

The protective effects of apocynin in hyperoxic lung injury in neonatal rats

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Abstract

Aim: Inflammation and oxidate stress are significant factors in the pathogenesis of bronchopulmonary dysplasia (BPD). The aim of this study is to investigate the efficacy of apocynin (APO), an anti-inflammatory, antioxidant, and antiapoptotic drug, in the prophylaxis of neonatal hyperoxic lung injury.

Method: This experimental study included 40 neonatal rats divided into the control, APO, BPD, and BPD + APO groups. The control and APO groups were kept in a normal room environment, while the BPD and BPD + APO groups were kept in a hyperoxic environment. The rats in the APO and BPD + APO groups were administered intraperitoneal APO, while the control and BPD rats were administered ordinary saline. At the end of the trial, lung tissue was evaluated with respect to the degree of histopathological injury, apoptosis, oxidant and antioxidant capacity, and severity of inflammation.

Result: The BPD and BPD + APO groups exhibited higher mean histopathological injury and alveolar macrophage scores compared to the control and APO groups. Both scores were lower in the BPD + APO group in comparison to the BPD group. The BPD + APO group had a significantly lower average of TUNEL positive cells than the BPD group. The lung tissue examination indicated significantly higher levels of mean malondialdehyde (MDA), total oxidant status (TOS), tumor necrosis factor- α (TNF- α), and interleukin-1 β (IL-1 β) in the BPD group compared to the control and APO groups. While the TNF- α and IL-1 β levels of the BPD + APO group were similar to that of the control group, the MDA and TOS levels were higher compared to the controls and lower compared to the BPD group. The BPD group demonstrated significantly lower levels/activities of mean total antioxidant status, glutathione reductase, superoxide dismutase, glutathione peroxidase in comparison to the control and APO groups. While the mean antioxidant enzyme activity of the BPD + APO group was lower than the control group, it was significantly higher compared to the BPD group.

Conclusion: This is the first study in the literature to reveal through an experimental neonatal hyperoxic lung injury that APO, an anti-inflammatory, antioxidant, and antiapoptotic drug, exhibits protective properties against the development of BPD.

KEYWORDS

anti-inflammatory, antioxidant, apocynin, bronchopulmonary dysplasia, chronic lung disease, newborn

1 | INTRODUCTION

Despite significant advances in perinatal care, bronchopulmonary dysplasia (BPD) remains one of the most common, complex, and intriguing diseases in preterm infants and perinatal medicine.^{1,2} The pathogenesis of BPD involves multiple factors including developmental arrest of the premature lung, infection, oxygen toxicity, and ventilation-induced lung injury.³

Inflammation is the common pathway leading to BPD. Supplemental oxygen, positive pressure ventilation, and postnatal sepsis all cause lung inflammation and are clinically associated with BPD.⁴ The inflammatory response in the developing lung causes vascular, alveolar, and bronchial growth changes and results in aberrant lung development with dysregulated angiogenesis and alveolarization.⁵ Inflammation accompanied by an accumulation of neutrophils and macrophages into the injured preterm lung results in the elaboration of proinflammatory and profibrotic growth factors, an imbalance of the proteolytic activity in the lung and increased vascular permeability.⁶ Numerous studies have shown that inhibition of inflammatory mediators, receptors, and signaling pathways improve the BPD in rats.⁷⁻⁹

On the other hand, infants at the greatest risk for developing BPD are often exposed to supplemental oxygen in the late canalicular or saccular phases of lung development.³ Hyperoxia is the most prominent and frequently examined postnatal risk factor for BPD. Its pathogenesis includes epithelial and endothelial cell death, inflammation, impaired mitochondrial function, and decreased resident lung and systemic progenitor cells.² Hyperoxia leads to exaggerated production of reactive oxygen species (ROS), including hydrogen peroxide and hydroxyl radicals, and causes oxidative stress. Oxidative stress indicates an imbalance between ROS and corresponding antioxidant counterparts; the resulting toxic levels of ROS causes damage to lipids, proteins, and DNA. ROS induce apoptotic cell death in various cell types, and deregulation of apoptosis causes clinical disorders. ROS are highly unstable, and their formation is typically regulated by various compounds called antioxidant agents, both enzymatic (such as superoxide dismutase [SOD], catalase, and glutathione peroxidase [GSH-Px]) and nonenzymatic.^{10,11}

The nicotinamide adenine dinucleotide phosphate oxidase (NOX) is the major enzyme responsible for ROS production, and inhibition of this enzyme represents an appealing therapeutic target for the treatment of many diseases.^{12,13} Apocynin (APO) (4-hydroxy-3-methoxy-acetophenone) is extracted from the roots of *Apocynum cannabinum* (Canadian hemp) and *Picrorhiza kurroa* (Scrophulariaceae). It is a natural occurring methoxy-substituted catechol that selectively and effectively inhibits NOX in activated leukocytes, preventing production of ROS.¹⁴ Several cell cultures and animal studies have

demonstrated that APO can diminish neutrophil chemotaxis and neutrophil oxidative burst, and thus reduce neutrophil-mediated cell injury.^{15,16} Contemporary studies have shown that APO inhibited NOX expression and NOX activity, reduced lipid peroxidation, suppressed the nuclear factor- κ B (NF- κ B) pathway, and subsequent transcription of proinflammatory cytokines in lung tissue, thus attenuating lung injury.¹⁷ APO also inhibited the production of proinflammatory cytokines such as tumor necrosis factor- α (TNF- α), Interleukin-1 β (IL-1 β), and IL-6.¹⁸

Many studies have shown the beneficial antioxidant, anti-inflammatory and antiapoptotic effects of APO in inflammatory airway/lung diseases like asthma, chronic obstructive pulmonary disease, lung fibrosis.¹⁷⁻²⁵ In the absence of effective interventions, the treatment of BPD is currently a major challenge. Therefore, considering the antioxidative and anti-inflammatory activities of APO, we hypothesized that APO could potentially have protective effects against BPD. In the current study, we evaluated the effects of APO on BPD through a hyperoxia-based model of BPD in the newborn rat.

2 | MATERIALS AND METHODS

2.1 | Animal model and treatment

This study was approved by the Experimental Animal Ethics Committee of Inonu University Faculty of Medicine (Protocol No: 2017/A-25) with respect to the National Institute of Health Guide for the Care and Use of Laboratory Animals. It was conducted at the Inonu University Experimental Animal Production and Research Center and laboratories of Physiology and Histology/Embryology.

Timed pregnant Wistar rats were kept in a 12-h dark/light cycle and fed a standard rat chow (Special Diet Services) ad libitum. Breeding pairs were allowed access for 1 h on the day female rats showed very specific sexual behaviors such as lordosis, hopping, and air-flapping. Pups were delivered spontaneously at full gestation and only pups delivered during a specified 24-h period were used. The pups were randomly distributed to the dams to achieve 10 pups per group. A total of 40 pups were derived from four dams. All pups were left with their dams to breastfeed freely. Experimental treatments began on postnatal Day 1 and lasted until postnatal Day 10, with the date of birth being Day 1.

