Alcohol exposure during late gestation: multiple developmental outcomes in sheep

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Abstract

Alcohol consumption during pregnancy remains common in many countries. Exposure to even low amounts of alcohol (ie ethanol) in pregnancy can result in the heterogeneous fetal alcohol spectrum disorders (FASD), while heavy alcohol consumption can result in the fetal alcohol syndrome (FAS). FAS is characterised by cerebral dysfunction, growth restriction and craniofacial malformations. However, the effects of lower doses of alcohol during pregnancy, such as those that lead to FASD, are less well understood. In this article, we discuss the findings of recent studies performed in our laboratories on the effects of fetal alcohol exposure using sheep, in which we studied the effects of late gestational alcohol exposure on the developing brain, arteries, kidneys, heart and lungs. Our studies indicate that alcohol exposure in late gestation can (1) affect cerebral white matter development and increase the risk of hemorrhage in the fetal brain, (2) cause left ventricular hypertrophy with evidence of altered cardiomyocyte maturations, (3) lead to a decrease in nephron number in the kidney, (4) cause altered arterial wall stiffness and endothelial and smooth muscle function, and (5) result in altered surfactant protein mRNA expression, surfactant phospholipid composition and pro-inflammatory cytokine mRNA expression in the lung. These findings suggest that alcohol exposure in late gestation can affect multiple organs, potentially increasing the risk of disease and organ dysfunction in later life.

Introduction

In this short review, we provide a summary of our recent findings from a series of related studies in sheep. In these studies we assessed fetal and early postnatal functional and structural effects on several organs of repeated maternal administration of alcohol (ie ethanol) during the third-trimester-equivalent of gestation. We have focused on organs that are likely susceptible to fetal alcohol exposure and that could result in developmental programming for later onset disease. Organs studied were the brain, heart, kidney, lung and arteries. We address the potential role of fetal alcohol exposure in developmental programming of postnatal health and disease, and suggest new avenues of research in this field.

Incidence of fetal alcohol spectrum disorders

Substantial numbers of infants are exposed to alcohol before birth. Recent surveys show that ~30% of women in the U.S.A[1] and ~60% of women in Australia[2] drink alcohol at some point during their pregnancy. Alcohol consumption is typically highest during the first trimester with less consumed as the pregnancy progresses; however 3% of women in the U.S.A consume alcohol throughout pregnancy[1]. These statistics are of concern because maternal consumption of alcohol during pregnancy can result in a spectrum of developmental defects in the offspring referred to collectively as fetal alcohol spectrum disorders (FASD). The incidence of FASD is 20-50[3] and 9[4] per 1,000 live births in the U.S.A and Canada respectively, but these rates may be under-estimated due to the reliance on mothers to self-report alcohol consumption; detection of fatty acid ethyl esters in meconium may be a more objective measure of prenatal

alcohol exposure[5]. The most severe manifestation of FASD is the fetal alcohol syndrome (FAS) which is caused by repeated, heavy alcohol consumption during pregnancy. FAS is diagnosed by a triad of birth defects: growth restriction (pre- and post-natal), craniofacial dysmorphology, and central nervous system (CNS) dysfunction[6]. The incidence of FAS ranges from 2-7 per 1,000 live births in the U.S.A.[3], but is highest in South Africa where it has been reported to be 68-83 per 1,000 live births[7].

Pre- and post-natal growth restriction

The developmental programming of adult-onset diseases, such as cardiovascular disease, due to low birth weight is now well-documented and accepted as a clinically important phenomenon[8]. Alcohol exposure during gestation can cause low birth weight[9] which can arise due to intrauterine growth restriction (IUGR)[10, 11] and/or preterm birth[12, 13]. Prolonged alcohol exposure (>10g alcohol or 0.7 U.S.A standard drinks per day) during early or late gestation may increase the risk of IUGR[10, 11]. Prenatal alcohol exposure also results in postnatal growth restriction in offspring[14, 15] with reductions in body weight and height still evident in some individuals at 20 years of age[16].

Animal studies indicate that alcohol exposure can restrict fetal and/or postnatal growth depending on the degree of exposure. For example, in sheep daily self-administered alcohol exposure throughout gestation can cause IUGR[17], whereas our own studies showed that prolonged, daily exposure in late gestation does not induce IUGR[18], but 3 days of alcohol exposure in late gestation apparently reduced fetal body weight[19]. In

rats, alcohol exposure throughout pregnancy (20% alcohol, 80% liquid diet) and lactation was associated with a reduction in postnatal growth, despite a normal birth weight [20]. Similarly, a mouse model of fetal alcohol exposure showed that mice born with a normal birth weight were growth restricted from 3 to 5 weeks postnatal age despite cross-fostering[21]. In Japanese medaka fish (Oryzias latipes), alcohol exposure can reduce head width at any point during gestation, however reductions in body length occur only following alcohol exposure in early or late gestation[22]. Together these findings suggest that there are many variables, such as the duration, gestational timing and maximal level of alcohol exposure, which can affect fetal and postnatal growth.

