Mesenchymal Stem Cells for Severe Intraventricular Hemorrhage in Preterm Infants: Phase I Dose-Escalation Clinical Trial

SO YOON AHN,[a,b,c,*,†] ORCID YUN SIL CHANG,[a,b,c,*,†] SE IN SUNG,[a] WON SOON PARK[a,b,c]

Key Words. Cell transplantation • Clinical trial • Infant, Newborn • Infant, Premature • Intracranial hemorrhages • Mesenchymal stromal cells

[a]Department of Pediatrics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea; [b]Stem Cell and Regenerative Medicine Institute, Samsung Medical Center, Seoul, South Korea; [c]Department of Health Sciences and Technology, SAIHST, Sungkyunkwan University, Seoul, South Korea

*Contributed equally.
†Co-first authors.

Correspondence: Won Soon Park, M.D., Ph.D., Department of Pediatrics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 06351, Korea. Telephone: 82.2-3410-3523; e-mail: wonspark@skku.edu

ABSTRACT

We previously demonstrated that transplanting mesenchymal stem cells (MSCs) improved recovery from brain injury induced by severe intraventricular hemorrhage (IVH) in newborn rats. To assess the safety and feasibility of MSCs in preterm infants with severe IVH, we performed a phase I dose-escalation clinical trial. The first three patients received a low dose of MSCs (5×10^6 cells/kg), and the next six received a high dose (1×10^7 cells/kg). We assessed adverse outcomes, including mortality and the progress of posthemorrhagic hydrocephalus. Intraventricular transplantation of MSCs was performed in nine premature infants with mean gestational age of 26.1 ± 0.7 weeks and birth weight of 808 ± 85 g at 11.6 ± 0.9 postnatal days. Treatment with MSCs was well tolerated, and no patients showed serious adverse effects or dose-limiting toxicities attributable to MSC transplantation. There was no mortality in IVH patients receiving MSCs. Infants who underwent shunt surgery showed a higher level of interleukin (IL)-6 in cerebrospinal fluid (CSF) obtained before MSC transplantation in comparison with infants who did not receive a shunt. Levels of IL-6 and tumor necrosis factor-α in initially obtained CSF correlated significantly with baseline ventricular index. Intraventricular transplantation of allogeneic human UCB-derived MSCs into preterm infants with severe IVH is safe and feasible, and warrants a larger, and controlled, phase II study. STEM CELLS TRANSLATIONAL MEDICINE 2018;7:847–856

SIGNIFICANCE STATEMENT

The present phase I clinical trial is the first in human study of mesenchymal stem cells transplantation for severe intraventricular hemorrhage in preterm infants. No infant died or showed serious adverse effects related with stem cell transplantation in this study. Intraventricular transplantation of mesenchymal stem cells in preterm infants with severe intraventricular hemorrhage might be safe and feasible and warrants further phase II study.

INTRODUCTION

Intraventricular hemorrhage (IVH), a condition resulting from the rupture of the germinal matrix through the ependymal into the lateral ventricle, mainly occurs in premature infants [1, 2]. The risk and severity of IVH directly correlate with degree of immaturity [3], and the improved survival of extremely preterm infants, enabled by recent advances in neonatal intensive care, has resulted in an increased number of preterm infants at high risk of developing severe IVH [4]. More than 50% of preterm infants affected by severe (grade ≥3) IVH die or develop posthemorrhagic hydrocephalus (PHH) ultimately requiring shunt surgery in up to 40%–70% of cases [5–7]. Brain injuries induced by severe IVH and exacerbated by PHH result in increased mortality, and neurologic morbidities in survivors such as seizures, cerebral palsy, and developmental delay [5, 8–10]. Currently, no effective treatment is available to attenuate brain injury or prevent the progress of PHH after severe IVH in preterm infants. Therefore, the development of new, safe, and effective therapeutic modalities to improve the prognosis of this intractable and devastating neonatal disorder is an urgent issue.

Various preclinical data support the therapeutic potential of transplantation with mesenchymal stem cells (MSCs) for treating various intractable neonatal disorders including bronchopulmonary dysplasia (BPD) [11–14] and hypoxic ischemic encephalopathy (HIE) [15].

Human umbilical cord blood (UCB) is a promising source of MSCs. In comparison to adult tissue-derived MSCs, its advantages include easier extraction, lower immunogenicity [16], higher in vitro proliferation capacity [17, 18], and better in vivo therapeutic efficacy [19]. Recently, we have shown that xenotransplantation of human UCB-derived MSCs significantly attenuates brain injury and the progress of PHH after severe IVH in immunocompetent newborn rats [15, 20, 21]. Furthermore, we have observed that treatment of BPD in premature infants with intratracheal transplantation of allogeneic human UCB-derived MSCs is safe, feasible, and not associated with adverse respiratory, growth, or neurodevelopmental effects [22]. This was confirmed by follow-up of the same infants for up to 2 years of corrected age (CA) [23]. Collectively, these findings suggest that the transplantation of allogeneic human UCB-derived MSCs may be a novel and promising therapeutic modality for treating severe IVH in premature infants. However, the safety of this approach has not yet been tested in a clinical setting. Here, we report a phase I dose-escalating clinical study assessing the safety and feasibility of intraventricular transplantation of human UCB-derived MSCs into premature infants with severe IVH.

EXPERIMENTAL PROCEDURES

Trial Design

This study was designed as a phase I, open-label, single-arm, single-center trial to evaluate the safety and feasibility of intraventricular allograft transplantation of UCB-derived MSCs into premature infants with severe IVH. The protocol was reviewed and approved by the Korean Food and Drug Administration (30261) and by the Institutional Review Board of the Samsung Medical Center in Seoul, Korea (IRB No. 2014–06-103). The study was registered on ClinicalTrials.gov (NCT02274428). The Clinical Trial Center of Samsung Medical Center (Seoul, South Korea) acted as the external monitor of the present study and performed intensive and cautious external monitoring. The informed consent document was reviewed at least 3 times by all parents, and the principle investigator or coinvestigator. Full written informed consent was obtained with particular attention to the understanding of potential safety issues, and the fact that therapeutic benefit was neither expected nor promised.

For determination of sample size, we used a "3 + 3" cohort design with an expansion cohort of six patients at the next highest dose. Therefore, a minimum of nine patients, in total, was needed to establish the safety profile of this phase I study. The first three patients were assigned to receive a low dose of MSCs (5×10^6 cells/kg), and the next six patients were assigned to receive a high dose of MSCs (1×10^7 cells/kg).

The primary outcome was establishing the feasibility and safety of escalating doses of hUCB-derived MSCs in preterm infants. This was assessed by monitoring for: (a) dose-limiting toxicity (DLT), defined as sudden death within 6 hours of MSC transplantation for no other known reason; (b) anaphylactic shock occurring immediately after MSC transplantation; and (c) brain tumor lesions occurring after MSC transplantation. The secondary outcome was defined as incidence of adverse events resulting from hUCB-derived MSC transplantation. These events were classified as shunt placement, death, culture-confirmed late-onset sepsis, surgery required to correct retinopathy of prematurity (ROP), necrotizing enterocolitis (>Bell's stage 2b), or seizure.