All animal experiments were done once but with several animals in each group. Forty rat pups were randomly divided into four groups. Both male and female rats were used in the all groups in equal numbers. The pups in one group ($n = 10$) were kept in a normal room environment; these pups served as the control group. The second group ($n = 10$) was exposed to hyperoxia (BPD group), while the third group ($n = 10$) was

exposed to hyperoxia but also treated with APO (BPD + APO group). The pups in the fourth group ($n = 10$) were kept in a normal room environment and also treated with APO (APO group). The pups exposed to hyperoxia were kept in transparent Plexiglas chambers in which the oxygen concentration (90%–95%) was monitored continuously with an oxygen sensor and CO_2 was eliminated through soda lime absorption. The oxygen concentration was sustained at 90%–95% using a flow of 2.5 L/min. Hyperoxia is administered continuously through 10 days to pups in corresponding groups. The control group was also kept in a similar chamber, as previously described.²⁶ For all groups, humidity and environmental temperature were maintained at >80% and 22–25°C, respectively. The rats were evaluated daily with regard to weight, signs of disease, and mortality.

All pups were weighed each morning and received daily intraperitoneal injections of either APO or placebo (normal saline) from Day 1 of life to Day 10. The pups in groups three and four received APO injections at a dose of 20 mg/kg body weight in 0.25 ml phosphate-buffered saline (Apocynin; Sigma-Aldrich),²⁰ whereas the pups in the control and BPD groups were given normal saline (0.25 ml) by intraperitoneal injection using a 1/2 in. (26 gauge, 13 mm) needle.

To avoid O_2 toxicity to dams exposed to hyperoxia, dams were switched every 24 h between the 21% O_2 and 90%–95% O_2 exposure groups. This addresses the oxygen toxicity issues in adult rats, which are highly susceptible to prolonged periods of hyperoxia. The injections were administered in the morning while changing bedding, water, and chow. All animals were kept in the same room with a light/dark cycle of 12 h. On postnatal Day 11, the animals from each group were killed.

2.2 | Tissue preparation

The tissue preparation process has been described in a previous study.²⁶

2.2.1 | Assessment of oxidative stress and antioxidant enzyme activity in lung tissue

The tissue homogenization process has been briefly described in our previous studies.^{8,26} Lipid peroxidation (as malondialdehyde [MDA]) levels in lung homogenate were measured with the thiobarbituric acid reaction by the method of Esterbauer and Cheeseman.²⁷ The quantification of thiobarbituric acid reactive substances was determined by comparing the absorption with a standard curve of MDA equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane. The values of MDA were expressed as nmol/mg protein. Total (copper–zinc and manganese) SOD (EC 1.15.1.1) activity was determined according to the method of Sun et al.²⁸ The principle of the method is based on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine–xanthine oxidase system as a superoxide generator. One unit of SOD was defined as the amount of enzyme causing 50% inhibition in the NBT reduction rate. SOD activity was also expressed as U/mg protein.

Determination of GSH-Px activity was measured by the method of Paglia and Valentine.²⁹ An enzymatic reaction in a tube containing NADPH, GSH-Px, sodium azide, and glutathione reductase was initiated by the addition of H_2O_2 , and the change in absorbance at 340 nm was observed by a spectrophotometer. Activity was given in U/mg protein. GSH content was determined according to Ellman.³⁰ GSH reacts with 5,5'-dithiobis-2-nitrobenzoic acid, and the absorbance spectra of the product have a maximum absorbance at 410 nm. The results were expressed as $\mu\text{mol/mg}$ protein. Total oxidant status (TOS) was determined using a novel automated measurement method, developed by Erel.³¹ Oxidants present in the sample oxidize the ferrous ion–O-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric iron makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide. Total antioxidant status (TAS) levels were determined using a novel automated colorimetric measurement method developed by Erel.³¹ In this method, the hydroxyl radical, the most potent biological radical, is produced by the Fenton reaction and reacts with the colorless substrate O-dianisidine to produce the dianisyl radical, which is bright yellowish-brown in color. Upon the addition of sample, the oxidative reactions initiated by the hydroxyl radicals present in the reaction mix are suppressed by the antioxidant components of the sample, preventing the color change and thereby providing an effective measure of the total antioxidant capacity of the sample.

2.3 | Assessment of lung inflammation

TNF- α and IL-1 β concentrations in lung tissue were measured in duplicates with a commercially available enzyme-linked immunosorbent assay kit in accordance with the manufacturer's instructions (SunRed Biotechnology).

2.4 | Histological evaluation

For light microscopic evaluation, as soon as the lung tissues were removed, they were placed in 10% formaldehyde solution. After the tissues hardened a little in formaldehyde, they were trimmed and left for 3 more days in formaldehyde. After complete fixation, the other tissue procedures were applied. The lung samples were processed through routine tissue techniques and were embedded in paraffin. Paraffin-embedded specimens were cut into 5 μm thick sections, mounted on slides, and stained with hematoxylin–eosin (HE). In addition, the periodic acid schiff (PAS) staining procedure was periodically applied. Stained sections were examined under a Leica DFC280 light microscope by Leica Q Win and Image Analysis System (Leica Micros Imaging Solutions Ltd.).

While scoring the groups, randomly selected preparations were evaluated blindly without knowing the group name. In each preparation, 10 different areas were randomly selected and evaluated. Tissue sections

were examined for alveolar wall thickness, vascular congestion, edema in vessels, hemorrhage, mononuclear cell infiltration, macrophages between alveoli and vacuoli in vessels. The histopathological damage was scored on a scale of 0-3: 0 for normal lungs, 1 for 25% injury involvement, 2 for 26%–75% injury involvement, and 3 for 75% injury involvement. In addition, the diameters of 20 alveoli were measured in randomly selected areas from each section. A total of 120 alveoli diameters were measured in each group at a magnification of $\times 40$. Two different (vertical and horizontal) diameters of each alveolus were measured to identify an average diameter. Alveolar macrophages were counted in 10 randomly selected areas in each section. The number of alveolar macrophages was determined in a total of 100 fields from each group. And the number of alveolar macrophages between groups was scored.

2.5 | Assessment of cell apoptosis in the lungs

Apoptotic cells in lung tissue was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining using apoptotic cell detection kits, as previously described.^{32,33} TUNEL positive cell nuclei were stained a black color. Light microscopy was used to count the number of TUNEL positive cells on ten randomly selected fields for each section. TUNEL positive cells were counted in 10 randomly selected areas from each of the TUNEL stained sections taken from the groups. Cell counts were made in a total of 90 fields in each group, and the average TUNEL positive cell count was calculated.

2.6 | Statistical analysis

2.6.1 | Analysis of histological and apoptosis data

The statistical analysis was carried out using SPSS 13.0 (SPSS Inc.) and MedCalc 11.0 (Ostend) software to test the histological data. All data were expressed as arithmetic mean $\pm$ SE. For comparisons between groups, the Kruskal-Wallis and Conover tests were used.

2.6.2 | Analysis of biochemical data

The IBM SPSS Statistics 24.0 for Windows package program was used for analysis of biochemical data. Quantitative data were expressed as arithmetic mean $\pm$ SD. The Shapiro-Wilk test was used to determine if the data were normally distributed. The Kruskal-Wallis H test was used to compare quantitative variables between groups. For data representing significant differences among groups, multiple comparisons were performed through the Bonferroni Corrected Mann-Whitney U test. The value $p < .05$ was accepted as statistically significant.