Fetal growth restriction caused by gestational exposure to alcohol is characterized by reduced body fat stores[23] and/or the number and type of skeletal muscle fibres[24]; these effects may be regulated by changes in insulin-like growth factors (IGFs). Increased or decreased levels of both IGF-1 and IGF-2 in offspring have been documented[25-30]. The reason for this variability in IGF levels following prenatal alcohol exposure is likely due to species differences in animal models used (ie human, rat, sheep). Differences in maximal blood alcohol concentration or the gestational age at the time of exposure may also be contributing factors, as there is little consistency between studies in the procedures used to expose offspring to alcohol. Increased oxidative stress may also contribute to alcohol-induced fetal growth restriction, as administration of anti-oxidative peptides (NAPVSIPQ and SALLRSIPA) can alleviate fetal growth restriction in alcohol-exposed pregnant mice[31].

Vascularisation and growth of the placenta are important for maintaining fetal growth, and several studies suggest that the placenta may be highly susceptible to alcohol exposure. For example, during human pregnancies, consuming more than 5 U.S.A. standard drinks per week doubles the risk of placental-associated syndromes, defined as the incidence of placental abruption, placental previa, preeclampsia, small for gestational age, preterm birth or stillbirth[32]. Studies in rats show that daily alcohol exposure (1 g and 5 g / kg of maternal weight) throughout pregnancy can cause irregular placental vascularisation[33] and that alcohol-induced growth restriction is associated with increases in placental weight[34]. A combination of alcohol and tobacco smoke exposure in pregnant rats produced decreased placental weight, altered placental histology, including an increased number of hemorrhages, and delayed fetal development, but the changes in fetal and placental growth were reversed by the concurrent administration of sodium ferulate, an antioxidant used to treat vascular disease[35]. Alcohol-induced IUGR may therefore be mediated, in part, by effects on placental growth and aberrant vascularisation. The placental effects of alcohol in inducing IUGR deserve further study.

Alcohol-related birth defects

The incidence of fetal structural malformations or alcohol-related birth defects (ARBD) is increased by prenatal alcohol exposure. Cardiac malformations, most commonly atrial and ventricular septal defects[6, 36], occur in ~20% of individuals exposed to alcohol *in utero* compared with 1.2% in control populations[37, 38]. In FAS or FASD, a range of distinct facial anomalies is common: these include a smooth philtrum (the medial cleft

between nose and upper lip), malformed noses (small, upturned, cleft and flat nasal bridge), “railtrack” ears, narrow ear canals, prominent/deformed pinna and otosclerosis (bone overgrowth in the middle ear) and ocular defects such as microphthalmos (small eyes), epicanthus (skin fold over the upper eyelid) and strabismus (crossed eyes)[39, 40]. Other malformations include kidney defects, such as renal hypoplasia, urethral obstructions and hydronephrosis[41, 42].

Alcohol-related neurodevelopmental disorders

In children, gestational alcohol exposure has been associated with decreased IQ, learning disabilities, memory impairment, visuo-spatial deficits, delayed language and motor development and increased risk of attention-deficit hyperactivity disorder[43-46]. Prenatal alcohol exposure can affect multiple brain regions and functions, and can cause alterations in brain mass and neuronal organisation during development[47, 48]. CNS dysfunction is a pre-requisite for the diagnosis of FAS and alcohol-related neurodevelopmental disorder (ARND), and is believed to be a result of CNS injury, although not all animal studies have detected clear evidence of brain damage in the fetus or neonate[18]. Alcohol-induced brain injury can vary from none to extreme and may, in part, be due to genetic differences, which can influence the susceptibility of the brain to alcohol-induced injury[49].

Maternal alcohol exposure in late gestation using a sheep model

Experimental studies of fetal alcohol exposure vary widely in terms of the dose, the temporal pattern (e.g. binge or acute) and duration of exposure, the gestational age of

the offspring and the animal model used. Although several species have been used for studying the developmental effects of gestational alcohol exposure[50], the sheep has numerous advantages, especially for physiological studies, over other species including rodents. Sheep have a long gestation (~147 days), give birth to one or two offspring that have a similar body weight and gestational ontogeny of major organs to humans, and have a non-reactant uterus that allows surgical implantation of probes and catheters that permit physiological monitoring of the intact fetus *in utero*. The pharmacokinetics, including distribution and biotransformation, of alcohol in the maternal-fetal unit are similar for ovine and human pregnancies[51]. Maternal-fetal weight of sheep is also similar to that of humans.

