Cord blood stem cell-derived Angptl7 ameliorates the severity of bronchopulmonary dysplasia via anti-inflammatory and proangiogenic effects

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Abstract. Perinatal exposure of the neonatal lung to inflammation leads to decreased lung angiogenesis and the development of bronchopulmonary dysplasia (BPD). Notably, autologous cord blood mononuclear cells (ACBMNCs) can substantially prevent severe BPD and decrease the inflammatory response in surviving very preterm neonates. Angiopoietin-like protein 7 (Angptl7) is one of the main paracrine cytokines in cord blood stem cells, and is capable of stimulating human hematopoietic stem and progenitor cell expansion. The present study compared Angptl7 levels between the ACBMNCs infusion and control groups (cohort 1). Subsequently, the association between cord blood Angptl7 levels and BPD incidence in a cohort of very preterm neonates was assessed (cohort 2). The hypothesis was further verified in a lipopolysaccharide (LPS)-induced lung injury mouse model. The mRNA expression levels and protein concentrations of inflammatory cytokines in the lung tissue and mouse serum were measured using reverse transcription-quantitative PCR and ELISA, respectively. The number and diameter of lung vessels and macrophage infiltration were assessed using immunofluorescence staining. Compared with in the control group, Angptl7 levels were significantly higher in the ACBMNCs infusion group in cohort 1. In cohort 2, the cord blood Angptl7 levels were significantly lower in infants who later developed BPD. Multiple linear regression analysis showed that higher Angptl7 level was an independent protective factor for BPD. The concentrations of interleukin-6 and monocyte chemoattractant protein-1 were negatively correlated with cord blood Angptl7 level; whereas, vascular endothelial growth factor-A levels were positively correlated with Angptl7 levels. In the LPS-induced lung injury mouse model, the LPS group presented with a significant loss of pulmonary vessels and smaller vessel diameters, which were ameliorated in the Angptl7 treatment group. Furthermore, LPS-induced lung inflammation and macrophage infiltration were alleviated by Angptl7 treatment ($P<0.05$). In conclusion, the anti-inflammatory and proangiogenic effects of Angptl7 derived from cord blood stem cells may ameliorate BPD severity. The trial for cohort 1 was registered at ClinicalTrials.gov (trial registration no. NCT02999373; date registered, December 21, 2016).

Introduction

Bronchopulmonary dysplasia (BPD) is the most critical complication in preterm neonates and is an independent risk factor affecting long-term cognitive development (1-6). Inflammation is a common pathway leading to the BPD phenotype (7,8). Current treatments, such as mechanical ventilation, dexamethasone therapy or diuretics, have shown limited improvement in the prevalence of BPD (4-6). Notably, stem cell-based paracrine cytokine treatment, with its anti-inflammatory and immunoregulatory ability (9,10) has been regarded as a promising therapy for BPD in preclinical models and clinical studies (10-12). Our previous study demonstrated that autologous cord blood mononuclear cells (ACBMNCs), which are rich in stem cells, could substantially prevent moderate or severe BPD in surviving very preterm neonates, and that the immunomodulatory effect of MNCs contributed in mitigating the severity of BPD (13). Stem cells exert paracrine effects by secreting various bioactive substances. Angiopoietin-like protein 7 (Angptl7) is a glycoprotein that shares sequence homology with angiopoietins, important modulators of angiogenesis (13,14). Angptl7 has been shown to be one of the most abundant paracrine cytokines secreted by stem cells, which is

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capable of stimulating human hematopoietic stem and progenitor cell expansion (15,16). In our previous study, it was revealed that Angptl7 was capable of stimulating human hematopoietic stem and progenitor cell expansion, and increasing the repopulation activities of human hematopoietic progenitors (15,16). In addition, Angptl7-deficient mice were generated using transcription activator-like effector nuclease-mediated gene targeting, and it was demonstrated that hematopoietic stem cell compartments in Angptl7-null mice were compromised (16). Parri *et al* (17) reported that Angptl7 is a proangiogenic factor in human differentiated endothelial cells and can be specifically upregulated under inflammatory conditions.

Inflammation-induced impaired lung angiogenesis and alveolar growth cause BPD (6,8). As Angptl7 potentially enhances angiopoiesis and has anti-inflammatory effects (16-18), it was hypothesized that Angptl7 may ameliorate BPD severity. The present study used serum samples from the ACBMNCs intervention study cohort (cohort 1) (13) to compare Angptl7 levels between the ACBMNCs infusion and control groups. In addition, the present study investigated the association between cord blood Angptl7 levels and BPD incidence in a cohort of very preterm neonates (cohort 2). The hypothesis was further verified in a mouse model: Angptl7 was administered to mice with lipopolysaccharide (LPS)-induced lung injury, and the protective effects of Angptl7 on lung angiopoiesis and inflammation was assessed.

Materials and methods

Ethics approval. Guangdong Women and Children Hospital Ethics committee approved the present study (approval no. 202101030 for the study involving humans; approval no. 202001031 for the animal experiments; Guangzhou, China).

Cohort 1: Angptl7 level detection in very preterm neonates treated with ACBMNCs. In our previous trial (13), an ACBMNCs infusion was administered to a very preterm neonate cohort (cohort 1). A decrease in moderate or severe BPD was observed in the ACBMNCs infusion group. Angptl7 levels were measured in preserved samples of cord blood and unused blood from routine clinical blood tests using an ELISA kit (cat. no. E8974h; Wuhan EIAab Science Co., Ltd.), according to the manufacturer's protocol. Patient inclusion and exclusion criteria for cohort 1 have been described previously (13). Briefly, 29 and 33 patients were enrolled in the ACBMNCs and control groups, respectively; the details of patient enrollment are shown in our previous study (13).

Cohort 2: Association of cord blood Angptl7 levels and BPD
Patient enrollment. Singleton infants (n=112) born between November 2017 and March 2020 at Guangdong Women and Children Hospital were included in the present study. The inclusion criteria were as follows: i) Born in the study hospital, ii) singleton birth, iii) <32 gestational weeks, and iv) consent was obtained from the parents. Exclusion criteria included: i) Major congenital abnormalities and ii) severe perinatal asphyxia (defined as an Apgar score of 0-3 for >5 min, cord blood gas pH <7.00, or both) (19).

