# Anesthesia Neurotoxicity in Congenital Heart Disease

The proposed studies will provide cellular/molecular insights into caspase-induced non-apoptotic neuronal degeneration and will establish a highly translational treatment aimed at reducing neurotoxicity due to neonatal cardiac surgery and improving neurological outcome in children with CHD. The proposed study is of broad relevance for not only for CHD but other populations who need to undergo major surgery early in life.
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**Introduction:** Significant neurological impairment is emerging as one of the most important challenges for survivors of complex congenital heart disease (CHD) repair. Prolonged anesthesia for CHD surgery early in life can damage the developing brain. Cardiopulmonary bypass (CPB) causes systemic inflammation: in addition both deep hypothermic circulatory arrest (HCA) and low flow cerebral perfusion cause relative or complete cerebral ischemia under hypothermia and expose neonatal brains to reperfusion/reoxygenation. Prolonged anesthesia and CPB are indispensable for the care of children with complex CHD: however the cellular and molecular mechanisms whereby anesthesia and cardiac surgery impact cortical development are poorly understood, thereby inhibiting development of effective treatment to minimize surgery-induced neurotoxicity.

Various operative factors such as anesthetic agents and inflammation activate caspases in the developing brain. Although caspase activation has been used widely as the standard measure for cell apoptosis, it does not always lead to cell death but rather alters cellular status at the levels of epigenetic marking on the cellular genome, organelle dynamics and cytoskeletal rearrangement. Indeed there has been little evidence for a reduction of cell numbers associated with neonatal anesthesia neurotoxicity. Consistently our studies have shown no reduction of cortical neurons after activation of caspases. We have also observed increases of markers for caspase-mediated non-apoptotic neurite degeneration in anesthesia-exposed cortices. The most common neurologic deficit seen in children after CHD repair is impairment of fine and gross motor skill development. Our data show that non-apoptotic caspase activation in the neonatal mouse brain impairs fine and gross motor skill learning. Consistent with these findings, we have found that extensive caspase activation after CPB did not change the number of neurons but did alter cortical microstructures. These results have led to our primary hypothesis that: extensive caspase activation due to the global impact of cardiac surgery leads to non-apoptotic neuronal degeneration thereby contributing to neurological deficits in CHD. Given that caspase is a ubiquitous housekeeping enzyme during development, long-term caspase inhibition potentially has undesirable side effects. Our proposed studies therefore will test a potent pan-caspase inhibitor, Q-VD-OPh, in a limited time-frame that specifically targets caspase activation. Notably inhibition of caspase in this crucial period improves impairments of fine and gross motor skill learning in mice. To test our central hypothesis and design new neuroprotective treatment in children with CHD, the proposed studies will use a cross-species approach in our unique piglet model as well as a well-established mouse model.

**Aim 1: Determine the effects of surgery on caspase-induced non-apoptotic neuronal degeneration by prolonged anesthesia.** Obvious difficulties prohibit distinguishing anesthesia neurotoxicity from the overall impact of surgery in children. We hypothesize that systemic inflammation and reoxygenation resulting from CPB/DHCA exacerbate caspase-induced non-apoptotic neuronal degeneration due to prolonged anesthesia. As observed in mice, our study in piglets shows extensive caspase activation after anesthesia. In the piglet model we will test whether CPB exacerbates the effects of anesthesia at the cellular level. Advanced diffusion MRI will estimate the complexity of neuronal dendrites/axons and determine structural cortical maturation. Finally, we will determine differences in long-term epigenetic changes between anesthesia and surgery.

**Aim 2: Determine the effects of brief caspase inhibition on neuronal degeneration and cortical dysmaturatation after cardiac surgery.** We will test the hypothesis that brief inhibition of caspase activation is a potential treatment to limit the adverse impact of neonatal cardiac surgery. Q-VD-OPh will be introduced in the piglet CPB model to determine the effects on caspase-induced neuronal degeneration and cortical dysmaturatation. The same methodologies in Aim 1 will be applied to define cellular and structural changes. Finally we will assess clinically-relevant physiological biomarkers to define the possible positive/negative effects of brief pan-caspase inhibition on multi-organ function after surgery.

**Aim 3: Determine the contribution of caspase-induced non-apoptotic neuronal degeneration to fine and gross motor deficits.** Leveraging sophisticated genetic tools and behavioral assays in the mouse model, we will examine poorly understood non-apoptotic caspase functions in developmental neurotoxicity. We will use anesthetics to induce extensive caspase activation in the neonatal mouse cortex. We will first determine cellular/molecular events in cortical neurons. Long-term epigenetic effects of non-apoptotic caspases will also be determined. Given that fine/gross motor deficits are commonly seen in children after complex CHD surgery as well as in our mouse model of anesthesia, the effects on neuronal plasticity will be analyzed in the primary motor cortex during motor skill learning. The pharmacological inhibition of caspases will be performed to determine the contribution on behavioral deficits.

The proposed studies will provide cellular/molecular insights into caspase-induced non-apoptotic neuronal degeneration and will establish a highly translational treatment aimed at reducing neurotoxicity due to neonatal cardiac surgery and improving neurological outcome in children with CHD.
KEYWORDS:
congenital heart disease
neurologic injury
neuroprotection
surgery
anesthesia
neurotoxicity
caspase
ACCOMPLISHMENTS:

Major goals of the project:
The major goals of the project are to provide cellular/molecular insights into caspase-induced non-apoptotic neuronal degeneration and establish a highly translational treatment aimed at reducing neurotoxicity due to neonatal cardiac surgery and improving neurological outcome in children with CHD. The proposed specific goals and major tasks with timeline are listed below as stated in the approved SOW.

Specific Goal 1: Determine the effects of surgery on caspase-induced non-apoptotic neuronal degeneration by prolonged anesthesia
Major Task 1: Define effects of cardiac surgery on caspase activation and neural degeneration due to prolonged anesthesia. (Proposed timeline: 1-36 months)
Major Task 2: Define effects of anesthesia and cardiac surgery on structural cortical maturation. (Proposed timeline: 1-36 months)
Major Task 3: Define effects of anesthesia and cardiac surgery on epigenetic changes. (Proposed timeline: 1-36 months)

Specific Goal 2: Determine the effects of brief caspase inhibition on neuronal degeneration and cortical dysmaturation after cardiac surgery
Major Task 4: Define the effects of brief caspase inhibition on neuronal degeneration after neonatal cardiac surgery. (Proposed timeline: 23-36 months)
Major Task 5: Define the effects of brief caspase inhibition on cortical dysmaturation after neonatal cardiac surgery. (Proposed timeline: 23-36 months)
Major Task 6: Determine the possible positive/negative effects of brief pan-caspase inhibition on multi-organ function after neonatal cardiac surgery. (Proposed timeline: 23-36 months)

Specific Goal 3: Determine the contribution of caspase-induced non-apoptotic neuronal degeneration to fine and gross motor deficits.
Major Task 7: Determine the contribution of caspase-mediated non-apoptotic cellular degeneration caused by anesthesia. (Proposed timeline: 1-12 months)
Major Task 8: Determine caspase-mediated long-term effects on neuronal plasticity. (Proposed timeline: 13-24 months)
Major Task 9: Determine the effect of extensive caspase activation and the inhibition on neuronal plasticity during motor skill learning. (Proposed timeline: 25-36 months)

Accomplishment under these goals:
Our goal in Year 3 remained to test our principal hypothesis, namely that: extensive caspase activation due to the global impact of cardiac surgery leads to non-apoptotic neuronal degeneration thereby contributing to neurological deficits in CHD. As proposed and discussed below, our activities during the Year 3 period focused on Specific Goal 1 (Task 3), 2 (Task 5, 6), and 3 (Task 8).

Major activities:
Goal 1: To accomplish the goal, we utilized a neonatal piglet model to determine the effects of surgery on caspase-induced non-apoptotic neuronal degeneration by prolonged anesthesia (Specific Goal 1). As observed in mice, our study in piglets showed extensive caspase activation after anesthesia. The hypothesis to be tested in Goal 1 was “systemic inflammation and reoxygenation resulting from CPB and HCA exacerbate caspase-induced non-apoptotic neuronal degeneration due to prolonged anesthesia”. In consistent with the proposed hypothesis, our studies in Year 2 have demonstrated that systemic inflammation and reoxygenation resulting from CPB and HCA decreases gyrencephaly and causes impairment of cortical expansion and maturation, which are pathological signatures observed commonly in survivors of neonatal cardiac surgery for complex CHD1. Our studies using RNA sequencing during this reporting period assessed molecular alterations in the frontal cortex resulting from anesthesia and cardiac surgery (see details in our accomplishment under Specific Goal 1).

Goal 2: Neural stem and progenitor cells (NSPCs) retain their mitotic and differentiation potential throughout their lifespan, as the brain can endogenously replenish damaged brain2-6. The subventricular zone (SVZ) represents the largest source of NSPCs7. Recent studies have discovered that neuroblasts in the human infant
SVZ migrate postnatally along the lateral ventricles to populate frontal cortex⁸,⁹. We have shown a direct relationship between the SVZ and cortical growth in the CHD population¹⁰. In the piglet brain, newly generated neurons from the early postnatal SVZ migrate to specific frontal cortices and differentiate into inhibitory interneurons¹⁰,¹¹, as observed in the human infant⁶. As described in the previous annual report, our studies in Year 2 identified the impact of CPB on postnatal SVZ and cortical development¹. Over a 4-week recovery period after CPB, there were significant alterations of the NSPC pool and neuroblast populations in the SVZ resulting in a depletion of a source of interneurons in the frontal cortex¹. Furthermore, CPB resulted in decreased gyrencephaly and microstructural alterations determined by diffusion tensor imaging (DTI)¹. Consistent with previous reports that demonstrated significant neuroprotective properties of mesenchymal stromal cells (MSCs)¹²,¹³, our ongoing studies found MSC-induced inhibition of caspase activation in cortical neurons (Figure 2). To determine the effects of brief caspase inhibition on neuronal degeneration and cortical dysmaturation after cardiac surgery (Specific Goal 2), we assessed the responses of MSC-induced neuronal caspase inhibitions using our piglet model of CPB in Year 3 (see details in our accomplishment under Specific Goal 2).

**Goal 3:** One of the significant aspects of our project is we examine poorly understood non-apoptotic functions of caspases. Leveraging sophisticated genetic tools, our Year 3 studies continued to use the mouse model of neonatal anesthesia to determine the contribution of caspase-induced non-apoptotic neuronal degeneration to fine and gross motor deficits (Specific Goal 3). Recent clinical studies identified a number of de novo mutations in the patients with CHD¹⁴,¹⁵. At least 50% of these genes are associated with primary cilia. Similarly, a large scale screen in the mouse model of CHD found the enrichment of primary cilia-related genes¹⁶. As described in our previous annual report, our studies supported by this grant found that primary cilia play an integral role in mitigating adverse impacts of environmental stressors such as anesthetics on perinatal brain development¹⁷. In order to establish a highly translational treatment aimed at reducing neurotoxicity and improving neurological outcome in children with CHD, our Year 3 studies further assessed the effect of extensive caspase inhibition and the inhibition on neuronal plasticity during motor skill learning (Major Task 9) using Emx1cre;Il18r1f mice, in which primary cilia are lost specifically in cortical excitatory neurons (see details in our accomplishment under Specific Goal 3).