3 | RESULTS

Of the 40 pups, 39 survived until the end of the study. One pup in group BPD died during the study period. At birth, on postnatal Day 1, the mean bodyweight of the pups were 5.4 ± 0.6 g. At the end of the study, on postnatal Day 11, the mean body weight of pups were similar in the four groups, that is, control group 12.9 ± 0.8 g, BPD group 12.58 ± 1.4 g, BPD + APO group 13.11 ± 1.1 g, and APO group 13.3 ± 1.2 g ($p > .05$). No significant toxicity was observed in the animals treated with APO.

3.1 | Histopathological findings

In the control (Figure 1A,C,E) and APO (Figure 1B,D,F) groups, lung tissues exhibited a normal histological appearance in terms of bronch, bronchiol, and alveoli. The lung tissues in the BPD group demonstrated alveolar wall thickness (Figure 2A,B,E), vascular congestion (black arrows) (Figure 2A,F), edema in vessels (white asterisks) (Figure 2A), hemorrhage (black asterisks) (Figure 2B), mononuclear cell infiltration (black arrows) (Figure 2C), macrophages between alveoli (thin black arrows) (Figure 2D), vacuoli in vessels (black arrows) (Figure 2F). The BPD group had severe histological alterations compared to the BPD + APO group. Therefore, APO treatment may have potentially decreased the side effects of BPD and may be beneficial against these alterations (Figure 3A–C). In addition, we observed a considerably increased mean histopathological damage score in the BPD group compared to the control and APO groups ($p \leq .05$). On the other hand, the mean histopathological damage score was statistically lower in the BPD + APO group in comparison to the BPD group ($p \leq .05$) (Figure 3D). Furthermore, we have observed prominently increased mean diameters in the BPD group compared to the control groups ($p \leq .05$). However, the mean alveolar diameters statistically decreased in the BPD + APO group in comparison to the BPD group ($p \leq .05$) (Figure 3E).

Alveolar macrophages were identified with the HE staining method, and were also observed through the PAS staining method (Figure 4). In comparison to the control (Figure 4A) and APO (Figure 4D) groups, a great number of alveolar macrophages were observed in the BPD (Figure 4B) group. In the BPD + APO (Figure 4C) group, alveolar macrophages were found to be less than the BPD (Figure 4B) group. Additionally, another notable finding in the BPD group was the increased mean alveolar macrophage score compared to the control and APO groups ($p \leq .05$). However, the mean alveolar macrophage score was significantly lower in the BPD + APO group in contrast to the BPD group ($p \leq .05$) (Figure 4E).

Sections were evaluated by TUNEL (Figure 5) method. The average of TUNEL-positive cells were significantly lower in the BPD + APO (Figure 5C) group compared with BPD (Figure 5B) group. There was a small amount of positive stained cells in the control (Figure 5A) and APO (Figure 5D) groups (Figure 5E).

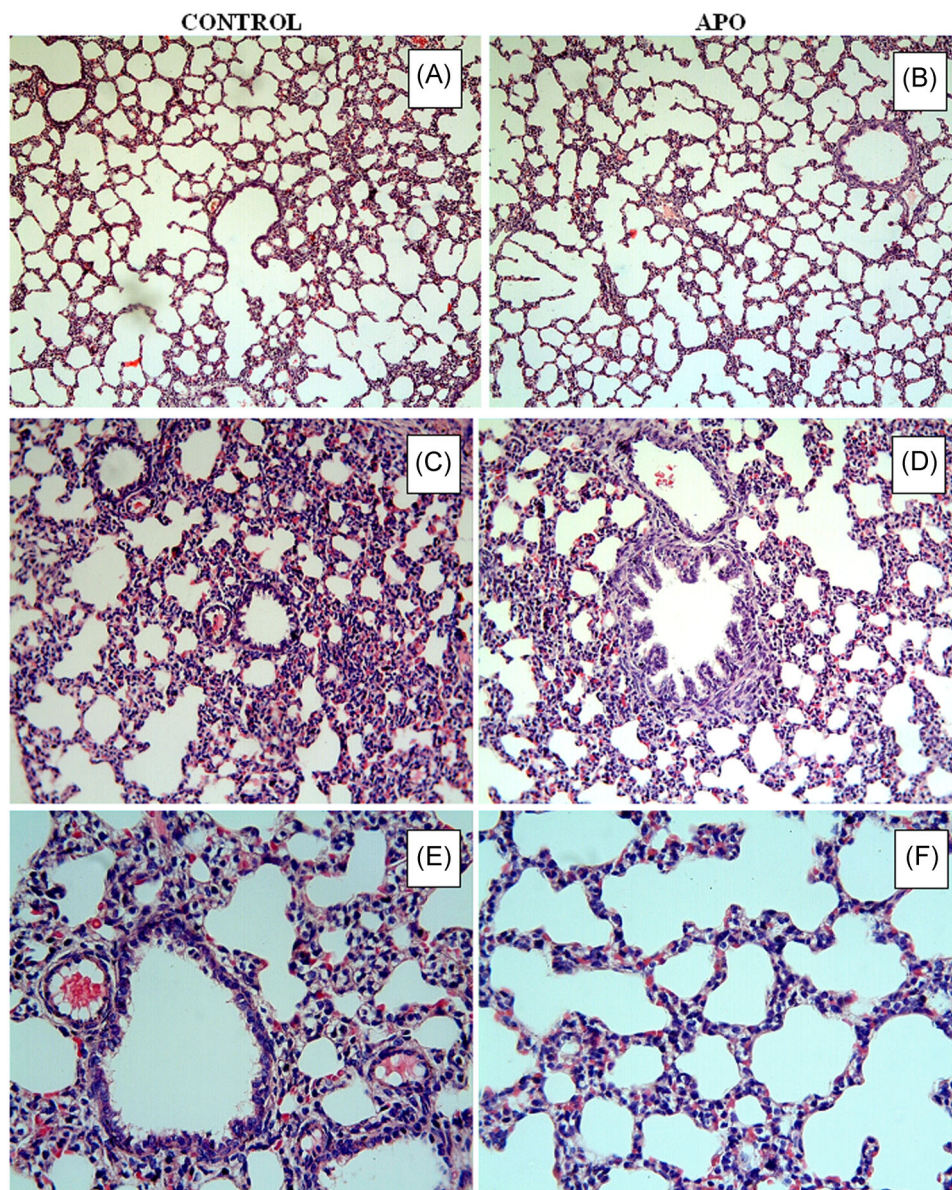


FIGURE 1 In the control (A, C, E) and APO (B, D, F) groups, lung tissue showed normal histological appearance. (A, B) HE; $\times 10$, (C, D) HE; $\times 20$, (E, F) HE; $\times 40$. APO, apocynin, HE, hematoxylin-eosin [Color figure can be viewed at wileyonlinelibrary.com]

3.2 | Biochemical findings

The BPD group exhibited significantly higher mean levels of MDA and TOS (oxidative stress markers) in lung tissue compared to the control and APO groups ($p < .05$). While the mean MDA and TOS levels of the BPD + APO group were higher than that of the control group, they were remarkably lower than the BPD group ($p < .05$) (Figure 6). The lung tissue of the BPD group demonstrated a significantly higher mean inflammatory cytokine level (TNF- α , IL-1 β) in comparison to the control and APO groups ($p < .05$). However, the mean level of TNF- α and IL-1 β were similar among the BPD + APO and control groups ($p < .05$) (Figure 7). The BPD group exhibited significantly lower mean levels/activities of TAS, GSH, SOD and

GSH-Px when compared with the control and APO groups ($p < .05$). While the mean levels of antioxidant enzyme activities were found to be lower in the BPD + APO group compared to the control group, they were significantly higher in comparison to the BPD group ($p < .05$) (Figure 8).

