



Creatine supplementation reduces the cerebral oxidative and metabolic stress responses to acute *in utero* hypoxia in the late-gestation fetal sheep

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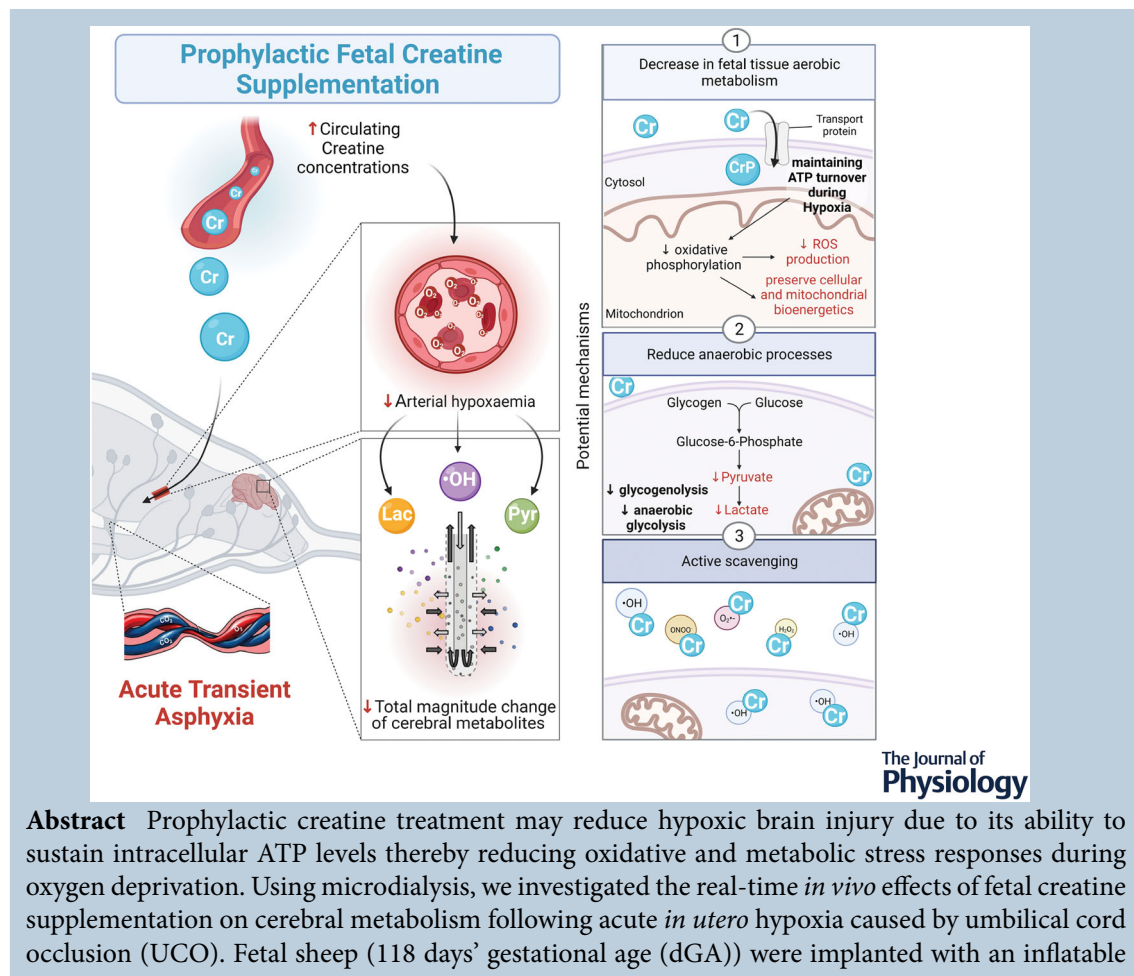
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Silastic cuff around the umbilical cord and a microdialysis probe inserted into the right cerebral hemisphere for interstitial fluid sampling. Creatine ($6 \text{ mg kg}^{-1} \text{ h}^{-1}$) or saline was continuously infused intravenously from 122 dGA. At 131 dGA, a 10 min UCO was induced. Hourly microdialysis samples were obtained from -24 to 72 h post-UCO and analysed for percentage change of hydroxyl radicals ($\cdot\text{OH}$) and interstitial metabolites (lactate, pyruvate, glutamate, glycerol, glycine). Histochemical markers of protein and lipid oxidation were assessed at post-mortem 72 h post-UCO. Prior to UCO, creatine treatment reduced pyruvate and glycerol concentrations in the microdialysate outflow. Creatine treatment reduced interstitial cerebral $\cdot\text{OH}$ outflow 0 to 24 h post-UCO. Fetuses with higher arterial creatine concentrations before UCO presented with reduced levels of hypoxaemia (P_{O_2} and S_{O_2}) during UCO which associated with reduced interstitial cerebral pyruvate, lactate and $\cdot\text{OH}$ accumulation. No effects of creatine treatment on immunohistochemical markers of oxidative stress were found. In conclusion, fetal creatine treatment decreased cerebral outflow of $\cdot\text{OH}$ and was associated with an improvement in cerebral bioenergetics following acute hypoxia.

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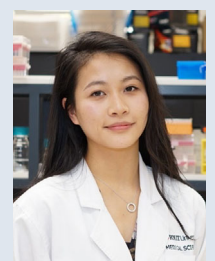
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Abstract figure legend Prophylactic fetal creatine supplementation is a proposed treatment strategy for acute transient asphyxia such as umbilical cord occlusion (UCO). Fetal sheep with higher circulating arterial creatine (Cr) concentrations before UCO and reduced levels of systemic hypoxaemia during UCO were associated with reduced accumulation of cerebral interstitial pyruvate (Pyr), lactate (Lac) and reactive oxygen species (specifically $\cdot\text{OH}$) following UCO as measured by microdialysis. There are three proposed mechanisms, which include maintaining ATP turnover during hypoxia and therefore reducing the need for aerobic metabolism, anaerobic processes, and active reactive oxygen species scavenging. Created with BioRender.com.

Key points

- Fetal hypoxia can cause persistent metabolic and oxidative stress responses that disturb energy homeostasis in the brain. Creatine in its phosphorylated form is an endogenous phosphagen; therefore, supplementation is a proposed prophylactic treatment for fetal hypoxia.
- Fetal sheep instrumented with a cerebral microdialysis probe were continuously infused with or without creatine-monohydrate for 10 days before induction of 10 min umbilical cord occlusion (UCO; 131 days' gestation). Cerebral interstitial fluid was collected up to 72 h following UCO.
- Prior to UCO, fetal creatine supplementation reduced interstitial cerebral pyruvate and glycerol concentrations.
- Fetal creatine supplementation reduced cerebral hydroxyl radical efflux up to 24 h post-UCO. Fetuses with higher arterial creatine concentrations before UCO and reduced levels of systemic hypoxaemia during UCO were associated with reduced cerebral interstitial pyruvate, lactate and $\cdot\text{OH}$ following UCO.
- Creatine supplementation leads to some improvements in cerebral bioenergetics following *in utero* acute hypoxia.

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Introduction

Perinatal asphyxia remains one of the most prominent causes of childhood morbidity and mortality, affecting up to 26 per 1000 live births around the globe each year (Kurinczuk et al., 2010; Lawn et al., 2010). Transient episodes of acute asphyxia such as those caused by nuchal cords, placental abruption or umbilical cord compression can range from <1 to 30 min or more (Anyaeibunam et al., 1986; Goldsmith, 2015; Nelson & Grether, 1998; Osak et al., 1997). The fetus can temporarily maintain adequate cellular oxidative metabolism through rapid, chemoreceptor-mediated changes in the distribution of cardiovascular output, ensuring that blood flow and oxygen delivery to the brain and heart are preserved, or even increased (Giussani, 2016). However, as the acute asphyxic event progresses, hypoxia and acidosis worsen, eventually leading to a compromise of cellular oxidative metabolism and, a cascade of metabolic and oxidative stress responses that can ultimately result in cell death (Ferriero, 2004; Gunn & Bennet, 2009). Even when normal oxygen supply to the tissues is reinstated, cellular metabolism remains disrupted, resulting in further pathological consequences arising from reperfusion injury as well as secondary and tertiary energy failure (Azzopardi et al., 1989; Gunn & Bennet, 2009; Roth et al., 1997; Wyatt, 2002).

At a cellular level, acute hypoxia causes a progressive loss of ATP and a shift to anaerobic respiration, and the resulting metabolic stress can cause increased reactive oxygen species (ROS), accumulation of anaerobic by-products, pyruvate and lactate, and depolarisation of the cellular membranes leading to the release of excitotoxic neurotransmitters such as glutamate (Ferriero, 2004; Volpe, 2008). While high-energy phosphates are transiently restored during the immediate recovery phase of hypoxic–ischaemic (HI) brain injury, another phase of decreased high-energy phosphate levels (i.e. ATP and phosphocreatine) occurs at about 8–24 h post-injury, a phenomenon known as secondary energy failure (Vannucci et al., 2004). This later phase of energy failure is associated with poorer prognoses and long-term neurodevelopmental impairment that characterises hypoxia–ischaemia encephalopathy (HIE) (Azzopardi et al., 1989; Hamilton et al., 1986; Hope, 1984; Kagan et al., 2021).

