

***Astragalus* polysaccharides exert protective effects in newborn rats with bronchopulmonary dysplasia by upregulating the expression of EGFL7 in lung tissue**

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Abstract. The aim of this study was to explore the effects of *Astragalus* polysaccharides (APS) on the mRNA expression of epidermal growth factor-like domain 7 (EGFL7) in lung tissue in newborn rats with bronchopulmonary dysplasia (BPD). For this purpose, a total of 96 newborn SD rats were randomly divided into 4 groups (n=24): the control group, air room plus APS group, BPD group and the APS group (20 mg/kg/day). Lung tissues were obtained on days 4, 10 and 14 after birth. Morphological changes were observed and the protein and mRNA expression levels of EGFL7, Bax and Bcl-2 were determined. The rats in the BPD group (BPD induced by hyperoxia) presented with an arrest in alveolar and vascular development and low mRNA and protein expression levels of EGFL7, Bcl-2 and high levels of Bax compared with the rats in the control group. However, lung damage in the APS intervention group was attenuated compared with the BPD group. The protein and mRNA expression levels of EGFL7 and Bcl-2 were also increased and the level of Bax was decreased in the APS intervention group ($P<0.01$) compared with the BPD model group after birth on days 4, 10 and 14. Our data demonstrate that APS reduce airway remodeling and alveolar damage by upregulating the expression of EGFL7 and exert protective effects against BPD in neonatal rats. Thus, APS may have potential for use as a therapeutic strategy for BPD.

Introduction

Bronchopulmonary dysplasia (BPD) was first reported and defined by Northway *et al* in 1967 as the most common form of chronic lung damage in premature infants, which includes barotrauma, volutrauma and oxygen toxicity (1). It is characterized by arrested lung growth, with decreased alveolarization

and a dysmorphic vasculature (2). It is the cause of prolonged hospitalization with serious social and economic consequences. A deeper understanding of the disease, such as future preventative measures, aiming at reducing its incidence, minimizing complications, diminishing hospital costs and improving infant health has become the prime objective of public health (3).

Although its exact etiology and pathogenesis have yet to be fully ascertained, several researches suggest that it results from the complex interplay between impairments in the premature lung, perinatal insults and resulting from the supportive care of the infant (from mechanical ventilation and supplemental oxygen administration) (4). In other words, the strongest association is with pre-term birth, although other variables, such as pre-natal and post-natal infection, inflammation, mechanical ventilation, oxygen toxicity, patent ductus arteriosus (PDA) also contribute to the pathogenesis of BPD, whereas anti-angiogenesis is known to contribute significantly to the disruption of lung development in animal models (5). An increasing number of studies has demonstrated impaired angiogenesis in the development of preeclampsia (6).

Few therapies are known to effectively prevent or treat BPD and corticosteroid therapy may decrease lung injury through a variety of mechanisms, such as decreasing the inflammatory response (7). However, this type of treatment has been found to cause serious short-term and long-term side-effects, such as gastrointestinal bleeding, gastrointestinal perforation, hyperglycemia and hypertension that give clinicians reason to severely limit the use of this type of treatment (8). The survival rate of premature neonatals has significantly increased; however, the morbidity of BPD also shows an increasing trend (9).

Epidermal growth factor-like domain 7 (EGFL7) is a protein secreted from endothelial cells which plays an important role in vascular tubulogenesis. It has been found that EGFL7 gene expression is significantly decreased in neonatal rat lungs following exposure to hyperoxic conditions and is important for cell survival (10). It has been identified as a potential therapeutic target for lung injury. Thus, antioxidant therapy has been considered as a potential preventive or treatment option for BPD (11-13).

Radix Astragali, a type of Chinese traditional herb, has been used in Traditional Chinese Medicine for over 20 centuries to strengthen the body against disease. It is officially listed in both the Chinese and Japanese Pharmacopoeia (14). Large numbers

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of pharmacological and clinical studies have demonstrated that *Radix Astragali* possesses a wide spectrum of activities, such as immunomodulatory, antioxidant and anti-inflammatory, cardioprotective, hepatoprotective, antihyperglycemic and anti-tumor activities (15-23). It is well known that the principle active constituents of *Astragalus* are polysaccharides, saponins and isoflavonoids (24). Several research groups have isolated and purified polysaccharides from *Astragalus* termed *Astragalus* polysaccharides (APS) or Astragalans (25). APS from *Radix Astragali*, have attracted much attention due to their outstanding antioxidant and anti-inflammatory effects (26). APS have also proven to have strong immunoregulatory properties (27).

However, the effects of APS on the mRNA expression of EGFL7 in newborn rats with hyperoxia-induced BPD are unknown. Thus, the aim of the present study was to determine the potent effects of APS using a rat model of BPD and to clarify the association between BPD and the expression of EGFL7.

