Prevention for moderate or severe BPD with intravenous infusion of autologous cord blood mononuclear cells in very preterm infants-a prospective non-randomized placebo-controlled trial and two-year follow up outcomes

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Summary

Background Bronchopulmonary dysplasia (BPD) is the primary severe complication of preterm birth. Severe BPD was associated with higher risks of mortality, more postnatal growth failure, long term respiratory and neurological developmental retardation. Inflammation plays a central role in alveolar simplification and dysregulated vascularization of BPD. There is no effective treatment to improve BPD severity in clinical practice. Our previous clinical study showed autologous cord blood mononuclear cells (ACBMNCs) infusion could reduce the respiratory support duration safely and potential improved BPD severity. Abundant preclinical studies have reported the immunomodulation effect as an important mechanism underlying the beneficial results of stem cell therapies in preventing and treating BPD. However, clinical studies assessing the immunomodulatory effect after stem cells therapy were rare. This study was to investigate the effect of ACBMNCs infusion soon after birth on prevention for severe BPD and long term outcomes in very preterm neonates. The immune cells and inflammatory biomarkers were detected to investigate the underlying immunomodulatory mechanisms.

Methods This single-center, prospective, investigator-initiated, non-randomized trial with blinded outcome assessment aimed to assess the effect of a single intravenous infusion of ACBMNCs in preventing severe BPD (moderate or severe BPD at 36 weeks of gestational age or discharge home) in surviving very preterm neonates less than 32 gestational weeks. Patients admitted to the Neonatal Intensive Care Unit (NICU) of Guangdong Women and Children Hospital from July 01, 2018 to January 1, 2020 were assigned to receiving a targeted dosage of 5×10^7 cells/kg ACBMNC or normal saline intravenously within 24 h after enrollment. Incidence of moderate or severe BPD in survivors were investigated as the primary short term outcome. Growth, respiratory and neurological development were assessed as long term outcomes at corrected age of 18–24 month-old. Immune cells and inflammatory biomarkers were detected for potential mechanism investigation. The trial was registered at ClinicalTrials.gov (NCT02999373).

Findings Six-two infants were enrolled, of which 29 were enrolled to intervention group, 33 to control group. Moderate or severe BPD in survivors significantly decreased in intervention group (adjusted p = 0.021). The number of patients needed to treat to gain one moderate or severe BPD-free survival was 5 (95% confidence interval: 3–20). Survivors in the intervention group had a significantly higher chance to be extubated than infants in the control group (adjusted p = 0.018). There was no statistical significant difference in total BPD incidence (adjusted p = 0.106) or mortality (p = 1.000). Incidence of developmental delay reduced in intervention group in long term follow-up (adjusted p = 0.047). Specific immune cells including proportion of T cells (p = 0.04) and $CD4^+$ T cells in lymphocytes (p = 0.03), and CD4+ CD25+ forkhead box protein 3 (FoxP3)+ regulatory T cells in CD4+ T cells increased

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significantly after ACBMNCs intervention ($p < 0.001$). Anti-inflammatory factor IL-10 was higher ($p = 0.03$), while pro-inflammatory factor such as TNF-a ($p = 0.03$) and C reactive protein ($p < 0.001$) level was lower in intervention group than in control group after intervention.

Interpretation ACBMNCs could prevent moderate or severe BPD in surviving very premature neonates and might improve neurodevelopmental outcomes in long term. An immunomodulatory effect of MNCs contributed to the improvement of BPD severity.

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Keywords: Bronchopulmonary dysplasia; Very preterm neonates; Autologous cord blood mononuclear cells; Immunomodulation; Outcomes

Research in context

Evidence before this study

Prior to this trial, abundant preclinical studies have reported the immunomodulation effect as an important mechanism underlying the beneficial results of stem cell therapies in preventing and treating bronchopulmonary dysplasia (BPD). Our previous clinical study showed autologous cord blood mononuclear cells (ACBMNCs) infusion could reduce the respiratory support duration safely. Mononuclear cell (MNC) is the main resource of stem and progenitor cell in cord blood A few recent clinical studies showed intratracheal transplantation of allogeneic human umbilical cord blood stem cells reduced the incidence of severer BPD in extremely preterm infants. However, until now, there is no study to evaluate the effect of intravenous autologous MNCs infusion to prevent severer BPD in very preterm infants and the follow-up outcomes were rare. Clinical studies assessing the immunomodulatory effect after stem cells therapy for BPD were seldomly reported.

Added value of this study

In this non-randomized, placebo-controlled trial with blinded outcome assessment, twenty-nine patients were assigned to receiving a targeted dosage of 5×10^7 cells/kg ACBMNC and 33 were assigned to receiving normal saline intravenously within 24 h after enrollment depending on their parents' consents. ACBMNC infusion early after birth reduced moderate or severe BPD in survivors significantly. Incidence of developmental delay reduced in intervention group in long term follow-up. Proportion of regulatory T cells in CD4+ T cells increased after ACBMNCs intervention. Anti-inflammatory factor IL-10 was higher, while inflammatory factor TNF-a and C reactive protein level was lower in intervention group than in control group after intervention.

Implications of all the available evidence

ACBMNCs could substantially prevent severer BPD in surviving very premature neonates and improve neurodevelopmental outcomes in long term. An immunomodulatory effect of MNCs contributed to the improvement of BPD severity. Further multi-center randomized trials were needed to prove the preventive effect of ACBMNCs on BPD in more premature infants.

Introduction

Bronchopulmonary dysplasia (BPD) is the primary major complication of very preterm infants (VPIs) causing neonatal mortality and morbidity.[1] Despite rapid advances in neonatal care, BPD remains a significant burden in the preterm population, resulting in long-term respiratory and cognitive disabilities.[2,3] The current BPD definition was used to assess infants less than 32 gestational weeks worldwide.[4,5] The most recently used severity based definition of BPD released in 2019 classified BPD among very preterm infants as grade 1, 2 and 3 according to the level of respiratory support at 36 weeks postmenstrual age (PMA) or discharge home, which could better predicted the late death or serious respiratory morbidity, and neurodevelopmental impairment compared with the older one in 2001.[4,5] Compared with children with no or mild BPD, those with severer BPD had more in-hospital mortality, more postnatal growth failure, poorer prognosis of respiratory system and long-term neurodevelopment outcome.[6–9]

The pathophysiology of BPD is the arrest of alveolar and vascular development.[1,10] Persistent immune dysregulation and inflammation are regarded as the main factors contributing to BPD.[10,11] Current therapies are lung protective ventilation strategies, pulmonary surfactant and steroid treatments.[1,2] However, these therapies do not address the underlying alveolar and vascular structural deficits, therefore only alleviating the

symptoms of BPD, but did not reduced the BPD severity or improved the long term outcomes.[2,12] New therapies focusing on dampening the inflammatory response at an early stage without harmful effects and promoting the lung structural repairing are warranted to prevent lung injury therefore ameliorating pathologic pulmonary injury in BPD.[11]

Mononuclear cell (MNC) is the main resource of stem and progenitor cell in cord blood.[13] MNC is mainly composed of mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs).[13] It is increasingly recognized that immunomodulation represents an important mechanism underlying the benefits of stem cell therapies.[14] Cord blood derived stem cells showed immunomodulatory effect and contributed to outcomes improvement in adults acute ARDS and COVID-19.[15,16] A previous study of our team showed cord blood MNCs collection in very preterm infants was feasible and autologous, cord blood MNCs intravenous infusion could reduce the respiratory support duration in preterm neonates,[17,18] our ten-year follow up study demonstrated the long term safety.[19] Another recent clinical study showed the intratracheal transplantation of allogeneic human umbilical cord blood MSCs reduced the incidence of severer BPD in infants less than 24 gestational age (GA) and decreased pro-inflammatory cytokines in airway.[20] This indicated autologous cord blood mononuclear cells (ACBMNC) infusion may have the potential to prevent severer BPD and its protective effect might be via immune modulation. However, until now, there is no study to evaluate the effect of intravenous autologous MNC infusion to prevent severer BPD in very preterm infants. And no clinical study investigated the effect on immune cells. In this prospective study, we analyzed whether early intravenous ACBMNCs could improve BPD severity in very preterm neonates and its long term outcomes. Specially, we detected the immune cells and inflammatory cytokines before and after intervention so as to investigate the potential mechanisms.