**Accomplishment under Specific Goal 1:** To assess temporal dynamics of caspase activation after CPB-induced oxidative and inflammatory stress, cleaved caspase-3+ cells were first compared between 3 hours and 1 day post deep hypothermic circulatory arrest (DHCA) in three frontal cortices. In traumatic neural injury caspase-3 is first activated at approximately 1 hour after damage and peaks at 4 hours¹⁸. Consistent with these findings, we observed increases in caspase-3 activation and activation of p53 and JAK-STAT3 pathways in the developing frontal cortex. **Figure 1.** Cardiopulmonary bypass causes acute caspase-3 activation and activation of p53 and JAK-STAT3 pathways in the developing frontal cortex. I-J. The number of Caspase3+ cells in upper (I) and lower (J) cortex layers. K-L, The number of NeuN+-Caspase3+ cells in upper cortex layer (K) and lower (L) cortex layers. M-P, Genome-wide RNA profiling from cortical tissues at 3 hours following CPB. Volcano plot showing genes differentially expressed between control and CPB. 229 upregulated, 74 downregulated genes in CPB vs control after DESeq2 analysis (M). Normalized expression of apoptosis related genes; Caspase8 and Acin1 between control and CPB (N). Top predicted transcription factors (TF) by binding motifs/sites detected in promoters of genes upregulated after CPB (O). Overlap between genes upregulated by CPB and genes differentially expressed after kinase enzyme genetic manipulations in vitro or in vivo. JAK2 (Janus kinase 2) as top predicted kinase whose knockdown leads to downregulation of genes found upregulated after CPB (P). Data are shown as mean ± standard error of mean (n=3-4 each). P values were determined by two-way (I-L) ANOVA with Bonferroni comparisons. *P < 0.05, **P < 0.01.
3+ cells at 3 hours after DHCA in both upper and lower cortical layers (Figure 1I,J). No regional differences were seen in the number of activated cells, consistent with a global effect of DHCA-induced caspase-3 activation. On the other hand, there were no differences in the caspase-3+ cell number on post-DHCA day 1, compared to control and 3 hours after surgery. Similarly, caspase-3 was activated at 3 hours but not at 1 day after DHCA in cortical neurons (Figure 1K,L). To further characterize molecular events occurring during acute caspase-3 activation after cardiac surgery, genome-wide RNA profiling was performed in the developing cortex at 3 hours following DHCA. Differential expression analysis between control and CPB conditions revealed 303 differentially expressed genes (DEGs) at 3 hours post-CBP (229 upregulated, 74 downregulated; Figure 1M). We focused on sub-setted down/upregulated DEG lists for ontology analyses to detect biologically meaningful patterns. Consistent with our findings of CPB-induced acute caspase activation (Figure 11-L), caspase 8 (Casp8) and apoptotic chromatin condensation inducer-1 (Acin1) were upregulated in the CPB group compared to control (Figure 1N). Gene ontology analysis was performed to predict transcription factors (TF) by binding motifs and sites detected in promoters of genes upregulated by CPB. The analysis identified TP53 as the top predicted TF (Figure 1O), suggesting a likely role for p53 activation after CPB. When we assessed overlap between CPB-induced upregulated genes and genes differentially expressed after TF loss of function (LOF) mutations in various cell lines, Heat Shock Factor-1 (HSF1) was the top predicted factor whose LOF induced similar gene expression changes to CPB. Together with the well-known interaction of HSF1 with the p53 pathway, the results support the possible involvement of HSF1 in response to CPB. Oxidative stress during CPB can activate the p53 pathway, which in turn regulates the production of intracellular reactive oxygen species (ROS) and functions as both a sensitizer and an activator of apoptosis. We also tested for overlap between genes upregulated by CPB and genes differentially expressed after various TF genetic manipulations, obtained from the Gene Expression Omnibus (GEO) database. In the analysis a statistically significant fraction of CPB-induced upregulated genes were found downregulated after Tp53 and/or Stat3 knockdown. These results suggest that the same gene sets that were identified as upregulated after CPB are also upregulated after p53/STAT3 pathway activation. Finally, our gene set enrichment analysis revealed Janus kinase 2 (JAK2) as the top predicted kinase whose knockdown leads to downregulation of genes found upregulated after CPB (Figure 1P). This data suggests a likely activation signature of JAK2-STAT3 signaling after CPB. Altogether, our transcriptomic profiling revealed previously uncharacterized links between CPB-induced brain insults and the activation of p53 and JAK2-STAT3 pathways, both of which are known to be critical in oxidative stress and inflammatory responses as well as cell survival, suggesting that suppressing these signaling events during this critical time window is beneficial for CPB-induced brain injury (Task 3).

Accomplishment under Specific Goal 2: When we assessed acute effects of MSC treatment on NSPC activities, our studies found that the treatment through CPB has the potential to mitigate effects of CPB on NSPCs and to promote migration of neuroblasts (see details in Appendix). In addition, our ongoing studies found MSC-induced acute caspase inhibitions in cortical neurons (Figure 2). To assess whether the short-term caspase inhibition due to MSC treatment during cardiopulmonary bypass. E-G, NeuN-caspase3+ cells in upper cortex layer among three groups tested. Scale bar, 50um. H-K, The number of caspase3+ cells in upper cortex layer (H) and lower cortex layer (I). The ratio of NeuN-caspase3+ double positive cells to total NeuN positive cells in upper cortex layer (J) and lower cortex layer (K). L, The relationship between p-stat3+ cell and NeuN-caspase3+ cell numbers in cortex. M, The relationship between the length of microglia process and NeuN-caspase3+ cell numbers in cortex. N, The relationship between the number of microglia process and NeuN-caspase3+ cell numbers in cortex. O, Relative mRNA expression by quantitative PCR from cortical tissues. Data are shown as mean ± standard error of mean (n=4 each). P values were determined by two-way ANOVA with Bonferroni comparisons (H-K,O) and Spearman correlation (L-N). *P < 0.05, **P < 0.01, vs Control, †P < 0.05, ††P < 0.01 vs control and CPB+MSC.
CPB affect cortical dysmaturation after neonatal cardiac surgery, animals were assessed up to 4 weeks after surgery. Similar to our previous studies\(^1\), the gyrification index at post-operative week 4 was lower after CPB compared to controls (Figure 3G-J). Notably, CPB-induced alterations of gyrification were improved in the brain with BM-MSC treatment (Figure 3G-J). To further assess cortical microstructure, high-resolution DTI was employed. Cortical FA after CPB was higher compared to control (Figure 3K-M), indicative of CPB-induced inhibition of the maturation-dependent decrease in cortical FA\(^22\). Decreased mean diffusivity (MD) and radial diffusivity (RD) in the CPB group also indicated a loss of structural complexity in the developing cortex (Figure 3N-S). On the other hand, there were no differences in axial diffusivity (AD) among groups (Figure 3T-V). Following MSC infusion, we found a reduction in CPB-induced microstructural alterations, as determined by FA and RD (Figure 3K-M,Q-S). Together, our results indicate that caspase inhibition during CPB mitigates structural dysmaturation in the developing cortex resulting from anesthesia and CPB (Task 5).

We next evaluated the dose effect of MSC-induced caspase inhibitions on multi-organ function and systemic inflammation after CPB. There were no differences in post CPB hemodynamics and other clinically-relevant biomarkers between the three experimental groups in the acute period after CPB. At 3hrs post CPB, a significant increase was observed in the plasma Interleukin-4 (IL-4) level in the high-dose MSC compared to CPB and low-dose MSC. On the other hand, Interleukin-8 (IL-8) was significantly reduced after high-dose MSC delivery compared to low-dose treatment. Together our results indicate that while there are no acute adverse reactions after both low- and high-dose MSC delivery, circulating cytokine levels are dose-dependently modulated by MSC-induced caspase inhibitions via CPB (Task 6, see details in Appendix\(^27\).

**Accomplishment under Specific Goal 3:** As proposed, the mouse model of neonatal anesthesia have been used for Specific Goal 3. Pups were weighed and intraperitoneally injected twice with 2.5 g/kg of EtOH (or PBS control) at 2-hour intervals, or once with 20 mg/kg ketamine (or PBS control) at postnatal day 7. The most common neurological deficits seen in infants with CHD after cardiac surgery are motor deficits\(^23,24\). Our studies in previous years have demonstrated impaired cognitive flexibility and gross motor skills in cilia-deficient mice exposed to neonatal anesthesia (Figure 4A and B). As previously described\(^25\), Q-VD-OPh was dissolved in 10% DMSO in PBS and introduced via subcutaneous injection at 30mins before the onset of anesthesia at 5 mg/kg per injection. As activation of caspase disappears by 24hrs post anesthesia in mice, we repeated the same
volume once at 2.5hrs post anesthesia. By using single-pellet reaching test, we measured fine motor skill functions. In addition to cognitive flexibility and gross motor skills Ift88 cKO mice treated with neonatal anesthesia displayed significant impairment in the fine motor skill (Figure 3C). Notably, our ongoing studies found that acute caspase inhibition by Q-VD-OPH rescued the anesthesia-induced impairment of fine motor functions (Task 9).

Additional accomplishment: The proposed project addresses a following FY19 PRMIP Area of Encouragement: Research both on the risk of neurologic injury and on enhanced neuroprotection before, during, and after surgery for CHD. As proposed and approved, children with CHD are at increased risk for neurodevelopmental challenges across the lifespan. These are associated with neurological changes and potential acquired brain injury, which occur across a developmental trajectory and which are influenced by an array of medical, sociodemographic, environmental, and personal factors. These alterations to brain development lead to an array of adverse neurodevelopmental outcomes, which impact a characteristic set of skills over the course of development. To disseminate the issue to academia and accelerate research on the risk of neurologic injury during surgery for CHD, our recent publication reviewed existing knowledge of aberrant brain development and brain injury alongside associated neurodevelopmental challenges across the lifespan. These provide a framework for discussion of emerging and potential interventions to improve neurodevelopmental outcomes at each developmental stage (see more details in Appendices).

Opportunities for training and professional development:
Nothing to Report

Dissemination to communities of interest:
Our four key audiences are: 1) Patients, patient families, and the public; 2) Cardiologists, cardiac surgeons, neuroscientists, and health care professionals working in the field of neuroprotection in the CHD population; 3) Academia; 4) Industry (who might be interested in possible therapeutic approach from this project). To ensure that this work is disseminated, we have developed the following dissemination plan based on the four key audiences.

Patients, Patient Families, and the Public: To educate patients, their families, and the public we worked with our Public Relations and Marketing team (see the section of PRODUCT).

Health care professionals working in the field of neuroprotection in the CHD population: To educate physicians about our findings we presented and discussed results from this project at a total of 14 regional, national, and international conferences during this reporting period (Details are shown in the section of PRODUCT).

Academia: The focus for academia is on publication of study results in a high-impact journal to support dissemination of this work. Our work from the Goal 2 studies was successfully published at Annals of Thoracic Surgery, respected journal in the field of cardiovascular surgery, in August 2022. In addition, one clinical original article and one review article were published at Cardiology in Young and Child Neuropsychology for dissemination of our work. (Details are shown in the section of PRODUCT)
Plan during the next reporting period to accomplish the goals:
Our goal during the approved NCE period is to continue testing our principal hypothesis, namely that: extensive caspase activation due to the global impact of cardiac surgery leads to non-apoptotic neuronal degeneration thereby contributing to neurological deficits in CHD.

