Chronic hypoxemia induces mitochondrial respiratory complex gene expression in the fetal sheep brain

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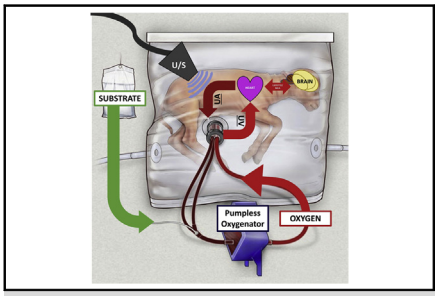
ABSTRACT

Objective: The molecular pathways underlying hypoxemia-induced alterations in neurodevelopment of infants with congenital heart disease have not been delineated. We used transcriptome analysis to investigate differential gene expression induced by hypoxemia in an ovine artificial-womb model.

Methods: Mid-gestation fetal sheep (median [interquartile range] 109 [107-112] days' gestation) were cannulated via the umbilical vessels, attached to a pumpless, low-resistance oxygenator circuit, and incubated in a sterile, fluid environment for 22 [21-23] days. Fetuses were maintained with an oxygen delivery of 20-25 mL/kg/min (normoxemia, n = 3) or 14-16 mL/kg/min (hypoxemia, n = 4). Transcriptional profiling by RNA sequencing was carried out on left frontal brains and hypoxemia-regulated genes were identified by differential gene expression analysis.

Results: A total of 228 genes whose expression was up or down regulated by ≥1.5-fold (false discovery rate ≤0.05) were identified. The majority of these genes were induced in hypoxemic animals compared to normoxemic controls, and functional enrichment analysis identified respiratory electron transport as a pathway strongly upregulated in the brain during chronic hypoxemia. Further examination of hypoxemia-induced genes showed robust induction of all 7 subunits of the mitochondrial NADH:ubiquinone oxidoreductase (complex I). Other hypoxemia-induced genes included cytochrome B, a component of complex III, and ATP6, ATP8, both of which are components of complex V.

Conclusions: Chronic fetal hypoxemia leads to upregulation of multiple mitochondrial respiratory complex genes critical for energy production and reactive oxygen species generation, including complex I. These data provide valuable insight into potential pathways involved in chronic hypoxemia-induced neuropathology and offers potential therapeutic targets for fetal neuroprotection in fetuses with congenital heart defects. (JTCVS Open 2022;10:342-9)



Study of brain transcriptomics in fetal hypoxemia with an ovine artificial womb model.

CENTRAL MESSAGE

Chronic fetal hypoxemia leads to upregulation of multiple mitochondrial respiratory complex genes critical for energy production and reactive oxygen species generation, including complex I.

PERSPECTIVE

Chronic fetal hypoxemia due to congenital heart diseases leads to impairment of brain development. We use an extrauterine support model to study the effects of chronic fetal hypoxemia on the brain. We aim to better understand chronic hypoxemia-induced neuropathology and potential future therapeutic targets for fetal neuroprotection in fetuses with congenital heart defects.

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Abbreviations and Acronyms

ATP = adenosine triphosphate
CHD = congenital heart disease
DO_2 = oxygen delivery
EXTEND = EXTrauterine Environment for Neonatal Development
FFPE = formalin-fixed, paraffin-embedded
ROS = reactive oxygen species

▶ Video clip is available online.

Approximately 6 in 1000 children are affected by severe congenital heart disease (CHD).[1,2] Improvements in perinatal and surgical care have improved the survival of these patient into adulthood to upwards of 90%.[3] Despite increased survival, outcomes in neurocognitive development remain poor.[4] In children with surgically corrected CHD, increased attention deficiency and hyperactivity at school age as well as poor academic performance and motor skills have been observed.[5-7] The persistent neurocognitive deficits despite early surgical correction, as well as magnetic resonance imaging evidence of brain abnormalities in CHD neonates before surgery,[8,9] suggest that the injury may begin during gestation. Advances in fetal imaging directly support this hypothesis, with evidence of brain dysmaturity on fetal magnetic resonance imaging of CHD fetuses.[10,11] In addition, histologic evidence of chronic diffuse white matter injury was seen in electively aborted fetuses with CHD consistent with previous imaging studies.[12]

