

ORIGINAL ARTICLE

Abnormal fetal cerebral and vascular development in hypoplastic left heart syndrome

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Abstract

Objective: To assess the cerebral and vascular development in fetuses with hypoplastic left heart syndrome (HLHS).

Methods: Pregnant women carrying fetuses diagnosed with HLHS who decided to interrupt their pregnancies were included in our study. Aortic size and blood flow were assessed based from fetal echocardiography. Immunohistochemical staining was performed in brain sections obtained from pathology in fetuses with HLHS and control fetuses without heart disease.

Results: Twenty-seven midgestation fetal HLHS were included (gestational age, 23.3 ± 3.4 weeks). Head circumference z scores were lower in HLHS fetuses. Middle cerebral artery pulsatility index, a measure of cerebrovascular resistance, was inversely correlated with the ascending aortic z score ($P < 0.05$). Fetuses with HLHS had lower capillary density in the germinal matrix and their capillaries were larger compared with control fetuses with ($P < 0.05$). The expression of neuronal differentiation marker, FGFR1, and oligodendrocyte precursor, O4, were lower in HLHS brains compared with controls ($P < 0.05$).

Conclusion: Our study identified abnormalities of vascular flow and structural brain abnormalities in fetal HLHS associated with impaired neuronal and oligodendrocyte differentiation, as well as cerebral growth impairment, early in gestation. These findings may be related in part to early vascular abnormalities.

1 | INTRODUCTION

Neurodevelopmental abnormalities (low motor scores, low IQ scores) are common in children with HLHS.¹ Improved survival following surgery has uncovered abnormalities in neurodevelopmental outcomes that manifest in late infancy and early childhood. Although initially thought to be secondary to postnatal and perioperative insult, neuro-radiological studies have shown abnormal cerebral structure in utero in association with delayed brain growth in neonates with HLHS.²⁻⁶

Studies have also detected cranial ultrasound abnormalities (cerebral edema, ventriculomegaly)⁷ and MRI abnormalities⁸ in infants with HLHS prior to cardiac surgery. Neonates with HLHS have been reported to have smaller head circumference emphasizing that prenatal factors may contribute to the cerebral growth impairment in HLHS.⁹

In fetuses with aortic atresia, brain perfusion is dependent on retrograde flow via the ductus arteriosus (Figure 1). Fetuses with severe HLHS (aortic atresia) have little or no antegrade blood flow in the aorta that supplies the head vessels, which limits fetal cerebral blood perfusion. Blood flow is a critical stimulus for vasculogenesis and

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induces angiogenic factors such as vascular endothelial growth factor (VEGF), to promote the formation of new blood vessels through stem cell recruitment and differentiation. The effect of reduced cerebral blood flow during fetal life on cerebrovascular development, in HLHS is not known. The aim of our study was to assess cerebral and vascular growth in fetuses with HLHS compared with normal controls.

While structural brain abnormalities and abnormalities in neurodevelopment have been reported in HLHS, our study describes abnormal vascular development that may account for abnormal neuronal and white matter differentiation in the developing fetus with HLHS.

2 | METHODS

The study was approved by the Hospital for Sick Children and Mount Sinai Hospital research ethics board. Pregnant women carrying fetuses diagnosed with HLHS who decided to interrupt their pregnancies and consented for fetal autopsies and inclusion in the study were enrolled prenatally. Fetal echocardiograms performed at the Hospital for Sick Children were reviewed by a single echocardiographer and de-identified data were analyzed. Pulsatility index of the middle cerebral artery (MCA) and direction of blood flow across the aortic arch was measured. Pulsatility index is a measure of vascular resistance in the circulatory bed downstream from the point of Doppler sampling.¹⁰ It is calculated according to the equation: Pulsatility index = (systolic velocity – diastolic velocity)/mean velocity. Fetal cardiac and brain pathology findings at autopsy were recorded including body weight, head circumference, and ascending aortic diameter.

