Protective Effects of Resveratrol Against Airway Hyperreactivity, Oxidative Stress, and Lung Inflammation in a Rat Pup Model of Bronchopulmonary Dysplasia

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Summary

Oxygen therapy provides an important treatment for preterm and low-birth-weight neonates, however, it has been shown that prolonged exposure to high levels of oxygen (hyperoxia) is one of the factors contributing to the development of bronchopulmonary dysplasia (BPD) by inducing lung injury and airway hyperreactivity. There is no effective therapy against the adverse effects of hyperoxia. Therefore, this study was undertaken to test the hypothesis that natural phytoalexin resveratrol will overcome hyperoxia-induced airway hyperreactivity, oxidative stress, and lung inflammation. Newborn rats were exposed to hyperoxia (fraction of inspired oxygen - FiO_2>95 % O_2) or ambient air (AA) for seven days. Resveratrol was supplemented either *in vivo* (30 $mg \cdot kg^{-1} \cdot day^{-1}$) by intraperitoneal administration or *in vitro* to the tracheal preparations in an organ bath (100 μM). Contractile and relaxant responses were studied in tracheal smooth muscle (TSM) using the *in vitro* organ bath system. To explain the involvement of nitric oxide in the mechanisms of the protective effect of resveratrol against hyperoxia, a nitric oxide synthase inhibitor – N^{ω}-nitro-L-arginine methyl ester (L-NAME), was administered in some sets of experiments. The superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities and the tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) levels in the lungs were determined. Resveratrol significantly reduced contraction and restored the impaired relaxation of hyperoxia-exposed TSM ($p<0.001$). L-NAME reduced the inhibitory effect of resveratrol on TSM contractility, as well as its promotion relaxant effect ($p<0.01$). Resveratrol preserved the SOD and GPx activities and decreased the expression of TNF-α and IL-1β in hyperoxic animals. The findings of this study demonstrate the protective effect of resveratrol against hyperoxia-induced airway hyperreactivity and lung damage and suggest that resveratrol might serve as a therapy to prevent the adverse effects of neonatal hyperoxia.

Keywords

Bronchopulmonary dysplasia • Hyperoxia • Airway hyperreactivity • Resveratrol • Pro-inflammatory cytokines

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Introduction

Bronchopulmonary dysplasia (BPD), also known as chronic lung disease of prematurity, contributes to the increase in morbidity and mortality rates in infants [1]. Oxygen therapy provides an important treatment for preterm and low-birth-weight neonates. However, it has been shown that prolonged exposure to high levels of oxygen (hyperoxia) is one of the factors contributing to the development of BPD by inducing lung injury and airway hyperreactivity [2,3]. In pathological aspect BPD is characterized by fewer and larger alveoli, accompanied by fibrosis [1].

Hyperoxic exposure of newborn rodents represents an established model that reproduces the BPD phenotype manifested with airway hyperreactivity and alveolar simplification, as well as lung inflammation [4-7]. The normal function of airways is maintained by the balance between contraction and relaxation of airway smooth muscle (ASM), and disturbance of this balance leads to airway hyperreactivity [8].

Neonatal hyperoxia increases airway smooth muscle (ASM) contractile responses by upregulating Ca^{2+} sensitization signaling in rats [4]. The increased contraction of ASM is associated with impaired relaxant responses due to reduced production of relaxant molecules such as nitric oxide (NO), a major component of bronchodilation [5-9]. Furthermore, in response to hyperoxia, the lungs elicit a pro-inflammatory response by upregulating the expression of cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β). Lung inflammation contributes to airway hyperreactivity and stimulates the release of reactive oxygen species (ROS), leading to oxidative stress, which is considered a risk factor for the development of BPD [2,7,10-13]. Under an oxidative environment, cellular survival and adaptation are dependent upon sufficient antioxidant defenses to counteract the effect of ROS in cells and tissues [14]. Antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) divert or dismutate ROS into harmless products [15], and their activity is altered by various factors. Mechanical ventilation was shown to reduce SOD activity in preterm animals, while mice lacking superoxide dismutase (SOD) are more sensitive to hyperoxia [16,17].

Despite the known pathophysiological mechanisms by which hyperoxic exposure leads to airway hyperreactivity and lung injury, unfortunately, no effective therapies for BPD have been developed for decades [18,19]. Therefore, a therapy that could prevent neonatal hyperoxia-induced airway hyperreactivity and lung injury is imperative. In this study, we sought to determine the effects of resveratrol on hyperoxia-induced airway hyperreactivity and lung injury in neonatal rats.

Resveratrol (3,4′,5-trihydroxystilbene), a phyto-alexin, occurs naturally in a number of dietary sources, including grapes and their products, such as red wines, and other plants, such as berries, plums, and peanuts [20]. Resveratrol has attracted significant attention in the scientific community due to the pharmacological activities that have been reported. The evidence shows that resveratrol is both a free radical scavenger and a potent antioxidant because of its ability to promote the activities of a variety of antioxidative enzymes [21,22]. In addition to its antioxidant effects, resveratrol has potential anti-inflammatory activity [23]. Besides the antioxidative and anti-inflammatory properties, resveratrol showed a protective effect against airway remodeling and hyperreactivity in a mouse model of allergic asthma [24]. No data show the effect of resveratrol on airway hyperreactivity induced by neonatal hyperoxia. Therefore, in this study, we aimed to investigate the effect of resveratrol on airway hyperreactivity as well as its antioxidative and anti-inflammatory effects in a rat model of BPD.