Animal studies are necessarily limited in terms of recreating a realistic model of human alcohol consumption, primarily because human alcohol intake during pregnancy is highly variable between subjects. Furthermore, nutritional deficits or alcohol tolerance may be observed in humans following chronic alcohol consumption prior to pregnancy, which is difficult to recreate in animal models. Therefore, our model of alcohol exposure did not attempt to mimic a common pattern of human alcohol consumption, but instead investigated the effects that may occur following alcohol exposure during latter pregnancy. We chose to expose fetuses to alcohol during the last trimester equivalent, as this is when key maturational processes occur in the fetus, such as white matter development in the brain[52], nephrogenesis in the kidney[53], maturation of cardiomyocytes[54], and maturation of the gas-exchanging region and surfactant system in the lung[55, 56].

Alcohol was administered *intravenously* to pregnant ewes at either 1 g or 0.75 g/kg maternal body weight over 1 hour (h), which gave a maximal plasma alcohol (ethanol) concentration (PEC) of 110-120 mg/dL in the ewe (range: 0.10-0.13 g/dL, coefficient of variation (CV): 0.11) and fetus (range: 0.09-0.12 g/dL, CV: 0.12) (Fig 1); this equates to ~3-4 standard U.S.A drinks ingested over 1h. The change in alcohol dose (from 1 g/kg to 0.75 g/kg) was made following the end of our first study, wherein our infusion protocol was improved and the alcohol dose lowered to maintain a similar maximal PEC across the studies. Maternal and fetal PEC reached similar peak values at the same time, and declined at approximately the same rate, indicating the rapid, bidirectional transfer of alcohol between the maternal and fetal circulations. Virtually all alcohol had been eliminated from maternal and fetal plasma by 8 h after the daily infusion onset. It is likely that maternal hepatic biotransformation of alcohol to acetaldehyde and acetate is the major mechanism for alcohol elimination from the maternal-fetal unit[51]. Alcohol was infused into the maternal jugular vein so that PEC could be reliably controlled.

Our laboratory has conducted three different experiments on the fetal and postnatal effects of fetal alcohol exposure during the third trimester-equivalent (Fig 2). The first of these examined the effects of administrating alcohol for 1 h per day to the pregnant ewe from 116-118 days of gestation age (DGA) on the fetal brain at 123 DGA (0.84 of term)[19]. The second study examined a longer period of maternal alcohol exposure with pregnant ewes exposed to alcohol infusions for 1 h per day from 95-133 DGA and fetal brain, lungs, kidneys, heart and small arteries examined at 134 DGA (0.9 of term)[18, 57-59]; alcohol infusions were stopped at 133DGA due to the risk of pre-term birth[12]. The third study examined the effects of this long alcohol exposure regimen used in our

second study on postnatal outcomes; that is, fetuses exposed to alcohol for 1 h per day from 95-133 DGA were allowed to be born naturally, and were then studied at nine weeks of age[60]. The duration of exposure and the PEC were similar between all three studies and the experiments were performed at the same facility, using the same equipment, and in sheep from the same supplier.

The effects of daily 1h exposure for three days

In this study, we exposed 8 fetuses to a daily 1 h alcohol infusion (1 g/kg) for three days, from 116-118 DGA, via the maternal jugular vein; 8 control fetuses received saline infusion[19, 27]. The maximal fetal and maternal PEC of 0.11+/-0.01 g/dL were reached at one hour after the start of the infusion. Fetal tissue was collected at 123 DGA, five days after the last alcohol infusion. Alcohol infusions did not affect fetal arterial partial pressure of oxygen (PaO_2) or carbon dioxide ($PaCO_2$), arterial saturation of oxygen (SaO_2), pH, blood glucose concentration or mean arterial pressure (MAP)[19]. Pro-inflammatory cytokines, interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) were also measured in fetal plasma, but their concentrations were similar to controls[19]. At necropsy, a mean decrease of 500g (19%) in body weight was seen in alcohol-exposed fetuses compared with controls[27]. There was a transient decrease in plasma IGF-1 in ewes at 30 h and IGF-2 in fetuses from 24-54 h after the first alcohol infusion, which could account for the decrease in fetal body weight[27].

The fetal brain was examined with particular emphasis on white matter development that occurs in late gestation in sheep[52]. Three days of alcohol exposure during this period resulted in subcortical white matter injury, as defined by microglia/macrophage

infiltration, axonal disruption, increased apoptosis, astrogliosis and altered glial cell morphology in 4/8 (50%) fetuses examined[19]. Three of the remaining four fetuses demonstrated astrogliosis and increased apoptosis in cerebral white matter[19]. Thus, 7/8 (88%) alcohol-exposed fetuses showed evidence of white matter vulnerability to alcohol, which could induce CNS dysfunction; fetal control brains did not show any signs of overt injury. Importantly, there was a positive correlation between the maximal fetal PEC and the degree of white matter injury between fetuses[19]. Other studies have demonstrated correlations between peak PEC and effects in offspring[61-64]. The relationship between maximal PEC and effects on the fetal brain may explain the variability in brain injury that occurs in individuals with FASD[65].

