Cullen-McEwen Luise (Orcid ID: 0000-0003-2229-5762)
Bertram John F. (Orcid ID: 0000-0001-5863-6464)
Moritz Karen (Orcid ID: 0000-0002-8085-0034)

Moderate prenatal ethanol exposure in the rat promotes kidney cell apoptosis, nephron deficits and sex-specific kidney dysfunction in adult offspring

Lisa K Akison[1,2], Megan E Probyn[1], Stephen P Gray[3], Louise A Cullen-McEwen[3], Karrona Tep[1], Sarah E Steane[1], Glenda C Gobe[1], Mary E Wlodek[4], John F Bertram[3] and Karen M Moritz[1,2]

[1]School of Biomedical Sciences, and [2]Child Health Research Centre, The University of Queensland, Brisbane, Australia

[3]Development and Stem Cells Program, Monash Biomedicine Discovery Institute, and Department of Anatomy and Developmental Biology, Monash University, Clayton, Australia

[4]Department of Physiology, The University of Melbourne, Parkville, Australia

Running title: Prenatal alcohol impairs nephrogenesis.

Address for correspondence:

Professor Karen Moritz

Child Health Research Centre, The University of Queensland, Centre for Children's Health Research (CCHR), 62 Graham St, South Brisbane, QLD 4101, Australia

Phone: 617-3069-7362, E-mail: k.moritz@uq.edu.au

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ar.24370

Grant Sponsors: This work was supported by a project grant and fellowship from the National Health and Medical Research Council of Australia (NHMRC).

Grant numbers: Project Grant 511162 and Fellowship APP1078164.

Abstract

Alcohol during pregnancy can impair fetal development and result in offspring with neurodevelopmental deficits. Less is known about how low to moderate alcohol exposure can affect other organs, such as the kidney. Here, the effects of moderate ethanol exposure throughout pregnancy on kidney development were examined using a rat model. Rats were fed a liquid diet containing 6% ethanol (vol/vol) or control (0% ethanol) throughout pregnancy. Kidneys were collected at embryonic day (E) 20 or postnatal day (PN) 30 and total glomerular (nephron) number determined using unbiased stereology. Kidney function was examined in offspring at 8 and 19 months. At E20, fetuses exposed to ethanol had fewer nephrons with increased apoptosis. Alcohol exposure caused kidney dysregulation of pro- (*Bax*) and anti- (*Bcl-2*) apoptotic factors, and reduced expression of the cell proliferation marker, *Ki67*. Prenatal alcohol decreased expression of *Gdnf* and *Tgfb1*, important regulators of branching morphogenesis, in male fetuses. At PN30, kidney volume and nephron number were lower in offspring exposed to prenatal alcohol. Urine flow and osmolality were normal in offspring exposed to alcohol however sodium excretion tended to be lower in females prenatally exposed to alcohol. Findings suggest exposure to moderate levels of alcohol during pregnancy results in impaired kidney development and leads to a permanent nephron deficit. Although the impact on adult kidney function was relatively minor, these data highlight that even at moderate levels, alcohol consumption during pregnancy can have deleterious long-term outcomes and should be avoided.

Key words: nephron endowment, prenatal alcohol exposure, apoptosis, kidney dysfunction

Introduction

Alcohol is a common exposure for the developing fetus in Western society, with estimates that around 60% of women consume alcohol between conception and pregnancy recognition (McCormack et al., 2017). Although the World Health Organisation recommends abstinence, many women continue to drink even when they know they are pregnant (Kesmodel, 2001; Muggli et al., 2016). It is well known that consumption of high levels of alcohol on a regular basis during pregnancy has teratogenic effects on the fetus. This may manifest as fetal alcohol syndrome (FAS), which is characterised by gross craniofacial abnormalities along with cardiovascular and nervous system defects and mental retardation (Abel and Sokol, 1987). However, lower amounts of alcohol may have more subtle effects leading to the term 'fetal alcohol spectrum disorders' (FASD), which includes a wide range of effects that can occur without the recognisable craniofacial deformities (Caley et al., 2005).

Little information exists on whether prenatal exposure to alcohol affects kidney development. A number of clinical case studies have found that heavy alcohol consumption increases the risk for kidney anomalies (Qazi et al., 1979). Two larger studies in which more moderate levels of alcohol exposure were examined also reported kidney abnormalities including kidney agenesis and hypoplasia, with outcomes dependent upon the level and timing of exposure (Taylor et al., 1994; Moore et al., 1997). In a small study examining kidney outcomes in children with FAS, kidney deficits in concentrating ability and electrolyte handling were observed (Assadi and Ziai, 1985, 1986; Assadi, 1990, 2008). In a pilot study, Australian Aboriginal children (studied

between 8-14 years of age) of mothers who drank routinely during pregnancy were found to have a higher incidence of albuminuria and smaller kidney volumes (measured by ultrasound) (Singh et al., 2005). In animal studies, the kidneys of 9 day-old rat pups exposed to ethanol throughout pregnancy weighed less than control pups and had decreased DNA and protein content (Gallo and Weinberg, 1986). A single binge exposure at embryonic day (E) 8 in the rat resulted in decreased kidney weight at birth (Schwartz and Carey, 2005) whilst mice exposed to prenatal alcohol had increased rates of urogenital malformations including hydroureter (Randall et al., 1977; Gage and Sulik, 1991).

We have shown nephron endowment is reduced in the sheep following exposure to moderate to relatively high doses of ethanol over the last third of pregnancy (Gray et al., 2008) and in rats exposed to a high dose of ethanol on days E14 and 15 of pregnancy (Gray et al., 2010). In the rat, we demonstrated the nephron deficit was due, at least in part, to impaired branching morphogenesis (Gray et al., 2010). As yet, no studies have examined nephron endowment in a model of low to moderate prenatal ethanol exposure. This is of great importance, as evidence suggests 25-30% of women reduce alcohol intake during pregnancy but continue to drink at low levels (Cameron et al., 2013; Anderson et al., 2014). A low nephron endowment is considered a risk factor for the development of kidney disease in adulthood (Amann et al., 2004; Hoy et al., 2006). Animal studies have shown that a reduction in nephron endowment may result from maternal insults at specific times during development, with the early period of kidney development being extremely susceptible (Moritz and Bertram, 2006). These prenatal insults

have included maternal exposure to glucocorticoids (Wintour et al., 2003), maternal calorie or protein restriction (Woods et al., 2004; Hoppe et al., 2007) and uteroplacental insufficiency (Schreuder et al., 2005; Wlodek et al., 2007). In the present study, we aimed to determine the effects of low to moderate alcohol exposure throughout pregnancy on nephron endowment and adult kidney function. We hypothesised that moderate alcohol consumption would result in offspring with a decreased nephron number, resulting in kidney dysfunction later in life.

