Effect of Antibiotic-induced Intestinal Dysbacteriosis on Bronchopulmonary Dysplasia and Related Mechanisms

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Research

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Abstract

Background

The modification of the gut microbiota by antibiotics may influence the disease susceptibility and immunological responses. Infants in the neonatal intensive care unit (NICU) subjected to frequent antibiotics and oxygen therapies, which may give rise to the local and systemic inflammatory reactions and progression of bronchopulmonary dysplasia (BPD). This study aimed to investigate the role of intestinal dysbacteriosis by antibiotic therapy before hyperoxia exposure in the progression of BPD.

Methods

Mice had been exposed to hyperoxia (85% O_2) since postnatal day 3 until day 16 for the BPD model establishment, treated with antibiotics from postnatal day 2 until day 8. Treated mice and appropriate controls were harvested on postnatal day 10 for 16S rRNA gene sequencing, or postnatal day 17 for assessment of alveolar morphometry and macrophages differentiation.

Results

Antibiotic-induced intestinal dysbacteriosis before hyperoxia exposure gave rise to deterioration of BPD evidenced by reduced survival rates and alveolarization, moreover, antibiotic-induced intestinal dysbacteriosis resulted in increased iNOS and decreased Arg-1 levels in lung homogenates.

Conclusion

Broad-spectrum antibiotic-induced intestinal dysbacteriosis may participate in BPD pathogenesis via alteration of the macrophage polarization status. Manipulating the gut microbiota may potentially intervene the therapy of BPD.

1 Introduction

As a multi-factorial chronic lung disease of preterm infants, Bronchopulmonary dysplasia (BPD) subjected to interrupted lung deterioration[1]. It most commonly contributed to long-term morbidity and mortality in effectively low birth body weight (ELBW) babies[2]. Therefore, here comes a necessity for the understanding of the mechanisms leading to BPD. Infants in the neonatal intensive care unit (NICU) frequently receive antibiotic therapy [3]. Exposure to antibiotics was one of the most critical factors altering the gut microbiota of neonates[4, 5]. Immunological homeostasis rely on the microbiota, the metabolites and components of microbiota influence how the host susceptible to many immunological-mediated diseases and disorders[6]. Intestinal dysbacteriosis may influence the pulmonary immunological response through the intestinal-pulmonary axis[7, 8]. However, the role of intestinal dysbacteriosis in the BPD development remains unclear. Hence, this study aimed to investigate the underlying mechanismand role of intestinal dysbacteriosis in BPD.

2 Materials And Methods

2.1 Animals

All animal experiments were carried out pursuant to the protocols approved by the Animal Care and Use Ethics Committee of the Chongqing Medical University (Chongqing, China). C57 BL/6 mice were bought from the Experimental Animal Center of Chongqing Medical University. All mice were under the environment where temperature and humidity were controlled.

2.2 Antibiotics treatment and BPD model establishment

We treated the neonatal C57BL/6 mice with broad-spectrum antibiotics from postnatal day two (PN2) to PN8. Antibiotics treatment (ABX) involved oral gavage of ampicillin (10 mg/kg; Sangon Biotech), vancomycin (5 mg/kg; Sangon Biotech), neomycin sulfate (10 mg/kg; Sangon Biotech) and metronidazole (10 mg/kg; Sangon Biotech) every 12 hours [9–11]. Saline perfused the control mice were every 12 hours. Intestinal samples were collected at PN10. Separate therapeutic experiment for mice with antibiotics and with saline was carried out in different cages and were fed by their mother.

In the case of the BPD model establishment where mice received antibiotics treatment on PN2, hyperoxia exposure was initiated on PN3. The mouse modeling of BPD was performed pursuant to prior study[12]. Random assignments of the neonates were made to hyperoxia (85% O_2) or room air (21% O_2) from PN3 to PN16 (Fig. 1).

2.3 Histopathological analysis

The left lung of every mouse subjected to immersion in 4% paraformaldehyde, fastening in paraffin, and sectioning at 5 μm. Sections were stainted with hematoxylin and eosin (HE) and evaluated by light microscopy.

