevated mean arterial pressure observed in both male and female offspring. Sex-specific changes in renal function were observed at 6 months of age: male ethanol-exposed offspring had elevated GFR and renal blood flow with evidence of proteinuria, while females demonstrated elevations in renal vascular resistance coupled with a reduction in renal blood flow and GFR. These results suggest the male offspring were in the early stages of renal dysfunction, while females had established renal dysfunction.

Whole rat metanephric organ culture studies demonstrated that culture in the presence of ethanol inhibited ureteric branching morphogenesis, and fewer glomeruli were formed. This suggests that the identified reduction in nephron number *in vivo*, may in part be due to attenuated ureteric branching morphogenesis. Co-culture of metanephroi with ethanol and RA protected against the adverse effects of ethanol. Quantitative real-time PCR analysis suggested a number of mechanisms whereby ethanol might inhibit metanephric development.

A schematic diagram summarising the effects observed in the present study of acute ethanol exposure on rat kidney development, as well as other possible processes/mechanisms, is presented in Figure 6.1.

Figure 6.1 Schematic diagram of the potential mechanisms through which acute alcohol exposure might act to disrupt kidney development, and the subsequent events that predispose to renal dysfunction. Solid lines represent findings from the present study in rats, while dashed lines represent possible associations that must be confirmed in future studies.

The effects of ethanol occur early in kidney development and include changes in gene expression. A nephron number deficit is observed, likely involving inhibition of ureteric branching morphogenesis. Male and female kidneys, at least in rats, appear to respond differently to prenatal ethanol exposure, leading to different effects on kidney size and nephron number. These sex-specific responses to ethanol likely contribute to sex-specific differences in renal function at 6 months of age, when male offspring show early stages of renal dysfunction, while female offspring have established renal dysfunction. These changes in renal structure and physiology may lead to renal disease, and further loss of glomeruli.


Ethanol Exposure at E13.5 and E14.5
In Vitro
Direct Effects of Ethanol
Changes in renal gene expression
↓ Ureteric Branching Morphogenesis
Direct Effects of Ethanol
Changes in renal gene expression
Potential postnatal adaptations, tubular hypertrophy?
↓ Glomerular Number
PN30
Male
↓ Kidney Weight
Female
↔ Kidney Weight
↓ Nephron Number
↑ Glomerular Volume
↑ Glomerular Volume
↓ Nephron Number
In Vivo
6 Months
Cardiovascular Physiology
↑ MAP
Renal Physiology
↑ RBF
↑ GFR
↔ RVR
↑ Urinary Protein
Cardiovascular Physiology
↑ MAP
Renal Physiology
↓ RBF
↓ GFR
↑ RVR
↔ Urinary Protein
Are these glomeruli in the early stages of hypertrophy?
Evidence of glomerular dysfunction
Have these kidneys entered renal insufficiency?
Perhaps endothelial nitric oxide dysfunction?
Renal Disease
Renal Disease
Further nephron loss
Further nephron loss

6.2 Ethanol Impairs Kidney Development

In the present study, prenatal ethanol exposure resulted in reduced nephron endowment in two species (sheep, rat) indicating that renal development had been impaired. This was despite the fact that two models of ethanol exposure (acute, chronic) were employed, and the timing of exposure relative to kidney development was very different in the two species (rat, in first 2 days of metanephric development; sheep, in final 35 days of nephrogenesis). Taken together, these results suggest that the kidney is susceptible to ethanol exposure throughout much, if not all of nephrogenesis. This raises the question as to whether the mechanisms contributing to the nephron deficit are the same in the two models or whether different pathways are involved. While the extent of the nephron deficit observed in the present study was not marked (11% in sheep, 15% in rat), it is similar to that reported following exposure to a low protein diet throughout pregnancy in rats (~15%), elevated maternal glucocorticoids in rats (~20%) and placental embolisation in sheep(~24%) [Woods *et al* 2004, Singh *et al* 2007, Zodhi *et al* 2008, Hoppe *et al* 2009]. In each of these cases, the nephron deficit was associated with abnormalities in renal and cardiovascular function.