Methods

Ethics statement: The trial was approved by the ethics committee of Guangdong Women and Children Hospital (201601079).

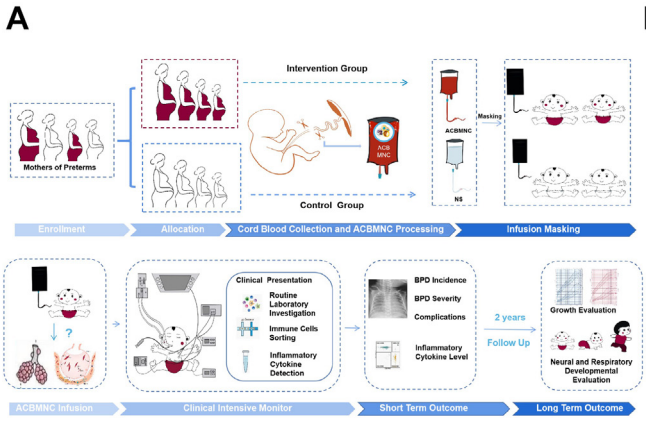
A non-randomized, placebo-controlled trial with blinded outcome assessment was conducted. The design and process of this study was shown in Fig. 1A. Written informed consent was obtained from the parents of all enrolled infants.

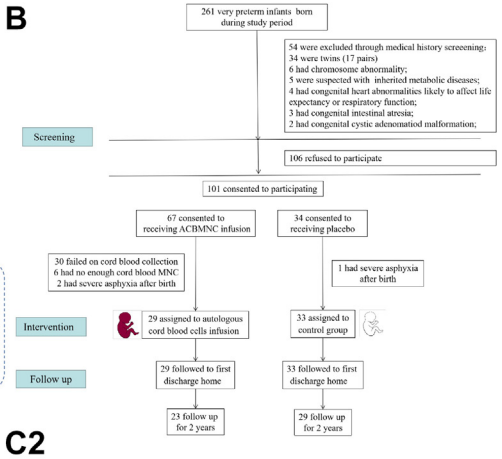
Study participants

Inborn infants less than 32 gestational weeks admitted to the Neonatal Intensive Care Unit (NICU) of Guangdong Women and Children Hospital from July 01, 2018 to January 1, 2020 were screened and enrolled.

Inclusion criteria

Infants fulfilling all the following inclusion criteria could be enrolled in this trial: (1) born at study hospital; (2) singleton birth; (3) gestational age (GA) <32 weeks (GA was calculated based on the date of the last menstrual period of the mother and an ultrasonographic screening performed during the first trimester of pregnancy)[21]; (4) enrolled within the first 24 postnatal hours; (5) free of





C1

Group	Complete cohort	Follow up	Arterial blood gas (postnatal days)							Serum electrolytes (postnatal days)					Peripheral blood cell counts (postnatal days)				
			1	3	5	7	14	21	28	1	7	14	21	28	1	7	14	21	28
Control (n)	33	29	33	30	28	28	23	22	17	33	30	29	24	23	33	33	32	32	32
Intervention (n)	29	23	29	28	25	22	25	16	12	29	23	25	17	15	29	29	29	29	29

C2

Group	Inflammatory cytokines Pannel				Immune cells Pannel			
	IL-6, TNF-a, IL-10		CRP		Lymphocyte subsets		Treg	
	CB	AI	CB	AI	CB	AI	CB	AI
Control (n)	20	20	33	33	33	33	30	30
Intervention (n)	20	20	29	29	29	29	27	27

Fig. 1: The design and process of this study. A. Schematic representation of subject cohorts and workflow; B. Patients enrollment process; C. The number of patients in control and intervention groups analyzed by various combination of detection. ACBMNC: autologous cord blood mononuclear cells; NS: normal saline; BPD: bronchopulmonary dysplasia; CB: cord blood; AI: after intervention.

severe perinatal asphyxia (defined as an Apgar score of 0–3 for more than 5 min, a cord blood gas pH < 7.00, or both); (6) free of severe congenital anomalies or genetic syndromes; (7) free of maternal sepsis; (8) infants receiving intravenous ACBMNC infusion have available umbilical cord blood (UCB), and the cell number was targeted to be 5×10^7 cells per kilogram, but should not be less than 1×10^7 cells per kilogram.

Exclusion criteria

Infants were excluded from the study if: (1) they exhibit severe congenital abnormalities (detected via prenatal ultrasound or found soon after birth); (2) expected to die within the first 24 h.

Patients allocation

Before very preterm neonates less than 32 weeks of GA were born, the neonatologists were informed beforehand, and they went through the patient medical history and confirmed with the obstetrician whether they might fulfill the enrollment criteria. In this screening stage, patients who were not singleton or suspected with severe congenital anomalies or genetic syndromes or with maternal sepsis were excluded. For those singleton birth without above medical history, we would talk with their parents before the delivery. If the consents for IV ACBMNC infusion were obtained from their guardians, the cord blood would be collected. If their umbilical cord blood cells after processing were available and they were free of severe perinatal asphyxia after birth, the infants were assigned to autologous cord blood mononuclear cells infusion group and received an infusion of ACBMNC with 24 h after cell process. Cell dose for all patients was targeted at 5×10^7 cells per kilogram, but no less than 1×10^7 cells per kilogram. If the consents for IV ACBMNC infusion were not obtained, the cord blood would not be collected for this trial, the parents could choose to deal with the cord blood as they preferred. If their guardians consented and the infants were free of severe perinatal asphyxia after birth, they were assigned to the placebo-controlled group and received 10 ml/kg normal saline with 24 h after birth.

Blinding, enrollment and cord blood collection, cord blood processing, MNC infusion, patients monitoring, assessment of safety and clinical management

The details were showed in supplemental file 1-study protocol, Fig. 2A and supplemental file 2-sheet for cord blood process step in the blood bank.

The details of laboratory investigation, immune cells sorting and inflammatory biomarkers detection were showed in supplemental file 1-study protocol.

Clinical and biological outcome assessment

The primary outcome was moderate or severe BPD in survivors. The secondary outcome was incidence of BPD, mild, moderate and severe BPD in survivors and mortality. BPD was defined as treatment with oxygen >21% for at least 28 days. BPD severity was categorized on the basis of the respiratory support administered at a PMA of 36 and 0/7 weeks or discharge, no BPD: no support; mild BPD: nasal cannula ≦2 L/min; moderate BPD: nasal cannula >2 L/min or noninvasive positive pressure ventilation; and severe BPD: invasive mechanical ventilation.[5] Other clinical outcomes included: 1. Incidence of other common preterm complications including IVH, NEC, retinopathy of prematurity (ROP), respiratory distress syndrome (RDS), LOS, and persistent pulmonary hypertension of newborn (PPHN); 2. Duration of mechanical ventilation and oxygen therapy; 3. Duration of hospitalization and hospitalization expense. The definitions used in this study were showed in Supplemental File 1-study protocol.

Biological outcomes

Biological markers of inflammation (CRP, TNF-a, IL-6 and IL-10) were measured in serum with ELISA kits and immune cells number and percent were calculated by flow cytometry at baseline and 72 h after infusion.

Long term follow up

We examined the growth of patients, their physical and neurological parameters when they were recalled to visit the clinic at corrected age of 18–24 months old, the details of the follow up methods were showed in study protocol.

Statistical analysis

Sample size

As this study was non-randomized study, we used the requirements of the logistic regression on sample size to estimate the study sample size based on the classic experience norms.[22] As indicated by previous studies, the strongest risk factors for BPD were prematurity (GA) and low birth weight. Male sex was also considered as the risk factor. Other maternal factors on BPD were inconsistently.[1,2] Since GA and birth weight usually showed collinearity, thus, we included GA or birth weight in the logistic regression analysis for calculating sample size. Therefore, the total sample size was 20 times the co-variables, in our study, the number of co-variables was 3 (MNCs intervention, GA or birth weight, and gender), thus, the estimated sample size was 60 totally.