Patients

Patients were enrolled at the Samsung Medical Center between November 13, 2014 and November 19, 2015. Inclusion criteria for preterm infants included gestational age of 23–34 weeks, and diagnosis with severe IVH (≥grade 3 by Papile's classification [24]) using cranial ultrasonography assessed independently by two radiologists. Transplantation of MSCs was performed within 7 days after the first diagnosis of severe IVH (≥grade 3). Patients were excluded for severe congenital anomalies, antenatal brain hemorrhage, intracranial infection, congenital infection, uncorrected thrombocytopenia (<50,000/ml), uncorrected severe metabolic acidosis (pH <7.1), surgery within 72 hours prior to intended enrollment, expectation of surgery or death within several days after enrollment, or enrollment in another clinical trial.

Transplantation of Human UCB-Derived MSCs

Human UCB-derived MSCs were obtained as described in our previous (first-in-human) phase I clinical trial for BPD in premature infants [22]. PNEUMOSTEM consists of ex vivo cultured allogeneic, unrelated, human umbilical cord-blood-derived mesenchymal stem cells (hUCB-MSCs). These cells are CD45-, CD14-, CD34-, and human leukocyte antigen (HLA)-DR-negative, and are also CD73-, CD105-, and CD90-positive. This immunophenotype is confirmatory for hUCB-MSCs and distinguishes them from hematopoietic stem cells. Processing of hUCB-MSCs involves isolation steps to remove hematopoietic elements, followed by MSC expansion from the nucleated cells in culture medium (minimum essential medium: Gibco BRL, Grand Island, New York) supplemented with 10% fetal bovine serum. The cells are cryopreserved at $-150°C$ or lower in 10% dimethyl sulfoxide. Before administration, frozen hUCB-MSCs were thawed and washed with culture medium and saline. The reconstituted hUCB-MSCs for transplantation were stored at $2°C–8°C$, with a shelf life of 24 hours from the time of manufacture. Pneumostem passage-six human UCB-derived MSCs were purchased from MEDIPOST Co., Ltd. (Seoul, Korea). The manufacturer confirms shelf-life stability of 24 hours by process validation and stability data for each batch. The reconstituted hUCB-MSCs were tested for viability, phenotype, and presence of endotoxins before infusion and this information was provided as a certificate of analysis by the manufacturer. For analysis of phenotype, viability, and endotoxins in reconstituted hUCB-MSCs, immunophenotyping was performed using flow cytometry, trypan blue exclusion, and chromogenic assays. When dispatching cells from GMP facility, the viabilities of all cell lots were over than 75% and thus every cell lot passed the acceptance criteria of cell viability >70%.

Stem cells were prepared in compliance with good manufacturing practices at a concentration of 5×10^6 cells/ml in normal saline. A low dose of 5×10^6 MSCs (1 ml/kg), or a high dose of 1×10^7 MSCs (2 ml/kg), was transplanted intraventricularly, via an anterior fontanelle tap, using a 24-gauge medicut catheter guided by cranial ultrasound. Briefly, after sedation with intravenous fentanyl, the anterior fontanelle skin area was prepared and draped for the aseptic procedure. The ultrasound probe was also covered with a sterile sheath. A neurosurgeon inserted a 22-gauge medicut needle into the

dilated lateral ventricle unilaterally via the fontanelle tap under direct ultrasound guidance. After withdrawing the needle stylet through the remaining catheter, we naturally drained a volume of cerebrospinal fluid (CSF) approximately equivalent to that of the prepared MSCs. After obtaining CSF, a syringe containing the MSCs was connected to the catheter, and MSCs were slowly injected into the lateral ventricle. The catheter was then removed, after flushing with normal saline (0.5 ml). The site of needle puncture was manually compressed for 5 minutes and aseptic dressing was applied.

Assessment of Safety

Safety was defined primarily as the absence of treatment-related serious adverse events (SAEs), assessed according to the Consolidated Standards of Reporting Trials [25], and secondarily as the absence of dose-limiting toxicity, defined as death within 6 hours after MSC transplantation, or anaphylactic shock related to the administration of MSCs. After a single intraventricular transplantation of MSCs, all patients were regularly and intensively assessed until either 36–40 weeks of corrected gestational age, or first discharge from NICU, whichever occurred first.

Immediately after MSC transplantation, patients were monitored for SAEs [22] and DLT. As part of routine NICU care, vital signs were continuously monitored with an electrocardiogram and recorded in an electronic medical record system. A cranial ultrasonogram was performed at baseline (screening point), then at the 2nd, 4th, 6th, and 8th week after MSC transplantation. Cranial ultrasonogram was also used for additional reassessment as needed. Brain MRI was performed at 36–40 weeks of corrected gestational age. Collection of CSF was carried out via the fontanelle tap immediately before MSC transplantation and via spinal tap at 36–40 weeks of corrected gestational age.

Ventricular Parameters

While performing cranial ultrasonography, standard views were obtained in the coronal and sagittal planes, with the anterior fontanel used as an acoustic window. To assess baseline ventricular enlargement induced by IVH, the first ultrasound images, which detected severe IVH ($\geq$grade 3), were used to measure the dimensions of the ventricles. Three ventricular parameters were used in the present study. The ventricular index (VI) was defined as the distance between the falx and the lateral wall of the anterior horn in the coronal plane; anterior horn width (AHW) was defined as the diagonal width of the anterior horn measured at its widest point in the coronal plane; and the thalamo-occipital distance (TOD) was defined as the distance between the outermost point of the thalamus at its junction with the choroid plexus and the outermost part of the occipital horn in the parasagittal plane [26]. Ventricular measurements were performed offline using ImageJ software (NIH) by a pediatric radiology specialist and neonatology staff, who were blinded to the study.

Temporal Profile of CSF Cytokines and Growth Factors

To assess changes in the levels of cytokines and growth factors associated with the extent of inflammation of the brain after severe IVH, CSF was collected via the anterior fontanelle tap immediately before MSC transplantation, and by lumbar puncture after MSC transplantation, at 36–40 weeks of corrected

gestational age. Supernatant was frozen at −70°C after centrifugation at 15,000 rpm for 10 minutes. The levels of the following cytokines and growth factors were measured according to the manufacturer's protocols: interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF). Measurements were performed using the Magnetic Luminex Performance Assay kit (R&D Systems, Inc., Minneapolis, MN). Transforming growth factor (TGF)-β1/2, brain-derived neurotropic factor (BDNF), and fibroblast growth factor (FGF) were assessed with a Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN). Cytokine analysis of CSF was performed by Seoul Clinical Laboratories (Yonginsi, Kyonggido, Korea), who also verified the inter-assay and intraassay reliability of the cytokine profiles.