Materials and methods

Animal experiments. A total of 96 Sprague Dawley newborn rats (weighing 7.82 ± 0.63 g) and fungible mother rats were obtained from the Experimental Animal Center of Southern Medical University (Guangzhou, China). The present study was approved by the Ethical and Research Committee of Southern Medical University. All investigations were conducted according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. These rats were randomly divided into 4 groups of 24 rats in each group ($n=8$ for each time point) as follows: the control group, air room (RA) plus APS group, BPD group and the APS group (20 mg/kg/day). BPD was induced by exposing the rats to hyperoxic conditions. Ten hours after birth, the pups in the control group and RA plus APS group were kept in room air containing 21% O₂. The rats in the RA plus APS group received daily injections of APS (Sainuo Pharmaceutical Co., Ltd., Tianjin, China) [intraperitoneally (i.p.) 20 mg/kg/day] throughout the post-natal 14 days, while those in the BPD and APS groups were placed in an oxygen chamber, into which oxygen was continuously delivered (FiO₂, 0.85 ± 0.03) and received daily injections of saline (i.p.) and APS (i.p. 20 mg/kg) throughout the post-natal 14 days, respectively. Temperature and humidity were maintained at 22-25°C and 60-70%, respectively. The chamber was opened for 1 h daily to switch dams between air and the O₂ environment to protect the dams from oxygen toxicity. These pups were then sacrificed on days 4, 10 and 14 after the experiments were completed and the lung tissues were collected.

Assessment of lung histological damage. Following anesthesia with pentobarbital (60 mg/kg, i.p.), the lungs of the pups were fixed with an intratracheal injection of 4% paraformaldehyde and post-fixed overnight at room temperature. The tissues were paraffin-embedded, sectioned to 5 μ m thickness and stained with hematoxylin and eosin (H&E). A quantitative analysis of the pulmonary mean linear intercept (MLI), the mean alveolar number (MAN) was carried out according to previously described methods (28).

Immunohistochemistry. Lung tissue was obtained at each time point and post-fixed overnight at room temperature. The tissues

Table I. List of oligonucleotides used as primers in the quantitative PCR analysis of gene expression in lung tissue.

Gene symbol	Primer	Primer sequence
Bax	Forward	5'-AGAGGATGGCTGGGGAGAC-3'
	Reverse	5'-CGCTCAGCTTCTTGGTGGAT-3'
Bcl-2	Forward	5'-ACCCCTGGCATCTTCTCCT-3'
	Reverse	5'-CGACGGTAGCGACGAGAG-3'
EGFL7	Forward	5'-CCGAACCATCTACCGGACTG-3'
	Reverse	5'-GCCTGTCTGTCACCCATTCA-3'
β -actin	Forward	5'-AGGGAAATCGTGC GTGACAT-3'
	Reverse	5'-GAACCGCTCATTGCCGATAG-3'

EGFL7, epidermal growth factor-like domain 7.

were then paraffin-embedded, sectioned to 5 μ m thickness and deparaffinized in xylene and hydrated in a series of graded alcohol. After dewaxing and rehydration, the sections were immersed in 3% hydrogen peroxide in methanol for 20 min at room temperature to abolish endogenous peroxidase activity and then antigen retrieval was carried out using a microwave for 15 min before blocking with 5% bovine serum albumin (Life Technologies Co., Carlsbad, CA, USA) at 37°C for 20 min. The sections were incubated with polyclonal CD31 antibody (diluted to 1/200; Novus Biologicals, Littleton, CO, USA) at 37°C for 2 h. After washing with PBS, the sections were incubated with a biotinylated peroxidase-conjugated secondary antibody and 0.1% DAB substrate, using the standard streptavidin-biotin-based method. A negative control was prepared by reacting a few sections with normal mouse IgG (Abcam Biotechnology, Cambridge, UK) at the same dilution instead of the specific antibody. A cytoplasmic brown granule was marked as a positive expression of CD31. We quantified vascular density by measuring the area of CD31 immunostaining relative to the total area of parenchymal cells using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA) of differential interference contrast images. The tissue sections analyzed contained mainly the alveolar parenchyma and excluded any large airways or blood vessels.

Reverse transcription quantitative PCR. Total RNA from total lung tissue was isolated using the TRIzol kit (Takara Bio, Dalian, China) following the manufacturer's instructions. Reverse transcription (RT) was performed using the PrimeScript RT reagent kit (Takara Bio). Real-time (quantitative) PCR was conducted using an Applied Biosystems 7500 Real Time PCR System and the relative quantification of mRNA expression was calculated using the 2- $\Delta\Delta$ Ct method, as previously described (29). The SYBR Premix Ex Taq (Tli RNaseH Plus) kit was purchased from Takara Bio. This was followed by priming with Oligo(dT) and subsequent amplification using specific oligonucleotide primers based on the rat corresponding gene sequence (Table I); β -actin served as a housekeeping gene. The primers were synthesized by Shanghai Invitrogen Biotechnology Co., Ltd. (Shanghai, China). Two microlitres of cDNA were amplified in 20 μ l of PCR solution.

