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Original Article

Aerosolized deferoxamine administration in mouse model of bronchopulmonary dysplasia improve pulmonary development

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Abstract: Bronchopulmonary dysplasia (BPD) is the most common complication in preterm newborns. It occurs due to early exposure to high-oxygen and ventilation therapy. The mechanisms of disrupted alveolarization and vascular development associated with BPD were unclear. Deferoxamine (DFO) has been reported to reduce mortality and lung injury in mice after chlorine exposure. The effect of DFO in the treatment of BPD has not been explored. This study aimed to investigate the effect of aerosolized DFO administration in a mouse model of BPD. A mouse model of oxygen-induced BPD was established by postnatal hyperoxia (75% oxygen for 7 days) and DFO [17 mg/(kg·day)] (BPD+D) or aerosolized vehicle (BPD+V) administered for 14 days. The mice were anesthetized and sacrificed after 14 days treatment before removing the lungs for analysis. An exogenous continuous aerosol of DFO exerted a biological effect on BPD mice. The BPD+DFO group showed a better weight gain compared with the BPD+V group. Furthermore, the treatment of DFO exhibited a reduced pathological severity and increase expression of hypoxia-inducible factor (HIF)-1α and CD31, and activated downstream vascular endothelial growth factor (VEGF)-induced angiogenesis. The results showed that C57BL/6 mice exposed to hyperoxic environment and treated with aerosolized of DFO solution, obviously promoted the pulmonary vascularization and alveolarization. The HIF-1α/VEGF signaling pathway mediated this process. The findings indicated that treatment with an exogenous continuous aerosol of DFO might be a potential therapeutic strategy for BPD.

Keywords: Bronchopulmonary dysplasia, deferoxamine, hypoxia-inducible factor-1α, vascular endothelial growth factor, aerosolize

Introduction

Bronchopulmonary dysplasia (BPD) was initially described by Northway in 1967 [1]. It is the most common complication in preterm newborns occurring due to early exposure to high oxygen and ventilation therapy. It is associated with high mortality and morbidity in infants and chronic lung diseases in adulthood [2-6]. BPD is characterized by arrested alveolarization and defective pulmonary angiogenesis, which derives from early exposure to high-oxygen environment, mechanical ventilation, inflammation and so on [7]. Lung development occurs in six stages, including embryonic, glandular, canalicular, saccular, alveolar and micro vascular maturation [7-10]. The saccular stage is always disrupted in preterm newborns due to early exposure to relatively hyperoxic environment compared with uterine environment. The main pathophysiology changes involved in this condition are arrest of alveolarization, decreased angiogenesis and fibrosis [2, 11].

The hypoxia-inducible factor (HIF)-1α is particularly important for the development of normal organs [12-14]. However, the expression of HIF-1α is immediately decreased in oxygen-exposed preterm infants, leading to impaired alveolarization and angiogenesis. In the presence of