The *in vivo* reduction in nephron number in rats in response to acute ethanol exposure indicates the early stages of kidney development can be affected by ethanol exposure. The timing of ethanol exposure corresponded to when the metanephros consists of metanephric mesenchyme and a ureteric tree with just a few branches [Burrow 2000]. Studies using whole kidney organ culture at the same developmental stage indicated ethanol-induced inhibition of ureteric branching morphogenesis, suggesting that the *in vivo* nephron deficit may be due, at least in part, to attenuated ureteric branching. Following ethanol exposure *in vivo*, altered expression of genes involved in ureteric branching morphogenesis and nephrogenesis (*GDNF, FGF7, Wnt11, TGFβ2, TGFβ3*) was identified, suggesting putative molecular mechanisms for the observed phenotypic changes. These findings are similar to those of Dickinson *et al*

(2007) and Singh *et al* (2007) who observed reduced nephron number following prenatal glucocoticoid exposure. In all studies, reduced ureteric branching morphogenesis and nephron endowment was associated with reduced renal expression of *GDNF*, as well as altered expression of *TGFβ1* and *BMP4*. As the actions of ethanol influence the activity of the hypothalamic-pituitary-adrenal axis, it is plausible that the findings observed in the current study are an indirect product of the actions of glucocorticoids on kidney development [Weinberg *et al* 2008]. Wilcoxon *et al* (2003) found that ethanol exposure resulted in elevated placental *11β-HSD* mRNA expression, which is driven by the actions of elevated maternal glucocorticoids. The phenotypic effects of ethanol were prevented by the removal of the maternal adrenals. This suggests that maternal hormone status driven by the actions of glucocorticoids may contribute to the ethanol-induced phenotype and is congruent with the similar gene changes found in glucocorticoid and ethanol exposed fetal kidneys.

Results from several previous studies suggest that the teratogenic properties of ethanol may be mediated in part by a reduction in RA concentrations in the developing fetus [Deltour *et al* 1996, Crabb *et al* 2001, Goodyer *et al* 2007]. As RA is a known promoter of kidney development, we hypothesized this may be a mechanism of action by which ethanol attenuates nephron endowment. RA supplementation experiments *in vitro* demonstrated that co-culture with ethanol and RA protected the kidney from the adverse effects of ethanol. These data provide preliminary evidence for a second potential mechanism through which ethanol disrupts kidney development.

It is not known if the mechanism through which chronic ethanol exposure results in a reduction in nephron number is the same as that caused by acute exposure. In the present study, pregnant sheep were administered ethanol daily between 90 and 134 days of gestation, the period of peak nephrogenesis. Slowing of ureteric branching morphogenesis and nephron induction could certainly be involved, but another potential mechanism could involve increased apoptosis of condensing mesenchyme. This may result in a lower number of

nephrogenic condensates, leading to a reduction in nephron number. Apoptosis is a critical process in metanephric development, and increased apoptosis has been shown to lead to reduced nephron number [Burrow 2000, Saikumar *et al* 2003]. Overall, the present findings suggest that multiple pathways may mediate the deleterious effects of ethanol on kidney development, and that the particular mechanism through which ethanol acts may be influenced by the duration and dose of ethanol exposure.

6.3 Birth Weight, Nephron Number and Hypertension

Low nephron number has been associated with an increased likelihood to develop hypertension in both humans and animal models [Brenner *et al* 1988, Langley-Evans *et al* 1999, Woods *et al* 2001, 2004, Wintour *et al* 2003, Hoy *et al* 2006, 2008, Dickinson *et al* 2007, Hoppe *et al* 2007, Singh *et al* 2007, Hughson *et al* 2008, Moritz *et al* 2008, McNamara *et al* 2009]. Low birth weight is often used as an indicator of low nephron number, and is also associated with increased predisposition to the development of hypertension later in life [Langley-Evans *et al* 1999, Hughson *et al* 2003, Woods *et al* 2004, Luyckx and Brenner 2005]. However, this is not always the case, with some studies reporting low nephron endowment and hypertension without evidence of low birth weight [Wintour *et al* 2003, Singh *et al* 2007]. In the present study, rat offspring acutely exposed to ethanol during gestation had reduced birth weight, whereas ovine fetuses chronically exposed to ethanol were of normal weight late in gestation. A recent study in sheep investigating acute prenatal ethanol exposure reported a reduction in body weight at autopsy (120DGA) [Gatford *et al* 2007]. Interestingly, the one major difference between the Gatford *et al* (2007) study and the present sheep study is the duration of alcohol exposure: 95DGA to 134DGA in the present study, compared with 116DGA to 118DGA in Gatford *et al* (2007). One interpretation of the differing effects on body weight in the two studies is the length of time the developing fetus had to adapt to the alcohol environment. Studies of fetal alcohol exposure in rodents have also reported conflicting outcomes on birth and body weight, with some studies reporting a reduction [Vavrousek-Jakuba *et al* 1991, Spohr *et al* 1993, Wilcoxon *et al* 2004, Dembele *et al* 2006] and other studies reporting no difference [Yao *et al* 2007, Jaddoe *et al* 2007]. Again, the lack of consistency on birth/body weight has been attributed to differences in the dose and timing of ethanol exposure.