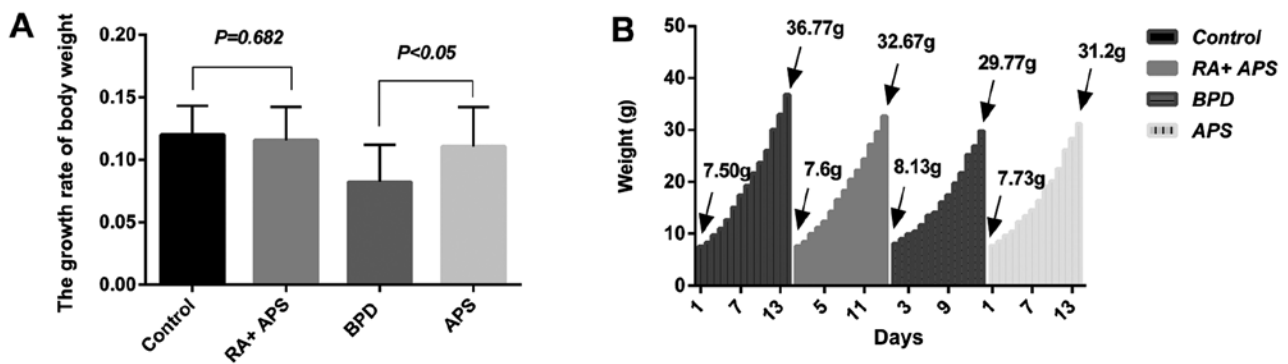


Figure 1. (A) The growth rate of body weight of the control group and *Astragalus* polysaccharides APS group was significantly higher than that of the bronchopulmonary dysplasia (BPD) group ($P<0.05$); no significant difference was found between the control group and RA plus APS group. (B) The body weight of the APS group and BPD group was slightly lower than that of the control group and RA plus APS group pups after 14 days of exposure, and the body weight of the APS group was slightly higher than that in the BPD group. Values are the means \pm SD ($n=8$). *Significantly different from time-matched BPD group ($P<0.05$).

Table II. The mean linear intercept value and the number of alveolar per square area in each group.

Group	MLI (μm)		MAN ($/10^4 \mu\text{m}^2$)	
	Day 10	Day 14	Day 10	Day 14
Control	32.13 ± 2.84^a	33.26 ± 0.74^a	34.03 ± 2.83^b	37.65 ± 4.12^b
RA + APS	31.96 ± 3.55	32.04 ± 2.32	33.60 ± 1.42	36.39 ± 1.53
BPD	38.43 ± 1.01	38.83 ± 1.14	22.45 ± 1.82	25.00 ± 2.19
APS	34.21 ± 0.98^b	35.31 ± 1.37^a	27.33 ± 1.93^a	34.59 ± 0.89^b

Values are the means \pm SD. Data were analyzed using one-way ANOVA and the post hoc Newman-Keuls test. ^aSignificantly different from the time-matched BPD group ($P<0.05$); ^bsignificantly different from the time-matched BPD group ($P<0.01$); $n=6$ separate samples. MLI, mean linear intercept; MAN mean alveolar number; RA, air room; APS, *Astragalus* polysaccharides; BPD, bronchopulmonary dysplasia.

The thermal cycling parameters consisted of 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec.

Western blot analysis. Protein was extracted from the lung tissue of the pups in the different groups with lysis buffer using the Total Protein Extraction Reagent kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). The protein concentration of each sample was measured using the KeyGen BCA Protein assay (Nanjing KeyGen Biotech). Protein extract samples ($30 \mu\text{g}/\text{lane}$) were analyzed by 12% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked in 5% non-fat milk in TBS + 0.1% Tween-20, and incubated with EGFL7 (Proteintech Group, Inc., Chicago, IL, USA), Bax (Epitomics-an Abcam Co., Burlingame, CA, USA) and Bcl-2 (Bioworld Technology, Inc., St. Louis Park, MN, USA) polyclonal antibody, and diluted at 1/500; anti- β -actin (ZSGB-Bio, Beijing, China) was used as the cytoplasmic endogenous control diluted at 1/2,000. Anti-rabbit (H+L) HRP and anti-mouse (H+L) HRP diluted at 1:2,000 was used as the secondary antibody with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA) for detection. The membrane was further incubated for 60 min at room temperature. The intensities of the protein bands were analyzed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis. The results are presented as the means \pm SD. Statistical analysis was performed using SPSS 13.0 statistical

software. One way analysis of variance and the Student-Newman-Keuls test were used for data analysis. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Body weight. The body weight of the hyperoxic pups was slightly lower than that of the normoxic (control and RA plus APS groups) pups; the body weight of the rats in the APS group was slightly lower than that of the rats in the control and RA plus APS groups, although higher than that of the rats in the BPD group after 14 days of exposure. The growth rate of body weight in the control group and RA plus APS group was slightly higher than that of the APS group and BPD group. The growth rate of the APS group was significantly higher than that of the BPD group ($P<0.05$) and there were no statistically significant difference among the APS group, RA plus APS group and the control group (Fig. 1).