Materials and methods

Ethical approval

Ethical approval was obtained from the Animal Experimentation Ethics Committees at Monash University and the University of Queensland. All experiments were performed in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (8th Edition, National Health and Medical Research Council, 2013).

Animal treatment and experimental cohorts

Female Sprague Dawley rats, at least 8 weeks of age and 230 g, were habituated to a liquid diet containing no ethanol for 3-5 days prior to mating. After mating, as determined by the presence of a vaginal plug, rats either remained on the control diet (CON, n = 25) or were placed on a diet containing 6% vol/vol ethanol (EtOH, n = 25). This provides 15% EtOH-derived calories in the diet, which is much lower than the typical 35% EtOH-derived calories in liquid diets used in previous studies (e.g. Elton et al. (2002)). The liquid-based diets were prepared fresh daily and offered ad libitum each day of pregnancy at midday (the onset of the dark cycle) for 21 h per day. For the remaining 3 h per day, dams were offered water ad libitum. Details of the animal model and diets, including diet composition, consumption rates, maternal weight gain and litter sizes, have been reported previously (Probyn et al., 2012). Briefly, dietary components in the EtOH diet were adjusted to ensure the diets were isocaloric following the addition of alcohol. Dams in both groups consumed similar volumes of the diets and thus similar calories. Maximal

consumption of the diet occurred during the first 15 minutes of the dark cycle, and an equal volume was consumed over the first 5 h of the dark cycle and the remaining 16 h (12 h of which was during the light cycle). Therefore, blood alcohol concentration (BAC) was measured in a subset of EtOH-treated dams at 15 and 30 minutes after the diet was first offered at midday, reaching 0.02 ± 0.01% and 0.03 ± 0.01% respectively (Probyn et al., 2012). BAC was also measured at 60 minutes after offering the diet and was below the limit of detection (0.0008%) (Probyn et al., 2012). These BAC levels were much lower than those previously reported in high dose models (e.g. 0.10-0.15% at 2 h after the diet was offered; Elton et al. (2002)). No control-treated animals had their BAC levels measured, but a subsequent study has found that untreated rats have levels below the limit of detection (Nguyen et al, Under Review). There were no significant differences in maternal weight gain or litter sizes between the two groups, and no growth restriction at birth (Probyn et al., 2012).

Dams were divided into two cohorts: a subset (9 CON and 11 EtOH) were killed at embryonic day 20 (E20). The remaining dams (16 CON and 14 EtOH) were returned to standard rat chow and water ad libitum following delivery of their litters. Offspring were weaned at postnatal day 28 (PN28). At PN30, one male and one female offspring from 8 litters (CON and EtOH) were culled for analysis of kidney growth and nephron number. A second subset of offspring was housed in metabolic cages at 8 months of age for analysis of kidney function. Finally, a third subset of offspring were maintained until approximately 19 months of age for analysis of kidney and cardiac function in aged adults. All offspring reported in this study were a subset of the

offspring previously reported in Probyn et al. (2012) at E20, PN30 and 8 months of age or reported in Probyn et al. (2013a) at 19 months of age. At all ages where kidneys were collected for subsequent analysis, the left kidney was fixed for histology and/or immunohistochemistry and the right kidney was frozen for molecular analysis.

Embryonic day 20

At E20, a subset of dams and their fetuses was culled as previously described (Probyn et al., 2012). Fetal kidneys were removed, weighed and either immediately frozen in liquid nitrogen for extraction of RNA, or placed in 10% buffered formalin for histological and stereological studies. Following fixation, kidneys were cut sagitally, embedded in paraffin and sectioned at 4 μm.

Glomerular number estimation

Total glomerular number was estimated using a validated methodology that allows determination of glomerular number prior to the completion of nephrogenesis (Cullen-McEwen et al., 2011). Glomerular numbers were estimated in kidneys from 6 to 9 fetuses across 5 to 6 litters for each sex. No more than 2 fetuses of each sex per litter were examined.

Kidney cell apoptosis and mitosis

Levels of apoptosis were examined in kidney sections using three methodologies: haematoxylin and eosin (H & E) staining, terminal dideoxyuridine transferase-mediated nick end labelling (TUNEL) assay and immunostaining for activated caspase-3. TUNEL analysis was performed

using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Merck Millipore, Bayswater, NSW, Australia, Product No. S7100) according to the manufacturer's instructions. Briefly, sections were dewaxed in xylene, rehydrated and pre-treated with proteinase K (20 μg/mL in 0.05M Tris-HCL, 0.01M $CaCl_2$ pH 8.0). Endogenous peroxidise activity was blocked by incubating the sections in 3% hydrogen peroxide (H_20_2) in PBS, washed and then incubated with terminal deoxyribonucleotidyl transferase (TdT) enzyme at 37^0C for 1 h. Sections were incubated with stop/wash buffer (1:34 dilution) for 10 minutes, washed and incubated with anti-digoxygenin conjugate for 30 minutes at room temperature. Colour was developed using Vector® *NovaRED*™ (Vector Laboratories Inc., Burlingame, CA, USA Product No. SK-4800) according to the manufacturer's instructions and sections counterstained with haematoxylin, dehydrated and coverslipped. Negative controls were prepared by excluding the TdT enzyme from the ApopTag reaction. For further biochemical verification of apoptosis, activated caspase-3 antibody (Santa Cruz Biotechnology Inc., Paso Robles, CA, USA) was used for immunohistochemistry (Burke and Gobe, 2005). Apoptotic bodies identified using the three staining methods were counted by one observer, blinded to treatment. Fields of view (10-15 per section) were taken in a clockwise direction around the nephrogenic zone in both halves of each kidney using a Nikon Eclipse E800 microscope (Nikon Instruments Inc, New York, NY, USA) at 400X magnification and digitally captured using a Nikon Digital Camera DXM1200F (Nikon Instruments Inc, New York, NY, USA). The morphological criteria used to identify apoptotic bodies were as used previously (Burke and Gobe, 2005).

Kidney sections were also assessed for cell proliferation using immunostaining for proliferating cell nuclear antigen (PCNA) as previously described (Dallemagne et al., 2000). All assessments of apoptosis and mitosis were done for one male and one female from n = 6 litters for each treatment/control group.