Materials and Methods

Animals and Experimental Design

On the fourth day of life, Wistar rat pups from two different litters were randomly mixed and assigned to either hyperoxia (FiO_2 > 95 % O_2; *n=72*) or ambient air (AA – 21 %O_2; *n=70*) groups for seven days (showed in the graphical scheme of time management of the study). Dams were provided with food and water *ad libitum*, and a 12 hour on/12 hour off light cycle was maintained. Hyperoxic groups were housed with their mothers in a commercial rat cage placed into a plexiglass box (38 liters) with continuous O_2 (2 L/min), and its concentration was monitored with an oximeter (MiniOX-1, Ohio Medical Corp., IL, USA). To avoid hyperoxic toxicity, mothers were swapped each day between groups. Subsets from each group were supplemented intraperitoneally (i.p.) with resveratrol (30 $mg \cdot kg^{-1} \cdot day^{-1}$; Sigma, Germany) (25µl) during the exposure time (H-res group, *n=19*; AA-res group, *n=19*). This dose was chosen based on our pilot experiments which showed to be more effective in airway hyperreactivity reversal than lower doses. Control animals received equal volumes of vehicle (25 µl; 2.5 % DMSO + saline (v/v)) (H-veh group, *n=19*; AA-veh group, *n=19*). Both sexes were included in this study. This study was conducted in compliance with the rules described in the Guide for Care and Use of Laboratory Animals (1985), Bethesda, as well as the European Guidelines on Laboratory Animal Care. The research protocol was approved by the Ethical Committee for Research and Doctoral Studies of the Faculty of Medicine of the University of Pristina, where the study was performed.

Tracheal smooth muscle preparation

After exposure, animals were euthanized with CO_2. The trachea was removed and freed of serosal connective tissue in an ice-cold oxygenated Krebs-Henseleit (KH) buffer (118.2 mM NaCl, 25 mM $NaHCO_3$, 4.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 10 % D-glucose, pH=7.4 (all from Sigma-Aldrich, Germany)). Rings of 3 mm in length were isolated from the mid-trachea and transferred

into a tissue-organ bath containing KH-buffer (10 ml) at 37 °C for *in vitro* force contraction measurements, as previously described [25].

Tracheal rings were suspended between a stainless-steel wire triangle at the bottom of the tissue bath and a force-displacement transducer. Tracheal smooth muscle (TSM) tension was measured by the four-channel organ bath system (DMT-750TOBS, Danish Myo Technology, Denmark) connected to Power Lab/8SP (AD Instruments Inc., CO, USA) and interfaced with computer-monitored recordings (Chart 8.0 software). The TSM tension was expressed in grams (g). An initial load of 0.3 g was applied, then equilibrated for 45 min. During the equilibration time, the KH buffer was changed every 15 minutes and continuously aerated with a 95 % O_2 + 5 % CO_2 gas mixture.

Methacholine-induced contraction of TSM experiments

Airway reactivity was studied by constructing a dose-response curve for methacholine (MCh, 10^{-8}–10^{-4} M; Sigma-Aldrich, Germany). After recording the control responses of TSM to MCh, the preparations were rinsed three to four times every five minutes with warm KH solution until the TSM tension returned to baseline and then allowed to rest for an additional 45 minutes. During the equilibration time, preparations were incubated with a single dose of resveratrol (100 μM) for 30 minutes before a second dose-response curve to MCh was recorded. Additionally, the effect of *in vivo* supplementation with resveratrol on contractile responses to MCh was studied in TSM obtained from animals treated with resveratrol or vehicles.

In order to explain the mechanisms of resveratrol effects to counteract the ASM hyperreactivity, tracheal rings in organ baths were incubated with the nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 100 μM) for 30 minutes or co-incubated with L-NAME and resveratrol (100 μM), followed by MCh administration. All records for every MCh dose response for every condition were obtained in duplicates.

Electrical field stimulation-induced relaxation of TSM experiments

To show the effect of hyperoxia on the relaxant responses of TSM, preparations obtained from both hyperoxia- and ambient air-exposed animals were placed in organ baths as described above. After equilibration of preparations, a cumulative dose-response curve of bethanechol (Sigma-Aldrich, Germany)- induced contraction of TSM was built, followed by washing outs. Bethanechol is a parasympathomimetic agent that stimulates muscarinic receptors, which is not hydrolyzed by cholinesterase and will therefore have a longer duration of action to keep tissues in a pre-contracted state. A concentration of 3 x 10^{-5} μM of bethanechol was found to be the optimal dose to elicit 75 % of the maximal response. Following the equilibration time, TSMs were pre-constricted using a single concentration of bethanechol (3 x 10^{-5} μM), then incremental electrical field stimulation (EFS) was applied through platinum electrodes [5-60 V alternating current (AC) at 50 Hz] for 10 s at 2-min intervals to induce relaxation. The relaxation of the TSM was expressed as a percentage (%) of the pre-constricted level for each preparation. In addition, in subsets of TSM, after the first recordings of EFS-induced relaxation responses, followed by washing outs and equilibration. Preparations were incubated in a single dose of resveratrol (100 μM) for 30 minutes, and then EFS was applied in the pre-constructed TSM. To explain the possible mechanisms through which resveratrol promotes relaxation, tracheal rings in organ baths were incubated with L-NAME (100 μM) alone for 30 minutes or co-incubated with L-NAME and resveratrol (100 μM), followed by pre-constriction and EFS application.

Antioxidant enzyme activity assay

To study the effect of resveratrol on SOD and GPx activity, lungs were harvested from hyperoxia- and ambient air-exposed pups supplemented with resveratrol (30 $mg \cdot kg^{-1} \cdot day^{-1}$) or vehicle (25 μl; 2.5 % DMSO + saline (v/v) as a control). SOD activity was determined according to the method described by Marklund and Marklund (1974), based on the ability of SOD to inhibit the auto-oxidation of pyrogallol, while GPx activity was determined according to the method described by Lawrence and Burk (1976). The detailed procedure is described in our previous publication [26].

TNF-α and IL-1β ELISA assay

Lung tissue obtained from resveratrol- or vehicle-supplemented animals exposed to hyperoxia or AA was homogenized, and concentrations were measured in duplicate with commercially available enzyme-linked immunosorbent assay (ELISA) kits for TNF-α and IL-1β (BMS622 and BMS630, respectively) (Bender MedSystems GmbH, Vienna, Austria) according to the manufacturer's instructions. The limits of detection assays were 11.0 pg/ml for TNF-α, and 4.0 pg/ml for IL-1β. Inter- and intra-assays CVs were 10 % and 5 % for

TNF-α, and 10 % and 10 % for IL-1β. The plate was read at 450 nm with a spectrophotometer (Bio-Rad Laboratories, CA, USA), and the results were expressed as pg/mg protein.