Hypoxia provokes changes in both neurons and glia that affect metabolite release and re-uptake, and fundamentally affects aerobic cellular metabolism (Logica et al., 2016). Previous studies in fetal sheep have described delayed and persistent increases in ROS and multiple cellular metabolites, particularly pyruvate, lactate, glucose and glutamate, in brain interstitial fluid following acute global hypoxia (Miller et al., 2005; Yan et al., 2009). ROS, such as the superoxide radical ($O_2^{\bullet-}$), hydrogen

peroxide (H_2O_2) and the hydroxyl radical ($\bullet OH$), are particularly implicated in hypoxia-mediated cellular injury due to their ability to directly damage cellular macromolecules such as proteins, lipids and nucleic acids (Ikeda et al., 1999; Myers, 1975; Rao et al., 2011; Yawno et al., 2017). The fetal brain is no exception and is highly vulnerable to ROS-mediated oxidative stress due to its high oxygen consumption, abundant levels of free iron and unsaturated fatty acids, as well as relatively immature development of antioxidant systems (Castagne, 1999; Mishra & Delivoria-Papadopoulos, 1988). Accordingly, increased ROS-induced cellular and macromolecule by-products have been reported in the plasma of newborns diagnosed with HIE (Chafer-Pericas et al., 2016; Raicevic et al., 2010; Rogers et al., 1997), as well as in animal models of HI (Coimbra-Costa et al., 2017; Lafemina et al., 2006; Rocha-Ferreira et al., 2016; Yawno et al., 2017). The combination of perturbed cellular energy metabolism and oxidative stress responses following hypoxia and associated energy failure suggests that preventing or reducing these responses may be an effective approach to improving perinatal HI outcomes.

Creatine supplementation is proposed as a prophylactic treatment in pregnancy for preventing hypoxia-mediated brain to the fetal brain during labour and birth (Ellery et al., 2016; Ireland et al., 2011; Muccini et al., 2021; Tran et al., 2021). Creatine in its phosphorylated form, phosphocreatine, acts physiologically in vertebrates as an ATP buffer through the creatine kinase circuit to maintain ATP-dependent cellular metabolism (Wyss & Kaddurah-Daouk, 2000). Creatine and phosphocreatine act as a spatial energy buffer and a cytosolic source of high-energy phosphates for rapid re-phosphorylation of ADP (temporal energy buffer), thus sustaining cellular ATP during processes of high metabolic demand such as during physical exertion (Clark, 1997; Muccini et al., 2021), and also during periods of tissue hypoxia (Alberti, 1977). Creatine and phosphocreatine's energy stabilising capacity has the potential to buffer cellular ATP depletion following HI, thereby helping to preserve metabolic and redox balance (Andres et al., 2005; Shen & Goldberg, 2012). In addition, creatine has been shown to have both direct and indirect antioxidant actions *in vitro* in cultured cells (Lawler et al., 2002; Sestili et al., 2006).

We have previously shown in fetal sheep that arterial creatine levels were significantly increase by 4 days of continuous intravenous creatine supplementation, and by 13 days there were increases in total creatine content (creatine plus phosphocreatine content) in several regions of the fetal brain (Tran et al., 2021). Therefore, in this study, we used cerebral microdialysis to investigate if intravenous creatine infusion could reduce the generation of oxidative and metabolic stress markers following transient (10 min) hypoxia induced by mechanical umbilical cord occlusion (UCO). We also investigated if any

associations existed between available arterial creatine concentrations prior to UCO, the changes in blood gas measurements experienced during UCO, and the accumulation of metabolites and $^{\circ}\text{OH}$ in brain interstitial fluid following UCO.

Methods

Ethical approval

Twenty-eight pregnant Border–Leicester/Merino cross ewes carrying singletons at 118 days' gestational age (dGA; term is ~ 145 – 147 dGA) were sourced from a private supplier who used induced ovulation and time mating procedures to allow calculation of gestation to ± 1 day. The use of animals was approved by Monash Medical Centre Animal Ethics Committee (MMCA-2017-02) and was conducted in accordance with the Australian Code of Practice for the care and use of Animals for Scientific Purposes established by the National Health and Medical Research Council of Australia. The ewes were placed separately in individual pens at the Monash Medical Centre animal house holding facility, always in the company of other sheep, and allowed to acclimatise for 6 days prior to surgery. During this time they were maintained in a 12 h light–dark cycle (08.00–20.00 h) at an ambient temperature of $\sim 20^{\circ}\text{C}$. Ewes were fed twice daily with a lucerne chaff mixture with access to water *ad libitum*. The well-being and food and water intake of each ewe was monitored daily throughout the entire experiment.

Animal surgery

Prior to surgery and using a computer random number generator, the pregnant ewes were randomly assigned to either control or UCO group and then randomly assigned to either saline or creatine infusion (saline control, $n = 6$; creatine control, $n = 7$; saline UCO, $n = 8$; creatine UCO, $n = 7$). In this study, microdialysis probes were only inserted into the saline- and creatine-treated UCO fetuses. Ewes and fetuses in this study were used in a previously published study detailing the experimental model and physiological outcomes of the creatine and cord occlusion protocols (Tran et al., 2021). Experimental protocols unique to this study are reported in detail below.

Briefly, at 118 dGA, after withdrawal of food for at least 18 h, each ewe was anaesthetised with intravenous sodium thiopentone (20 mg kg^{-1}), intubated, and the anaesthesia then maintained by mechanical ventilation and inhalation of 1.5–3.5% isoflurane in oxygen:air (70:30). After sterile exteriorisation of the fetus during surgery and following the placement of an inflatable Silastic cuff around the umbilical cord, the fetuses were placed in the prone

position and the head positioned for insertion of a microdialysis probe. An incision was made along the midline of the fetal head to expose the skull, and connective tissue cleared to reveal skull landmarks. A hole 1 mm in diameter was drilled into the skull using external co-ordinates of 5 mm anterior of the coronal suture and 10 mm lateral to the midline of the right hemisphere. A microdialysis probe (CMA 20 Elite; 10 mm polyarylethersulfone membrane, 14 mm shaft length, 20 kDa cutoff; CMA Microdialysis, Kista, Sweden), connected to 1 m-long inlet and outlet tubing (outer diameter: 2.5 mm, internal diameter: 1.7 mm; Microtube Extrusions, North Rocks, Australia), was inserted according to the manufacturer's instruction to a depth of 12 mm for sampling of interstitial fluid from parasagittal cortical grey matter and sub-cortical white matter. The probe was held in place using cyanoacrylate glue and dental acrylic. All fetal incisions were then closed using a continuous suture and all catheters, including a brachial artery and vein catheter and the umbilical cuff catheter, were exteriorised through the right flank of the ewe before closing all maternal incisions. Post-surgery, a fentanyl patch (12.6 mg Durogesic 75, Sandoz, Boucherville, Canada) that provided $75\text{ }\mu\text{g h}^{-1}$ of analgesia was placed on the ewe's left inner thigh (Varcoe et al., 2019). The ewe was allowed 3 days of post-operative recovery during which time antibiotics were administered daily (ewe: oxytetracycline, 50 mg in 5 ml, intravenous injection; fetus: ampicillin, 100 mg in 1 ml suspended in 25 IU ml^{-1} heparinised saline, intravenous injection; intra-amniotic ampicillin, 400 mg in 4 ml suspended in 25 IU ml^{-1} heparinised saline) before the experimental procedures began.

Microdialysis experimental procedures

Ewes were housed in pens that restricted their sideways movement as soon as they had recovered from effects of post-surgery anaesthesia and were moving freely, eating and drinking (usually, 3–4 h). The microdialysis probe inlet and outlet were identified and both catheters extended with pre-sterilised 0.5 m-long vinyl tubing (outer diameter: 2.5 mm, internal diameter: 1.7 mm; Microtube Extrusions) using 100% ethanol-soaked tubing adapters (CMA Microdialysis). The inlet catheter was connected to a syringe pump and the outlet connected to a fraction collector (BASi, West Lafayette, Indiana, USA). Freshly made artificial cerebrospinal fluid (aCSF; 148 mM NaCl, 4 mM KCl, 10 mM NaH_2PO_4 , 40 mM Na_2HPO_4 in sterile water, pH 7.2–7.4) was passed through a $0.22\text{ }\mu\text{m}$ filter, and perfusion of the microdialysis probe at $1\text{ }\mu\text{l min}^{-1}$ was commenced. Care was taken to maintain patency within microdialysis probes by continually perfusing aCSF throughout the entire experimental protocol. Prior to starting the experiment,

the probes were perfused at $1 \mu\text{l min}^{-1}$ and dialysate fluid was collected every 12 h and stored at -80°C . Terephthalic acid (5 mM TA; Sigma-Aldrich, St Louis, MO, USA), a $\cdot\text{OH}$ trapping agent as used by our group previously (Yan et al., 2005), was added to the aCSF 42 h prior to commencing the experiment at 129 dGA to allow for equilibration with the interstitial fluid. Perfusion rate was then increased to $2 \mu\text{l min}^{-1}$ ~ 50 h prior to performing the UCO at 130 dGA to increase dialysate volume collection to $120 \mu\text{l h}^{-1}$. Microdialysis fluid was collected hourly from 24 h prior to UCO and then continuously for 72 h after UCO. Dialysate was collected in capped borosilicate glass sample vials (BASi) using a fraction collector (BASi) and stored at -80°C until biochemical analysis was performed. Transit time from the probe tip to the outlet at the collection vial was determined to be 15 min, and therefore hourly samples were timed to account for this delay, i.e. an hourly sample collection began 15 min after the induction of UCO.