4 | DISCUSSION

This study is the first to describe the effects of APO in an experimental neonatal hyperoxic lung injury. We evaluated the effects of intraperitoneal administration of APO, an inhibitor of NOX, in protecting against hyperoxic induced lung injury in neonatal rats. In the

BPD

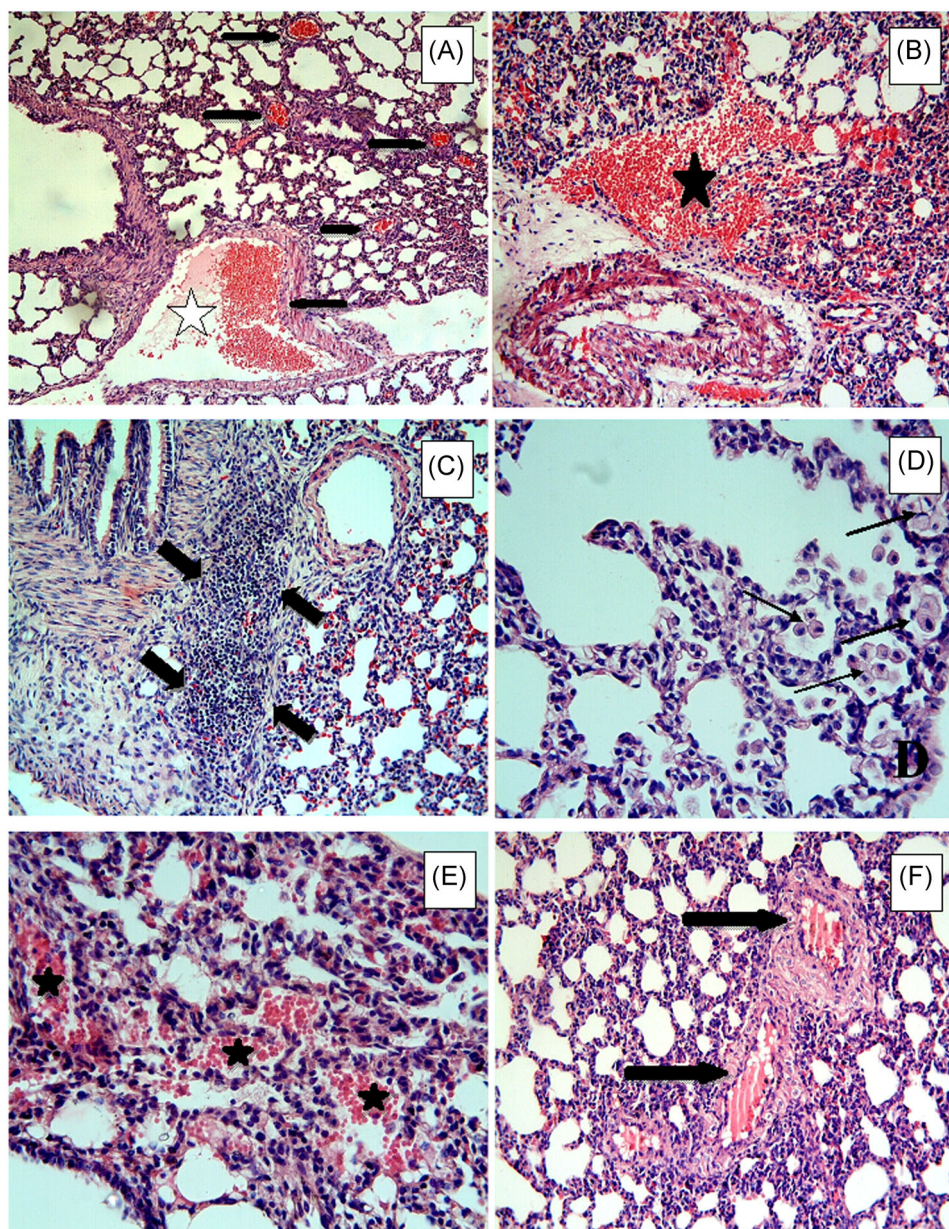


FIGURE 2 The BPD group demonstrating thickness of alveolar wall (A, B, E), vascular congestion (black arrows) (A, F), edema in vessels (white asterisks) (A), hemorrhage (black asterisks) (B, E), mononuclear cell infiltration (black arrows) (C), macrophages between alveoli (thin black arrows) (D), vacuoli in vessels (black arrows) (F). (A) HE; $\times 10$, (B, C, F) HE; $\times 20$, (D, E) HE; $\times 40$. BPD, bronchopulmonary dysplasia; HE, hematoxylin-eosin [Color figure can be viewed at wileyonlinelibrary.com]

current study; when treated with APO, the hyperoxic neonatal rats have demonstrated a significant improvement in lung development in terms of histopathological evaluation, increased antioxidant levels/activities (TAS, GSH, SOD, GSH-Px), decreased lipid peroxidation and oxidative stress (MDA, TOS), decreased inflammatory cytokines (IL-1 β , TNF- α) and inhibition of apoptosis.

BPD, the most common chronic respiratory disease in infants, is characterized by the presence of impaired alveolarization and dysregulated vascularization. High-concentration oxygen inhalation results in restriction of alveolarization and angiogenesis in the

developing lung via increased production of ROS.^{10,34} NOX, a superoxide anion-generating enzyme, is considered to be the main source of ROS, and NOX dependent ROS generation has been described in pulmonary endothelial cells, Type II pneumocytes, airway epithelia cells, and smooth muscle cells.^{12,13} APO is a well characterized inhibitor of NOX.^{14,35} Impellizzeri et al. found that APO has potent effects on carrageenan-induced acute lung inflammation through inhibition of NOX activity.¹⁵ Similarly, many studies have shown that inhibition of NOX using APO protects against LPS as well as ventilation-induced lung injury.^{21,36,37}