Histological assessment of the lungs of rat pups

The lungs of rat pups were removed by thoracotomy. Bleeded tissue samples were rinsed with physiological saline prior to mounting in fixative. They were placed in wide-mouthed plastic containers in 10 % formalin basic universal fixative for routine examination. Fixation was carried out at room temperature for a period of 24 hours. The dehydration process has gone through several stages: dehydration, cleaning (with xylene), and placing the tissue in paraffin at a temperature of 60 °C, through the machine called "Automatic Process". The cuts were made with a manual microtome at a thickness of 5 μm, and at the end, the tissues were stained with routine hematoxylin-eosin dyes [27].

Statistical analysis

Results are expressed as means±SEM. Statistical significance was determined by a two-way *ANOVA* with repeated measurements for TSM responses and a post hoc Tukey-Kramer multiple comparison test for group and dose differences. For TNF-α and IL-1β levels, a one-way ANOVA was used to determine statistical significance. A p-value<0.05 was considered statistically significant.

Results

Effect of resveratrol on TSM contraction

MCh induced a dose-response contraction of TSM, and the responses were significantly higher in the hyperoxic group (n=10) compared to the AA group (p<0.001; n=10). The contractile responses differed significantly at concentrations of 10^{-6} – 10^{-4} M of MCh, and the maximal responses in the hyperoxic and AA groups were 1.80±0.09 g and 1.10±0.10 g, respectively (Fig. 1A).

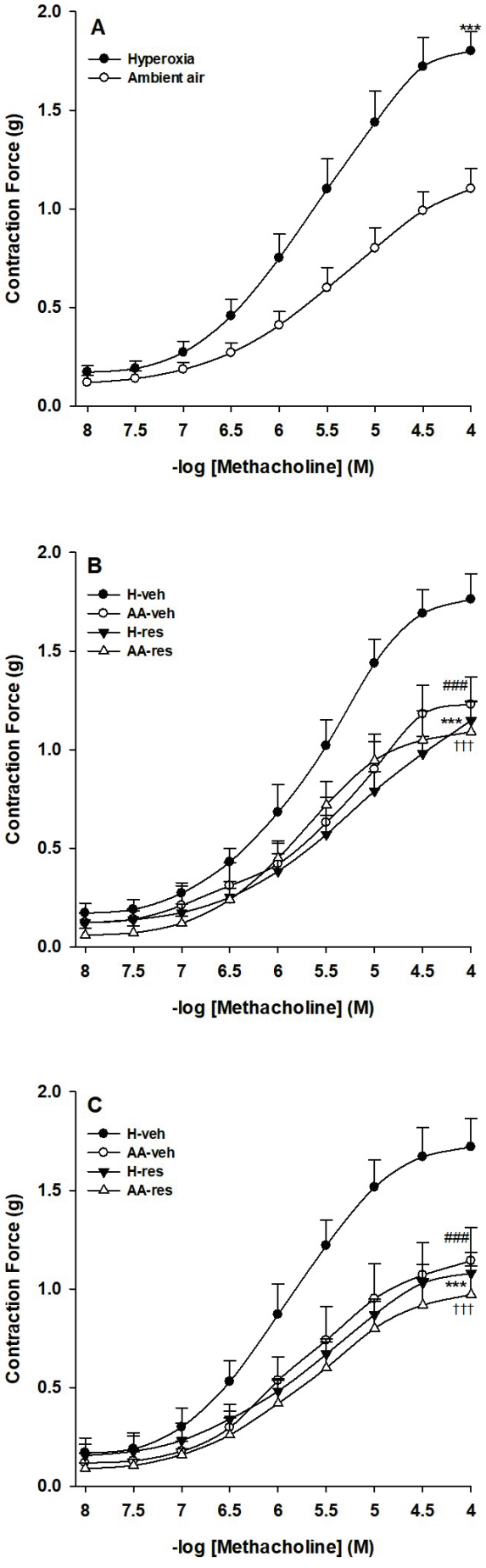

Fig. 1. The effect of *in vitro and in vivo* resveratrol supplementation on the methacholine-induced contraction of TSM of rat pups. **A**: TSM contractile responses in hyperoxic and ambient air control groups (*** p<0.001; n=10). **B**: TSM contractile responses in hyperoxic and ambient air groups in the absence or presence of resveratrol supplemented *in vitro* (n=7). **C**: TSM contractile responses in hyperoxic and ambient air groups in the absence or presence of resveratrol supplemented *in vivo* (n=7). (****P<0.001 – H-res vs. H-veh; †††P<0.001 – AA-res vs. H-veh; $^{###}$P<0.001 – AA-veh vs. H-veh*). H-veh: hyperoxia-vehicle; AA-veh: ambient air-vehicle; H-res: hyperoxia+resveratrol; AA-res: ambient air+resveratrol. Data are reported as means±SEM.