The effects of a prolonged period of daily alcohol exposure

To determine the effects of a longer period of daily alcohol exposure on fetal development we infused alcohol from 95-133 days of gestation, with necropsy at 134 days (term ~147 DGA). A similar protocol of daily maternal alcohol administration (0.75 g/kg) was used as in the 3-day study to achieve a maximal PEC of ~0.11-0.12 g/dL (Fig. 1).

Physiological effects: When measured at 131-133 DGA, there were no significant effects of alcohol exposure on fetal heart rate (HR), MAP[18], electrocorticographic (ECoG) activity and sagittal sinus blood flow (unpublished observations). Prenatal alcohol exposure can initially impair fetal biophysical parameters, such as fetal breathing movements, electrooccular activity (EOG) and/or ECoG, in humans and

sheep[66, 67]. However variables such as the gestational maturity of the fetus and the duration of alcohol exposure can impact upon biophysical parameters such as fetal breathing movements[67, 68], which may have occurred in our long term alcohol exposure model.

In relation to the start of the daily alcohol infusion maternal pH was decreased at 2-4 h, blood glucose concentration was decreased at 1-4 h and blood lactate concentration was increased at 1-6 h before returning to baseline values[18]. In the fetus, there was a similar increase in blood lactate concentration at 4-10 h from the start of the daily alcohol infusion, whereas fetal pH and blood glucose concentration were unaffected[18]. We further observed that fetal PaO_2 and SaO_2 were decreased at 10 h and 6-10 h, respectively, whereas maternal oxygenation was unaffected[18]. All fetal arterial blood measurements returned to baseline values by 23 h after the end of the daily alcohol infusion[18].

Fetal and maternal responses to daily alcohol infusion were more pronounced in this model of repeated alcohol exposure than in the 3-day exposure studies. This may be due to either the duration of maternal alcohol exposure or the greater gestational age of the fetus (131-133 *vs* 123 DGA) at the time of measuring responses. Our study showed for the first time that maternal daily alcohol exposure could cause delayed and prolonged changes in maternal and fetal homeostasis which could affect fetal development. Thus, the main physiological effects of this more prolonged daily exposure to alcohol were transient increases in maternal and fetal lactate concentrations, decreases in maternal blood glucose concentrations and pH and decreases in fetal oxygenation, effects not seen with the shorter (3 days) treatment.

Surprisingly, there was no evidence of fetal growth restriction after this longer (39 days) period of daily alcohol exposure; fetal body weight was not different between alcohol-exposed and control groups at 134 DGA[18], or at birth when animals proceeded to term[60]. In this latter group, postnatal growth up to nine weeks was also similar in the alcohol and control groups[60]. In alcohol-exposed fetuses at 134 DGA, the ponderal index (body weight/crown-rump length^3) was lower than in controls, indicating that these fetuses were thin relative to length[18]. However, there was no decrease in ponderal index in the cohort of alcohol-exposed offspring that were born naturally at term, indicating that the prenatal reduction in body fat and/or muscle does not persist following the cessation of alcohol exposure at 133 DGA. Thus, fetal growth restriction was not a feature of this model of prolonged, daily alcohol exposure in late gestation.

Brain development: The fetal brain was examined at 134 DGA following 39 days of alcohol exposure[18]. Based on our previous finding that 3 days of alcohol exposure causes white matter injury[19], we expected that 39 days of exposure would result in more severe white matter injury. We evaluated astrocyte and microglia/macrophage density, percentages of activated microglia, myelinating oligodendrocytes and apoptotic cells, and the number of blood vessels per unit area of brain tissue, but found no differences between alcohol-exposed and control fetuses[18]; although the presence of more subtle brain injury may have been revealed by the use of more quantitative methods (e.g. stereology). In 3/8 (38%) alcohol-exposed fetuses, there were small subarachnoid hemorrhages in the cerebral cortex and/or cerebellum (Fig 3)[18]; we did not detect any hemorrhages in control fetal brains. Blood vessel rupture could be due to

changes in blood vessel wall composition and/or function, for which we have some evidence (see “small artery development” section). Cerebral hemorrhage following pre-natal alcohol exposure, especially during development, could put the neonate at an increased risk of cerebral dysfunction, a key manifestation of the CNS injury of FAS and ARND[69]. The differences seen in our 3 and 39 day alcohol models with respect to white matter injury and hemorrhage are worthy of further investigation, as they raise the possibility of white matter repair following initial alcohol exposure, but increasing risk of cerebrovascular injury with prolonged exposure .

Small artery development: Direct application of alcohol to isolated smooth muscle cells from mature pig coronary arteries or intact coronary arteries results in an increase of intracellular Ca^{2+} and smooth muscle contraction[70]. These effects are modified by the presence of the endothelium, indicating complex interactions of alcohol with vascular tissue that include smooth muscle contraction via increases in intracellular free Ca^{2+} and endothelial release of vasodilator agents.

