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Time and sex dependent effects of magnesium sulphate on post-asphyxial seizures in preterm fetal sheep

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Key points

- We evaluated the effect of magnesium sulphate ($MgSO_4$) on seizures induced by asphyxia in preterm fetal sheep.
- $MgSO_4$ did not prevent seizures, but significantly reduced the total duration, number of seizures, seizure amplitude and average seizure burden.
- Saline-asphyxia male fetuses had significantly more seizures than female fetuses, but male fetuses showed significantly greater reduction in seizures during $MgSO_4$ infusion than female fetuses.
- A circadian profile of seizure activity was observed in all fetuses, with peak seizures seen around 04.00–06.00 h on the first and second days after the end of asphyxia.
- This study is the first to demonstrate that $MgSO_4$ has utility as an anti-seizure agent after hypoxia–ischaemia. More information is needed about the mechanisms mediating the effect of $MgSO_4$ on seizures and sexual dimorphism, and the influence of circadian rhythms on seizure expression.

Abstract Seizures are common in newborns after asphyxia at birth and are often refractory to anti-seizure agents. Magnesium sulphate ($MgSO_4$) has anticonvulsant effects and is increasingly given to women in preterm labour for potential neuroprotection. There is limited information on its effects on perinatal seizures. We examined the hypothesis that $MgSO_4$ infusion would reduce fetal seizures after asphyxia *in utero*. Preterm fetal sheep at 0.7 gestation (104 days, term = 147 days) were given intravenous infusions of either saline ($n = 14$) or $MgSO_4$ ($n = 12$, 160 mg bolus + 48 mg h^{-1} infusion over 48 h). Fetuses underwent umbilical cord occlusion (UCO) for 25 min, 24 h after the start of infusion. The start time for seizures did not differ between groups, but $MgSO_4$ significantly reduced the total number of seizures ($P < 0.001$), peak seizure amplitude ($P < 0.05$) and seizure burden ($P < 0.005$). Within the saline-asphyxia group, male fetuses had significantly more seizures than females ($P < 0.05$). Within the $MgSO_4$-asphyxia group, although both sexes had fewer seizures than the saline-asphyxia group, the greatest effect

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of $MgSO_4$ was on male fetuses, with reduced numbers of seizures ($P < 0.001$) and seizure burden ($P < 0.005$). Only 1 out of 6 $MgSO_4$ males had seizures on the second day post-UCO compared to 5 out of 6 $MgSO_4$ female fetuses ($P = 0.08$). Finally, seizures showed a circadian profile with peak seizures between 04.00 and 06.00 h on the first and second day post-UCO. Collectively, these results suggest that $MgSO_4$ may have utility in treating perinatal seizures and has sexually dimorphic effects.

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Introduction

There is strong clinical and preclinical evidence in adults that $MgSO_4$ has significant anticonvulsant effects, primarily by binding to a specific site on the *N*-methyl-D-aspartate (NMDA) receptor in a voltage-dependent manner (Traynelis *et al.* 2010). $MgSO_4$ may also reduce excitation of the glutaminergic kainate and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors (Hallak *et al.* 2000). It has been used for many years to reduce the risk of maternal seizures during moderate to severe eclampsia, and may be more effective than anticonvulsant agents such as phenytoin (Duley *et al.* 2010; Roy *et al.* 2013). Meta-analysis suggests that $MgSO_4$ may also be effective for non-eclamptic refractory epilepsy and status epilepticus (Zeiler *et al.* 2015). Similarly, animal experiments have confirmed that $MgSO_4$ has marked anticonvulsant effects in seizures induced by NMDA, electrical stimulation and eclampsia, including reducing seizure duration and number (Wolf *et al.* 1990; Hallak *et al.* 1992; Cotton *et al.* 1993; Standley *et al.* 1995*a*; Decollogne *et al.* 1997; Oliveira *et al.* 2011; Liu *et al.* 2013; Huang *et al.* 2014).

In newborn infants, hypomagnesaemia is associated with seizures, and it is standard practice to correct this with $MgSO_4$ infusions (Silverstein & Jensen, 2007). However, there is little direct evidence about the effect of $MgSO_4$ on other neonatal seizures. Hypoxic–ischaemic encephalopathy (HIE) at birth is the commonest cause of seizures in newborns (Silverstein & Jensen, 2007). The effect of boluses of $MgSO_4$ (250 mg kg^{-1} or 125 mg kg^{-1}) given in the days after birth in infants with HIE had either no anti-seizure effect (Groenendaal *et al.* 2002; Ichiba *et al.* 2002; Bhat *et al.* 2009), or a modest effect (Gathwala *et al.* 2010). Levene and colleagues observed that bolus doses produced a rapidly tapering plasma concentration, and that cerebrospinal fluid levels are lower, with a delay in transfer of $MgSO_4$ from blood to the brain (Levene *et al.* 1995). Broadly consistent with these findings, $MgSO_4$ (250 mg kg^{-1}) given within 24 h of birth did not reduce the levels of excitatory amino acids in cerebrospinal fluid in term infants with HIE (Khashaba *et al.* 2006).

$MgSO_4$ is increasingly given to women at risk of preterm labour because it may provide neuroprotection (Galinsky *et al.* 2014; Shepherd *et al.* 2017). Extremely preterm infants have much higher risks of perinatal HIE than term infants (Manuck *et al.* 2016), with correspondingly high risk of clinical seizures (Gale *et al.* 2017). Further, it is important to note that a much higher proportion of seizures in preterm infants are subclinical than at term (Glass *et al.* 2017). At present, there is limited information on the effect of antenatal $MgSO_4$ treatment on preterm seizures after a hypoxic–ischaemic insult.

Thus, the primary aim of the current study was to examine the effect of antenatal $MgSO_4$ on the development of seizures after acute asphyxia induced by complete umbilical cord occlusion in preterm fetal sheep at 0.7 of gestation. At this gestational age, the neural maturation of fetal sheep is broadly equivalent to 28–32 weeks of human development (Barlow, 1969). Based on the evidence from adult animal experiments, we hypothesised that $MgSO_4$ would significantly reduce the total time spent seizing and seizure number. A secondary aim of the study was to examine whether there were sex-specific effects of $MgSO_4$ on seizure activity. There is sexual dimorphism in seizure-control networks within the brain, and males have a greater susceptibility to seizures (Akman *et al.* 2014). Indeed, limited data suggest that $MgSO_4$ may be more effective in males (Standley *et al.* 1995*b*).

Methods

Ethical approval

All animal procedures and animal facilities were approved by the Animal Ethics Committee of The University of Auckland, New Zealand, in accordance with the Code of Ethical Conduct of The University of Auckland, and the New Zealand Animal Welfare Act 1999. These studies conform to the principles and regulations described by Grundy (2015).