Clinical data collection for cohort 2. Maternal clinical information included age, gestational diabetes mellitus, pregnancy-induced hypertension, antenatal steroids administration, preeclampsia, histological chorioamnionitis and cholestasis (20). Neonatal clinical data included the following: i) Demographic data, including sex, gestational age (GA), delivery mode, birth weight, small for GA, length, head circumference, Apgar score in 1 and 5 min; ii) clinical outcomes data, including BPD and its severity, necrotizing enterocolitis (NEC), retinopathy of preterm (ROP), late-onset sepsis, anemia, invasive mechanical ventilation and total respiratory support duration, postnatal steroids administration, red blood cells infusion and length of hospital stay.

Clinical definitions used in the present study. BPD was defined as treatment with oxygen >21% for ≥28 days using the diagnostic criteria proposed in 2001 by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (21). Cranial ultrasonography was recommended between the 14 and 28th day of life, and between 34 and 36 weeks of postmenstrual age if the infant was still hospitalized in the study center at that time. NEC was diagnosed during surgery, at autopsy, or by the detection of pneumatosis intestinalis, hepatobiliary gas or free intraperitoneal air on radiography. All stages of ROP were recorded according to the international classification (19). Late-onset sepsis was defined as positive blood or cerebrospinal fluid cultures 72 h after birth. Anemia was defined as a hemoglobin level of <140 mg/ml. All clinical diagnoses were made according to standard reference (19). Respiratory support included invasive and non-invasive ventilation and oxygen therapy.

Cord blood collection and quantification of cytokine levels. Cord blood was collected after cord clamping and before the delivery of the placenta as described in a previous study (22). Cord blood was then centrifuged at 500 x g for 8 min at 4˚C (Beckman Coulter, Inc.). Serum was separated from cord blood for the assessment of Angptl7 (ng/ml), vascular endothelial growth factor A (VEGF-A; ng/ml), interleukin-6 (IL-6; pg/ml), and monocyte chemoattractant protein-1 (MCP-1; pg/ml) concentrations. A minimum of 5 μl serum was used for analysis using the following ELISA kits: Angptl7, (cat. no. E8974h), VEGF-A (cat. no. E0143h), IL-6 (cat. no. E0079h) and MCP-1 (cat. no. E0087h) (all from Elabscience Biotechnology, Inc. Cytokine detection by ELISA was performed as described previously (23).

Animal model: Effect of Angptl7 on LPS-induced lung injury
Establishment of LPS-induced lung injury mouse model. All animal experiments were conducted after approval by Guangdong Women and Children Hospital Ethics committee, which conforms to the Guide for the Care and Use of Laboratory Animals (24). A total of 18 C57BL/6J wild-type (WT) mice (age, 3 days; weight, 3-4 g; n=6/group) were obtained from the Guangzhou Institute of Biomedicine and Health Laboratory. Neonatal mice were used for all experiments. The mice were fed standard mouse food and water *ad libitum*, and were maintained at 21-28˚C, 50-60% humidity, and under 12-h light/dark cycles.

LPS was used to mediate inflammation-induced lung injury (25,26). Neonatal WT mice were injected intraperitoneally with 10 mg/kg LPS derived from *Escherichia coli* serotype O26:B6 (MilliporeSigma) in the LPS group, or with an equivalent volume of the vehicle control (normal saline) in the control group once on postnatal day (PND)3 in the saccular stage of lung development (24). The mice in the LPS + Angptl7 group were then injected intraperitoneally with Angptl7 (500 ng/g; R&D Systems, Inc.). Serum was separated from cardiac blood samples, which were obtained by cardiac puncture following pentobarbital sodium injection (50 mg/kg; intraperitoneal), -via centrifugation at 500 x g for 20 min at 4˚C. Mice in the vehicle, LPS and LPS + Angptl7 groups were euthanized using CO_2, with the volume displacement rate being ~50% CO_2 on PND7 (n=6/group). The lung tissues were then collected and stored at -80˚C.

Fluorescence immunohistochemical analysis. The lungs from the mice in each group (n=6/group) were washed with phosphate-buffered saline, fixed for 24 h in 4% paraformaldehyde in phosphate buffer solution (NaCl, 13.7 mM; KCl, 2.7 mM; Na_2HPO_4, 0.9 mM; KH_2PO_4, 1.8 mM; pH 7.4) at 4˚C, and embedded in paraffin. For immunohistochemistry, 5-μm sections were prepared from these paraffin-embedded tissues. Paraffin-embedded lung tissues were deparaffinized in xylene, rehydrated in a descending series of alcohol and subjected to antigen retrieval at 95˚C for immunohistochemical analysis. After blocking of nonspecific binding with 5% BSA (cat. no. G5001; Wuhan Servicebio Technology Co., Ltd.) in room temperature for 30 min, the lung sections were incubated overnight at 4˚C with the following primary antibodies: Anti-von Willebrand factor (vWF; endothelial-specific marker; cat. no. GB11020; 1:600) and anti-F4/80 (macrophage-specific marker; cat. no. GB113373;1:1,000) (all from Wuhan Servicebio Technology Co., Ltd.). Subsequently, sections were incubated with secondary antibodies (for vWF: Cyanine 3-conjugated antibody; cat. no. GB21303; 1:300; and for F4/80: FITC-conjugated antibody, cat. no. GB22303; 1:500; both from Wuhan Servicebio Technology Co., Ltd.) at room temperature 50 min. Antigen-antibody reactions were visualized using the diaminobenzidine reaction, and image analysis was performed using Image-Pro Plus software (6.0 version; Media Cybernetics, Inc.). Three randomly selected high-power fields (magnification, x400) of the peripheral pulmonary tissue on each slide were analyzed. The observers analyzing the slides were blinded to the experimental conditions. Fluorescence signals were detected at excitation-emission wavelengths of 590 nm (CY3, red) and 515-555 nm (FITC, green). The imaging was performed using a confocal microscope (Nikon Eclipse C1; Nikon Corporation).

Analysis of lung vessel development. Pulmonary blood vessel development was determined using immunofluorescence (IF) staining for vWF, as aforementioned. At least three counts from three random non-overlapping fields (original magnification, x400) were performed for each animal (n=6/group). The number of vessels per field and mean vessel diameter were calculated and analyzed manually. The observers who performed the measurements were blinded to the specimen.