In addition, we also conducted a priori power analysis. We calculated sample size by using comparing 2 proportions: 2-Sample Non-Inferiority or Superiority method, as we presumed that the MNCs intervention was not inferior to the placebo control. We used a type I error-rate (α) of 5% and a power of 80%, non-inferiority or superiority margin was –0.1. The priori power analysis of sample size, controlling for the set α, power and non-inferiority or superiority margin was 30 infants in each group, based on an expected moderate or severe BPD rate of 16% in the control group (based on the data

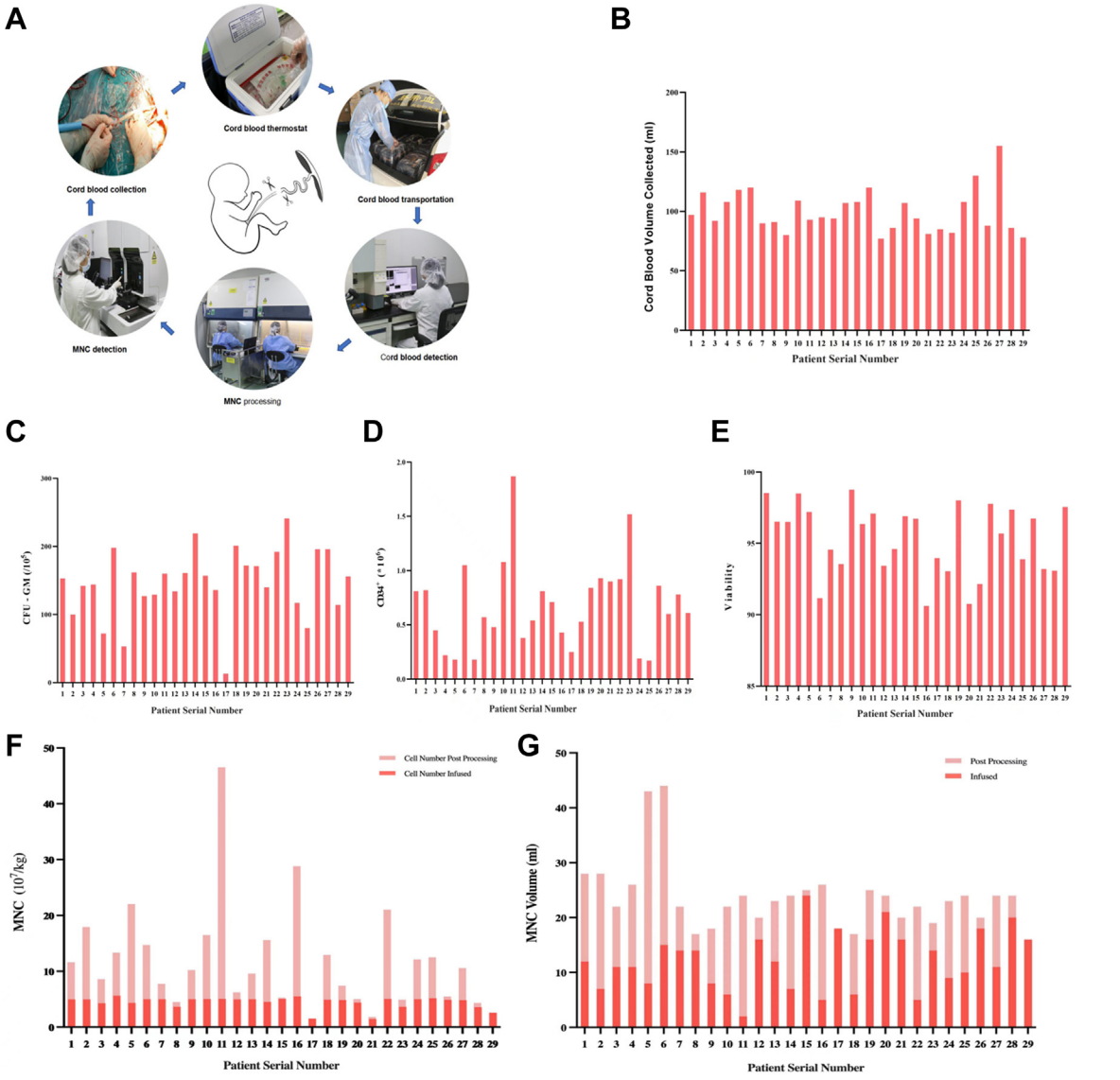


Fig. 2: The Umbilical Cord-blood processing and cord blood cells characteristics. A. Cord blood collection, transportation, processing and detection. B. Cord blood volume after collection. C. CFU-GM number in the cord blood. D. CD34 positive cells number in the cord blood. E. Cell viability of the cord blood mononuclear cells. F. Cord blood mononuclear cells number after processing and infused to the patients. G. Cord blood volume after processing and infused to the patients. CFU-GM: granulocyte Macrophage colony forming unit; MNC: mononuclear cells; kg: kilogram; ml: milliliter.

in our center) and 6% in the MNC group (based on the results from "Stem Cells Transl Med. 2021 Aug;10(8):1129–1137" that severe BPD was significantly reduced from 53% (8/15) to 19% (3/16) with MSC transplantation intratracheally, a more than 60% reduction). As both of the two methods showed a minimum 30 infants in each group could detect a hypothesised effect size, therefore, we estimated the sample size was 60 totally.

Means (standard deviation) and unpaired Student's t-test were used for continuous variables with normal distribution, and median and interquartile range (IQR) and non-parametric analysis was used for data with non-normal distribution. The variables' distribution characteristics were estimated with Kolmogrov–Smirnov test. The number and percentage were reported for categorical variables. Group comparisons of categorical variables for outcomes and co-interventions including death, BPD, and BPD severity were performed using the Fisher's exact test, or chi-square test, as appropriate. Single factor regression analysis was conducted to assess the possible confounding factors for the main short and long term outcomes. Then multiple logistic regression was used to estimate the contribution of intervention to outcomes

after adjusting for confounders. The relative risk (RR) was calculated as the ratio of the cumulative incidence rates of an event occurring in the intervention infusion group to the control group, and the number needed to treat as the inverse of these two cumulative incidence rates. For continuous variables, the mean values at each time point in each group for variables were derived from a generalized linear model and differences in values over time were compared using an unpaired Student's t-test between the two groups. Kaplan–Meier cumulative incidence plots were generated to show time-to-event end points including being extubated and being weaned to room air, log-rank test was used for assessing variables that may affect the chance to be extubated and weaned to room air in the baseline characters. Cox regression was used to adjust this analysis for potential confounding variables. All statistical tests were two-tailed, and p-values <0.05 were deemed statistically significant. All statistical analysis was done using SPSS 21.0 (IBM).

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. No authors have been paid to write this article by a pharmaceutical company or other agency. Authors were not precluded from accessing data in the study, and they accept responsibility to submit for publication. Yang Jie and Ren Zhuxiao had access to the dataset. Yang Jie and Feng Zhichun had final decision to submit for publication.

Results

Study population

During the experimental period from July 01, 2018 to January 1, 2020, 261 preterm neonates less than 32 GA were born in our center. 54 infants were excluded in the medical history screening process because of being not singleton or suspected with severe congenital anomalies or genetic syndromes. Among the remaining 207 infants, the informed consents for participation were not obtained from the guardians of 106 infants. Then, informed consents were obtained from the guardians of the remaining 101 infants, among which, 67 of them consented to receiving ACBMNCs, but 36 had not available MNCs and 2 had severe asphyxia after birth, thus, finally twenty-nine infants were assigned to ACBMNCs intervention group. The guardians of 34 infants consented to receiving placebo, 1 had severe asphyxia after birth, thus, 33 infants were assigned to placebo-controlled group. The patient enrollment processes were shown in Fig. 1B.

Totally, 199 infants were excluded, among which 142 infants were patients intent-to treat (ITT) but excluded (261 infans-54 (twins or with congenital malformation)-3 (severe asphyxia)- 62(enrolled patients) = 142 infants). We compared the primary outcome (moderate or severe BPD in survivors) between 142 patients (ITT but were excluded) and 33 infants enrolled in control group, and found they were comparable (22/137, 16.1% vs 8/32, 25.0%, p = 0.233).