By using MSC treatment we started testing our hypothesis in the approved Goal 2 studies that “brief inhibition of non-apoptotic caspase activation is a potential treatment to limit the adverse effect of cardiac surgery”. Specific goal for our studies during the NCE period will be to determine the effects of brief caspase inhibition on neuronal degeneration and cortical dysmaturation after cardiac surgery (Specific Goal 2). Q-VD-OPh will be introduced in the piglet model to determine dose-related effects on caspase-induced neuronal degeneration and cortical dysmaturation as we did for MSC treatment. Q-VD-OPh will be introduced at 30min before the onset of anesthesia. Because of its short half-life, Q-VD-OPh dosing will be repeated every 8hrs following initial administration during caspase activation. In our previous studies we have defined the time course of CPB-induced caspase activation (Figure 1). The effects of Q-VD-OPh will be compared with animals with CPB after administration of vehicle in same amount. The results will also be compared with naïve control animals used in the Goal 1 studies to define the effects of brief caspase inhibition on: 1) neuronal degeneration (Major Task 4); and 2) cortical dysmaturation (Major Task 5) and to determine the possible positive/negative effects of brief pan-caspase inhibition on multi-organ function (Major Task 6).

Leveraging sophisticated genetic tools, our studies will continue to use the mouse model to determine the contribution of caspase-induced non-apoptotic neuronal degeneration to fine and gross motor deficits (Specific Goal 3). In previous years, we successfully accomplished Major Task 7 and 8. Thus, our studies during this reporting period focused on determining the effect of extensive caspase activation and the inhibition on neuronal plasticity during motor skill learning (Major Task 9). Notably, our results in Year 3 found that acute caspase inhibition by Q-VD-OPh rescued the anesthesia-induced impairment of fine motor functions (See accomplishment). Using in vivo imaging, studies during the NCE period will determine spine formation and elimination in layer II and V neurons in the primary motor cortices. The relation between fine/gross motor function and neuronal plasticity will be assessed to define the effects of caspase inhibition on neuronal plasticity in fine/gross motor skill learning.

REFERENCES


IMPACT:

Impact on the development of the principal discipline of the project:
Anesthetics are an indispensable component of complex pediatric surgeries, for instance, corrective heart surgery in patients with CHD. Our study published during this reporting period has characterized the dose dependent effect of MSC delivery through CPB in a porcine model which is anatomically and physiologically similar to humans. Our analysis has shown CPB as an efficient administration system for MSC delivery and demonstrated that MSC treatment via CPB is safe up to 100 million cells per kg. In addition, we have shown that high-dose MSC treatment significantly increases IL-4 expression post CPB, which is one of the critical cytokines regulating immune and inflammatory responses. The studies have also indicated both low- and high-dose MSC treatment shifts the microglial phenotype to a less pro-inflammatory state based on morphological observations. Finally, we observe a unique dose-dependent reaction to the MSC treatment in the most active neurogenic niche of the infant brain. This unique therapeutic approach for caspase inhibition during cardiac surgery has the potential to reduce systemic and cerebral inflammation and modulate responses of the active neurogenic niche to CPB in the infant with CHD. As proposed, our future studies will determine the effects of brief caspase inhibition on neuronal degeneration and cortical dysmaturation after cardiac surgery using the piglet model of CPB.

To disseminate the issue to academia and accelerate research on the risk of neurologic injury for CHD, our article published at Child Neuropsychology during this reporting period discussed existing knowledge of aberrant brain development and brain injury alongside associated neurodevelopmental challenges across the lifespan. Our article now provides a framework for discussion of emerging and potential interventions to improve neurodevelopmental outcomes at each developmental stage.

Original copy of our articles published on Annals of Thoracic Surgery and Child Neuropsychology has been attached as APPENDICES.

Impact on other disciplines:
As addressed a following FY19 PRMRP Area of Encouragement “Research both on the risk of neurologic injury and on enhanced neuroprotection before, during, and after surgery for CHD”, neurological deficits are a serious and common sequelae of CHD. While their underlying mechanisms have not been fully characterized, their manifestations are well-known and understood to persist through adulthood. Development of therapies to address or prevent these deficits are critical to attenuate future morbidity and improve quality of life including our ongoing DoD-approved project. The Mesenchymal Stromal Cell Delivery through Cardiopulmonary Bypass in Pediatric Cardiac Surgery study is a prospective, open-label, single-centre, dose-escalation phase 1 trial assessing the safety and feasibility of delivering mesenchymal stromal cells to neonates and infants during cardiac surgery. Outcomes will be compared with historical data from a similar population. In our original article published at Cardiology in Young during this reporting period, we defined an optimal control group for use in the Mesenchymal Stromal Cell Delivery through Cardiopulmonary Bypass in Pediatric Cardiac Surgery trial. Our studies confirmed max inotropic and vasoactive-inotropic scores as important quantitative measures after neonatal and infant cardiac surgery. This initial study will help to determine the sample size of future efficacy and effectiveness studies.

Impact on technology transfer:
Nothing to report

Impact on society beyond science and technology:
Nothing to report
CHANGES/PROBLEMS:
As described in the previous annual reports, several key experiments were delayed in the last three years due to COVID. To complete all aims proposed, we have requested 12-month no cost extension of Grant #W81XWH2010199 that has been approved, started from May 1st, 2023, and scheduled to end on April 30th, 2024. Our goals during the next study period remain same and are to test our principal hypothesis, namely that: extensive caspase activation due to the global impact of cardiac surgery leads to non-apoptotic neuronal degeneration thereby contributing to neurological deficits in CHD.
PRODUCTS

Publications, conference papers, and presentations:

Journal publications.
Title: The dose-effect of mesenchymal stromal cell delivery through cardiopulmonary bypass in a juvenile porcine model.
Status: published
Acknowledgement of federal support: yes

Title: Defining Historical Control Group for Phase 1 Trial of Mesenchymal Stromal Cell Delivery through Cardiopulmonary Bypass in Neonates and Infants.
Status: published
Acknowledgement of federal support: yes
Authors: Kobayashi K, Maeda T, Ayodeji MA, Tu SC, Chen A, Hanley PJ, Ishibashi N.
Authors: Sanz JH, Cox S, Donofrio MT, Ishibashi N, McQuillen P, Peyvandi S, Schlatterer S.

Title: Trajectories of Neurodevelopment and Opportunities for Intervention Across the Lifespan in Congenital Heart Disease.
Status: published
Acknowledgement of federal support: yes

Books or other non-periodical, one-time publications.
Nothing to report

Other publications, conference papers, and presentations.
Title: Primary cilia loss renders pyramidal neurons susceptible to perinatal ketamine-induced dendritic degeneration and learning deficits.
Presentation: 24th Biennial Meeting of the International Society for Developmental Neuroscience, May 7-10, 2022, Vancouver, Canada.

Authors: Kobayashi K, Bansal A, Saric N, Hanley PJ, Hashimoto-Torii K, Ishibashi N.
Title: Exosomal MicroRNA mir-21-5p as Potential Molecular Entity for Neuroprotective Effects of Mesenchymal Stromal Cells during Cardiopulmonary Bypass.
Presentation: The Congenital Heart Surgeons’ Society (CHSS) 49th Annual Meeting, October 23-24, 2022, Chicago, IL.

Title: Mesenchymal Stromal Cell Delivery via Cardiopulmonary Bypass Provides Neuroprotection in A Juvenile Porcine Surgical Model.
Presentation: American Heart Association (AHA)’s Annual Scientific Sessions, November 5-7, 2022, Chicago, IL.

Title: Primary cilia loss renders pyramidal neurons susceptible to perinatal ketamine-induced dendritic degeneration and learning deficits.
Presentation: Biomedical Engineering Society Annual Meeting, October 12-15, 2022, San Antonio, TX.
Authors: Strauss M, Ballón N, Salameh S, Ishibashi N.
Title: Histone-3 Lysine-4 Methylation and Neonatal White Matter Injury in Congenital Heart Disease.
Presentation: 51st Annual Meeting of the Society for Neuroscience, November 12-16, 2022, San Diego, CA.

Authors: Lam VK, Kobayashi K, Li J, Ayodeji M, Tu SC, Agaronyan A, Lin S, Wang PC, Tu TW, Ishibashi N.
Title: The Effects of Tetrahydrobiopterin on Structural Connectivity in a Piglet Model of Chronic Hypoxia.
Presentation: 51st Annual Meeting of the Society for Neuroscience, November 12-16, 2022, San Diego, CA.

Title: Primary cilia loss renders pyramidal neurons susceptible to perinatal ketamine-induced dendritic degeneration and learning deficits.
Presentation: 51st Annual Meeting of the Society for Neuroscience, November 12-16, 2022, San Diego, CA.

Authors: Lam VK, Li J, Xu S, Shinha A, Agaronyan A, Lin S, Tu TW, Ishibashi N.
Title: Structural connectome analysis in a piglet model of chronic hypoxia.
Presentation: The Pediatric Academic Societies 2023 Meeting, April 28 – May 1, 2023, Washington, DC.

Authors: Saric N, Reddy N. Somaa F, Sade, FC. Wang L, Hashimoto-Torii K, Ishibashi N.
Title: Primary cilia loss renders pyramidal neurons susceptible to perinatal ketamine-induced dendritic degeneration and learning deficits.
Presentation: The Pediatric Academic Societies 2023 Meeting, April 28 – May 1, 2023, Washington, DC.

Title: The effects of cardiopulmonary bypass to neonatal piglet brain connectivity.
Prentation: Research Education & Innovation Week 2023, April 17-22, 2023, Washington, DC.

Authors: Lam VK, Li J, Xu S, Shinha A, Agaronyan A, Lin S, Tu TW, Ishibashi N.
Title: Structural connectome analysis in a piglet model of chronic hypoxia.
Presentation: Research Education & Innovation Week 2023, April 17-22, 2023, Washington, DC.

Authors: Prasad JD, Li Z, Lam VK, Henmi S, Haydar TF, Ishibashi N.
Title: The Spatial and Temporal Characterisation of Neural Precursor Cells in the Developing Göttingen Minipig Brain.
Presentation: Research Education & Innovation Week 2023, April 17-22, 2023, Washington, DC.

Title: Mesenchymal Stromal Cell Delivery through Cardiopulmonary Bypass in Pediatric Cardiac Surgery - MedCaP Phase I Trial.
Presentation: Research Education & Innovation Week 2023, April 17-22, 2023, Washington, DC.

Authors: Saric N, Reddy N. Somaa F, Sade, FC. Wang L, Hashimoto-Torii K, Ishibashi N.
Title: Primary cilia loss renders pyramidal neurons susceptible to perinatal ketamine-induced dendritic degeneration and learning deficits.
Presentation: Research Education & Innovation Week 2023, April 17-22, 2023, Washington, DC.

Website or other Internet site:
To educate patients, their families, and the public and to identify potential industry partners we worked with our Public Relations and Marketing team. Our research activities as well as the results have been published through our website "innovation district".

Technologies or techniques: Nothing to report

Inventions, patent applications, and/or licenses: Nothing to report
**Other Products:** Nothing to report
## PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

### Individuals on the project:

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<tr>
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<td>PD/PI</td>
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<td>Dr. Ishibashi has supervised all aspects of the experiments and contributed to analyze the data as well as prepare manuscripts. He is responsible for communication with DoD.</td>
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Each person has worked at least one month per year on the project during the reporting period.

### Change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period:

The change of active support for the PD/PI and key personnel are described below.