The leading theory is that cerebral hypoxemia due to altered circulatory anatomy in CHD leads to impairment of brain development. Nevertheless, until recently, in utero investigation of isolated chronic fetal hypoxemia has been difficult. While multiple models of fetal hypoxemia exist, few have been able to isolate the effects of chronic fetal hypoxemia without affecting maternal or placental oxygenation and circulations as well. With the development of an EXTrauterine Environment for Neonatal Development (EXTEND) system—an ovine artificial womb model, however, we have now been able to study the effects of isolated chronic fetal hypoxemia on fetal brain development in depth.[13-15] Histopathologic studies in this model have shown chronic fetal hypoxemia results in white matter hypervascularity, decreased neuronal density, and impaired myelination.[13] Nevertheless, the molecular pathways involved in chronic fetal hypoxemia-induced brain injury remain unclear. The EXTEND system does not mimic the complex in vivo uterine and fetal environment; however, it allows investigation of the impact of modification of a single factor (eg, hypoxemia) while holding other factors constant.

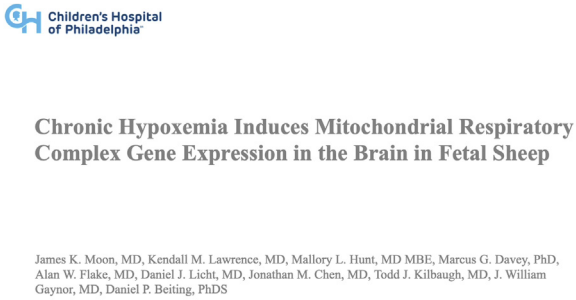
In this study, we compared the brain transcriptome of fetal sheep in the EXTEND system supported under normoxemic versus chronically hypoxemic conditions, which are similar to those found in a human fetus with CHD. While there have been previous fetal brain transcriptome studies looking at the effects of acute hypoxemia on fetal brains in multiple animal models,[16-18] to our knowledge this is the first transcriptomics study investigating the effects of chronic hypoxemia at levels similar to those seen in severe CHD on the fetal brain (Video 1).

METHODS

Animal Experiments

All animal experiments were conducted in accordance with approved protocols by the institutional animal care and use committee of The Children's Hospital of Philadelphia. All ewes tested negative for Q fever serology both at the farm before delivery and after arrival in the laboratory facility. Seven lambs born premature were delivered via hysterectomy and placed into the EXTEND system as previous described in detail by Partridge and colleagues.[19] In short, fetal lambs median 109 ([interquartile range], [107-112]) days' gestation—similar in brain white matter maturity to approximately 28- to 30-week gestation in human fetus[20]—were cannulated via the umbilical vessels onto a low-resistance pumpless oxygenator circuit (Maquet Quadrox-ID Pediatric Oxygenator: Maquet Cardiopulmonary AG). Each animal was then transitioned to a temperature-controlled, sterile fluid-filled environment with continuous exchange of artificial amniotic fluid and supported in the system for 22 [21-23] days (Figure 1).

After 24 hours of initial stabilization in the system, the animals were supported under normoxemic or hypoxemic conditions as described by Lawrence and colleagues.[13] Normoxemia was defined as oxygen delivery (DO_2) 20 to 25 mL/kg/min and hypoxemia was defined as DO_2 14 to 16 mL/kg/min to simulate physiologic normoxemia[21] and pathologic[11,22] hypoxemia at levels seen similar to CHD. DO_2 was continuously monitored in real time throughout the run and recorded (LabChart 5, AD Instruments, Inc) using the formula:



VIDEO 1. Summary of methods and results. Chronic fetal hypoxemia leads to upregulation of multiple mitochondrial respiratory complex genes critical for energy production and reactive oxygen species generation, including complex I. These data provide valuable insight into potential pathways involved in chronic hypoxemia–induced neuropathology and offer potential therapeutic targets for fetal neuroprotection in fetuses with congenital heart defects. Video available at: https://www.jtcvs.org/article/S2666-2736(22)00207-8/fulltext.