2.4 Immunofluorescence

Lung tissue sections were submerged in the xylene and graded alcohols to deparaffinize and rehydrate, santigen retrieval was done using ethylenediaminetetraacetic acid (EDTA, pH 8.0). Sections blocking with blocking solution containing 10% donkey serum was performed at room temperature for 30 min. Then Sections incubation was performed with mouse anti-CD68 (1:200, Zenbio), rabbit anti-iNOS (1:200, Zenbio), rabbit anti-Arg-1 (1:200, Zenbio) at 4 °C overnight. The sections washing and incubation were performed with the secondary fluorescein Alexa Fluor 488 (1:400, green, Beyotime) and Cy3 (1:300, red, Beyotime) antibodies at roomtemperature for 50 min. Furthermore, nuclei was stained with 4', 6-diamidino-2-phenylindole (DAPI, Leagene) at room temperature for 10 min. Finally, the sections washing and incubation were performed with AutoFluo Quencher (Beyotime) for 5 min and sections were viewed by fluorescent microscopy (Nikon, Japan).

2.5 Western blot analysis.

Western blotting analysis was made to evaluate the expressions of iNOS and Arg-1 in the lung tissues. Lung tissues lysing in protein lysis buffer and protein concentration were evaluated with the Bradford approach. Lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and lysates transfer to polyvinylidene difluoride (PVDF) membranes (Millipore) were made. After 1 h membranes blocking with TBST buffer containing 5% non-fat dry for 1 h at room temperature, membranes incubation was performed with 1:800 anti-iNOS (Zenbio), 1:1000 anti-Arg-1(Zenbio) and 1:1000 anti-β-actin(Zenbio) antibody overnight at 4 °C. After membranes washing with TBST (5 min x 3), 1 h membranes incubation was performed at room temperature with a secondary antibody (anti-rabbit IgG, 1:5000; Proteintech) diluted in TBST. Then immunoblots were visualized using ECL kit (Bio-Rad, USA).

2.6 Fecal sample collection and assay of commensal bacteria in the intestine of neonatal mice

Due to the difficulty of gathering sufficient intestinal contents from neonates for 16 s sequencing, we chose to collect intestinal contents from 6–8 neonates per group[13]. We collected the intestinal contents as done previously[14]. QIAamp DNA stool Mini Kit (Qiagen) was applied for the extraction of bacterial DNA from the intestinal contents as instructed by the manufacturer [15]. Illumina MiSeq platform was applied for the sequencing of the 16S rRNA V3–V4 hypervariable region of bacteria according to protocols. The microbial community analysis was conducted as previously reported[16]. The community structure was analyzed by R package vegan 2.0[17].

2.7 Statistical Analysis

Statistical analysis was made with SPSS version 22.0. Expression of quantitative data is denoted as mean ± standard deviation. In addition, unpaired two-tailed Student's t-test or ANOVA or Wilcoxon signed-rank test was applied for the comparison of differences between groups. $P < 0.05$ was deemed significant in terms of statistics.

3 Results

3.1 Composition analysis of gut microbiota

After seven days of antibiotics therapy, *Proteobacteria* accounted for a higher proportion at the phylum level, whereas *Firmicutes* and *Bacteroidetes* accounted for a reduced proportion in the antibiotics treated mice than that in the saline treated mice(Fig. 2A, $P < 0.05$). At the genus level, the proportion of *Citrobacter* and *unclassified_f__Enterobacteriaceae* elevated, whereas the proportion of *Bacteroides* and *norank_f__Muribaculaceae* reduced in the antibiotics treated mice than that in the saline treated mice (Fig. 2B, $P < 0.05$).

3.2 Effect of antibiotics treatment (ABX) on survival and alveolarization in BPD mice

The survival rate of BPD mice reduced in the antibiotics treated mice than that in the saline treated mice (Fig. 3A, $P < 0.05$). Figure 3B and C showed that, the lungs of BPD mice had an elevated MLI and decreased RAC after exposed to hyperoxia, moreover, lungs of mice in the ABX + O_2 group had an elevated MLI and reduced RAC compared to the Saline + O_2 group(Fig. 3B and C, $P < 0.05$).