In addition, we compared the GA, birth weight and gender in 142 infants (ITT but were excluded) and 33 infants in control group and 29 infants in intervention group (male gender: control-20/33, 60.6%, intervention-11/29, 37.9%, 142 patients-76/142, 53.5%, p = 0.183; GA (median, IQR): control-29.86 (2.14), intervention-29.57 (2.14), 142 patients-29.96 (2.29), p = 0.340; birth weight (median, IQR): control-1.35 (0.34), intervention-1.32 (0.47), 142 patients-1.35 (0.43), p = 0.945), they were also comparable. Therefore, we assumed the included cohort were considered to be representative of the wider population intent to treat.

The main baseline characteristics of the mothers and infants were similar between the intervention and control groups (Table 1). All patients were followed up to their first discharge home. Twenty-three patients in intervention group and 29 in control group were followed up on corrected age between 18 and 24 months.

We also noticed the incidence of gestational diabetes was 72.7% in the control and 55.2% in the intervention groups, which was higher than the general pregnant women. During the study period, a second-child population policy was started in China.[23] Pregnant women with more pregnancy associated complications were referred to our hospital. Additionally, pregnant women with gestational diabetes may deliver more preterm infants compared with the general pregnant women population.[24] These factors may contribute to higher incidence of gestational diabetes in this study.

Study intervention and co-interventions

The characteristics of the ACBMNCs are shown in Fig. 2B–G and in Supplemental Table S1. All infants in the intervention group received the ACBMNC infusion within 24 h after cord blood processing. The mean number of obtained ACBMNCs after processing was $14.71 \pm 2.75 \times 10^7$ cells per kilogram (/kg) and the mean number of infused MNCs was $4.48 \pm 1.05 \times 10^7$ cells/kg. Furthermore, the mean volume transfused was 12.33 ± 5.24 ml. In all cases, the infusion duration was within 4 h and the infusion speed was less than 10 ml/kg/h. The MNC viability was 95.32 ± 2.45%.

No patients experienced any of the pre-specified adverse hemodynamic or respiratory safety events during or within 24 h from the start of the infusion. There were no significant differences between the two groups in the use of co-interventions (Table 2). All infants in both groups received assisted oxygen therapy.

Outcomes

Primary outcome

One (3.6%) of the 28 surviving infants assigned to the intervention group and eight (25.0%) of 32 surviving

Characteristics	Control group (N = 33)	Intervention group (N = 29)	p value
Mothers			
Age (years), median (IQR)	32 (8)	32 (7)	0.955
Antenatal glucocorticoids, n, (%)	30 (90.9)	27 (93.1)	1.000
Preeclampsia, n, (%)	1 (3.0)	4 (13.8)	0.278
Gestational hypertension, n, (%)	6 (18.2)	5 (17.2)	0.490
Gestational diabetes, n, (%)	24 (72.7)	16 (55.2)	0.509
Infants at birth			
CS, n, (%)	15 (45.5)	14 (48.3)	0.824
Apgar score			
1 min, median (IQR)	8 (2)	8 (1)	0.506
5 min, median (IQR)	9 (1)	9 (1)	0.432
GA (weeks), median (IQR)	29.86 (2.14)	29.57 (2.14)	0.961
GA < 28 weeks, n, (%)	4 (12.1)	4 (13.8)	0.845
Male, n, (%)	20 (60.6)	11 (37.9)	0.075
Birth weight (kg), median (IQR)	1.35 (0.34)	1.32 (0.47)	0.794
Body length (cm), median (IQR)	38 (6)	38 (7)	0.629
Head circumference (cm), median (IQR)	27 (3)	28 (3)	0.123
HsPDA, n, (%)	0 (0%)	0 (0%)	1.000
Respiratory condition before intervention			
Intubated, n, (%)	19 (57.6)	16 (55.2)	0.849
PS treatment, n, (%)	13 (39.4)	15 (51.7)	0.330
RDS ≧3, n, (%)	5 (15.2)	5 (17.2)	1.000
Selective HFOV, n, (%)	7 (21.2)	3 (10.3)	0.312

IQR: interquartile range; n: number; CS: caesarean section; GA: gestational age; kg: kilogram; cm: centimeter; HsPDA: hemodynamically significant patent ductus arteriosus; PS: pulmonary surfactant; RDS: respiratory distress syndrome; HFOV: high frequency oscillatory ventilation.

Table 1: **Baseline characteristics and status of the infants and their mothers in the two groups.**

infants assigned to the control group were diagnosed as moderate or severe BPD (relative risk: 0.112, 95% confidence interval: 0.013–0.956, p = 0.029). After single factor regression analysis for the possible confounding factors of the primary outcome, we found birth weight and PS treatment before intervention were the two main confounding factors, other baseline or maternal factors showed no significant effect on the primary outcome (Supplemental Table S2). After adjusting for the two main confounding factors, MNCs intervention could still reduce the incidence of moderate or severe BPD (adjusted p = 0.021). The number of patients needed to treat to gain one moderate or severe BPD-free survival was 5 (95% confidence interval: 3–20) (Table 2).

Secondary outcomes

One patient in intervention group died on 102 days of life because of septic shock; his GA was 30 weeks. One patient in control group died on 9 days of life owing to persistent pulmonary hypertension and shock. His GA was 25 + 4 weeks. Three of the 28 surviving infants (10.7%) assigned to the intervention group and 9 of the 32 surviving infants (28.1%) assigned to the control group were diagnosed with BPD. The difference between the groups failed to reach significance (adjusted p = 0.106) (Table 2, Fig. 3A). There was no significant difference on incidence of mild or moderate or severe BPD in two group survivors.

Other outcomes

There were no significant differences between the groups in terms of other preterm-associated complication events or potential adverse effects of treatment. Survivors in the intervention group required less endotracheal intubation (p = 0.029) and assisted positive pressure ventilation therapy than did those in the control group within 28 days after birth (p < 0.001) (Fig. 3B). Among the surviving infants during the study, those in the intervention group had a significantly higher chance to be extubated than infants in the control group (p = 0.004, adjusted p = 0.005) (Fig. 3C). There was no significant difference on chance to be weaned to room air between two groups (p = 0.199, adjusted p = 0.153) (Fig. 3D).

The vital signs for survivors before and after infusion as well as during the first 28 days were showed in Fig. 4A–D. There was no significant difference in any of these variables between the groups. During the first four weeks after enrollment, no significant differences in weight gain or calorie intake were observed in survivors between the two groups (Fig. 4E and F).

Outcomes	Control (n = 33)	Intervention (n = 29)	p	Adjusted p	RR (95% CI)
Primary outcomes					
Moderate or severe BPD in survivors, n, (%)	8 (25.0%, n = 32)	1 (3.6%, n = 28)	***0.029***	***0.021***[a]	***0.048 (0.004,0.636)***
Secondary outcomes					
BPD in survivors, n, (%)	9 (28.1%, n = 32)	3 (10.7%, n = 28)	0.116	0.106[b]	0.271 (0.056,1320)
Death, n, (%)	1 (3.3)	1 (3.4)	1.000		
Mild BPD in survivors, n, (%)	1 (3.1%, n = 32)	2 (7.1%, n = 28)	0.594	0.364[c]	2.356 (0.371, 14.982)
Moderate BPD in survivors, n, (%)	6 (18.8%, n = 32)	1 (3.6, n = 28)	0.109	0.064[d]	0.103 (0.009, 1.141)
Severe BPD in survivors, n, (%)	2 (6.3%, n = 32)	0 (0%, n = 28)	0.494		
Other outcomes					
IVH, n, (%)	11 (33.3)	12 (41.4)	0.513		
NEC, n, (%)	14 (42.4)	7 (24.1)	0.129		
ROP, n, (%)	9 (27.3)	9 (31.0)	0.745		
LOS, n, (%)	4 (12.1)	1 (3.4)	0.211		
PPHN, n, (%)	3 (9.1)	2 (6.9)	1.000		
Duration of endotracheal intubation (day), median (IQR)	3.5 (10)	1.5 (5.75)	0.161		
Duration of assisted oxygen therapy (day), median (IQR)	38 (34)	29 (17)	0.208		
Duration of Hospitalization (day), median (IQR)	57 (28)	50 (18)	0.266		
Hospitalization expense (RMB), median (IQR)	125,193 (83,601)	102,163 (55,290)	0.145		
Co-interventions					
Re-intubation, n, (%)	1 (3.0)	3 (10.3)	0.515		
Postnatal corticosteroids, n, (%)	1 (3.0)	0 (0)	1.000		
Inhalted nitric oxide, n, (%)	1 (3.0)	1 (3.4)	1.000		
PDA ligation, n, (%)	0 (0)	0 (0)	1.000		
Transfusions of red cells, n, (%)	26 (78.8)	18 (58.6)	0.086		
Daily fluid at the end of a week after birth (ml), median (IQR)	124 ± 50	127 ± 43	0.784		