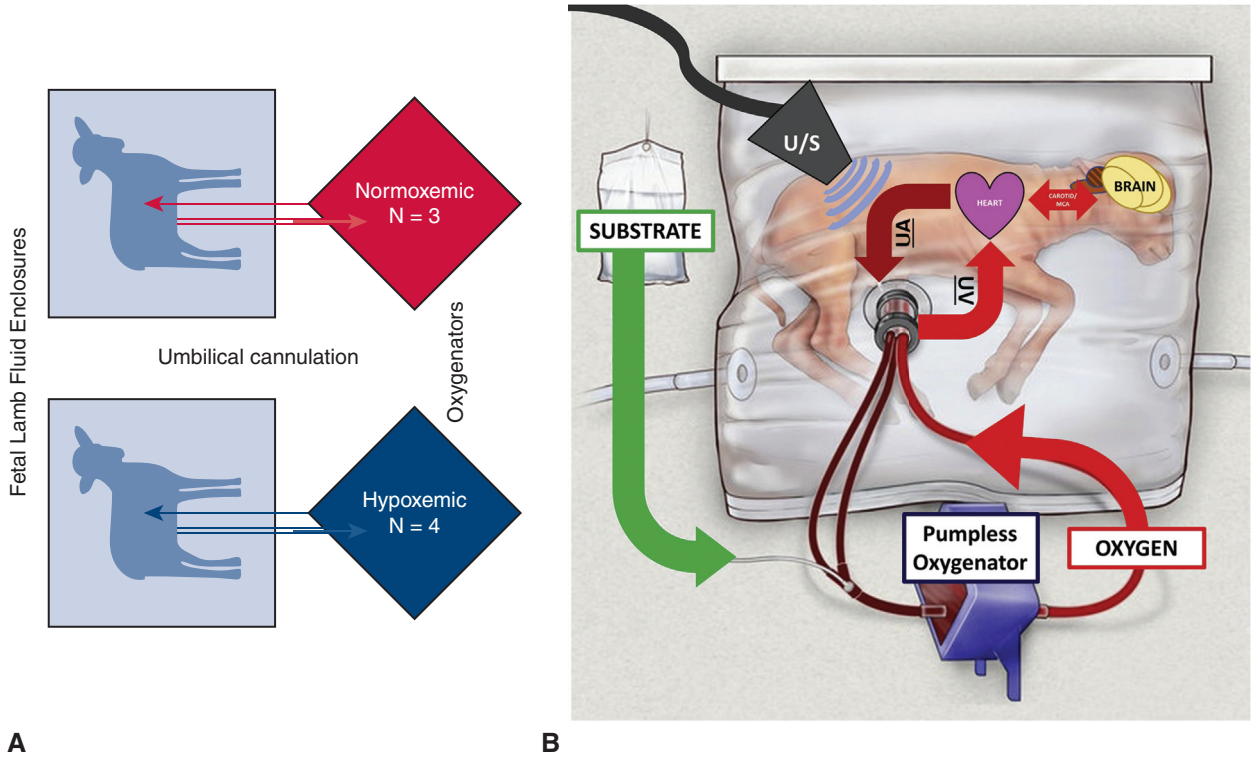


FIGURE 1. Experimental schematic and diagram. A, Experimental schema. B, System enclosure and fetal oxygenation circuit/circulation. *U/S*, Ultrasound; *UA*, umbilical artery; *UV*, umbilical vein. Figure adapted from McGovern and colleagues.[15]

$$DO_2 = \frac{\text{circuit blood flow} \times (1.39\times \text{Hb} \times \text{postoxygenator oxygen saturation})}{\text{Daily body weight}}$$

Weight-based umbilical blood flow (HXL Tubing Flowsensor, Transonic Systems, Inc) and postoxygenator saturation and hematocrit concentration (M2-Sensor, Spectrum Medical) were measured in real-time. Body weight was measured at the time of cesarean delivery and necropsy and estimated daily weights were calculated based on an exponential growth.[19] Target DO_2 was achieved by adjusting fraction of inspired oxygen in sweep gas to the oxygenator. Nutrition was provided by total parental nutrition with dextrose adjusted to a goal blood glucose between 20 and 30 mg/dL, TrophAmine (B. Braun Medical) titrated to a goal blood urea nitrogen between 20 and 30 mg/dL, and intralipid 20% given at 0.01-0.02 g/kg/d.

Statistical Analysis

Analysis of animal characteristics was performed using Stata, version 16 (StataCorp). Sex, age at cannulation, duration of support, and DO_2 were compared between the normoxemic and hypoxemic groups. For categorical variables, a Fisher exact test was performed. Mann–Whitney *U* test was performed for continuous variables. All data are presented as median (interquartile range).