Hearts and brains from fetal autopsies with and without HLHS were accessed from pathology. Cardiac and brain paraffin-embedded sections were incubated overnight at 4°C with a polyclonal vWF antibody, CD133, and VEGF (1:400, 1:200, and 1:100, respectively) (Abcam, Cambridge, Massachusetts). FITC conjugated anti-rabbit IgG was used as secondary antibody (1:400) (Sigma-Aldrich, St. Louis, Missouri). Slides were counterstained with DAPI. Images were captured using a Nikon Eclipse E1000 microscope (400× magnification). Immunohistochemistry was performed for Fibroblast Growth Factor Receptor 1 (FGFR1) (neuronal differentiation marker) and Oligodendrocyte 4 (O4) (oligodendrocyte progenitor marker). Monoclonal FGFR1 (1:100) (Abcam) and anti-O4 (1:200) (R&D Systems Inc., Minneapolis, Minnesota) were used as primary antibodies. A horseradish peroxidase conjugated streptavidin and 3'-diaminobenzidine (DAB) method (Histostain Kit, Zymed Laboratories, Life Technologies, Carlsbad, California) was used according to the manufacturer's protocol. FGFR1 and O4 stains were visualized using a Leica DC 500 microscope (600× and 400× magnification, respectively).

2.1 | Statistical analysis

Echocardiographic data obtained from HLHS fetuses was analyzed by converting measurements into z scores using published normative data from a large population of healthy fetuses.¹¹ Doppler indices were compared between fetuses with and without antegrade aortic flow. Capillary density, capillary area, intensity of CD133 fluorescence, O4 expression, and proportion of VEGF or FGFR1 positive cells in four

What is known about this topic?

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What does this study add?

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brain layers (germinal matrix, intermediate layer, subcortex, and cortex) were compared between HLHS and control brains. Student *t* test or one-way analysis of variance (ANOVA) with post hoc Bonferroni testing was used to determine differences between groups. A *P* value of <0.05 was considered significant.

3 | RESULTS

Twenty-seven midgestational fetuses with HLHS (mean gestational age, 23.3 ± 3.4 weeks) were evaluated for cardiac and brain findings at autopsy. Fetal echocardiograms were available in 16 HLHS fetuses

Hypoplastic left heart syndrome

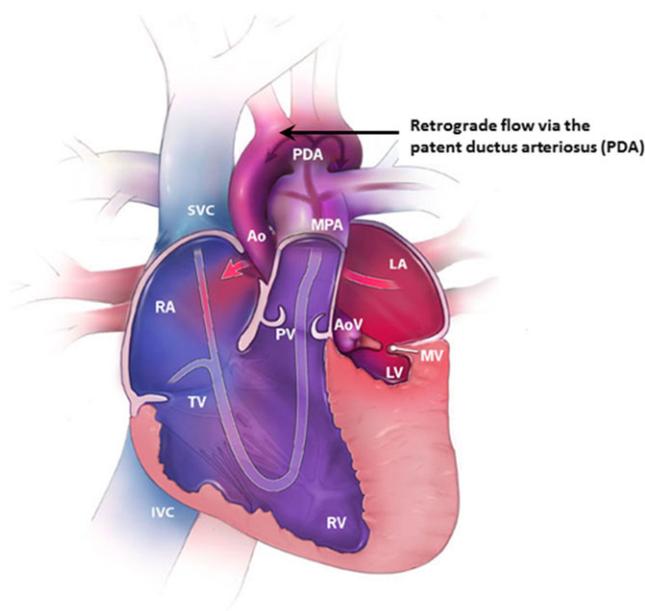


FIGURE 1 Anatomy of the HLHS heart. The diagram illustrates an atretic aortic and mitral valve, hypoplasia of the ascending aorta, and a hypoplastic left ventricle. The black arrow shows retrograde blood flow in the head vessels via a patent ductus arteriosus. We acknowledge the Centers for Disease Control and Prevention, National Center on Birth Defects and Developmental Disabilities for the image. RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle; PDA, patent ductus arteriosus; SVC, superior vena cava; IVC, inferior vena cava; MPA, main pulmonary artery; Ao, aorta; TV, tricuspid valve; MV, mitral valve; PV, pulmonary valve; AoV, aortic valve