Histological evaluation. As shown in Fig. 2, no significant histological damage was observed in the control group and RA plus APS group. In the pups with hyperoxia-induced BPD, a number of inflammatory cells infiltrating the interstitial lung was observed, and there were fewer and larger simplified alveoli, variable interstitial fibroproliferation, and fewer and dysmorphic capillaries. Treatment with APS significantly attenuated the extent and severity of the histological signs and

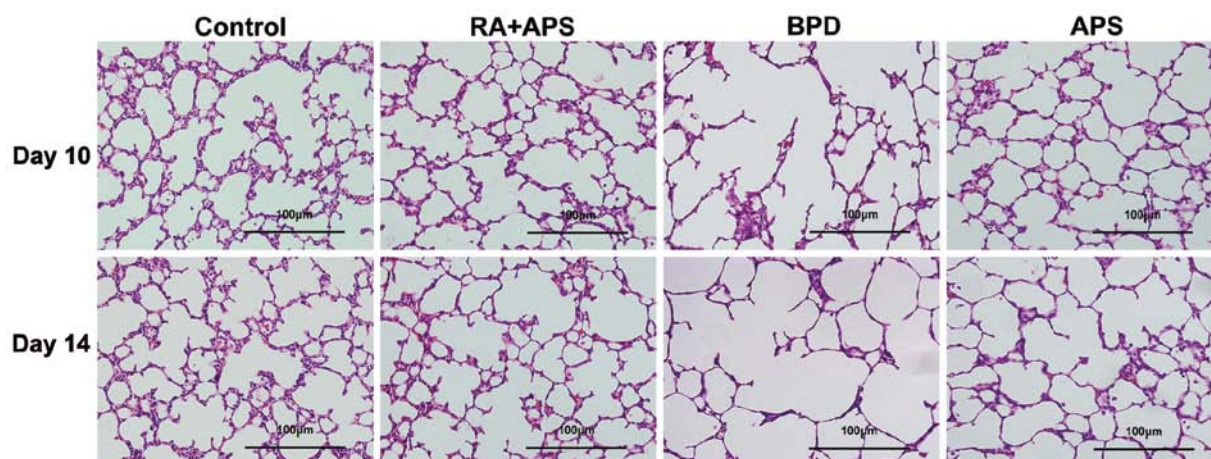


Figure 2. Haematoxylin and eosin staining of lung tissue (magnification, $\times 200$). There was almost no damage observed in the control group and RA plus APS group. There were changes in histology in the BPD group on days 10 and 14; in the lung tissue, cell infiltration was observed, and alveolar and vascular development was arrested. The extent of inflammation, lung injury and alveolar and lung vascular development in the APS group on days 10 and 14 were improved compared with the BPD group. RA, air room; APS, *Astragalus* polysaccharides; BPD, bronchopulmonary dysplasia.

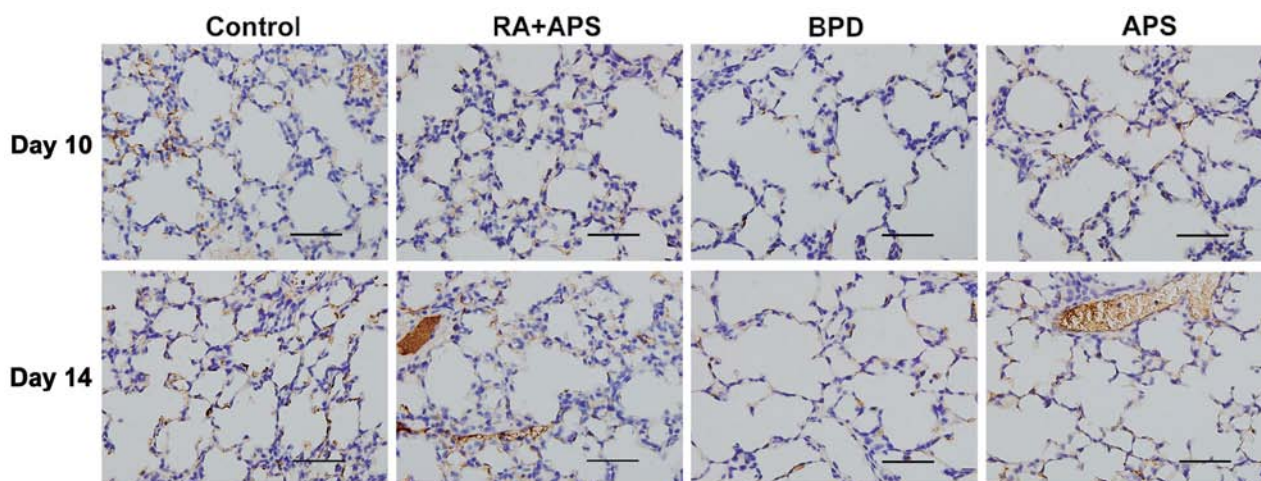


Figure 3. CD31 immunohistochemistry of lung tissue from each groups of pups. Tissue was immunostained with a monoclonal antibody to CD31 (scale bars, $50\ \mu\text{m}$; magnification, $\times 400$). RA, air room; APS, *Astragalus* polysaccharides; BPD, bronchopulmonary dysplasia.

evidently prevented the development of lung damage compared to the BPD group. As compared with the BPD group, the MLI was significantly low and the MAN per square area was significantly high in the APS group ($P < 0.05$ or $P < 0.01$) (Table II).