Tissue Preservation

All animals were humanely euthanized at gestational age 131 (130-135) days using an intravenous barbiturate overdose (EUTHASOL; Virbac). The brains were immediately perfusion-fixed via the carotid arteries with 10% formalin at 55 cmH_2O to achieve a perfusion pressure equivalent to a mean arterial pressure of 40 mm Hg. The cerebrum and cerebellum were removed from the skull and consecutive coronal slices (4 mm) were sectioned using a brain matrix (Ted Pella Inc), dehydrated through graded alcohol and embedded in paraffin (formalin-fixed, paraffin-embedded [FFPE]).

Transcriptomic Analysis

Anatomically equivalent coronal sections of the left frontal brain for all experimental animals were identified by a board-certified veterinary neuropathologist, and five 10-μM thick slices from each animal were used for RNA analysis. Samples were sent to GENEWIZ, LLC for RNA extraction, purification, and sequencing. RNA extraction was performed using RNeasy FFPE kit (Qiagen). Ribosomal RNA depletion was carried out using ribozero depletion kit (Illumina) and sequencing libraries were prepared using NEBNext Ultra II RNA library Preparation Kit (New England BioLabs, Inc). Sequencing was carried out using the HiSeq platform (Illumina) to produce paired-end, 150 base pair sequences. Mean sequencing depth was approximately 200 million reads per sample (range of 182.5-208.1 million).

Raw reads were mapped to the sheep (Texel breed) cDNA reference transcriptome, version 3.1 (Ensembl, release 102) using Kallisto, version 0.45. The quality of raw reads, as well as results of Kallisto mapping, were summarized using fastqc and multiqc. Mean percentage of reads mapping was 6.7% (range of 2.6%-12%). Relatively low percent read mapping may be due to incomplete removal of ribosomal sequences and/or poor annotation of the sheep transcriptome. Transcript-level expression data were imported into the R environment (version 4.0.0; R Foundation for Statistical Computing) and summarized to the gene-level using TxImport with annotations provided by BioMart. Filtering was carried out to remove lowly expressed genes, and data were normalized using the Trimmed Mean of M values method in EdgeR. For differential expression analysis, precision weights were applied to each gene based on its mean-variance relationship using the VOOM function from the Limma package. Linear

modeling and Bayesian statistics were employed via Limma to identify genes that were up or down regulated in the hypoxemic animals, compared to normoxemic, by 1.5-fold or more, with a Benjamini–Hochberg adjusted *P* value (false-discovery rate) of less than or equal to .05.

RESULTS

Animal Groups

Seven total animals were included in this study. Four animals were maintained under hypoxemic conditions, whereas 3 animals were maintained under normoxemic conditions. Characteristics of the 2 animal groups are summarized in Table 1. There was no difference in sex ($P = 1.00$), age at cannulation ($P = .12$), duration of support on circuit ($P = .58$), and hemoglobin concentration ($P = .15$) between the 2 groups. After 24 hours of stabilization on circuit, the mean DO_2 was significantly lower for the hypoxemic animals versus the normoxemic animals (14.9 [14.6-15.3] vs 22.1 [20.9-22.7] mL/kg/min, $P = .002$) throughout the study period.

Transcriptomic Analysis

Mapping of RNA-seq reads to the sheep transcriptome reference allowed measurement of the expression of 23,113 annotated transcripts that associated with 14,139 genes. Filtering based on low or no expression further reduced the number of genes to 12,111. Based on the cutoffs described in the Methods ($\geq$1.5-fold up or down regulation with a false-discovery rate $\leq$0.05), 228 genes were differentially expressed between the hypoxemic and normoxemic groups (Online Data Supplement). A volcano plot was then generated (Figure 2, *A*) showing that the majority of these differentially expressed genes were induced in the brain by hypoxemia. Gene Ontology enrichment analysis of these hypoxemia-induced genes identified respiratory electron transport as a pathway strongly upregulated in the brain during chronic hypoxemia (Figure 2, *C*). Further examination of hypoxemia-induced genes showed robust induction of components of the mitochondrial NADH:ubiquinone oxidoreductase, also known as complex I (Figure 2, *D*), including all 7 subunits of the mitochondrially encoded NADH dehydrogenase (ND1, ND2, ND3, ND4, ND5, ND6, and ND4L). ND6 was the most highly upregulated gene, increasing nearly 5-fold in hypoxemic compared with normoxemic animals (Figure 2, *A* and *B*). Other hypoxemia-induced genes included cytochrome B, a component of complex III, and mitochondrially encoded adenosine triphosphate (ATP) synthase membrane subunit-6 and -8 (ATP6, ATP8), both of which are components of complex V (Figure 2, *A* and *B*).