Little is known of the effects of alcohol exposure during pregnancy on the development of the vascular system. Although vascular development begins early, it is not until very late in gestation and early postnatal life that collagen and elastin (which provide strength and elasticity in vessel walls) reach maximal production[71, 72]. Alterations to blood vessel wall structure, such as changes in collagen and elastin content or in extracellular matrix cross-linking, can alter endothelial and smooth muscle function and arterial wall stiffness[73]. In pregnant sheep a long (30-82 days gestation) exposure to a relatively high level of alcohol (maximal PEC of ~0.22 g/dL) once a day (5 days on, 2 days off)

resulted in increased cerebrovascular relaxation responses when isolated vessels were tested *in vitro*[74]. In human umbilical arteries taken from newborn infants exposed to alcohol during pregnancy (17-20 standard drinks per trimester), endothelium-dependent relaxation was not affected, but the sensitivity of smooth muscle cells to vasoconstrictors was reduced[75]. Furthermore, nine-year-old children, exposed to alcohol *in utero* but not diagnosed with FASD*,* had increased aortic wall stiffness when compared with control children[76]; increased arterial wall stiffness is a clinical predictor of cardiovascular disease[77]. Together, these studies indicate that prenatal alcohol exposure effects arterial function and stiffness which persist into childhood and may be the early expression of vascular dysfunction leading to cardiovascular disease in later life.

We have examined passive mechanical properties and endothelial and smooth muscle function in fetal and postnatal small arteries from five vascular beds (cerebral, coronary, renal, femoral and mesenteric) following daily alcohol exposure for 39 days during late gestation. At 134 DGA and nine weeks after birth, we observed widespread changes in endothelial and smooth muscle function and passive mechanical properties, including arterial wall stiffness[78-81]. The changes that we observed in vascular function and vessel wall mechanics differed between vessels, and also differed between the fetus and postnatal lamb. Changes observed in endothelial and smooth muscle function and arterial wall stiffness in arteries from the brain, heart and kidneys are summarised in Table 1. These changes, if they persist into later life are likely to alter cardiovascular function and increase the risk of cardiovascular disease; for this reason they are the subject of on-going investigation. Changes in arterial mechanics and function could

explain the cerebral hemorrhages that we observed in some fetuses after 39 days of alcohol exposure. Importantly, such changes could have serious consequences if they persist into postnatal life; the effects of challenges, such as exercise or stress, may further exacerbate the cardiovascular phenotype. Arterial dysfunction can restrict regional blood flow, suggesting that key organs such as the brain, kidneys and heart may be at increased risk of damage following alcohol exposure in late gestation.

Heart development: The fetal heart was the only organ in which we observed evidence of altered growth following 39 days of daily alcohol exposure. In this study, we observed that the fetal heart-to-body weight ratio was greater in alcohol-exposed fetuses than in controls[57]. Specifically, there was a greater left ventricular and septal wall volume in alcohol-exposed fetuses[57]. This left ventricular hypertrophy was attributed to an increase in cardiomyocyte size and a 12% increase in the proportion of mature, binucleated cardiomyocytes relative to immature, mononucleated cardiomyocytes (Fig 4)[57]. The stimulus for the increase in heart growth appears to be a direct trophic effect on the cardiomyocytes rather than a hemodynamic effect, as fetal and postnatal MAP were not affected. Other drugs, including angiotensin II, have been found to induce cardiac hypertrophy in the absence of hemodynamic changes[82].

Toxicants including cocaine and acetaldehyde (the principal metabolite of ethanol) have also been shown to induce cardiac hypertrophy[83]. *In vitro* studies show that alcohol exposure can increase the number of mature cardiomyocytes by increasing the number of cells in the GO/G1 phase of the cell cycle (indicative of mature cells that have exited the cell cycle)[84], which is consistent with the findings of our *in vivo* pregnant sheep

study. As mature binucleated cardiomyocytes are larger than immature mononucleated cardiomyocytes (Fig 4B), the increased proportion of binucleated cardiomyocytes likely contributes to the overall increase in left ventricular and septal size in the alcohol-exposed lambs. In addition, mRNA expression of the cardiomyocyte growth factor *IGF-1* was increased in alcohol-exposed fetal hearts, which may also contribute to the increased size of the cardiomyocytes[57].

We observed an increase in the mRNA expression of the pro-apoptotic genes (caspase 3 and BAX) in the alcohol-exposed hearts[57]. Alcohol exposure during pregnancy in rats can increase the expression of markers of cardiomyocyte apoptosis, decrease the heart to body weight ratio and increase cardiac contractile function in offspring[85]. However in our model the increase in caspase-3 and BAX mRNA expression was not associated with an increase in cardiac cell death. The functional consequences of these effects on cardiomyocyte growth and subsequent left ventricular hypertrophy remain to be investigated.