Inflammatory cytokine detection and mRNA expression. A minimum of 5 μl serum was used for analysis using the following ELISA kits: IL-6 (cat. no. E0079m) and MCP-1 (cat. no. E0087m) (both from Wuhan EIAab Science Co., Ltd.). The extent of lung inflammation was assessed by quantifying lung cytokine/chemokine gene expression. Total RNA was extracted from frozen lung tissues using the Direct-zol RNA MiniPrep Kit (cat. no. R2052; Zymo Research Corp.) and was reverse transcribed into cDNA using Revert Aid First Strand cDNA Synthesis Kit (cat. no. K1622; Thermo Fisher Scientific, Inc.). Reverse transcription-quantitative PCR (RT-qPCR) analysis was performed using a 7900HT Real-Time PCR System with TaqMan Gene Expression Master Mix (cat. no. 4369016) and TaqMan Gene Expression Assays (all from Applied Biosystems; Thermo Fisher Scientific, Inc.) for IL-6, MCP-1, IL-10 and GAPDH. GAPDH was used as the reference gene. The samples were denatured at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. The $2^{-\Delta\Delta Cq}$ method was used to calculate fold changes in mRNA expression (27).

The primers used were as follows: m-IL-6-forward (F)1, 5'-CAGAAGGAGTGGCTAAGGACC-3'; m-IL-6-reverse (R)1, 5'-GCACTAGGTTTGCCGAGTAG-3'; m-MCP-1-F1, 5'-GAGCTCTCTGGTACTCTTTG-3'; m-MCP-1-R1, 5'-GTGCATTACAGGGAACAAAC-3'; m-IL-10-F1, 5'-GCTCCAAGAvCCAAGGTGTCT-3'; m-IL-10-R1, 5'-CGGAGAGAGGTACAAACGAGG-3'; m-GAPDH-F1: 5'-GGCCTCCAAGGAGTAAGAAA-3'; m-GAPDH-R1: 5'-GCCCCTCCTGTTATTATGG-3'.

Statistical analysis. Continuous variables are presented as the mean ± SD, and numbers and percentages are presented for categorical variables. Differences in continuous variables between two groups were compared using unpaired Student's t-test. Pearson correlation coefficients were used to determine the correlation between Angptl7 levels and other variables. Multiple linear regression analysis was used to estimate the predictive contribution of neonatal/maternal factors on Angptl7 levels or the contribution of factors on BPD. A two-way mixed ANOVA and post hoc Tukey's test was used for comparisons of Angptl7 levels before and after the intervention in our previous trial. The distribution characteristics of the variables were estimated using a single-sample Kolmogorov-Smirnov test. All statistical tests were two-tailed, and $P<0.05$ was considered to indicate a statistically significant difference.

For animal studies, all experiments were performed at least three times in triplicate. Results are expressed as the mean ± SEM. One-way ANOVA followed by Bonferroni's multiple comparison test was used to compare groups. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS 21.0 software (IBM Corp.).

Results

Angptl7 levels in very preterm neonates after ACBMNCs intervention in cohort 1. At baseline, no difference in Angptl7 levels was detected between the groups (control, 4.76±1.82 ng/ml vs. ACBMNC, 4.01±2.45 ng/ml; P=0.281; Fig. 1). In the control group, Angptl7 levels decreased after intervention (P=0.011); however, Angptl7 levels were significantly higher after intervention in the ACBMNCs infusion group compared with those in the control group (control, 2.35±1.20 ng/ml vs. ACBMNC,

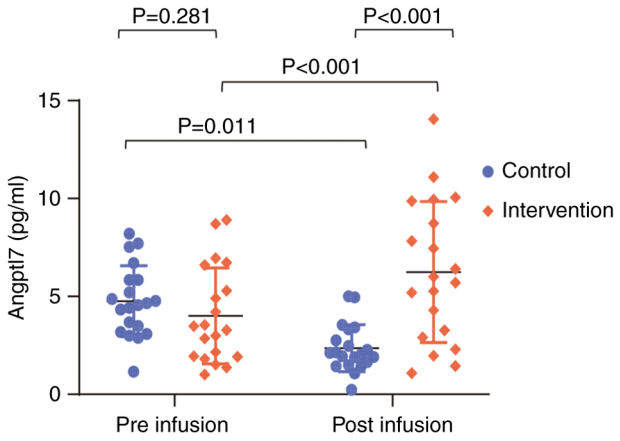

Figure 1. Angptl7 levels in very preterm infants in the control and ACBMNCs infusion groups. Angptl7 levels were higher in very preterm infants treated with ACBMNCs. ACBMNCs, autologous cord blood mononuclear cells; Angptl7, angiopoietin-like protein 7.

6.24±3.20 ng/ml; P<0.001; n=20/group with available results). The clinical characteristics of this cohort have been described previously (13).

Angptl7 levels in very preterm neonates in cohort 2

Study population. A total of 370 preterm neonates (<32 gestational weeks) were screened between November 2017 and March 2020. Among them, 120 were twins, 8 had severe asphyxia, 2 had major congenital abnormalities, and 128 did not provide consent. Ultimately, 112 patients were enrolled in the present study (Fig. 2). The maternal and neonatal clinical characteristics are shown in Table I.

Effect of perinatal factors on cord blood Angptl7 levels. The present study investigated perinatal factors that may affect cord blood Angptl7 levels in very preterm neonates. Multiple regression analysis showed that perinatal factors did not affect the cord blood levels of Angptl7 (Table II). Since GA and birth weight showed a positive correlation (r=0.238, P=0.015), although this correlation was weak, only GA was included in the multiple regression analysis.

Association between cord blood Angptl7 levels and outcomes in very preterm neonates. To investigate the association between Angptl7 levels and outcomes in very preterm neonates, the levels of Angptl7 were compared in the very preterm groups between those who were diagnosed with the following common complications: BPD, NEC, intraventricular hemorrhage, ROP and late-onset sepsis, and those who were not. Notably, the cord blood levels of Angptl7 were lower in neonates who later developed BPD than in those without BPD (P<0.01; Table III). Whereas, Angptl7 expression did not differ significantly in other complications (n=112).

Cord blood Angptl7 levels and BPD in very preterm neonates. To further investigate the possible protective contribution of cord blood Angptl7 against BPD in very preterm neonates, multiple regression analysis was used, which included perinatal factors that may affect the incidence of BPD. The results showed that higher cord blood levels of Angptl7 were an independent protective factor against BPD (P=0.049, n=112; Table IV).

Table I. Maternal and neonatal clinical characteristics.