Animals and surgical instrumentation

Romney ewes were time mated with Suffolk rams and pregnancies and parity were identified using ultrasound. Ewes were brought to the University laboratory a week before surgery to allow acclimatisation to the laboratory. Ewes and their fetuses underwent surgical procedures between

97 and 98 days gestation (term = 147 days) (Quaedackers *et al.* 2004; Galinsky *et al.* 2017). Food, but not water, was withdrawn 12–18 h before surgery to reduce aspiration during surgery. Prior to surgery, ewes were weighed and given an intramuscular injection of the antibiotic oxytetracycline (20 mg kg^{-1}, Phoenix Pharm, Auckland, New Zealand) for prophylaxis. Anaesthesia was induced by intravenous injection of propofol (5 mg kg^{-1}; AstraZeneca Limited, Auckland, New Zealand), the ewes intubated, and anaesthesia maintained using 2–3% isoflurane in O_2 (Bomac Animal Health, NSW, Australia). During surgery, ewes received isotonic saline ($\sim$250 ml h^{-1}) by intravenous drip to maintain fluid balance. All anaesthetic protocols were undertaken and monitored by trained anaesthetic technicians.

Instrumentation

Following maternal laparotomy and a uterine incision the fetus was partially exteriorised for instrumentation. Two pairs of electroencephalograph (EEG) electrodes (AS633-5SSF; Cooner Wire, Chatsworth, CA, USA) were placed through burr holes onto the dura over the parasagittal parietal cortex (5 and 10 mm anterior to, and 5 mm lateral to, bregma) and secured with cyanoacrylate glue. A reference electrode was sewn over the occiput. Additional electrodes were placed across the fetal chest to measure the fetal electrocardiogram (ECG) to derive fetal heart rate. Polyvinyl catheters (SteriHealth, Dandenong South, VIC, Australia) were inserted in the right femoral artery, and right brachial artery and vein for measurement of blood pressure and for fetal blood sampling and drug administration. A catheter was placed in the amniotic cavity for measurement of amniotic pressure. An inflatable silicone occluder (In Vivo Metric, Healdsburg, CA, USA) was placed loosely around the umbilical cord to facilitate cord occlusion at a later time. The fetus was returned to the uterus, the uterus and abdominal wounds closed, and antibiotics (80 mg gentamycin; Rousell Ltd, Auckland, New Zealand) administered into the amniotic sac. The maternal midline skin incision was infiltrated with a local analgesic (10 ml 0.5% bupivacaine plus adrenaline, AstraZeneca Ltd). All fetal leads were exteriorised through the maternal flank and a maternal long saphenous vein to provide access for post-operative care and humane killing.

Post-surgical care

When surgery was completed the ewes were taken off anaesthesia, extubated and returned to their home cages where they were continuously monitored until they were stable and had been observed to stand and eat and drink. Sheep were housed together in separate metabolic cages with access to water and food *ad libitum* in a temperature-controlled room (16 $\pm$ 1°C, humidity 50 $\pm$ 10%) with a 12 h:12 h light–dark cycle (lights off at 18.00 h). Fetuses were allowed a 4–5 days post-operative recovery period before experiments commenced. During this time welfare monitoring was undertaken several times each day and ewes received intravenous antibiotics daily for 4 days (benzylpenicillin sodium; 600 mg; Novaris, Auckland, New Zealand and gentamycin; 80 mg). Fetal catheters were maintained patent by continuous infusion of heparinised saline (20 IU ml^{-1}) at a rate of 0.2 ml h^{-1} and the maternal catheters were flushed daily with heparinised saline.

Experimental recordings

Fetal mean arterial blood pressure (MAP), corrected for movement by subtraction of amniotic pressure, EEG and ECG were recorded continuously for offline analysis using custom data acquisition software (LabView for Windows, National Instruments, Austin, TX, USA). The blood pressure signal was recorded using a Novatrans II, MX860 pressure transducer (Medex Inc., Hilliard, OH, USA) collected at 64 Hz and low-pass filtered at 30 Hz. The raw ECG signal was used to derive fetal heart rate (FHR) and was analogue filtered with a first order high-pass filter set at 0.05 Hz and an 8th order low-pass Bessel filter set at 100 Hz and digitised at 512 Hz. EEG signals were recorded via leads through a head-stage with an overall gain of 10,000. Signals were then processed with a 6th order low-pass Butterworth filter set to 500 Hz. The EEG signal was low-pass filtered with a cutoff frequency set with the -3 dB point at 30 Hz, and digitised at a sampling rate of 1024 Hz for seizure analysis (Davidson *et al.* 2011; Koome *et al.* 2013; Abbasi *et al.* 2017).

Experimental protocol

At 104 days fetuses were randomly allocated to receive an intravenous infusion of magnesium plus asphyxia (magnesium sulphate heptahydrate, $MgSO_4.7H_2O$, 500 mg ml^{-1}; Phebra, NSW, Australia; n = 12: 6 females and 6 males), or isotonic saline plus asphyxia (n = 14: 7 females and 7 males). Fetuses were infused with $MgSO_4$ from 24 h before until 24 h after fetal asphyxia (Galinsky *et al.* 2016). The sheep placenta rapidly metabolises magnesium and thus to achieve a clinically relevant steady-state fetal plasma concentration $MgSO_4$ was given directly to the fetus. Fetuses received a 160 mg loading dose over 5 min followed by a 48 mg h^{-1} maintenance infusion for 48 h. By 24 h, this produced a steady-state plasma concentration of around 1.88 mmol l^{-1} (Galinsky *et al.* 2016), consistent with the neonatal plasma levels after antenatal treatment for neuroprotection (Borja-Del-Rosario *et al.* 2014).

Fetal asphyxia was induced by rapid, complete inflation of the umbilical cord occluder for 25 min (Quaedackers

et al. 2004). Umbilical cord occlusion (UCO) was started at 09.30 h. Successful occlusion was confirmed by the rapid onset of bradycardia, a rise in MAP and changes in blood chemistry. Fetal blood samples were taken from the brachial artery for analysis of $MgSO_4$ (Roche/Hitachi 902 clinical chemistry analyser, Hoffman-La Roche, Basel, Switzerland) 15 min before $MgSO_4$ or saline infusion started (baseline), and 1, 4, 6 and 24 h before asphyxia, then 1, 2, 4, 6, 24, 48 and 72 h after asphyxia. Pre-ductal blood was also taken for pH, blood gas (ABL 800, Radiometer, Copenhagen, Denmark), glucose and lactate measurements (model 2300, YSI, Yellow Springs, OH, USA) before the start of $MgSO_4$ infusion, 1 h pre-asphyxia, 17 min of asphyxia, and 1, 2, 4, 6, 24, 48 and 72 h post-asphyxia. Ewes and fetuses were killed 3 days post-asphyxia by an overdose of pentobarbitone sodium to the ewe (9 g, Pentobarb 300; Chemstock International, Christchurch, New Zealand).

Data analysis and statistics

Off-line physiological data analysis was performed using Labview-based customised programs. Seizure analysis was undertaken blinded to the treatment group (V. Draghi and L. Bennet). Each minute of the raw EEG traces of all fetuses was assessed for the appearance of high amplitude stereotypic evolving seizures consisting of rhythmic repetitive waves occurring for at least 10 s, and varying in frequency or amplitude as the seizure progresses (Shellhaas & Clancy, 2007; Davidson *et al.* 2012; Koome *et al.* 2013). Data were averaged hourly for analysis. The numbers of high amplitude seizures per hour, the individual duration of seizures, their peak amplitude, the duration spent seizing per hour (seizure burden), and the total duration of seizures (from first to last seizure) was calculated for the groups as a whole and by sex.