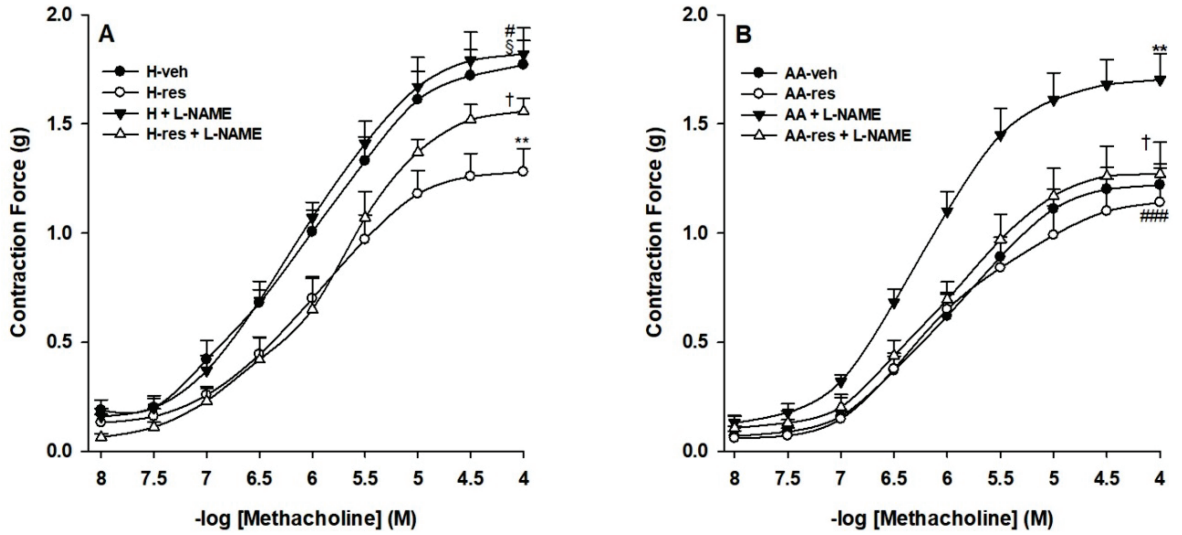

Fig. 2. Effect of L-NAME on MCh-induced airway hyperreactivity. **A**: TSM contractile responses in hyperoxia-exposed rat pups in absence or presence of L-NAME and/or resveratrol (n=8; $^{**}P<0.001$ – *H-res vs. H+L-NAME / H-veh;* $^{\dagger}P<0.05$ – *H-res+L-NAME vs. H-res;* $^{\S}P<0.05$ – *H-veh vs. H-res+L-NAME*; $^{\#}P<0.05$ – *H+L-NAME vs. H-res+L-NAME*). **B**: TSM contractile responses in ambient air-exposed rat pups in the absence or presence of L-NAME and/or resveratrol (n=6; $^{**}P<0.01$ - *AA+L-NAME vs. AA-veh;* $^{\dagger}P<0.05$ – *AA-res+L-NAME vs. AA+L-NAME;* $^{\#\#\#}P<0.001$ – *AA-res vs. AA+L-NAME).* H-veh: hyperoxia-vehicle; AA-veh: ambient air-vehicle; H-res: hyperoxia+resveratrol; AA-res: ambient air+resveratrol. Data are reported as means ± SEM.

In vitro supplementation of resveratrol to the tissues in an organ bath reversed the hyperoxia-induced airway hyperreactivity to MCh. In the presence of resveratrol, TSM contractile responses were significantly reduced at concentrations of 10^{-6}-10^{-4} M of MCh ($p<0.001$; n=7); the maximal contractile force in H-res was 1.14±0.09 g. Resveratrol had no significant effect on the contractile responses of TSM in the AA group of animals, and the maximal contractile responses in the AA-veh and AA-res were 1.28±0.14 g and 1.1±0.16 g, respectively (Fig. 1B; n=7).

In vivo supplementation of animals with resveratrol during hyperoxic exposure prevented airway hyperreactivity. The TSM contractile responses were significantly decreased in H-res compared to H-veh ($p<0.001$; n=7, each condition) (Fig. 1C). Supplementation of resveratrol normalized the responses in hyperoxic animals such that they did not significantly differ from the AA-veh ($p=0.307$). The maximal values of responses in H-veh and H-res groups were 1.72±0.14 g and 1.08±0.11 g, respectively. The contractile responses of AA-res did not significantly change compared to AA-veh (n=7) (Fig. 1C). The maximal values of contractile responses in the AA-veh and AA-res groups were 1.14±0.17 g and 0.97±0.15 g, respectively.

Effect of L-NAME on resveratrol-attenuated TSM contraction

The TSM contraction of H-veh animals did not change with L-NAME administration, but NOS inhibition diminished the effect of resveratrol and attenuated contractile responses, particularly at higher doses of MCh. The presence of L-NAME shifted to the left the dose-response curve of MCh-induced contractions of TSM in the H-res group, such that they significantly differed from responses when resveratrol was administered alone ($p<0.05$; n=8). The responses were significantly different at concentrations of $10^{-4.5}$-10^{-4} M of MCh, and the maximal values of contraction in H-res+ L-NAME and H-res were 1.56±0.06 g and 1.28±0.11 g, respectively. Although inhibition of NOS reduced the protective effect of resveratrol against airway hyperreactivity, it did not completely overturn its effect, and again, the contractile responses in the H-res+ L-NAME were significantly lower compared to both H-veh and H+L-NAME ($p<0.05$) (Fig. 2A). The differences were significant at concentrations of 10^{-6}-10^{-4} M of MCh, and the maximal values of H-veh and H+L-NAME were 1.77±0.11 g and 1.82±0.12 g, respectively.

NOS inhibitors significantly increased the TSM contractile responses of the preparations obtained from ambient air-exposed rat pups (AA+L-NAME) compared to both AA-veh and AA-res ($p<0.01$ and $p<0.001$, respectively; n=6) (Fig. 2B). The maximal values of responses in AA+L-NAME, AA-veh, and AA-res were 1.70±0.12 g, 1.22±0.09 g, and 1.14±0.15 g, respectively. The differences were significant at concentrations of

$10^{-6.5}$-10^{-4} M of MCh. The co-incubation of TSM with L-NAME and resveratrol did not significantly affect the contractile responses of TSM in ambient air-exposed rat pups (AA-res+L-NAME) compared to either AA-veh or AA-res. Thus, contractile responses in AA-res+L-NAME were significantly lower compared to AA+L-NAME ($p<0.05$). The maximal values of contraction in AA-res+L-NAME were 1.27±0.14 g.

Effect of resveratrol on relaxation of TSM

EFS-induced relaxation of TSM was significantly decreased in the hyperoxia-exposed animals compared to AA-exposed animals ($p<0.001$; n=8, each group). The differences were significant at 20-60 V, and the maximal relaxant responses in the hyperoxia and ambient air-exposed animals were 21.93±1.26 % and 55.40±2.29 %, respectively (Fig. 3A). Pre-incubation of tracheal preparations with a single concentration of resveratrol restored the impaired relaxation in hyperoxia-exposed TSM, and the relaxant responses in H-res were significantly increased compared to H-veh ($p<0.001$; n=8). The differences were significant at 20-60 V, and the maximal value of TSM relaxation in H-res was 51.30±4.05 % (Fig. 3B). The incubation of tracheal preparations in L-NAME did not affect the relaxation of TSM in the H-veh group but slightly reduced the relaxant effects of resveratrol. Even though the inhibition of NOS altered the promoting relaxant effect of resveratrol, the measured relaxation in H-res+L-NAME was significantly higher compared to H-veh and H+L-NAME ($p<0.001$). The differences in relaxation were significant at 20-60 volts, and the maximal value of relaxation in H-res+L-NAME was 39.30±2.99 %. ($p<0.05$) (Fig. 3B).