Gene expression

Relative expression of apoptosis/proliferation related genes (*Bcl-2, Bax, Ki67*) and branching morphogenesis genes (*Gdnf, Ret, Wnt4, Tgfb1*), was quantified using real-time PCR. Total RNA was isolated from frozen E20 kidneys (n = 5-10/sex across 5 litters per treatment) using an RNeasy Micro Kit (Qiagen Pty Ltd, Doncaster, VIC, Australia) according to the manufacturer's instructions. After treatment with deoxyribonuclease I, 0.5 μg of total RNA was reverse transcribed into cDNA using Taqman Reverse Transcription Reagents Kit (Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Australia) as described previously (Singh et al., 2007). Samples excluding multiscribe in the cDNA synthesis were included to confirm no genomic DNA contamination. Relative levels of mRNA were determined using 50 ng of cDNA per 20 μl reaction using Taqman reagents (Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Australia) and Assay-On-Demand primer–probe sets or custom-designed primer-probes. The following Taqman Assay-on-Demands were employed: *Bcl-2* (B-cell lymphoma 2), *Bax* (Bcl-2-associated X protein), *Ki67* (antigen Ki-67), *Gdnf* (glial cell derived neurotrophic factor) *Ret* (ret proto-oncogene), and *Wnt4* (Wnt family member 4). Custom primer-probe for *Tgfb1* (transforming growth factor beta 1): Forward primer, 5'-TCGACATGGAGCTGGTGAAA-3';

reverse primer, 5'-GAGCCTTAGTTTGGACAGGATACTG-3'; probe, 5'-AAGCGCATCGAAGCCTCCGTG-3'. Real-time PCR was performed using an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) as described previously (Singh et al., 2007). All primer/probe sets were analysed using multiplexed reactions with 18S ribosomal RNA (Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Australia, Cat. No. 4308329) used as the reference gene. Multiplex testing was completed to verify that the amplification efficiency of multiple targets was not impaired compared with single-plex reactions. The reference gene was stably expressed across sex and treatment (data not shown). Fold-change relative to the average of the male control group was calculated using the ΔΔCt method (Livak and Schmittgen, 2001). For comparison of gene expression across sex and treatment group, fold-change data were normalized to the male control group, such that the mean fold-change for this group was set to 1.0.

Postnatal day 30 (PN30)

A subset of animals was culled at PN30 and in this study, we determined kidney volume and nephron number using stereology of fixed kidneys from one male and one female offspring from 8 litters across each treatment group. Kidneys were sliced into 1 mm sections and every second slice was processed to glycolmethacrylate (Technovit 7100, Emgrid Australia Pty Ltd, Gulfview

Heights, SA, Australia) and sectioned exhaustively at 20μm. Every 10th and 11th sections were selected for further analysis with the first section chosen at random. Total kidney volume was estimated using the Cavalieri principle (Gundersen et al., 1988). Total glomerular (nephron) number was estimated using the validated physical disector/fractionator methodology (Bertram et al., 1992), with the observer blinded to treatment group.

Adult (8 month) and aged (19 month) offspring

Adult and aged offspring were assessed for kidney function, with body weight and total kidney weight obtained at necropsy shortly afterwards.

Kidney function

To examine urinary excretion rates, separate cohorts of offspring were placed in metabolic cages for 24 h for collection of urine at 8 and 19 months of age. At 8 months, 9-12 animals of each sex across 6 to 8 litters and at 19 months, 7-13 animals of each sex across 6-8 litters from control and ethanol-exposed dams respectively were used (1-2 animals/sex/litter). Prior to the 24 h measurement, rats were habituated to the cages on at least two occasions for 3-4 h. Food and water intake (g or ml/g body weight per day) along with urine flow (ml/g body weight per day) were measured in all animals. Osmolality was measured in urine samples (all animals) and plasma samples (8 month only) using a Vapro Vapor Pressure Osmometer (Wescor Inc., Logan, UT, USA). Urinary sodium, chloride and potassium concentrations were measured in 8 month

and 19 month animals and total protein was measured in 19 month animals only using a COBAS Integra 400 Plus Analyzer (Roche Diagnostics Australia Pty Ltd, Castle Hill, NSW, Australia).

Statistical analyses

All data were analysed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± SEM. A two-way ANOVA, with treatment and sex as factors, was used to test for differences in body/organ weights as well as gene expression levels and quantified morphometric data. For analysis of kidney function in adult offspring (8 and 19 months), a two-way ANOVA, with treatment and age as factors, was conducted separately in each sex. Where data were not normally distributed and/or had unequal variances between groups, a non-parametric Kruskal Wallis test was used across all groups. Urinary total protein was analysed in 19-month offspring only using either an unpaired t-test (parametric) or a Mann-Whitney Rank Sum test (non-parametric) within each sex. Where possible, when raw data were non-parametric, data were log-transformed prior to analysis. Post-hoc analysis was performed when there was a significant interaction between factors in the two-way ANOVA (Sidak's multiple comparison test) or a significant difference between groups in the Kruskal Wallis Test (Dunn's multiple comparison test). Statistical significance was set at $P<0.05$.

Results

Fetal and postnatal kidney and body weights

As previously reported (Probyn et al., 2012), fetal body weight was significantly reduced at E20 in prenatal EtOH-exposed compared to CON pups, but there were no significant differences in absolute or relative kidney weights (Supplemental Table 1). For the subset of PN30 offspring used in this study, there was a trend for reduced body weight in EtOH-exposed pups (P = 0.052), but no effect of EtOH exposure on absolute or relative kidney weights (Supplemental Table 1). By 8 months of age, mean body weights were still lower in EtOH-exposed offspring of both sexes, but this was not statistically significant (P = 0.093). There was a trend for reduced kidney weights in EtOH-exposed offspring (P = 0.056), but this was no longer significant when adjusted for body weight (P = 0.204). In aged (19-month old) offspring, there were no significant effects of prenatal EtOH exposure on body weight or absolute/relative kidney weights (Supplemental Table 1).

Fetal nephron endowment and gene expression at E20

Glomerular number was significantly reduced at E20 following prenatal EtOH treatment (Fig. 1A). This was most prominent in females, where the deficit was ~20% (compared to 5% in males), although there was no significant interaction.

Relative gene expression of *Gdnf* was significantly lower at E20 in the kidneys of ethanol-exposed fetuses (Fig. 2A). A similar trend was seen in expression of *Wnt4* (Fig. 2B) although

this did not reach statistical significance. mRNA expression of *cRET* (Fig. 2C) was not different between groups. Expression of *Tgfb1* was variable, although a significant non-parametric test suggested expression was reduced in the kidneys of fetuses exposed to alcohol (Fig. 2D); however, no significant difference between individual groups were observed through post-hoc testing.