Hourly averages were used to evaluate temporal changes in activity, and analysed in 6 h bins to account for temporal changes. Data were compared within and between groups using two-way analysis of variance (ANOVA, SPSS 22.0 for Windows (SPSS, Chicago, Il, USA) followed by a Fisher's protected least-significant difference (LSD) *post hoc* test when a significant effect of group was found. The data were also averaged over the total period (total group data) for group, and by sex and group, and these and the blood chemistry data were analysed by one-way ANOVA with group as the independent factor. Statistical significance was accepted when $P < 0.05$. Data are median (interquartile range) or mean ± SEM as appropriate.

Results

General group data and plasma magnesium

The effects of the $MgSO_4$ infusion on fetal plasma magnesium have been reported earlier for a subset of the fetuses in the present study (Galinsky *et al.* 2017). $MgSO_4$ infusion increased fetal plasma magnesium rapidly after the start of infusion, and levels remained significantly elevated throughout the experiment, and for 2 days after the end of the infusion. Peak concentrations occurred on the day of occlusion (Galinsky *et al.* 2017). There were no differences between groups in pH, blood gases, glucose and lactate before or during the occlusion (Table 1). There was a small reduction in P_{aCO_2} during recovery in the $MgSO_4$-asphyxia group (Table 1), but there were no other significant differences between groups. The nadir of FHR in the final minute of UCO was 57 ± 2.7 beats min^{-1} in the vehicle group *vs.* 56.0 ± 2.1 beats min^{-1} in magnesium (not significantly different (NS)). The nadir of MAP was 11.2 ± 0.7 mmHg *vs.* 11.3 ± 0.6 mmHg (NS).

General seizure analysis

The start time for seizures after UCO was not significantly different between groups (9.7 (7.4–14.5) h *vs.* 6.7 (5.6–7.7) h post-UCO, $MgSO_4$-asphyxia *vs.* saline-asphyxia, $P = 0.08$, Fig. 1*A*). $MgSO_4$ reduced the total

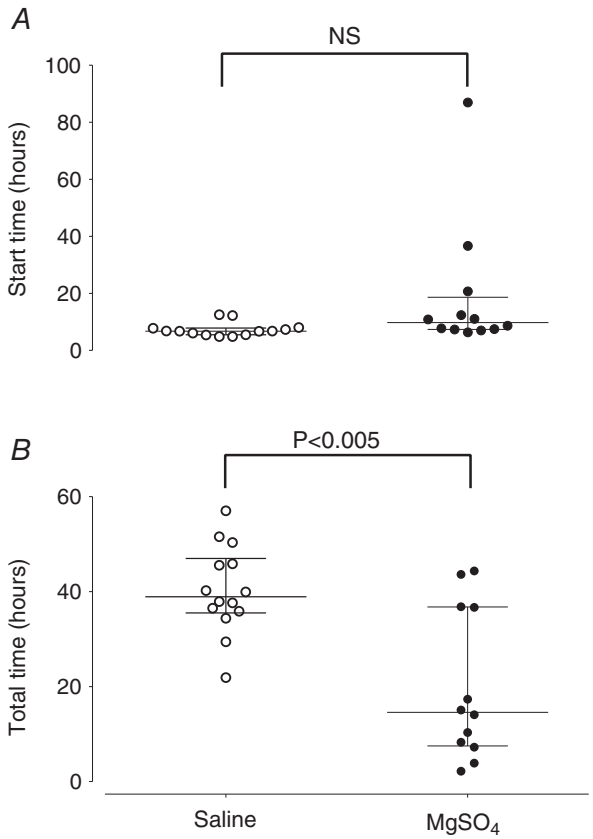

Figure 1. The onset time of seizures after the end of umbilical cord occlusion (*A*) and the total time spent seizing from first to last seizure (*B*) in saline-asphyxia (open circles) and $MgSO_4$-asphyxia (filled circles) treated fetuses
Data are median (interquartile ranges). NS, not significant.

Table 1. Fetal arterial pH, blood gases, glucose and lactate concentrations before and after UCO before magnesium sulphate infusion begins (−24 h), 1 hour prior to UCO (−60 min), 17 min during umbilical cord occlusion, and at designated time points during recovery from occlusion[a]

	Time	−24 h	−60 min	17 min	+1 h	+2 h	+4 h	+6 h	+24 h	+48 h	+72 h
pH	Saline	7.37 ± 0.01	7.37 ± 0.01	6.80 ± 0.01	7.29 ± 0.2	7.33 ± 0.03	7.40 ± 0.01	7.39 ± 0.01	7.36 ± 0.01	7.37 ± 0.1	7.35 ± 0.1
	$MgSO_4$	7.36 ± 0.01	7.34 ± 0.01	6.82 ± 0.01*	7.29 ± 0.01	7.32 ± 0.01	7.38 ± 0.01	7.38 ± 0.01	7.36 ± 0.01	7.36 ± 0.1	7.34 ± 0.2
P_{CO_2} (mmHg)	Saline	48.5 ± 2.0	46.5 ± 0.3	135.0 ± 2.1*	50.1 ± 2.6	49.5 ± 1.7	46.2 ± 0.9	47.9 ± 1.7	48.3 ± 1.1	46.2 ± 0.7	49.4 ± 0.7
	$MgSO_4$	45.7 ± 1.1	44.1 ± 1.2	129.5 ± 4.6*	41.5 ± 1.6*	42.0 ± 2.0*	42.3 ± 1.3*	43.4 ± 1.6*	44.6 ± 1.3	42.3 ± 1.3*	44.5 ± 2.0
P_{O_2} (mmHg)	Saline	24.2 ± 0.6	25.2 ± 1.8	6.9 ± 0.8*	28.7 ± 2.4	25.0 ± 1.9	24.1 ± 1.4	24.4 ± 1.6	25.3 ± 1.7	25.8 ± 1.4	26.0 ± 1.6
	$MgSO_4$	24.6 ± 1.1	24.6 ± 1.5	6.5 ± 1.1*	30.5 ± 2.5	28.6 ± 1.6	27.1 ± 2.1	27.0 ± 1.5	30.1 ± 1.6	30.1 ± 3.0	28.1 ± 2.7
Lactate (mmol l^{-1})	Saline	0.8 ± 0.1	1.0 ± 0.1	6.8 ± 0.3*	4.2 ± 0.6	4.0 ± 0.8	2.6 ± 0.6	2.3 ± 0.5	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
	$MgSO_4$	0.9 ± 0.1	0.9 ± 0.1	6.3 ± 0.3*	4.1 ± 0.3	3.5 ± 0.2	2.0 ± 0.2	1.9 ± 0.2	1.3 ± 0.3	1.0 ± 0.1	0.9 ± 0.1
Glucose (mmol l^{-1})	Saline	1.1 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
	$MgSO_4$	1.0 ± 0.0	0.9 ± 0.1	0.7 ± 0.1	1.3 ± 0.1	1.2 ± 0.0	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.0 ± 0.1