Creatine supplementation and UCO experimental procedures

The creatine supplementation and UCO protocol are described in detail elsewhere (Tran et al., 2021). Briefly, sterilised and filtered creatine monohydrate (Sigma-Aldrich, St Louis, MO, USA) in saline was prepared at a concentration of 12 mg ml^{-1} and was delivered intravenously at 1.5 ml h^{-1} from 121 dGA at 09.00 h until 134 dGA; fetal weight was estimated to be 3 kg, thus, the infusion rate was $\sim 6 \text{ mg kg}^{-1} \text{ h}^{-1}$. Saline fetuses received an isovolumetric administration of 0.9% NaCl, pH 7.4 for the same duration. At 131 dGA, at 09.00 h, the Silastic cuff was inflated for 10 min using a predetermined volume of sterile water that would cause complete occlusion of the blood vessels in the umbilical cord; the internal cuff pressure was $>100 \text{ mmHg}$. Successful UCO was determined by immediate hypertension and bradycardia (Tran et al., 2021). Control fetuses were not subjected to UCO.

Ewes were euthanised at 134 dGA with intravenous pentobarbital sodium (100 mg kg^{-1} ; Lethabarb; Virbac, Milperra, NSW, Australia) and allowing approximately a further 3 min for euthanasia of the fetus. The right hemisphere of the fetal brain was immediately immersion fixed in 4% paraformaldehyde (Merck) in 0.1 M phosphate buffer for 5 days and then coronally sectioned into 5 mm blocks for paraffin embedding.

2-Hydroxy-terephthalic acid HPLC

Terephthalic acid (TA) is an inert, non-fluorescent molecule that forms a single stable isomer, 2-hydroxy-terephthalic acid (2-OH-TA) when oxidised

with $\cdot\text{OH}$. 2-OH-TA is highly fluorescent and can be detected at low concentrations at an excitation of 326 nm and emission at 432 nm (Yan et al., 2005), thereby providing a measurement of extracellular $\cdot\text{OH}$ concentration. Microdialysis samples were measured for 2-OH-TA efflux using HPLC as described previously (Yan et al., 2005). Due to technical issues, microdialysis samples from two animals from the saline UCO group and one animal from the creatine UCO group were not suitable for analysis owing to low and intermittent outflow; thus, the revised group numbers for successful microdialysis experiments are saline UCO, $n = 6$; creatine UCO, $n = 6$.

Briefly, a HPLC consisting of a solvent delivery system (LC-10AT, Shimadzu, Kyoto, Japan), an automatic injector (SIL-20AC, Shimadzu), an online degasser (DGPU-14A, Shimadzu), a C18 reverse-phase column ($5 \mu\text{m}$ particle size, 100 \AA pore size, $250 \text{ mm} \times 4.6 \text{ mm}$; Luna, Phenomenex, Lane Cove, NSW, Australia), a fluorescent detector set at excitation 326 nm and emission 432 nm (RF-20A, Shimadzu), and data collection software (LabSolutions, Shimadzu) was used. Microdialysis samples were diluted to 10% with mobile phase (v/v), and $10 \mu\text{l}$ of diluted sample was automatically injected into the HPLC system with a filtered mobile phase, 50 mM KH_2PO_4 in 30% (v/v) methanol (Sigma-Aldrich); and pH adjusted to 3.2 with 1 M H_3PO_4 (Sigma-Aldrich) at a flow rate of 0.7 ml min^{-1} . Elution of 2-OH-TA occurred at 10.13 min, and 2-OH-TA concentration was determined from a standard curve generated from dissolved 2-OH-TA standards (Merck) after integration of the peak area under the curve (AUC). Assays of samples and standards were run with a calculated inter-assay variability of 1.96% and intra-assay variability of 0.58%.

GC-MS

Targeted metabolomics was performed on microdialysis samples using GC-MS. Due to low volumes caused again by inadequate outflow, one animal from each group was further removed from these analyses, i.e. revised group number for GC-MS analysis is saline UCO, $n = 5$; creatine UCO, $n = 5$. In addition, not all of the hourly samples were analysed, but all time point samples that were measured are reported. An Agilent HP 6890N system equipped with a VF-5 ms capillary column with 10 m Eziguard (J&W Scientific, Folsom, California, USA, 30 m, $250 \mu\text{m}$ inner diameter, $0.25 \mu\text{m}$ film thickness) and an Agilent 5975 MSD (Agilent Technologies, Santa Clara, CA, USA) in the electron ionisation mode with helium as the carrier gas were used. Using 1 ml glass vials (Verex, Phenomenex), $50 \mu\text{l}$ of sample, extraction blanks (MillQ water) or series of serially diluted external unlabelled standards (lactate, pyruvate, glutamate, glycine and glycerol) were

mixed with 50 μl of analytical grade methanol (Fisher Scientific, Waltham, Massachusetts, USA) containing a mixture of stable isotope labelled internal standards (14.4 mM sodium [$^{13}\text{C}_3$]pyruvate (Sigma-Aldrich), 23.7 mM sodium [$^{13}\text{C}_3$]L-lactate (Sigma-Aldrich), 10 mM [$^{13}\text{C}_3$]glycerol (Sigma-Aldrich) and 2.5 mM ^{13}C - and ^{15}N -labelled amino acid mix (MSK-A2-1.2; Cambridge Isotope Laboratories, USA, Tewksbury, Massachusetts, USA)). Samples were dried under vacuum for 40 min at 37°C. Following the drying procedure, which aided in precipitation of salt and small proteins, a second extraction step was performed which involved the addition of 100 μl of cold analytical grade ethanol (Fisher Chemicals) to all samples. The glass vials were then capped, vortexed and centrifuged for 5 min at 4°C 37,000 g. Approximately 100 μl of supernatant was transferred to 250 μl glass inserts in GC glass vials (Agilent) and again dried under vacuum for 30 min at 37°C. Samples were then first derivatised with 25 μl of pyridine containing 20 mg ml $^{-1}$ methoxyamine hydrochloride (Sigma-Aldrich), and then incubated for 1.5 h at 40°C. A second derivatisation step was performed by the addition of 25 μl of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide with 1% *tert*-butyl-dimethyl-chlorosilane (Sigma-Aldrich), followed by incubation for 30 min at 60°C. Two microlitres of the derivatised sample was injected in the splitless mode for a total GC run time of 18 min at a constant helium flow rate of 1 ml min $^{-1}$. The *tert*-butyl-dimethylsilyl (TBDMS) derivatised samples were analysed by selected ion monitoring of lactate (261, 264 *m/z*), pyruvate (174, 177 *m/z*), glycerol (377, 380 *m/z*), glycine (246, 249 *m/z*) and glutamate (432, 438 *m/z*) ions. Absolute metabolite quantification was performed by integration of peak AUC for specific ions using Agilent Mass Hunter Quantitative analysis software (Agilent Technologies) and calculated from linear regression of the serially diluted external (unlabelled) standards using the isotope dilution technique. Assays of samples and standards were run with a calculated inter-assay variability as follows: pyruvate, 8.4%; lactate, 31.6%; glycerol, 14.3%; glycine, 5.2%; and glutamate, 13.4%; and intra-assay variability as follows: pyruvate, 1.3%; lactate, 0.5%; glycerol, 4.8%; glycine, 8.1%; and glutamate, 16.7%.

Immunohistochemistry

Immunohistochemistry was carried out on 8 μm paraffin-embedded sections. Two sections from the parietal-temporal lobe were sampled corresponding to section 720 and section 1120 according to the Michigan State University Sheep Atlas (Johnson et al., 2013). Tissue slides were dewaxed in xylene, rehydrated in decreasing concentrations of ethanol, and antigen retrieval was conducted by heating in citrate buffer (10 mM tri-sodium

citrate in dH $_2$ O, pH 6.0; Sigma-Aldrich). Sections were then rinsed with phosphate buffer saline (PBS), blocked for endogenous peroxidases with 0.3% hydrogen peroxide in methanol for 30 min at room temperature, washed in PBS again then blocked for non-specific binding with 4% BSA in PBS for 4-hydroxynonenal (4-HNE) immunohistochemistry, or 10% normal rabbit serum in PBS for 3-nitrotyrosine (3-NT) immunohistochemistry for 1 h. Sections were then incubated overnight at 4°C with primary rabbit anti-4-HNE antibody (1:500, Merck Millipore, Burlington, Massachusetts, USA), or anti-3NT antibody (1:200, Merck Millipore) in antibody diluent. Negative controls (no primary antibody) were included in all runs and demonstrated no positive staining. The sections were washed and incubated in secondary biotinylated IgG antibody (1:200 in PBS raised in corresponding animal). Antigen-antibody complex was visualised with avidin-biotin complex (ABC) (Vectastain, Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine (MP Biomedicals, Seven Hills, NSW, Australia). Immunohistochemistry stains were counterstained with haematoxylin accordingly.