Statistical Analyses

Data are expressed as mean $\pm$ standard error of the mean. To compare continuous variables, statistical comparisons between groups were performed using the Mann–Whitney test. The paired t test was used for assessment of the temporal profile of growth factors and cytokines in the CSF. Correlation between the ventricular parameters measured in the baseline cranial ultrasound images and the initial levels of growth factors and cytokines in the CSF was evaluated using Spearman rank correlation. The p values of <.05 were considered statistically significant. The software package SPSS, version 17 (SPSS Inc., Chicago, IL), was used for all statistical analyses.

RESULTS

Clinical Characteristics

Out of the total of nine premature infants with severe (grade 4) IVH enrolled in this trial, three received a low-dose (5×10^6 cells/kg) of human UCB-derived MSCs, and the remaining six received a high-dose (1×10^7 cells/kg), (Table 1). Gestational age, birth weight, and postnatal age of MSC transplant recipients were 26.1 $\pm$ 2.1 weeks (range 24–30 weeks), 767 $\pm$ 231 g (range 440–1,310 g), and 11.6 $\pm$ 2.6 days (range 7–15 days), respectively. Clinical variables, including gestational age, birth weight, sex, Apgar scores, and antenatal findings of low and high dose groups are presented in Table 2. In the low dose group, all enrolled infants were female, and only male infants were enrolled in the high dose group.

Serious Adverse Events

Details of SAEs, recorded until first discharge from the NICU, are shown in Table 1. All nine infants who received MSC transplantation were discharged alive. Intraventricular transplantation of human UCB-derived MSCs was performed aseptically under ultrasound guidance. All the patients tolerated the procedure well, without immediate complications such as anaphylaxis or death within 6 hours after transplantation. However, eight patients developed SAEs including severe BPD, sepsis, NEC, ROP requiring surgery, inguinal hernia requiring surgery, and seizures (Table 1). The most common SAE was inguinal hernia, which was surgically corrected in four of the nine patients (44%). All surgeries for hernia were performed at least 3–4 months after MSC transplantation. Two infants (A1 and A2) received laser treatment for stage 3 ROP, which was diagnosed 3 months after MSC transplantation. NEIIb was diagnosed at 16 and 115 days

Table 1. Clinical data of enrolled patients

Patient	A1	A2	A3	B1	B2	B3	B4	B5	B6
Gestational age (weeks)	24+5	24+3	28+1	30+5	25+6	26+0	25+4	25+3	28+6
Birth weight (g)	660	700	730	1,310	440	630	900	880	1,020
Apgar score, 1 min	5	5	4	4	2	2	7	5	8
Apgar score, 5 min	7	6	7	7	4	6	8	7	8
Gender	F	F	F	M	M	M	M	M	M
Delivery	CS	CS	NV	CS	NV	CS	CS	CS	CS
Pathologically confirmed chorioamnionitis	–	–	–	–	Y	–	Y	–	–
Antenatal steroid use	Y	–	Y	Y	Y	Y	Y	Y	Y
IVH grade	4	4	4	4	4	4	4	4	4
Age at MSC injection (postnatal day)	12	10	15	7	12	12	12	9	15
Ventricular parameters[a]									
Ventricular index	0.20	0.19	0.20	0.29	0.23	0.18	0.21	0.22	0.21
Anterior horn width	0.13	0.10	0.12	0.20	0.11	0.13	0.14	0.13	0.15
Thalamo-occipital distance	0.18	0.29	0.21	0.35	0.23	0.25	0.25	0.34	0.36
Serious AEs with major morbidities									
Death within 6 h after MSC transplantation	–	–	–	–	–	–	–	–	–
Anaphylaxis after MSC injection	–	–	–	–	–	–	–	–	–
VP shunt operation	Y	–	–	Y	–	–	Y	Y	Y
Operation postnatal day				40			119	63	83
Seizure	Y[b]	Y[b]	–	Y[b]	Y[b]	–	–	Y	–
Medication duration (days)	92	101	–	60	89	–	–	75	–
BPD severity	mild	mild	none	severe	mild	severe	severe	mild	severe
Late-onset sepsis	–	–	–	–	–	–	–	Y[c]	–
NEC (≥stage 2)	–	–	–	–	–	Y[d]	Y[d]	–	–
Laparoscopic operation	–	–	–	–	–	–	–	–	–
ROP required laser op	Y	Y	–	–	–	–	–	–	–
Inguinal hernia operation	–	–	–	–	Y	Y	Y	Y	–

[a]Ventricular parameter was assessed with brain ultrasound images taken right before MSCs transplantation.
[b]Seizure was started before enrollment.
[c]Blood-culture proven sepsis was detected at postnatal day 29 for the first time.
[d]NEC was diagnosis at P28 and P127 in B3 and B4 case infants, respectively.

after MSC transplantation in infants B3 and B4, respectively. One patient (B5), in the high-dose MSC-transplantation group, developed late-onset sepsis and meningitis, caused by *Enterobacter cloacae*, and seizures requiring anticonvulsant medication, at postnatal day (P) 29 (Table 1). Because of the extended time interval of 20 days between the transplantation of MSCs at P9, and the onset of late-onset sepsis and meningitis at P29, it seems likely that these SAEs are not directly related to the intraventricular transplantation of MSCs. Because seizures in this patient developed with the onset of meningitis, and because seizures were observed in the other four infants before enrollment, this SAE may not be attributable to MSC transplantation. Last, although there was no mortality, five out of the nine enrolled infants (56%) with grade 4 severe IVH received a ventriculoperitoneal shunt before first NICU discharge (P40–

P127). Major SAEs including rates of shunt placement, late-onset sepsis, NEC (≥stage 2), and seizures are displayed in Table 3. All infants who received shunt operations in the present study had the surgery within the first 6 months of life and before their first NICU discharge.

Changes in IVH-Induced Brain Injury

Representative cranial ultrasound images of the nine patients with MSC transplantation, acquired pretransplantation and at 1, 2, and 3 months posttransplantation, as well as MRI images acquired at 36–40 weeks of gestational age, are displayed in Figure 1. The infant designated A1 showed an initial improvement in hemorrhage area including attenuation of periventricular hemorrhagic infarct, for up to 2 months after

Table 2. Clinical characteristics of the MSC group

	MSC transplantation group		
	Low-dose (5×10^6 cells/kg) (*n* = 3)	High-dose (1×10^7 cells/kg) (*n* = 6)	Total (*n* = 9)
Baseline characteristics			
Gestational age (weeks)	25.3 ± 2.3	26.5 ± 2.1	26.1 ± 2.1
Birth weight (g)	697 ± 35	863 ± 303	767 ± 231
Apgar score, 1 min	4.7 ± 0.6	4.7 ± 2.5	4.7 ± 2.0
Apgar score, 5 min	6.7 ± 0.6	6.7 ± 1.5	6.7 ± 1.2
Female sex (number, %)	3/3 (100%)	0/6 (0%)	3 (33%)
SGA (number, %)	1/3 (33%)	1/6 (17%)	2 (22%)
PIH (number, %)	0/3 (0%)	1/6 (17%)	1 (11%)
Antenatal corticosteroids (number, %)	2/3 (67%)	6/6 (100%)	8 (89%)
Pathologic chorioamnionitis (number, %)	0/3 (0%)	2/6 (33%)	2 (22%)

transplantation with MSCs (Fig. 1a). However, delayed-progressive ventriculomegaly developed at 3 months after MSC transplantation, requiring shunt surgery at 127 postnatal days (Fig. 1a). This infant was discharged home at 135 postnatal days. The infants A2, A3, B2, and B3 showed continual improvement and regression in hemorrhage area, throughout the observation period and were discharged without shunt placement (Fig. 1a). The infants B1, B4, B5, and B6 received shunt placement because of progressive ventriculomegaly before being discharged (Fig. 1a).