3.3 Effects of antibiotics treatment (ABX) on polarization of macrophages in BPD mice

As the macrophages polarization have potential relevance with pathogenesis of BPD[18], we next observed whether the antibiotics treatment aggravated BPD via M1/M2 polarization pathways. The polarization of macrophages was identified by double immunofluorescence staining using anti-iNOS (M1) antibodies, anti-Arg-1 (M2) antibodies (Fig. 4B). The iNOS expression increased in BPD mice and then was upregulated when ABX was added (Fig. 4B). The Arg-1 expression decreased in BPD mice and then was inhibited when ABX was added (Fig. 4B). Then, the protein levels of iNOS and Arg-1 were detected by westernblot. Consistent with the above results, iNOS increased in BPD mice and antibiotics treatment upregulated the expression of iNOS only when mice exposed to hyperoxia (Fig. 4A, $p < 0.05$). Arg-1 decreased in BPD mice and ABX inhibited Arg-1 expression only when mice exposed to hyperoxia (Fig. 4A, $p < 0.05$). Therefore, with the antibiotics treatment, the ratio of M1 macrophages increased and the ratio of M2 macrophage decreased in BPD mice, which indicates that ABX upregulated the ratio of M1/M2 macrophages as a pro-inflammatory factor in BPD mice.

4 Discussion

In this study we found that antibiotic-induced intestinal dysbacteriosis prior to hyperoxia exposure increased the susceptibility of mouse BPD, evidenced by decreased RAC, increased MLI and increased mortality in the antibiotics treated BPD mice. These findings supported the previous hypothesis that the preterm infants who frequently received antibiotics therapy often developed more severe BPD[19].

BPD is the most common complicating disease of premature birth. Neonates with BPD usually subjected to respiratory sequelae [20, 21]. BPD remains a substantial challenge to the neonatologist. Therefore, in this study, we researched the potential mechanisms of BPD through animal experiments.

Infants in NICU often receive antibiotics treatment. Antibiotics change the composition of gut microbiota. Gut microbiota composition is related to the immunological response. There are increasing evidences that support the concept of cross-talk between the gut microbiota and the lung. For example, Intestinal dysbacteriosis in mice results in abnormal airway allergic responses[22]. Microbiota depletion aggravate ventilator-induced lung injury[23]. These evidences supported that the gut microbiota may affect the lung mucosa by influencing the immunological response. But the participation of antibiotic-induced intestinal dysbacteriosis in the progression of BPD remains unclear. This study researched the effect of intestinal dysbacteriosis on hyperoxia exposure induced the BPD mouse model.

To investigate this interaction, neonates were therapied with a broad-spectrum antibiotic regimen for the induction of intestinal dysbacteriosis. The administration of antibiotics was showed significantly changed the composition of gut microbiota in neonatal mice.We found the relative abundance of the phylum *Firmicutes*, *Bacteroidetes* and the genus *Bacteroides* and *norank_f__Muribaculaceae* significantly decreased in the antibiotics treated mice. In contrast, the relative abundance of the phylum *Proteobacteria* and the genus *Citrobacter* and *unclassified_f__Enterobacteriaceae* significantly increased in the antibiotics treated mice. The findings were consistent with the previous studies, which showed that antibiotics induced intestinal dysbacteriosis causing decreased relative abundance of phylum *Bacteroidetes* and *Firmicutes* and increased relative abundance of *Proteobacteria* [24, 25].

According to the previous reports, macrophage polarization may play important roles in the development of BPD [26, 27]. Activated macrophages can be M1- or M2-polarized[28]. Modulating the M1/M2 polarization status of macrophages can affect the severity of acute inflammatory conditions of the lung[29]. In this study, we found that antibiotics treatment promoted M1 markers iNOS expression and inhibited M2 markers Arg1 expression in the BPD mice. Hence, antibiotics treatment promoted the macrophages transformation towards pro-inflammatory M1 phenotype and inhibited the macrophages transformation towards anti-inflammatory M2 phenotype. So antibiotics treatment may promote inflammations and inhibit anti-inflammations resulting in the aggravation of BPD. Meanwhile, another study showed that after the composition of gut microbiota were changed and their metabolites were depleted during antibiotic administration, the supplementation of antibiotics with the metabolites could promote the macrophages transformation towards anti-inflammatory M2 phenotype[30]. Based on the above evidences, we hypothesised that antibiotics treatment may aggravate the mouse BPD via modulating the M1/M2 polarization status of macrophages.