**Nobuyuki Ishibashi, M.D., Principal Investigator (PD/PI):**

- **Closed grant:**
  - Nothing to report

- **New active grant:**
  - Nothing to report

**Kazue Hashimoto-Torii Ph.D., Co-investigator:**

- **Closed grant:**
  - Nothing to report

- **New active grant:**
  - Nothing to report

**Richard A. Jonas, M.D., Co-investigator:**

- **Closed grant:**
  - Nothing to report

- **New active grant:**
  - Nothing to report
New active grant:
Nothing to report

**Paul Wang, Ph.D., Consultant:**
Closed grant:
Nothing to report

New active grant:
Nothing to report

**David Zurakowski, Ph.D., Consultant:**
Closed grant:
Nothing to report

New active grant:
Nothing to report

**Nina Deutsch, M.D., Other Significant Contributor:**
Closed grant:
Nothing to report

New active grant:
Nothing to report

**Vesna Jevtovic-Todorovic, MD, PhD, MBA, Other Significant Contributor:**
Closed grant:
Nothing to report

New active grant:
Nothing to report

**Other organizations as partners:**

Organization Name: Howard University
Location of Organization: 2041 Georgia Ave, NW, Washington, DC, 20059
Partner's contribution to the project: Facilities
Animal imaging has been performed at Molecular Imaging Laboratory Facility at Howard University which functions as preclinical neuroimaging core of the District of Colombia Intellectual and Developmental Disabilities Research Center (DC-IDDRD) at Children’s National Hospital.

Organization Name: Boston Children’s Hospital
Location of Organization: 300 Longwood Avenue, Boston, MA 02115
Partner's contribution to the project: Collaboration
Dr. Zurakowski, Director of Biostatistics at Boston Children’s Hospital, serve as Biostatistician in the project.

Organization Name: University of Colorado School of Medicine
Location of Organization: 12401 East 17th Avenue, Aurora, CO 80045
Partner's contribution to the project: Collaboration
Dr. Jevtovic-Todorovic, a Professor of Anesthesiology at University of Colorado School of Medicine, is Other Significant Contributor and guides the preclinical studies of neonatal anesthesia.
SPECIAL REPORTING REQUIREMENTS:
Nothing to report
APPENDICES:

Original copy of our articles published on *Annals of Thoracic Surgery* and *Child Neuropsychology* has been attached as APPENDICES.
ABSTRACT

BACKGROUND Neurologic impairments are a significant concern for survivors after pediatric cardiac surgery with cardiopulmonary bypass (CPB). We have previously shown that mesenchymal stromal cell (MSC) delivery through CPB has the potential to mitigate the effects of CPB on neural stem/progenitor cells. This study assessed the dose effects of MSCs.

METHODS Piglets (n = 20) were randomly assigned to 1 of 4 groups: control, CPB, or CPB followed by MSC administration with low and high doses (10^3 to 10^6 and 100^3 to 10^6 cells per kilogram). We assessed acute dose effect on cell distribution, multiorgan functions, systemic inflammation, microglia activation, and neural stem/progenitor cell activities.

RESULTS By magnetic resonance imaging, approximately 10 times more MSCs were detected within the entire brain after high-dose delivery than after low-dose delivery. No adverse events affecting hemodynamics, various biomarkers, and neuroimaging were detected after high-dose MSC delivery. High-dose MSCs significantly increased circulating levels of interleukin 4 after CPB. Both MSC groups normalized microglia activation after CPB, demonstrating MSC-induced reduction in cerebral inflammation. There was a significant increase in neuroblasts in the subventricular zone in both treatment groups. The thickness of the most active neurogenic area within the subventricular zone was significantly increased after high-dose treatment compared with CPB and low-dose MSCs, suggesting dose-dependent effects on the neurogenic niche.

CONCLUSIONS MSC delivery through CPB is feasible up to 100 x 10^6 cells per kilogram. MSC treatment during cardiac surgery has the potential to reduce systemic and cerebral inflammation and to modulate responses of an active neurogenic niche to CPB. Further investigation is necessary to assess the long-term effects and to develop a more complete dose-response curve.

(Ann Thorac Surg 2022; ■ ■ ■ ■)
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Many children with complex congenital heart disease (CHD) who require surgical correction with cardiopulmonary bypass (CPB) during the early postnatal period have developmental

The Supplemental Material can be viewed in the online version of this article [https://doi.org/10.1016/j.athoracsur.2022.07.035] on https://www.annalsthoracicsurgery.org.
Abbreviations and Acronyms
ANOVA = analysis of variance
CHD = congenital heart disease
CPB = cardiopulmonary bypass
DCX = doublecortin
DHCA = deep hypothermic circulatory arrest
HD = high dose
IL-4 = interleukin 4
LD = low dose
MRI = magnetic resonance imaging
MSC = mesenchymal stromal cell
SPIO = superparamagnetic iron oxide
SVZ = subventricular zone

delay, neurologic impairment, or behavioral problems. However, few treatment options are currently available. Mesenchymal stromal cells (MSCs) are remarkable nonhematopoietic cells with a high potential for treating a wide range of diseases including ischemic brain injury. In the damaged rodent white matter, MSCs promote endogenous oligodendrocyte proliferation and differentiation and increase myelin formation. In addition, MSC administration significantly augments neurogenesis in the subventricular zone (SVZ), the largest source of neural stem/progenitor cells. Moreover, MSCs regulate microglia activation after ischemic brain injury. Finally, MSCs have been widely reported to have extensive anti-inflammatory and immunomodulatory properties. We have previously demonstrated that MSC administration through CPB at $10^6$ cells per kilogram mitigates effects of CPB on SVZ neural stem/progenitor cells and promotes migration of neuroblasts. Our ongoing studies also found that MSCs at this dose level reduce microglia activation caused by CPB in both cortex and white matter. The aim of this study was to test the feasibility of higher dosage cell delivery through CPB and to assess the acute dose-response relationship in a postnatal porcine model.

MATERIAL AND METHODS

EXPERIMENTAL MODEL. This study involved a total of 20 male Yorkshire piglets (4.1 ± 0.3 kg) at 2 weeks of age. Animals were randomly assigned to 4 groups: control (no surgical procedure, n = 5); 18 °C deep hypothermic

![Figure 1](image_url)

**FIGURE 1** Methods. (A) Experimental protocol of cardiopulmonary bypass (CPB) operation. (B) Sagittal plane of the porcine brain. Coronal section of line C was used for immunohistological analysis. (C) Coronal section and the subventricular zone (SVZ; box D). The cortex and white matter are subdivided and analyzed within 3 and 5 regions, respectively. (D) Magnified image from the boxed region in C and subdivision of the SVZ with doublecortin stain. Scale bar = 100 μm. (CBC, complete blood count; CC, corpus callosum; DHCA, deep hypothermic circulatory arrest; DL, dorsolateral; HD, high dose; Ht, hematocrit; IC, insular cortex; IHC, immunohistochemistry; IWM, insular white matter; MRI, magnetic resonance imaging; MSC, mesenchymal stromal cell; NT, normothermia; PBS, phosphate-buffered saline; PMC, premotor cortex; PMWM, premotor cortex white matter; PSSC, primary somatosensory cortex; PVWM, periventricular white matter; RW, rewarming; SSWM, somatosensory white matter; V, ventral.)
circulatory arrest (DHCA) for 60 minutes (CPB, n = 5); and 18°C DHCA with MSC administration (MSC, n = 10 total). MSCs were manufactured by the same methods used for clinical trials at Children’s National Hospital. In the MSC group, animals were subdivided into 2 different cell dose groups, low dose (MSC-LD, n = 5) or high dose (MSC-HD, n = 5), to determine the dose effect. After initial perfusion, animals were cooled to a temperature of 18°C and then underwent DHCA for 60 minutes (Figure 1A). After 20 minutes of rewarming, either normal saline (CPB group) or MSCs (10 × 10^6 cells per kilogram for the MSC-LD group, 100 × 10^6 cells per kilogram for the MSC-HD group) were administered through the aortic cannula during 10 minutes. Preoperative data and experimental conditions are described in Supplemental Tables 1 and 2. At 3 hours after CPB, the brain was harvested (Figure 1A). For magnetic resonance detection of MSCs, cells were labeled with superparamagnetic iron oxide (SPIO) co-labeled with a green fluorescence protein. We performed all experiments in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was approved by the Animal Care and Use Committee of Children’s National Hospital.
**TABLE Cytokine Assay**

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*Statistically significant. P values were determined by 1-way ANOVA with Tukey comparison (n = 5 in CPB and MSC low-dose groups, n = 4 in MSC high-dose group). ANOVA, analysis of variance; CPB, cardiopulmonary bypass; MSC, mesenchymal stromal cell; TNF-α, tumor necrosis factor α.*

**CELLULAR ANALYSIS.** Antibodies for Iba1 and double-cortin (DCX) were used to identify microglia and young neurons (neuroblasts), respectively. Radial glia-like cells (ie, neural stem cells) were identified with an anti-glial fibrillary acidic protein antibody with the morphologic feature. Cellular analysis was performed with coronal sections, which included 3 cortical regions, 5 white matter regions, and the SVZ (Figure 1B, C). The SVZ was divided into 3 tiers as previously described for human SVZ (Figure 1D).11 The SVZ was further subdivided into dorsolateral and ventral regions as previously described (Figure 1D).12 Given high cell density and homogeneous cell distribution, the process length of glial fibrillary acidic protein-positive radial glia-like cells was co-labeled with DCX to determine the thickness of a cell-dense band of young neurons around the lateral ventricle (tier 1) in each SVZ region.13 The branch length and number of terminal points were semiautomatically quantified with Imaris software (Bitplane).

**STATISTICAL ANALYSIS.** One-way analysis of variance (ANOVA) with Tukey post hoc comparisons was used to compare pericentral variables, cytokine and chemokine levels, and density of SPIO signals. Two-way ANOVA was applied with Tukey post hoc comparisons for multiple comparison. The comparison of SPIO spots between low- and high-dose groups was performed by the Student t-test. We also applied 2-way ANOVA with Bonferroni post hoc comparisons for a region as 1 factor to compare the proportion of SPIO signals. The outlier was identified by ROUT method with 0.1% false discovery rate.14 Statistical analysis was performed with the PRISM9 software package (GraphPad Software). P values < .05 were considered statistically significant.

The detailed methods are described in the Supplemental materials.

**RESULTS**

**CPB EFFICIENTLY DISTRIBUTES HIGH-DOSE MSCS WITHIN THE ENTIRE BRAIN.** Distribution of cells was analyzed with magnetic resonance imaging (MRI) and compared between low dose and high dose to assess the feasibility of low- and high-dose cell delivery through CPB. The average number of cells delivered was 35.6 ± 0.5 × 10⁶.
cells for MSC-HD and $41.0 \pm 0.5 \times 10^7$ cells for MSC-LD. At 3 hours after administration of MSCs, T2*-weighted brain imaging showed 493.0 $\pm$ 94.3 SPIO signals in MSC-LD and 4888.9 $\pm$ 765.5 SPIO signals in MSC-HD (Figure 2A, B), that is, approximately 10 times higher signals in MSC-HD. When the brain was divided into 7 structures, there were no differences in the density of SPIO signals between regions after high-dose delivery (Supplemental Figure). Furthermore, the proportion of SPIO signals in each region after high-dose delivery was similar to the low-dose group (Figure 2C). These findings indicate that CPB efficiently distributes MSCs within the entire brain even after high-dose administration. In addition, no signs of stroke were observed by MRI after high-dose administration (Figure 2D), confirming the safety of MSC delivery through CPB.

MSC DELIVERY THROUGH CPB DOSE DEPENDENTLY ALTERS CYTOKINE LEVELS AFTER CARDIAC SURGERY. MSCs are known to have significant immunomodulatory properties.15,16 We therefore evaluated

![Figure 3](image-url)
the dose effect of MSCs on multiorgan function and systemic inflammation after CPB. There were no differences in post-CPB hemodynamics and other clinically relevant biomarkers between the 3 experimental groups in the acute period after CPB (Supplemental Tables 1, 2). At 3 hours after CPB, a significant increase was observed in the plasma interleukin 4 (IL-4) level in the MSC-HD group compared with CPB and MSC-LD groups. On the other hand, interleukin 8 was significantly reduced after high-dose MSC delivery compared with low-dose treatment (Table). Together our results indicate that whereas there are no acute adverse reactions after both low- and high-dose MSC delivery, circulating cytokine levels are dose dependently modulated by MSC delivery through CPB.