Detection of CD31 protein expression and vascular density by immunohistochemistry. The cells positive for CD31 show a brown-yellow cytoplasm and the negative control shows an absence of staining. CD31 protein was mainly expressed in the cytoplasm of the pulmonary microvascular endothelial cells. Both CD31 and vascular density in the BPD group were decreased as compared with the control group. The expression of CD31 and vascular density in the APS group was significantly increased compared with the BPD group at each time point ($P < 0.05$ or $P < 0.01$) (Figs. 3 and 4).

Changes in the mRNA expression of Bax, Bcl-2 and EGFL7 detected by quantitative (real-time) PCR. The mRNA expression of EGFL7 and Bcl-2 in the rats in the BPD group was significantly low, while the Bax mRNA level was significantly

high compared with the control group at each time point ($P < 0.01$). In the APS group, the expression of EGFL7 and Bcl-2 was significantly increased and that of Bax was significantly decreased compared with the BPD group on days 4, 10 and 14 ($P < 0.05$ or $P < 0.01$). There was no significant difference observed between the RA plus APS group and the control group (Fig. 5).

Detection of the Bax, Bcl-2 and EGFL7 protein expression in lung tissue by western blot analysis. The protein levels of Bax, Bcl-2 and EGFL7 in the lung tissue the pups in each group were examined on days 4, 10 and 14. The results from western blot analysis revealed that the protein expression of Bcl-2 and EGFL7 in the BPD group was decreased, while the protein expression of Bax was increased compared with the control group on days 4, 10 and 14 ($P < 0.01$). In the APS group, the protein expression of Bcl-2 and EGFL7 was significantly increased, while that of Bax was significantly decreased compared with the BPD group. The protein expression of Bax, Bcl-2 and EGFL7 in the RA plus APS groups did not differ significantly from that of control group at any time point (Fig. 6).