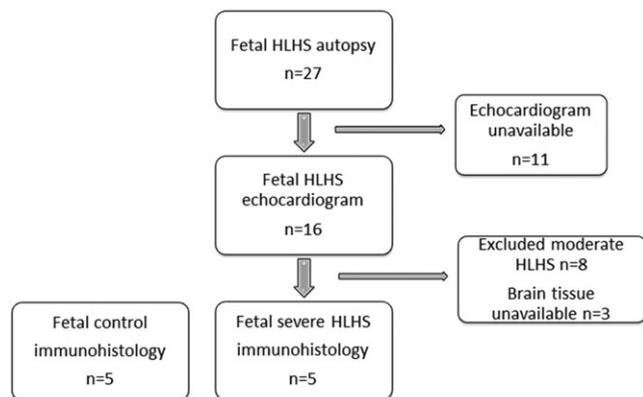


FIGURE 2 Flow chart of study population. Cardiac and brain anatomy and morphology were obtained from 27 fetuses with hypoplastic left heart syndrome (HLHS) at autopsy including body weight, head circumference, and ascending aortic diameter. Echocardiograms were available from 16 fetal HLHS cases. Immunohistology was focused on fetal brains with severe HLHS ($n = 5$) and compared with age-matched control fetuses with normal hearts ($n = 5$)

performed at our center. Brain sections were available in five severe HLHS cases (moderate HLHS were excluded for this analysis). A flow chart of the study population is summarized in Figure 2.

Autopsy revealed that the severity of HLHS varied from moderate (mitral and aortic stenosis) ($n = 12$) to severe (mitral and/or aortic atresia) ($n = 15$). Associated cardiovascular lesions included endocardial fibroelastosis ($n = 2$), persistent left posterior cardinal vein ($n = 1$), ventricular septal defect ($n = 5$), and anomalous pulmonary venous connection ($n = 1$). Karyotype was normal in 22 HLHS fetuses. Chromosomal abnormalities were detected in five fetuses, 45 XO ($n = 2$), trisomy 18 ($n = 2$), and trisomy 21 ($n = 1$). Mean body weight z score was -0.17 ± 1.02 . Mean head circumference z score was -0.57 ± 0.76 . Although within the normal range, the lower z scores for head circumference suggests that cerebral growth impairment begins in early gestation, ie, second trimester. Central nervous system findings showed that 6 out of 27 fetuses with HLHS demonstrated structural brain abnormalities, and only one had severe HLHS and only

TABLE 1 Structural brain abnormalities on fetal autopsy in HLHS

Neurological Findings	Cardiac Diagnosis
Inferior cerebellar hypoplasia ($n = 1$)	Moderate HLHS
Absent olfactory bulbs, tracts; absent corpus callosum; retrocerebellar cyst; ventriculomegaly; patent, enlarged central canal, spinal cord ($n = 1$)	Moderate HLHS
Frontal encephalocele, right; polymicrogyria, localized; cerebellar dysplasia ($n = 1$)	Severe HLHS
Polymicrogyria, ventriculomegaly, inferior olivary dysplasia, fasciculation of descending tracts, basis pontis (anatomical variant?) ($n = 1$)	Moderate HLHS
Nonspecific finding: rounding of lateral angle of lateral ventricles, generous cerebral aqueduct ($n = 1$)	Moderate HLHS
Microcephaly with delayed cortical development; hypoplasia temporal lobes; excess early gyration, parasagittal cortex; moderate autolytic artefact in fetus with trisomy 18 ($n = 1$)	Moderate HLHS

Abbreviation: HLHS, hypoplastic left heart syndrome.

one had chromosomal abnormalities (Table 1). There was no association of structural brain abnormalities with severity of HLHS or concomitant cardiac lesions.