Slides were scanned at $\times 20$ magnification using an Aperio Scanscope AT Turbo (Leica Biosystems, Nußloch, Baden-Württemberg, Germany). Regions of interest included the cortical grey matter (GM), subcortical white matter (SCWM), periventricular white matter (PVWM), corpus callosum (CC), striatum, putamen, internal capsule, thalamic nuclei and dorsal hippocampus (CA1-CA3 and dentate gyrus). Regions were outlined using Aperio Image Scope (Leica Biosystems) and positive-stained antigens were manually counted for cell density (cells per μm^2) analysis in 6-10 fields of view of 200 \times 200 μm (except for hippocampal CA1-CA3 and CC, where it was 100 \times 100 μm). All analyses were completed blinded to experimental treatment groups.

Data processing and statistical analyses

To determine the stability of basal microdialysis 2-OH-TA and cerebral metabolite efflux over the repeated microdialysis sampling period, baseline collections were compared using repeated measures two-way ANOVA to assess for time or treatment main effects. No significant time effect on efflux levels was found, thereby demonstrating that basal efflux for each parameter was stable across the baseline period (-24 to -1 h). Thus, for each fetus, the means of baseline collections were calculated, and data presented as a percentage change from average basal levels to allow for inter-individual and group comparisons.

To assess changes across time periods, the experimental timeline was separated *a priori* into specific time blocks: early recovery (0-8 h post-UCO), delayed recovery

(9–24 h post-UCO; to account for secondary energy failure (Gunn & Bennet, 2009)), and late recovery (24–72 h post-UCO); these time blocks correspond to that used previously (Tran et al., 2021). Each time block includes a pre-UCO time point (–2 h post-UCO) to assess significant changes from baseline. For each microdialysis analyte, a two-way repeated measures ANOVA was conducted to assess treatment (P_{TREAT}) and time following UCO (P_{TIME}) effects and interaction (P_{INT}) across each of these time blocks. Any significant main effect for time or interaction was followed up using Šidák's multiple comparisons *post hoc* testing.

For immunohistochemistry analyses, two sections per animal were analysed for greater sampling across the fetal brain. Regions that were analysed in both sections (i.e. SCWM, PVWM, GM, CC) were averaged across both sections if no significant sampling effect was found. Data sets were assessed for main effects of UCO (P_{UCO}), main effects of creatine treatment (P_{TREAT}) and interactions between the main effects (P_{INT}) by two-way ANOVA. Where a significant interaction was observed, *post hoc* analysis was performed using Tukey's multiple comparison test.

To determine the correlation between the plasma creatine concentration, blood gas measurements obtained during UCO, and the resulting changes in oxidative and metabolic cerebral metabolites, multiple linear regression modelling was conducted. The methodology and results of plasma creatine concentration and blood gas measurements have been reported elsewhere (Tran et al., 2021). The percentage change from baseline of blood gas parameters was calculated from the value obtained –30 min prior to the start of the UCO and the value at the 9 min time point of the 10 min duration of UCO, and was used to establish the degree of hypoxaemia, hypercapnia and lactic acidosis each fetus experienced. Raw arterial blood gases and metabolites (–30 min, +9 min and +72 h relative to UCO) are reported in Supplementary Data S2. The oxidative and metabolic cerebral metabolite changes were calculated as the AUC of the relative (percentage) change from average basal levels from 0 to 72 h after UCO. This approach was taken to represent the total impact of the hypoxia on the specific metabolite as a result of UCO. The concentration of arterial creatine prior to UCO was calculated as the average creatine plasma concentration across days 4–8 of creatine infusion, i.e. days 6–0 before the UCO. Within this analysis, the saline UCO and creatine UCO groups were combined to assess the effect of arterial creatine concentrations alone irrespective of treatment group. The assumption of little to no multi-collinearity was confirmed if the variance inflation factor was <3.

Data are presented as means \pm SD. Data were assessed for normality using the Shapiro–Wilk test. Statistical significance was accepted for $P \leq 0.05$. ANOVA statistical

analysis and regression modelling was computed using GraphPad Prism (Version 8.4.3 for Windows, GraphPad Software, San Diego, CA, USA). 2-OH-TA and metabolite efflux concentrations are reported uncorrected for probe membrane efficiency.

Results

Effects of creatine supplementation and UCO on interstitial •OH concentrations

Basal levels of 2-OH-TA. Basal cerebral levels of 2-OH-TA efflux were stable across the 24 h prior to commencement of the UCO (Table 1). The mean basal concentration of 2-OH-TA (uncorrected for probe membrane efficiency) for saline- and creatine-treated fetuses was 0.40 ± 0.02 and $0.39 \pm 0.03 \mu\text{mol l}^{-1}$, respectively (Table 1).

Effects of UCO on 2-OH-TA. There were no time or interaction effects for 2-OH-TA concentrations in the dialysate outflow across any of the post-UCO time intervals (Supplementary Data S1). However, creatine treatment led to a decrease in the 2-OH-TA concentration compared to saline fetuses during the early recovery phase (0–8 h post-UCO; $F(1, 10) = 4.96$, $P_{\text{TREAT}} = 0.050$; Fig. 1; Supplementary Data S1). A similar effect for creatine treatment to reduce 2-OH-TA concentrations below saline-treated fetuses occurred in the delayed recovery phase (9–24 h post-UCO; $F(1, 10) = 4.90$, $P_{\text{TREAT}} = 0.050$). By late recovery, 24–72 h after UCO, there were no differences of 2-OH-TA levels between the treatment groups ($F(1, 10) = 1.29$, $P_{\text{TREAT}} = 0.283$).

Effects of creatine supplementation and UCO on cerebral metabolism

Basal concentration of metabolites. Basal interstitial concentrations of lactate, glycine and glutamate during the 24 h prior to UCO (i.e. at –24, –18, –12, –6, –2 h prior to UCO) did not differ between the saline or creatine treatment groups ($P_{\text{TREAT}} > 0.05$; Table 1). In contrast, basal levels of pyruvate and glycerol efflux during the 24 h prior to UCO were significantly lower in creatine-treated fetuses than in saline-treated fetuses (both $P_{\text{TREAT}} = 0.049$; Table 1). As no significant fluctuations with time during the baseline microdialysis sampling period were found for any of the metabolites (–24 to –2 h; $P_{\text{TIME}} > 0.05$; Table 1), the pre-UCO data were used to calculate the relative percentage change from the average baseline levels for each animal (Fig. 2A–E).

Effects of UCO on interstitial metabolite concentrations. There were no interaction or creatine treatment effects for any of the metabolite concentrations across any

Table 1. Two-way ANOVA and combined average baseline concentrations of cerebral interstitial 2-OH-TA and metabolites in saline ($n = 5-6$)- or creatine ($n = 5-6$)-treated fetuses in dialysate (uncorrected for dialysate recovery efficiency) during the 24 h prior to UCO

	Group		Two-way ANOVA		
	Saline	Creatine	P_{TREAT}	P_{TIME}	P_{INT}
2-OH-TA ($\mu\text{mol l}^{-1}$)	0.40 \pm 0.02	0.39 \pm 0.03	0.893	0.261	0.474
Pyruvate ($\mu\text{mol l}^{-1}$)	31.32 \pm 16.98	21.70 \pm 12.34	0.049*	0.659	0.767
Lactate ($\mu\text{mol l}^{-1}$)	671.00 \pm 164.70	748.10 \pm 181.00	0.597	0.201	0.636
Glycerol ($\mu\text{mol l}^{-1}$)	8.52 \pm 2.74	6.52 \pm 3.29	0.049*	0.979	0.866
Glycine ($\mu\text{mol l}^{-1}$)	29.05 \pm 1.76	29.55 \pm 6.65	0.922	0.135	0.060
Glutamate ($\mu\text{mol l}^{-1}$)	9.75 \pm 2.63	7.55 \pm 2.66	0.470	0.678	0.921

Data are means \pm SD. * $P \leq 0.05$: statistically significant creatine treatment effects.

recovery time period following UCO (Supplementary Data S1). There were significant time effects for pyruvate, lactate and glycerol occurring mainly during the early recovery period. The UCO induced a rise in pyruvate, lactate and glycerol concentrations in the cerebral interstitial fluid with a similar temporal pattern identified in both saline- and creatine-treated fetuses during the early recovery stage (0–8 h post-UCO; $F(1.72,$

12.45) = 7.39, $P_{TIME} = 0.009$; $F(2.05, 14.35) = 4.79$, $P_{TIME} = 0.025$ and $F(2.26, 16.38) = 6.24$; $P_{TIME} = 0.008$ respectively, Fig. 2A–C, Supplementary Data S1). Pyruvate concentrations were significantly increased up to ~69% compared to baseline and remained elevated up to 8 h post-UCO in both treatment groups (Fig. 2A). Lactate and glycerol concentrations were significantly increased up to 6 h post-UCO with efflux reaching peak increases