TABLE 1. Animal characteristics by group (N = 7)

	Normoxemic (n = 3)	Hypoxemic (n = 4)	*P* value
Male sex, n	2	2	1.00
Age at cannulation, d	107 (107-109)	110.5 (108.5-113.5)	.11
Duration of support, d	22 (21-27)	22 (21-23)	.71
Hemoglobin, g/dL	12.1 (11.9-12.3)	13.6 (12.7-14.6)	.15
Oxygen delivery, mL/kg/min	22.1 (20.9-22.7)	14.9 (14.6-15.3)	.034

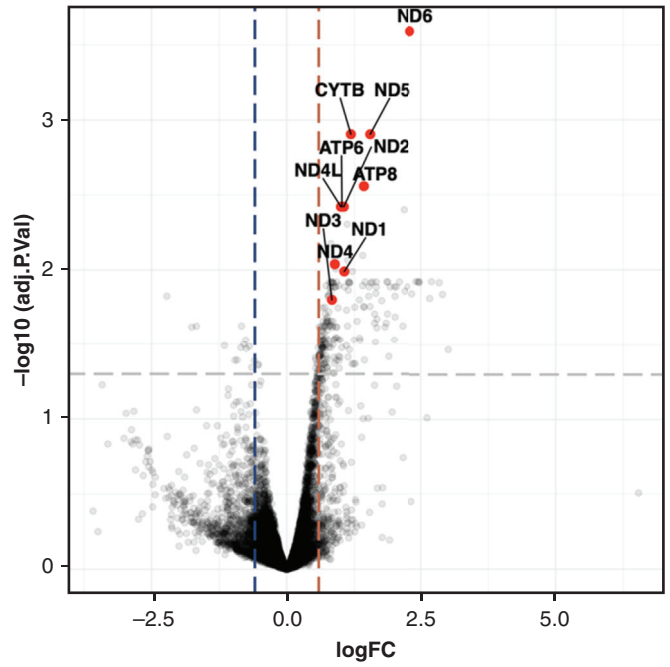
All data shown as median (interquartile range).

DISCUSSION

This study shows that chronic fetal hypoxemia induces upregulation of multiple genes in the brain including a robust upregulation of mitochondrial complex I. Previous studies from our group have shown the effects of chronic intrauterine hypoxemia on neurodevelopment on a histopathologic level. Using the same animal model as this study, we demonstrated that chronic, sustained fetal hypoxemia led to altered cerebrovascular resistance and loss of brain mass.[15] Histologically, white matter hypervascularity, decreased neuronal density, and impaired myelination was seen in chronically hypoxemic fetal ovine brains, similar to that observed in children with congenital heart disease.[13] Nonetheless, the molecular mechanism underlying these histopathologic changes had not yet been studied. Here for the first time, we demonstrate upregulation of mitochondrial complex I under conditions of chronic hypoxemia at levels similar to those seen in CHD.

Previous efforts have largely focused on the effects of acute hypoxemic injury to fetal and neonatal animal models. Limited studies have shown the effects of acute hypoxemia on fetal and neonatal brain transcriptomic profiles including upregulation of chemokines,[17] purine binding and signaling,[18] and hypoxia-inducible transcription factor–dependent genes and apoptosis-promoting factors.[16] However, these studies were not designed to accurately model the effects of chronic hypoxemia in a fetus with CHD. Our unique model by no means exactly replicates the complex fetal and intrauterine environment of CHD. The effects of altered cardiac circulation, placental response to fetal hypoxemia, as well as maternal contributions are not modeled in our system. Nonetheless, to our knowledge, it is the first model to truly isolate the effects of chronic fetal hypoxemia while keeping all other maternal and placental variables constant. We believe first understanding potential effects of isolated components without confounding factors is essential in eventually understanding a more complex disease process such as CHD.