**FIGURE 4** Immunohistochemistry in the subventricular zone (SVZ). (A) Immunostains of doublecortin-positive (DCX⁺) cells in tiers 2-3 of the dorsolateral SVZ (DL-SVZ). Scale bar = 20 μm. (B) Immunostains of glial fibrillary acidic protein (GFAP) in the DL-SVZ. Scale bar = 50 μm. (C) Quantification of the average density of DCX⁺ cells. (D) Quantification of the average length of GFAP⁺ processes. Data values are shown as mean ± standard error of mean. DCX analysis: n = 5 in control and CPB groups, n = 4 in MSC groups; GFAP analysis: n = 5 in control, CPB, and MSC-HD groups, n = 4 in MSC-LD group. P values were determined by 2-way analysis of variance with Tukey comparisons. *P < .05, **P < .01, ***P < .001. (CPB, cardiopulmonary bypass; MSC, mesenchymal stromal cell; MSC-HD, MSC high dose; MSC-LD, MSC low dose; V-SVZ, ventral SVZ.)
under normal conditions, activated microglia undergo structural remodeling and adopt an amoeboid shape with highly retracted processes, a hallmark of brain inflammation. Our data showed that the CPB insult significantly reduced branch length and the number of branches (ie, amoeboid shape of microglia) compared with control in both cortex and white matter (Figure 3A-C). We found that both low- and high-dose MSC treatments normalized the reduction of branch length as well as the number of branches after CPB (Figure 3A-C), indicative of MSC-induced reduction of microglial activation. There were no differences between 2 dosages and brain regions (Figure 3B, C), suggesting dose-independent and pancerebral effects.

MSC TREATMENT CHANGES DISTRIBUTION OF YOUNG NEURONS WITHIN THE SVZ. SVZ neuroblasts are observed moving tangentially close to the walls of the lateral ventricles and along blood vessels in human infants. We have previously shown that MSC treatment at 10 × 10^6 cells per kilogram increases the number of SVZ neuroblasts shortly after CPB, probably through acute migration of neuroblasts toward outer regions of the SVZ. Consistent with previous findings, a significant increase in the neuroblast number at the outer region was seen after both low- and high-dose MSC treatments compared with control and CPB (Figure 4A, C). No regional differences in the increase of neuroblasts were observed between the dorsolateral and ventral SVZ after MSC delivery (Figure 4A, C), similar to our previous observations. Notably, the thickness of tier 1, a dense cell band of neuroblasts, in the dorsolateral SVZ region was significantly increased in the MSC-HD group compared with the CPB and MSC-LD groups (Figure 4B, D). The dorsolateral SVZ is the most active neurogenic niche in the postnatal piglet brain. Thus, results in this study suggest a dose-dependent acute response of SVZ neurogenic activity to MSC delivery during CPB.

COMMENT

This study in a porcine model has characterized the dose-dependent effect of MSC delivery through CPB. Our analysis has shown CPB as an efficient administration system for MSC delivery and demonstrated that MSC treatment through CPB is safe up to 100 × 10^6 cells per kilogram. In addition, we have shown that after CPB, high-dose MSC treatment significantly increases expression of IL-4, one of the critical cytokines regulating immune and inflammatory responses. These studies have also indicated that both low- and high-dose MSC treatment shifts the microglial phenotype to a less proinflammatory state based on morphologic observations. Finally, we observe a unique dose-dependent reaction to the MSC treatment in the most active neurogenic niche of the infant brain.

Our previous ex vivo study has demonstrated that the administration of MSCs does not interfere with oxygenator function during CPB. In addition to oxygenator performance, our studies using the translational piglet model reveal no adverse effects on post-CPB hemodynamics and other clinically relevant biomarkers after high-dose MSC treatment. An MRI study demonstrated that intra-arterial cell infusion through CPB efficiently distributes SPIO-labeled MSCs throughout the entire brain. There were no signs of stroke with MSC-HD, confirming the feasibility of MSC treatment through CPB up to 100 × 10^6 cells per kilogram.

MSCs can be enriched and expanded from multiple sources. Because most clinical trials in stroke have used bone marrow-derived MSCs, the safety of bone marrow-derived MSCs has been well established compared with other sources. MSC treatment may be either autologous or allogeneic. The autologous cell treatment requires a significant period to expand cells and to confirm the quality and safety before transplantation, limiting use during neonatal cardiac surgery. On the other hand, the use of an allogeneic source of MSCs allows the availability of cells from healthy donors “off the shelf,” which would be a major advantage for clinical practice.

MSCs have significant anti-inflammatory properties. Indeed, MSC delivery through CPB during the rewarming period changed the microglial activation state from activating to inactivating phenotype. Overactivated microglia, so-called reactive microgliosis, may cause neurotoxic effects and ultimately induces neuronal death. Therefore, the microglial phenotype switch caused by MSCs probably inhibits microglia-induced neurotoxicity after CPB. Our studies also found MSC-induced modulation in plasma cytokine and chemokine levels after CPB and DHCA. Notably, high-dose MSCs increased the IL-4 level at 3 hours after CPB, even though there was no significant increase after the administration of low-dose MSCs. IL-4 is known for regulating a variety of immune responses, including cell proliferation, apoptosis, and expression of numerous genes in various cell types. IL-4 has also been characterized as a promoter of M2 polarization, an anti-inflammatory phenotype, in microglia and microphages. In vivo, IL-4 promotes M2-like macrophage phenotypes after stroke, stimulates astrocytes to secrete growth factors, and improves neurocognitive function in a mouse model of brain ischemia. Interleukin 8 is one of the proinflammatory cytokines that affects chemotaxis, recruitment, and adhesion of neutrophils. Interestingly, there was a significant difference between MSC-HD and MSC-LD at 3 hours after CPB, with a
decrease in MSC-HD and an increase in MSC-LD. We also found a trend toward an increase of interleukin 10, the anti-inflammatory cytokine, in the MSC-LD group. Cytokines are highly complex regulatory systems, and a proper balance between various cytokines is necessary in tissue repair and regenerative processes. The complete role of cytokines during CPB has yet to be clarified, although our results along with these previous studies suggest that high-dose MSC treatment has the potential to mitigate systemic and brain-specific inflammation caused by CPB.

In addition to an increase of neuroblasts in outer regions of the SVZ as we have previously reported,8 significant expansion of tier 1 was also observed after high-dose MSC delivery. Although the contribution to postnatal brain maturation is largely unknown, the area is recognized as a unique neurogenic niche including various cell populations, such as radial glia-like cells (ie, neural stem cells), neural and glial progenitors, neuroblasts, and vasculatures.14,27 Jinnou and coworkers18 demonstrated that fibers of radial glia-like cells in this region can act as a scaffold for neuroblasts that migrate to the lesion site after neonatal brain injury in mice. Notably, time-lapse imaging in the study has indicated rapid growth of the fibers in the mouse radial glia-like cells with a mean velocity of about 5 μm/h. In addition to cell migration within the SVZ, it is possible that extension of radial glia fibers may contribute to the rapid expansion of the tier 1 after high-dose MSC treatment. Our recent studies have demonstrated that CPB causes prolonged alterations in SVZ neurogenic properties.13 We certainly need to assess the long-term effect of MSC treatment on SVZ neurogenic activities and the dose-response curve using our survival model.

In addition to a lack of long-term assessment, small sample size is another limitation of this study. Although we conducted a well-controlled procedure in the model, it is possible that the small numbers contributed to the lack of difference between groups in circulating cytokine levels. In this study, the MRI data were acquired by 2 MRI scanners, according to availability of the imaging resource during experiments. To account for the potential difference between 2 MRI systems, we standardized our quantification of the SPIO counts using an unbiased image processing pipeline for all 3T and 7T. Our results were able to show the spatial distribution of MSCs across the brain. SPIO particles co-label with a green fluorescence protein.12 Our future studies will determine the dose-response relationship on unique migration dynamics of MSCs delivered through CPB. These studies investigate the dose effect of MSCs after ischemia-reperfusion injury and reoxygenation injury of brain that can be reproduced in the model used. On the other hand, it is possible that acute reactions of MSCs may be altered according to CPB with or without DHCA. A more common and less risky scenario, such as CPB only, should be assessed in future studies.

A prospective open-label, single-center, phase 1 clinical trial of MSC delivery through CPB in pediatric cardiac surgery (NCT04236479) is currently ongoing. The trial will determine not only the safety and feasibility but also the appropriate dosage level of MSCs in infants with CHD.

In conclusion, MSC delivery through CPB is feasible up to 100 × 10⁶ cells per kilogram. This unique therapeutic approach during cardiac surgery has the potential to reduce systemic and cerebral inflammation and to modulate responses of the active neurogenic niche to CPB in the infant with CHD. Further investigation is necessary to assess the effect of MSCs and the dose-dependent response in a long-term model.

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DISCLOSURES
Patrick J. Hanley is a cofounder and on the board of directors of Mana Therapeutics. He is on the scientific advisory board of CellEvolve and an advisor to MaxCyte and Cellerion.


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REVIEW ARTICLE

Trajectories of neurodevelopment and opportunities for intervention across the lifespan in congenital heart disease

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ABSTRACT

Children with congenital heart disease (CHD) are at increased risk for neurodevelopmental challenges across the lifespan. These are associated with neurological changes and potential acquired brain injury, which occur across a developmental trajectory and which are influenced by an array of medical, socio-demographic, environmental, and personal factors. These alterations to brain development lead to an array of adverse neurodevelopmental outcomes, which impact a characteristic set of skills over the course of development. The current paper reviews existing knowledge of aberrant brain development and brain injury alongside associated neurodevelopmental challenges across the lifespan. These provide a framework for discussion of emerging and potential interventions to improve neurodevelopmental outcomes at each developmental stage.

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KEYWORDS

Congenital heart disease; neurodevelopmental outcomes; neurodevelopmental interventions

Advances in prenatal diagnosis, neonatal and perioperative care, and medical, educational, and psychosocial care have led to increased survival rates and improved outcomes for individuals with Congenital Heart Disease (CHD). Within the context of these remarkable gains, there are clear patterns of changes in brain development, brain injury risk, and associated neurodevelopmental challenges observed across the lifespan, particularly for those children requiring surgery within the first year of life (Marino et al., 2012).

These neurological differences have been referred to as a unique “encephalopathy of CHD,” with intertwined influences of alterations in typical brain development and acquired brain injury (Volpe, 2014). That is, changes to the trajectory of brain development begin in the fetal period and are further compounded by neonatal and perioperative...
brain injuries. There is a multitude of medical, environmental, personal, and socio-demographic risk factors that impact these trajectories. This is reflected in the resulting spectrum of neurodevelopmental outcomes associated with CHD, which are heterogeneous but impact a characteristic set of skills (Cassidy et al., 2017; Sanz et al., 2021).

Most notably, neurological changes and associated neurodevelopmental challenges in CHD occur over the course of development, likely in a developmental cascade, with earlier delays or impairments impacting the continued course of both skill development and brain development (Sanz et al., 2021). This paper will review our existing knowledge of brain development, injury, and recovery over the course of the lifespan in individuals with CHD, along with associated neurodevelopmental challenges. These are used as a frame to explore potential key timepoints for interventions that might interrupt the cascade of changes described, to review those care strategies and interventions that are currently in use or under investigation, and to reveal understudied opportunities for change.