Echocardiographic data were available in a subset of 16 HLHS fetuses. Ascending aortic size was smaller in 11 fetuses with retrograde aortic flow (0.17 ± 0.04 cm; z score, -5.53) compared with five fetuses with antegrade aortic blood flow (0.33 ± 0.07 cm; z score, -0.72) ($P = 0.003$). MCA pulsatility index, a measure of cerebral vascular resistance, in five fetuses with severe HLHS was 1.71 ± 0.7 with an inverse correlation between the MCA pulsatility index and the ascending aortic z score ($r^2 = 0.89$, $P = 0.015$); ie, resistance was higher in those with smaller aortas.

Brain sections from five fetuses with HLHS and from five gestational age-matched control fetuses with normal hearts as determined by fetal echocardiography and autopsy were analyzed. The capillary density, ie, proportion of vWF positive cells, was significantly lower in the germinal matrix of HLHS compared with controls (0.36 ± 0.09 vs 0.91 ± 0.25 , $P = 0.0006$) (Figure 3). The capillary density was also lower in the cortex, although the difference did not reach statistical significance (0.19 ± 0.14 vs 0.46 ± 0.29 , $P = 0.08$). Cross-sectional area assessment showed that capillaries were significantly larger in HLHS brains compared with controls ($P = 0.005$) (Figure 4). This may indicate compensatory vessel dilation in response to reduced cerebral blood flow. The expression of CD133, an early stem cell marker, was lower in blood vessels in all layers of HLHS brains compared with controls suggesting abnormal vascular development (Figure 4). There was however no difference in angiogenic factor, VEGF, expression between the two groups (data not shown).

Nuclear FGFR1 induces neuronal differentiation. The percentage of FGFR1 positive neurons was lower in the cortex of HLHS compared with controls ($11 \pm 16.8\%$ vs $58.7 \pm 6.6\%$, respectively; $P = 0.002$) and in the subcortex of HLHS compared with controls ($13.3 \pm 11.5\%$ vs $40.3 \pm 8.6\%$; $P = 0.009$) (Figure 5). Oligodendrocyte progenitors are important for axonal myelination and were measured using O4 axonal staining (Figure 6). Scores from 0 (for no expression) to 5 (for highest expression) were given, which showed lower scores in HLHS intermediate layer compared with controls (1.5 ± 0.5 vs 4.3 ± 0.8 , respectively; $P = 0.005$) and in HLHS subcortical layer compared with controls (1.8 ± 0.3 vs 3.3 ± 0.6 , respectively; $P = 0.007$).

4 | DISCUSSION

A high proportion of neonates born with complex congenital heart diseases have neurodevelopmental problems prior to surgery raising concern about in utero cerebral injury.¹²⁻¹⁴ In our study, structural brain abnormalities were seen predominantly in moderate rather than severe HLHS suggesting that the neuronal and vascular maturation abnormalities seen on histopathology in severe HLHS in our study were independent of the structural brain abnormalities.

The hypothesis is that in utero hypoxia or altered cerebrovascular, hemodynamics leads to delayed brain growth and maturation.¹⁵⁻¹⁹ This is a particular concern in HLHS fetuses in whom cerebral blood flow and oxygen delivery is often via retrograde flow through the ductus arteriosus due to aortic stenosis or atresia. Fetal auto