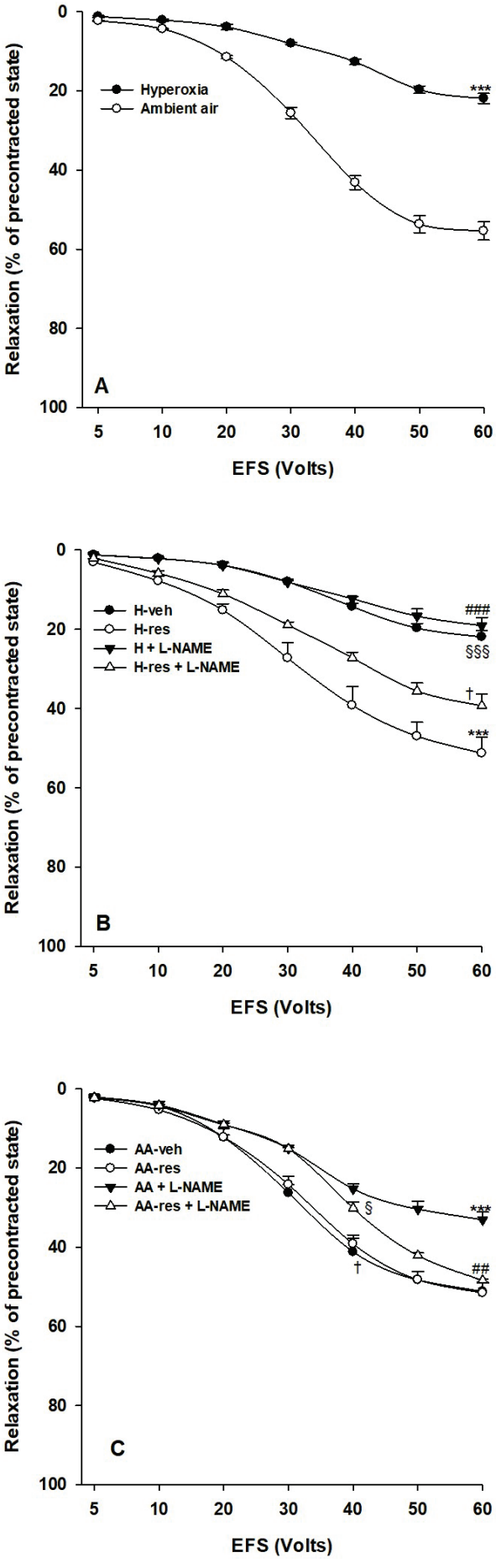

Fig. 3. Effect of *in vitro* resveratrol supplementation on TSM relaxation of rat pups. **A**: TSM relaxation in hyperoxic and ambient air control groups (****P<0.001*; n=8). **B**: TSM relaxation in hyperoxia-exposed rat pups in the absence or presence of resveratrol and/or L-NAME (n=8; ****P<0.001 – H-res vs. H-veh/H+L-NAME; †P<0.05 – H-res+L-NAME vs. H-res; §§§P<0.001 – H-veh vs. H-res+L-NAME; ###P<0.001 – H+L-NAME vs. H-res+L-NAME)*. **C**: TSM relaxation in ambient air groups in the absence or presence of resveratrol and/or L-NAME (n=8; ****P<0.001 – AA+L-NAME vs. AA-veh; ##P<0.01 – AA-res+ L-NAME vs. AA+L-NAME; §P<0.05 – AA-res+L-NAME vs. AA-res; †P<0.05 – AA-res+L-NAME vs. AA-veh.* H-veh: hyperoxia-vehicle; AA-veh: ambient air-vehicle; H-res: hyperoxia+resveratrol; AA-res: ambient air+resveratrol. Data are reported as means ± SEM.

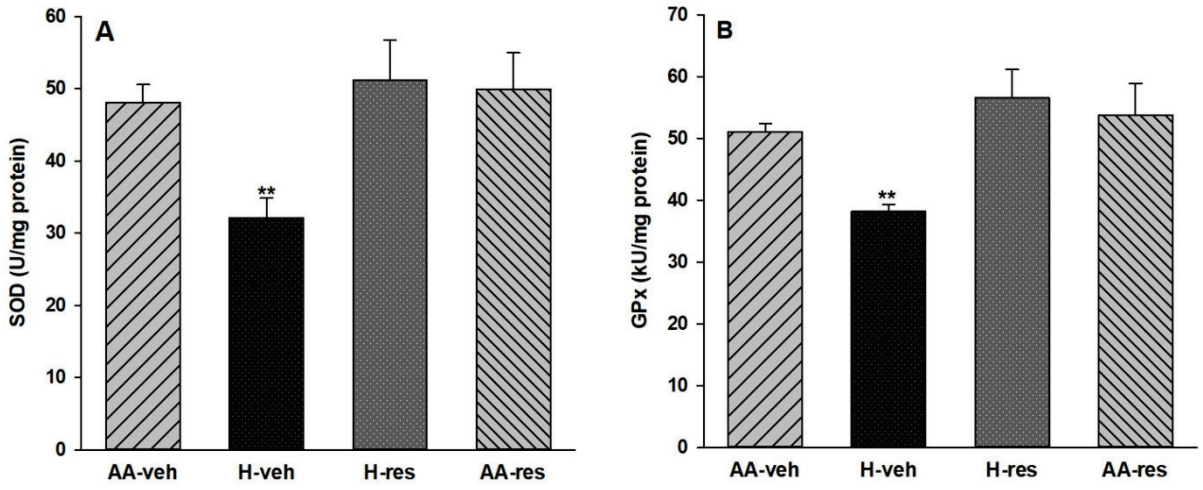

Fig. 4. Effect of resveratrol on SOD and GPx activity in lung tissue of rat pups. **A**: SOD activity in lung tissue of rat pups exposed to hyperoxia or ambient air in the absence (n=10) or presence of resveratrol (n=8). **B**: GPx activity in lung tissue of rat pups exposed to hyperoxia or ambient air in the absence (n=7) or presence of resveratrol (n=8). ***P<0.01; H-veh vs. AA-veh/ H-res/ AA-res.* H-veh: hyperoxia-vehicle; AA-veh: ambient air-vehicle; H-res: hyperoxia+resveratrol; AA-res: ambient air+resveratrol. Data are presented as means ± SEM.