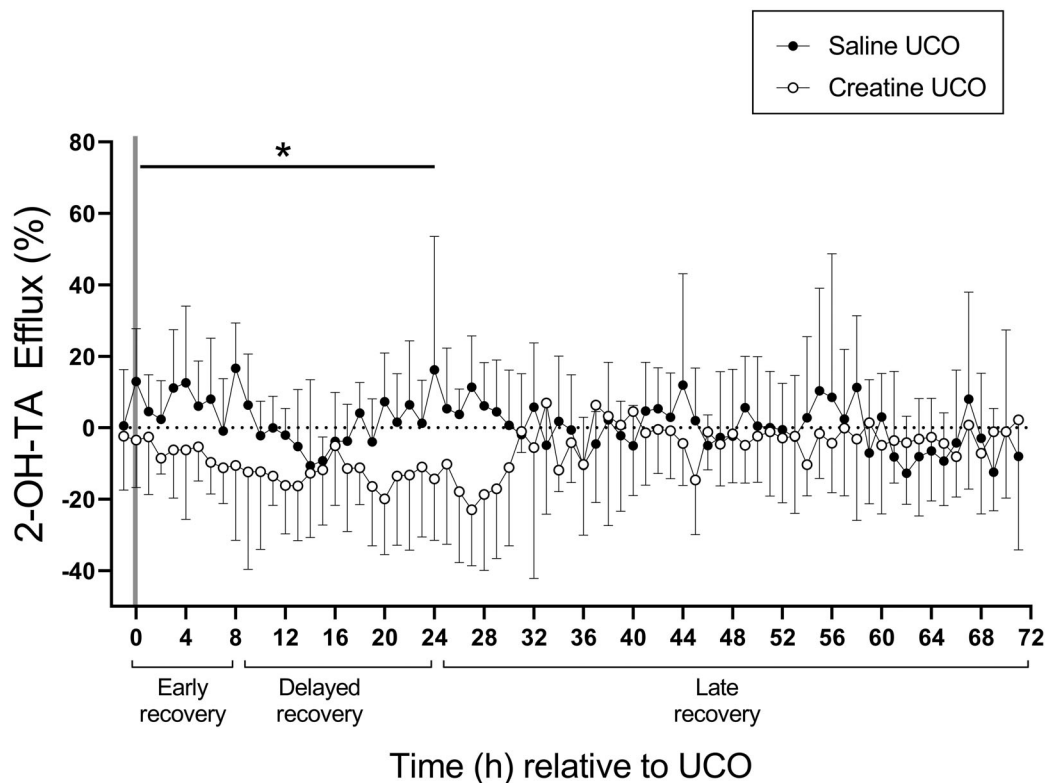


Figure 1. Percentage change from baseline 2-OH-TA microdialysis efflux in the fetal subcortical white matter and cortical grey matter of saline-treated (filled circles; $n = 6$) and creatine-treated (open circles; $n = 6$) fetuses following 10 min UCO occurring at 0 h (grey bar)

First data point represents 1 h pre-UCO. Data calculated as percentage change from pre-UCO values and presented as means \pm SD (saline-UCO, $n = 6$; creatine-UCO, $n = 6$). * $P \leq 0.05$ indicates statistically significant main effect of creatine treatment.

of ~34% and ~201%, respectively (Fig. 2B and C). By the delayed recovery stage (9–24 h post-UCO), both pyruvate and lactate concentrations had returned to near baseline in both treatment groups ($P_{\text{TIME}} > 0.05$). Glycerol concentrations remained affected 9–24 h following UCO irrespective of treatment ($F(3.55, 27.49) = 2.94$, $P_{\text{TIME}} = 0.043$; Supplementary Data S1), and returned by late recovery (>24 h post-UCO). Levels of glycine and glutamate were not affected by UCO, though there was a near-to-significant interaction between time and creatine treatment during the late recovery period for glycine concentrations ($F(8, 63) = 1.98$, $P_{\text{INT}} = 0.064$) (Fig. 2D and E, Supplementary Data S1).

Relationship between arterial creatine concentration and blood gas measurements during UCO on metabolic changes within the fetal brain

Potential relationships between the circulating arterial creatine concentrations prior to UCO, the degree of hypoxaemia, hypercapnia and lactic acidosis experienced by each fetus, and changes in cerebral 2-OH-TA and metabolites following UCO were then assessed with multivariate regression analyses. Overall, there were significant associations between fetal arterial creatine concentrations, arterial P_{O_2} and S_{O_2} , and the cumulative percentage change of cerebral interstitial pyruvate, lactate

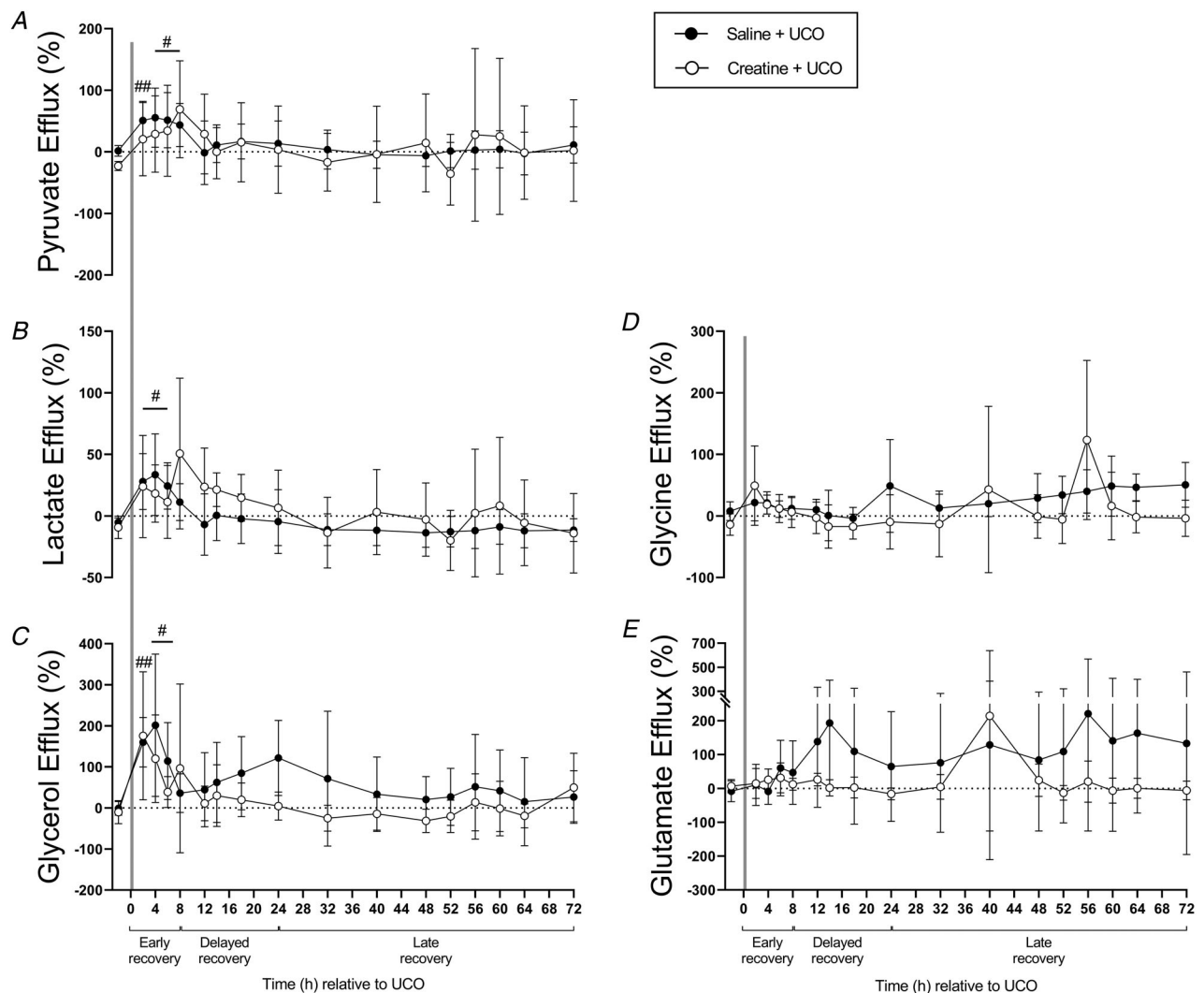


Figure 2. Metabolite efflux from microdialysis sampling in the fetal subcortical white matter and cortical grey matter following UCO

Saline-treated (filled circles; $n = 5$) and creatine-treated (open circles; $n = 5$) fetuses underwent a 10 min UCO occurring at 0 h (grey bar). Levels of pyruvate (A), lactate (B), glycerol (C), glycine (D), and glutamate (E) efflux are presented as percentage change from baseline. There were no significant interaction effects of time and treatment. # $P \leq 0.05$, ## $P \leq 0.01$ refer to a time main effect and indicate statistically significant differences compared to baseline in both saline- and creatine-treated fetuses. Data are means \pm SD.

and 2-OH-TA (Fig. 3; Supplementary Data S3). The level of arterial creatine was positively correlated with the percentage change of P_{O_2} and S_{O_2} , which were then negatively associated with percentage change in cerebral pyruvate accumulation, suggesting that higher levels of arterial creatine and reduced changes in arterial P_{O_2} and S_{O_2} during UCO resulted in lower levels of pyruvate accumulation ($P = 0.006$ and $P < 0.0001$, respectively; Fig. 3; Supplementary Data S3). A similar relationship between higher levels of creatine and reduced changes in arterial S_{O_2} during UCO resulting in lower levels of cerebral lactate accumulation was also found ($P = 0.017$; Fig. 3; Supplementary Data S3). In addition, a significant relationship between arterial creatine, arterial P_{O_2} and cerebral 2-OH-TA was observed, in which higher arterial creatine and reduced changes in P_{O_2} during UCO was associated with lower cerebral 2-OH-TA levels ($P = 0.050$; Fig. 3; Supplementary Data S3).