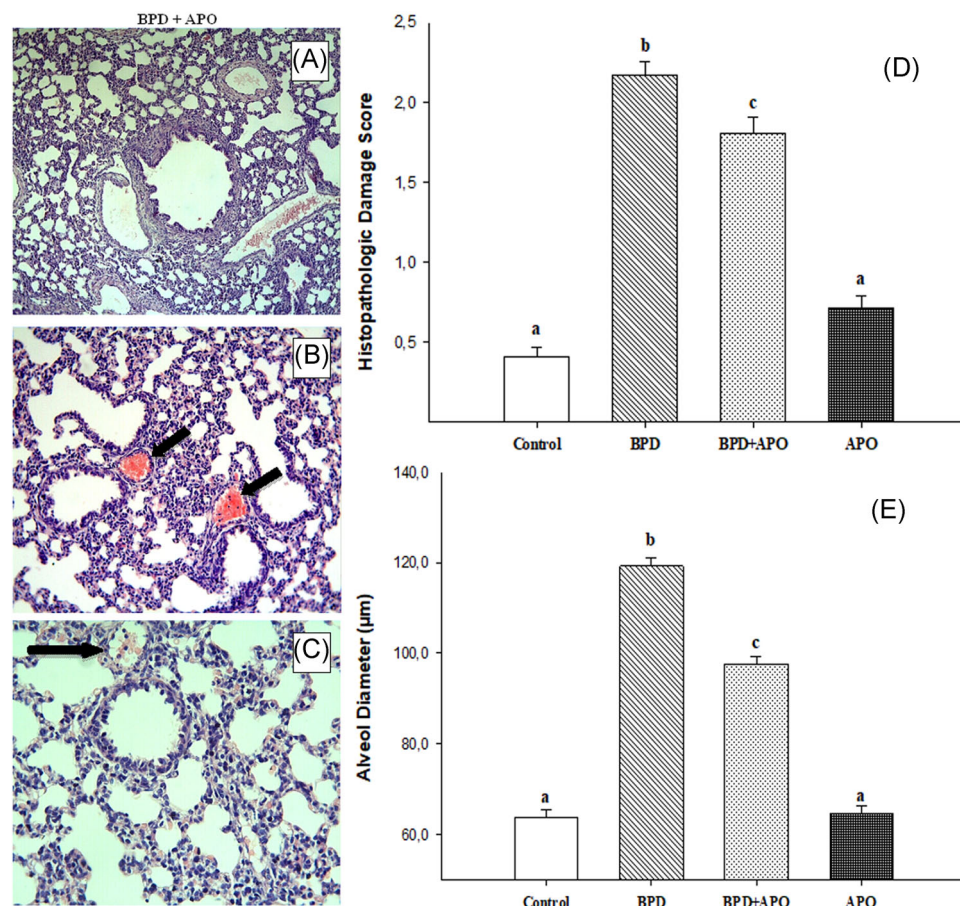


FIGURE 3 In the BPD + APO group, alveolar wall thickness (A–C) and vascular congestion (black arrows) (B) vacuolization (C) were decreased compared to the BPD group, histopathological damage score of the groups (E), alveolar diameter measurement (F). (A) HE; $\times 10$, (B) HE; $\times 20$, (C) HE; $\times 40$. APO, apocynin; BPD, bronchopulmonary dysplasia; BPD + APO, bronchopulmonary dysplasia + Apocynin; HE, hematoxylin-eosin. ^{a,b,c}Different superscript letters are statistically different from each other ($p \leq .05$) [Color figure can be viewed at wileyonlinelibrary.com]

A balance between oxidants and antioxidants is essential for normal pulmonary homeostasis. Generation of ROS can lead to imbalances between prooxidant and antioxidant states, resulting in oxidative stress and cell injury. The defense system of enzymatic antioxidants provide innate protection against lipid peroxidation and consists of CAT, SOD, GSH, and GSH-Px.³⁴ SOD catalyses the dismutation of superoxide anions to oxygen and H_2O_2 . The GSH redox system, which includes GSH, plays an important role in scavenging of ROS and lipid peroxidation. Some innate or synthetic compounds with antioxidant features may reduce lung injury. In the current study, a significant decrease in the levels/activities of TAS, GSH, SOD, GSH-Px was observed in the lung tissue of BPD group rats when compared with the control group, whereas antioxidant enzyme activity levels displayed a significant increase in rats receiving APO prophylaxis (Figure 8). Several previous studies have reported that APO had protective effects in different organs and tissues by increasing the activity of these antioxidant enzymes, which had decreased due to various toxic agents. Abdelmageed et al. showed that APO significantly increased SOD and GSH activities in lung tissue in a model of LPS-induced acute lung injury in rats.²¹ Tanriverdi. et al.

reported that APO increased SOD, CAT, GSH, and TAC levels in heart tissue in isoproterenol-induced myocardial damage.³⁸ In another study, Liu et al. demonstrated that administration of APO in mice with gestational diabetes mellitus significantly enhanced the levels of SOD, GSH, GSH-Px.³⁹ Our results are in accordance with these observations. By enhancing antioxidant enzyme activity, APO may potentially promote beneficial effects in reducing tissue damage. Because, it is well known that oxidative stress leads to excessive production of ROS in the lungs. And, overproduction of ROS has been found to reduce the antioxidant activity of SOD, CAT, TAC, GSH, and GSH-Px in patients.⁴⁰ On the other hand, NOX is the major enzyme responsible for ROS generation in the lungs. And, APO possesses powerful antioxidant and anti-inflammatory properties, associated with its ability to block the activity of NOX; this is achieved by interfering with the binding of cytosolic NOX components to its membrane components.^{14,41}

ROS is widely known to cause injury to lipids, protein, and DNA and therefore may cause a decrease in enzyme activity or structural damage in hyperoxia-induced lung tissue. Our observations, which are supported by this hypothesis, showed a significant increase in