BPD: bronchopulmonary dysplasia; RR: relative risk; CI: confidence interval; GA: gestational age; n: number; IVH: intraventricular hemorrhage; NEC: necrotizing enterocolitis; ROP: retinopathy of prematurity; RDS: respiratory distress syndrome; LOS: late onset sepsis; PPHN: persistent pulmonary hypertension of the newborn; IQR: interquartile range; RMB: Renminbi; PS: pulmonary surfactant; PDA: patent ductus arteriosus. The bold and italic values mean these values had statistical difference. [a]p-adjusted for birth weight and PS treatment before intervention. [b]p-adjusted for intubation and PS treatment before intervention. [c]p-adjusted for GA. [d]p-adjusted for birth weight (multiple logistic regression was used to estimate the contribution of intervention to outcomes after adjusting for potential confounding factors based on the results of single factor regression analysis).

Table 2: **Short term outcomes and co-intervention.**

Laboratory investigations

The number of patients in control and intervention groups analyzed by various combination of detection is shown in Fig. 1C. The two groups were comparable in blood gases (PH, PO_2, PCO_2), serum electrolytes (sodium, potassium, chloride, calcium), glucose, lactic acid, blood urea nitrogen and peripheral blood cells (white blood cells, hematocrit and platelet) before intervention and during the first 4 weeks of the study (Fig. 3E–G and Fig. 4G–P).

Biological markers and immune cells detection

At baseline, B cells proportion were higher, and CRP level and T cell proportion was lower in intervention group than in control group, and no other biomarkers or immune cells differed between groups at baseline. IL-10 level increased significantly after intervention in MNC infusion group compared with control group ($p = 0.03$). CRP and TNF-a level were lower in intervention group after MNC infusion ($p < 0.05$). T cells, $CD4^+$ T cells and Treg proportion increased dramatically after MNC infusion ($p < 0.05$), no difference was noticed in other immune cells or cytokines between two groups after intervention (Fig. 5A–K, Supplemental Table S3).

Two-year follow up outcomes

Study sample

All patients were followed up to their first discharge home. Four in control group and 6 in intervention group were lost in follow up study, two of them were examined in one-year old in clinic and were healthy, and the other 8 patients were without severe complications when firstly discharged home, therefore we assumed they were alive. Finally, 23 (82.1%) infants in intervention and 29 (90.6%) infants in control group were followed up successfully and all of them survived (Table 3).

Follow up information of patients

The BW, GA, follow up time, main caregivers education level, rate of parents as main caregivers and smoking rate in main caregivers were similar in two groups. There were more male patients in control group than in intervention group in follow up cohort

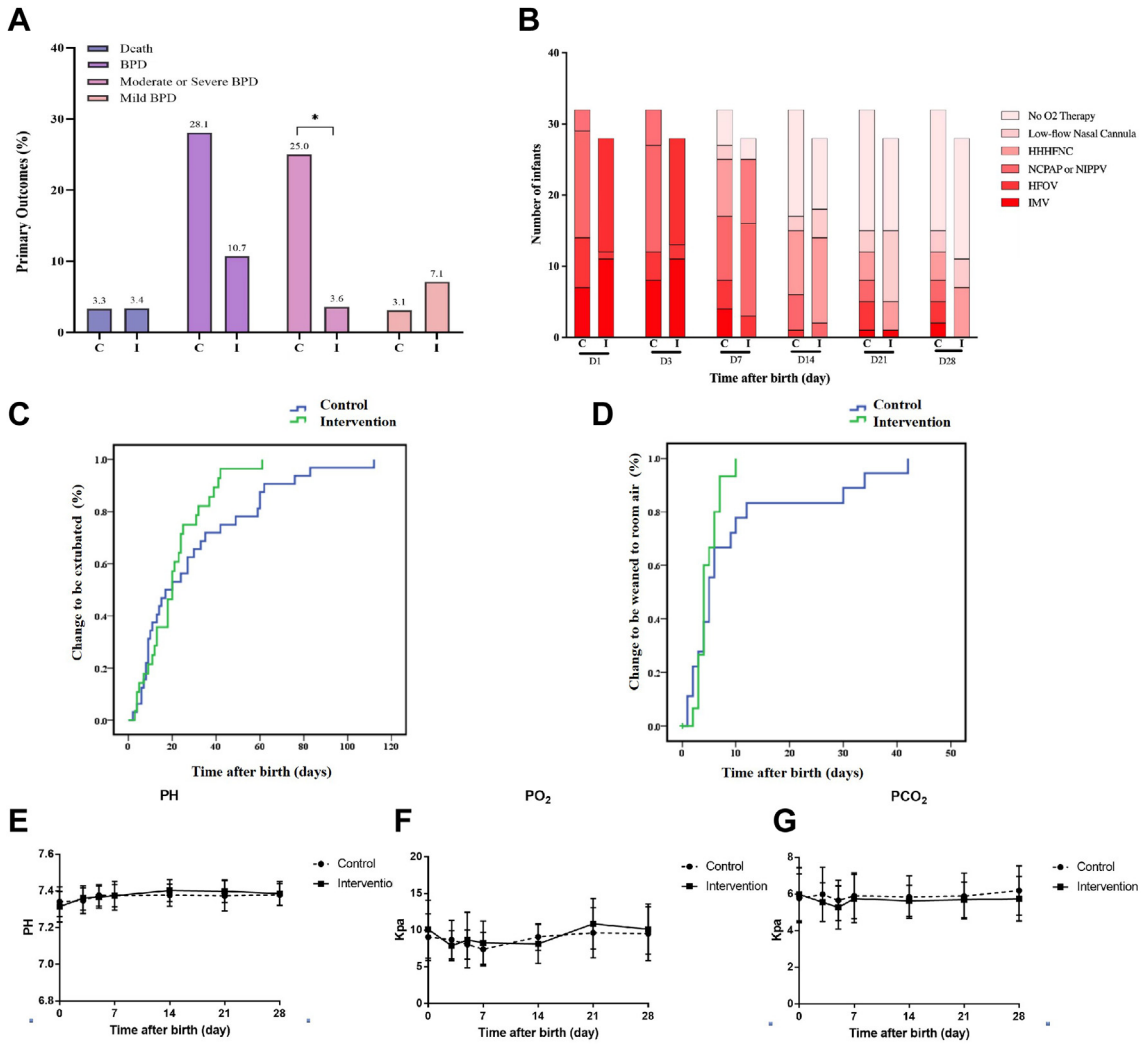


Fig. 3: The main outcomes and respiratory support conditions in two group. A. The incidence of main outcomes; B. Number of infants needing assisted positive pressure ventilation therapy before 28 days. Assisted positive pressure ventilation therapy was defined as a requirement for any of the following: NCPAP, NIPPV, HFOV or IMV. Survivors in the intervention group required less endotracheal intubation (control: 5/32 vs intervention: 0/28, p = 0.029) and assisted positive pressure ventilation therapy than did those in the control group on Day 28 (control: 8/24 vs intervention: 0/28, p < 0.001). C, D. Proportion of survivors extubated (C) or survivors weaned to room air (D) over time during the study. Infants in the intervention group had a significantly higher chance to be extubated than infants in the control group (p = 0.004, adjusted p = 0.005). There was no significant difference on assisted O_2 therapy between two groups (p = 0.199, adjusted p = 0.153). GA, birth weight, preeclampsia, gestational hypertension, gestational diabetes showed significant effect on the extubation, and GA, birth weight, maternal age showed significant effect on chance to be weaned to room air in log-rank analysis. Cox regression was then applied, after adjusting for these potential confounding variables. E–G. Comparison of blood gases between the control and intervention groups during the first 4 weeks of the study. There was no significant difference in any of these variables between the groups. BPD: bronchopulmonary dysplasia; O_2: oxygen; GA: gestational age; D: day; HHHFNC: heated, humidified high-flow nasal cannula; NCPAP: nasal continuous positive airway pressure; NIPPV: nasal intermittent positive pressure ventilation; HFOV: high-frequency oscillatory ventilation; IMV: intermittent mandatory ventilation; PH: pondus hydrogenii; PO_2: oxygen pressure; PCO_2: partial pressure of carbon dioxide.