**Prenatal brain development in CHD**

Human brain development is a complex process with morphologic events occurring across developmental stages, and a prolonged period of refinement of connections that occurs in the third trimester and extends into the early postnatal period. Technical advancements in brain magnetic resonance imaging (MRI) have revealed an increased frequency of structural and developmental abnormalities of the fetal and neonatal brains in the setting of CHD (Peyvandi et al., 2019). It was long thought that developmental abnormalities in children with CHD were secondary to surgical and perioperative factors, such as the need for cardiopulmonary bypass in infancy. However, in one of the first neonatal brain MRI studies performed, Miller et al. observed that neonates with complex forms of CHD (d-transposition of the great arteries and single-ventricle physiology) have evidence of delayed brain development even before going to the operating room (S. P. Miller et al., 2007). Subsequently, Limperopoulos et al. observed these same patterns in the 3rd trimester fetus with complex CHD, demonstrating that delayed brain development begins in late gestation (Limperopoulos et al., 2010). Importantly, this divergence from typical patterns of brain development begins in the 3rd trimester of fetal life – a time of rapid brain growth and development that presumably requires a significant increase in oxygen and substrate delivery. Overall, late gestation fetuses and newborns with significant CHD have smaller brains (Limperopoulos et al., 2010) with simplified cortical gyriﬁcation (Clouchoux et al., 2013), less organized white matter tracts (S. P. Miller et al., 2007), and immature biochemistry (Limperopoulos et al., 2010). With advancements in fetal MRI, including technology to account for fetal and maternal motion, there is new evidence that fetuses with CHD have decreased cerebral oxygenation (Peyvandi et al., 2021). Importantly, perinatal impairments in brain growth appear to affect subsequent brain growth trajectories (C. M. Ortinau et al., 2018) and neurodevelopmental outcomes in infancy (Sadhwani et al., 2022). The etiology of delayed brain development in the late gestation fetus with CHD is likely multifactorial, with contributions from genetic abnormalities, cardiovascular physiology, and environmental factors.
**Cardiovascular physiology**

The third trimester of fetal life is marked by a period of rapid brain growth and refinement of connections. Consequently, blood flow to the fetal brain increases and is estimated to be approximately 25% of the combined ventricular output in the third trimester (Rudolph, 2018). In the normal fetus, cerebral blood flow is supplied by highly oxygenated blood from the ductus venosus preferentially streaming across the foramen ovale to the left atrium and ventricle. Depending on the sub-type of CHD, cerebral blood flow and thus oxygen delivery can be impaired. For example, in D-transposition of the great arteries, the aorta and pulmonary artery are transposed, and thus the higher oxygenated blood reaches the pulmonary vasculature as opposed to the aorta. In hypoplastic left heart syndrome, inadequate left heart structures lead to reversal of blood flow in the foramen ovale, with mixing of oxygenated and deoxygenated blood in the right ventricle and in the cases of aortic atresia, retrograde flow in the ascending aorta.

Multi-modal fetal imaging techniques have demonstrated how this altered circulation can lead to flow disturbances affecting in-utero growth and brain development. Cerebral Doppler ultrasound can assess cerebral vascular resistance in the middle cerebral artery (MCA). Several studies have observed altered cerebral blood flow patterns in the fetus with CHD (Donofrio et al., 2003; Kaltman et al., 2005). In particular, fetuses with hypoplastic left heart syndrome consistently have the most abnormal patterns, with decreased resistance in the MCA thought to be an autoregulatory phenomenon in response to decreased flow and oxygenation to the brain, similar to the growth restricted fetus. However, it is unclear if fetuses with CHD have normal autoregulatory capacities of the brain. A recent study measuring MCA vascular resistance at baseline and after brief administration of maternal hyperoxia demonstrated variable response across different sub-types of CHD (Hogan et al., 2021). This may reflect differences in cerebral autoregulatory capacities in the setting of complex CHD. More recently, novel fetal cardiac MRI techniques have been developed, which enables measurements of flow and oxygen saturation in fetal blood vessels. By combining fetal brain MRI and cardiovascular magnetic resonance, Sun et al. found a correlation between fetal cerebral oxygen consumption and brain size (estimated brain weight) among fetuses with complex CHD in late gestation (Sun et al., 2015). Specifically, there was a direct correlation between estimated brain weight and cerebral oxygen consumption and a modest association between cerebral oxygen delivery and brain size. Both ultrasound- and MRI-based studies support the hypothesis that aberrations in cardiovascular physiology can result in decreased perfusion and oxygen delivery to the brain, affecting brain development and increasing susceptibility to brain injury (Peyvandi et al., 2019).

**Environmental influences on the developing fetal brain and the role of the placenta**

Natural experimental studies and other observational studies have demonstrated a link between prenatal maternal stress and developmental outcomes in offspring among children without congenital anomalies (Scheinost et al., 2016, 2017). The biological mechanism of these findings is hypothesized to be through epigenetic mechanisms at the level of the placenta acting as a mediator of maternal and environmental signals to the
developing fetus (Bale, 2015; Nugent & Bale, 2015). Not surprisingly, maternal stress levels are noted to be significantly higher after the diagnosis of a fetal anomaly including fetal CHD (Rychik et al., 2013). In a study of pregnant women carrying a fetus with CHD, 65% experienced significant stress, 44% reported anxiety, and 29% reported depression. Furthermore, maternal stress and anxiety were associated with smaller hippocampal and cerebellum volumes in the fetus with CHD (Wu et al., 2020).

Placental pathology is known to be abnormal in pregnancies affected by significant CHD, and there is a relationship between cardiac development and placental health, which in combination can contribute to in utero brain development. Common abnormalities on pathologic exams have included thrombosis, infarction, chorangiosis, immature villi, and abnormal placental perfusion (Rychik et al., 2018; You et al., 2020). In addition, recent studies demonstrate abnormalities in placental vasculature in the setting of CHD with high rates of vascular malperfusion lesions (Leon et al., 2022). It is unclear whether placental abnormalities precede the development of CHD or if the placental pathology develops secondary to abnormal cardiovascular physiology. There is likely a complex interplay between maternal health, placental function, and developmental programming in the fetus that can have long-term effects on outcomes such as neurodevelopment, though future studies are necessary to identify these causal pathways.

Possible prenatal intervention strategies

Given the multi-factorial nature of delayed fetal brain development in utero, identifying an intervention to optimize outcomes is challenging. For certain complex forms of CHD, fetal cardiac interventions to change the natural history of disease have been in use for several years (Schidlow et al., 2017), though the impact of altering cardiovascular physiology on long-term developmental outcomes remains unclear (Laraja et al., 2017). Trials and experiments are underway to assess the utility of maternal hyper-oxygenation therapy, which is thought to increase oxygen flow to the brain, though it is unclear if this therapy holds promise given the potential impact on the placenta as well as flow patterns in the fetus (Edwards et al., 2019; Li et al., 2022). Behavioral interventions in utero to manage maternal stress hold great promise in the general population (Li et al., 2022) and can be applied to mothers with a fetal anomaly. Finally, animal models are underway to understand complex and cumulative events in the developing cortex and white matter and for the development of a potential neuroprotective approach (Leonetti et al., 2019; Morton et al., 2015). Studies using a large animal model found that neural stem/progenitor cells contribute to perinatal corticogenesis and suggest that restoration of the neurogenic potential of the unique cell population is a candidate therapeutic target for improving cortical growth (Morton et al., 2017). In a rodent model of chronic hypoxia, treatment with tetrahydrobiopterin (BH4) mitigated the deleterious effects of chronic hypoxia on the developing white matter (Romanowicz et al., 2019). Because BH4 is already approved by the FDA and shown to be safe during pregnancy, there is translational potential for BH4 to become a new neuroprotective therapy for fetuses with CHD. In addition, genetic etiologies are now recognized as a very important contributor. Integrative approaches involving genetics, cell biology, and molecular biology to model brain development in CHD will play a key role in defining the underlying
causes of brain dysmaturation and optimal windows for treatment to improve neurodevelopmental outcomes in CHD.

**Neonatal brain development and injury in CHD**

As outlined previously, differences in brain development begin in the fetal period in CHD, and may be related to alterations in brain perfusion and oxygenation (Petit et al., 2009; Sethi et al., 2013). Neonates with TGA, ToF, and HLHS have significantly smaller head circumference (HC) than children without CHD (Barbu et al., 2009; Manzar et al., 2005; Rosenthal, 1996; Shillingford et al., 2007), which may be related to these differences in fetal brain development. Newly acquired brain injury is common among neonates with critical CHD, and this population is known to be at high risk for neurologic and neurodevelopmental differences (Donofrio et al., 2011; Licht et al., 2009; S. P. Miller et al., 2007; Sarajuuri et al., 2012; Shillingford et al., 2007). Brain injury and neurologic outcomes are due to multiple, cumulative influences beginning in the fetal period and extending throughout a survivor’s lifetime (Donofrio & Massaro, 2010; Limperopoulos et al., 1999, 2000; Mulkey et al., 2013).

**Trouble transitioning: fetal predictors of brain injury**

Delayed brain development, in particular, has been shown to correlate with abnormal postnatal brain development and pre- and postoperative brain injury in neonates with CHD (Andropoulos et al., 2010; Beca et al., 2013; Brossard-Racine et al., 2016; Claessens, Khalili, et al., 2019; Dimitropoulos et al., 2013). Preoperative brain injury in CHD appears to be strongly related to microstructural and metabolic brain development (Dimitropoulos et al., 2013) and may be related to differences in the cerebral circulation (C. Ortinau et al., 2012). Specific markers of delayed brain development, including enlarged ventricular and extra-axial CSF spaces (Brossard-Racine et al., 2016; Claessens, Khalili, et al., 2019), brain maturation scores (Andropoulos et al., 2010; Beca et al., 2013), and abnormal microstructural and metabolic brain developments (Dimitropoulos et al., 2013) are associated with more severe pre-and postoperative brain injury.

Cardiac physiology and timing of diagnosis have also been associated with the degree of brain injury (Peyvandi et al., 2016; Peyvandi, Kim, et al., 2018). Single-ventricle physiology has been most clearly associated with a higher risk for postoperative brain injury compared to newborns with TGA (Beca et al., 2013; Limperopoulos et al., 2000; Mcquillen et al., 2007; Mulkey et al., 2013; Peyvandi, Kim, et al., 2018). Interestingly, prenatal diagnosis of single-ventricle heart disease or transposition of the great arteries may be protective, as prenatally diagnosed neonates with CHD have a less severe postnatal brain injury than postnatally diagnosed neonates (Peyvandi et al., 2016).

**Contributing factors to neonatal brain injury**

There are multiple risk factors for pre- and postoperative brain injury in neonates with CHD (Table 1). Physiologic factors that have been most commonly associated with brain injury include hypoxemia and time to surgery (Lynch et al., 2014; Petit et al., 2009),
Table 1. Risk factors and proposed interventions for brain injury in complex congenital heart disease.