A, Maternal characteristics

Clinical characteristic	Value
Mean ± SD age, years	32.01±5.64
Gestational diabetes mellitus, n (%)	29 (25.89)
Pregnancy-induced hypertension, n (%)	24 (21.43)
Antenatal steroids administration, n (%)	49 (43.75)
Preeclampsia, n (%)	22 (19.64)
Histological chorioamnionitis, n (%)	7 (6.25)
Cholestasis, n (%)	4 (3.57)

B, Neonatal characteristics

Clinical characteristic	Value
Mean ± SD gestational age, weeks	29.65±1.55
Gestational age <28 weeks, n (%)	18 (16.07)
Male sex, n (%)	64 (57.14)
Cesarean delivery mode, n (%)	58 (51.79)
Mean ± SD birth weight, kg	1.35±0.32
Small for gestational age, n (%)	15 (13.39)
Mean ± SD birth length, cm	38.01±3.64
Meedian (IQR), Apgar score in 1 min	9.00 (1.00)
Meedian (IQR), Apgar score in 5 min	10.00 (1.00)
RDS, n (%)	107 (95.54)
Grade 1	64 (57.14)
Grade 2	27 (24.11)
Grade 3	16 (14.29)
BPD, n (%)	35 (31.25)
Grade 1	9 (8.04)
Grade 2	19 (16.96)
Grade 3	7 (6.25)
NEC, n (%)	19 (16.96)
ROP, n (%)	31 (27.68)
Late-onset sepsis, n (%)	33 (29.46)
IVH, n (%)	35 (31.25)
Mean ± SD intubation duration, days	3.64±8.37
Mean ± SD respiratory support duration, days	16.96±18.16
Postnatal steroids administration, n (%)	18 (16.07)
Mean ± SD length of hospital stay, days	47.46±22.09

RDS, respiratory distress syndrome; BPD, bronchopulmonary dysplasia; NEC, necrotizing enterocolitis; ROP, retinopathy of prematurity; IVH, intraventricular hemorrhage.

Correlation of cord blood Angptl7 levels with inflammatory cytokines and VEGF-A. Perinatal inflammation serves a crucial role in the pathogenesis of BPD and the pro-inflammatory process affects premature lungs. Angptl7 has important roles in inflammation and angiogenesis. Therefore, the present study analyzed the correlation between cord blood Angptl7 levels and common pro-inflammatory

Table II. Multiple regression analysis model for perinatal factors on the cord blood levels of Angptl7.

A, Neonatal variables

Variable	B	95% CI for B	P-value
Male sex	0.058	(-1.196, 2.229)	0.551
Gestational age	0.072	(-0.386, 0.793)	0.495
Small for gestational age	0.177	(-0.374, 4.923)	0.091
Birth length	0.076	(-0.176, 0.358)	0.499
Cesarean delivery mode	-0.134	(-2.912, 0.566)	0.184

B, Maternal variables

Variable	B	95% CI for B	P-value
Maternal age	-0.200	(-0.323, 0.012)	0.068
Histological chorioamnionitis	-0.027	(-3.836, 2.861)	0.773
Cholestasis	-0.141	(-7.677, 1.054)	0.135
Preeclampsia	0.111	(-5.131, 7.579)	0.703
Gestational diabetes mellitus	0.125	(-0.678, 3.163)	0.202
Pregnancy-induced hypertension	0.248	(-3.643, 8.927)	0.406
Antenatal steroids administration	-0.064	(-2.267, 1.136)	0.511

Angptl7, angiopoietin-like protein 7; B, unstandardized regression coefficient.

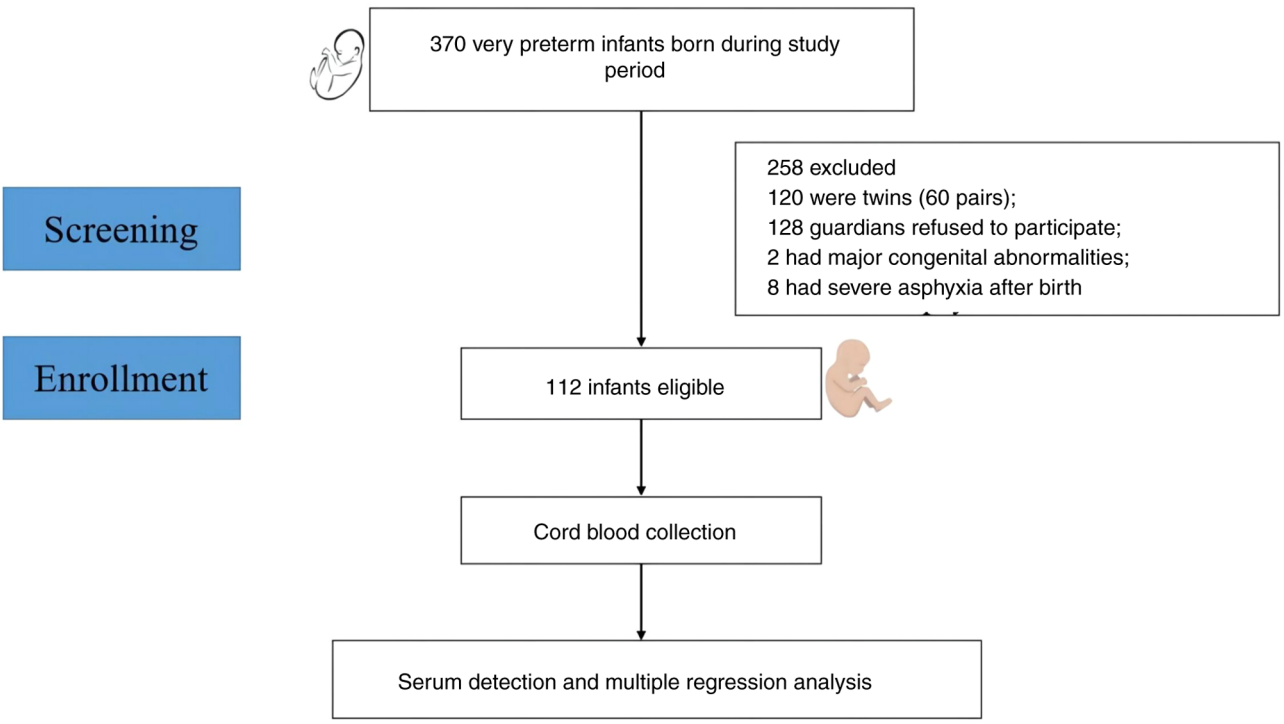

Figure 2. Flow chart of case enrollment and analysis.

cytokines contributing to the development of BPD, including IL-6, MCP-1 and VEGF-A. It was observed that the IL-6 levels (r=-0.284, P=0.002, n=112; Fig. 3A) and MCP-1 levels (r=-0.387, P<0.001, n=112; Fig. 3B) were inversely correlated with Angptl7 levels. However, the correlation with IL-6 was weak. By contrast, VEGF-A levels were positively correlated with Angptl7 levels (r=0.295, P=0.002, n=112; Fig. 3C), although this correlation was also weak.