There was no significant difference in baseline ventricular parameters, including AHW, VI, and TOD, between infants who received shunt operations and those who did not (Fig. 1b).

Temporal Profiles of Changes in the Levels of CSF Cytokines and Growth Factors

Temporal profiles of changes in the levels of CSF cytokines and growth factors measured before and after MSC transplantation are shown in Figure 2. The average level of IL-6 measured at 36–40 weeks corrected age was significantly decreased when compared to the baseline CSF level obtained before MSC transplantation (*p* = .008). Levels in CSF of TGF-β1/2, TNF-α, IL-1β, and VEGF, measured at 36–40 weeks CA, showed a tendency to decrease. The exception was infant B5, who developed late-onset sepsis and meningitis caused by *E. cloacae*; this infant showed increased levels of TGF-β1/2, TNF-α, IL-1β, VEGF, and BDNF compared with the respective baseline values measured in CSF before MSC transplantation. The infant B1, who developed prolonged severe acidosis and hypotension before the transfer to our NICU, and received shunt placement 1 month after MSC transplantation, showed the highest CSF levels of IL-6, TGF-ß1, and TNF-α compared with baseline values (Fig. 2). Baseline CSF IL-6 levels in infants with shunt placement were significantly higher compared with those of infants without shunt placement (*p* = .03) (Fig. 3a). Baseline levels of other proinflammatory cytokines, such as TGF-β1/2, TNF-α, and IL-1β, in the infants with shunt placement were elevated compared with those in infants without shunt placement. However, these differences were not statistically significant. Spearman rank correlation tests revealed significant correlations between baseline IL-6 and TNF-α levels in CSF and initial VI and AHW (Fig. 3b).

Table 3. Outcomes of the MSC group

	MSC transplantation group		
	Low-dose (5×10^6 cells/kg) (*n* = 3)	High-dose (1×10^7 cells/kg) (*n* = 6)	Total (*n* = 9)
Age at MSC transplantation	12.3 ± 2.5	11.2 ± 2.8	11.6 ± 2.6
Ventricular parameters			
Ventricular index	19.7 ± 0.4	22.3 ± 3.7	21.4 ± 3.2
Anterior horn width	11.7 ± 1.5	14.3 ± 3.0	13.4 ± 2.8
Thalamo-occipital distance	22.8 ± 5.5	33.1 ± 7.1	27.4 ± 6.3
Major outcomes			
Shunt operation or death	1/3 (33%)	4/6 (67%)	5 (56%)
Death at discharge	0/3 (0%)	0/6 (0%)	0 (0%)
Shunt operation	1/3 (33%)	4/6 (67%)	5 (56%)
Late-onset sepsis	0/3 (0%)	1/6 (17%)	1 (11%)
ROP required operation	2/3 (33%)	0/6 (0%)	2 (22%)
NEC (≥stage 2)	0/3 (0%)	2/6 (33%)	2 (22%)
Seizure	2/3 (67%)	3/6 (50%)	5 (56%)
Medication (days)	$64.3 + 55.9$	$37.3 + 41.9$	$46.3 + 45.4$

DISCUSSION

To the best of our knowledge, this is the first clinical trial to test the safety and feasibility of MSC transplantation for severe IVH in premature infants. In this study, intraventricular transplantation with low-dose (5×10^6 cells/kg) or high-dose (1×10^7 cells/kg) of allogeneic human UCB-derived MSCs was
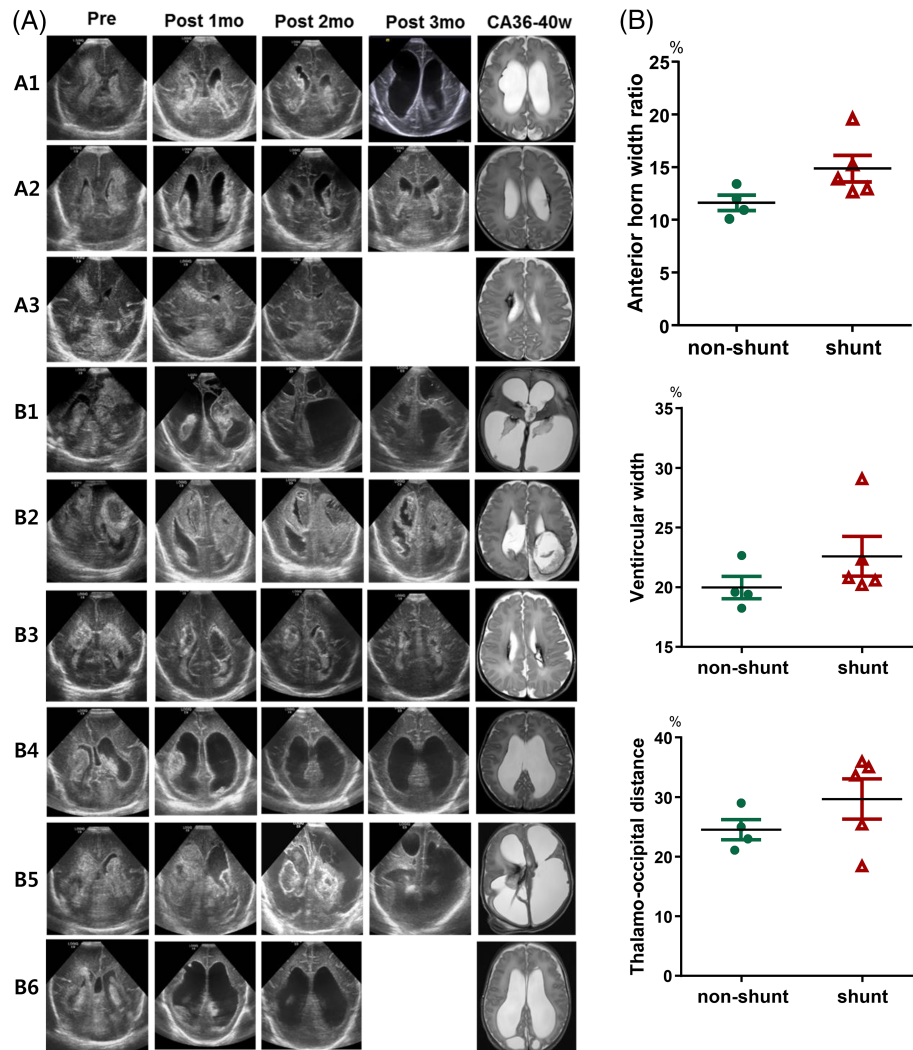
Figure 1. Serial images of brain injury induced by intraventricular hemorrhage (IVH). **(A)** Representative coronal-view images of a serial cranial ultrasonogram obtained before, and 1, 2, and 3 months after the transplantation of mesenchymal stem cells (MSCs). Representative brain magnetic resonance (MRI) images were obtained at 36–40 weeks CA. **(B)** The baseline brain ventricular index, assessing anterior horn width ratio, ventricular width ratio, and trans-occipital distance ratio were measured using cranial ultrasonogram images, which were first obtained after the onset of severe IVH. The baseline brain ventricular index is according to shunt placement in the MSC-transplanted group. Data are represented as mean ± SEM. Abbreviations: CA 36–40w, corrected age of 36–40 weeks; Pre, pre-MSC transplantation; Post1mo, 1 month after MSC transplantation; Post2mo, 2 months after MSC transplantation; Post3mo, 3 months after MSC transplantation.