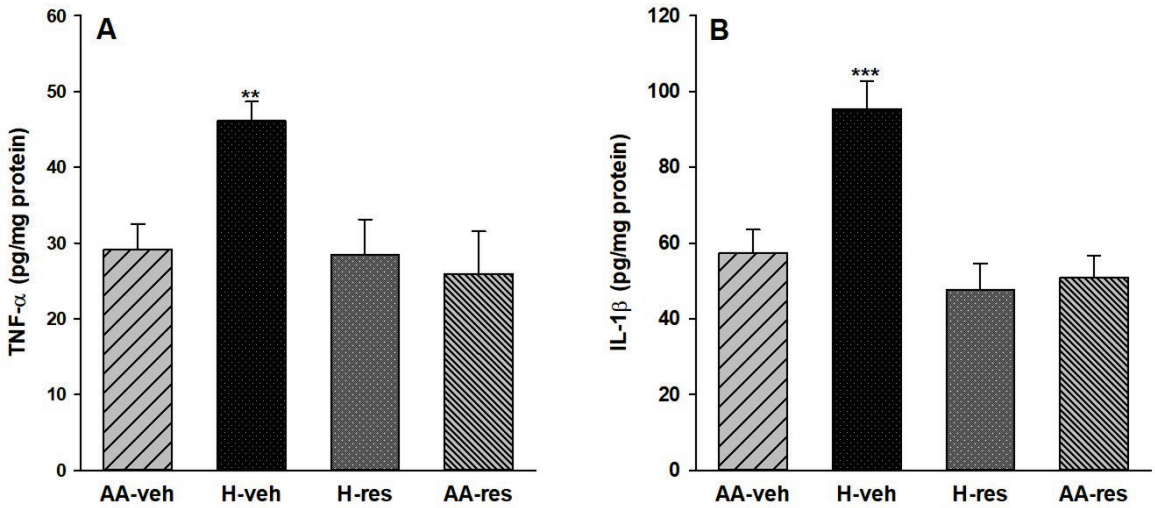

Fig. 5. Effect of resveratrol on proinflammatory cytokines in the lung tissue of rat pups. **A**: TNF-α level in lung tissue of rat pups exposed to hyperoxia or ambient air (n=6). **B**: IL-1ß level in lung tissue of rat pups exposed to hyperoxia or ambient air (n=6). (***P<0.01;* ****P<0.001, H-veh vs. other groups).* H-veh: hyperoxia-vehicle; AA-veh: ambient air-vehicle; H-res: hyperoxia+resveratrol; AA-res: ambient air+resveratrol. Data are presented as means ± SEM.

Resveratrol supplementation did not affect the relaxation of TSM in the AA group, and the maximal value of relaxation in the AA-res group was 51.50±2.40 %. Incubation of tracheal preparations with L-NAME significantly reduced the relaxation compared to AA-veh and AA-res ($p<0.001$; n=8) (Fig. 3C). The differences in relaxant responses were significant at 30-60 V, and the maximal value of responses in AA+L-NAME was 33.20±1.97 %. Interestingly, in the presence of L-NAME, resveratrol did not show any effect at lower voltages. In comparison, at higher voltages (40–60 V), it did overcome the inhibitory effect of L-NAME on relaxation; thus, at 60 V, the relaxation was at the level of AA-veh and AA-res (48.41±0.37 %). The relaxation of AA-res+L-NAME tracheal preparations significantly differed at 50–60 V ($p<0.01$) compared to AA+L-NAME, while compared to AA-veh and AA-res, it was significantly reduced at 30–50 V ($p<0.05$).

Effect of resveratrol on antioxidant enzymes activity

In the hyperoxic group (H-veh) of rat pups, the activity of both enzymes, SOD and GPx, was significantly decreased ($p<0.01$) compared to the ambient air group (AA-veh). The mean values of SOD activity in

H-veh and AA-veh animals were 32.1±2.7 U/mg protein and 48.04±2.5 U/mg protein, respectively (n=10 in each group). *In vivo* supplementation with resveratrol significantly increased the SOD activity in hyperoxia-exposed animals (H-res) ($p<0.01$; 51.20±5.5 U/mg protein; n=8), while it had no significant effect in the AA-exposed animals (AA-res) (49.90±5.02 U/mg protein; n=8) (Fig. 4A). Resveratrol also preserved GPx activity, which was significantly increased in H-res compared to H-veh ($p<0.01$; 56.60±4.50 kU/mg protein and 38.20±1.10 kU/mg protein, respectively; n=7 and n=8) (Fig. 4B). Resveratrol did not have any effect on GPx activity in the AA group, and the activity of this enzyme in the AA-res and AA-veh groups was 53.71±5.07 kU/mg protein and 51.73±1.30 kU/mg protein, respectively (n=7 and n=8).