Table III. Cord blood levels of Angptl7 and preterm complications.

	Mean ± SD cord blood ANGPTL7, ng/ml (n)		
Complication	With	Without	P-value
BPD	7.54±3.88 (n=35)	9.91±4.43 (n=77)	<0.01
IVH	9.00±4.66 (n=35)	9.25±4.29 (n=77)	0.78
ROP	10.28±4.90 (n=31)	8.75±4.13 (n=81)	0.10
NEC	8.82±4.73 (n=19)	9.25±4.34 (n=93)	0.07
Sepsis	8.19±4.20 (n=33)	9.58±4.43 (n=79)	0.13

Angptl7, angiopoietin-like protein 7; BPD, bronchopulmonary dysplasia; IVH, intraventricular hemorrhage; ROP, retinopathy of prematurity; NEC, necrotizing enterocolitis.

Table IV. Multiple regression analysis model for perinatal factors on BPD.

Variable	B	95% CI for B	P-value
Male sex	-0.580	(-0.200, 1.571)	0.271
Gestational age	-0.313	(0.512, 1.044)	0.085
Small for gestational age	-0.291	(0.148, 3.782)	0.725
Birth length	-0.148	(0.733, 1.015)	0.074
Cesarean delivery mode	-0.210	(0.289, 2.279)	0.691
Maternal age	0.037	(0.934, 1.153)	0.489
Histological chorioamnionitis	-1.035	(0.061, 2.086)	0.252
Cholestasis	0.427	(0.116, 20.291)	0.746
Preeclampsia	0.901	(0.022, 281.497)	0.710
Gestational diabetes mellitus	-0.237	(0.259, 2.401)	0.676
Pregnancy-induced hypertension	-0.622	(0.005,60.261)	0.796
Antenatal steroids administration	-0.745	(0.174, 1.292)	0.145
Late-onset sepsis	-0.445	(0.222, 1.852)	0.411
Angptl7	-0.130	(0.771, 0.999)	0.049

Angptl7, angiopoietin-like protein 7; BPD, bronchopulmonary dysplasia; B, regression coefficient.

Effect of Angptl7 on LPS-induced lung injury in mice

Angptl7 treatment and pulmonary blood vessel development. To explore the potential effect of Angptl7 on LPS-induced peripheral pulmonary vascular impairment, lung sections prepared from PND7 mice post-LPS-induced lung injury were analyzed for the expression of vWF. There was an obvious decrease in the mean vessel diameter in the LPS group (control, 35.00±1.41 μm vs. LPS, 14.33±1.12 μm; P<0.001; n=6; Fig. 4A, B and D). Angptl7 treatment ameliorated the LPS-induced vascular diameter impairment (LPS, 14.33±1.12 μm vs. LPS + Angptl7, 25.33±1.65 μm; P<0.001; Fig. 4B-D). Compared with mice in the control group, LPS exposure led to significant loss of small (peripheral) vessels <50 μm in diameter (control, 8.50±0.43 vs. LPS, 2.33±0.42; P<0.001; n=6), whereas Angptl7 treatment increased the number of these vessels (LPS, 2.33±0.42 vs. LPS + Angptl7, 5.50±0.43; P<0.001) (Fig. 4A-C and E).

Angptl7 intervention and lung inflammation. The present study also evaluated the extent of lung inflammation following treatment with LPS and Angptl7. On PND7, the number of inflammatory macrophages infiltrating the lungs were detected. To compare the number of infiltrating macrophages between the treatment groups, the lung tissues were stained with fluorescent antibodies against F4/80 and analyzed by IF microscopy. WT mice treated with LPS exhibited increased infiltration of macrophages in their lung tissues compared with vehicle-treated mice (vehicle, 1.33±0.50 vs. LPS, 8.83±0.31; P<0.001; Fig. 5A, B and D). Angptl7 significantly suppressed pulmonary macrophage infiltration compared with that in the LPS group (LPS, 8.83±0.31 vs. LPS + Angptl7, 5.50±0.43; P<0.001; Fig. 5B-D).

The present study also determined the extent of lung inflammation by quantifying the production of the proinflammatory cytokine genes IL-6 and MCP-1, and the anti-inflammatory cytokine gene IL-10 in lung tissues by RT-qPCR. LPS (10 mg/kg) increased the mRNA expression levels of IL-6 (control, 1.09±0.05 vs. LPS, 2.43±0.11; P<0.001), and MCP-1 (control, 1.35±0.19 vs. LPS, 3.83±0.44; P=0.0067), whereas Angptl7 lowered the mRNA expression levels of IL-6 (LPS, 2.43±0.11 vs. LPS + Angptl7,

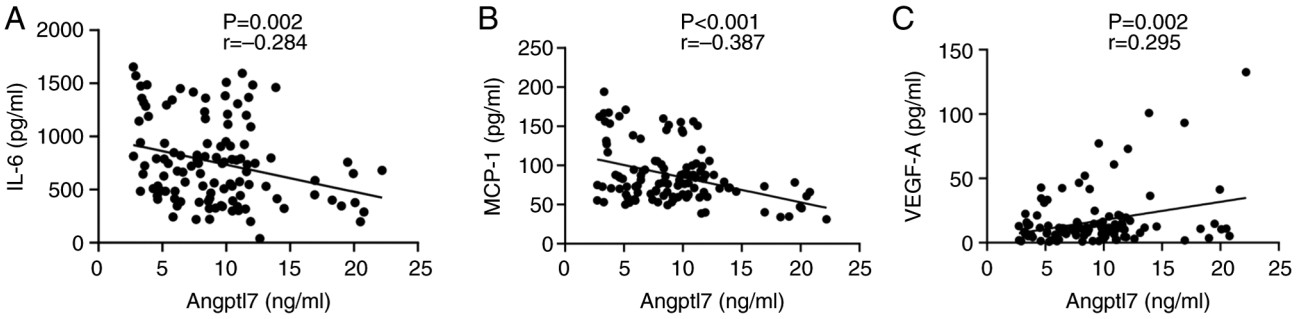

Figure 3. Correlation between cord blood Angptl7, and inflammatory cytokines (IL-6 and MCP-1) and VEGF-A. (A) IL-6 and (B) MCP-1 levels was inversely correlated with Angptl7 levels. (C) VEGF-A levels were positively correlated with Angptl7 levels. Angptl7, angiopoietin-like protein 7; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; VEGF-A, vascular endothelial growth factor-A.