Data are mean ± SEM, *$P < 0.05$ saline *vs.* $MgSO_4$. [a]Magnesium sulphate infusion ended after the +24 h sample.

time spent seizing (14.6 (8.0–36.7) h *vs.* 39 (36.0–46.0) h, $P < 0.005$, Fig. 1*B*). $MgSO_4$ also significantly reduced the total number of seizures (30.0 (17.0–37.0) *vs.* 138.0 (100–168.5), $P < 0.001$, Fig. 2*A*), peak seizure amplitude (86.0 (70.0–118.0) μV *vs.* 120.0 (90–156.0) μV, $P < 0.05$, Fig. 2*B*), and seizure burden (124.0 (92.3–156.0) s h^{-1} *vs.* 201.0 (159.1–267.0) s h^{-1}, $P < 0.005$, Fig. 2*C*). However, there was no significant effect on the mean duration of individual seizures (68.0 (56.4–85.0) s *vs.* 61.1 (53.2–77.5) s, $P = 0.33$, data not shown).

Sex differences in seizure activity

Data for sex and treatment are shown in Table 2. There were no significant sex differences in the onset time of seizures (Fig. 3*A*). In the saline-asphyxia group, males had more seizures than females ($P < 0.05$). Overall, both $MgSO_4$ females and males had significantly fewer

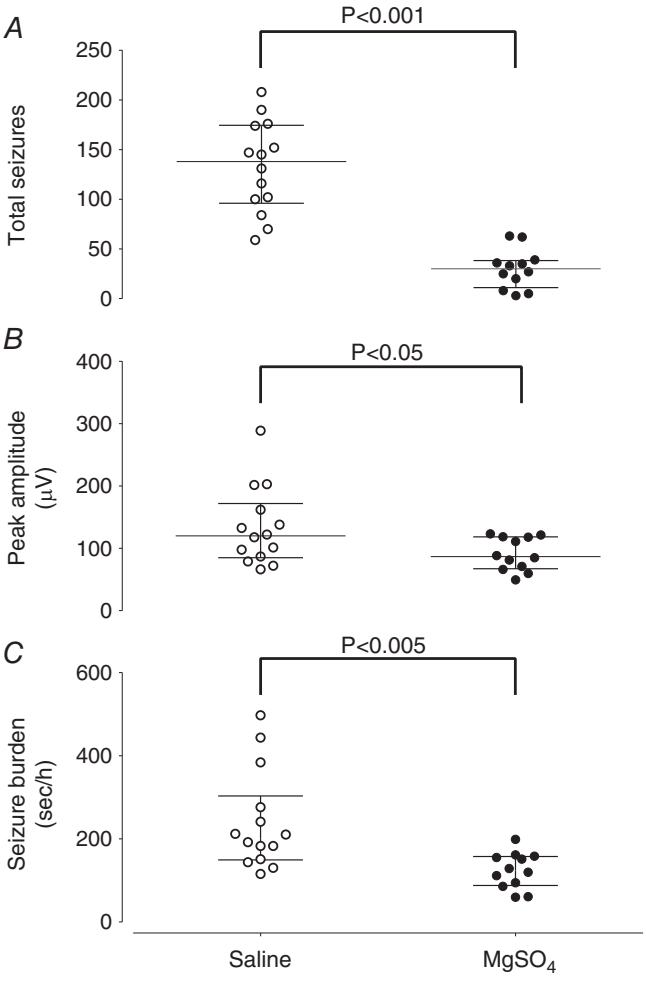

Figure 2. The total number of seizures (*A*), peak seizure amplitude (*B*) and seizure burden (*C*) in saline-asphyxia (open circles) and $MgSO_4$-asphyxia (filled circles) treated fetuses
Data are median (interquartile ranges).

seizures than their saline-treated counterparts ($P < 0.001$, Fig. 4*A*), and reduced total seizure burden ($P < 0.05$, Fig. 4*C*). The total duration of seizures was not different between saline-asphyxia and $MgSO_4$-asphyxia females (Fig. 3*B*), whereas it was shorter in $MgSO_4$-asphyxia males than saline-asphyxia males ($P < 0.001$, Fig. 3*B*) and $MgSO_4$-asphyxia females ($P < 0.005$). There were no significant sex differences for individual seizure duration (Table 2), or seizure amplitude (Table 2, Fig. 4*B*).

Temporal pattern of seizures

The saline-asphyxia group showed two distinct phases of seizures in the first and the second 24 h after asphyxia (Fig. 5). During the first phase, seizure number and burden peaked around 21 h post-UCO (06.30 h). Seizures then progressively fell until around 36 h post-UCO, before increasing again to a second peak at around 43 h post-UCO (04.30 h). In the saline-asphyxia group, seizure amplitude was higher in the first 12 h compared to subsequent 12 h time bins ($P < 0.05$, Fig. 5) and the individual duration of a seizure was longer in the first 12 h than in subsequent 12 h time bins ($P < 0.05$, data not shown). There was a significant decrease in seizure number in the $MgSO_4$-asphyxia group between 6 and 37 h ($P < 0.001$, Fig. 5*A*), peak seizure amplitude between 6 and 18 h ($P < 0.05$, Fig. 5*B*), and seizure burden between 6 and 36 h ($P < 0.005$, Fig. 5*C*). The individual length of a seizure was significantly longer between 7 and 20 h in the saline-asphyxia group *vs.* $MgSO_4$-asphyxia group (data not shown).

Compared to saline-asphyxia female fetuses, $MgSO_4$-asphyxia female fetuses had significantly fewer seizures between 7 and 20 h ($P < 0.005$, Fig. 6*A*), reduced seizure amplitude between 6 and 18 h ($P < 0.05$, Fig. 6*B*), and reduced seizure burden between 7 and 20 h ($P < 0.005$, Fig. 6*C*). Compared to saline-asphyxia males,

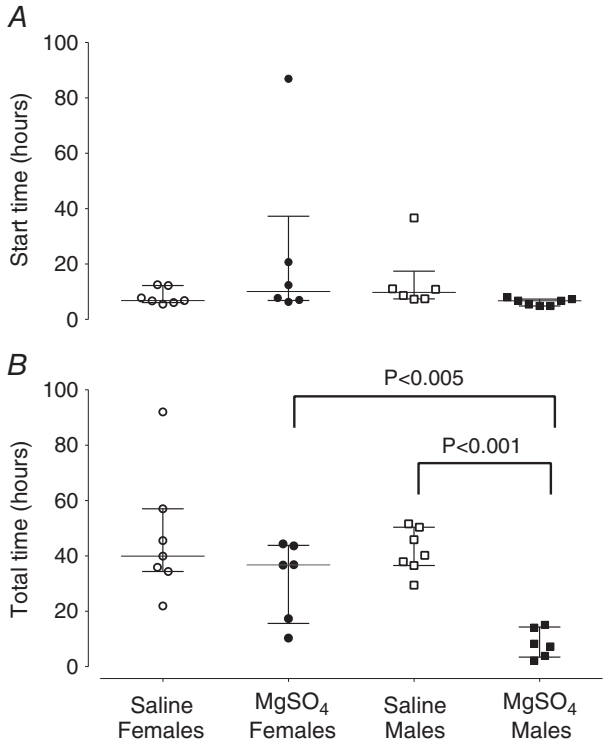

Figure 3. Start time of seizures after the end of umbilical cord occlusion (*A*) and the total time spent making seizures (*B*) in female and male saline-asphyxia fetuses (open and filled circles), and female and male $MgSO_4$-asphyxia fetuses (open and filled squares)
Data are median (interquartile ranges).