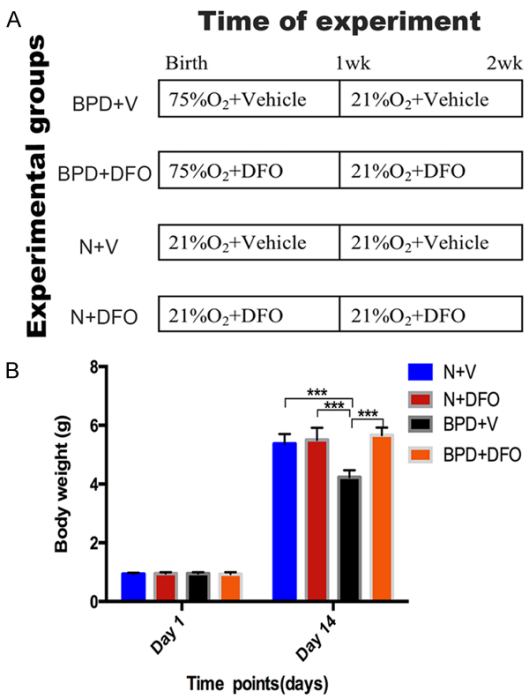

Figure 1. DFO induced weight gain in the BPD groups. A. Study design, four experimental groups were set up as followed: BPD+DFO, BPD+V, N+V, N+DFO. BPD groups were exposed to 75% oxygen from postnatal day 1 (P1) to P7 and subsequently kept in room air for another 7 days until sacrifice. Other two groups were kept in room air until sacrifice on day 14. And aerosol of 17 mg/ml DFO 10% solution prepared in sterile water was performed in a compressor nebulizer. B. All groups were weighted at P1 and P14. Data were shown are means ± SD (n=5). The body weight of BPD+V group was obviously lower than other three groups after 14th days' treatment. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ were considered significant. Typical differences are shown in groups.

oxygen, ferrous ion, vitamin C and prolylhydroxylase domain-containing protein (PHD) participate in the degradation of HIF-1α. HIF promotes angiogenesis by upregulating the vascular endothelial growth factor (VEGF), thereby promoting lung and alveolar development [10, 14-16]. The HIF-1α/VEGF pathway is vital in the lung development. Pulmonary angiogenesis is closely linked with VEGF. In a baboon model of BPD, the mRNAs level of vascular endothelial growth factor (VEGF)-α and vascular endothelial growth factor receptor (VEGFR)-1 both decreased [17, 18]. In human infants of BPD, the mRNA levels of VEGF-α and VEGFR1 were also reduced [19]. Novel methods to reverse the immature endothelium and assist lung angiogenesis may help relieve clinical symptoms and improve a long-term prognosis.

DFO is a bacterial siderophore. It is commonly used in clinical application to chelate iron and then is metabolized in a nontoxic chelate form. Lalonde proposed a treatment using deferoxamine atomization treatment to prevent smoking caused by lung and systemic injury [20]. Zarogiannis S G found that administering DFO and ascorbate after chlorine exposure alleviated lung injury and reduced mortality in a mouse model, mainly by reducing alveolar capillary permeability, anti-inflammation, lipid peroxidation, and epithelial cell sloughing [21]. The effect of DFO in treating BPD has not been explored extensively.

A previous study, we demonstrated the involvement of DFO in angiogenesis [22]. This study hypothesized that DFO atomization therapy could upregulate HIF-1α and VEGF levels and promote lung development in a mouse model of BPD.

Material and methods

Mouse model of oxygen-induced BPD and experiment design

C57BL/6 mice on gestation day 14 were purchased from Shanghai Laboratorial Animal Centre, Chinese Academy of Sciences (Shanghai, China). Nursing Mice were bred in the specific pathogen-free animal facility of Xinhua Hospital, Shanghai Jiao Tong University School of Medicine. Four experimental groups were set up as followed: BPD+DFO, BPD+V (vehicle), N (normal)+V, N+DFO. (**Figure 1A**) A mouse model of oxygen-induced BPD was established by postnatal hyperoxia (75% oxygen for 7 days) and D [17 mg/(kg·day)] (BPD+D) or aerosolized vehicle (BPD+V) administered for 14 days. Other two groups were kept in room air until sacrifice on day 14. Nursing mouse was rotated between 75% O_2 and room air every 24 hours to avoid oxygen toxicity. The mice were anesthetized and sacrificed after 14 days of treatment before removing the lungs for analysis. And aerosol of 17 mg/ml DFO (Sigma-Aldrich, St. Louis, MO, USA) 10% solution prepared in sterile water was performed in a compressor nebulizer (OPARI, Shenzhen, China) using a 5 L/min flow rate and the nebulizer generates particles

of 3 to 5 μm mean mass diameter. The nebulizer was connected to the animal-specific atomizing mask (Read Eagle Technology Co., Ltd., Beijing, China). Mice received aerosolized DFO via animal-specific atomizing mask inhalation for 60 minutes per day after birth for 14 days. And two normoxia groups were given an equal volume of vehicle (sterile water). The dose of DFO was according to the study by Lalonde C [20]. The Animal Ethical and Welfare Committee of Xinhua Hospital approved the protocols. All efforts were made to minimize suffering and all surgical procedures were performed after anaesthesia.