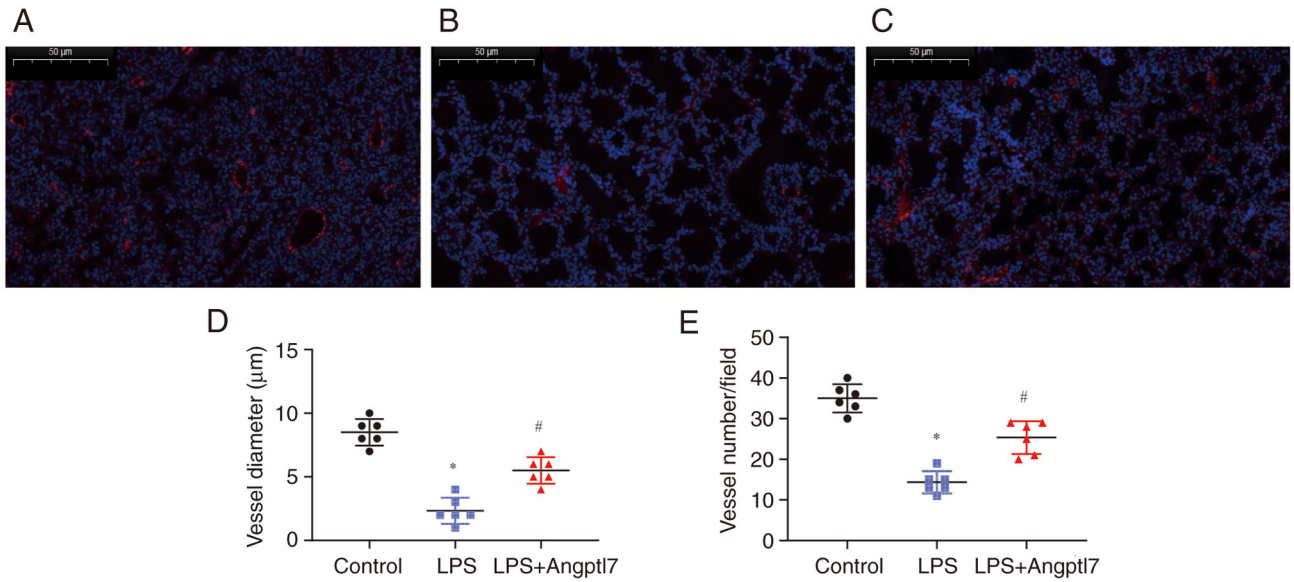

Figure 4. Angptl7 treatment ameliorates the LPS-induced reduction in lung vessel diameter and number. Representative slides are shown. (A) Control, (B) LPS and (C) LPS + Angptl7 groups (magnification, x400; scale bar, 50 μm). (D) Vascular diameter and (E) vascular number. *P<0.05 vs. Control; #P<0.05 vs. LPS, n=6. Angptl7, angiopoietin-like protein 7; LPS, lipopolysaccharide.

1.46±0.03; P<0.001) and MCP-1 (LPS, 3.83±0.44 vs. LPS + Angptl7, 2.01±0.11; P=0.016) (Fig. 6A and B). Furthermore, the mRNA expression levels of the anti-inflammatory cytokine gene IL-10 were detected in the lung tissues. It was observed that exposure to 10 mg/kg LPS decreased the mRNA expression levels of lung IL-10 (control, 1.09±0.05 vs. LPS, 0.35±0.06; P<0.001), whereas Angptl7 intervention increased the mRNA expression levels of lung IL-10 compared with those in the LPS group (LPS, 0.35±0.06 vs. LPS + Angptl7, 0.67±0.06; P=0.018 (Fig. 6C). These results indicated that Angptl7 may suppress lung inflammation via IL-10 expression.

The concentrations of IL-6 and MCP-1 were also detected in the serum of mice post-LPS-induced lung injury. LPS stimulation increased the serum levels of IL-6 (control, 359.30±32.32 pg/ml vs. LPS, 1,338.00±83.85 pg/ml; P<0.001) and MCP-1 (control, 167.00±9.14 pg/ml vs. LPS, 1,239.00±103.00 pg/ml, P<0.001), whereas Angptl7 decreased the serum levels of IL-6 (LPS, 1,338.00±83.90 pg/ml vs. LPS + Angptl7, 520.90±113.70 pg/ml; P<0.001) and MCP-1 (LPS, 1,239.00±103.00 pg/ml vs. LPS + Angptl7, 799.90±148.60 pg/ml; P=0.036) induced by LPS stimulation (Fig. 7).

Discussion

Inflammatory response-induced impaired angiogenesis serves an important role in the pathogenesis of BPD (28-31). Mesenchymal stem cells (MSCs) are potent immunomodulatory cells capable of alleviating inflammation in experimental BPD (10,32-35). One important protective mechanism of stem cells is their ability to reduce lung vascular injury and promote angiogenesis by secreting and regulating angiogenic proteins (10,36). Angptl7 is a member of the angiogenin-like protein family that is abundantly expressed in cord blood stem and angiogenic progenitor cells (14,37,38). Important pathological processes involving Angptl7 include inflammation, apoptosis and angiogenesis (39,40). Our previous study reported that ACBMNCs could substantially prevent severe BPD in surviving very preterm neonates and that the immunomodulatory effect of MNCs contributed to the mitigation of BPD severity (13). The present study first investigated Angptl7 levels among patients in our previous trial, and then measured the cord blood level of Angptl7 in very preterm neonates and its association with preterm complications in a prospective