not associated with immediate SAEs or DLT and showed no mortality in our cohort of extremely preterm infants with grade 4 IVH. These findings suggest that intraventricular transplantation of up to 1×10^7 cells/kg of human UCB-derived MSCs into preterm infants with severe IVH may be safe and feasible.

Identifying the most vulnerable preterm infants, with the highest rates of mortality and morbidity, by cautious risk–benefit evaluation is important for clinical use of these potentially beneficial rescue therapies. As over 50% of preterm infants with severe (grade ≥ 3) IVH die or develop PHH, ultimately requiring shunt placement in 40%–70% of cases [5–7], the extremely preterm infants with severe grade 4 IVH enrolled in the present study may provide a good indication of the feasibility and potential of stem cell therapy.

In the present study, there was no mortality in the MSC transplantation group. This finding may suggest that

intracerebroventricular MSC transplantation might not be considered as an aggravating factor for mortality in preterm infants with severe IVH, who are at risk for mortality. However, due to the inevitable limitations of phase I study design, including small sample size, further well-designed controlled studies are necessary to confirm the safety and therapeutic efficacy of MSC transplantation.

Clinical courses vary widely, and death or requirement for shunt placement does not always occur even in preterm infants with severe IVH [7]. Therefore, identifying additional biomarkers for the early identification of neuronal injury, in addition to cranial ultrasonography, is crucial for improving therapeutic outcomes [27]. Various CSF biomarkers, including TGFß-1 [28], VEGF [29], BDNF [20], GDNF [30], and proinflammatory cytokines, such as IL-1α [31], IL-1ß [32], CCL-3, and CCL-19 [31] have been used to diagnose brain injury and predict neurodevelopmental outcomes. In a previous study of
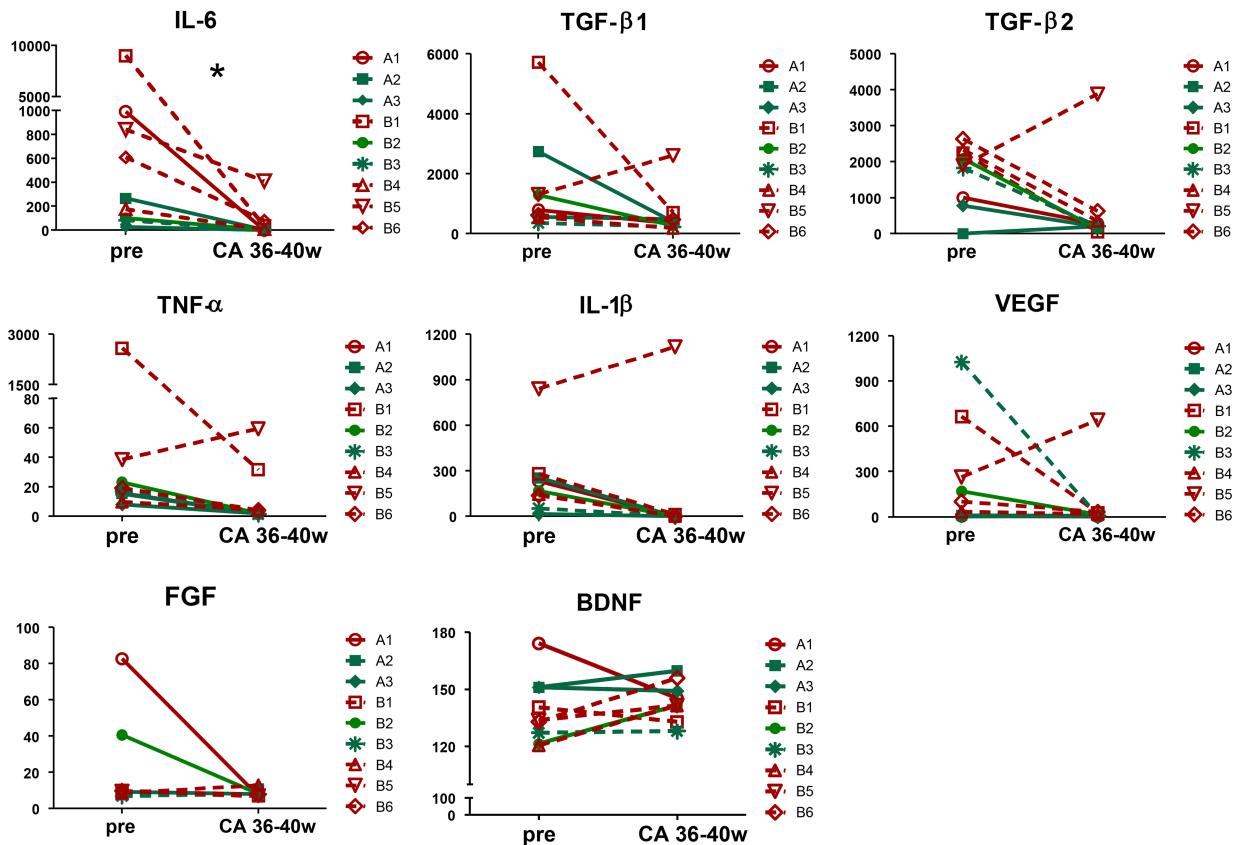
Figure 2. Temporal profiles of cytokines and growth factors in cerebrospinal fluid (CSF). Levels of interleukin (IL)-6, transforming growth factor (TGF)-β1/2, tumor necrosis factor (TNF)-α, IL-1β, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and brain-derived neurotropic factor (BDNF), in CSF obtained before MSC transplantation, and at 36–40 weeks CA, are shown for each infant transplanted with MSCs. *$p < .05$; Abbreviations: CA 36–40w, corrected age of 36–40 weeks. Pre, pre-MSCs transplantation.