Lung tissues preparation

Fourteen-day old mice were anesthetized and sacrificed, and left lungs were inflated and kept in 10% paraformaldehyde for histological analysis. Right lungs were immediately stored at -80°C for biochemical analysis. The lungs were embedded in paraffin after washing. Then lung tissue sections were cut into 5 μm thickness per slice and subjected to H&E staining and immunofluorescence (IF). The Alveolar size (volume) was determined on H&E-stained lung sections by digital camera system (Leica DMI-3000B, Wetzlar, Germany) and by fluorescence microscopy (Olympus BX-FLA, Japan). Mean linear intercept (MLI) and radial alveolar count (RAC) were measured as parameters of alveolarization.

Immunofluorescence staining

Lung sections were stained with anti-HIF-1α antibody (1:50, NB100-105, NOVUS, US). The CD31 (PECAM-1) antibody (1:200, sc-1506r, Santa Cruz, US) was used to detect the vascular density of lung tissue. These slides were then stained with secondary antibodies as follows: anti-rabbit Alexa 564-conjugated secondary antibody (#A-11010, Life Technologies) and anti-rabbit Alexa 484-conjugated secondary antibody. All slides were stained with 40,6-diamidino-2-phenylin-dole (DAPI) (Beyotime, China). Fluorescence microscopy (Olympus BX-FLA, Japan) was used for photograph.

Western blot analysis

The lung tissue was lysed by Ultrasonic cell disruptor machine and total protein was extracted. BCA protein assay kit was adopted for quantified. Load samples containing equal amounts of protein (50 μg/lane protein from purified protein) into SDS-PAGE wells and then transferred by polyvinylidene difluoride membrane (0.45 μm, Millipore, US). Incubate membrane in blocking solution for 1 hour at room temperature. Incubate membrane with anti-VEGF (Cat: NB100-664, Novus), anti-Hif-1α (Cat: NB100-105, Novus), anti-Actin (Cat: AA128, Beyotime) in 5 ml primary antibody dilution buffer with gentle agitation overnight at 4°C. Wash the membrane with 1×TBST three times for 10 minutes each. Incubate membrane with appropriate diluted HRP-conjugated secondary antibody for western blot analysis. Expose the membrane to image with Image Lab, Bio-Rad.

Real-time quantitative RT-PCR

RNAiso Plus (TAKARA, Japan) was used to extract total RNA from lung tissue. Thermo NANODROP 2000 spectrophotometer was used to test the concentration and purity of the RNA. Then cDNA was synthesized by using PrimeScript™ RT Master Mix (TAKARA, Japan). The expression of HIF-1α, VEGF and GAPDH genes were determined by real-time PCR and performed by the ABI Prism 7500 sequence detection system (Applied Biosystems, USA). The following gene primers were purchased from generay biotechnology: GAPDH: GGTTGTCTCCTGCGATTCA (Forward) and TGGTCCAGGGTTTCTTACTCC (Reverse), HIF-1α: TGTGTTTGATTTTACTCATCCATGT (Forward) and CTCCGCTGTGTGTTTAGTTCTT (Reverse), VEGF: GAAGAGTGGAGCACAGGCGAAC (Forward) and CCAGGGTGGTTAAGTGAGAGAGTAC (Reverse). Quantification of gene expression was determined by the comparative $2^{-\Delta\Delta CT}$ methods.

Statistical analysis

We adopted two-tailed t tests to compare between two groups. And we analysed multiple groups by one-way or two-way ANOVA with Bonferroni post-test with SPSS 21.0 version. The GraphPad prism (version 6.) was used for figures. For all statistical analysis, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ were considered significant.