Measures of apoptosis and mitosis at E20

Prenatal ethanol exposure caused significant increases in levels of apoptosis, as evidenced by apoptotic cells visualised using H & E staining (Fig. 3B,C compared to Fig. 3A), increased numbers of apoptotic 'bodies' (TUNEL-positive staining within cells, Fig. 3D-F) and levels of activated caspase-3 (Fig. 3G). When quantified, although there was no effect of treatment or sex on the number of apoptotic bodies, there was a significant interaction (Fig. 3K). Post-hoc analysis revealed a significant increase in apoptotic bodies in the kidney cells of females exposed to ethanol compared to controls of the same sex ($P<0.01$). Levels of activated caspase-3 were higher in both sexes exposed to prenatal ethanol (Fig. 3J). In addition to increased levels of apoptosis, there was evidence of increased mitosis in the ethanol exposed offspring, with increased levels of PCNA immunoreactivity (Fig. 3L). The relative expression of both *Bax* (Fig. 3M) and *Bcl-2* (Fig. 3N) was significantly lower in the kidneys of EtOH-exposed fetuses. *Ki67* labelling was also reduced in the kidneys from fetuses exposed to alcohol (Fig. 3O).

Glomerular number and kidney volume at PN30

Although kidney weights were similar in the two groups at PN30 (Supplemental Table 1), prenatal EtOH exposure significantly reduced total kidney volume (Fig. 1B). Total glomerular number was also significantly reduced following prenatal ethanol exposure (Fig. 1C).

Kidney function

To test if kidney function was affected in adulthood (i.e. 8 months of age) by prenatal alcohol exposure and worsened with age (i.e. at 19 months of age), water intake, urine flow and osmolality were assessed separately in males and females with treatment and age used as the factors in a two-way ANOVA. In both sexes, there was an age-related decrease in water intake (Fig. 4A-B). In females, the interaction between treatment and age approached statistical significance ($P = 0.06$), with animals exposed to alcohol tending to drink less water as they aged. In both sexes, age resulted in a decrease in urine flow but this was not affected by prenatal alcohol treatment (Fig. 4C-D). In females but not males, urine osmolality also decreased with age, suggesting an inability to concentrate urine in older animals (Fig. 4E-F). In aged (i.e. 19 months) female offspring exposed to alcohol, the urine osmolality was ~20% lower than in controls (Fig. 4F), although not statistically significant.

Urinary excretion rates of sodium were variable and in both sexes, decreased with age (Fig. 4G-H). At both ages, sodium excretion tended to be lower (~40%) in female offspring exposed to alcohol, although this was not statistically significant using a non-parametric Kruskal-Wallis test. In males, a tendency for lower sodium excretion was only seen at 8 months of age (~40%), although there was no statistically significant interaction in the two-way ANOVA (Fig. 4G). Chloride excretion was also significantly reduced in female EtOH-exposed offspring at both ages (~33% decrease, Table 1) but was not significantly different in males. This may be attributed in part to reduced sodium and chloride intake, as females exposed to alcohol tended to eat ~10%

less than controls (P = 0.072, Table 1). Potassium excretion was similar in the two groups. Protein excretion measured at 19 months was similar in males of both treatment groups. Although the protein excretion was statistically lower in female offspring exposed to alcohol, values in both groups were in the low-normal range.

Discussion

This study demonstrates for the first time that exposure of the rat dam to a modest amount of ethanol during pregnancy results in a reduced nephron endowment in the offspring. This nephron deficit was present in late embryogenesis, prior to completion of nephrogenesis and was sustained in the postnatal period. In both sexes, this represents a permanent nephron deficit. Importantly, we have determined that the mechanisms contributing to the nephron deficit may include enhanced kidney cell apoptosis during fetal life. Changes in gene expression also suggest a role for decreased branching morphogenesis in contributing to the nephron deficit. Together, results suggest that even relatively low levels of ethanol during pregnancy can impair kidney development and result in a permanent nephron deficit. Whilst the outcomes on kidney function were minor, our data suggest that even low amounts of alcohol during pregnancy can have deleterious effects on long-term health.

Consumption of low doses of alcohol during pregnancy is relatively common, with many women consciously decreasing pre-pregnancy drinking levels but not abstaining once they become

pregnant (Anderson et al., 2014; Muggli et al., 2016). Our model has been designed to mimic consumption of a small amount of alcohol daily during pregnancy and results in a peak BAC of ~0.03% (Probyn et al., 2012). The major finding of this study was that chronic low dose alcohol during pregnancy impacted kidney development. At E20, the number of glomeruli formed was lower in the alcohol treated fetuses, this being most evident in females, but by PN30, the nephron deficit was similar in both sexes. As nephrogenesis continues postnatally in the rat, it is possible that the nephron deficit we observed at PN30 is due to postnatal factors. It has been shown that impaired lactation in a model of placental insufficiency does indeed contribute to a reduction in nephron endowment, which can be reversed by improved lactation when pups are cross-fostered (Wlodek et al., 2007). We have reported previously that low dose prenatal alcohol alters milk proteins and offspring consume less milk during lactation (Probyn et al., 2013b). Other studies have also found ethanol exposed rat offspring take longer to attach to the nipple (Chen et al., 1982) and have a reduced number of rhythmic sucks per minute (Rockwood and Riley, 1986). In another study in rats, prenatal alcohol exposure reduced the amount of milk produced by the dam (Murillo-Fuentes et al., 2001). This highlights that prenatal alcohol exposure may indirectly affect the latter stages of kidney growth and maturation through impaired lactation.

This study has given considerable insight into the mechanisms through which prenatal ethanol exposure may reduce nephron number. Increased levels of apoptosis were evident in the kidneys at E20, particularly in the females where the nephron deficit was ~20%. In utero alcohol exposure is well known to cause apoptosis in many other tissues, including the developing brain

(for review see Olney et al. (2002)) and heart (Yan et al., 2017). The doses used in these previous studies were much higher than in our study. However, our results suggest that even relatively low alcohol concentrations can cause cells to undergo apoptosis. Increased apoptosis has been identified as a key mechanism contributing to a low nephron endowment in other models of prenatal exposures including maternal low protein (Welham et al., 2002) and uteroplacental insufficiency (Cuffe et al., 2018).

In addition, we demonstrated some changes in genes regulating branching morphogenesis. This is consistent with our previous work using higher, acute doses of alcohol (Gray et al, 2009). There we demonstrated *in vitro* (using metanephric organ culture), that alcohol reduced the number of ureteric branch points and branch tips and resulted in formation of less nephrons. Similar to the current study, expression of genes controlling this process (Costantini, 2006), including GDNF, was reduced by the alcohol although c-RET which is the mediating receptor for GDNF, was unaltered. Nevertheless, this raises the possibility that ethanol-induced kidney dysfunction is in part due to interactions between the metabolism of Vitamin A and alcohol. Vitamin A and its derivatives are known to promote kidney development in a dose-dependent fashion (Moreau et al., 1998). Alcohol dehydrogenase, part of the oxidation pathway for alcohol, is able to inhibit retinal dehydrogenase, thus preventing the metabolism of Vitamin A to retinoic acid. In turn, this causes a reduction in c-RET expression (Batourina et al., 2001). We have shown, in vitro, that addition of retinoic acid is able to ameliorate the effects on branching morphogenesis and kidney development caused by ethanol (Gray et al., 2012).