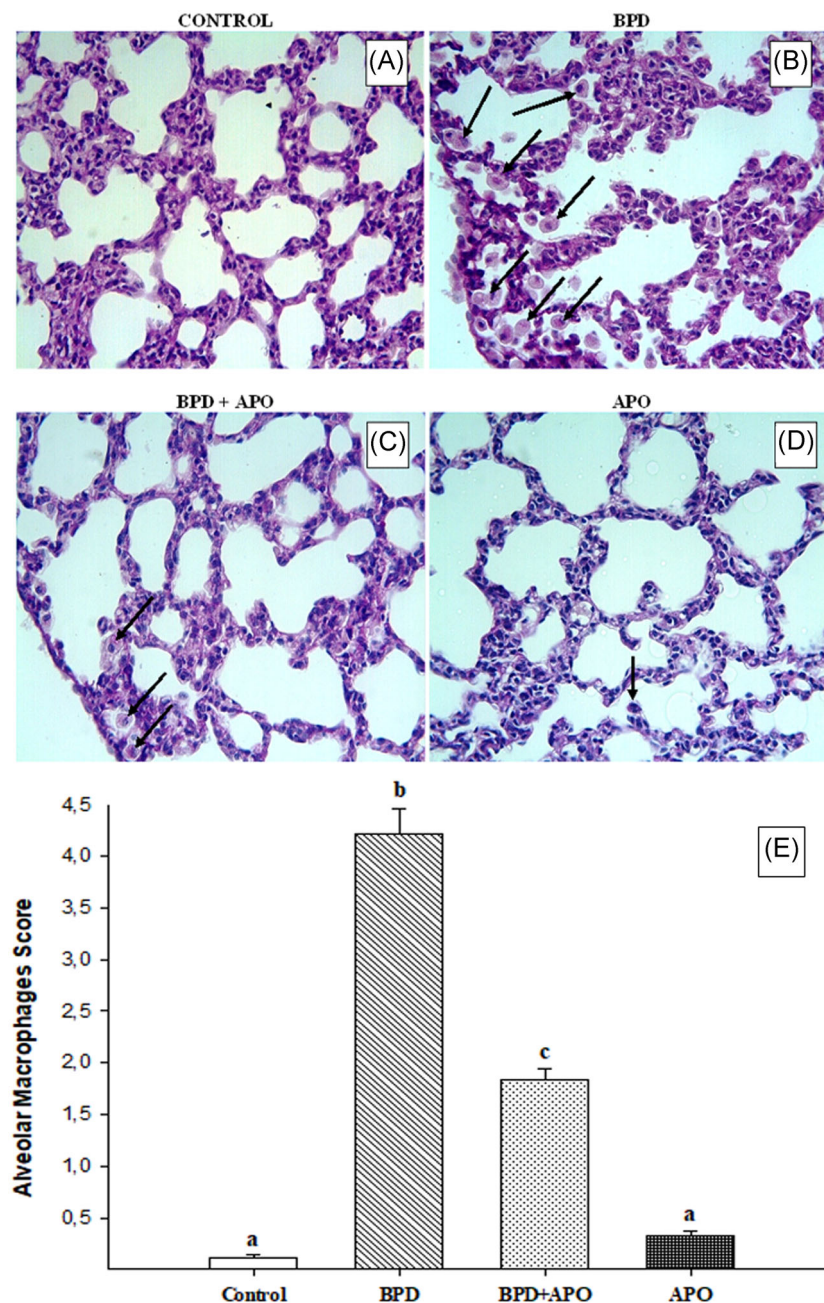


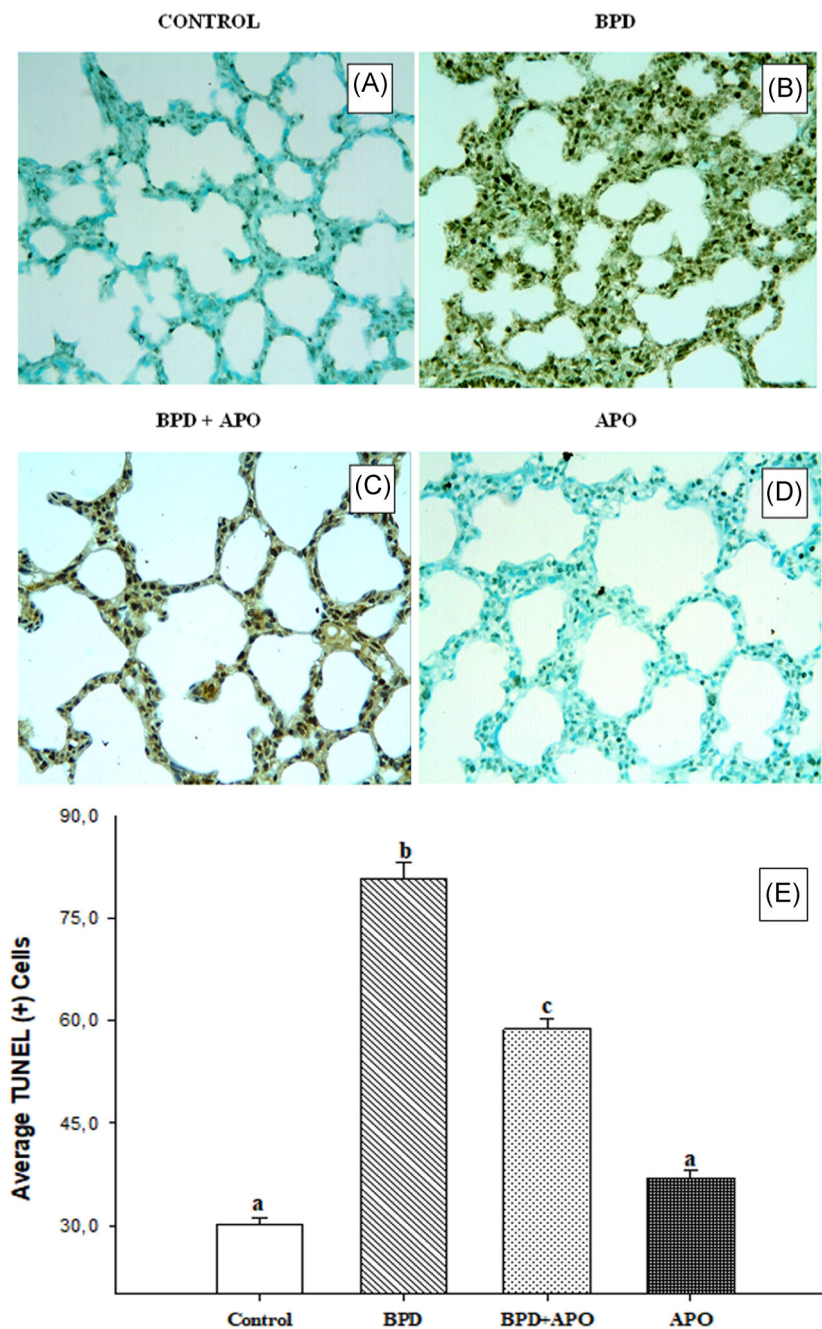
FIGURE 4 Alveolar macrophages were shown in control (A), BPD (B), BPD + APO (C), and APO (D) groups, alveolar macrophages score between the groups (E). (A–D) PAS; $\times 40$. APO: apocynin; BPD, bronchopulmonary dysplasia; BPD + APO, bronchopulmonary dysplasia + Apocynin; PAS, periodic acid schiff. ^{a,b,c}Different superscript letters are statistically different from each other ($p \leq .05$) [Color figure can be viewed at wileyonlinelibrary.com]

MDA and TOS levels, which are very important novel indexes of oxidative stress, in the lung tissue. Many experimental studies have also shown the increase in lipid peroxidation products, such as MDA, and TOS in lung tissue.^{8,20,42,43} On the other hand, in the current study, APO treatment reversed the increase in MDA and TOS induced by hyperoxia (Figure 6). Further damage is probably elicited by augmented amounts of ROS and reactive nitrogen species produced by the activated inflammatory cells recruited into the damaged lung induced by hyperoxia. We believe that the protective role of APO in BPD development is through its inhibitory effects on NOX activity which, in turn, inhibits ROS generation.