Results

DFO induced weight gain in the BPD groups

The body weight was used as an indicator to determine the effects of aerosol of DFO in treating the BPD group after birth. The mice were

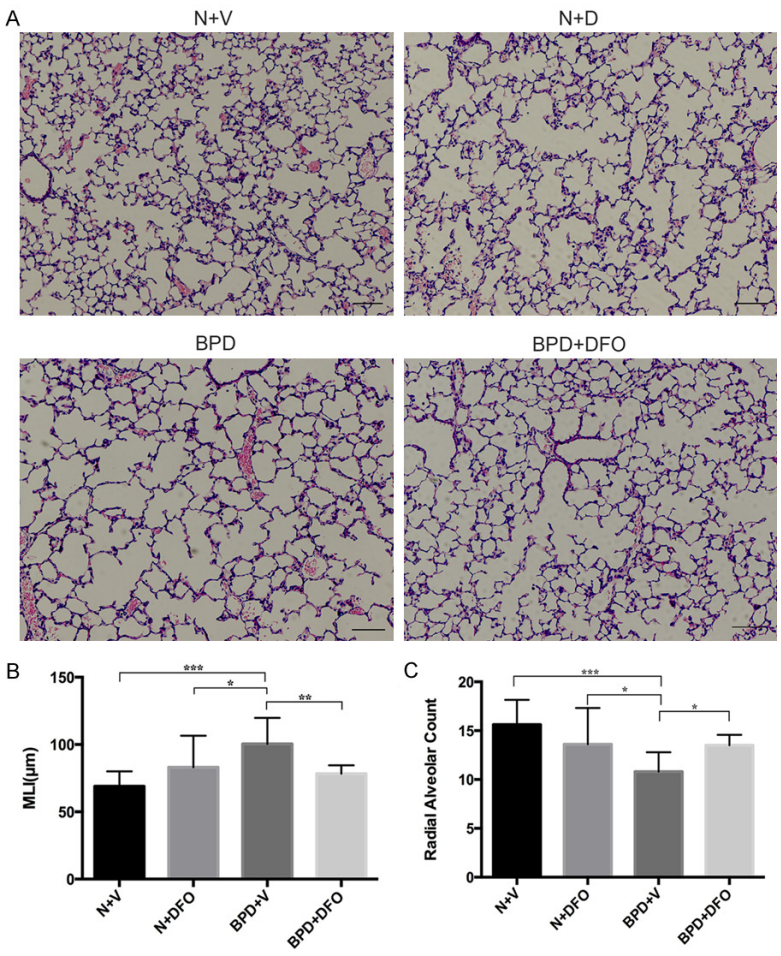

Figure 2. DFO promoted the alveolar development in the BPD group. A. Representative photomicrographs of mouse lungs on postnatal day 14. H&E staining, magnification, 100×. Scale bar: 200 μm. Data represent one of three independent experiments. B. Mean linear intercept (MLI) were compared between four groups. C. Radial alveolar count (RAC) were compared between four groups. Four experimental groups were set up as followed: BPD+DFO, BPD+V, N+V, N+DFO. Data were shown are means ± SD (n=5). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ were considered significant.

weighed on the first day after birth and on 14th day of age, the body weight significantly lowered in the BPD group than in other three groups (**Figure 1B**). This result indicated that exogenous continuous aerosolized of DFO exerted a biological effect on BPD mice.

DFO promoted the alveolar development in the BPD group

The morphological changes in lung tissue were identified using hematoxylin and eosin staining. As shown in **Figure 2**, the distal air space was lager and simpler in the BPD group treated with vehicle (BPD+V) compared with other groups. The radical alveolar count (RAC) value decreased significantly in the BPD+V group compared with other groups. No obvious difference was found between the N+V and the N+D groups. MLI, another indicator of average alveolar size, was significantly higher in the BPD+V group, compared with the other groups. DFO lowered MLI and increased RAC in the BPD group. However, it showed no reaction in the other two normal groups (**Figure 2A-C**).

DFO upregulated the expression of HIF-1α and CD31

The expression of HIF-1α and CD31 was investigated by immunofluorescence staining. Their expression substantially decreased in the lung tissue of the BPD+V group. The results also indicated that the BPD group treated with DFO (BPD+DFO) exhibited increased levels of HIF-1α and CD31 compared with the BPD+V group (**Figure 3**). Furthermore, mice receiving DFO treatment showed no obvious changes in the N+D group compared with the N+V group (**Figure 3**).