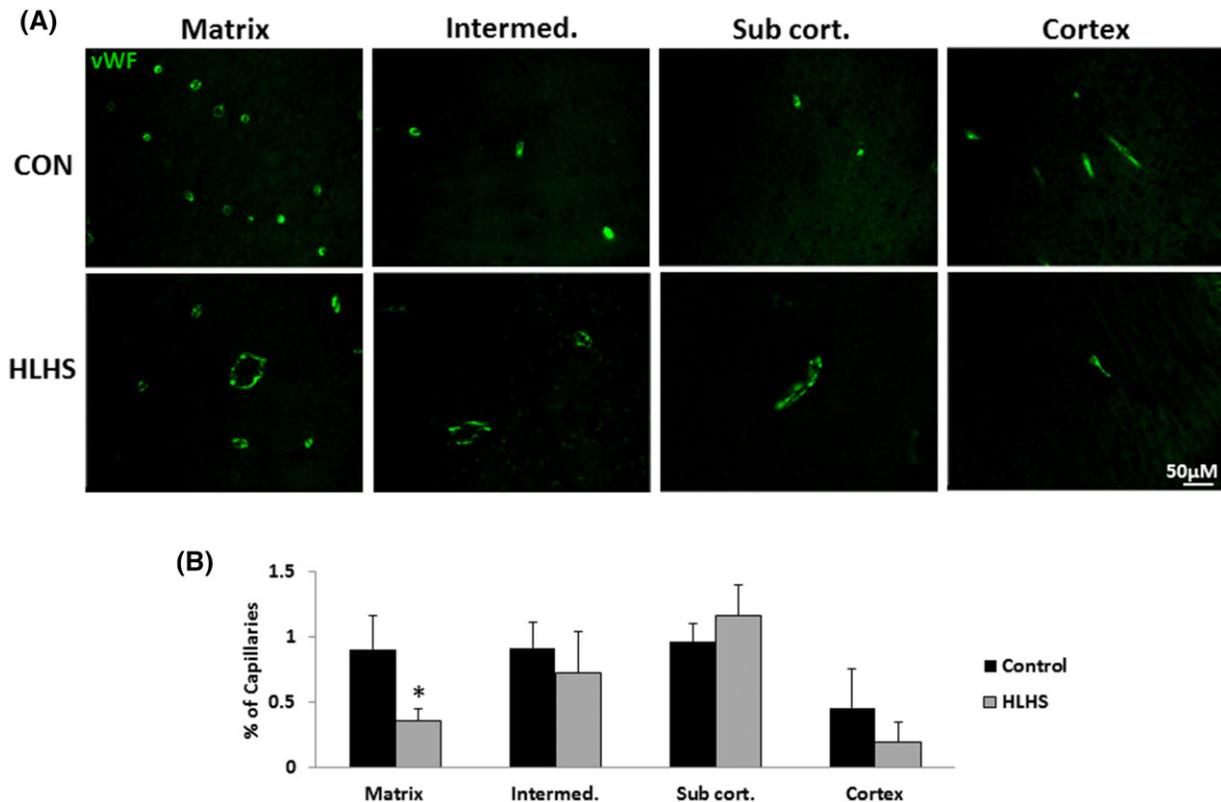


FIGURE 3 Capillary density in fetal brains. (A) Immunofluorescence staining for vWF (green), endothelial marker, in fetal brains with HLHS ($n = 5$) and controls without congenital heart disease ($n = 5$). (B) Capillary density measurements in control (black bars) and HLHS (grey bars) brain layers showed the percentage of capillaries was lower in the germinal matrix of HLHS vs controls ($P < 0.05$). vWF, von Willebrand factor; HLHS, hypoplastic left heart syndrome