Kidney function is minimally affected

Kidney function has been rarely studied in children affected by prenatal alcohol exposure. A series of four clinical studies examined kidney dysfunction in a small group of infants with FAS (Assadi and Ziai, 1985, 1986; Assadi, 1990, 2008). The infants with FAS were found to have deficits in kidney acidification and a decreased ability to concentrate urine during a period of water deprivation. FAS usually results from exposure to high levels of alcohol and thus represents a much more severe exposure than our animal model. We have not identified any studies that examine kidney function in children with FASD, which is an area that warrants further investigation. A small number of studies have examined impacts on kidney function using animal models. We have reported previously that a 'binge' exposure for two days during early kidney development in the rat results in an altered glomerular filtration rate (GFR) and proteinuria (Gray et al., 2010). Other studies have demonstrated prenatal alcohol results in increased water intake and diuresis, which may be related to changes in the release or sensitivity to arginine vasopressin (Dow-Edwards et al., 1989; Bird et al., 2006). Other preclinical studies have shown disruption in electrolyte excretion rates (Dow-Edwards et al., 1989; Assadi et al., 1991; Knee et al., 2004). Although not significant, we found sodium excretion was 20-40% less in female offspring exposed to alcohol. Together, these studies suggest that, in addition to altering nephron endowment, prenatal alcohol may affect the development and/or functioning of the kidney tubules. We have recently optimised methods to examine renal tubular morphology

(Walton et al., 2016) and in the future, this can be used to examine tubular development following prenatal alcohol exposure. Previous studies have shown that offspring from dams exposed to high levels of ethanol prenatally (35% of calories derived from ethanol) demonstrate ultrastructural changes in the distal tubules and collecting ducts at 90 days of gestation (Assadi and Zajac, 1992) although a similar exposure did not cause any observable morphological changes by light microscopy (Assadi et al., 1991). In addition, we were unable to measure GFR in our study. In future, this should be examined using recently developed methods that allow for non-invasive measurement of GFR in conscious animals (Schock-Kusch et al., 2011).

A low nephron endowment is often associated with increased blood pressure (see Briffa et al. (2018) for review). In our rat binge model, we observed increased blood pressure in offspring at 6 months of age (Gray et al., 2010). We have recently reported blood pressure outcomes in the chronic low dose model and, surprisingly, found no difference between treatment groups in males and a reduction in blood pressure only in female offspring exposed to prenatal alcohol (Walton et al., 2019). However, this same study showed impaired cardiac function in offspring exposed to chronic low dose prenatal alcohol, suggesting the cardiovascular system is still affected. It is likely these differing outcomes reflect the low dose used in the current study resulting in a more subtle phenotype (Probyn et al., 2013a; Probyn et al., 2013c) and overt disease may not develop unless offspring are exposed to a secondary or further insult. Indeed, recent studies using prenatal alcohol exposure in rats have demonstrated that additional early life insults, such as poor maternal care, exacerbated the outcomes of prenatal alcohol (Raineki et al.,

2017). We have also demonstrated, in rodent models, that other prenatal insults, such as maternal hypoxia (Walton et al., 2017) and uteroplacental insufficiency (Gallo et al., 2018), result in deficits in renal structure and function that are exacerbated by a postnatal high salt diet. In contrast to nephrogenesis in humans, which is completed at birth, our rodent model of chronic alcohol exposure would not have exposed the developing kidney to alcohol during the final stages of nephrogenesis, as this process continues during the first week postnatally. We predict that continuing alcohol exposure until the completion of nephrogenesis may result in a more severe phenotype. This is an important consideration in a clinical setting, where a woman drinking low levels throughout pregnancy would result in fetal exposure to alcohol throughout the entirety of nephrogenesis.

In summary, we have shown for the first time that exposure of the pregnant rat dam to low-moderate levels of alcohol during pregnancy results in a nephron deficit in offspring. This occurred in part due to increased rates of cell apoptosis and altered expression of genes regulating branching morphogenesis during fetal development. Despite minimal changes in basal renal function, this finding of a nephron deficit is of significant clinical importance as it may render individuals more susceptible to hypertension and kidney failure in adulthood, particularly if combined with other lifestyle factors such as a high salt diet (Barker et al., 2006; Moritz and Bertram, 2006).

References

Abel EL, Sokol RJ. 1987. Incidence of fetal alcohol syndrome and economic impact of FAS-related anomalies. Drug Alcohol Depend 19:51-70.

Amann K, Plank C, Dotsch J. 2004. Low nephron number--a new cardiovascular risk factor in children? Pediatr Nephrol 19:1319-1323.

Anderson AE, Hure AJ, Forder PM, Powers J, Kay-Lambkin FJ, Loxton DJ. 2014. Risky drinking patterns are being continued into pregnancy: a prospective cohort study. PLoS One 9:e86171.

Assadi F. 1990. Renal tubular dysfunction in fetal alcohol syndrome. Pediatr Nephrol 4:48-51.

Assadi F. 2008. Effects of prenatal alcohol exposure on the developing kidneys. Iranian Journal of Pediatrics 18:5-10.

Assadi F, Ziai M. 1985. Impaired renal acidification in infants with fetal alcohol syndrome. Pediatric research 19:850-853.

Assadi F, Ziai M. 1986. Zinc status of infants with fetal alcohol syndrome. Pediatric research 20:551-554.

Assadi FK, Manaligod JR, Fleischmann LE, Zajac CS. 1991. Effects of prenatal ethanol exposure on postnatal renal function and structure in the rat. Alcohol 8:259-263.

Assadi FK, Zajac CS. 1992. Ultrastructural changes in the rat kidney following fetal exposure to ethanol. Alcohol 9:509-512.

Barker DJ, Bagby SP, Hanson MA. 2006. Mechanisms of disease: in utero programming in the pathogenesis of hypertension. Nat Clin Pract Nephrol 2:700-707.

Batourina E, Gim S, Bello N, Shy M, Clagett-Dame M, Srinivas S, Costantini F, Mendelsohn C. 2001. Vitamin A controls epithelial/mesenchymal interactions through Ret expression. Nat Genet 27:74-78.

Bertram JF, Soosaipillai MC, Ricardo SD, Ryan GB. 1992. Total numbers of glomeruli and individual glomerular cell types in the normal rat kidney. Cell Tissue Res 270:37-45.

Bird DN, Sato AK, Knee DS, Uyehara CFT, Person DA, Claybaugh JR. 2006. Effects of prenatal ethanol exposure and sex on the arginine vasopressin response to hemorrhage in the rat. American Journal of Physiology - Regulatory Integrative and Comparative Physiology 291:R77-R82.

Briffa JF, Wlodek ME, Moritz KM. 2018. Transgenerational programming of nephron deficits and hypertension. Semin Cell Dev Biol.