Kidney development: An examination of the fetal kidney at 134 DGA following prolonged (39 days) alcohol exposure during late gestation revealed no overt signs of renal injury or changes in kidney weight or glomerular volume[58]. Furthermore, there were no differences in mRNA expression of several key genes responsible for kidney development and function such as the renin-angiotensin system, IGFs, and sodium transporters[58]. Renal function was not assessed in this study, but amniotic fluid composition, which can be used as an indicator of fetal renal function, showed no differences between control and alcohol-exposed groups with respect to concentrations

of Na^+, K^+, Cl^-, creatinine, urea, uric acid and total protein, suggesting that fetal renal function was not overtly compromised.

Nephrogenesis is normally complete by 120-130 DGA in sheep[53], and so we were able to assess the effects of the prolonged alcohol exposure on final nephron number. We observed that total nephron number was decreased by 11% in the alcohol-exposed fetuses (Fig 5)[58]. This reduction may have been due to a reduction in renal branching morphogenesis, which is actively occurring at ~100 DGA in the fetal sheep[53], as alcohol has been shown to reduce branching in organ-culture studies of kidney[86]. As no new nephrons are produced following the completion of nephrogenesis, it is expected that the observed reduction in nephron number will persist into postnatal life. Previous studies have shown that a reduction of 20-30% in nephron number is associated with elevated blood pressure in later life in sheep[87] and rodents[88-90], but it is unclear what effects an 11% reduction in nephron number will have on blood pressure in later life. As mentioned previously, there was no difference in MAP or HR in our alcohol-exposed nine-week-old lambs compared with controls[57]. However in rats, two doses of alcohol, resulting in similar maximal PEC and nephron deficit to that obtained in our study, resulted in elevated blood pressure in offspring at 6 months of age[91]. Potentially, a small decrease in nephron number when combined with other cardiovascular risk factors, such as obesity and/or a poor diet, could increase the risk of hypertension in later life.

Lung development: In sheep as in humans, lung development predominantly occurs *in utero*, but continues after birth. In both species, alveolarization begins during late

gestation and continues into postnatal life[55, 56]; however, in sheep it is likely that a greater proportion of the final number of alveoli are formed before birth than in humans. Repeated daily alcohol exposure did not alter lung morphology or growth in our fetuses at 134 DGA or in postnatal lambs at nine weeks of age[59, 60]. We observed an increase in collagen Iα1 mRNA and an increase in collagen protein deposition in fetal lungs, which could affect lung compliance; although this increase in collagen did not persist postnatally[59, 60], if present at term or preterm birth, it could affect lung compliance at the onset of gaseous ventilation. We also measured mRNA expression of pro-inflammatory cytokines and observed that IL-1β and interleukin 8 (IL-8) mRNA expressions were reduced in the fetal lungs; however, there was no change in cytokine mRNA expression at nine weeks after birth[59, 60]. The alcohol-induced reductions in fetal IL-1β and IL-8 mRNA expression suggest impairment in the innate immune status of the lung during development which could affect the ability of the lung to clear pathogens.

Lung surfactant comprises ~90% lipids (90-95% of which are phospholipids) and ~10% surfactant proteins, and acts to decrease surface tension and to assist in the immune response to respiratory pathogens. In amniotic fluid collected at 134 DGA, we observed that 39 days of alcohol exposure led to an overall decrease in phospholipid concentration, with a decrease in the proportion of the phospholipids, phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) (Fig. 6)[60]. Amniotic fluid is comprised of fetal lung liquid and fetal urine and thus changes in lung liquid secretion rates, fetal swallowing and/or fetal urine production and could account for the decrease in the proportion of phospholipids. Such a change in phospholipids could result in increased surface tension in the lung, which could

contribute to respiratory distress at birth; however, alcohol-exposed lambs that were born at term (approximately 12 days after the final maternal alcohol infusion), showed no respiratory dysfunction at birth. The proportion of phosphatidylcholine (PC), which is the major phospholipid in surfactant and is involved in decreasing surface tension, was increased in amniotic fluid taken from alcohol-exposed fetuses; this may have compensated, in part, for the reduced phospholipid concentration[60]. In bronchoalveolar lavage fluid from postnatal lambs, surfactant phospholipid composition was not different from that of controls[60], suggesting that recovery of surfactant production occurs after alcohol exposure is discontinued.