There are limitations need to be considered. Firstly, there was a relatively high mortality in the experimental mice. the reason may be that experiments were conducted in neonatal mice subjected at 2–3 d of life. Secondly, antibiotics administration may effect the results of this study, the administration of antibiotics with fecal microbiota transplants (FMT) may help us to understand the role of microbiota in the development of BPD.

5 Conclusion

In summary, our study showed that antibiotic-induced intestinal dysbacteriosis aggravated the hyperoxia induced the mouse BPD, possibly via alteration of the macrophage polarization status. The findings highlight a potentially role of intestinal dysbacteriosis in the BPD pathogenesis.Manipulating the gut microbiota may be a potential therapy of BPD.

Abbreviations

BPD	bronchopulmonary dysplasia
ELBW	extremely low birth weight
PN	postnatal day
NICU	neonatal intensive care unit
ABX	antibiotics treatment
HE	hematoxylin and eosin
EDTA	ethylenediaminetetraacetic acid
DAPI	4', 6-diamidino-2-phenylindole
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
PVDF	polyvinylidene difluoride
RAC	radial alveolar count
MLI	mean linear intercept
FMT	fecal microbiota transplants

Declarations

Ethics approval and consent to participate

The studies were approved by the Animal Care and Use Ethics Committee of the Chongqing Medical University (Chongqing, China).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

We confirm that none of the authors has any conflict of interest.

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Authors' contributions

XR and YS designed the research and drafted the manuscript. YH revised the manuscript. XR and QA made the figures and conducted information collection. All authors read and approved the final manuscript.

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Figures

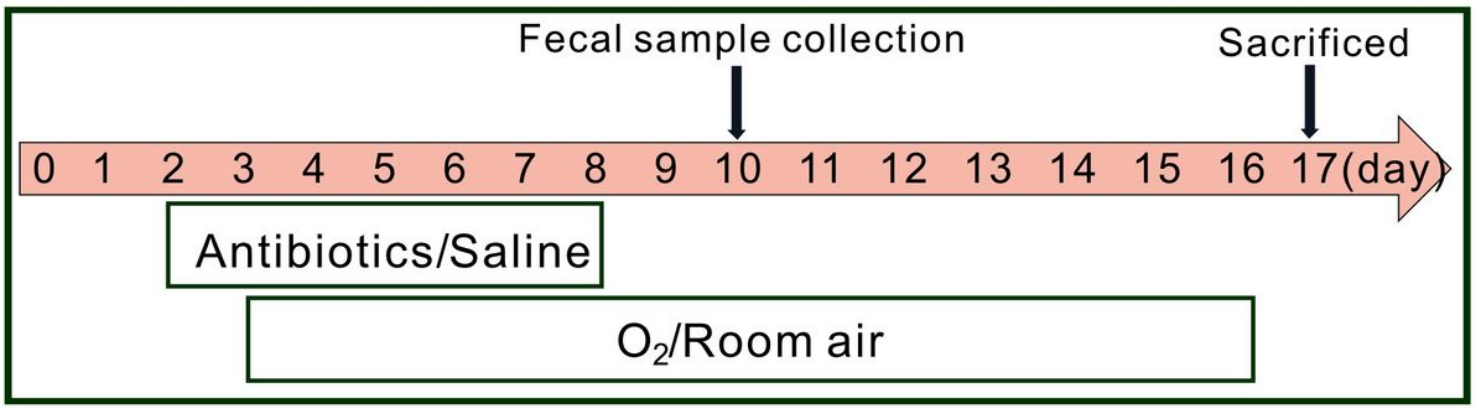

Figure 1

Treatment regime schematic for the bronchopulmonary dysplasia (BPD) model establishment and administration of antibiotics. The mouse model of BPD was conducted and treated with broad-spectrum antibiotics. The newborn C57BL/6 mice were treated with broad-spectrum antibiotics(ampicillin (10 mg/kg), vancomycin (5 mg/kg), neomycin sulfate (10 mg/kg) and metronidazole (10 mg/kg))every 12 hours by gavage from postnatal day two(PN2) to PN8. The control mice were perfused with saline every 12 hours from PN2 to PN8. In the case of the BPD model establishment, the newborn mice were exposed to hyperoxia (85% O2) from PN3 to PN16. The control mice were exposed to room air (21% O2) from PN3 to PN16.