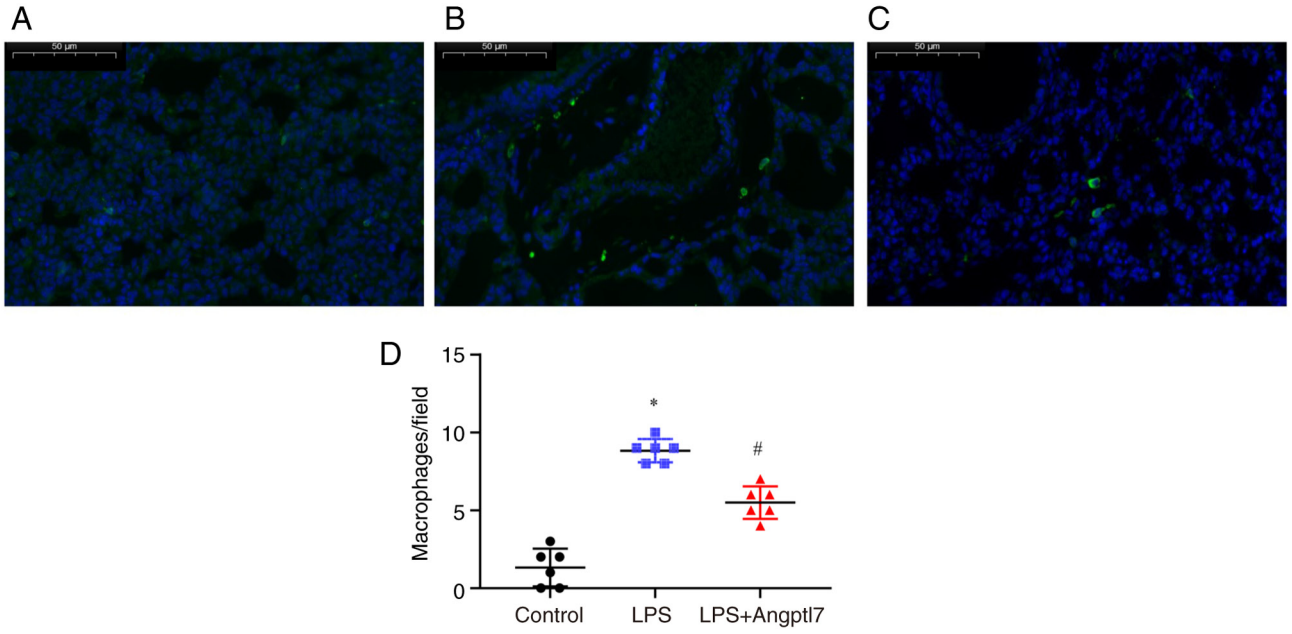

Figure 5. Angptl7 treatment ameliorates the LPS-induced lung macrophage infiltration. Representative slides are shown. (A) Control, (B) LPS and (C) LPS + Angptl7 groups (magnification, x400; scale bar, 50 μm). (D) Quantification of the lung macrophage number. *P<0.05 vs. Control; #P<0.05 vs. LPS, n=6. Angptl7, angiopoietin-like protein 7; LPS, lipopolysaccharide.

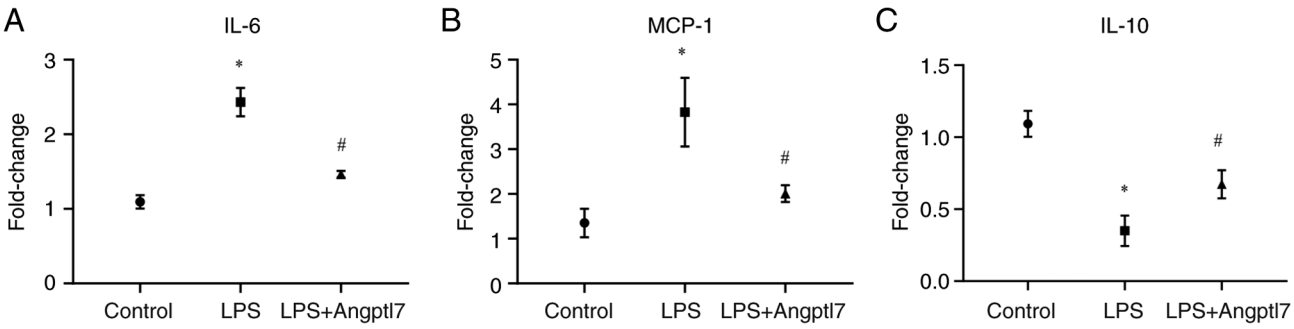

Figure 6. mRNA expression levels of proinflammatory cytokines IL-6 and MCP-1, and the anti-inflammatory cytokine IL-10, in lung tissues as determined by reverse transcription-quantitative PCR. (A) IL-6, (B) MCP-1 and (C) IL-10. *P<0.05 vs. Control; #P<0.05 vs. LPS, n=6. Angptl7, angiopoietin-like protein 7; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1.

cohort. The relationship between Angptl7 and inflammatory cytokines, as well as VEGF-A, were further analyzed. This clinical study showed that: i) Angptl7 levels were higher in the ACBMNCs infusion group than those in the control group post-intervention; ii) higher cord blood levels of Angptl7 were an independent protective factor for developing BPD; and iii) pro-inflammatory cytokines IL-6 and MCP-1 were inversely correlated with Angptl7 levels, whereas VEGF-A was positively correlated with Angptl7 levels. However, it was noted that some of the correlations were not very strong; therefore, further studies with larger samples are required to verify the findings.

In a murine model, the present study investigated the effects of Angptl7 intervention on systemic LPS exposure-induced lung injury during the saccular phase of lung development. The results revealed that Angptl7 treatment rescued the LPS-induced loss of peripheral pulmonary blood vessels, ameliorated lung macrophage cell infiltration and attenuated LPS-induced inflammation. An inflammation-induced mouse model in air containing 21% oxygen is a well-known model of BPD-like pulmonary phenotype (41,42). The present study provides additional evidence for the translational implementation of stem cells and the derived cytokine Angptl7 in preventing and/or treating BPD.

Previous studies have explored the relationship between Angptl7 and multiple pathological processes and diseases. Parri *et al* (17) demonstrated that Angptl7 is a proangiogenic factor in differentiated human endothelial cells and can be specifically upregulated by hypoxia. By contrast, Toyono *et al* found that Angptl7 may act as an antiangiogenic protein to maintain corneal transparency (43). Xiao *et al* reported that Angptl7 promoted the regenerative capacity of hematopoietic stem and progenitor cells (15,16). Angiogenesis is a complex biological process that is known to be involved in multiple preterm diseases (44,45). However, Angptl7 may have various effects in different disease conditions. Until now, to the best of our knowledge, there has been no investigation of the relationship between Angptl7 and BPD or its underlying mechanisms.