Surfactant proteins (SP)-A and SP-D have immunoregulatory roles, whilst SP-B and SP-C help to decrease surface tension in the lung. The gene expression of SP-A and SP-B was reduced in fetal lung tissue[60] suggesting that the innate immunity of fetal lung may be compromised and lung compliance may be reduced. In nine-week-old alcohol-exposed lambs the mRNA expression of SP-D was increased in lung tissue (Fig. 7)[60]. This increase in SP-D may be associated with an increased pro-inflammatory state, since previous studies have shown that SP-D not only controls pro-inflammatory responses[92], but SP-D expression can be stimulated by an increase in pro-inflammatory cytokines[93]. Furthermore, SP-D can control T-helper (Th)2-type inflammation in the airways and hence is involved in regulating allergic airway inflammation[94]. Given the known roles of SP-D in controlling pulmonary immunity, further studies are required to determine if susceptibility to infection and allergic sensitization is affected by prenatal ethanol exposure later in life. The alcohol-induced

changes that we observed in the innate immune status of the fetal and postnatal lung may alter the risk of lung infection after birth, and warrant further investigation.

Relevance of the pregnant sheep models to FASD and FAS

In the 3 day alcohol model, alcohol-exposed fetuses were lighter than controls. However, neither fetal nor postnatal growth restriction occurred in our 39 day maternal alcohol administration model, with a reduction in ponderal index, but not body weight, observed at 134 DGA. The lack of persistent IUGR may indicate that placental function or maternal nutrition were not seriously affected by the alcohol protocol that we used. Structurally, many of the organs collected at necropsy were not different. Indeed, the only organ grossly affected was the fetal heart, which displayed signs of left ventricular hypertrophy that was attributed to an increase in cardiomyocyte size or maturity, rather than in the number of cardiomyocytes. We observed white matter injury in the 3 day alcohol model, but there was less evidence of overt cerebral injury in the 39 day model. Therefore, our exposure regimen of ~3-4 standard U.S.A drinks over 1h for 39 days in late gestation appears to reflect the more subtle effects associated with FASD, rather than the more severe phenotype of FAS.

Repeated maternal alcohol administration decreased maternal blood glucose concentration and arterial pH, and increased blood lactate concentration[18]. In the fetus, these alcohol treatments caused increased blood lactate concentration and decreased arterial oxygenation[18]. Such changes in arterial blood composition were not seen in the 3-day alcohol-exposure study, which may be the result of the shorter sampling protocol used in the 3 day study. It is likely that the changes observed in blood lactate and pH

were caused by the metabolism of alcohol or acetaldehyde, the major metabolite of alcohol metabolism. Our finding that alcohol-exposure resulted in a mild reduction in fetal blood oxygenation beginning at 6 h from infusion onset is novel and suggests a modest impairment of placental function, perhaps as a result of acetaldehyde or other metabolites, as the PEC in both the ewe and fetus was negligible at this time[18]. The presence of only mild hypoxemia following prolonged alcohol exposure is consistent with our failure to detect IUGR and more overt structural changes in the fetal brain, which might have been expected if more profound chronic fetal hypoxemia had occurred.

Pre-natal alcohol exposure in late gestation and cardiovascular risk

Prenatal alcohol exposure may affect heart and vascular development, thereby increasing the risk of cardiovascular disease in offspring. We observed that fetal alcohol exposure inhibited nephrogenesis, and together with left ventricular hypertrophy and altered small artery wall stiffness and function, these changes could impact adversely on systemic cardiovascular function in later life. Prolonged daily fetal alcohol exposure was also associated with cerebral haemorrhage, which suggests alterations in cerebral artery structure and/or function. Currently, there are no data on the prevalence of cardiovascular disease in older individuals affected by FASD. However one study has suggested an association between FAS and mid-aortic syndrome, which is an uncommon syndrome characterised by the narrowing of the abdominal aortic walls and branches resulting in hypertension[95]. Based on our findings, the risk of cardiovascular

disease in individuals exposed to alcohol prenatally in late gestation could be increased, and this should be a focus of future epidemiological studies.

Prenatal alcohol exposure in late gestation and the risk of compromised pulmonary immunity

Our findings that prenatal alcohol exposure altered surfactant in the fetal lung are consistent with other studies in sheep[96, 97]. In our model, we observed an 80% increase in SP-D mRNA expression in alcohol-exposed nine-week-old lambs[60]. SP-D primarily acts to protect the lungs from airborne pathogens by maintaining innate immunity in the lung; such an increase in SP-D could enhance the innate immunity of the lung. It is possible that our observed increase in SP-D in postnatal lambs may be a response to an overall reduction in pulmonary immune function following prenatal alcohol exposure. This speculation is supported by our finding that SP-A mRNA expression in lung tissue of alcohol-exposed fetuses was ~1/3 of control levels at 134 DGA, suggesting a compromised fetal immune status[59]. The expression of SP-A, which also plays an immunoregulatory role, and IL-1β and IL-8 mRNA were decreased in the fetal lungs[59] and TNF-α mRNA expression was increased in the placenta[98]. It is unclear what effect these changes could have on overall immunity, but they may result in postnatal changes to immune function which could increase the risk of infection. Children with FAS are known to suffer more frequently from respiratory and other infections[99, 100].