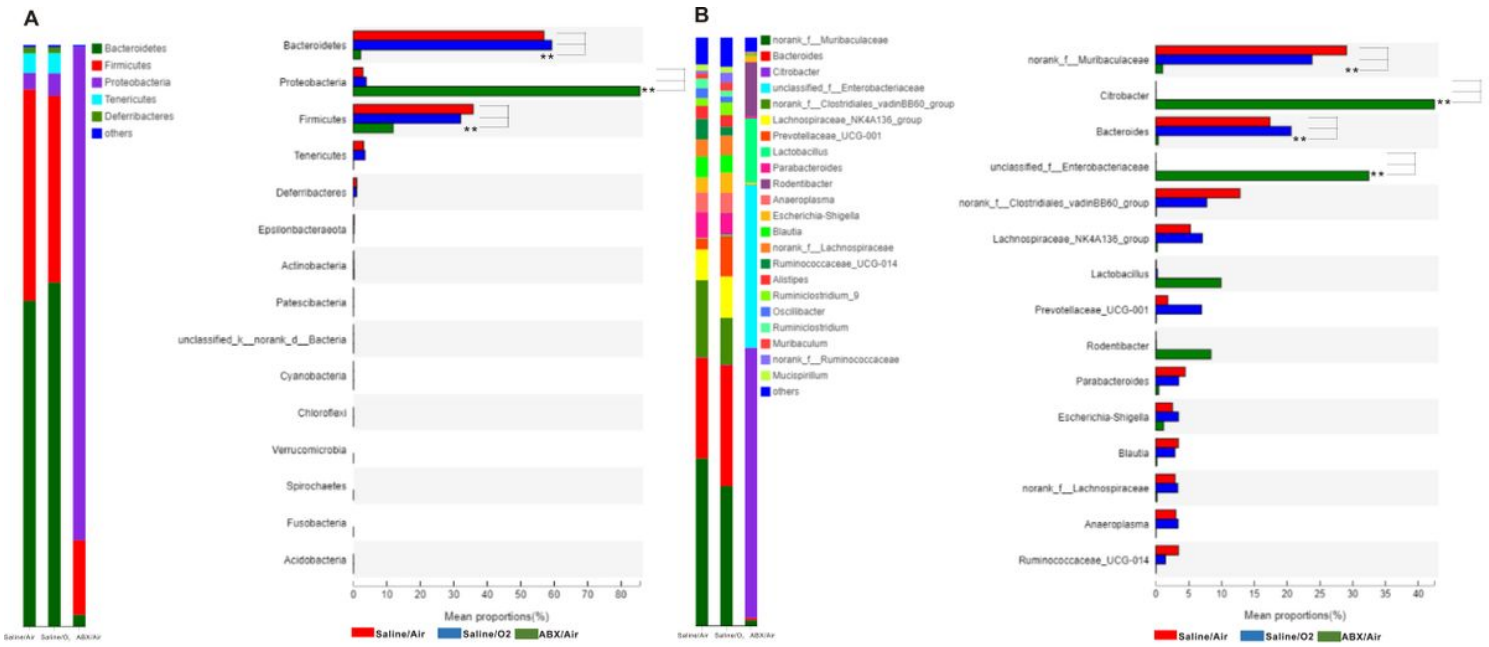

Figure 2

Antibiotic treatment changes the microbiota composition in neonatal mice. (A-B) after seven days treatment with antibiotics or saline, the relative abundance of microbial communities at the phylum level (A) and genus level (B); Relative abundance>1%, *P<0.05.