(55.2% vs 26.1%, p = 0.049) (Table 3). Single factor regression analysis showed no potential confounding factors had significant effect on the long term respiratory and neurodevelopmental outcome. Thus, we only adjusted for male gender in the multiple logistic regression.

General outcomes

The general characteristics of studied infants at 18–24 months corrected age (CA) of follow-up assessment were showed in Table 3. No differences were found in body weight or length. The percent of low-body weight or short stature was similar in two groups. No patients

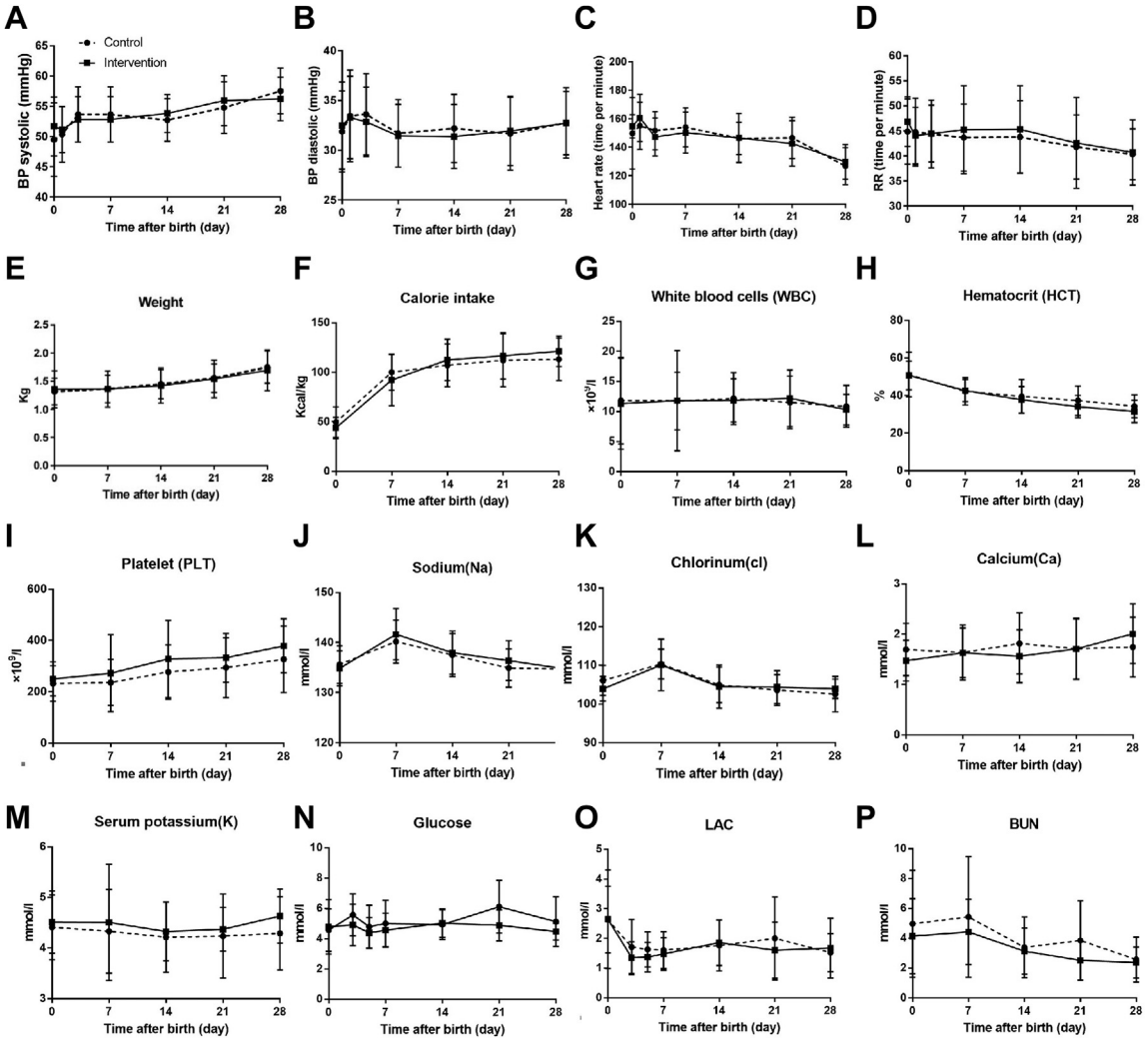


Fig. 4: Comparison of heart rate, respiratory rate (RR), systolic and diastolic blood pressure (BP), body weight (BW), calorie intake, blood glucose, serum electrolytes, lactic acid, blood urea nitrogen and peripheral blood cells between the control and intervention groups during the first 4 weeks of the study. Day 0: before intervention. LAC: lactic acid; BUN: blood urea nitrogen.

had rickets or delay fontanelle close. No supplemental oxygen was needed in any of the patients. More pneumonia related hospitalizations happened in control group ($p = 0.044$). After adjusted for gender, the difference was not significant (adjusted $p = 0.092$) (Table 3).

Neurodevelopmental outcomes

The incidence of infants with mental or psychomotor development delay was higher in control group, although without a statistically significant difference. The incidence of neurodevelopmental delay (with either cognitive or motor development impairment) was obviously higher in control than in MNC infusion group, even after correcting for gender ($p = 0.046$, adjusted $p = 0.047$, Table 3).

Discussion

In this study, we performed a prospective, non-randomized, placebo-controlled trial to investigate the effect of autologous, plasma and RBC-reduced, non-cryopreserved cord blood MNC infusion soon after birth on severe BPD prevention. Our results showed that one intravenous dose of ACBMNCs could significantly decreased incidence of moderate or severe BPD in surviving very preterm neonates. There were no MNC-related predefined hemodynamic or respiratory adverse events. Although one patient who received an infusion of ACBMNCs died 102 days after birth, the safety monitoring board judged the death as unlikely to be related to the administration of ACBMNCs. A two-year long term follow up outcome showed incidence of developmental delay was reduced in intervention