Burke C, Gobe G. 2005. Pontosubicular apoptosis ("necrosis") in human neonates with intrauterine growth retardation and placental infarction. Virchows Arch 446:640-645.

Caley LM, Kramer C, Robinson LK. 2005. Fetal alcohol spectrum disorder. J Sch Nurs 21:139-146.

Cameron CM, Davey TM, Kendall E, Wilson A, McClure RJ. 2013. Changes in alcohol consumption in pregnant Australian women between 2007 and 2011. Med J Aust 199:355-357.

Chen JS, Driscoll CD, Riley EP. 1982. Ontogeny of suckling behavior in rats prenatally exposed to alcohol. Teratology 26:145-153.

Costantini F. 2006. Renal branching morphogenesis: concepts, questions, and recent advances. Differentiation 74:402-421.

Cuffe JSM, Briffa JF, Rosser S, Siebel AL, Romano T, Hryciw DH, Wlodek ME, Moritz KM. 2018. Uteroplacental insufficiency in rats induces renal apoptosis and delays nephrogenesis completion. Acta Physiol (Oxf) 222.

Cullen-McEwen LA, Armitage JA, Nyengaard JR, Moritz KM, Bertram JF. 2011. A design-based method for estimating glomerular number in the developing kidney. Am J Physiol Renal Physiol 300:F1448-1453.

Dallemagne C, Ooi S-Y, Brown L, GobÉ G, Endre Z. 2000. Renal impairment in deoxycorticosterone acetate-salt hypertensive rats. Nephrology 5:277-284.

Dow-Edwards DL, Trachtman H, Riley EP, Freed LA, Milhorat TH. 1989. Arginine vasopressin and body fluid homeostasis in the fetal alcohol exposed rat. Alcohol 6:193-198.

Elton CW, Pennington JS, Lynch SA, Carver FM, Pennington SN. 2002. Insulin resistance in adult rat offspring associated with maternal dietary fat and alcohol consumption. J Endocrinol 173:63-71.

Gage JC, Sulik KK. 1991. Pathogenesis of ethanol-induced hydronephrosis and hydroureter as demonstrated following in vivo exposure of mouse embryos. Teratology 44:299-312.

Gallo LA, Walton SL, Mazzuca MQ, Tare M, Parkington HC, Wlodek ME, Moritz KM. 2018. Uteroplacental insufficiency temporally exacerbates salt-induced hypertension associated with a reduced natriuretic response in male rat offspring. J Physiol 596:5859-5872.

Gallo PV, Weinberg J. 1986. Organ growth and cellular development in ethanol-exposed rats. Alcohol 3:261-267.

Gray SP, Cullen-McEwen LA, Bertram JF, Moritz KM. 2012. Mechanism of alcohol-induced impairment in renal development: Could it be reduced by retinoic acid? Clin Exp Pharmacol Physiol 39:807-813.

Gray SP, Denton KM, Cullen-McEwen L, Bertram JF, Moritz KM. 2010. Prenatal exposure to alcohol reduces nephron number and raises blood pressure in progeny. J Am Soc Nephrol 21:1891-1902.

Gray SP, Kenna K, Bertram JF, Hoy WE, Yan EB, Bocking AD, Brien JF, Walker DW, Harding R, Moritz KM. 2008. Repeated ethanol exposure during late gestation decreases nephron endowment in fetal sheep. Am J Physiol Regul Integr Comp Physiol 295:R568-574.

Gundersen HJ, Bendtsen TF, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sorensen FB, Vesterby A, et al. 1988. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. Apmis 96:379-394.

Hoppe CC, Evans RG, Moritz KM, Cullen-McEwen LA, Fitzgerald SM, Dowling J, Bertram JF. 2007. Combined prenatal and postnatal protein restriction influences adult kidney

structure, function, and arterial pressure. Am J Physiol Regul Integr Comp Physiol 292:R462-469.

Hoy WE, Hughson MD, Singh GR, Douglas-Denton R, Bertram JF. 2006. Reduced nephron number and glomerulomegaly in Australian Aborigines: a group at high risk for renal disease and hypertension. Kidney Int 70:104-110.

Kesmodel U. 2001. Binge drinking in pregnancy--frequency and methodology. Am J Epidemiol 154:777-782.

Knee DS, Sato AK, Uyehara CFT, Claybaugh JR. 2004. Prenatal exposure to ethanol causes partial diabetes insipidus in adult rats. American Journal of Physiology - Regulatory Integrative and Comparative Physiology 287:R277-R283.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

McCormack C, Hutchinson D, Burns L, Wilson J, Elliott E, Allsop S, Najman J, Jacobs S, Rossen L, Olsson C, Mattick R. 2017. Prenatal alcohol consumption between conception and recognition of pregnancy. Alcohol Clin Exp Res 41:369-378.

Moore CA, Khoury MJ, Liu Y. 1997. Does light-to-moderate alcohol consumption during pregnancy increase the risk for renal anomalies among offspring? Pediatrics 99:E11.

Moreau E, Vilar J, Lelievre-Pegorier M, Merlet-Benichou C, Gilbert T. 1998. Regulation of c-ret expression by retinoic acid in rat metanephros: implication in nephron mass control. Am J Physiol 275:F938-945.

Moritz KM, Bertram JF. 2006. Barker and Brenner: a basis for hypertension? Current Hypertension Reviews 2:179-185.

Muggli E, O'Leary C, Donath S, Orsini F, Forster D, Anderson PJ, Lewis S, Nagle C, Craig JM, Elliott E, Halliday J. 2016. "Did you ever drink more?" A detailed description of pregnant women's drinking patterns. BMC Public Health 16:683.

Murillo-Fuentes L, Artillo R, Carreras O, Murillo L. 2001. Effects of maternal chronic alcohol administration in the rat: lactation performance and pup's growth. Eur J Nutr 40:147-154.

Olney JW, Wozniak DF, Jevtovic-Todorovic V, Farber NB, Bittigau P, Ikonomidou C. 2002. Drug-induced apoptotic neurodegeneration in the developing brain. Brain Pathol 12:488-498.

Probyn ME, Cuffe JS, Zanini S, Moritz KM. 2013a. The effects of low-moderate dose prenatal ethanol exposure on the fetal and postnatal rat lung. J Dev Orig Health Dis 4:358-367.

Probyn ME, Lock EK, Anderson ST, Walton S, Bertram JF, Wlodek ME, Moritz KM. 2013b. The effect of low-to-moderate-dose ethanol consumption on rat mammary gland structure and function and early postnatal growth of offspring. Am J Physiol Regul Integr Comp Physiol 304:R791-798.

Probyn ME, Parsonson KR, Gardebjer EM, Ward LC, Wlodek ME, Anderson ST, Moritz KM. 2013c. Impact of low dose prenatal ethanol exposure on glucose homeostasis in Sprague-Dawley rats aged up to eight months. PLoS One 8:e59718.