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<thead>
<tr>
<th>Risk Factors</th>
<th>Supporting Studies</th>
<th>Interventions under investigation</th>
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<td>Maternal Hyperoxygenation</td>
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<td>Cardiac Physiology (Single vs Two Ventricle)</td>
<td>Limperopoulos et al. (2000)</td>
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<td>Timing of Diagnosis of Cardiac Disease*</td>
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<td>Decreased time to surgery/Delivery planning</td>
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<td>Maternal Stress</td>
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<td>Preoperative</td>
<td>Lower O2 Saturation</td>
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<td>Petit et al. (2009)</td>
<td>Precision monitoring/therapy to optimize blood pressure, oxygenation, and cerebral perfusion</td>
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<td>Hypotension</td>
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<td>Length of Time to Surgery</td>
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<td>Postoperative</td>
<td>Lower O2 saturation</td>
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<td>Lower Cerebral rSO2**</td>
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<td>Promoting a more stimulating home environment</td>
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* Peyvandi et al. (2016) found infants prenatally diagnosed with CHD had less brain injury (Peyvandi et al., 2016).
** Claessens, N.H.P et al. (2019) found lower cerebral rSO2 was NOT associated with brain injury (Claessens, Jansen, et al., 2019).
hypotension (Dimitropoulos et al., 2013; Galli et al., 2004; Mcquillen et al., 2007), and decreased regional cerebral oxygen saturation (rSO2) (Dent et al., 2005; Mcquillen et al., 2007). Lower preoperative oxygen saturation has been associated with more severe preoperative brain injury in studies evaluating neonates with heterogeneous CHD diagnoses, and in TGA, specifically (Block et al., 2010; Dimitropoulos et al., 2013; Petit et al., 2009). Preoperative hypotension is predictive of preoperative brain injury scores in CHD (Dimitropoulos et al., 2013). Autonomic function is immature in infants with CHD (Mulkey et al., 2019), and worse autonomic function correlates with preoperative brain injury scores in this population (Schlatterer et al., 2021).

Aberrant electrocortical activity, as measured by EEG and amplitude-integrated EEG (aEEG), is common among neonates with CHD, and is associated with abnormal brain development and brain injury (Meibius et al., 2018; Mulkey et al., 2015). Abnormal background activity on aEEG is highly associated with preoperative brain injury (60% in one study) (3,4) as well as brain atrophy (low brain volume) prior to surgery (Mulkey et al., 2015). Immature structural and microstructural brain development also correlates with abnormal brain electrical activity (Birca et al., 2016). Neonates with immature structural brain development and preoperative brain injury had increased high-frequency connectivity on EEG, and neonates with delayed microstructural brain development had weaker low-frequency connectivity on EEG (Birca et al., 2016). Moreover, failure to recover continuous background activity on aEEG by 48 hours postoperatively is associated with mortality and worse neurodevelopmental outcomes on BSID-III at age 2 years (Gunn et al., 2012). Thus, early differences in aEEG and EEG are highly associated with developmental outcomes and mortality, indicating long-term impact (Birca et al., 2016; Gunn et al., 2012; Meibius et al., 2018; Mulkey et al., 2015).

Postoperatively, brain injury is associated with lower postoperative oxygen saturation (Galli et al., 2004), hypotension in the first 24–48 hours following surgery (Dimitropoulos et al., 2013; Galli et al., 2004; Mcquillen et al., 2007), and lower postoperative regional cerebral rSO2 (Galli et al., 2004; Mcquillen et al., 2007). However, one recent study did not find an association between lower postoperative cerebral rSO2 and postoperative brain injury (Claessens, Jansen, et al., 2019).

Surgical considerations associated with neonatal brain injury in CHD include time to surgery (Lynch et al., 2014; Petit et al., 2009), need for balloon atrial septostomy (BAS) (Block et al., 2010; Dimitropoulos et al., 2013; Kelly et al., 2019; Mcquillen et al., 2006), and prolonged bypass and circulatory arrest times (Beca et al., 2013). Both pre- and postoperative white matter injuries are associated with a longer time between birth and surgery (Lynch et al., 2014; Petit et al., 2009). This association may be explained by a progressive decrease in cerebral tissue oxygenation between birth and surgery in TGA or HLHS (Lynch et al., 2018). BAS is associated with stroke in several studies (Block et al., 2010; Dimitropoulos et al., 2013; Kelly et al., 2019; Mcquillen et al., 2006), and prolonged cardiopulmonary bypass and circulatory arrest times correlate with new postoperative white matter injury (Beca et al., 2013). Overall, infants with CHD undergo a complex clinical course during which they are likely to experience multiple physiologic changes that may contribute to the development of brain injury.
Neuroimaging predictors of later outcomes

Neonates with CHD may experience two separate but related processes that contribute to neurodevelopmental outcomes, namely, delayed brain development and brain injury. Structural brain immaturity during the neonatal period is associated with neurodevelopmental outcomes in survivors of CHD at 2 years of age (Beca et al., 2013), and another study revealed an association between smaller cortical gray matter and cerebellar volumes and lower fine motor scores at 9 and 18 months (Stegeman et al., 2022).

The relationship between brain injury and neurodevelopmental outcomes in CHD is an area of active investigation. Neonatal white matter injury, particularly involvement of the posterior limb of the internal capsule, is associated with lower IQ and more severe motor and attention deficits in school-age survivors of CHD (Claessens et al., 2018). One study showed that preoperative white matter injury correlates with lower Bayley Scales of Infant and Toddler Development III scores at age 12 months in CHD (Andropoulos et al., 2012). Another study found that moderate-to-severe WMI before or after surgery is associated with lower motor scores at 2.5 years but not 12 months (Peyvandi, Chau, et al., 2018). Clinically silent, small neonatal strokes were not associated with adverse ND outcomes and even trended to better outcomes in TGA (Peyvandi, Chau, et al., 2018). However, there does appear to be an association between acute ischemic stroke in the corticospinal tract and abnormalities of muscle tone and gross motor delay, and severe ischemic brain injury is associated with a diagnosis of cerebral palsy in CHD (Stegeman et al., 2022). In addition, less is known regarding the association between early alterations in brain development or brain injury and longer-term neurodevelopmental outcomes in school age, adolescence, or adulthood.

Genetic influences on neurodevelopment

CHD has strong heritability, and a genetic abnormality can be identified in up to 50% of syndromic CHD and 10% of non-syndromic sporadic cases (Homsy et al., 2015). Genetic anomalies include chromosomal duplication or deletion (aneuploidy, 13%), copy number variation (10%), de novo single nucleotide variant (10%) (Zaidi & Brueckner, 2017). Aneuploidies were the earliest genetic anomalies to be linked to CHD and have a well-described association with adverse neurodevelopmental outcome (e.g., Down syndrome and Turner syndrome). Gaynor et al. were the first to focus on the importance of patient-specific factors, including genetic syndromes and ApoE genotype for neurodevelopmental outcomes in early single-center studies (Gaynor et al., 2007) subsequently confirmed in secondary analyses of prospective trials (Newburger et al., 2012). The review of published outcome studies over two decades found the presence of genetic/extracardiac anomaly as a significant risk factor for worse neurodevelopmental outcomes (Gaynor et al., 2015). With improvements in genomic sequencing technology, multicenter projects have embarked on large-scale sequencing efforts (Hoang et al., 2018). De novo variants in genes with high heart and brain expression were identified in 20% of subjects with CHD and neurodevelopmental delay but only 2% with isolated CHD (Homsy et al., 2015). Rare de novo variants in isolated CHD overlap substantially with those identified in children with autism (Jin et al., 2017). These include genes involved in chromatin remodeling, cilia genes, and signaling pathways involving Notch and Ras known to be
important for both brain and heart development (Zaidi & Brueckner, 2017). Additionally, de novo damaging variants were associated with postoperative outcomes in CHD (Boskovski et al., 2020). Together, this suggests that genetic variants can affect neurodevelopmental outcomes independently, but also as potential modifiers of neurodevelopmental outcomes in CHD patients. This highlights the need for a systematic search and inclusion of genetic risk factors in neurodevelopmental outcome prediction models in CHD.

**Neurobehavioral outcomes in the neonatal period**

Neurodevelopmental impairments are present even in the earliest stages of development. Neonatal evaluations have suggested a range of impairments that are present even prior to surgical intervention. Compared with typically developing term neonates, neonates with CHD are known to have poorer automatic and motor regulation, lower attention and arousal scores, poorer self-regulation, and poor feeding (Butler et al., 2017, 2019; Desai et al., 2023; Hogan et al., 2018). Neonatal attentional and self-regulation scores have been of particular interest since they may relate to improved feeding at discharge (Gakenheimer-Smith et al., 2019) and to later motor delays, but not later cognitive or language delays (Campbell et al., 2022). Poor feeding also predicts worse motor outcomes (Medoff-Cooper et al., 2016).

**Interventions in the neonatal and perioperative period**

Identified risk factors for brain injury and altered development can suggest opportunities for neuroprotective interventions that may prevent injury and promote development, leading to improved neurodevelopmental outcomes.

Potential roles of acute and chronic hypoxic ischemic and inflammatory mechanisms have led to trials of maternal progesterone in the fetal period (NCT02133573) and perioperative allopurinol (NCT04217421). An initial single-center trial of allopurinol before, during and after surgery did not show significant benefit to infants with two-ventricle physiology but may have decreased adverse cardiovascular events (R. R. Clancy et al., 2001). The consistent finding that increased time to surgery is associated with the risk of WMI in both TGA and HLHS newborns (Lim et al., 2019; Lynch et al., 2014; Petit et al., 2009) suggests that early surgery, when feasible, would be an effective non-pharmacologic intervention.

Lower postoperative blood pressure has been associated with an increased risk of new postoperative white matter injury (Galli et al., 2004; Mcquillen et al., 2007), suggesting the simple intervention of targeting higher blood pressures with preload, inotropic support, or vasopressors. While this is likely to be well tolerated and beneficial in newborns with TGA, concern arises for the postoperative management of HLHS where intraoperative and postoperative afterload reductions have improved survival after the Norwood procedure by preventing episodes of elevated systemic vascular resistance and low cardiac output syndrome (Hoffman et al., 2021). To achieve the optimal balance between cerebral perfusion and overall cardiac output with single-ventricle physiology will likely require individualized precision therapy guided by new methods for bedside
monitoring of cerebral blood flow, oxygenation, and autoregulation along with cardiac output.

A final area of concern and opportunity relates to exposure of the vulnerable neonatal brain to repeated anesthetics, narcotics, and benzodiazepines that have been associated with neurotoxicity and programmed neuronal cell death (Ing et al., 2022; Sarić et al., 2022). These findings, while well established in animal models, are a topic of multiple clinical research studies to establish their significance in humans (Andropoulos et al., 2014; McCann et al., 2019). Nevertheless, neuroprotective clinical trials have been proposed using putative non-neurotoxic and possibly direct neuroprotective anesthesia and sedation with dexmedetomidine. Trials are also underway examining the delivery of mesenchymal stromal cells through cardiopulmonary bypass as a neuroprotective measure, as this has been shown to protect stem/progenitor cells in the subventricular zones and to promote migration of neuroblasts in animal models (Maeda et al., 2020). A major challenge to neuroprotective trials in CHD newborns is the need for early surrogate outcome measures that reliably predict long-term childhood and adult neurodevelopmental outcomes.

**Developmental care for neonates and infants**

Recently, more focus has been placed on neurodevelopmental care in the CICU setting (Lisanti et al., 2016; Torowicz et al., 2012). Individualized developmental care in the ICU setting is known to improve neurobehavioral functioning and structural development in preterm infants (Als et al., 2004). Currently, developmental care is variable among CICUs within North America (Sood et al., 2016), in part because critically ill infants with CHD face unique challenges (Lisanti et al., 2019). Proposed models for developmental care in the CICU include the following elements: parental engagement/family-centered care; an individualized, cue-based plan; and an emphasis on a supportive hospital environment, with particular attention to circadian rhythms, noise/light levels, feeding, social interaction/play, kangaroo care, and developmentally considerate approaches to medical procedures (Desai et al., 2023; El-Farrash et al., 2020; Lisanti et al., 2019; Peterson & Evangelista, 2017). Interdisciplinary engagement, including parents, speech/language pathologists, physical/occupational therapy, child/family life therapists, psychology, social work, dieticians, nurses, and physicians is critical for implementation of any successful developmental care program in the CICU (Butler et al., 2017; Peterson & Evangelista, 2017), and a well-planned process of implementation with a strong commitment from leadership and staff is necessary for success (Sood et al., 2016). The inclusion of parents, families, and children with CHD in the design of supportive psychosocial interventions is also critically important to ongoing research (Sood et al., 2022). Many CICUs are beginning to implement their own developmental care programs based on the above ideas, and the community of caregivers and families of CHD patients await further studies to determine their long-term impact.