Inflammation is the common pathway that initiates the lung injury that can potentially progress to BPD.⁴ Lung inflammation can

cause aberrated alveolarization.^{2–4} Inflammation is typically known to be initiated by early proinflammatory cytokines, including TNF- α , IL-1 β , IL-6, and IL-8. Hyperoxia is a powerful proinflammatory stimulus. Increased oxidative stress plays a key role in the progression of the inflammatory process. It has been shown that lung cells release these cytokines in response to oxidative stress.^{24,44} Our study has demonstrated that pups in the control and APO groups had lower mean levels of TNF- α , IL-1 β in lung tissue along with fewer alveolar macrophages than the pups in the BPD group. More importantly, we have observed that mean levels of TNF- α , IL-1 β in lung tissue, and the mean alveolar macrophage score significantly decreased in the rats after APO treatment (Figures 4 and 7). Kim et al. reported that APO inhibited the production of proinflammatory

FIGURE 5 Representative images of lung sections showing the TUNEL staining. Control (A), BPD (B), BPD + APO (C), and APO (D) groups, average TUNEL positive cells between the groups (E). (A–D) TUNEL $\times 40$. APO, apocynin; BPD, bronchopulmonary dysplasia; BPD + APO, bronchopulmonary dysplasia + Apocynin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling. ^{a,b,c}Different superscript letters are statistically different from each other ($p \leq .05$) [Color figure can be viewed at wileyonlinelibrary.com]



cytokines including TNF- α , IL-1 β , and IL-6 in experimental asthma mice.¹⁸ Similarly, recent studies have demonstrated that APO decreased TNF- α and IL-1 β gene expression or downregulation of these cytokines through experimental animal studies or cell cultures on the different inflammatory lung diseases.^{17,24,45,46} NOX is an enzyme responsible for ROS production, and proinflammatory stimulus induces intracellular ROS via enzymatic activation of NOX, which ultimately induces activation of NF- κ B.^{47,48} NF- κ B is a robust regulator of various proinflammatory cytokine gene expressions in response to external stimuli. Several important proinflammatory cytokines such as IL-6, IL-1 β , and TNF- α were also activated by the NF- κ B signaling pathway.^{17,18,45} Although the differential inhibitory effects by APO on the production of inflammatory cytokines could

not be addressed, we believe that APO effectively inhibited proinflammatory signaling, which prevented activation of NF- κ B and potentially inhibited production of proinflammatory cytokines. Thus, we speculate that pharmacological inhibition of the NF- κ B pathway had an impact on lung alveolarization in newborn pups.

ROS can act directly on oxidized nucleic acid which will lead to DNA strand breaks, thereby inducing cell necrosis or apoptosis.⁴⁹ Because, DNA damage may lead to alteration in cellular signaling pathways or apoptosis of the cell if not fully repaired.⁴⁰ Many studies have shown that levels of apoptosis are elevated in BPD, and that this increase has a crucial effect on alveolar maldevelopment in BPD.^{50,51} Therefore, reducing apoptosis is of significant importance for the treatment of BPD. Many researchers have shown the antiapoptotic

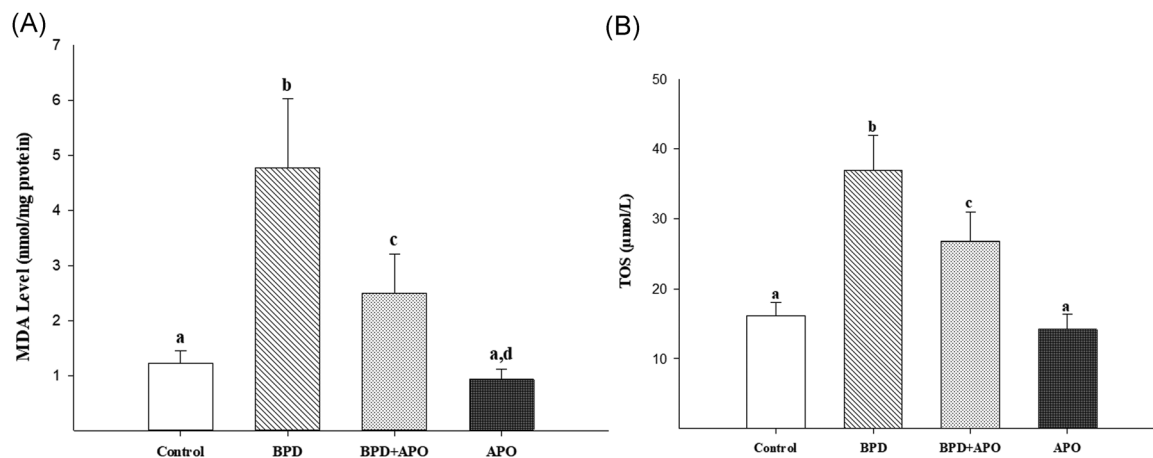


FIGURE 6 The levels of (A) malondialdehyde (MDA) and (B) total oxidant status (TOS) in lung tissue. APO, apocynin; BPD, bronchopulmonary dysplasia; BPD + APO, bronchopulmonary dysplasia + Apocynin. Values are expressed as mean $\pm$ SD. ^{a,b,c,d} Different superscript letters are statistically different from each other ($p \leq .05$)

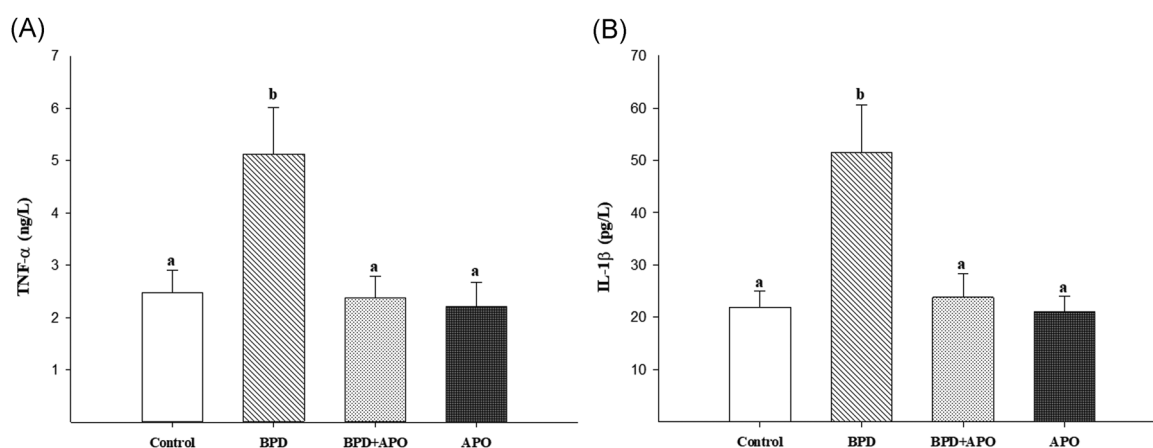


FIGURE 7 The levels of (A) tumor necrosis factor-α (TNF-α) and (B) interleukin-1β (IL-1β) in lung tissue. APO, apocynin; BPD, bronchopulmonary dysplasia; BPD + APO, Bronchopulmonary dysplasia + Apocynin. Values are expressed as mean $\pm$ SD. ^{a,b,c,d} Different superscript letters are statistically different from each other ($p \leq .05$)

effect of APO on various cells. In the current study, TUNEL immunohistochemical staining was performed to analyze lung damage and apoptosis. Our study results showed that increased cell apoptosis induced by hyperoxia was substantially suppressed by APO (Figure 5). It is known that NF-κB plays a pivotal role in cell apoptosis, and that APO inhibits the NF-κB pathway.^{52,53} We believe that APO prevented lung damage by inhibiting apoptosis. In a related study, pharmacological inhibition of NF-κB signaling had a pronounced impact on lung alveolarization in newborn mouse pups.⁵⁴ Besides, the results suggest that APO prevents DNA damage by inhibiting ROS formation through NOX, therefore contributes to inhibition of cellular necrosis and apoptosis. It is noteworthy that the reduced TUNEL results by APO includes both apoptotic cells and cells with oxidative DNA damage.