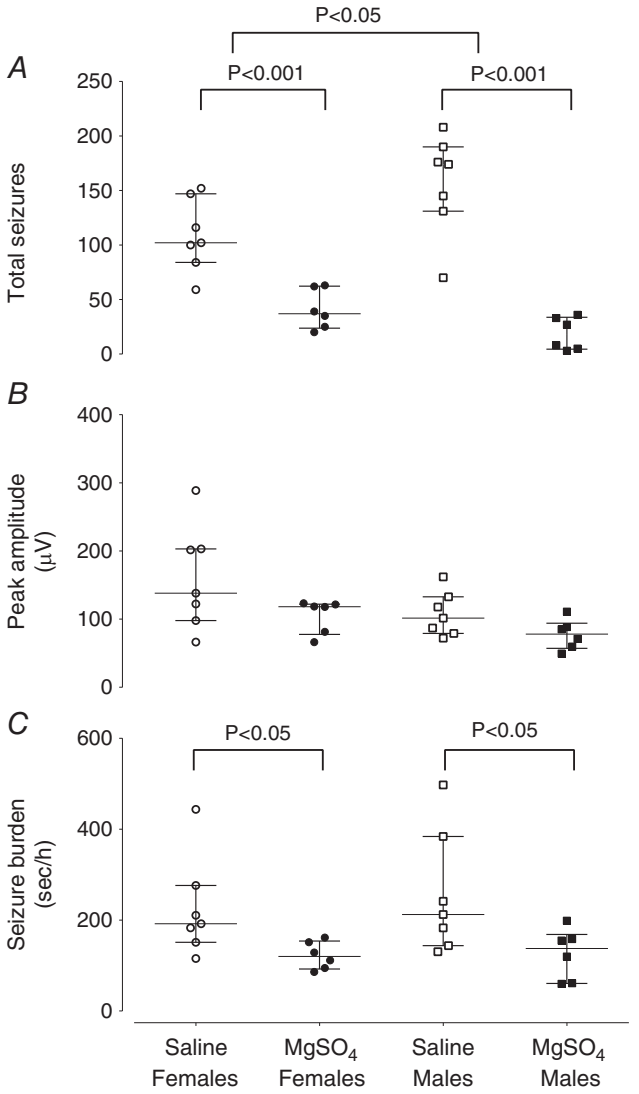

Figure 4. The total number of seizures (*A*), peak seizure amplitude (*B*) and seizure burden (*C*) in female and male saline-asphyxia fetuses (open and filled circles), and female and male $MgSO_4$-asphyxia fetuses (open and filled squares)
Data are median (interquartile ranges).

$MgSO_4$-asphyxia male fetuses had a significant reduction in seizure number between 18 and 36 h ($P < 0.001$, Fig. 7*A*), peak seizure amplitude between 6 and 18 h ($P < 0.05$, Fig. 7*B*), and seizure burden between 12 and 25 h ($P < 0.005$, Fig. 7*C*).

Saline-asphyxia female fetuses had total fewer seizures than saline-asphyxia male fetuses between 13 and 34 h ($P < 0.05$), but there were no other differences between sexes in the saline-asphyxia group (Table 2). $MgSO_4$-asphyxia males had a shorter total duration of seizures compared to $MgSO_4$-asphyxia females ($P < 0.005$, Table 2) and fewer seizures ($P < 0.05$, Table 2). It is notable that $MgSO_4$-asphyxia male fetuses had no seizures between 23 and 30 h (Fig. 7), whereas 3 out of 6 $MgSO_4$-asphyxia female fetuses had seizures during this period (Fig. 7). Only one male from the $MgSO_4$-asphyxia group had seizures in the second phase of seizures compared to 5 out of 6 female $MgSO_4$-asphyxia fetuses ($P = 0.08$, Fisher exact test).

Discussion

This study is the first to dissect in detail the effects of a clinically relevant plasma concentration of $MgSO_4$ on seizures in preterm fetuses after an acute asphyxial insult *in utero*. The data show that while $MgSO_4$ did not prevent or delay the onset of seizures, it significantly reduced the duration of seizures, seizure burden, and numbers and amplitude of seizures. Further, we have made the novel observation that while $MgSO_4$ was beneficial for both sexes, it had a greater impact on male fetuses. Unexpectedly, the effects of $MgSO_4$ were time dependent, with the maximal effect of $MgSO_4$ seen on the ramp up

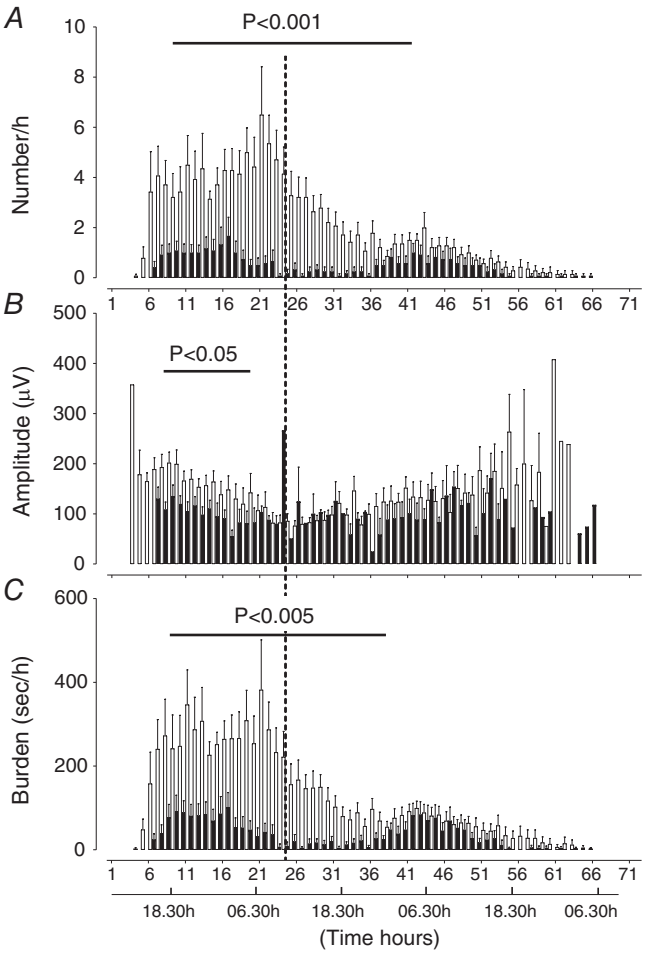

Figure 5. Time sequence of changes in numbers of seizures (*A*), peak seizure amplitude (*B*) and seizure burden (*C*) in saline-asphyxia (open bars) and $MgSO_4$-asphyxia (filled bars) fetuses
Data are mean ± SEM.