Effects of creatine supplementation and UCO on histochemical markers of oxidative stress

4-Hydroxynonenal. Lipid peroxidation (4-HNE immunoreactivity) was found in the cytoplasm in all of the

fetal brain regions examined, with some cells displaying intense positive staining within the nucleus (Fig. 4). The level of 4-HNE immunoreactivity was increased in both saline- and creatine-treated fetuses following UCO in the SCWM and thalamus ($F(1, 24) = 6.06$, $P_{UCO} = 0.021$; $F(1, 24) = 4.84$, $P_{UCO} = 0.038$, respectively; Table 2 and Fig. 4). Creatine treatment did not alter the intensity of 4-HNE immunoreactivity or the cell density in any of these regions in either control or UCO fetuses.

3-Nitrotyrosine. Free radical-mediated cell damage can result in 3-NT modification of proteins (Fig. 5). The cellular density of 3-NT positive immunoreactivity increased following UCO in the cortical GM and thalamus in both saline- and creatine-treated fetuses ($F(1, 24) = 4.96$, $P_{UCO} = 0.036$; $F(1, 24) = 4.63$, $P_{UCO} = 0.042$, respectively; Table 2). 3-NT immunoreactivity was predominately within the cytosol, with some positive staining also observable along axons, especially in the cortical GM (Fig. 5). In the hippocampus dentate gyrus (DG), there was a significant interaction between UCO and creatine treatment on 3-NT ($F(1, 24) = 4.50$, $P_{INT} = 0.045$), with 3-NT immunoreactivity almost entirely absent in creatine-treated controls, but elevated in creatine-treated

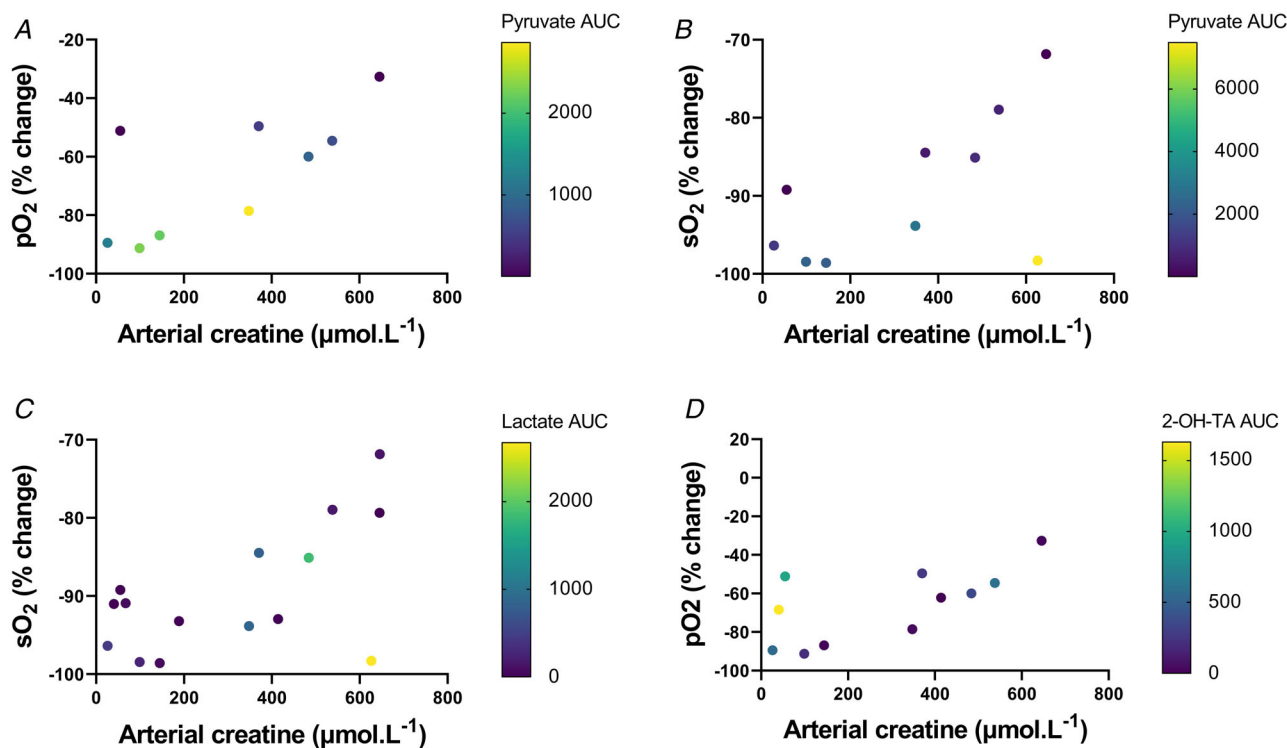


Figure 3. Significant relationships between arterial creatine concentration prior to UCO and percentage change of arterial P_{O_2} (A) and arterial S_{O_2} (B) during UCO to predict cerebral pyruvate accumulation following UCO; arterial S_{O_2} to predict cerebral lactate levels (C); and arterial P_{O_2} to predict cerebral 2-OH-TA levels (D)

Each data point represents a fetus; note there is no measurement of P_{O_2} for one fetus due to technical error. n (number of cases analysed) = 9–11. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 2. Cell density counts (cells mm⁻²) and two-way ANOVA of main effects and interaction of immunohistochemical staining of 4-hydroxynonenal (4-HNE) and 3-nitrotyrosine (3-NT)

Brain region	Control		UCO		Statistics		
	Saline (n = 6)	Creatine (n = 7)	Saline (n = 8)	Creatine (n = 7)	P _{UCO}	P _{TREAT}	P _{INT}
4-HNE							
SCWM	118.75 ± 64.65	138.39 ± 48.13	186.88 ± 78.93	194.46 ± 67.58	0.021*	0.594	0.813
GM	114.38 ± 37.89	74.11 ± 34.68	121.09 ± 62.81	137.50 ± 42.53	0.061	0.510	0.125
PVWM	143.13 ± 99.72	171.61 ± 86.86	203.75 ± 86.78	174.64 ± 98.71	0.375	0.993	0.422
Hipp DG	50.00 ± 32.17	86.31 ± 58.67	74.48 ± 32.77	102.98 ± 43.88	0.223	0.060	0.814
Hipp CA1–3	222.22 ± 223.03	264.29 ± 220.57	420.83 ± 277.42	390.48 ± 218.34	0.086	0.949	0.693
Thalamus	63.89 ± 37.79	64.29 ± 26.34	92.19 ± 47.37	112.5 ± 62.27	0.038*	0.557	0.572
CC	125.00 ± 133.65	95.24 ± 93.15	102.08 ± 65.12	85.71 ± 74.80	0.648	0.517	0.850
Internal capsule	14.58 ± 14.61	88.10 ± 70.85	43.75 ± 65.16	51.19 ± 70.99	0.870	0.100	0.171
Putamen	36.81 ± 41.95	56.55 ± 43.56	38.54 ± 37.38	35.71 ± 33.67	0.527	0.575	0.455
Striatum	18.06 ± 13.35	26.77 ± 13.36	25.00 ± 11.36	29.76 ± 16.91	0.354	0.211	0.709
3-NT							
SCWM	45.83 ± 40.73	9.64 ± 11.40	61.72 ± 67.98	53.93 ± 32.03	0.088	0.206	0.410
GM	31.88 ± 14.81	20.89 ± 12.54	35.16 ± 18.76	45.36 ± 18.01	0.036*	0.951	0.102
PVWM	24.58 ± 28.89	18.04 ± 28.87	43.28 ± 57.71	37.68 ± 28.74	0.214	0.689	0.975
Hipp DG	12.50 ± 22.82	0.00 ± 0.00	9.90 ± 6.66	23.81 ± 24.26	0.102	0.911	0.045*
Hipp CA1–3	2.78 ± 6.81	0.00 ± 0.00	10.42 ± 17.68	33.33 ± 60.09	0.102	0.411	0.296
Thalamus	21.53 ± 19.62	4.17 ± 4.81	38.02 ± 30.41	23.81 ± 23.04	0.042*	0.072	0.853
CC	5.56 ± 8.61	9.52 ± 16.26	43.75 ± 63.58	11.11 ± 13.61	0.177	0.326	0.213
Internal capsule	2.78 ± 5.05	8.33 ± 11.54	3.65 ± 4.69	8.33 ± 8.74	0.890	0.111	0.889
Putamen	9.72 ± 18.38	2.98 ± 3.96	10.42 ± 13.36	3.47 ± 4.87	0.900	0.145	0.983
Striatum	2.78 ± 5.05	10.12 ± 7.93	17.19 ± 25.63	16.07 ± 11.64	0.100	0.605	0.484

Data are means ± SD. Two-way ANOVA: * $P \leq 0.05$ indicates statistically significant main effects or interaction. CC: corpus collosum; GM: grey matter; Hipp CA1–3: hippocampus cornu ammonis; Hipp DG: hippocampus dentate gyrus; PVWM: periventricular white matter; SCWM: subcortical white matter.