Effect of resveratrol on TNF-α and IL-1β Levels

Neonatal hyperoxic exposure significantly increased the expression of TNF-α compared to AA-exposed animals ($p<0.01$; n=6). The mean TNF-α levels in the lung tissues of H-veh and AA-veh were 46.1±2.4 pg/mg protein and 29.1±3.4 pg/mg protein, respectively. Supplementation of animals with resveratrol during hyperoxic exposure (H-res) significantly reduced the expression of TNF-α ($p<0.01$); however, it did not have a significant effect on the TNF-α levels in the lungs of AA-exposed animals (AA-res) (n=6) (Fig. 5A). The

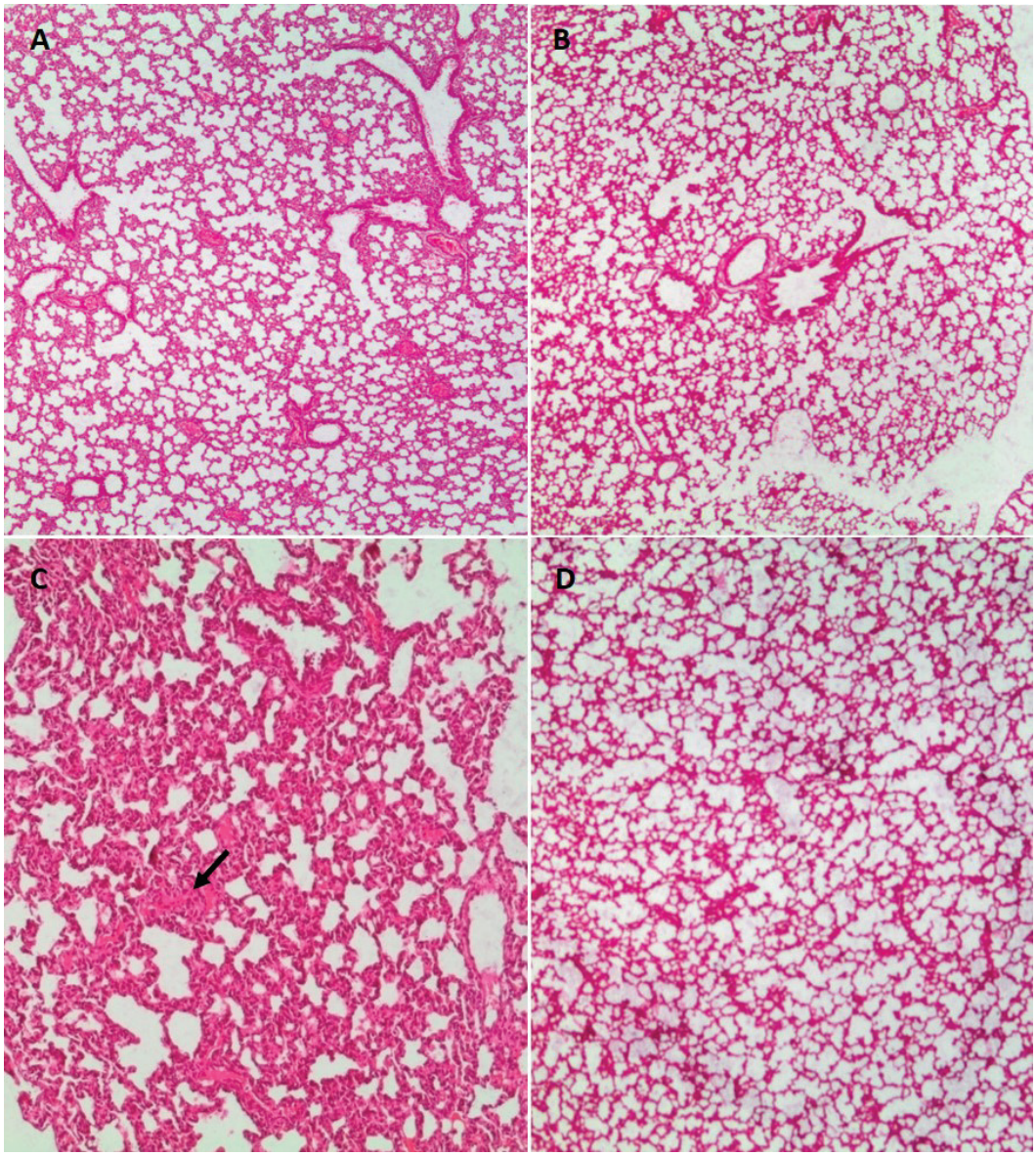

Fig. 6. Representative photomicrographs of histological sections of the lungs of neonatal rats showing normalization of alveolar spaces and interalveolar fibroelastic septas by resveratrol. **A**: AA-veh group; **B**: AA-res group; **C**: H-veh group (the arrow is pointing to the thickening of the interalveolar septa with interstitial proliferative changes); **D**: H-res group, (10x; n=4 / per group).

mean TNF-α levels in lung tissue were 28.5±4.6 pg/mg and 25.9±5.6 pg/mg, respectively. In addition to TNF-α, a significant increase in IL-1β levels was also confirmed in the lungs of hyperoxic animals (H-veh) compared to AA-exposed animals (AA-veh) ($p<0.001$; n=6). Resveratrol treatment reduced the expression of IL-1β ($p<0.001$) in the lungs of hyperoxic animals (H-res), while in AA-exposed animals (AA-res), there was no significant effect (n=6) (Fig. 5B). The mean values of IL-1β levels in lung tissues of the H-veh and AA-veh groups or those treated with resveratrol (H-res and AA-res) were 95.4±7.3 pg/mg, 57.3±6.2 pg/mg, 47.6±7.1 pg/mg, and 50.8±5.8 pg/mg, respectively.

Effect of resveratrol in histopathology of lungs

Pups exposed to hyperoxia (H-veh) demonstrated thickening of the interalveolar septa with interstitial proliferative changes and the presence of mixed inflammatory infiltrate was observed in the obtained samples (Fig. 6C). In hyperoxic animals treated with resveratrol (H-res) (Fig. 6D) the alveolar spaces and interalveolar fibroelastic septa show a regular configuration. A marked reduction of proliferative and inflammatory changes is observed in the interalveolar interstitium. In the AA-veh and AA-res groups (Fig. 6A and 6B), alveolar spaces are separated by thin fibroelastic septa, regular terminal bronchioles, blood capillaries, and a small amount of scar tissue are identified.

Discussion

To the best of our knowledge, this is the first study showing the protective effect of resveratrol against neonatal hyperoxia-induced airway hyperreactivity. The experimental model of BPD was developed in rat pups exposed to hyperoxia. The study reveals that resveratrol effectively attenuates the increased contractile responses and promotes the relaxation of ASM, resetting the balance between these processes disturbed by hyperoxic exposure. Moreover, resveratrol abrogated hyperoxia-induced oxidative stress and inflammatory responses in the lungs of rat pups.