**Neurodevelopmental outcomes from infancy to adulthood**

The broad range of influences on the brain, beginning in fetal life and continuing through the neonatal and perioperative periods, fundamentally alter the trajectory of brain
development and associated neurodevelopmental skills. Just as CHD is heterogeneous, outcomes can be quite varied, and often involve a range of neurocognitive domains (Cassidy et al., 2017).

For infants and toddlers, early difficulties with feeding, motor development, language acquisition, and self-regulation are observed (Brosig et al., 2007; Latal, 2016; T. Clancy et al., 2020; Ware et al., 2020). As children enter school, which presents increased cognitive, academic, and social-emotional demands, additional challenges become evident. Specifically, weaknesses in attention, executive functioning, visual-spatial processing, and academic achievement emerge (Bellinger & Newburger, 2010; Cassidy et al., 2015, 2017; Griffin et al., 2003; Sanz et al., 2017), as well concerns for social cognition, mood, emotional, and behavioral regulation, and/or anxiety (Bellinger et al., 2009; Kovacs et al., 2009; Wilson et al., 2015). There are also higher rates of neurodevelopmental disorder diagnoses (ADHD, autism spectrum disorder, learning disabilities) in children with CHD compared to the general population (Loblein et al., 2022; Razzaghi et al., 2015; Ryan et al., 2019; Tsao et al., 2017; Verrall et al., 2019), with increased risks and poorer outcomes for those with comorbid genetic conditions (Latal, 2016; Marino et al., 2012; Wernovsky, 2006). Further, deficits in these areas can hinder the development of daily living (adaptive) skills needed to successfully navigate the transition to adulthood and ultimately achieve independence (Cassidy et al., 2017; Ilardi et al., 2017).

In addition, with improving care, there is now a growing population of adults with CHD, and adults with CHD now outnumber children with CHD (Marelli et al., 2014). Despite this, we have an extremely limited understanding of the continued evolution of neurodevelopmental concerns over the lifespan, particularly during the aging process. Although mechanisms are poorly understood, underlying disease factors present increased risk for vascular problems and acquired cardiovascular events (Keir et al., 2019; Melazzini et al., 2019). These presumably contribute to substantially increased rates of dementia and neurocognitive decline in older adults with CHD, a topic that remains woefully understudied (Keir et al., 2019).

Though research up to this point has generally focused on specific skills sets or diagnoses in a cross-sectional fashion, researchers and clinicians have been encouraged to shift their conceptual framework away from static models that define a unitary, “neurodevelopmental signature,” toward a dynamic conceptual framework that includes a broader range of outcomes that interact and evolve over time, in a developmental cascade (Sanz et al., 2021). When viewed this way, interventions across the lifespan become important to interrupting these cascades and promoting improvement in neurodevelopmental trajectories. An emerging lifespan perspective also becomes critically important.

**Interventions across the lifespan**

Despite this, to date there has been notably less focus on the development and implementation of interventions for the CHD population, with only a small number of single-center investigations focused on interventions for children, adolescents, and young adults with CHD (Calderon & Bellinger, 2015; Cassidy et al., 2021). The efficacy of intervention programs developed and implemented for high-risk populations such as children born preterm and those diagnosed with chronic medical conditions and/or
neurodevelopmental disorders is well documented in the literature (Case-Smith, 2013; Spittle et al., 2007). As there are many specific areas of concern that are shared between chronic medical conditions, including CHD, these interventions offer a reasonable starting point for developing intervention strategies.

**Interventions in infancy and early childhood**

The importance and efficacy of direct early intervention services (e.g., physical therapy, occupational therapy, and speech and language therapy) for children who are at increased risk for or diagnosed with neurodevelopmental disability is well established (Majnemer, 1998; Nores & Barnett, 2010). While these services are frequently utilized by young children with CHD, little is known about the longitudinal impact of these child-directed therapies for this specific population. Parent-oriented psychoeducational interventions for young children with CHD, investigated through a series of controlled trials conducted as part of the Congenital Heart Disease Intervention Project (CHIP), have demonstrated improved cognitive development, maternal adjustment, and family functioning (McCusker et al., 2010, 2012; van der Mheen et al., 2019).

**Interventions for attention/executive function**

Across medical and neurodevelopmental disorder populations, there is a growing interest in neurocognitive interventions, particularly to address executive functioning deficits; however, to date, the effectiveness of these programs is mixed (Diamond & Ling, 2016; Melby-Lervåg & Hulme, 2013). A single-center study examining the effectiveness of a computerized neurocognitive intervention (Cogmed) for adolescents with CHD found no improvement in the primary outcome measure (working memory), but improvements in inhibitory control and parent-report of cognitive regulation were reported at 3-month follow-up (Calderon et al., 2020). Pharmacological treatment of symptoms of ADHD (inattention, hyperactivity, impulsivity) is often the most effective treatment for children and adolescents (Wolraich et al., 2019). The AHA released a scientific statement in 2008 (Vetter et al., 2008), with considerations for individuals with CHD recommending thorough evaluation and continued monitoring due to the cardiovascular effects of stimulant medication. However, to date there have not been any studies investigating the efficacy of these medications for individuals with CHD with or without diagnosis of ADHD. This is also the case for pharmaceutical treatment of mood and anxiety disorders in this population, which has not been directly studied.

**Social–emotional functioning and transition to adulthood**

Interventions to address social-emotional functioning are especially crucial during adolescence and adulthood given the bidirectional relationship between psychosocial and medical health (Kovacs & Bellinger, 2021), though few programs have been evaluated. Aerobic/physical activity programs have been shown to have positive effects on psychosocial outcomes, cognitive functioning, and quality of life based on self- and proxy-report (Dulfer et al., 2017). While some psychosocial intervention protocols have been described
and determined feasible for adolescents and adults with CHD, there is limited evidence for the efficacy of these interventions (Tesson et al., 2019).

In addition to interventions that directly target psychosocial and cognitive health in adolescents and young adults, interventions to address loss to medical follow-up have become more important. Namely, transition to continued specialized medical care is critical to reduce morbidity and mortality, and additional invasive interventions, which presumably would also improve psychological and neurocognitive outcomes over the remainder of the lifespan (Kollengode et al., 2018; Moceri et al., 2015; Nitta et al., 2021). There is also a more recent focus on developing interventions focused on preparing adolescents with CHD to enter adult care. One such nurse-led program has demonstrated efficacy in improving transition readiness (Mackie et al., 2018). Implementation of another CHD transition clinic intervention, including focused teaching, completion of self-assessment questionnaires, and tracking through a clinical registry, resulted in improved follow-up rates and self-ratings of transition readiness (Gaydos et al., 2020). Additional clinical trials geared toward improving independence and mental health over the course of the transition to adulthood are underway (Saarijärvi et al., 2021).

Despite the rapidly increasing population of older adults with CHD, the topic of monitoring and intervention in this group remains understudied and in its early phases (Keir et al., 2019). From a practical standpoint, few centers offer neurodevelopmental monitoring past adolescence and young adulthood; indeed, most centers still focus care on early childhood (T. A. Miller et al., 2020). While the expansion of neurodevelopmental care into adulthood presents logistical challenges and would require collaboration across specialties, this should be viewed as a critical opportunity to improve long-term care and outcomes.

**Dilemmas and future directions**

In individuals with CHD, alterations to the trajectory of brain development begin in utero, evolve over time, and are compounded by additional injury. These interact with a multitude of medical and demographic factors and lead to a heterogeneous array of adverse neurodevelopmental outcomes. Improved understanding of brain development in the context of these factors provides us with a conceptual framework for the development of targeted interventions that might improve neurodevelopmental trajectories and outcomes.

Despite the promising interventions described above, there are several gaps in our understanding of neurodevelopment in CHD that limit our progress. Firstly, the majority of research on neurodevelopment in CHD is cross-sectional, with fewer longitudinal studies in limited cohorts (for a review, see Sanz et al., 2021). As a result, we have a poor understanding of the relationship between early neurological and neurodevelopmental findings and later outcomes at school age, adolescence, and young adulthood. It is likely that many of the impacts of these early alterations to fetal and brain development manifest in later development, though these connections have not yet been clearly characterized. We also have limited data regarding the efficacy of earlier interventions on subsequent neurodevelopmental trajectories and outcomes. Additionally, the heterogeneity of this patient population presents a significant research challenge. These factors highlight the need for larger, collaborative clinical data registries that will allow for more
complex analyses of factors that contribute to neurodevelopmental trajectories across the lifespan. Fortunately, these collaborative efforts are underway, with the Cardiac Neurodevelopmental Outcome Collaborative and Cardiac Networks United leading efforts to establish longitudinal, multisite registries to answer these questions (Gaies et al., 2019; Marino et al., 2020).

Additionally, social determinants of health have consistently been a significant contributor to both medical and neurodevelopmental outcomes in CHD (Lopez et al., 2022). These social determinants of health impact even the earliest stages of care, with lower rates of prenatal diagnosis (Krishnan et al., 2021) and poorer surgical outcomes (Gallegos et al., 2022) in specific demographic groups. Emerging research also strongly suggests that socioeconomic factors are important, primary drivers of neurodevelopmental outcomes in CHD (Bucholz et al., 2020; Favilla et al., 2021), and that the impacts of socioeconomic factors not only persist but become more pronounced over the course of a child’s development (Bucholz et al., 2021). Our understanding of racial and socioeconomic contributors to neurodevelopmental outcomes is inadequate and needs to become a focus of continued research. Important barriers to address include improved inclusion of diverse populations in research, development of improved culturally and linguistically appropriate measurement tools for neurodevelopmental and psychological outcomes in diverse populations and improved representation of diverse populations in our clinical and research teams (Sanz et al., 2021). Though addressing these barriers will improve our understanding of the issue, we also need clear, actionable changes to our care pathways and specific interventions to address these inequities in neurodevelopmental and health outcomes. Examples of this may include interventions to improve access to neurodevelopmental evaluations, which may improve access to needed therapies or early intervention services that are currently underutilized in CHD (Mussatto et al., 2018; Soto et al., 2011). Other possible interventions include supporting parents in providing a more stimulating home environment to promote cognitive development (Bon throine et al., 2021).

Along these lines, we suspect that addressing disparities in access to clinical care, specifically equitable participation in formal neurodevelopmental follow-up programs, is critical. This is needed to provide earlier identification and clear documentation of neurodevelopmental problems, which are often required in order to access needed therapies and services in the community. Despite this, there are likely financial barriers (e.g., insurance coverage), language barriers, and cultural barriers that reduce access to needed care and thus create disparities in neurodevelopmental trajectories. Breaking down these barriers to care across the lifespan will also be critical to improving outcomes for all individuals with CHD.

Overall, we are now developing a better understanding of neurological and neurodevelopmental trajectories in individuals with CHD. This understanding should now begin to serve the development and evaluation of specific intervention strategies to improve the trajectory, with targets for intervention across the lifespan, beginning in fetal life and continuing through adulthood. The development of large-scale registries and collaborations will be necessary to better understand the long-term consequences of these interventions. Finally, improved inclusion of diverse populations in our clinical and research efforts will be critical to begin to weave in an understanding of the impacts of social determinants of health.
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