Alcohol dosing regimen and cerebral injury

The 3 day maternal alcohol administration protocol showed that 7/8 fetuses had some form of brain injury[19]. Surprisingly however, following 39 days of alcohol exposure, we found little evidence of brain injury, although subarachnoid and cerebellar hemorrhages were observed[18]. This finding is interesting as it suggests that grey and white matter in the fetal brain may adapt to episodic, long-term exposure to alcohol, but cerebral blood vessels may become progressively more vulnerable to damage. The molecular basis of these putative repair, adaptation, and cerebrovascular changes are currently unknown, and further investigation is clearly needed.

Conclusions

Based on our findings, it appears that moderate, daily maternal alcohol consumption during late gestation can cause a wide range of subtle effects on the fetal brain, heart, small arteries, lungs and kidneys, some of which persist after birth. It appears that fetal adaptation to repeated alcohol exposure can occur. Our data, together with those of other studies, suggest that the cardiovascular and immune systems may be at higher risk of dysfunction in postnatal life. Additional research into the long-term cardiovascular and immunological effects of moderate prenatal alcohol exposure, particularly when challenged by lifestyle factors and infection, is warranted. It is important to recognize that many of the changes we have observed in fetal organ development will likely have long-term effects on health and disease during postnatal life. In order to fully understand the effects of alcohol exposure on the developing fetus it is essential to examine

multiple organs at the microscopic and molecular levels. Finally, in view of our findings, pregnant women should be advised to abstain from consuming alcohol.

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Statement of interest

None

Figure legends

Figure 1: Maternal and fetal plasma ethanol concentration (PEC) over time in relation to infusion onset. Shaded area indicates the administration of the 1 hour daily infusion (0.75 g/maternal kg). *p<0.05 between alcohol and control cohorts. Reproduced and adapted with permission of the publisher (Kenna et. al., 2011).

Figure 2: The experimental protocols used in our three studies of fetal alcohol exposure in late gestation. Alcohol (1 g/maternal kg in study 1; 0.75 g/maternal kg in studies 2 and 3) was administered for 1 hour daily over the alcohol infusion period. DGA: Days of gestational age. Term is equivalent to ~147 DGA.

Figure 3: Subarachnoid (*A* - *C*) and cerebellar hemorrhages (*D* - *F*) following 39 days of daily alcohol (0.75 g / kg of maternal weight) exposure in late gestation (95-134 days of gestation). Hematoxylin and eosin (H&E) stained section of the cerebrum hemisphere (A) shows a subarachnoid hemorrhage. The square in (*A*) is magnified to demonstrate (*B*) astroglial (Glial fibrillary acidic protein (GFAP)) and (*C*) microglial (ionized calcium binding adaptor molecule 1 (IBA-1)) responses surrounding the hemorrhage. H&E section of the cerebellar hemisphere (*D*) shows a parenchymal hemorrhage and subsequent damage to the cerebellar cortex. The parachymal hemorrhage is also associated with astroglial (GFAP; *E*) and microglial (IBA-1; *F*) responses. Scale bars: (*A*) 200μm, (*B* - *F*) 100μm. Reproduced with permission of the publisher (Kenna et al, 2011).

Figure 4: Percentage of binucleated (*A*) cardiomyocytes within the left ventricle and septum in saline and EtOH-exposed fetal hearts at 134 days of gestation following 1 hour daily alcohol (0.75 g/maternal kg) infusions from 95-133 DGA. *p<0.05 between alcohol and control cohorts. Representative left ventricular section (*B*) showing mono-nucleated (smaller) and bi-nucleated (larger) cardiomyocytes (yellow arrows). Cardiomyocytes are stained with wheat germ agglutinin-Alexa Fluor 488 (green) and TO-PRO-3 (blue). Scale bar = 8 μm. Reproduced and adapted with permission of the publisher (Goh et. al., 2010).

Figure 5: Total nephron number in saline and EtOH-exposed fetal kidneys at 134 days of gestation after 39 days of daily 1 hour EtOH infusions (0.75 g/kg of maternal weight). *p<0.05 between alcohol and control cohorts. Reproduced and adapted with permission of the publisher (Gray et. al., 2008).

Figure 6: Surfactant phospholipid component concentrations (A) and relative percentages of surfactant phospholipid classes (B) in amniotic fluid at 134 days of gestation following 39 days of daily alcohol (0.75 g /maternal kg) infusions. Phospholipid classes: phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE). *p<0.05 between alcohol and control cohorts. Reproduced and adapted with permission of the publisher (Sozo et. al., 2011).

Figure 7: Surfactant protein mRNA expression levels (SP-A (A), SP-B (B), SP-C (C) and SP-D (D)) in 9 week old lamb lung tissue from controls or fetuses exposed to daily 1 hour alcohol (0.75 g / maternal kg) infusions for 39 days during late gestation (95-133 DGA). *$p<0.05$ between alcohol and control cohorts. Reproduced and adapted with permission of the publisher (Sozo et. al., 2011).

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