In our investigation of the chronically hypoxemic fetal brain transcriptome, while numerous individual genes were upregulated, gene onotology enrichment analysis identified up-regulation of several mitochondrial genes including complex I. Mitochondria are the fulcrum



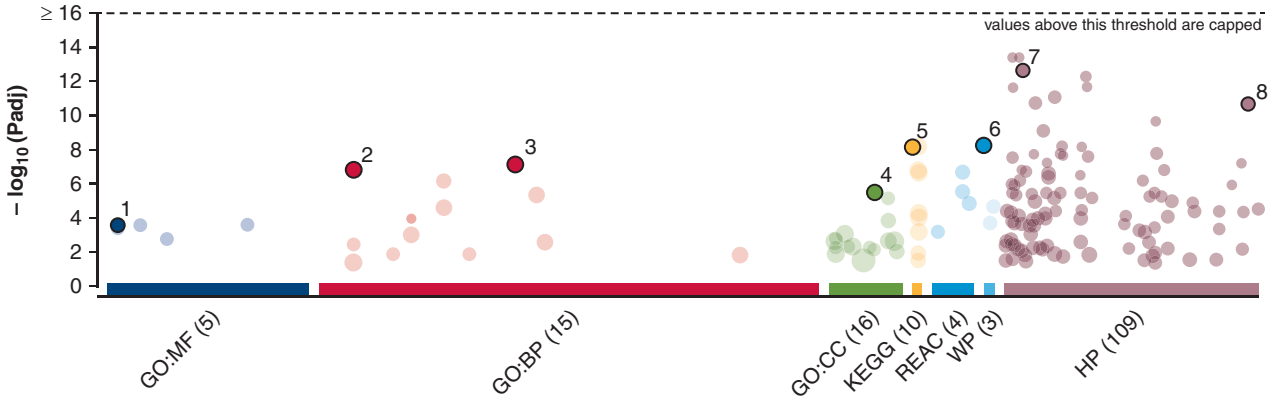
A

Mitochondrial genes
induced by hypoxemia

geneID	normoxemic.AVG	hypoxemic.AVG	LogFC
ND6[1]	10.0	12.3	2.3
ND5[1]	12.4	13.9	1.5
ATP8[2]	8.2	9.6	1.4
CYTB[3]	12.2	13.4	1.2
ND1[1]	11.8	12.9	1.1
ND2[1]	12.0	13.1	1.1
ATP6[2]	12.0	13.0	1.0
ND4L[1]	9.7	10.7	1.0
ND4[1]	13.1	13.9	0.9
ND3[1]	9.9	10.8	0.8

[1]component of Complex I
[2]component of Complex V
[3]component of Complex III

B



C

FIGURE 2. Elevated expression of mitochondrial respiratory genes in the lamb brain during hypoxemia. A, Volcano plot comparing hypoxemic to normoxemic animals. Genes differentially up-versus downregulated by a log2 fold change of 0.59 or greater are marked by vertical red and blue line, respectively. *Horizontal gray line* marks a false discovery rate of 0.05. Key mitochondrial respiration genes are indicated in red and labeled. B, Table showing average expression and log2-fold change of the top 10 mitochondrial respiratory genes from (A) (*red points*) and their association with either complex I, III, or V. C, Gene Ontology (*GO*) enrichment plot showing enriched terms for the 215 genes upregulated in hypoxemic versus normoxemic brain. Eight of the most enriched terms across distinct ontologic sources are highlighted and described in (D). *ND*, NADH Dehydrogenase; *CYTB*, cytochrome B; *ATP*, adenosine triphosphate; *FC*, fold change; *REAC*, reactome.

controlling the life and death of the cell, especially in organs with high energy requirements, and thus constitute a critical area of investigation. Recent studies have shown the importance of mitochondria, both in terms of function and dynamics, on brain development.[23] Furthermore, the interplay between mitochondrial bioenergetics, cellular metabolism, and mitochondrial gene regulation following ischemia may underlie the pathology of secondary brain injury in the immature brain.[24-26] Complex I in particular plays a vital role in energy production as the key driver of the proton motive force that is used by ATP synthase for ATP production and is highly susceptible to functional and structural damage. Beyond the immediate loss of energy reservoirs, acute brain injury causes dysregulation of the respiratory chain, disruption of the cellular metabolome that produces respiratory chain substrates/