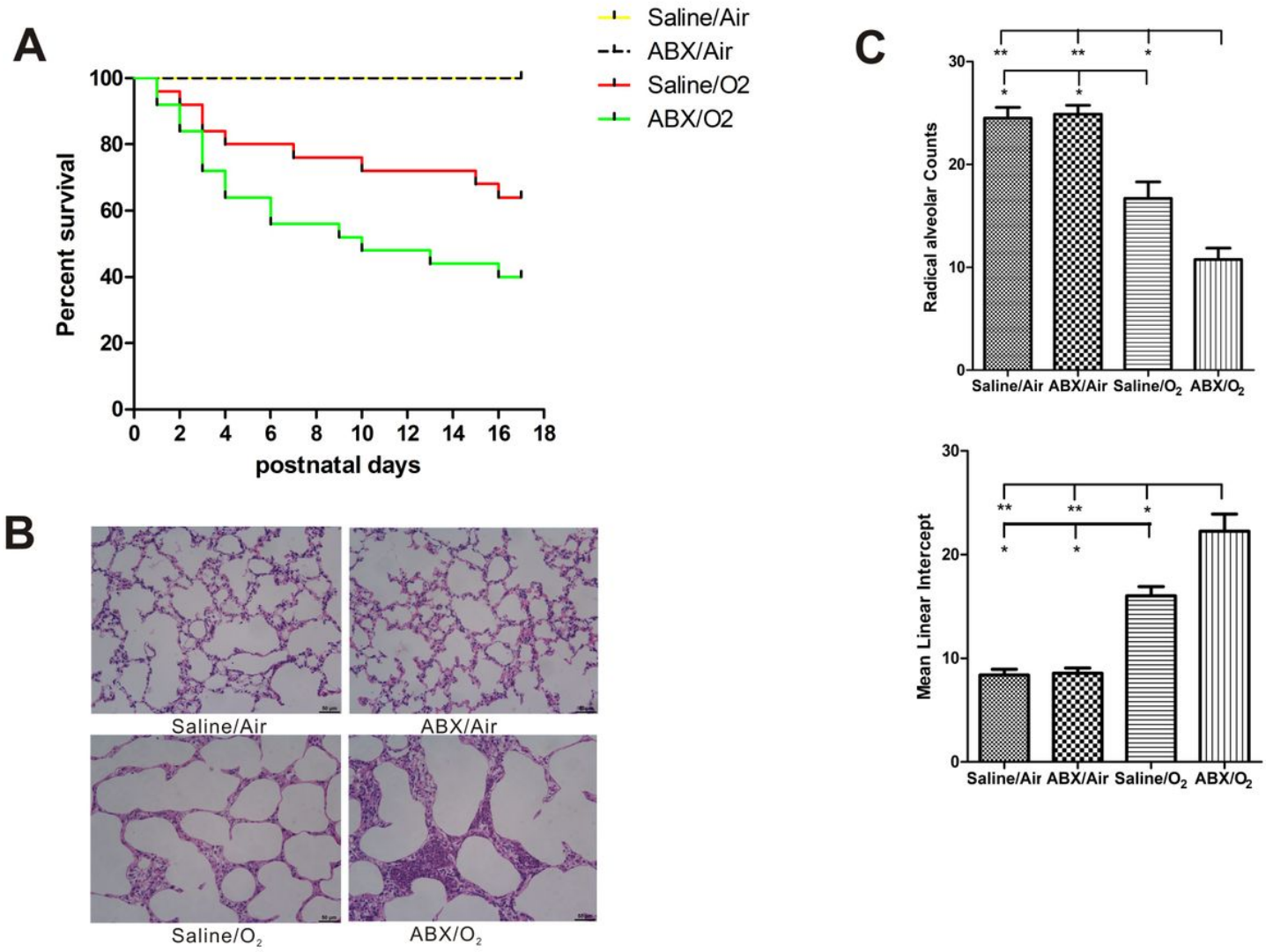

Figure 3

Effect of antibiotics treatment (ABX) on survival and alveolarization in BPD mice. (A) Survival of mice in the Saline+Air, ABX+Air, Saline+O2, ABX+O2 groups.antibiotic-treated or antibiotic-free mice. The data represent 25 mice per group. (B) Representative H&E- stained sections of neonatal lungs in different groups. (C) Radial alveolar count (RAC) and mean linear intercept (MLI) of mice in different groups. Data were displayed as mean ± standard deviation. *P<0.05, **P<0.01. Results were analyzed using ANOVA.