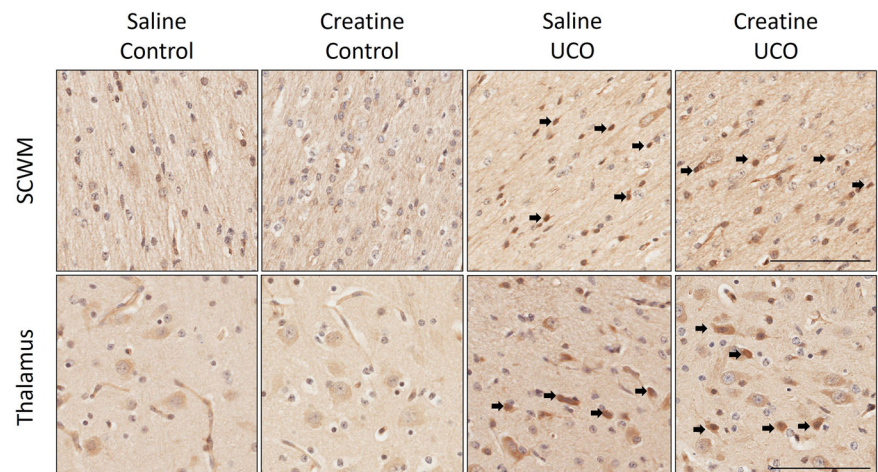
fetuses subjected to UCO ($P = 0.055$), but not compared to the saline-treated fetuses after UCO. There were no correlations between the total magnitude of change of 2-OH-TA following UCO on the cell density of 4-HNE and 3-NT positive immunostaining within the SCWM and GM (Supplementary Data S4).

Discussion

In this study we used microdialysis probes inserted into the near-term fetal sheep brain to capture shifts in interstitial fluid metabolites following acute *in utero* hypoxia. The key findings of this study were, firstly, that creatine

Figure 4. Representative immunohistochemical staining of 4-hydroxynonenal (4-HNE) in subcortical white matter (SCWM) and thalamus

Cell nuclei were counterstained with haematoxylin. Black arrows indicate positive staining of 4-HNE within cells. Bar corresponds to 100 μ m. [Colour figure can be viewed at wileyonlinelibrary.com]



supplementation reduced basal pyruvate and glycerol levels within the cerebral interstitial fluid in the absence of hypoxia; secondly, that creatine treatment reduced hydroxyl radical ($\cdot\text{OH}$) efflux up to 24 h following UCO. However, creatine supplementation did not alter lipid and protein oxidation that was observable in specific brain regions at 72 h following UCO. Lastly, we report a relationship where fetuses with higher arterial creatine concentrations displayed smaller (reduced) changes in arterial P_{O_2} and S_{O_2} during UCO and reduced pyruvate, lactate and 2-OH-TA accumulation within the fetal cerebral interstitial fluid following UCO.

Creatine and oxidative stress

This is the first study to demonstrate an *in vivo* reduction in $\cdot\text{OH}$ in the fetal brain following creatine treatment and UCO. Further, multivariate regression analysis revealed that fetuses with higher circulating creatine levels were associated with reduced 2-OH-TA and a reduced degree of hypoxaemia during the UCO. We hypothesise that this relationship results from a decrease in fetal tissue aerobic metabolism due to the increased creatine levels maintaining ATP turnover for longer under hypoxic conditions, thereby reducing the need for oxidative phosphorylation (Scopes, 1973). The relationship between reduced hypoxaemia during UCO and the smaller changes of cerebral pyruvate and lactate (discussed further below) also supports the hypothesis that there is a reduced reliance on mitochondrial bioenergetics during UCO

conferred by increased creatine levels (Bouillaud et al., 2021). We have previously reported from the same cohort of fetuses that creatine supplementation had no effect on state I, IV or III mitochondrial respiration in the cerebral grey or white matter 72 h after UCO (Muccini et al., 2022). While the relationship between oxygen deprivation and ROS production in the brain is well established (Abramov et al., 2007; Niatetskaya et al., 2012), our results suggest there is now a need to ascertain in real time if creatine preserves mitochondrial bioenergetics during acute hypoxia and investigate the actual cellular and mitochondrial oxygen flux and subsequent ROS rates (Tran et al., 2021).

Improved mitochondrial bioenergetics alone would not explain why 2-OH-TA was consistently below baseline during the immediate and early recovery period in the creatine-treated fetuses. This may not be due to increased $\cdot\text{OH}$ scavenging by intracellular antioxidant defence systems, given that creatine treatment does not enhance the expression and activity of the main antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalases in skeletal muscle (Guimarães-Ferreira et al., 2012; Lawler et al., 2002). A possibility is that creatine can directly scavenge ROS moieties. In a cell-free paradigm, Lawler et al. (2002) and Guimarães-Ferreira et al. (2012) both showed that creatine had direct scavenger effects on the superoxide radical ($\text{O}_2^{\cdot-}$) and the peroxynitrite (OONO^-) anion, but not on the generation of hydrogen peroxide (H_2O_2), although Sestili et al. (2006) was able to demonstrate this capacity for direct scavenging of H_2O_2 in cultured cells.

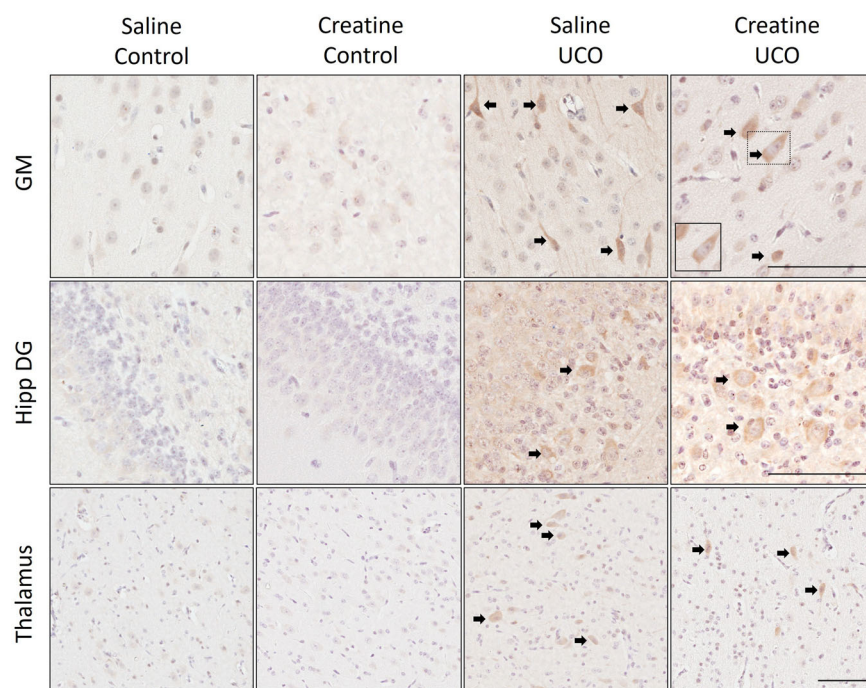


Figure 5. Representative immunohistochemical staining of 3-nitrotyrosine (3-NT) in cortical grey matter (GM), hippocampus dentate gyrus (Hipp DG) and thalamus

Cell nuclei were counterstained with haematoxylin. Black arrows indicate positive staining of 3-NT within cells. Bar corresponds to 100 μm . [Colour figure can be viewed at wileyonlinelibrary.com]

The effects of creatine treatment on the efflux of these other ROS were not assessed in this study, however, as $\cdot\text{OH}$ is formed by successive reduction of oxygen (Chen & Schopfer, 1999), the potentially reduced production of these oxidative metabolites may explain why the average percentage change of $\cdot\text{OH}$ was consistently below zero.

Another potential mechanism for direct reduction of $\cdot\text{OH}$ could involve creatinine, the non-enzymatic breakdown product of creatine, and its capacity to also scavenge $\cdot\text{OH}$ radicals (Ienaga & Yokozawa, 2011; Nakamura et al., 1991). Given we have shown total creatine content is increased in the fetal brain with supplementation (Tran et al., 2021), there may also be a relative increase in the presence of creatinine within the cerebral parenchyma. This would require direct measurement as it remains unknown if creatine supplementation increases brain creatinine levels, and there are conflicting studies on whether serum creatinine levels increase following creatine supplementation (Taes, 2003; Williamson & New, 2014). While it has been established that creatine and creatinine have a lower direct scavenging capacity compared to other enzymatic antioxidants (Lawler et al., 2002), our findings suggest it could support active scavenging of excess ROS in the fetal brain given the limited antioxidant capacity of fetal tissues (Gitto et al., 2009; Saugstad, 1996).