D

Label Source	Term ID	Term Name	Adjusted *P* value
1 Gene Ontology: Molecular Function (GO:MF)	GO:0003954	NADH dehydrogenase activity	3.110 × 10^–4
2 Gene Ontology: Biological Processs (GO:BP)	GO:0006119	oxidative phosphorylation	1.719 × 10^–7
3 Gene Ontology: Biological Processs (GO:BP)	GO:0042773	ATP synthesis coupled electron transport	9.936 × 10^–8
4 Gene Ontology: Cellular Component (GO:CC)	GO:0070469	respirasome	3.864 × 10^–6
5 Kyoto Encyclopedia of Genes and Genomes (KEGG)	KEGG:00190	Oxidative phosphorylation	8.298 × 10^–9
6 WikiPathways (WP)	WP111	Electron Transport Chain (OXPHOS system in mitochondria)	6.205 × 10^–9
7 Human Phenotype Ontology (HP)	HP:0001427	Mitochondrial inheritance	2.479 × 10^–13
8 Human Phenotype Ontology (HP)	HP:0200125	Mitochondrial respiratory chain defects	2.496 × 10^–11

FIGURE 2. Continued.

intermediates, production of reactive oxygen species (ROS), and triggers the imbalance of mitochondrial dynamics (fusion, fission, biogenesis, mitophagy). This cascade leads to cell death and an ongoing neuroinflammatory processes that cause long-term neuronal, encephalopathic damage and life-long disability.[27,28]

How chronic hypoxemia affects mitochondria—in particular, complex I—in the neonatal brain is unknown. While no directly comparable studies exist, certain conformational states of complex I have been shown to be upregulated in a model of neonatal acute hypoxemia,[29] consistent with our results. Studies of the structure of complex I support the hypothesis that conformational dynamics of complex I play a role in response to hypoxemia by regulating ROS production via reversed electron transfer.[30] Thus, the response of complex I to chronic hypoxemia represents a potential focal point for intervention. Therapies that target various aspects of mitochondrial function and ROS production have been shown to decrease synaptic plasticity, improve communication deficits, and motor impairments in patients with congenital mitochondrial deficits[31] and thus may represent a potential focal point for future interventions in children with CHD. Furthermore, how complex I adapts to additional stressors such a cardiopulmonary bypass[32] or sudden increase in oxygen tension in the brain postpartum, either during mechanical ventilation and perioperative, is a critical knowledge gap, as increased oxygen tension postoperatively could provide more cellular fuel for ROS production following conformational dynamic changes of complex I discovered in this study.

Interestingly, hypoxia inducible factor 1-alpha, a master regulator of transcriptional responses to hypoxemia[33,34] was not upregulated in our study. However, this is consistent with our previous study that demonstrated an acute elevation of hypoxia inducible factor 1-alpha in peripheral blood mononuclear cells in response to hypoxemia that normalized by the third week of hypoxemia,[35] indicating an adaptive response to hypoxemia in the chronic setting.

As an exploratory pilot study, there were limitations to this study, including the small sample size in each group, due to the significant resources needed to sustain each animal on the EXTEND circuit long-term. In addition, the sample brain tissue was preserved in FFPE blocks for the primary histopathologic analysis which limited the efficiency and quality of RNA sequencing. Furthermore, caution must be taken, as this initial study does not include protein level correlation of the RNA expression. However, all samples across the 2 groups were processed using the same protocol, eliminating any bias due to sample processing, and gene ontology enrichment analysis aided in strengthening our findings by highlighting pathways with multiple upregulated genes rather than focusing on individual gene expression. In addition, the hypoxemia was induced starting midgestation, rather than starting with organogenesis. Despite these limitations, transcriptomics analysis showed statistically significant differential expression of multiple genes between the 2 groups, which may direct future follow up studies with goals of larger sample sizes. Finally, in our model, while the DO_2 mimics that of CHD, other physiologic conditions associated with CHD are not replicated (ie, placental insufficiency, flow pattern abnormalities, impaired substrate delivery). Instead, we were able to isolate out the effects of chronic hypoxemia on a fetal brain while holding all other parameters constant.