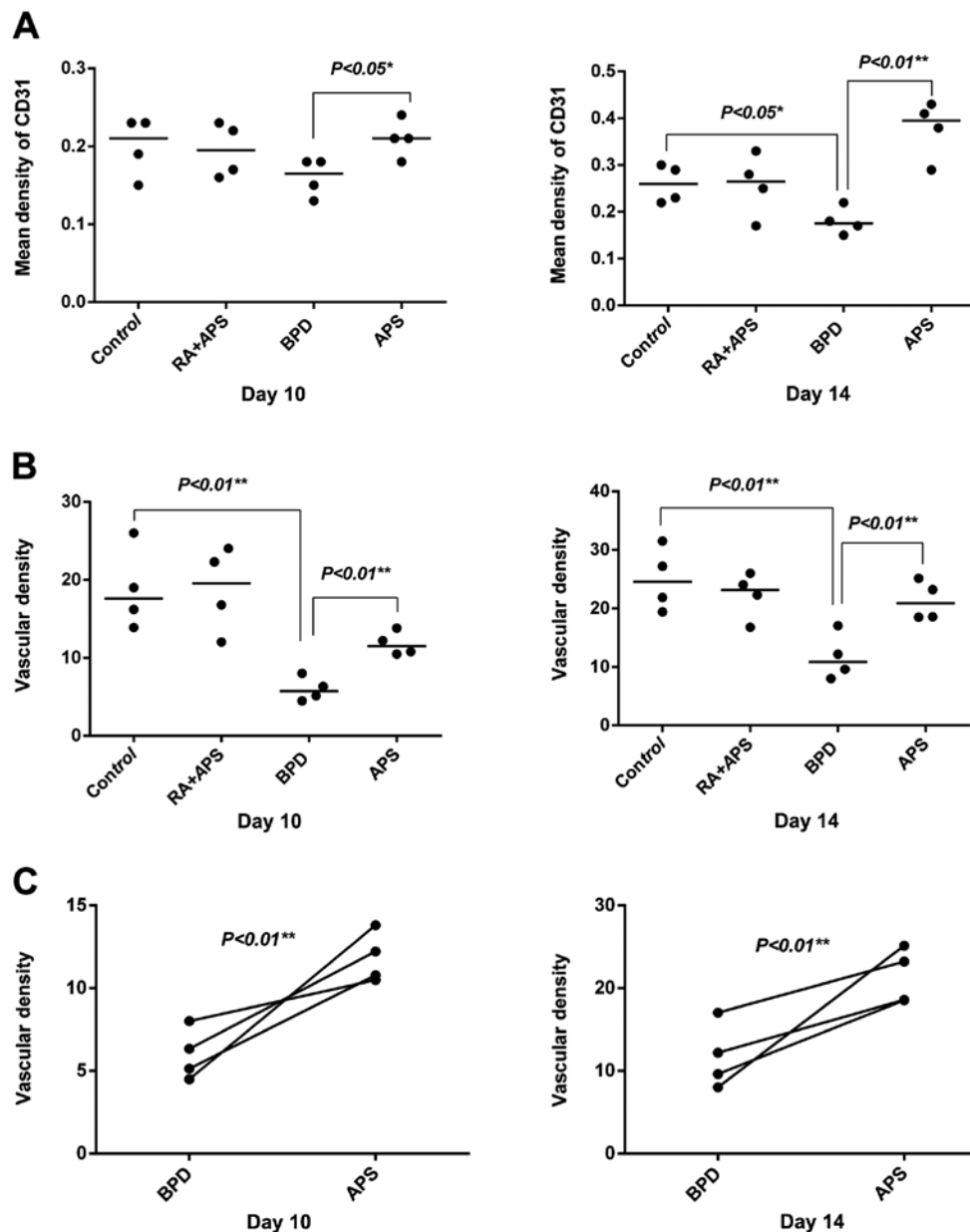


Figure 4. Quantification of immunohistochemistry. (A) Mean density of CD31 was measured to represent relative protein expression of CD31. (B) Comparison of vascular density in each group. The area of CD31 immunostained endothelial cells as a proportion of total lung parenchymal cell area was measured. (C) The contrast of vascular density between the BPD group and APS group. Values are the means \pm SD (n=4). *Significantly different from time-matched BPD group ($P < 0.05$). **Significantly different from time-matched BPD group ($P < 0.01$). RA, air room; APS, *Astragalus* polysaccharides; BPD, bronchopulmonary dysplasia.

Discussion

Bronchopulmonary dysplasia (BPD) is the product of a heterogeneous group of lung disorders that begin in the neonatal period. The overall incidence of BPD has not changed over the past decades. What is more, it remains a challenge for the future (3).

There is increasing evidence indicating that oxidative stress plays a key role in the development of BPD. In this study, we found serious damage to the lung tissue in the BPD model group. The balance of the production of reactive oxygen species (ROS) and the antioxidant defenses disturbed by exposure to high oxygen concentrations, leads to biochemical and histological effects being observed in the lung tissue. Furthermore, hyperoxia has the ability to modify cellular macromolecules, thereby promoting cell death (30).

Previous studies have demonstrated that the disruption of normal lung vascular growth plays a vital role in the pathogenesis of BPD (31). CD31, a marker for endothelial cells, is decreased in lung tissues in BPD (32). However, in the present study, the damage to the lung tissue of newborn rats in the APS group was mild, with vascular development, compared with the BPD model group. CD31 expression in lung tissue increased by almost 2-fold in the APS group compared to the BPD group, and vascular density also significantly improved in the APS group compared with the BPD group. This indicated that APS markedly improved pulmonary injury in the newborn rats with BPD by reducing oxidative damage. The antioxidant effects of *Astragalus* in brain and kidney tissue following ischemia-reperfusion injury have also been demonstrated (33).