Finally, the known vasodilator effects of increased creatine could have contributed to lower 2-OH-TA following UCO with creatine treatment (Prass et al., 2006). Vasodilatation may have resulted in an effective washing out of $\cdot\text{OH}$ from the extracellular space, but not before this ROS had been able to oxidatively modify cell proteins and lipids. Clearly, a study investigating the effects of increased circulating creatine on cerebral blood flow in the fetal brain should be conducted, perhaps using microspheres so that regional effects on blood flow can be revealed.

Despite the implied antioxidant effects discussed above, and our observed changes in brain metabolites, there was no indication that creatine treatment had ameliorated the increased lipid (4-HNE) and protein (3-NT) oxidation in the SCWM, GM and thalamus observed at the end of the experiment, at 72 h after UCO. The dynamic changes in $\cdot\text{OH}$ efflux found in the current study were not different between groups after 24 h post UCO, i.e. 2 days before histochemical assessments were made. This suggests that despite the seeming return to normal oxidative metabolism, there are possibly other persistent cellular perturbations, or remnants of cellular damage that were not ameliorated by the creatine treatment.

Creatine and cellular metabolites

The efflux of cellular metabolites such as pyruvate, lactate and glycerol occurred in clearly defined phases. Pyruvate and lactate efflux increased soon after the UCO and had

returned to baseline levels by 8 h post-UCO, while glycerol levels remained increased from UCO induction up to 24 h post-UCO. The time to metabolite concentration recovery to baseline is markedly slower compared to other studies in rats that report recovery within 1 h following reoxygenation (Roehl et al., 2012; Zoremba et al., 2007). This slow recovery in the fetal sheep brain might be due to a longer reliance on glycolysis after oxygenation had been restored in this *in utero* ovine model.

The increased pyruvate changes following UCO in the fetuses that experienced greater hypoxaemia during UCO is consistent with the fact that the greater the hypoxia, the greater the need for anaerobic glycolysis with associated increases in pyruvate and lactate production (Siesjö & Nilsson, 1971). Fetuses with higher arterial creatine concentrations and reduced decreases in P_{O_2} and S_{O_2} during the UCO were associated with lower cerebral pyruvate and lactate accumulation suggesting a reduced need for this metabolic shift during and early in recovery following hypoxia. These observations are consistent with studies in intensely contracting skeletal muscle which together support that elevated creatine reduced the shift to glycogenolysis and anaerobic glycolysis during hypoxia and thus reduced changes in pyruvate and lactate flux (Balsom et al., 1995; Scopes, 1973).

Another interesting outcome of this study was an observed reduction in basal pyruvate and glycerol levels in creatine-treated fetuses, prior to UCO. Pyruvate is generated via glycolysis and other cytosolic sources but serves ultimately as a substrate for the mitochondrial citric acid cycle for oxidative phosphorylation and ATP generation. Flux between intracellular and extracellular pyruvate is rapid, with levels between the compartments demonstrated to be closely comparable, at least *in vitro* (O'donnell-Tormey et al., 1987; Quek et al., 2016). Accordingly, the decrease in extracellular pyruvate observed with creatine treatment most likely reflects reduced intracellular pyruvate production. Indeed, increased intracellular phosphocreatine inhibits key glycolytic enzymes – phosphofructokinase and pyruvate kinase – which would subsequently reduce pyruvate production (Kemp, 1973; Storey & Hochachka, 1974). This feedback control is integral to the buffering capability of the creatine kinase phosphagen system as depletion of phosphocreatine and ATP following increased metabolic demand would then lead to the activation of glycolysis to maintain cellular bioenergetics. The decrease in basal cerebral interstitial pyruvate concentration occurred in the absence of changes to basal lactate levels with creatine treatment, suggesting no adverse changes to basal cellular metabolism (Kuhr & Korf, 1988) and maintenance of lactate shuttling between astrocytes and neurons (Pellerin et al., 1998).

The decrease in the basal cerebral interstitial levels of glycerol with creatine supplementation was an

unexpected finding, particularly as 2-OH-TA levels were not changed, nor were those of any other marker suggestive of oxidative cell damage. The reduced glycerol concentrations within the interstitial fluid could have occurred because of reduced lipolysis or increased neuronal glycerol utilisation via phosphorylation or oxidation (Nguyen et al., 2003). Indeed, *in vitro* and *in vivo* studies of creatine supplementation have observed reduced accumulation of triacylglycerol in the liver (Da Silva et al., 2014; Da Silva et al., 2017; Earnest et al., 1996), suggesting that creatine might reduce circulating fatty acids and/or increase fatty acid oxidation. Whether similar mechanisms due to creatine occur in the brain is not known. Fatty acids are actively catabolised for energy production in the fetal brain and are important for fetal brain development as well as during the perinatal transition (Clandinin et al., 1980; Desoye & Herrera, 2021; Steiner, 2019). Therefore, the effect of creatine supplementation during pregnancy on fetal cerebral cellular energy metabolism and fatty acid requirements, even in the absence of hypoxia, needs to be investigated further.

Methodological considerations

The key methodological consideration of this study is the use of microdialysis itself. The insertion of the microdialysis probe into tissue causes mechanical injury and after a time will induce gliosis, ultimately reducing the efficiency and recovery rate of dialysates (Bungay et al., 2003). While there are mathematical modelling approaches to determine microdialysis efficiency, they do not take into account endogenous microvasculature remodelling which must occur after such wounding by implantation of a probe, degree of brain tissue sensitivity, blood flow, tissue resistance and metabolism (Chen et al., 2002; Justice, 1993; Morrison et al., 1991), and also in the circumstance of the fetal brain which continues to develop over time. However, the long duration of the probe implantation in the current study (16 days) is less of a confounding factor because a degree of wound repair must have occurred, as shown by the relative stability of the efflux measurement in the days before the UCO was undertaken. Thus, while probe efficiency and therefore actual metabolite concentrations could not be calculated, the relative changes in metabolite flux allowed us to examine transient and dynamic changes over time.

It is important to note that *in vivo* recovery of microdialysis dialysate is influenced by local blood flow which may have been affected by the UCO itself. As mentioned above, increases in local blood flow may lower *in vivo* dialysate recovery due to increased clearance of molecules from the tissue bed, thereby underestimating measurements within interstitial fluid. This is of particular importance as changes in cerebral blood flow have been

identified up to 4 days post asphyxia, corresponding also to the severity of hypoxic injury (Bennet et al., 1998; Van Bel et al., 1987), as well as an impairment of cerebral vasoreactivity caused by oedema or vascular endothelial injury (Rosenberg, 1988). Whether creatine supplementation affects cerebral blood flow in the fetal brain is unknown but, as mentioned, creatine is known to have vasodilatory effects in the adult brain (Prass et al., 2007). Therefore, relative changes between treatment groups, i.e. saline UCO vs. creatine UCO, may be under- or overestimated. Nonetheless, the data from this study demonstrate that the consequences of altered cerebral metabolism can be measured robustly by the efflux of specific cellular metabolites in the fetal brain using microdialysis.

We report here, as well as in our previous study of the same animals (Tran et al., 2021), that average arterial plasma creatine concentrations were significantly higher in fetuses supplemented with creatine for 10 days, though there remained a range of initial arterial plasma creatine concentrations irrespective of creatine treatment as well as a range of arterial blood gas and metabolite absolute values during UCO. Thus, a strength of conducting multiple variable regression modelling is that it accounts for subject variability, such as that often observed clinically. Furthermore, the multivariate analysis revealed that denoting the degree of hypoxaemia by the relative changes of blood gases, rather than from the absolute values, was predictive of cerebral metabolic changes that followed the UCO. While measurement of relative or absolute changes of fetal oxygenation is unlikely to be clinically feasible, the potential relationships we have identified warrant further investigation.

Conclusion

This is the first study to investigate the *in vivo* temporal metabolic and oxidative milieu of the fetal cerebral interstitial space following creatine supplementation and acute mild hypoxia arising from UCO. While histological assessments 72 h after UCO may not have been able to fully capture beneficial changes afforded by fetal creatine treatment within the brain, our results demonstrate that fetuses with increased levels of arterial creatine prior to UCO displayed a lower degree of hypoxaemia and were likely to have improved cellular bioenergetics, i.e. reduced cerebral pyruvate, lactate and 2-OH-TA accumulation. Further studies are warranted to investigate the effects of these reduced metabolic and oxidative stress responses at different time points relating to the peak metabolic and oxidative stress response and long-term postnatal effects. Overall, the findings of this study provides evidence for creatine supplementation to the fetus to improve cellular metabolic stability as well as reduce reactive oxygen species efflux, specifically $\cdot\text{OH}$, within the fetal brain following an acute asphyxic event.

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Additional information

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Author contributions

Conceptualisation: N.T.T., R.J.S., D.W.W. and S.J.E.; methodology: N.T.T., G.M.K., I.N., N.H., R.J.S., D.W.W. and S.J.E.; data curation: N.T.T. and G.M.K.; data analysis: N.T.T.; writing – original draft preparation: N.T.T.; writing – review and editing: N.T.T., G.M.K., A.M.M., I.N., R.J.S., D.W.W. and S.J.E. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

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Statistical Summary Document

Supplementary Data S1.

Supplementary Data S2.

Supplementary Data S3.

Supplementary Data S4.