Administering D activated the HIF-1α/VEGF signaling pathway associated with BPD

The levels of HIF-1α and VEGF were measured to evaluate whether continuous administration of aerosol of DFO activated HIF-1α/VEGF signaling in BPD (**Figure 4**). The expression of HIF-1α and VEGF significantly decreased in the BPD+V group compared with the other groups. Additionally, continuous administration of aerosol of DFO showed no impact on the N+DFO group compared with the N+V group. In this study, real-time polymerase chain reaction (PCR) was used to evaluate mRNA levels of HIF-1α and VEGF in all four groups. A significant

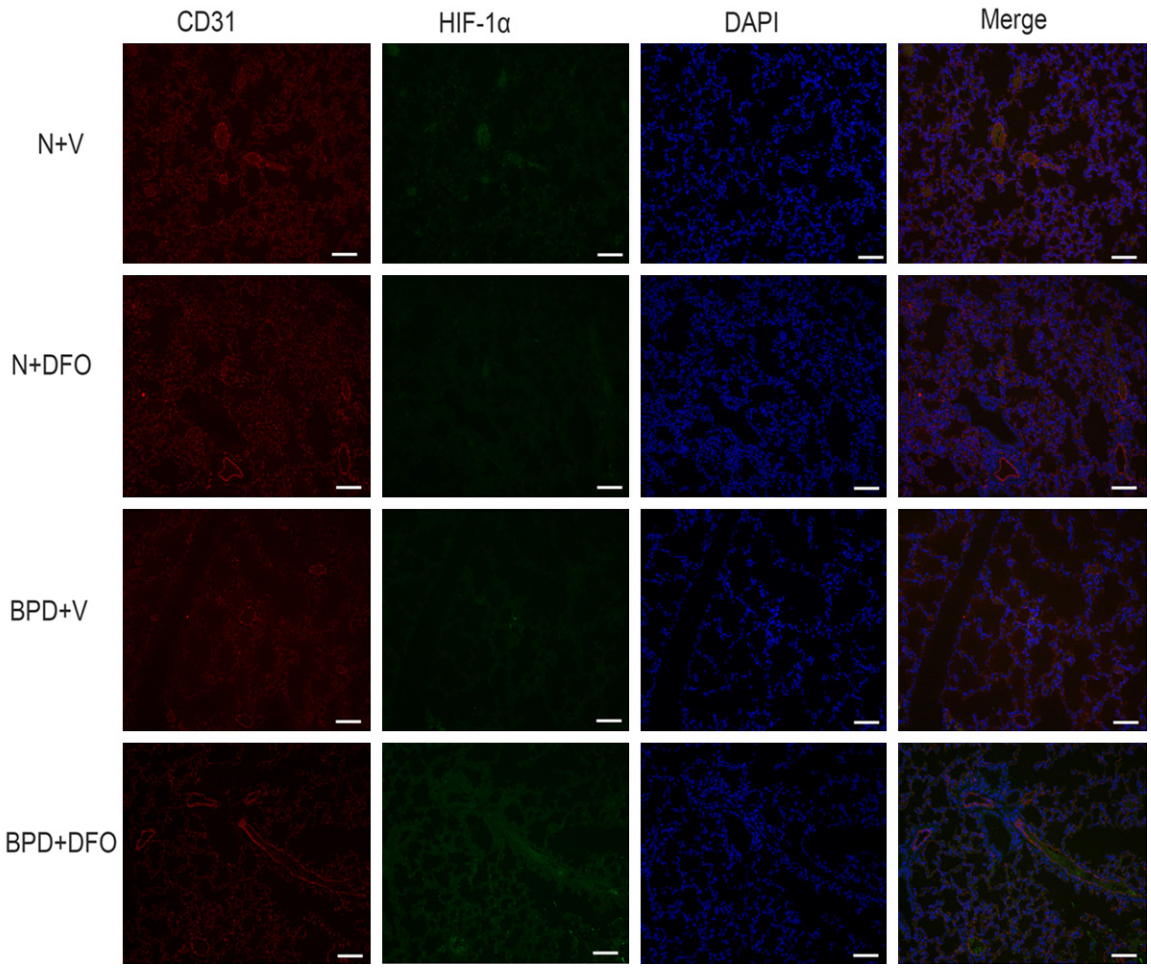

Figure 3. Immunofluorescence analysis for lung tissues. Lung tissues were processed by anti HIF-1α and anti-CD31. The expression of HIF-1α and CD31 was investigated by immunofluorescence staining on day 14 after treatment. Tissues were cut at 4 μm and subjected to immunofluorescence staining of HIF-1α and CD31. DFO, deferoxamine; HIF-1α, hypoxia-inducible factor-1α; BPD, bronchopulmonary dysplasia; N, normoxia; V, vehicle; DAPI, 4',6-diamidino-2-phenylindole. Scale bars are 100 μm.

decrease was found in mRNA levels of HIF-1α and VEGF in the BPD+V group (**Figure 4B** and **4C**). These findings indicated that DFO signaling activated the HIF-1α/VEGF-signaling pathway in BPD.

Discussion

BPD is closely related to infancy and childhood health problems, including pulmonary hypertension, exercise intolerance, asthma and chronic obstructive pulmonary disease, and neurodevelopmental disorders [23]. Pulmonary microvascular development and alveolar septal enlargement are the main features of BPD, often leading to clinical symptoms [24, 25]. As the formation of pulmonary branches depends on the development of vascular growth, abnormal angiogenesis needed oxygen therapy and mechanical ventilation in preterm newborns. Fetal development was in a relatively hypoxic environment, while the HIF family was involved in embryogenesis and oxygen-regulated events of vascular development [8-10, 22]. Previous studies demonstrated that HIF could promote angiogenesis, thereby improving the lung development of premature in a baboon model [11, 15, 26, 27]. This study hypothesized that DFO atomization therapy could upregulate HIF-1α and VEGF levels and promote lung development in a mouse model of BPD. Therefore, the study aimed to explore the mechanism underlying the effect of continuous administration of aerosol of DFO in an *in vivo* mouse model of