BPD remains a significant complication of premature birth with long-term consequences [1]. The pathogenesis of BPD is multifactorial, and with respect to this, hyperoxia plays a critical role in the development of BPD that causes persistent airway hyperreactivity, lung inflammation, and histological changes [28-30]. While oxygen therapy remains clinically essential for preterm survival, finding new therapies to treat or prevent airway hyperreactivity is imperative. It was reported that hyperoxia increases airway hyperreactivity via various signaling pathways. Beqiraj *et al*. [4] showed the active role of Rho/Rho-kinase signaling in hyperoxia-induced airway hyperreactivity in neonatal rats and elucidated the involvement of prostaglandin $F_{2\alpha}$ to upregulate this pathway. In addition to airway hyperreactivity, RhoA activation is involved in the increased synthesis of collagen-I, which is a key process in pulmonary fibrosis that occurs in hyperoxia-induced lung injury [31]. In human mammary epithelial cells, resveratrol inhibited cyclooxygenase-1 (COX-1) activity and COX-2 transcription, thereby reducing prostaglandin synthesis [32]. Resveratrol attenuated airway reactivity and remodeling in a mouse model of allergic asthma [24]. In the present study, resveratrol effectively reversed and prevented hyperoxia-induced airway reactivity when administered either *in vitro* or *in vivo*.

In addition to the increase in contraction, neonatal hyperoxia impairs relaxation of ASM *via* depletion of NO/cyclic-guanosine monophosphate (cGMP) signaling in the airways of rat pups, thus shifting the balance toward contraction and inducing airway hyperreactivity [5]. This occurs due to the limited bioavailability of the NOS substrate L-arginine, which was reversed by supplementing the animals with L-arginine or L-citrulline as well as by inhibiting the arginase activity, a competing enzyme of NOS for a common substrate, thereby decreasing the NO production [5,9,25]. Hyperoxia also decreases the cyclic adenosine monophosphate (cAMP) levels in lung tissue and tracheal preparations, which is an important molecule in the alternate signaling pathway that promotes the relaxation of ASM [33,34]. Resveratrol activates the NO-cGMP signaling pathway to inhibit platelet activation [35]. A relaxant effect of resveratrol mediated by NO was confirmed in porcine retinal micro-vessels, mesenteric and uterine arteries of guinea pigs, and the rat aorta [36-38]. Furthermore, resveratrol could upregulate the expression and activity of endothelial NOS (eNOS) and NO production from human umbilical vein endothelial cells (HUVEC) [39,40]. In addition to the increase in eNOS expression, high doses of resveratrol increased L-citrulline production, which in turn can be converted into L-arginine, thus increasing the bioavailability of L-arginine [39]. In this study, resveratrol-attenuated contractile responses were significantly reduced by the NOS inhibitor L-NAME. Although NOS inhibition

reduced the effect of resveratrol, it did not overturn its effect, suggesting that resveratrol uses other mechanisms as well, as it was shown that resveratrol activates potassium channels and also inhibits phospholipase C (PLC), leading to the sequestration of Ca^{2+} in intracellular stores [35,36]. The results of this study show that resveratrol restores the impaired EFS-induced relaxation of TSM exposed to hyperoxia. In line with other studies in different experimental models, the inhibition of NOS significantly reduced the promoting relaxant effect of resveratrol, but it did not abolish it. We speculate that resveratrol could involve other signaling mechanisms to trigger relaxant mechanisms, as was reported for the ability of resveratrol to directly inhibit phosphodiesterases (PDE) and subsequently increase the production of cAMP [41].

Lungs exposed to hyperoxia are prone to generating free radicals. When they exceed the capacity of antioxidant defenses, they cause oxidative stress, resulting in lung damage [42]. The antioxidant and anti-inflammatory effects of resveratrol were reported in numerous rodent models [43-45]. In a rat pup model of BPD, hyperoxia decreased SOD activity and GSH levels in lung tissue, which were restored by resveratrol [44]. In addition to preventing oxidative stress, resveratrol has demonstrated anti-inflammatory effects as well. Neonatal hyperoxia increases pro-inflammatory cytokine production in rats, such as TNF-α, IL-1β, and IL-6, which are reversed by resveratrol [44,45]. This study shows that hyperoxia induces oxidative stress by impairing the antioxidant enzyme (SOD and GPx) activities and dramatically increasing the expression of pro-inflammatory cytokines (such as TNF-α and IL-1β) in rat lungs. In line with other studies, supplementing animals with resveratrol showed protective effects against neonatal hyperoxia-induced oxidative stress and lung inflammation.

Prolonged neonatal hyperoxia causes alveolar and capillary hypoplasia, and variable cellularity and fibroproliferation [27]. Resveratrol showed an anti-fibrotic effect via downregulation of α-smooth muscle actin [45]. In our study, hyperoxia induced the thickening of interalveolar septa with interstitial proliferative changes and the presence of mixed inflammatory infiltrate is observed in the obtained samples, while resveratrol revealed to have a protective effect against these adverse effects of hyperoxia.

Although the high effectiveness of resveratrol in preventing adverse effects of neonatal hyperoxia and its linkage to many other health-promoting properties have been concluded to date, there are some limitations to be considered. A combination of several limiting factors might be a challenge, including poor aqueous solubility, limited stability, and high metabolization that decreases resveratrol's bioavailability. Therefore, the development of a pharmaceutical formulation to overcome these limitations will make resveratrol a prospective target therapy that can be used in multiple directions.

In summary, this study provides novel findings showing the capability of resveratrol to counteract the hyperoxia-induced airway hyperreactivity, the most persistent feature of BPD, by resetting the balance between contraction and relaxation processes. In addition, resveratrol prevented oxidative stress and inflammation in lungs exposed to hyperoxia. This makes resveratrol a multi-target active agent for protecting against neonatal hyperoxia. Following studies in clinical settings will contribute to shedding light on the comparable effects of resveratrol on the targeted population.

Conflict of Interest

The authors declare no conflict of interest with respect to the research, authorship, and/or publication of this article.

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