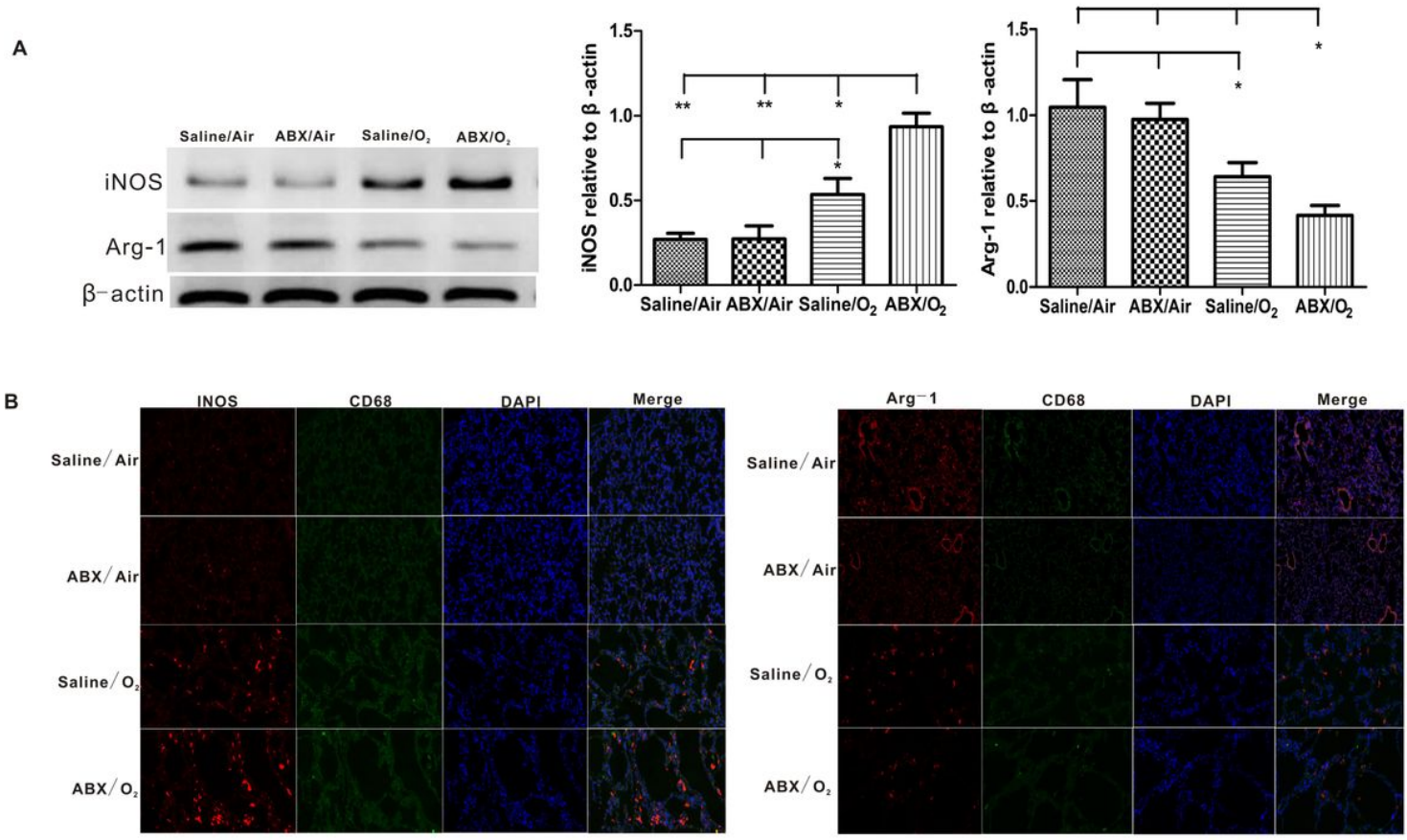

Figure 4

Effects of antibiotics treatment (ABX) on polarization of macrophages in BPD mice. (A)The iNOS and Arg-1 expressions were evaluated by western blot and relative gray intensity to β-actin was calculated. (B)Lung sections were stained by double immunofluorescence with anti-CD68 (macrophage marker, green), anti-Arg-1 (M2 marker, red) and anti-iNOS (M1 marker, red) antibodies. *P<0.05, **P<0.01. Results were analyzed using ANOVA.