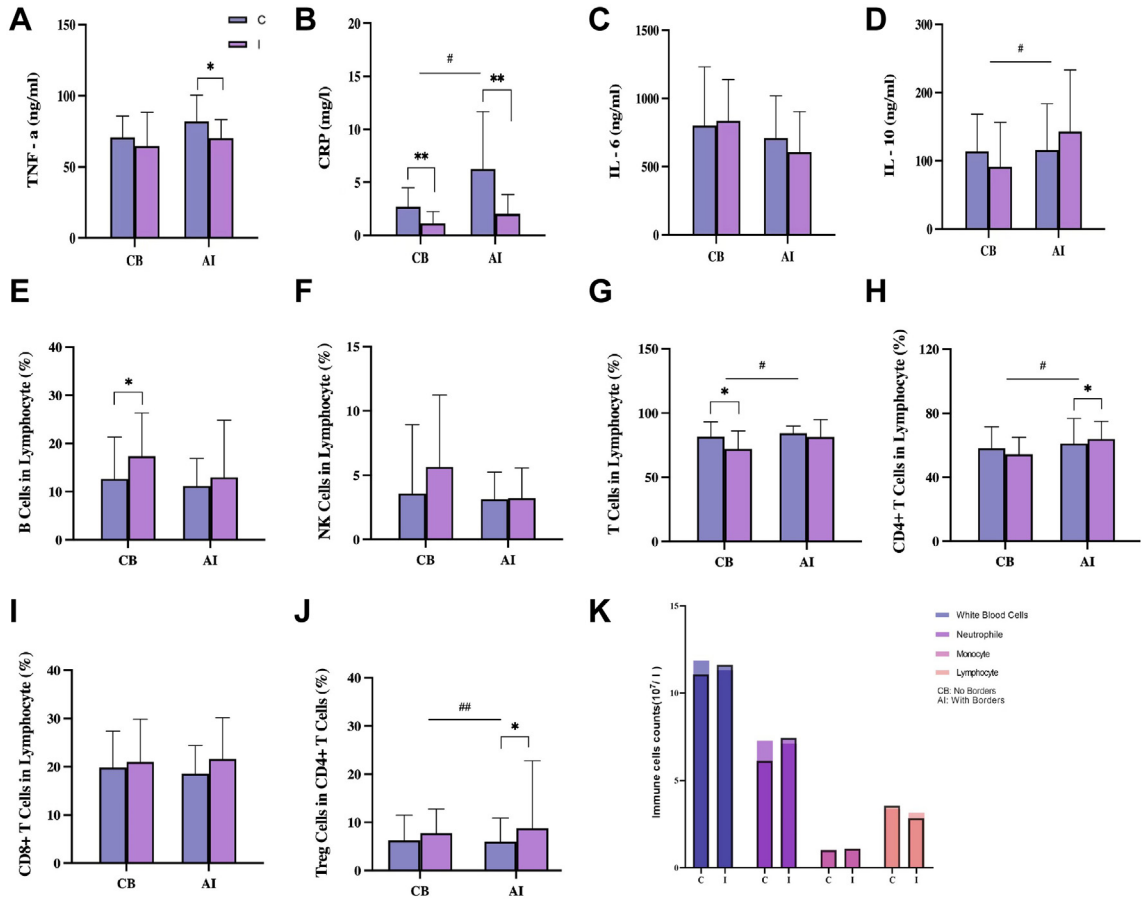


Fig. 5: Comparison of immune cells and biomarkers in cord blood and after intervention in two groups. *p < 0.05, **p < 0.01, ***p < 0.001-difference between two groups in cord blood or in serum after intervention; #p < 0.05, ##p < 0.01-difference on changes before and after intervention in each group. C: control group, I: intervention group, CB: cord blood, AI: after intervention. TNF-a: tumor necrosis factor α; CRP: C reactive protein; IL-6: interleukin-6; IL-10: interleukin-10; Treg: the regulatory T cell; NK: natural killer cell.

group. Immune cells sorting and cytokine detection results indicated early ACBMNCs infusion had an anti-inflammatory effect and immunoregulatory activity.

The infants less than 32 gestational weeks (GW) were still faced with high risk of developing BPD.[1,2,25–27] The incidence of BPD was 29.2% reported by Chinese Neonatal Network, 25.9% reported by US Vermont Oxford Network, and around 20% reported by Effective Perinatal Intensive Care In Europe (EPICE) cohort.[25–27] A recent study on trends in outcomes for neonates born very preterm in high-income countries showed the BPD incidence was increasing.[28] BPD was still the major and severe complication affecting a wide population of very preterm infants, which was found to be a crucial factor for adverse neurodevelopmental outcome of very preterm birth.[3] In several recent reviews on BPD management, stem cell therapy was considered to have great potential in prevention and treatment.[1,2] However, so far there is no data reported in BPD prevention with cell intervention. The results of this study laid important foundation for the safe clinical translation of cord blood stem cell therapy for preventing BPD in very preterm neonates.

Before us, only a korea team performed a clinical trial aiming to preventing BPD, in their study, allogeneic MSCs were administered endotracheally to extremely preterm neonates, and the result showed decreased BPD severity in infants less than 24 GA, and inflammation cytokine level in airway reduced significantly.[20] However, intratracheally administered umbilical cord blood-derived MSCs at around 2 weeks postnatally, at doses of 10–20 million cells per kilogram showed highly varied results regarding the incidence of severe BPD in extremely preterm infants in another two previous studies.[29,30] The major mechanisms underlying the beneficial effect of stem cell therapy appears to be the secretion of paracrine factors; thus, an intravenous infusion may better allow paracrine bioactive factors to act on the pulmonary tissue, with high deposition in the pulmonary capillary beds.[14,31] Consistent with this,

Characteristics		Control group (29)	Intervention group (23)	p value	
Gestational age (week), median (IQR)		30 (2)	30 (2)	0.438	
Birth weight (kg), median (IQR)		1.3 (0.3)	1.3 (0.6)	0.905	
Male, no/total no. (%)		16/29 (55.2)	6/23 (26.1)	***0.049***	
Follow up duration (months), median (IQR)		24.7 (4.7)	23.9 (3.4)	0.103	
Main caregiver as parents, no/total no. (%)		22/29, 75.9%	19/23, 82.6%	0.735	
Main caregiver with college education, no/total no. (%)		13/29, 44.2%	13/23, 55.8%	0.577	
Main caregiver smoking, no/total no. (%)		7/29, 24.1%	3/23, 13%	0.482	
Lost follow-up, no/total no. (%)		3/32, 9.4%	5/28, 17.9%	0.454	
Outcomes					**Adjusted p**[a]
Anthropometric characteristics	W (kg), median (IQR)	11 (1.5)	10.5 (2.0)	0.663	
	W < 3%, no/total no. (%)	6/29, 20.7%	4/23, 17.4%	1.000	
	L (cm), median (IQR)	83 (6)	83 (5)	0.534	
	L < 3%, no/total no. (%)	7/29, 24.1%	5/23, 21.7%	1.000	
	Fontanelle closed, no/total no. (%)	29/29,100%	23/23,100%	1.000	
	Rickets, no/total no. (%)	0/29, 0%	0/23, 0%%	1.000	
Respiratory outcomes	Wheezing, no/total no. (%)	6/29, 20.7%	2/23, 8.7%	0.278	
	Nocturnal cough, no/total no. (%)	1/29, 3.4%	1/23, 4.3%	1.000	
	Pneumonia related hospitalizations, no/total no. (%)	11/29, 37.9%	3/23, 13.0%	***0.044***	0.092
	Supplemental oxygen, no/total no. (%)	0/29, 0%	0/23, 0%	1.000	
Neurodevelopmental outcomes					
	Auditory impairment, no/total no. (%)	0/29, 0%	3/23, 13%	0.080	
	Visual impairment, no/total no. (%)	1/29, 3.4%	0/23, 0%	1.000	
Bayley score	PDI, median (IQR)	105 (19.5)	108 (14)	0.658	
	Psychomotor development delay, no/total no. (%)	5/29, 17.2%	1/23, 2.3%	0.210	
	MDI, median (IQR)	94 (40.5)	101 (19)	***0.018***	0.108
	Mental development delay, no/total no. (%)	8/29, 27.6%	2/23, 8.7%	0.075	
	Neurodevelopmental delay, no/total no. (%)	10/29, 34.5%	2/23, 8.7%	***0.046***	***0.047***
	Seizure, no/total no. (%)	2/29, 6.9%	1/23, 4.3%	1.000	
	Cerebral palsy, no/total no. (%)	3/29, 10.3%	1/23, 4.3%	0.621	

IQR: interquartile range; kg: kilogram; no: number; W: weight; L-length; cm: centimeter; CA: corrected age; PDI: psychomotor developmental index; MDI: mental developmental index. The bold and italic values mean these values had statistical difference. [a]p-adjusted for gender.

Table 3: **Long term follow-up outcomes.**

preclinical evidence indicates that the intravenous delivery of stem cells is significantly superior to intratracheal administration.[31] Although an intratracheal administration of stem cells allows direct pulmonary access and does not cause a rejection reaction with an allogeneic cell resource,[29,30] there remains confusion regarding how these cells can pass through the airway. Furthermore, unlike surfactants located in pulmonary alveoli, the survival duration of stem cells in the airway may be very short, as they are originally located in the blood. This may indicate an unstable effect with intratracheal delivery. Another issue to consider is that for non-intubated infants, intratracheal delivery is impossible, even though such infants still have a high risk of BPD.