Probyn ME, Zanini S, Ward LC, Bertram JF, Moritz KM. 2012. A rodent model of low- to moderate-dose ethanol consumption during pregnancy: patterns of ethanol consumption and effects on fetal and offspring growth. Reprod Fertil Dev 24:859-870.

Qazi Q, Masakawa A, Milman D, McGann B, Chua A, Haller J. 1979. Renal anomalies in fetal alcohol syndrome. Pediatrics 63:886-889.

Raineki C, Bodnar TS, Holman PJ, Baglot SL, Lan N, Weinberg J. 2017. Effects of early-life adversity on immune function are mediated by prenatal environment: Role of prenatal alcohol exposure. Brain Behav Immun 66:210-220.

Randall CL, Taylor J, Walker DW. 1977. Ethanol-induced malformations in mice. Alcohol Clin Exp Res 1:219-224.

Rockwood GA, Riley EP. 1986. Suckling deficits in rat pups exposed to alcohol in utero. Teratology 33:145-151.

Schock-Kusch D, Xie Q, Shulhevich Y, Hesser J, Stsepankou D, Sadick M, Koenig S, Hoecklin F, Pill J, Gretz N. 2011. Transcutaneous assessment of renal function in conscious rats with a device for measuring FITC-sinistrin disappearance curves. Kidney Int 79:1254-1258.

Schreuder MF, Nyengaard JR, Fodor M, van Wijk JA, Delemarre-van de Waal HA. 2005. Glomerular number and function are influenced by spontaneous and induced low birth weight in rats. J Am Soc Nephrol 16:2913-2919.

Schwartz J, Carey LC. 2005. Programming effects of moderate and binge alcohol consumption. In: Wintour EM, Owens JA, editors. Early life origins of health and disease. New York: Landes Bioscience. p 187-194.

Singh GR, Sayers SM, Hoy WE. 2005. Maternal alcohol ingestion during pregnancy predisposes to smaller kidneys and albuminuria in Aboriginal children: findings from an Aboriginal birth cohort. Nephrology 9
(suppl 1):75.

Singh RR, Cullen-McEwen LA, Kett MM, Boon WM, Dowling J, Bertram JF, Moritz KM. 2007. Prenatal corticosterone exposure results in altered AT1/AT2, nephron deficit and hypertension in the rat offspring. J Physiol 579:503-513.

Taylor CL, Jones KL, Jones MC, Kaplan GW. 1994. Incidence of renal anomalies in children prenatally exposed to ethanol. Pediatrics 94:209-212.

Walton SL, Bielefeldt-Ohmann H, Singh RR, Li J, Paravicini TM, Little MH, Moritz KM. 2017. Prenatal hypoxia leads to hypertension, renal renin-angiotensin system activation and exacerbates salt-induced pathology in a sex-specific manner. Sci Rep 7:8241.

Walton SL, Moritz KM, Bertram JF, Singh RR. 2016. Lengths of nephron tubule segments and collecting ducts in the CD-1 mouse kidney: an ontogeny study. Am J Physiol Renal Physiol 311:F976-F983.

Walton SL, Tjongue M, Tare M, Kwok E, Probyn M, Parkington HC, Bertram JF, Moritz KM, Denton KM. 2019. Chronic low alcohol intake during pregnancy programs sex-specific cardiovascular deficits in rats. Biol Sex Differ 10:21.

Welham SJ, Wade A, Woolf AS. 2002. Protein restriction in pregnancy is associated with increased apoptosis of mesenchymal cells at the start of rat metanephrogenesis. Kidney Int 61:1231-1242.

Wintour EM, Moritz KM, Johnson K, Ricardo S, Samuel CS, Dodic M. 2003. Reduced nephron number in adult sheep, hypertensive as a result of prenatal glucocorticoid treatment. J Physiol 549:929-935.

Wlodek ME, Mibus A, Tan A, Siebel AL, Owens JA, Moritz KM. 2007. Normal lactational environment restores nephron endowment and prevents hypertension after placental restriction in the rat. J Am Soc Nephrol 18:1688-1696.

Woods LL, Weeks DA, Rasch R. 2004. Programming of adult blood pressure by maternal protein restriction: role of nephrogenesis. Kidney Int 65:1339-1348.

Yan X, Pan B, Lv T, Liu L, Zhu J, Shen W, Huang X, Tian J. 2017. Inhibition of histone acetylation by curcumin reduces alcohol-induced fetal cardiac apoptosis. J Biomed Sci 24:1.

Figure Legends:

Figure 1. Prenatal exposure to alcohol reduces kidney volume and/or nephron number in fetal kidneys at embryonic day 20 and postnatal kidneys at day 30. Total glomerular number at E20 (A), kidney volume at PN30 (B) and total glomerular number at PN30 (C) are shown for male and female offspring from dams fed a control (open bars) or 6% ethanol (closed bars) diet throughout pregnancy. For glomerular counts at E20, 6-9 animals across 5-6 litters were counted for each sex. At PN30, 1 male and 1 female was measured per litter (8 per group). All data are expressed as mean + SEM. P values were calculated using a two-way ANOVA.

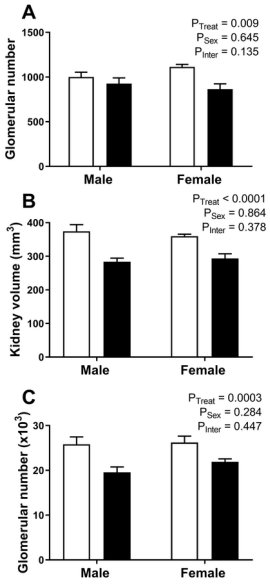
Figure 2. Prenatal exposure to alcohol dysregulates branching morphogenesis genes in fetal kidneys at embryonic day 20. mRNA expression of *Gdnf* (A), *Wnt4* (B), *Cret* (C) and *Tgfb1* (D) are shown for male and female offspring from dams fed a control (open bars) or 6% ethanol

(closed bars) diet throughout pregnancy. Data are expressed as mean + SEM, with n = 8-10 per sex per group. Data in (A) were log-transformed prior to analysis. P values were calculated using a two-way ANOVA. $^{+}$ indicates non-parametric Kruskal-Wallis test used due to non-normal data or unequal variances.