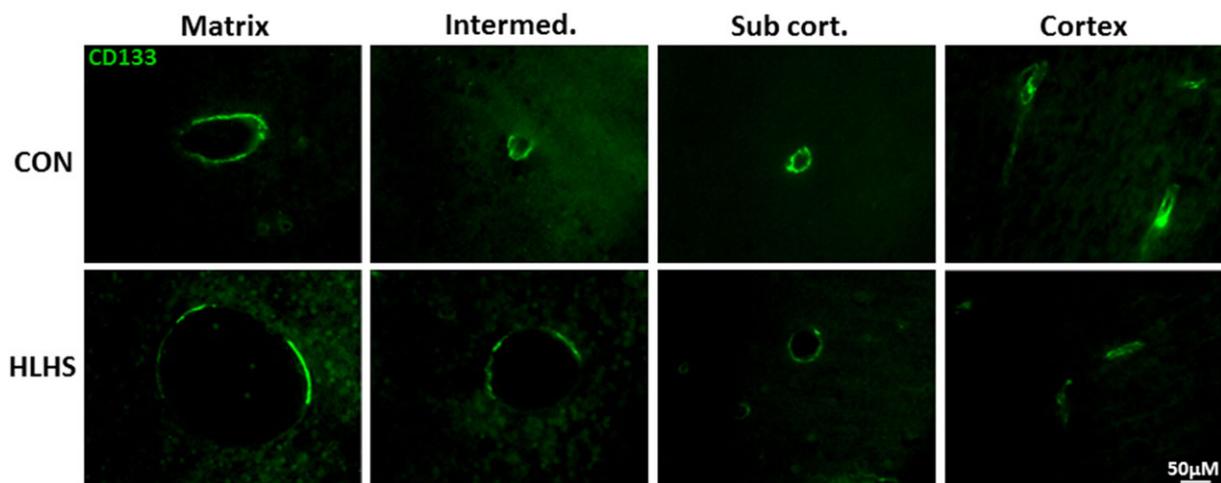


FIGURE 4 Vessel size in fetal brains. Immunofluorescence staining for stem cell marker, CD133 (green), expression in fetal HLHS ($n = 5$) versus control ($n = 5$) brains. CD133 expression was lower in the brains of HLHS vs controls. Vessel area was larger in HLHS compared to controls. CON, control; HLHS, hypoplastic left heart syndrome; Intermed, intermediate; Sub cort., subcortex

regulatory mechanisms are expected to counteract reduced cerebral oxygen delivery by decreasing cerebrovascular resistance and improving cerebral flow to allow brain growth to occur, ie, brain sparing effect.²⁰ However, in our study, head circumference in the second trimester was lower in HLHS compared with controls. A smaller ascending aorta was associated with a higher MCA pulsatility index, ie, higher cerebral vascular resistance suggesting that vascular autoregulation may be impaired. This may be related to an immature or

underdeveloped cerebral vascular bed, which may contribute to elevated cerebrovascular resistance and prevent a brain sparing effect in HLHS. These findings are consistent with other published reports that suggest impaired brain growth^{9,16,18,21,22} including smaller head circumference in midgestation and third trimester in fetal HLHS,^{9,16,21} delay in cerebral development and volume growth in the third trimester on fetal MRI,²³ and abnormal cerebral blood flow characteristics in midgestational fetuses with evolving HLHS.^{16,19,24,25}