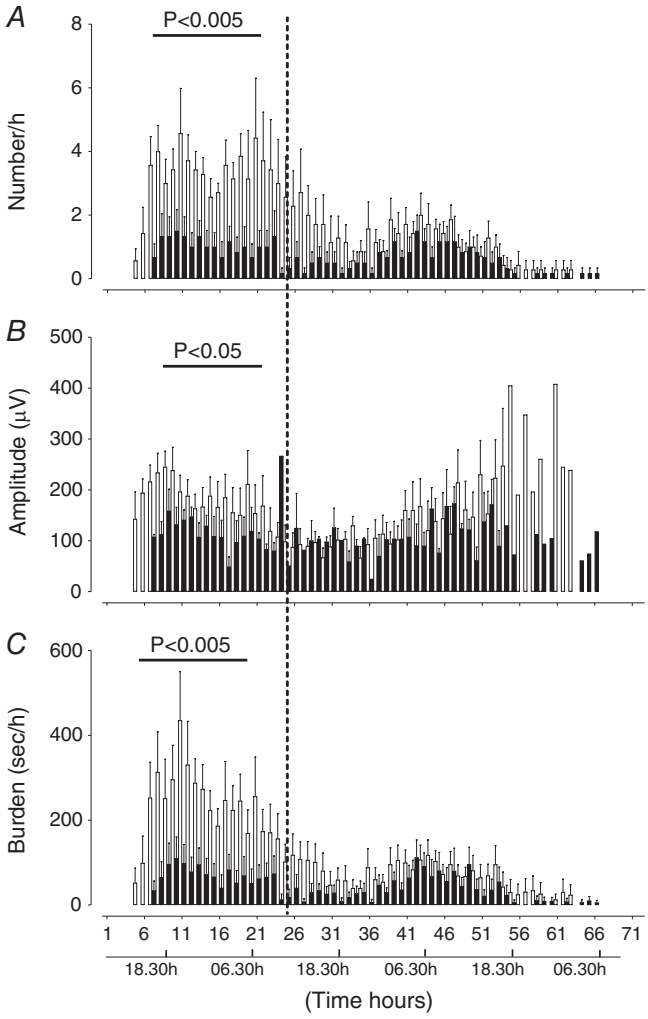

Figure 6. Time sequence of changes in number of seizures (*A*), peak seizure amplitude (*B*) and seizure burden (*C*) in female saline-asphyxia fetuses (open bars) and female $MgSO_4$-asphyxia fetuses (filled bars)
Data are mean ± SEM.

Table 2. Seizure activity grouped by sex and treatment

	Female saline-asphyxia	Female $MgSO_4$-asphyxia	Male saline-asphyxia	Male $MgSO_4$-asphyxia
Start (h)	6.8 (6.4–10.0)	10.1 (7.2–18.6)	6.7 (5.3–7.0)	9.7 (7.8–11.0)
Total time (h)	40.0 (35.1–51.3)	36.8 (22.2–42.0)	40.2 (37.0–48.1)	7.8 (4.7–12.6) $P < 0.001$ *vs.* male saline-asphyxia $P < 0.005$ *vs.* female $MgSO_4$-asphyxia
Seizure number	102 (92.0–132.0) $P < 0.05$ *vs.* male saline-asphyxia	37 (27.5–56.3) $P < 0.001$ *vs.* female saline-asphyxia	174 (152.3–183)	17.5 (5.8–31.5) $P < 0.001$ *vs.* male saline-asphyxia $P < 0.05$ *vs.* female $MgSO_4$-asphyxia
Average duration (s)	73 (58.1–79.4)	69 (61.0–88.2)	58.1 (57.2–61.1)	64.1 (60.1–79.1)
Peak amplitude (μV)	138.1 (110.1–202.3)	118.2 (91.0–121.0)	101.4 (90.5–126)	78.0 (62.0–87.5)
Seizure burden (s h^{-1})	192.0 (110–202.3)	125.5 (99–148.4)	212.2 (190.3–313.0)	137.5 (76.0–158.0) $P = 0.05$ *vs.* male saline-asphyxia

Data are median (interquartile range).

to peak seizure numbers. A second key observation was that there were two distinct peaks in seizure activity on the first and second days after occlusion, occurring around the same time in the early hours of the morning. A circadian pattern in seizure activity has been observed in adults, but this is the first report in the fetus.

The asphyxia protocol used in the present study is associated with subcortical neuronal injury and diffuse white matter loss, but no cortical neuronal injury (Bennet *et al.* 2006; Dean *et al.* 2006*b*; Galinsky *et al.* 2017). Using this protocol, we have recently shown that $MgSO_4$ is not neuroprotective, and thus any effects on seizures would be independent of changes in neuronal survival (Galinsky *et al.* 2017). Seizures in the current study in all fetuses were single, brief and relatively infrequent events as demonstrated previously (Quaedackers *et al.* 2004). The primary phase of seizures in the saline-asphyxia group occurred in the first 24 h after asphyxia. While little is known about the temporal evolution of preterm seizures, the present data are consistent with limited clinical data on the evolution of seizure activity in normothermic term infants, which show seizures are maximal in the first 24 h after a hypoxic–ischaemic insult (McBride *et al.* 2000; Lynch *et al.* 2012).

Consistent with the overall reduction of delayed seizures, we have previously observed a reduction in pre-seizure epileptiform transient activity during $MgSO_4$ infusion (Lakadia *et al.* 2016). $MgSO_4$ primarily ameliorates seizures by binding to glutamate receptors as demonstrated in adult rodent models (Hallak *et al.* 1992; Cotton *et al.* 1993; Mason *et al.* 1994; Decollogne *et al.* 1997). Glutamate plays a key role in mediating seizures in fetal sheep (Tan *et al.* 1992; Dean *et al.* 2006*a*). The immature brain is considered more susceptible to seizures due to the density of calcium permeable, GluR2-subunit-deficient AMPA receptors, which contribute to a lower threshold for seizures, and expression of NMDA glutamate receptor subunits (GluN) such as GluN2B, which mediate longer-duration excitatory postsynaptic potentials, and GluN3A, which are relatively magnesium insensitive (Jensen, 2009). Further, hypoxia can change the proportional expression of NMDA receptor subunits, and so promote increased neuronal excitability and seizures in the immature brain (Dean *et al.* 2005; Rakhade *et al.* 2008; Zhou *et al.* 2009, 2015).

γ-Aminobutyric acid (GABA) may also play a role, as chloride efflux promotes depolarisation instead of hyperpolarisation as seen in the adult brain (Nardou *et al.* 2013). While the GABAergic promotion of excitation is important for development of neural circuitry, it can increase susceptibility to seizures (Nardou *et al.* 2013). Moreover, in neonatal rodents, depolarisation of GABA receptors can facilitate the removal of the voltage-dependent magnesium block from NMDA channels (Leinekugel *et al.* 1999).