In the present study, acute ethanol exposure in the rat resulted in an elevation in MAP at 6 months of age in both male and female offspring. Similar studies investigating prenatal glucocorticoid exposure have demonstrated an elevation in MAP coupled with a reduction in nephron number [Dodic *et al* 1998, 2003, Oritz *et al* 2001, 2003, Wintour *et al* 2003, Singh *et al* 2007, Dickinson *et al* 2007]. Likewise, rats chronically exposed to ethanol (6.36% [vol/vol]), from gestation day 2 until birth have elevated MAP [Turcotte *et* al 2002], although no information on nephron number was reported in this study.

Whilst it would have been desirable in the present study to analyse MAP in adult sheep following chronic fetal ethanol exposure, the fetuses were culled in late gestation for alternate studies (not part of this thesis). Studies investigating the effects of prenatal glucocorticoid exposure in sheep have identified both reduced nephron number and an elevation in MAP [Wintour *et al* 2003]. However, the reduction in nephron number in the glucocorticoid-exposed animals was substantially larger (39%) than that observed in the current study (11%), and it is therefore difficult to predict whether the 11% reduction in nephron number observed here would have resulted in a change in adult MAP [Wintour *et al* 2003, Gray *et al* 2008]. Using the same model of chronic ethanol exposure in sheep, Parkington *et al* (2007) observed detrimental effects on the developing cardiovascular system. For example, the endothelium-dependant vasodilator function of coronary vessels was impaired, with constrictor capacity of the vessels increased. The vessels of ethanol-exposed animals were stiffer and thinner than vessels in control animals [Parkington *et al* 2007]. These changes in vessel function would contribute to alterations in blood flow to the heart, which could contribute to an increased likelihood of vascular dysfunction under situations of physiological stress. Coupled with the reduction in nephron number, these ethanol-exposed animals may be predisposed to develop vascular dysfunction that could contribute to the development of hypertension.

6.4 Sex-Specific Effects of Ethanol Exposure

An unexpected finding from the current study was the sexual dimorphic effect of acute ethanol exposure in the rat. The first evidence of sex-specific effects was observed at PN30, when a greater reduction in nephron number was observed in male offspring (10% in females and 20% in males). Kidneys of ethanol-exposed male rats weighed less than control kidneys at PN30, but kidney weights were similar in the female groups. The cause of this sexual dimorphism is unknown, although recent evidence indicates sex hormones can influence kidney development [Walker *et al* 2008]. It is well known that in early postnatal life the kidney undergoes significant developmental and physiological changes in order to adapt to postnatal life [Robillard *et al* 1988, 1992, Jose *et al* 1994]. At this time, nephrons undergo rapid growth and maturation, and there is upregulation of tubular transporters to cope with the increased amount of sodium consumed [Holtback and Aperia 2003]. While it is unknown what specifically occurred within the female rats exposed to ethanol in the present study to "normalise" kidney weight, there was evidence of glomerular hypertrophy. This glomerular hypertrophy may be coupled with tubular hypertrophy, resulting in normal kidney weight.

Sex-specific differences in renal function following prenatal ethanol exposure were observed in rats at 6 months of age. Despite the lesser effects on kidney weight and nephron endowment in females, renal dysfunction was more pronounced in female ethanol-exposed offspring than in males as only females had a reduced GFR. Interestingly, there was no change in plasma electrolyte levels in either sex following ethanol exposure, suggesting there may have been a shift in the pressure-natriuresis curve, returning sodium levels to normal. The resetting to a higher pressure status would contribute to the elevated MAP observed in the ethanol-exposed rats, suggesting that the elevation in MAP may have occurred earlier than at 6 months when it was measured.