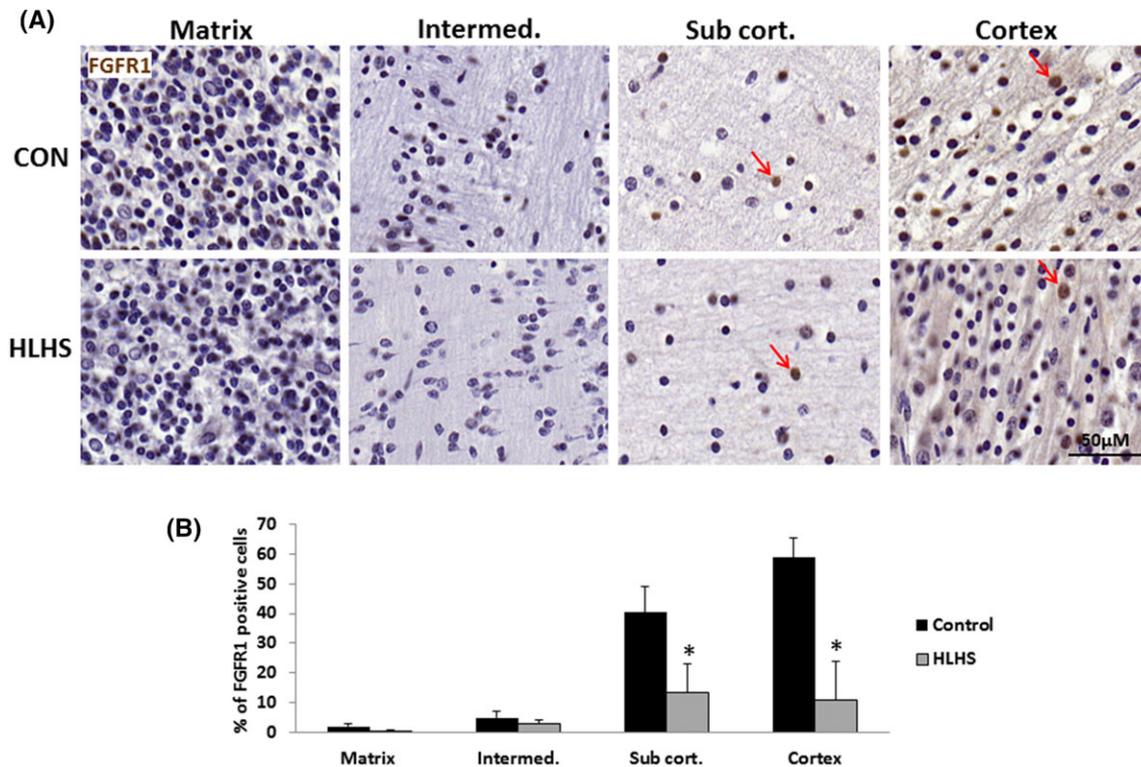


FIGURE 5 Neuronal differentiation in fetal brains. (A) Immunohistochemistry staining for FGFR1, neuronal differentiation marker (brown staining as pointed by arrows) expression in fetal HLHS ($n = 5$) versus control ($n = 5$) brains. Nuclei were counterstained with Mayer's hematoxylin (blue). (B) FGFR1 positive cells were less abundant in cortex and subcortex of HLHS vs controls ($P < 0.05$). HLHS, hypoplastic left heart syndrome; FGFR1, fibroblast growth factor receptor 1

