Pediatrics and Neonatology (2014) 55, 352–357

Available online at www.sciencedirect.com

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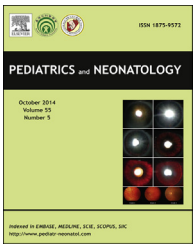

ORIGINAL ARTICLE

The Effects of Resveratrol on Hyperoxia-induced Lung Injury in Neonatal Rats

Özmert M.A. Özdemir [a], Ersin Gözkeser [a,*], Ferda Bir [b], Çiğdem Yenisey [c]

[a] Department of Pediatrics, Faculty of Medicine, Pamukkale University, Denizli, Turkey
[b] Department of Pathology, Faculty of Medicine, Pamukkale University, Denizli, Turkey
[c] Department of Biochemistry, Faculty of Medicine, Adnan Menderes University, Aydın, Turkey

Received May 21, 2013; received in revised form Nov 13, 2013; accepted Nov 21, 2013
Available online 11 March 2014

Key Words
hyperoxia-induced lung injury;
nitric oxide;
oxidative stress;
resveratrol

Background: Bronchopulmonary dysplasia (BPD) is a chronic lung disease that causes significant morbidity and mortality in premature infants. Inflammation and oxidative injury play an important role in the pathogenesis of BPD. Resveratrol is an antioxidant and anti-inflammatory agent. In this study, the histopathological and biochemical effects of resveratrol on a hyperoxia-induced lung injury model in newborn rats were investigated.
Methods: The experiment was performed on newborn rat pups from the 3rd to 13th postnatal day and they were randomly divided into four groups: Group 1 (air-exposed + saline, $n = 10$), Group 2 (air-exposed + resveratrol, $n = 11$), Group 3 (hyperoxia-exposed + saline, $n = 6$) and Group 4 (hyperoxia-exposed + resveratrol, $n = 7$). Resveratrol was administered (30 mg/kg/day) intraperitoneally. The histopathological effects of resveratrol on lung tissue were assessed by alveolar surface area, fibrosis, and smooth muscle actin (SMA) score, and the biochemical effects on lung tissue were assessed by glutathione (GSH), superoxide dismutase (SOD), nitric oxide (NO), tumor necrosis factor-α (TNF-α), and nuclear factor kappa B (NF-κB) levels.
Results: The alveolar surface area, fibrosis, SMA score, and NO levels were found to be significantly higher in Group 3 compared with Group 1 ($p < 0.05$). In addition, it was found that resveratrol treatment significantly reduced the SMA score and the NO and TNF-α levels, and increased the GSH and SOD levels in the hyperoxia group ($p < 0.05$).
Conclusion: This experimental study showed that oxidative stress and NO contributed to the pathogenesis of hyperoxia-induced lung injury, and that resveratrol had a preventive effect on hyperoxic lung injury through its anti-inflammatory and antioxidant properties.

* Corresponding author. Department of Pediatrics, School of Medicine, Pamukkale University, Çocuk Poliklinik Binası, Kat 2, Kınıklı 20070, Denizli, Turkey.
E-mail address: ersingozkeser@hotmail.com (E. Gözkeser).

http://dx.doi.org/10.1016/j.pedneo.2013.11.004

1. Introduction

Bronchopulmonary dysplasia (BPD), also referred to as chronic lung disease of prematurity, is a major cause of long-term morbidity and mortality in premature infants. Despite advances in neonatal critical care including antenatal steroids, surfactant replacement therapy, gentle ventilation/nasal continuous positive airway pressure, and reduced oxygen concentration, the number of infants with BPD has increased as the survival time of very small premature infants has increased.[1] Various factors contribute to the pathogenesis of this disease, including a susceptible host with immature lung structure, and the developmental deficiencies of factors crucial to lung development and function, such as surfactant, nitric oxide (NO), innate immune defense, and antioxidant capability.[1] Oxidant injury and inflammation are thought to be dominant mechanisms in the pathogenesis of BPD.[2] Although there are no safe and effective preventive therapies, new treatment strategies remain promising for the prevention of BPD.[1]

Resveratrol (3,5,4′-trans-trihydroxystilbene) is a natural phytoalexin present in grapes, peanuts, mulberries, and red wine. The anti-inflammatory and antioxidant activities of resveratrol have been well documented in many different studies.[3–5] Its anti-inflammatory effect is related to inhibiting oxidation, leukocyte priming, and expression of inflammatory mediators. It has been also reported in experimental studies that resveratrol reduced lung injury, such as sepsis or bleomycin-induced lung injury.[4,5] However, no report exists related to its activity on an experimental model of BPD in rats; therefore, we investigated the histopathological and biochemical effects of resveratrol on hyperoxia-induced lung injury in neonatal rats.