The lack of difference in kidneys weights from female offspring exposed to ethanol *in utero* compared to controls suggests that either the kidney has been "spared" from the growth restricting effects of ethanol or that they have undergone a greater degree of renal compensation (such as glomerular hypertrophy and growth) than males. The female ethanol exposed animals appear to have maintained renal growth prior to postnatal day 30 whereas males have kidneys that are small in proportion to their body weight. This is unexpected because compensatory kidney growth following adult uninephrectomy is known to be more marked in males and to be driven by testosterone [Zeier *et al* 1998, Mulroney *et al* 1999]. The effects in females may have been advantageous in the short-term, but these adaptations appear to have contributed to the earlier development of renal dysfunction, compared to males. Maladaptive compensatory changes in renal structure have been proposed in a model of ovine uninephrectomy, in which age-related decline in renal function occurs. Singh *et al* (2009) proposed that hypertrophy of the remnant kidney in response to *in utero* uninephrectomy, enhanced renal function in the short-term, but contributed to the decline in renal function with age. As for why the compensatory growth occurred in the females before the males is unknown, although it does suggest that sex steroid hormones could be involved. Interestingly, the males did eventually undergo compensatory renal growth as at 6 months of age kidney weights were similar to controls. This suggests that compensatory renal growth does occur in males following prenatal ethanol exposure, but this hypertrophy occurs between PN30 and 6 months, somewhat later than in females.

Steroid sex hormones may also be contributing to the elevation in renal vascular resistance observed in female rats, but not males, exposed prenatally to ethanol. Nitric oxide (NO), a potent vasodilator, is critically involved in the regulation of vascular tone within the kidney and its functions are influenced by steroid sex hormones [Baylis *et al* 1990, Vallon *et al* 2003]. The present results suggest possible dysfunction in the regulation of NO and its actions in females, causing vascular constriction and in turn increased renal vascular resistance. This

would result in a decrease in GFR and renal blood flow [Baylis *et al* 1990, Johnson *et al* 1992, Majid *et al* 1993, Denton and Anderson 1994]. It is interesting to postulate that the adaptations in the fetal/neonatal female kidney to "normalise" renal function in the short term resulted in a defect in NO production or activity leading to the defects identified. It has been demonstrated previously that prenatal alcohol exposure can result in vascular dysfunction [Turcotte *et al* 2002].

6.5 Health Implications of the Present Findings

The results of the present studies provide strong evidence that alcohol can affect fetal kidney development which is associated with altered physiological outcomes in offspring. Evidence of this kind may ultimately play an important role in the advice given to pregnant women on alcohol consumption, particularly the affects of acute alcohol exposure such as binge drinking. The timing of acute alcohol administration in our rat studies corresponds to approximately the 7th week of human gestation, well within the first trimester [Burrow 2000, Zoetis *et al* 2003]. This timeframe is relevant because many pregnant women continue to consume alcohol at their normal drinking patterns as they are unaware of their pregnancy, leaving the developing fetus vulnerable to alcohol exposure [Nayak *et al* 2004, Caetano *et al* 2006].

In the present chronic study, repeated ethanol exposure in the latter half of gestation also resulted in defects in kidney development. While it may seem unrealistic that a pregnant woman would consume alcohol every day in the third trimester it is important to note that epidemiological studies have demonstrated that while most pregnant women reduce their alcohol consumption once pregnancy is confirmed, they are more likely to resume their normal drinking patterns in the third trimester [Malet *et al* 2006, Colvin *et al* 2007]. There is evidence that a percentage of pregnant women believe that the fetus is less vulnerable to the teratogenic effects of alcohol in the third trimester compared with other trimesters [Lelong *et al* 1995, Chang *et al* 2006].

The BACs achieved in the current studies were between 0.10g/dl and 0.15g/dl, which equates to an average-sized woman consuming 3 to 4 standard alcoholic drinks over a 2 to 3 hour period [Caetano *et al* 2006]. While it is conceivable to suggest that a pregnant woman is unlikely to consume enough alcohol to raise her BAC to levels greater than 0.1g/dl, many women are unaware of how many standard drinks are contained within some commercially available drinks. For example, an average 600 ml premixed spirit-based drink can contain as

much as 1.6 to 1.9 standard units of alcohol [Kerr *et al* 2005, Barnett *et al* 2009]. Therefore, while a woman may think she has only consumed 1 to 2 drinks, she may have actually consumed up to 4 standard drinks, which is enough to raise her BAC to approximately 0.15g/dl. Epidemiological studies have shown that pregnant women do consume alcohol-based drinks at these volumes [Caetano *et al* 2006, Colvin *et al* 2007, Giglia *et al* 2007, Tsai *et al* 2007, Kristjason *et al* 2007]. Therefore, some pregnant women who consume alcoholic drinks are unaware of how much pure alcohol they are consuming.