brain-specific proteins in the CSF, glial fibrillary acidic protein level was found to be substantially higher in infants with IVH and PHH than in normal infants. It was also correlated with disability and parenchymal lesions [33]. Concentrations of amyloid precursor proteins (APP), soluble APPα, and L1 cell adhesion molecule have also been shown to be selectively increased in PHH infants [34]. In this study, the baseline level of IL-6 in the initial CSF obtained before MSC transplantation was correlated with initial ventricular dilatation measured as VI and AHW, and was higher in infants who received shunt operations. However, our small sample size and lack of an appropriate control group urge caution for inferences from our results relating to biomarkers. Further research is necessary to understand whether any biomarkers in the CSF [35] can be used, in addition to cranial ultrasonography, to predict neuronal injury, progress of PHH, and poor neurodevelopmental outcomes. Such biomarkers will help identify preterm infants with severe IVH who are most likely to benefit from stem cell therapy. Another limitation of our study stems from the small sample volumes of CSF obtained from extremely tiny premature infants. Assessment of technical variation was consequently impractical, though laboratory has verification of interassay and intraassay reliability for the cytokine profiles in previous other samples.

Choosing the correct cells for transplantation is a critical issue for successful clinical translation in stem cell therapy. In this study, we used the allogeneic human UCB-derived MSCs from the same 6th passage as in our previous phase I clinical trial for BPD, approved by both Korean [22] and United States Food and Drug Administrations (NCT02381366). These cells, manufactured in strict compliance with criteria for good manufacturing practice, were transplanted into premature infants with severe IVH. Allogeneic transplantation of MSCs may have a logistic advantage because they are ready to use "off the shelf" in the clinical setting. Moreover, UCB-derived MSCs exhibit several advantages over adult tissue-derived MSCs including lower immunogenicity [16, 36] and higher proliferation capacity, paracrine potency, and therapeutic efficacy both in vitro [17, 18] and in vivo [37]. These MSCs show karyotypic stability and no senescence up to the 11th passage. No transplantation-related adverse effects, including tumorigenicity, were observed at 70 days post-transplantation in rats [38], and at 2 years CA in preterm infants [23], after intratracheal administration of MSCs. Taken together; these findings suggest that human UCB-derived MSCs might be appealing choice of cells for transplantation into preterm infants with severe IVH.

Determining the optimal route for MSC transplantation is an important issue that needs to be addressed for successful clinical translation. In newborn rats with severe IVH, we found that local intraventricular transplantation of MSCs was fivefold more effective for attenuating brain injury than systemic intravenous administration, although the two routes are not
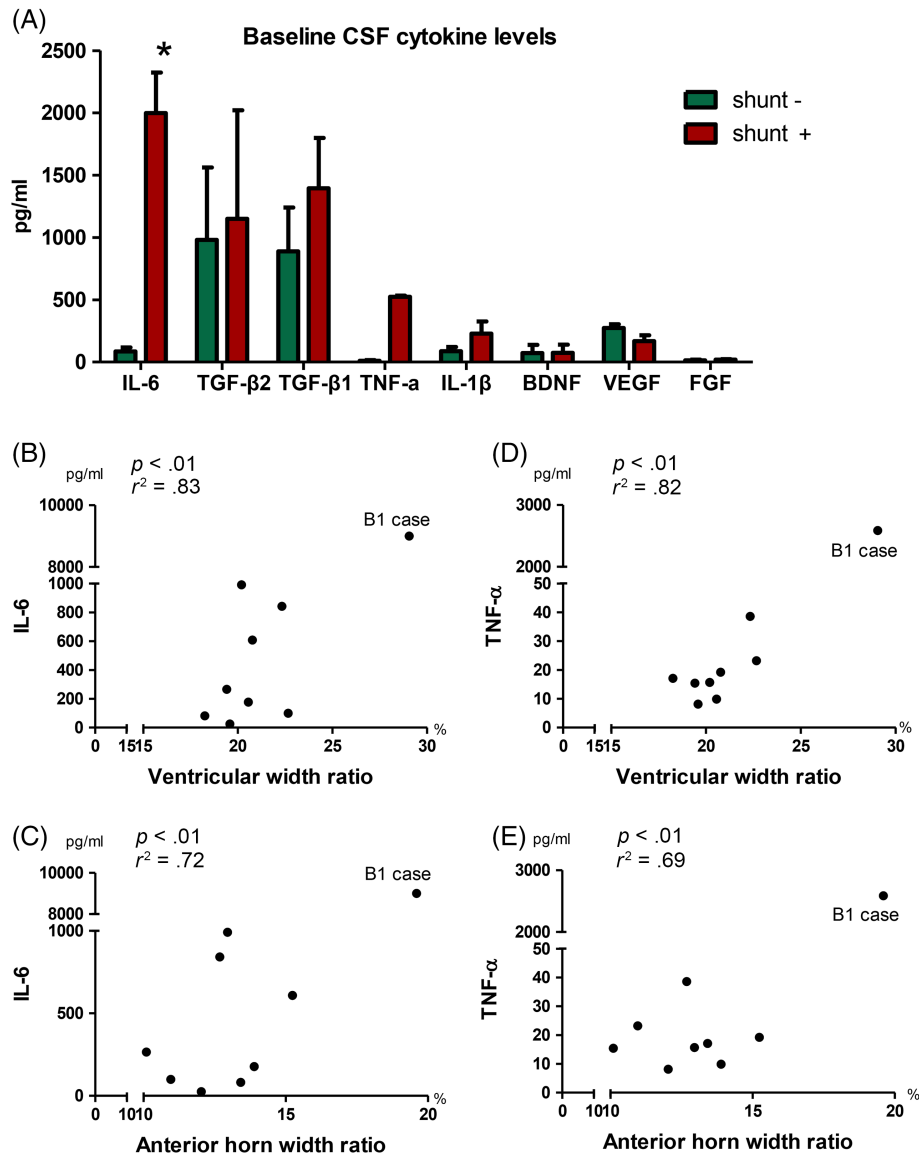
Figure 3. Baseline levels of cerebrospinal fluid (CSF) cytokines and growth factors. **(A)** Baseline levels of CSF cytokines and growth factors were grouped and compared with those of infants who received shunt placement. Data are represented as mean ± SEM. The correlations between initial brain ventricular index including anterior horn width ratio **(C)** and **(E)** and ventricular width ratio **(B)** and **(D)** measured with cranial ultrasonogram and the baseline levels of IL-6 and TNF-α are shown, respectively. Abbreviations: BDNF, brain-derived neurotropic factor; FGF, fibroblast growth factor; IL, interleukin; r^2, squared value of Spearman's rank correlation coefficient; TGF, Transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; *$p < .05$.

mutually exclusive [15]. In this study, MSCs were administered intraventricularly under ultrasonography guidance via a bedside ventricular tap placed through the open anterior fontanel. This approach did not require further invasive surgery in our cohort of premature infants with severe IVH. The procedure was well tolerated even by the smallest infant weighing 400 g. No clinical instability, SAEs, DLT, or complications related to MSC transplantation were observed in any of the nine infants. Collectively, these findings suggest that local intraventricular transplantation of MSCs in preterm infants with severe IVH is safe and feasible, and may be a therapeutically more effective delivery route than systemic intravenous transplantation.