The time-dependent anticonvulsant effects of $MgSO_4$ in the present study likely reflected the voltage-dependent nature of magnesium binding. Under physiological conditions, NMDA receptor activation requires glutamate and glycine plus initial membrane depolarisation, typically through AMPA receptor activation, to relieve the magnesium mediated voltage-dependent NMDA receptor channel block (Traynelis *et al.* 2010; Nikolaev *et al.* 2012). The influx of calcium plus internal calcium release during glutamatergic activation prolongs depolarisation and further extends the initial release of magnesium blockade (Traynelis *et al.* 2010; Nikolaev *et al.* 2012). At the onset of seizures in the present study, seizures were relatively infrequent but each event was of greater magnitude than subsequent events. Thus, speculatively, the greater magnitude of depolarisation-elevated levels of magnesium were insufficient to completely maintain the magnesium block, although the extracellular levels

attained during infusion were at a level that facilitated seizure modulation. As seizures evolved, their amplitude fell, likely sufficient to allow magnesium to further block NMDA receptors.

In the current study, fetal sex affected both seizure expression in saline-asphyxia animals and the impact of $MgSO_4$. Strikingly, $MgSO_4$ not only significantly attenuated the occurrence of seizures in the first phase of seizures (first 24 h), but in the second period of seizures (i.e. the second 24 h), only one male fetus developed further seizures, compared with nearly all of the female fetuses. These data suggest that the effect of $MgSO_4$ on males was not dependent on circulating plasma levels of $MgSO_4$, but rather that $MgSO_4$ had a sustained effect on the mechanisms that increase the threshold for seizure induction. In the saline-asphyxia group, female fetuses had significantly fewer seizures than males, consistent with observations in neonates (Jensen, 2009; Giorgi *et al.* 2014).

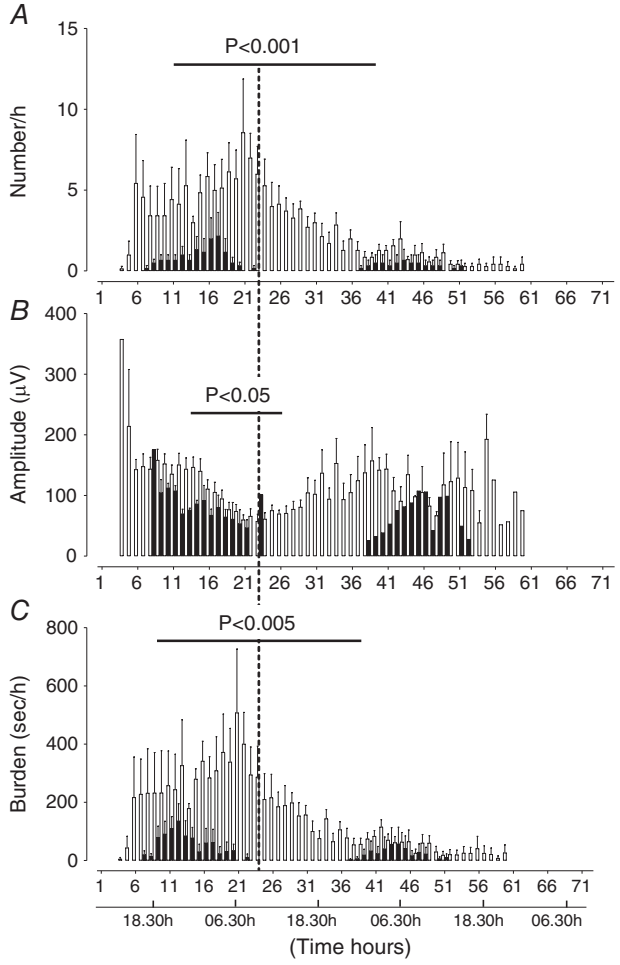

Figure 7. Time sequence of changes in number of seizures (*A*), peak seizure amplitude (*B*) and seizure burden (*C*) in male saline-asphyxia fetuses (open bars) and male $MgSO_4$-asphyxia fetuses (filled bars)
Data are mean ± SEM.

Sex-specific differences in seizures in the saline-asphyxia group may relate to GABA receptors. Numerous studies show that GABA switches from depolarisation to hyperpolarisation, as seen in adults, much sooner in females (Galanopoulou & Moshe, 2003; Veliskova *et al.* 2004; Nunez & McCarthy, 2007; Stafstrom, 2008; Murguia-Castillo *et al.* 2013). This is due to an earlier switch to the potassium chloride cotransporter 2 (KCC2), which is active in transporting chloride out of the cell (Galanopoulou & Moshe, 2003; Veliskova *et al.* 2004; Nunez & McCarthy, 2007; Stafstrom, 2008; Murguia-Castillo *et al.* 2013). However, GABA receptors, like glutamate receptors, can be labile during seizures and limited data suggest that repetitive seizures may cause GABA to switch from initiating neuronal depolarisation to inducing hyperpolarisation, and this may occur earlier in males (Stafstrom, 2008).

There are surprisingly few studies on sex differences in glutamate receptors in the developing brain. Newborn rodent studies show that there are developmental changes in NMDA receptor subunit composition with a switch from a predominance of NR2B-containing to NR2A-containing receptors (Monyer *et al.* 1994; Jensen, 2009; Traynelis *et al.* 2010). Damborsky and colleagues reported that neonatal male rodents have a higher expression of GluN2A, and lower mRNA expression of GluN2B, compared to females (Damborsky & Winzer-Serhan, 2012), which would lead to shorter NMDA receptor current durations (Matta *et al.* 2011). Further, excitotoxic brain damage in neonatal mice was associated with greater long-term motor and cognitive deficits in males than females, which were alleviated by $MgSO_4$ (Daher *et al.* 2017), supporting the possibility of sex-specific neuroprotection. In the current study, while individual seizures tended to be shorter in $MgSO_4$ treated males, this was not significant. Potentially, a sex dependent switch to more GluN2A, and a reduction in other receptor subtypes such as GluN3A, would increase the threshold to seizures leading to fewer seizures and the sex dependent reduction in seizure burden seen in males.

Alternatively, a role for sex hormones should also be considered. Our data on the effect of magnesium on male fetuses are consistent with a study in adult rats, which showed that intracerebroventricular administration of NMDA caused greater seizures in males, but magnesium treatment reduced total seizure duration and number only in males (Standley *et al.* 1995*b*). In this study, the authors speculated that there may be a role for sex hormones in modulating these differences, as is described in adults (McCarthy, 2008; Reddy, 2017). In neonatal rat pups, androgens exacerbate seizures induced in males by administration of the GABA agonist muscimol; this effect is prevented by castration (Giorgi *et al.* 2007). Oestradiol,

metabolised from testosterone, further reduces KCC2, thus exacerbating chloride clearance (Galanopoulou & Moshe, 2003). Additionally, androgens are associated with reduced clearance of calcium after a seizure, and a sustained calcium response to subsequent seizures in neonatal pups (Nunez & McCarthy, 2008).