6.6 Future Studies

The novel findings of this study present many new avenues for investigation. The present findings suggest that reduced ureteric branching morphogenesis may contribute to the reduction in nephron number observed following fetal alcohol exposure. However, the mechanism whereby ethanol inhibits ureteric branching remains unclear. While in the present study co-culture of ethanol-exposed kidneys with RA was able to ameliorate the effects of ethanol, further *in vivo* experiments are needed to determine if there is a reduction in maternal/fetal RA levels following ethanol exposure and whether this contributes to the disruption in kidney development. Temporospatial analyses of gene signalling pathways involved in kidney development such as the *GDNF* and *TGFβ* superfamily pathways would indicate whether the actions of ethanol/RA on kidney development are direct or involve these signalling pathways. Future studies utilizing RA receptor knockout mice would provide insights into mechanistic pathways, as eliminating the actions of RA-mediated signalling will provide a better understanding of the direct effects of ethanol on the developing kidney. As alcohol is known to influence the actions of glucocorticoids, and knowing that prenatal glucocorticoid exposure results in similar phenotypic outcomes, it would also be of interest to further investigate this mechanism. Studies employing a maternal adrenalectomy model would demonstrate if the renal phenotype elicited by ethanol exposure is influenced in any way by the actions of maternal glucocorticoids.

There were sexually dimorphic effects of ethanol on the phenotypic outcome. In future studies it would be worthwhile to identify the sex of the cultured kidneys, to determine if the sexual dimorphism observed *in vivo* is also seen *in vitro*.

Analysis of average nephron tubule length and volume in offspring following prenatal ethanol exposure would define the presence and site of tubular hypertrophy. A combined stereological/immunohistochemical approach could be used for this purpose. Furthermore, the

present findings suggest that acute prenatal ethanol exposure can result in adult renal dysfunction, with the effects more pronounced in females. Both male and female offspring demonstrated elevated MAP. Further experimentation is needed to determine the temporal relationship between elevated MAP and renal dysfunction, in order to define any causal relationships. As there is sexual dimorphism in the effects of prenatal alcohol exposure on renal function, this finding also warrants further investigation. An *in vitro* study of the effects of ethanol on male and female metanephroi would be a sensible way to begin. The identification of elevated renal vascular resistance in female offspring suggests a defect in renal NO production. This needs to be further investigated, possibly through studies involving administration of exogenous NO.

As sheep are very similar to humans in terms of kidney development, it would be of interest to analyse adult sheep following prenatal alcohol exposure to identify effects on renal and cardiovascular parameters. The nephron deficit of 11% observed in the present study does not seem particularly large, but may still lead to abnormal physiological outcomes under basal and/or stressed conditions.

6.7 Conclusion

Results from many studies, in both experimental animals and humans, have implicated reduced nephron number in the development of elevated blood pressure and renal disease. This thesis investigated the effects of both acute and chronic prenatal alcohol exposure on kidney development, nephron endowment, and adult renal and cardiovascular function. Both acute (rats) and chronic (sheep) ethanol exposure lead to reduced nephron endowment. Given that the process of nephrogenesis is complex and involves many morphological stages regulated by multiple signalling pathways, this finding of reduced nephron endowment indicates that at least one stage in metanephric development is susceptible to alcohol exposure. The present morphological and quantitative RT-PCR findings suggest that ureteric branching morphogenesis is one of these processes. Exogenous RA was able to prevent the adverse effects of ethanol on kidney development *in vitro*. Acute prenatal ethanol exposure in rats resulted in an elevation in MAP in adulthood. Male ethanol-exposed offspring showed early signs of renal dysfunction at 6 months of age, while female ethanol-exposed offspring already exhibited renal dysfunction. Taken together, the present findings indicate a previously unappreciated susceptibility of the developing kidney to exposure to low levels of ethanol, even for short durations. Further studies are warranted to shed more light on this apparent susceptibility, in order to better inform pregnant women, and those women considering becoming pregnant.

Chapter 7

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