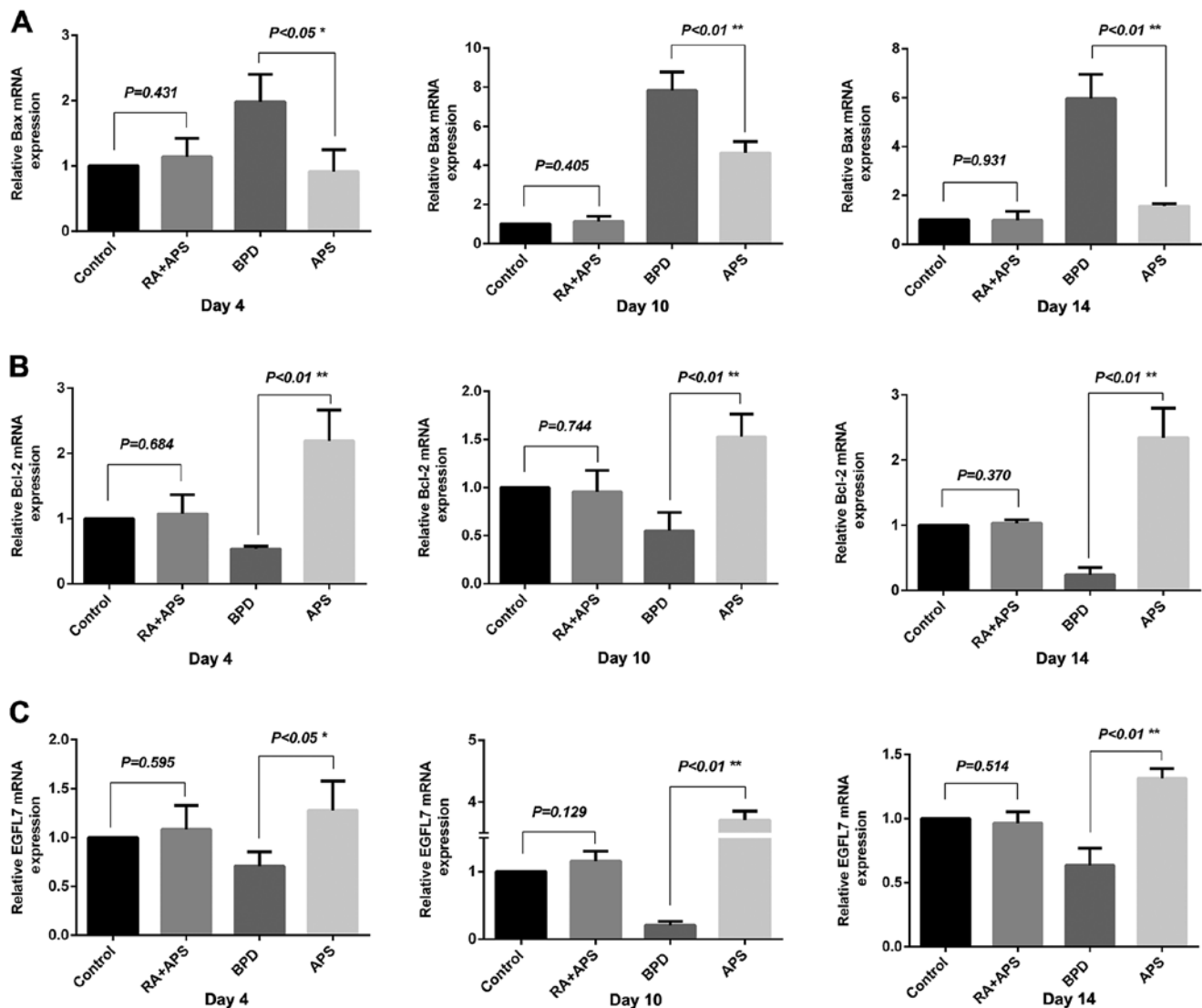


Figure 5. The mRNA expression of Bax, Bcl-2 and epidermal growth factor-like domain 7 (EGFL7) was measured using the $2^{-\Delta\Delta CT}$ method. Real-time PCR analysis revealed that the mRNA levels of Bcl-2 and EGFL7 in the BPD model group were significantly low compared with the control group on days 4, 10 and 14 (** $P<0.01$), while in the APS group the expression of Bcl-2 and EGFL7 was significantly upregulated compared with the BPD model group at each time point (** $P<0.01$). The mRNA expression of Bax showed an opposite trend. Values are the means \pm SD ($n=8$). *Significantly different from time-matched BPD group ($P<0.05$). **Significantly different from time-matched BPD group ($P<0.01$). RA, air room; APS, *Astragalus* polysaccharides; BPD, bronchopulmonary dysplasia.

In the present study, both the mRNA and protein levels of EGFL7 in the BPD model group were significantly decreased compared with the control, as shown by RT-qPCR and western blot analysis. However, the mRNA and protein expression of EGFL7 was significantly increased in the APS intervention group compared with the BPD model group. It is known that EGFL7 is a secreted protein specifically expressed by endothelial cells and is highly expressed in the lungs, heart, kidneys, spleen and uterus (34,35), and is an important tubulogenic factor in the process of vasculogenesis. Xu *et al* (10) proposed that the endothelial-specific growth factor, EGFL7, may play a role in hyperoxia-induced vascular injury. The knockdown of the gene in zebrafish has been shown to result in a severe impairment of arterial and venous endothelial cell cord segregation, leading to the formation of midline angioblast aggregates (36,37). The overexpression of EGFL7 reduces the expression of the pro-apoptotic protein, Bax, and increases the expression of the anti-apoptotic protein, Bcl-2, which prevents hyperoxia-induced

endothelial cell death and promotes lung vascular development (10).

Hockenbery *et al* (39) found that the overexpression of Bcl-2 completely suppressed lipid peroxidation. In other words, Bcl-2 functions in an antioxidant pathway to prevent apoptosis. It is known that Bax, a pro-apoptotic member of the Bcl-2 family of proteins, promotes apoptosis by binding Bcl-2 and inhibiting its anti-apoptotic function during mitochondria-regulated programmed cell death (40). Previous studies have demonstrated that the exposure of newborn mice to hyperoxia increased the mRNA expression pro-apoptotic Bax and the number of apoptotic lung cells (41). In this study, we found that the expression of Bcl-2 was significantly decreased in the BPD group and was significantly increased in the APS group both at the mRNA and protein level, while the expression of Bax showed an opposite trend. This suggests that APS exert protective effects against BPD. The protective effect of APS against hyperoxia-induced lung injury may occur through the