The cell source used in this study was autologous cord blood MNCs.[13] Cord blood is regarded as a convenient source of stem cells, as the stem cells are readily available, without risk to the donor.[13,17] Umbilical cord blood derived stem cells showed lower immunogenicity, and higher proliferation capacity compared with other cell sources.[13,32] There are several advantages of using ACBMNCs. First, the MNCs were acquired through centrifugation alone (i.e., physical separation) without adding any reagent,[18] thereby avoiding possible toxicity in preterm neonates.[32] Second, as the infused MNCs in the present study were fresh and not cryopreserved, they showed high cell viability. A study using MSCs for treating adult acute RDS indicated that higher cell viability is associated with better efficacy.[15] After cord blood processing, cell viability of ACBMNCs was 95.32% on average, which may be owing to the simple MNC separation procedure. As previously reported, umbilical cord blood cells that were processed up to 96 h from collection and stored at room temperature maintained a satisfactory functionality for cell therapy,[33] thus, in this study, the cell viability could be maintained well over this infusion period. Third, unlike allogeneic, in vitro expanded stem cells used in several previous studies, there is no concern for a rejection reaction or

infection or cell mutation issues.[20,29,30] Finally (and importantly), as umbilical cord blood from preterm infants, especially those with GA <34 weeks, is currently discarded due to an inadequate volume for banking, the use of these cord blood MNCs for preventing BPD reduced the waste of this valuable stem cell resource.[13] However, there is also potential limitation in the use of autologous cord blood MNCs. This treatment is not suitable for patients without available cord blood MNCs because of failed collection or not enough cell number.

The cell dosage in our study was 5×10^7 cell/kg, which was based on our and others previous studies.[18,32] In such a targeted cell dose, the administration volume to be as high as 19 ml/kg. We intended to deliver the highest possible concentration within a safe volume, and no volume-related cardiac accident was observed in our study.

In our study, the cord-blood cells were infused within 14.45 h after processing averagely. Preclinical studies indicate that cell therapies are most efficacious shortly after lung inflammation begins but is not yet overwhelming.[14] Therefore, compared with when the BPD was established in the later stage of altered lung development, a prophylactic administration before excessive lung inflammation happened might be more efficient. Therefore, there may be more necessity for a "prophylactic administration" than a rescue therapy. In Powell and colleagues study, infants were administered with human UCB-MSCs at a mean of 10.6 days of life. Although Powell et al. recognized the importance of administering cells as early as possible (based on animal research), they experienced difficulty in promptly recruiting patients, thus creating an obstacle for earlier cell therapy.[29] In this study, we obtained parental consent before birth allowing us to process the cord blood faster. The transport duration from cord blood bank to our center was around half an hour to an hour depending the traffic situation. These strategies ensured the ability to conduct the cell infusion very early.

Although this study was no randomized, it firstly assessed the effect of intravenous ACBMNCs intervention in very preterm infants for the purpose of preventing severe BPD and provided detailed short and long term outcomes. Specially, immune cells change and inflammatory cytokines in serum were firstly detected and compared before and after intervention. Inflammation is regarded to be the critical denominator in all these multi-factorial origins resulting in BPD.[10,11] Corticosteroids was used for several decades in preventing or treating BPD, as it may prevent the harmful effects of inflammation on the developing lung. However, several large RCTs had showed limited improvement in BPD, on the contrary, the benefits may not outweigh its short-term and long-term adverse effects including increased risk of infection and gastrointestinal bleeding, weakened immune system, further, it may affect long term neurodevelopmental outcomes.[2,34] New methods targeting alleviating immune dysregulation and reducing pro-infammatory cytokines but did not cause adverse outcomes might overcome the above shortages.

Preclinical study had demonstrated the immunomodulatory effects of stem cells without side effect.[14,35] Multiple studies have shown that IL-6 and TNF-α are elevated very early in the respiratory course of the human preterm population that ultimately develop BPD.[36] Previous preclinical studies showed stem cells could induce a change in the secretome of T-lymphocyte therefore reducing production of the pro-inflammatory cytokines while increasing anti-inflammatory cytokines such as IL-10.[37]

Our study showed cord blood MNCs infusion soon after birth smoothed inflammatory response in preterm neonates including reducing pro-inflammatory cytokines TNF-a, CRP and increasing anti-inflammatory cytokines IL-10. Previous study showed CD4+ T-cells decreased in infants with BPD.[38] Further, a pro-inflammatory CD4 (+) T cell status characterized by secreting IL-6 and decreased Treg proportion is associated with the development of BPD.[36] MNCs act on the adaptive immune system, particularly T cells.[37] In our study, we found T cells and CD4+ T-cells proportion increased after infusion, but IL-6 level did not increase. Among the substes of CD4+ T-cells, the regulatory T cells (Tregs) acts to prevent overwhelming inflammation, which was characterized by secretion of IL-10.[39] Tregs play an important role during the suppression of immune response and resolution of lung injury.[39] In our study, we found the Treg proportion among CD4+ cells increased significantly after MNC intervention. The main mechanisms of CD4+CD25+Foxp3+Tregs underlying resolution of lung injury are mediated by inducing secretion of IL-10 and suppression of anti-inflammatory cytokine secretion such as TNF-a and IL-6.[36,39] IL-10 is critical for resolution of inflammation and promotion of new tissue growth eventually leading to healing. Addition of an anti-IL-10 neutralizing antibody compromised the inhibitory effects of Treg on reducing lymphocytes-derived pro-inflammatory cytokines such as TNF-a.[39] The low expression level of IL-10 may indicate an insufficient adaptive immune response in response to the injury. The obviously increased Treg cells and IL-10 level after MNC infusion indicated an anti-inflammatory effect of MNC which may contribute to resolving of BPD severity. Our study firstly confirmed the preclinical findings that-the immunomodulatory ability of stem cells could promote resolution of inflammation and facilitate lung tissue repair in clinical setting, therefore demonstrating MNCs immunomodulatory effect as the important mechanisms in improving BPD severity.

The current study has some limitations. Firstly, the study was non-randomized and the sample size was still small to reliably assess the efficacy of BPD prevention. Secondly, this study enrolled infants <32 GW and the

number of patients <28 GW was limited (only 4 patients per group). The proportion of extremely preterm infants with GA <28 weeks was lower in china than that in developed countries like US.[25,26] For the enrolled infants in this study, the proportion of infants <28 GW was 12.9% (8/62), which was consistent with the reports from China national investigation in very preterm infants.[25] We are now conducting a multi-center randomized placebo-controlled trial (RCT) (ClinicalTrials.gov Identifier: NCT04440670). We hope the completion of this further RCT could provide more evidence for effect of ACBMNC therapy for BPD prevention in extremely preterm neonates. Thirdly, the time point for biological markers detection was only conducted on 72 h after intervention. In the future study, various time-point tests should be performed to evaluate the effect of ACBMNC infusion on different time points after intervention. And the resolution of inflammation in ACBMNCs intervention groups needs further verification.

In conclusion, this prospective, non-randomized, placebo-controlled study showed ACBMNCs cell infusion could prevent moderate or severe BPD in very premature survivors as well as may improve neurodevelopmental outcomes. The regulation of MNC on inflammation contributed to the protective effect.

Contributors

Conceptualization, Y.J and F.ZC; Methodology, W.W and L.YS; Software, L.QP and P.JJ; Validation, X.F and Y.SM; Formal Analysis, R.ZX and W.JY; Investigation, N.C; Writing—Original Draft Preparation, R.ZX and W.JL; Writing—Review & Editing, Y.J; Supervision, Y.J and F.ZC; Project Administration, L.YS; Funding Acquisition, L.QP and Y.J. †These authors contributed to this work. All authors have read and agreed to the published version of the manuscript. *To whom correspondence should be addressed. Dr R.ZX and Dr. Y.J have directly accessed and verified the underlying data reported in the manuscript.

Data sharing statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of interests

The authors have no competing interests to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.eclinm.2023.101844.

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