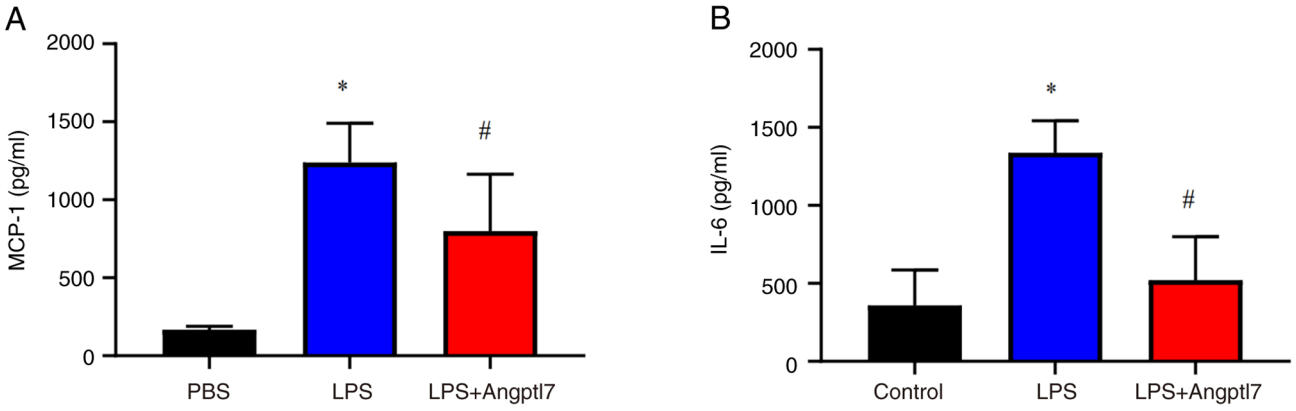

Figure 7. Concentrations of proinflammatory cytokines IL-6 and MCP-1 in serum as determined by ELISA. (A) MCP-1 and (B) IL-6. *P<0.05 vs. Control; #P<0.05 vs. LPS, n=6. Values are presented as the mean ± SEM. Angptl7, angiopoietin-like protein 7; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1.

In our previous non-randomized study, 29 patients with very preterm infants were enrolled in the ACBMNCs infusion group and 33 were enrolled in the control group. The severity of BPD in survivors significantly decreased in the ACBMNCs intervention group (13). MNCs have been reported to exert immunomodulatory effects by reducing the levels of inflammatory cytokines, and the association between inflammatory responses and aberrant lung vascular development has been well established (45,46). A previous study showed that proangiogenic factors were decreased in the lungs of infants dying from BPD and in animal models of BPD induced by LPS and/or hyperoxia exposure (2,47). Angptl7 is an important paracrine bioactive factor that serves a role in inflammatory response regulation (37,38). Macrophages are critical mediators of the lung inflammatory response (31,46) and MSCs can alleviate lung inflammation by inhibiting macrophage accumulation (10). Whether Angptl7 can alleviate the excessive lung inflammatory response and repair lung vascular injury is still unclear. The present study measured the concentrations of MCP-1 and IL-6 in the cord blood of preterm neonates; the correlation analysis results showed a negative relationship between MCP-1, IL-6 and Angptl7. In addition, the mRNA expression levels of inflammatory cytokines were further assessed in the lungs of a mouse model, as were their serum concentrations. Consistent with previous findings (26), LPS exposure increased the mRNA expression and protein levels of pro-inflammatory cytokines, such as MCP-1 and IL-6, which can accumulate and activate macrophages, resulting in an augmented inflammatory cascade (47). By contrast, LPS exposure reduced the levels of the anti-inflammatory chemokine, IL-10 (26). The present study revealed that Angptl7 upregulated IL-10 expression, reduced MCP-1 and IL-6 mRNA expression and serum levels, and reduced macrophage infiltration after LPS exposure in premature lungs. This indicated that the protective effect of Angptl7 against impaired pulmonary angiogenesis may be associated with its anti-inflammatory function.

Lung hypoplasia is a result of the disruption of angiogenesis, and loss of VEGF signaling between the epithelium and endothelium (48,49). Previous studies have reported significantly reduced VEGF-A levels in the bronchoalveolar lavage fluid of patients with BPD (50,51). The present study demonstrated that VEGF-A levels were positively correlated with Angptl7. In the LPS-induced lung injury mouse model, Angptl7 restored lung microvascular number and diameter, thereby enlarging the lung perfusion area. Endothelial cell mitogens and the survival factor VEGF-A are essential for normal blood vessel development (48). Considering the role of Angptl7 in regulating angiogenesis, this finding indicated that Angptl7 may have a proangiogenic effect and could contribute to the improvement of BPD in very preterm neonates.

The present study had several limitations. First, among the 112 very preterm neonates (<32 gestational weeks), only 18 had a GA of <28 weeks at birth. Further studies should include extremely preterm infants who are at a greater risk of developing BPD and its complications. Additionally, the correlations between Angptl7 and inflammatory cytokines were not very strong, further studies with larger samples were needed to verify the findings.

Second, further *in vitro* studies are needed to verify the effect of Angptl7 on the co-culture system of macrophages and vascular endothelial cells. Third, while inflammation-induced models in air containing 21% oxygen can cause a BPD-like pulmonary phenotype, hyperoxia-induced lung injury models have also been well described for mimicking BPD (25,26). In future studies, development of an additional model by hyperoxia stimulation could be used to verify the effect of Angptl7 on BPD. Furthermore, an Angptl7 knock-out mouse model and an additional control + Angptl7 group may aid in demonstrating the protective effect of Angptl7 on inflammation-induced lung injury.

In conclusion, increased levels of Angptl7 in cord blood are an independent protective factor against BPD development. The anti-inflammatory and proangiogenic effects of Angptl7 may be associated with its protective effects against BPD. These results provide a clinical foundation for the translational application of Angptl7 for the prevention and treatment of BPD in very preterm neonates.

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Availability of data and materials

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

Authors' contributions

JY, LY and ZR were involved in study conception and design. All authors provided administrative support, and study materials or patients. SL, JH and YY acquired and collated data. JY, JW and CD analyzed and interpreted data. LY and JW wrote the manuscript. JY and ZR confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Guangdong Women and Children Hospital Ethics committee approved the present study (approval no. 202101030 for the study involving humans; approval no. 202001031 for the animal experiments). Written informed consent was obtained from the parents of the infants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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