In adults, testosterone has both pro- and anticonvulsant effects, and the pro-convulsant effect may in part be due to aromatisation of testosterone to oestrogen (Reddy, 2017). Oestradiol can potentiate NMDA receptor activity through sex-specific mechanisms, with oestradiol increasing release and sensitivity to glutamate in males (Oberlander & Woolley, 2016). Further, seizures can induce neurosteroid oestradiol synthesis, which in turn escalates seizure activity (Sato & Woolley, 2016). Testosterone aromatase activity can be downregulated in the presence of increased magnesium (Balthazart *et al.* 2001) and thus magnesium may have modulated seizures by acting as an aromatase inhibitor. There is considerable, unsurprising, evidence that testosterone is elevated in fetal life in males compared to females (Pomerantz & Nalbandov, 1975; Roselli *et al.* 2011).

Finally, we report the novel observation that post-asphyxial seizure activity in the preterm fetal sheep shows an apparently circadian profile, with a ramp-up in seizures late in the evening/early morning and a peak around 04.00–06.00 h. In the context of the present study, it is not possible to rule out the possibility that it might be partly related to differential evolution of progressive secondary mitochondrial failure between regions of the brain (Bennet *et al.* 2006). Future studies could examine how the seizure profile is modulated by initiating asphyxia at night for example instead of during the day. Although this is the first description of a circadian pattern of seizures in the immature brain, diurnal changes in seizure activity were first described in postnatal life by Gowers in 1885. Subsequent studies in adults have shown that seizures can be either nocturnal or diurnal, depending on their type or seizure locus (Anderson *et al.* 2015; van Campen *et al.* 2015), and a similar pattern has been observed in children (Loddenkemper *et al.* 2011).

The mechanisms mediating nocturnal seizures are multifactorial, but may relate to changes in GABA and associated glutamate release (Avoli *et al.* 2013; Moore *et al.* 2017). In adults, GABA is a key circadian time-keeper, and within the suprachiasmatic nucleus (SCN) exhibits bipolar activity, promoting excitability during the day and inhibition at night (Wagner *et al.* 1997; Choi *et al.* 2008). However, deficits in GABAergic inhibition are associated with abnormal activity and within a hyper-excitable network GABA can promote seizure activity (Moore *et al.* 2017).

The fetus exhibits circadian patterns in behaviour, which are entrained by maternal rhythms and melatonin passage through the placenta (Dalton *et al.* 1977; Simonetta *et al.* 1991; Breen *et al.* 1996; Seron-Ferre *et al.* 2012). Maternal signals are in part co-ordinated by signals from the fetal central and peripheral clocks (Seron-Ferre *et al.* 2012). In the immature brain, activity in the neurons in the SCN are more active during the day than night, with SCN activity potentially suppressed by melatonin (Breen *et al.* 1996).

In the current study, fetuses do not yet show sleep-state cycling (Mellor *et al.* 2005). Nevertheless, the preterm fetal brain activity does change during the night, with loss of quiescent periods such as interburst intervals (Davidson *et al.* 2011). Further, we have demonstrated increased neural cytochrome oxidase activity at night, suggesting greater neural metabolic activity (Bennet *et al.* 2006). Neural circadian activity in the immature fetus is poorly understood but, like adults (Raven *et al.* 2017), the fetus may preferentially use night-time to promote neural network development. Certainly, sleep is important for normal perinatal maturation of the brain (Mirmiran *et al.* 2003) and GABA, along with synergistic glutamate activity, is postulated to play a key role in mediating development of the perinatal neural network (Dehorter *et al.* 2012). Speculatively, in our experiments, in a setting where there is high risk of epileptiform activity, increased release of GABA and glutamate at night may promote greater seizure activity.

Finally, there is a potential dose effect to consider. $MgSO_4$ did not modulate overall seizure activity in the second 24 h period, particularly in female fetuses who showed less effect during the first seizure phase. This may reflect falling levels of $MgSO_4$ after the end of infusion at 24 h post-UCO (Galinsky *et al.* 2017). Our data suggest that not only is there a need to maintain treatment until the risk of seizures has abated, but that a higher dose will likely be required to optimise control of seizures. Clinically, there are safety concerns with increasing the dose of $MgSO_4$ for both mother and infant (Levene *et al.* 1995; Zeng *et al.* 2016), including potential for increased risk of intraventricular haemorrhage and neonatal morbidity (Narasimhulu *et al.* 2017). Studies of $MgSO_4$ given to newborns suggest that bolus doses of between 125 and 250 mg kg^{-1} given repeatedly every 24 h have little effect on seizures, possibly because of the relatively limited transfer of $MgSO_4$ from blood to the brain (Ichiba *et al.* 2002; Gathwala *et al.* 2010). Further, in term infants with hypoxic–ischaemic encephalopathy, increasing the dose to 400 mg was associated with hypotension and respiratory depression (Levene *et al.* 1995). In the current study we have demonstrated the efficacy of a clinically relevant dose, given over a longer duration of treatment, on seizure activity without fetal hypotension or any evidence of mortality (Galinsky *et al.* 2016, 2017). Potentially, a relatively modest increase in the dose may have provided better control of seizures. Alternatively, we may speculate

that antenatal $MgSO_4$ treatment continued into the post-natal period at the current dose may facilitate better seizure management with anti-epileptic therapies.

In conclusion, our study has demonstrated for the first time that $MgSO_4$ may have utility as an anti-seizure agent after an acute fetal hypoxic–ischaemic insult. Importantly, our study has shown that seizures in the immature brain exhibit an apparently circadian profile, and that while both sexes benefitted from $MgSO_4$ treatment, the effect on male fetuses was greater. Further work is now required to determine the optimal therapeutic anti-seizure dose regime for $MgSO_4$, and to examine whether $MgSO_4$ given after seizures start can stop seizure activity.

Perspective

Seizures are common after perinatal hypoxic–ischaemic insults, and it is recognised that they have been significantly under-reported, in part due to a lack of EEG monitoring and training (Boylan *et al.* 2013; Hellstrom-Westas *et al.* 2015). Whether all seizures increase brain injury, and thus should be treated, remains debated (Shetty, 2015). However, resolving this issue is difficult because anti-seizure treatments for neonates are limited in number, potentially injurious themselves, and for many infants simply ineffective or effectiveness is lost over time (Hellstrom-Westas *et al.* 2015; Shetty, 2015; Thoresen & Sabir, 2015). There is a need for more evidence-based studies to guide neonatal seizure management (Hellstrom-Westas *et al.* 2015).

Importantly, however, as highlighted in this paper, to make advances in designing new treatments requires greater information to address significant gaps in our knowledge about the mechanisms mediating perinatal seizures, how they change over time and as a function of factors such as the seizures themselves, perinatal age and sex, time of day, and other clinical conditions and treatments. Collectively, this information, in turn, will inform us about which drugs may be effective, when they are effective and who will benefit, including those treated before birth.

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Additional information

Competing interests

None declared.

Author contributions

L.B., A.J.G. and R.G. conceived and designed the experiments; V.D., R.G., C.A.L., J.O.D. and L.B. collected the data; L.B. and V.D. analysed the data; L.B., C.P.U. and A.J.G. interpreted the data and drafted the article. R.G., V.D., C.A.L., J.O.D. and C.P.U. contributed to drafting the article and/or revising it critically for intellectual content. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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