Prolonged oxidative stress impedes alveolar development in premature infants. Its histology is characterized by hemorrhage and irregularly shaped, enlarged, and saccular-like air spaces surrounded

by thickened septae.⁵⁵ In addition, larger alveolar diameter values correspond to greater disruption of normal alveolar growth. In the present study, the BDP group was observed to have alveolar wall thickening, alveolar hemorrhage, vascular congestion, edema in vessels, mononuclear cell infiltration, increased of alveolar macrophages and vacuoli in vessels (Figure 2). The BDP group had severe histological alterations compared to the BPD + APO group (Figure 3). Moreover, the mean histopathological damage score, mean alveolar macrophage score, and mean alveolar diameters of the BPD group were notably greater than that of the BPD + APO group. Thus, APO prophylaxis could significantly ameliorate lung injury. We speculate that the antioxidant, anti-inflammatory, and antiapoptotic effects of APO have the potential to give substantial results in the treatment of ongoing alveolarization.

A few limitations of our study are important to consider. First, our model has eliminated other possible risk factors (such as immaturity, mechanical ventilation, infection, antenatal steroids,

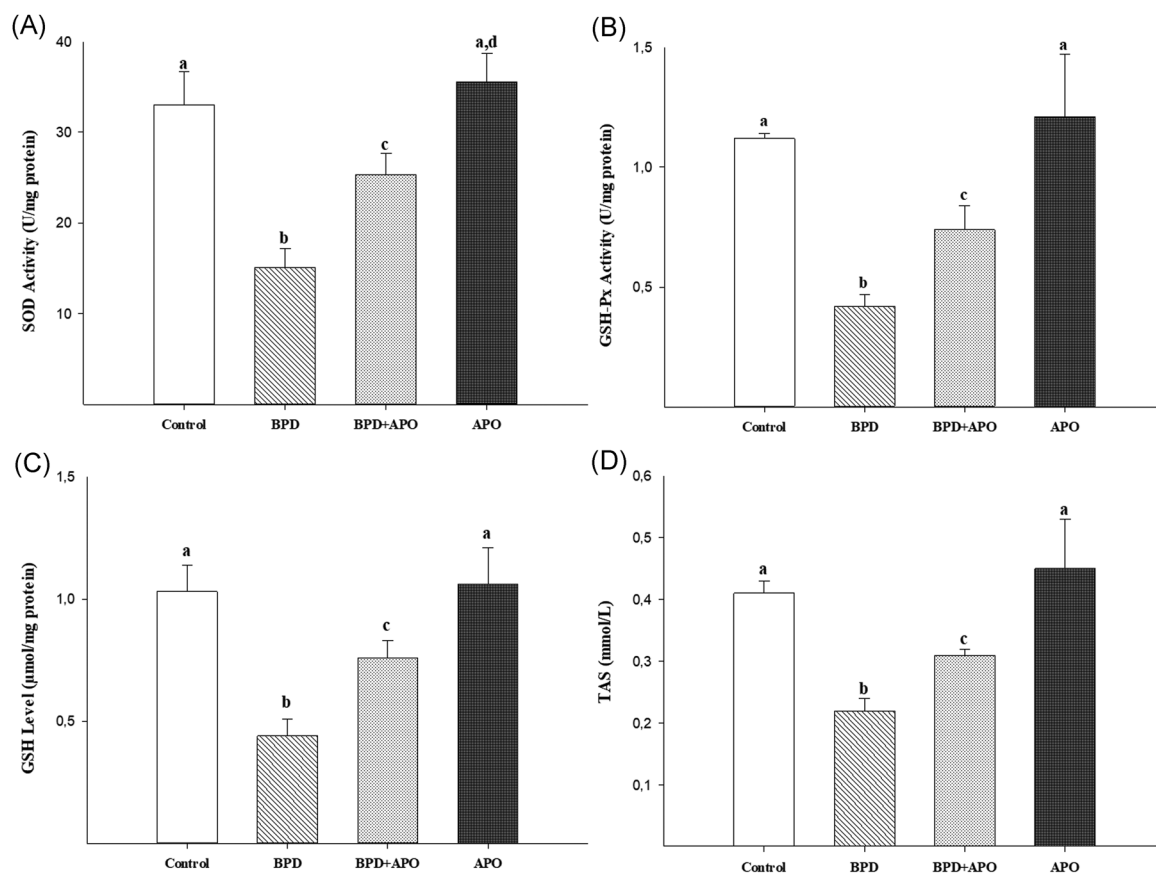


FIGURE 8 The activities of (A) superoxide dismutase (SOD), (B) glutathione peroxidase (GSH-Px), and the levels of (C) glutathione reductase (GSH), (D) total antioxidant status (TAS). APO, apocynin; BPD, bronchopulmonary dysplasia; BPD + APO, bronchopulmonary dysplasia + Apocynin. Values are expressed as mean $\pm$ SD. ^{a,b,c,d}Different superscript letters are statistically different from each other ($p \leq .05$)

maternal smoking, antenatal and postnatal nutrition, genetic background) and has solely depended on large amount of oxygen in the creation of BPD. Second, and correspondingly, this hinders the possibility of identifying the relationship between APO, the basic means of our treatment, and other risk factors. Finally, we did not measure NOX, ROS, NF- κ B, and biomarker of oxidative damage to DNA levels/activities due to our limited equipment.

5 | CONCLUSION

Our results determined for the first time that APO significantly attenuated lung damage via inhibition of oxidative stress as well as augmentation of endogenous antioxidants, suppression of proinflammatory mediators and inflammation, development of better alveolarization, and reduction of apoptosis in an experimental neonatal hyperoxic lung injury. However, further studies are required to evaluate the role of different doses of APO in the treatment of BPD and the effect of systemic administration. If our data is supported by further studies, APO may offer a new and promising therapy for BPD, which has no curative treatment, in the future.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ETHICS STATEMENT

All procedures performed in studies involving animals were in accordance with the ethical standards of the Ethical Committee of Experimental Animals of the Faculty of Medicine in Inonu University, at which the studies were conducted. The manuscript is approved by all authors for publication.

AUTHOR CONTRIBUTIONS

Ramazan Ozdemir: conceptualization (equal); data curation (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (lead); writing original draft (equal). **Ismail Kursat Gokce:** data curation (equal); investigation (equal); methodology (equal); validation (equal); writing review & editing (equal). **Suat Tekin:** formal analysis (equal); methodology (equal); visualization (equal); writing review & editing (equal). **Asli Cetin**

Taslidere: formal analysis (equal); methodology (equal); visualization (equal); writing review & editing (equal). **Hatice Turgut:** conceptualization (equal); resources (equal); software (equal); writing review & editing (equal). **Kevser Tanbek:** data curation (equal); methodology (equal); validation (equal); visualization (equal); writing review & editing (equal). **Cemile Ceren Gul:** formal analysis (equal); investigation (equal); validation (equal); visualization (equal); writing review & editing (equal). **Mehmet Fatih Deveci:** conceptualization (equal); investigation (equal); methodology (equal); resources (equal); writing review & editing (equal). **Mehmet Aslan:** conceptualization (equal); investigation (equal); supervision (equal); writing review & editing (equal).

DATA AVAILABILITY STATEMENT

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

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