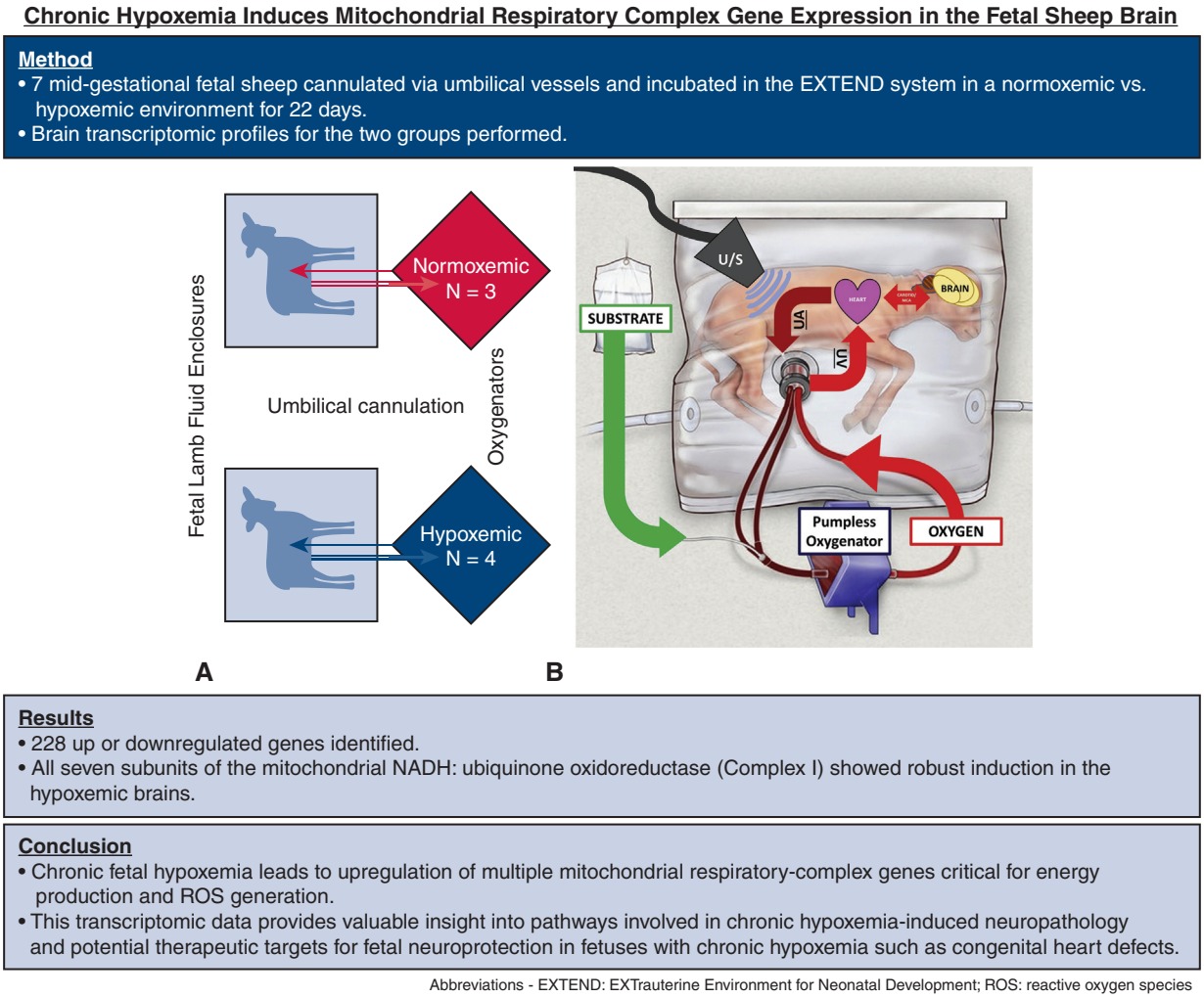


FIGURE 3. Chronic hypoxemia induces mitochondrial respiratory complex gene expression in the fetal sheep brain.

CONCLUSIONS

In conclusion, chronic fetal hypoxemia leads to upregulation of multiple mitochondrial respiratory complex genes critical for energy production and reactive oxygen species generation, including complex I. This transcriptomic data provides valuable insight into potential pathways involved in chronic hypoxemia-induced neuropathology and potential future therapeutic targets for fetal neuroprotection in fetuses with congenital heart defects (Figure 3).

Conflict of Interest Statement

Alan W. Flake holds multiple patents related to the EXTEND technology and is a Clinical Advisor for the Vitara Biomedical Inc. Marcus G. Davey holds multiple patents related to the EXTEND technology and is the Vice President of preclinical research at Vitara Biomedical Incorporated. All other authors reported no conflicts of interest.

The *Journal* policy requires editors and reviewers to disclose conflicts of interest and to decline handling or reviewing manuscripts for which they may have a conflict of interest. The editors and reviewers of this article have no conflicts of interest.

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