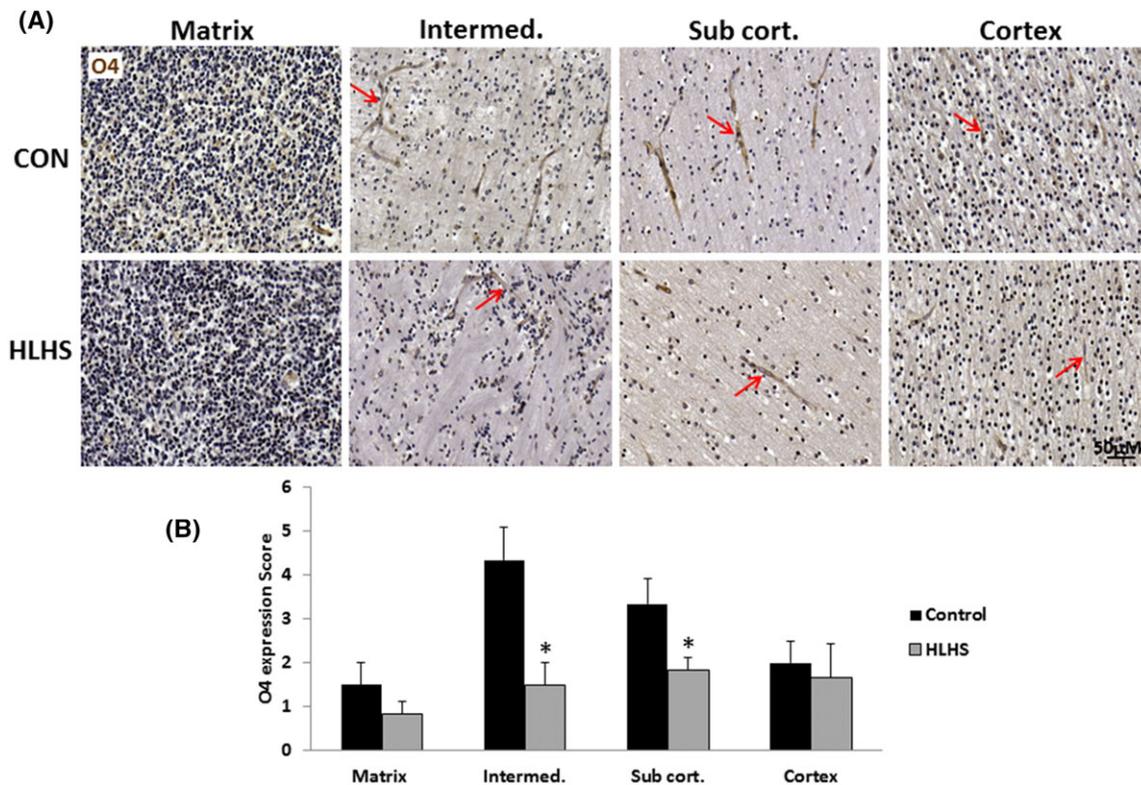


FIGURE 6 Oligodendrocyte differentiation in fetal brains. (A) Immunohistochemistry staining for O4, oligodendrocyte progenitor, (brown staining illustrated by arrows) expression in fetal HLHS ($n = 5$) versus control ($n = 5$) brains. Nuclear staining is shown in blue. (B) O4 expression was scored from 0 to 5 as per expression levels detected. O4 expression was lower in the intermediate and subcortical layers of HLHS vs controls ($P < 0.05$). CON, control; HLHS, hypoplastic left heart syndrome; Intermed, intermediate; Sub cort., subcortex; O4, oligodendrocyte marker

The potential mechanism of this impaired autoregulation has not been well studied. We therefore explored fetal brain and vascular pathology to understand the pathogenesis of this dysregulation. In our study, brain capillaries were significantly larger in HLHS vs controls, but the capillary density was reduced in the germinal matrix and the cortical layers of HLHS, and VEGF, a key angiogenic factor necessary for new vessel growth, was not increased. Failure to upregulate VEGF in HLHS as well as in other cyanotic CHD has been reported by us previously^{26,27}; however, the vascular abnormalities identified by us provide a potential mechanism for the inability of the fetal brain in HLHS to activate compensatory pathways of hypoxia and ischemia. This was further reflected in reduced expression of CD133, an early stem cell marker regulated by hypoxia and mitochondrial dysfunction,²⁸ in the brains of HLHS fetuses. Whether the failure to upregulate hypoxic and angiogenic response in fetal brains is a feature of fetal immaturity or is secondary to an intrinsic or genetic impairment²⁹⁻³² in these pathways remains to be explored.

An important finding was that vascular changes in HLHS fetal brains were associated with markers of impaired neuronal differentiation as seen by reduced FGFR1 expression, as well as reduced oligodendrocyte progenitors, which are important for myelination. This may explain in part the susceptibility to white matter injury observed in HLHS infants undergoing cardiac surgery and highlights the need for neuroprotective strategies especially in those with severe HLHS.

5 | CONCLUSION

Despite the small sample size, our study uncovers novel findings that may explain the mechanism of failed cerebrovascular autoregulation in fetal HLHS and its impact on grey and white matter development that starts prenatally. Further studies are needed to determine whether early fetal intervention to augment antegrade aortic blood flow can improve vascular development and cerebral growth.

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CONFLICTS OF INTEREST

None declared

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