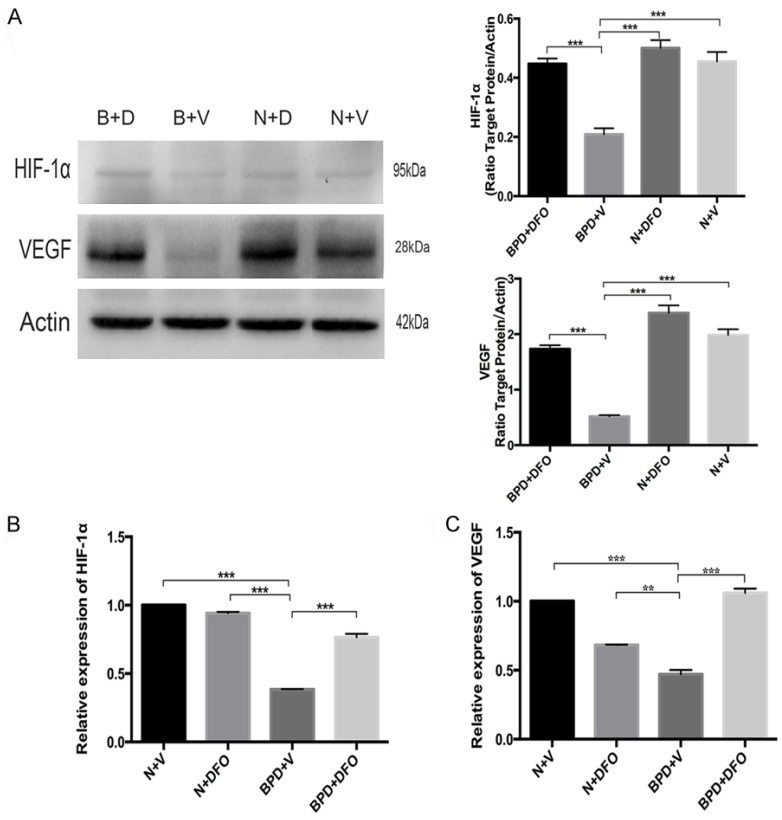

Figure 4. Administering D activated the HIF-1α/VEGF signaling pathway associated with BPD. A: Whole cell lysate (50 ug) of lung tissue were probed with anti-HIF-1α and anti-VEGF antibody. Actin was used as a loading control. Western blot analysis showed that DFO induce HIF-1α, VEGF protein expression in BPD group compared with the group treated with vehicle (sterile water). B: Real-time quantitative RT-PCR analysis showed that DFO-induce HIF-1α mRNA expression in BPD group compared with the group treated with vehicle (sterile water), Quantitative analysis of the RT-PCR results. C: Real-time quantitative RT-PCR analysis showed that DFO-induce VEGF mRNA expression in BPD group compared with the group treated with vehicle (sterile water), Quantitative analysis of the RT-PCR results. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ were considered significant. Data were shown are means ± SD (n=5).

BPD. The mouse model of BPD had similar changes in lung pathology as associated with the BPD in humans [8, 28].

The pulmonary morphology analysis revealed a lager and simpler distal air space in the BPD+V groups, compared with the other groups. The RAC value decreased significantly in the BPD+V group compared with other groups. No significant difference was found between the N+V group and the N+DFO group. MLI was significantly higher in the BPD+V group compared with the other groups (**Figure 2A-C**). Moreover, DFO could lower MLI and increase RAC in the BPD group, indicating that DFO promoted alveolar development. These results showed that administering DFO could relieve the pathological changes associated with arrested lung development.

DFO is a medication that binds iron and is especially, used in iron overdose due to multiple blood transfusion. It protects against the iron-mediated neurotoxicity and the depletion of low-molecular antioxidants after asphyxia in newborns. In the normoxia environment, HIF-1α is hydroxylated at a proline residue by PHD in a ferrous iron- and vitamin C-dependent manner. DFO as ferrous chelator, inhibits the PHD function and significantly upregulates HIF-1α to induce angiogenesis [21, 29].

This study found that the mRNA and/or protein levels of HIF-1α and VEGF are both increased in lungs after DFO treatment. (**Figure 4B** and **4C**) Vadival found that administering adenovirus intratracheally could upregulate HIF-1α and VEGF, and promote alveolarization and capillary growth in a rat model [30]. Zarogiannis SG established a mouse model of acute lung injury. After exposure to lethal concentrations of chlorine, the combined management of DFO and ascorbate intramuscularly and via aerosols increased the survival time by fourfold [21]. Moreover, lung tissue immunofluorescence also demonstrated upregulation of HIF-1α and CD31 in lungs after DFO treatment. This study found a significant increase in vessels group after immunofluorescence staining of endothelial cells using an anti-CD31 antibody in the BPD+D group compared with the vehicle control group (**Figure 2**). Asikainen also found that HIF in the preterm lung could enhance angiogenesis effectors in an *in vivo* experiment [15]. FG-4095, an inhibitor of PHDs, increased the expression of platelet endothelial cell adhesion molecule (PECAM-

1)/CD31 and VEGF mRNA and/or protein. These results suggested that the increased expression of HIF by inhibiting PHD activity could promote pulmonary angiogenesis in the primate models of BPD. These research conclusions were consistent with the findings of this study.

To summarize, the results showed that C57BL/6 mice exposed to hyperoxic environment and treated with aerosolized of DFO solution, obviously promoted the pulmonary vascularization and alveolarization. The HIF-1α/VEGF signaling pathway mediated this process. The findings indicated that treatment with an exogenous continuous aerosol of DFO might be a potential therapeutic strategy for BPD.

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Disclosure of conflict of interest

None.

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