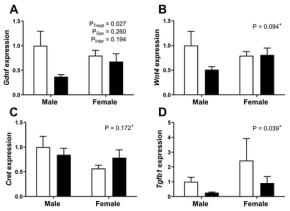
Figure 3. Apoptotic markers are dysregulated in the kidneys of neonates exposed to prenatal alcohol. Histological, immunohistochemical and gene expression analysis of apoptosis in the kidney at E20. Representative haematoxylin and eosin stained sections from female control (A), male ethanol (B) and female ethanol (C) kidneys. In A, arrows with asterisk indicate mitosis. In B and C, arrows indicate apoptotic cells. D-F are a panel of ApopTag-labelled (i.e. TUNEL positive) sections for female control (D), male ethanol (E) and female ethanol (F). Arrows show examples of labelled apoptotic cells. Apoptosis was also verified with immunohistochemistry (IHC) for cleaved caspase-3 (G). Positive cells are indicated by arrows in a representative female ethanol section. Mitosis was verified with proliferating cell nuclear antigen (PCNA) IHC (H) with examples of positive nuclei in a female ethanol section indicated with arrows. The arrow with the asterisk beside it indicates mitosis as separating metaphase plates. Negative control for the IHC is shown in (I). Quantification of cleaved caspase 3 IHC (J), Apoptag-labelled apoptotic bodies (K) and PCNA IHC staining (L) in kidney sections from male and female control (open bars) and ethanol exposed (closed bars) fetuses at E20. Data expressed as mean + SEM (n = 6 per group). Relative expression in kidneys of male and female fetuses at

E20 of *Bax* (M), *Bcl2* (N) and *Ki67* (O). Bar shading as above. Values are mean + SEM (n = 5-10 per group). P values were calculated using a two-way ANOVA. ** indicates P<0.01 from post-hoc Sidak's multiple comparison test (compared to control group of the same sex). Data in (J, M - O) were log-transformed prior to analysis.

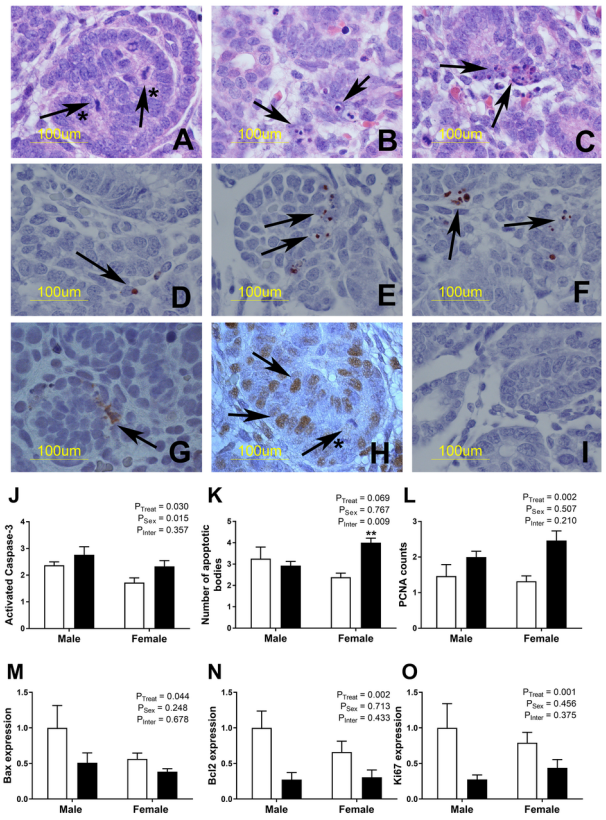
Figure 4. Water intake is reduced in aged female offspring from dams exposed to alcohol during pregnancy with no concomitant changes in urine flow or osmolality. Animals (8 and 19 months of age) were housed in metabolic cages for 24 h and data recorded for water intake (A and B). Urine was collected and urine flow (C and D), urine osmolality (E and F) and urinary sodium (Na) excretion (G and H) measured. Data are expressed as mean + SEM for male and female offspring from dams fed a control (open bars) or 6% ethanol (closed bars) diet throughout pregnancy (n = 7-13 per group). P values were calculated using a two-way ANOVA. $^{+}$ indicates non-parametric Kruskal-Wallis test used due to non-normal data or unequal variances. ## P<0.01 and ### P<0.001 indicates age difference within treatment group by post-hoc Dunn's multiple comparison test.



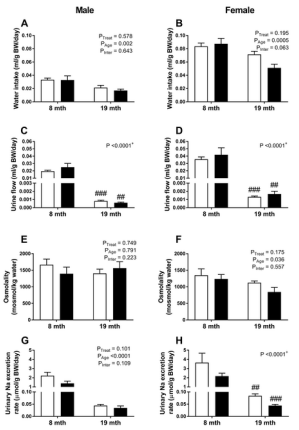
AR_24370_Fig1_NEW.tif



AR_24370_Fig2_NEW.tif



AR_24370_Fig3 NEW.tif



AR_24370_Fig4_NEW.tif

Table 1 – Kidney metabolite excretion (Cl and K), total protein excretion and food intake in adult offspring (8 and 19 month old) exposed to control (CON) or ethanol (EtOH) diet during pregnancy.

	8 month		19 month		
	CON	**EtOH**	**CON**	**EtOH**	**P Values**
Males	n = 11	n = 9	n = 13	n = 7	
UClV (mol/g BW/day)	3.18 ± 0.48	2.85 ± 0.65	0.076 ± 0.006	0.065 ± 0.007	P_T = 0.342, **P_A <0.001**
UKV (mol/g BW/day)	3.48 ± 0.35	3.50 ± 0.48	0.107 ± 0.011	0.090 ± 0.014	P_T = 0.995, **P_A <0.001**
FI (g/g BW/day)	0.036 ± 0.004	0.030 ± 0.004	0.017 ± 0.002	0.013 ± 0.003	P_T = 0.124, **P_A <0.001**
TP (mg/L)	-	-	2897 ± 664.1	3442 ± 1257	P = 0.678
Females	n = 9	n = 10	n = 6	n = 9	
UClV (mol/g BW/day)	4.50 ± 0.60	3.83 ± 0.43	0.152 ± 0.020	0.106 ± 0.010	**P_T = 0.020, P_A <0.001**
UKV (mol/g BW/day)	6.60 ± 0.59	6.42 ± 0.62	0.167 ± 0.011	0.124 ± 0.010	P_T = 0.813, **P_A <0.001**
FI (g/g BW/day)	0.063 ± 0.005	0.058 ± 0.004	0.038 ± 0.004	0.026 ± 0.005	P_T = 0.072, **P_A <0.001**
TP (mg/L)	-	-	619.1 ± 247.6	195.1 ± 45.35	P = 0.114

Mean ± SEM. Data analysed by two-way ANOVA with treatment (P_T) and age (P_A) as factors. There were no significant interactions between treatment and age in the analyses. UClV, urinary chloride excretion rate; UKV, urinary potassium excretion rate; FI, food intake; TP, urinary

total protein; BW, body weight. TP analysed by unpaired t-test (males) or a Mann-Whitney Rank Sum test (females) in 19 month animals only.