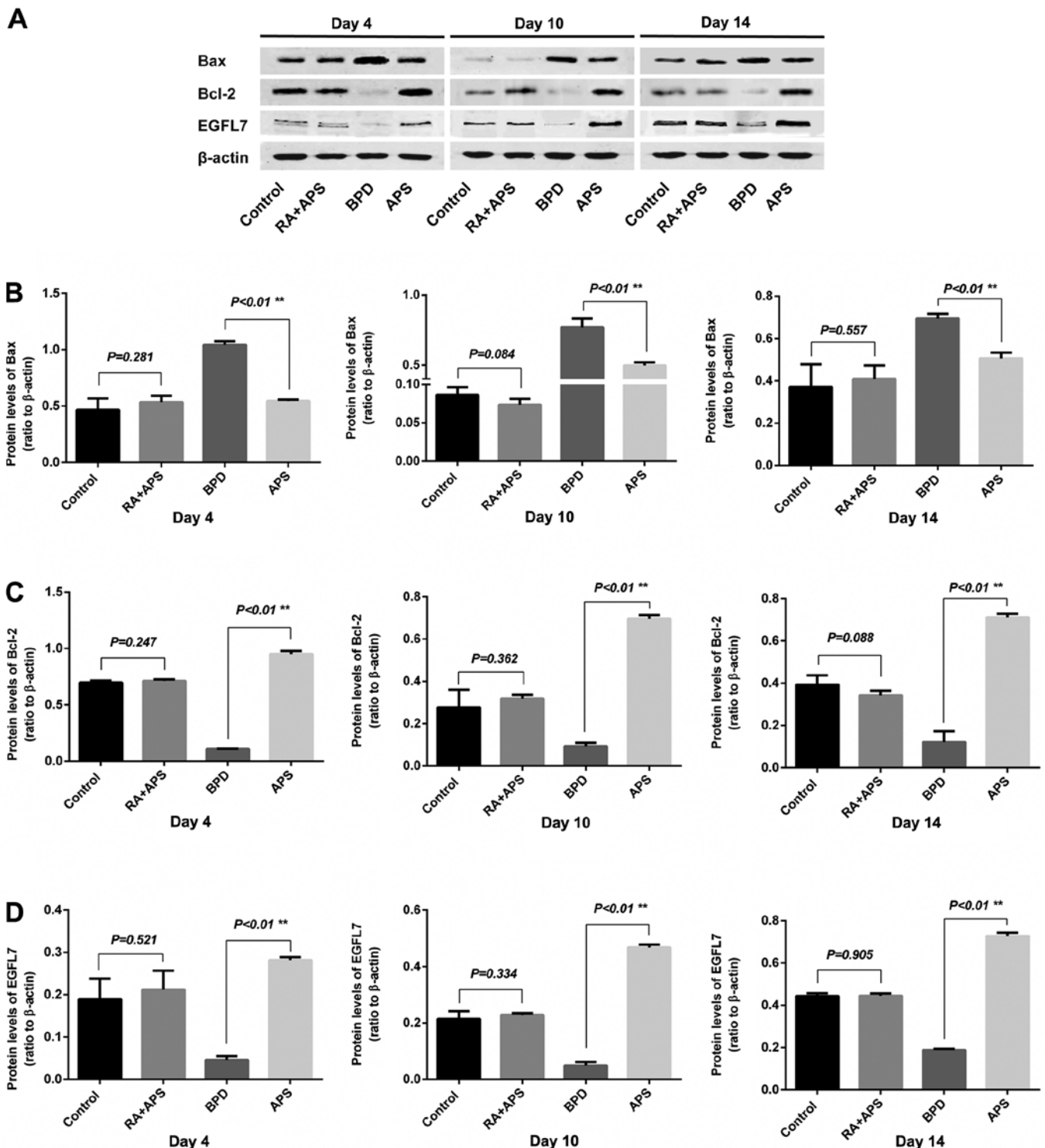


Figure 6. (A) The protein expression of Bax, Bcl-2 and epidermal growth factor-like domain 7 (EGFL7) in lung tissues from each group was examined by western blot analysis. (B) Quantification of western blot analysis measured by the mean ratios of Bax/ β -actin, Bcl-2/ β -actin and EGFL7/ β -actin. β -actin was used to verify equivalent loading. Values are the means \pm SD (n=8). *Significantly different from time-matched BPD group ($P<0.05$). **Significantly different from time-matched BPD group ($P<0.01$). RA, air room; APS, *Astragalus polysaccharides*; BPD, bronchopulmonary dysplasia.

upregulation EGFL7 expression. Emerging evidence links the pathophysiology of BPD to an imbalance between anti-apoptotic and pro-apoptotic signaling pathways and an arrest in alveolar and vascular development (42).

The present study demonstrates that APS upregulate the expression of EGFL7 and Bcl-2 and downregulates Bax expression and significantly reduces alveolar damage, exerting

protective effects on lung tissue in BPD, which was closely related to the inhibition of the endothelial cell apoptotic pathway. Our results demonstrated that APS exerted beneficial effects (antioxidant and anti-inflammatory effects) in the rats with hyperoxia-induced BPD. The exposure of newborn rats to a high oxygen environment caused lung injury which was very similar to human BPD. We speculate that the upregulation of

the expression of EGFL7 may be the target of potential drugs for BPD. Thus, APS may have potential for use as a therapeutic strategy for BPD in neonates.

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