Determining the optimal timing of MSC transplantation is another key issue that requires resolution. In our previous preclinical study, conducted to optimize the timing of MSC transplantation in severe IVH, early intraventricular transplantation of MSCs protected against IVH-induced brain injuries and progressive PHH, when delivered 2 days after induction of severe IVH, but not at 7 days [39]. Based on these preclinical data showing a narrow therapeutic time window, this clinical trial was designed to transplant MSCs within 7 days following the first detection of severe IVH (≥3) with cranial ultrasonography, on average at postnatal day 12. Further studies are needed to clarify whether MSC transplantation close to the onset of severe IVH provides the best therapeutic outcomes.

The optimal dose of MSCs to be used for transplantation is also unclear. We previously demonstrated that local intraventricular transplantation of 1×10^5 MSCs significantly

attenuated brain injury and the progress of PHH after severe IVH in newborn rats [21]. With systemic intravenous administration, doses fivefold higher were required for equivalent therapeutic efficacy [15]. These findings suggest that the site of injury and the delivery route of MSCs are the major determinants of optimal dose. Currently, no guidelines are available for the extrapolation of preclinical data [21] into clinical trials. We extrapolated from the cell doses used in animal studies, based on body weight and ventricular volume; thus, in this study we performed an intraventricular transplantation of 5×10^6 cells/kg for the low dose, and 1×10^7 cells/kg for the high dose. Further studies will be necessary to ascertain the optimal MSC doses for transplantation into preterm infants with severe IVH.

Severe brain injury induced by IVH can persist into childhood with increased risk of developmental delay, cerebral palsy, and cognitive impairment [40]. Therefore, a long-term follow-up safety assessment is necessary to prove the safety of MSC transplantation [41–43]. Such an assessment of the nine preterm infants treated with MSCs in this study is currently underway (NCT02673788). The planned duration of this study is 2 years.

There have been various interventions to prevent PHH in neonates with IVH, including repeated lumbar puncture, drug treatment to reduce CSF, and intraventricular fibrinolytic therapy, but no randomized trial has been conducted to investigate their efficacy and safety [44]. A pioneering randomized trial for neonatal IVH using drainage, irrigation and fibrinolytic therapy showed severe cognitive disability in survivors and overall death or severe disability, when neurodevelopmental outcome was assessed at 2-year follow-up [45]. Hence, to prove the safety and efficacy of MSCs as a therapeutic strategy, a double-blind randomized controlled phase II clinical trial with follow-up evaluation is necessary. A phase II trial to determine the therapeutic efficacy of MSC treatment of IVH in premature infants is currently underway (NCT02890953).

CONCLUSION

Intraventricular transplantation of human UCB-derived MSCs into extremely premature infants with severe IVH was found to be safe and feasible. This promising therapeutic modality warrants further evaluation for safety and therapeutic efficacy in a larger and well-controlled prospective phase II study.

AUTHOR CONTRIBUTIONS

S.Y.A., Y.S.C., W.S.P.: conception and design, provision of study material or patients, analysis and interpretation of data, manuscript writing and revision, and final approval of manuscript; S.I.S.: administrative support, provision of study patients, collection and/or assembly of data, editing the manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

W.S.P. and Y.S.C. declare potential conflicts of interest arising from a filed or issued patent titled "Composition for treating intraventricular hemorrhage in preterm infants comprising mesenchymal stem cells" as co-inventors, but not to date as patentees. The other authors indicated no potential conflicts of interest.

REFERENCES

1 Thorp JA, Jones PG, Clark RH et al. Perinatal factors associated with severe intracranial hemorrhage. Am J Obstet Gynecol 2001;185:859–862.

2 Payne AH, Hintz SR, Hibbs AM et al. Neurodevelopmental outcomes of extremely low-gestational-age neonates with low-grade periventricular-intraventricular hemorrhage. JAMA Pediatr 2013;167:451–459.

3 Walsh MC, Bell EF, Kandefer S et al. Neonatal outcomes of moderately preterm infants compared to extremely preterm infants. Pediatr Res 2017;82:297–304.

4 Stoll BJ, Hansen NI, Bell EF et al. Neonatal outcomes of extremely preterm infants from the NICHD neonatal research network. Pediatrics 2010;126: 443–456.

5 Murphy BP, Inder TE, Rooks V et al. Posthaemorrhagic ventricular dilatation in the premature infant: Natural history and predictors of outcome. Arch Dis Child Fetal Neonatal Ed 2002;87:F37–F41.

6 de Vries LS, Groenendaal F, Liem KD et al. Treatment thresholds for intervention in posthaemorrhagic ventricular dilation: A randomised controlled trial. Arch Dis Child Fetal Neonatal Ed 2018.

7 Ahn SY, Chang YS, Sung DK et al. Optimal route for mesenchymal stem cells transplantation after severe intraventricular hemorrhage in newborn rats. PLoS One 2015;10:e0132919.

8 International PHVD Drug Trial Group. International randomised controlled trial of acetazolamide and furosemide in posthaemorrhagic ventricular dilatation in infancy. Lancet 1998;352:433–440.

9 Kennedy CR, Ayers S, Campbell MJ et al. Randomized, controlled trial of acetazolamide and furosemide in posthemorrhagic ventricular dilation in infancy: Follow-up at 1 year. Pediatrics 2001;108:597–607.

10 Pinto-Martin JA, Whitaker AH, Feldman JF et al. Relation of cranial ultrasound abnormalities in low-birthweight infants to motor or cognitive performance at ages 2, 6, and 9 years. Dev Med Child Neurol 1999;41:826–833.

11 Ahn SY, Chang YS, Sung DK et al. Cell type-dependent variation in paracrine potency determines therapeutic efficacy against neonatal hyperoxic lung injury. Cytotherapy 2015;17:1025–1035.

12 Chang YS, Choi SJ, Sung DK et al. Intratracheal transplantation of human umbilical cord blood-derived mesenchymal stem cells dose-dependently attenuates hyperoxia-induced lung injury in neonatal rats. Cell Transplant 2011;20:1843–1854.

13 Chang YS, Oh W, Choi SJ et al. Human umbilical cord blood-derived mesenchymal stem cells attenuate hyperoxia-induced lung injury in neonatal rats. Cell Transplant 2009;18:869–886.

14 Chang YS, Ahn SY, Jeon HB et al. Critical role of VEGF secreted by mesenchymal stem cells in hyperoxic lung injury. Am J Respir Cell Mol Biol 2014;51:391–399.

15 Park WS, Sung SI, Ahn SY et al. Hypothermia augments neuroprotective activity of mesenchymal stem cells for

neonatal hypoxic-ischemic encephalopathy. PLoS One 2015;10:e0120893.

16 Rocha V, Wagner JE Jr, Sobocinski KA et al. Graft-versus-host disease in children who have received a cord-blood or bone marrow transplant from an HLA-identical sibling. Eurocord and International Bone Marrow Transplant Registry Working Committee on Alternative Donor and Stem Cell Sources. N Engl J Med 2000;342:1846–1854.

17 Yang SE, Ha CW, Jung M et al. Mesenchymal stem/progenitor cells developed in cultures from UC blood. Cytotherapy 2004;6:476–486.

18 Kern S, Eichler H, Stoeve J et al. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 2006;24: 1294–1301.

19 Jin HJ, Bae YK, Kim M et al. Comparative analysis of human mesenchymal stem cells from bone marrow, adipose tissue, and umbilical cord blood as sources of cell therapy. Int J Mol Sci 2013;14:17986–18001.

20 Ahn SY, Chang YS, Sung DK et al. Pivotal role of brain derived neurotrophic factor secreted by mesenchymal stem cells in severe intraventricular hemorrhage in the newborn rats. Cell Transplant 2016;26(1): 145–156.

21 Ahn SY, Chang YS, Sung DK et al. Mesenchymal stem cells prevent hydrocephalus after severe intraventricular hemorrhage. Stroke 2013;44:497–504.

22 Chang YS, Ahn SY, Yoo HS et al. Mesenchymal stem cells for bronchopulmonary dysplasia: Phase 1 dose-escalation clinical trial. J Pediatr 2014;164:966–972.

23 Ahn SY, Chang YS, Kim JH et al. Two-year follow-up outcomes of premature infants enrolled in the phase I trial of mesenchymal stem cells transplantation for bronchopulmonary dysplasia. J Pediatr 2017; 185:49–54. e42.

24 Papile LA, Burstein J, Burstein R et al. Incidence and evolution of subependymal and intraventricular hemorrhage: A study of infants with birth weights less than 1,500 gm. J Pediatr 1978;92:529–534.

25 Ioannidis JP, Evans SJ, Gotzsche PC et al. Better reporting of harms in randomized trials: An extension of the CONSORT statement. Ann Intern Med 2004;141: 781–788.

26 Brouwer MJ, de Vries LS, Groenendaal F et al. New reference values for the neonatal cerebral ventricles. Radiology 2012;262:224–233.

27 Limbrick DD Jr, Castaneyra-Ruiz L, Han RH et al. Cerebrospinal Fluid Biomarkers of Pediatric Hydrocephalus. Pediatr Neurosurg 2017;52:426–435.

28 Whitelaw A, Christie S, Pople I. Transforming growth factor-beta1: A possible signal molecule for posthemorrhagic hydrocephalus? Pediatr Res 1999;46:576–580.

29 Koehne P, Hochhaus F, Felderhoff-Mueser U et al. Vascular endothelial growth factor and erythropoietin concentrations in cerebrospinal fluid of children with hydrocephalus. Childs Nerv Syst 2002;18: 137–141.

30 Rajkumar R, Bhaya B, Mamilla D et al. A preliminary evaluation of glial cell line-derived neurotrophic factor (GDNF) levels in cerebrospinal fluid across various gestational ages and clinical conditions of the neonate. Int J Dev Neurosci 2017;65:61–65.

31 Habiyaremye G, Morales DM, Morgan CD et al. Chemokine and cytokine levels in the lumbar cerebrospinal fluid of preterm infants with post-hemorrhagic hydrocephalus. Fluids Barriers CNS 2017;14:35.

32 Schmitz T, Heep A, Groenendaal F et al. Interleukin-1beta, interleukin-18, and interferon-gamma expression in the cerebrospinal fluid of premature infants with posthemorrhagic hydrocephalus–Markers of white matter damage. Pediatr Res 2007;61:722–726.

33 Whitelaw A, Rosengren L, Blennow M. Brain specific proteins in posthaemorrhagic ventricular dilatation. Arch Dis Child Fetal Neonatal Ed 2001;84:F90–F91.

34 Morales DM, Silver SA, Morgan CD et al. Lumbar cerebrospinal fluid biomarkers of posthemorrhagic hydrocephalus of prematurity: Amyloid precursor protein, soluble amyloid precursor protein alpha, and l1 cell adhesion molecule. Neurosurgery 2017;80:82–90.

35 Merhar S. Biomarkers in neonatal posthemorrhagic hydrocephalus. Neonatology 2012;101:1–7.

36 Le Blanc K. Immunomodulatory effects of fetal and adult mesenchymal stem cells. Cytotherapy 2003;5:485–489.

37 Amable PR, Teixeira MV, Carias RB et al. Protein synthesis and secretion in human mesenchymal cells derived from bone marrow, adipose tissue and Wharton's jelly. Stem Cell Res Ther 2014;5:53.

38 Ahn SY, Chang YS, Kim SY et al. Long-term (postnatal day 70) outcome and safety of intratracheal transplantation of human umbilical cord blood-derived mesenchymal stem cells in neonatal hyperoxic lung injury. Yonsei Med J 2013;54:416–424.

39 Park WS, Sung SI, Ahn SY et al. Optimal Timing of Mesenchymal Stem Cell Therapy for Neonatal Intraventricular Hemorrhage. Cell Transplant 2016;25:1131–1144.

40 Wildrick D. Intraventricular hemorrhage and long-term outcome in the premature infant. J Neurosci Nurs 1997;29:281–289.

41 Sutsko RP, Young KC, Ribeiro A et al. Long-term reparative effects of mesenchymal stem cell therapy following neonatal hyperoxia-induced lung injury. Pediatr Res 2013;73:46–53.

42 Pierro M, Ionescu L, Montemurro T et al. Short-term, long-term and paracrine effect of human umbilical cord-derived stem cells in lung injury prevention and repair in experimental bronchopulmonary dysplasia. Thorax 2013;68:475–484.

43 Can A, Celikkan FT, Cinar O. Umbilical cord mesenchymal stromal cell transplantations: A systemic analysis of clinical trials. Cytotherapy 2017;19:1351–1382.

44 Whitelaw A, Aquilina K. Management of posthaemorrhagic ventricular dilatation. Arch Dis Child Fetal Neonatal Ed 2012;97: F229–F223.

45 Whitelaw A, Jary S, Kmita G et al. Randomized trial of drainage, irrigation and fibrinolytic therapy for premature infants with posthemorrhagic ventricular dilatation: Developmental outcome at 2 years. Pediatrics 2010;125:e852–e858.