2. Materials and methods

2.1. Animals

This study was approved by the Pamukkale University Animal Research Committee and was performed on newborn (3–13 days old) Wistar albino rat pups whose mothers had been kept under standard conditions.

3. Experimental design

Wistar albino rat pups were delivered spontaneously and reared with their dams ($n = 4$) until the time of the experimentation. Afterward, the rat pups were randomly divided into four groups: air-exposed + saline group (Group 1, $n = 10$), air-exposed + resveratrol group (Group 2, $n = 11$), hyperoxia-exposed + saline group (Group 3, $n = 10$), and hyperoxia-exposed + resveratrol treated (Group 4, $n = 11$). The experiment began on postnatal day 3 and continued until postnatal day 13 (day of birth = day 0). A hyperoxia-induced lung injury rat model of BPD was used.[6] Groups 3 and 4 (hyperoxia-exposed groups) were placed in an oxygen chamber (Plexiglas chamber), into which oxygen was continuously delivered ($FiO_2 = 0.90 \pm 0.02$) using a flow of 2 L/minute, and the groups were monitored twice daily (Anesthetic Gas Monitor, Drager 1996). The rat pups in Groups 2 and 4 received resveratrol (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) intraperitoneally at a dose of 30 mg/kg beginning on postnatal Day 3 and daily through to postnatal Day 13.[4] The other rat pups in Groups 1 and 3 received only saline (0.9%) intraperitoneally at the same dose and time. All animals were returned to their mothers, kept in a normothermic environment and humidity (at 22–25°C and 60–70% humidity), and breast-fed. CO_2 was removed by soda lime absorption.[7] Nursing mothers were not treated but were rotated between room air and hyperoxia every 24 hours. All animals were raised in the same room and all other conditions were the same. On postnatal Day 14, all animals were killed by intraperitoneal injection of pentobarbital sodium (200 mg/kg). Alveolar surface area (ASA) score, lung fibrosis score, smooth muscle actin (SMA) score, glutathione (GSH), superoxide dismutase (SOD), and NO activities, tumor necrosis factor-α (TNF-α) and nuclear factor kappa B (NF-κB) were measured on the lung tissue samples. The weight of the rats was also evaluated throughout the experiment.

3.1. Preparation of specimens

The lungs of the rats were removed by thoracotomy and the right lungs were inflation-fixed via a tracheal cannula using 10% neutral formaldehyde solution for histological evaluation. Each lobe of fixed lung tissue was separated, placed in cassettes, and embedded in paraffin after tissue processing. The tissues were paraffin sectioned (5 μm) and later stained with hematoxylin and eosin (H&E), and Masson trichrome stain after deparaffinization. These stained lung tissue samples were evaluated for histopathological changes by a pathologist in a blind fashion. The left lungs were gently perfused with 10 mL of 0.9% saline to remove blood, and then placed in Eppendorf tubes and stored in a freezer at −80°C for biochemical analyses.[7]

4. Histological assessment of lung fibrosis and ASA

The tissue sections of the right middle and lower lung lobes were stained with H&E and Masson trichrome stain for the evaluation of alveolar fibrosis. Fields in which large vessels and airways were present were not included. The images from the nonoverlapping peripheral zone of the samples, a minimum of 10 lung fields, were examined at ×10 magnification. Each lung section was evaluated histologically and scored as follows: 0: absence of alveolar fibrosis; 1: mild fibrosis; 2: moderate fibrosis; and 3: marked fibrosis.[8]

The tissue sections of the right middle lobe were stained with H&E for the evaluation of ASA. ASA was measured by a pathologist using a computer-assisted image analyzer system consisting of a microscope. The images from the peripheral zone of the samples were examined at ×4 magnification and 10 nonoverlapping fields were measured semiquantitatively.[6] The decrease in ASA was evaluated histologically and scored as follows: 0: no decrease; 1: mild decrease; 2: moderate decrease; and 3: marked decrease in ASA.[9]

5. Immunohistochemical assessment of lung SMA expression

The lung SMA expression was visualized using the avidin–biotin–peroxidase method. Embedded lung tissues from the right lobes were sectioned on poly-L-lysin-coated slides. The tissue sections were deparaffinized in xylene, and rehydrated and immersed in distilled water. Endogenous peroxidase activity was blocked using a 0.3% solution of hydrogen peroxidase in phosphate-buffered saline (PBS). The primary antibody against SMA (prediluted, Ventana Medical Systems Inc, Tucson, AZ, USA) was applied for 30 minutes at room temperature and washed in PBS. The peroxidase activity was visualized with 0.03% 3,30-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) applied for 5 min. After rinsing in deionized water and counterstaining with hematoxylin, the slides were dehydrated and mounted. Appropriate tissue sections were also labeled as positive and negative controls for the primary antibody. Ten nonoverlapping microscopic fields were selected at ×10 magnification in a random manner for immunohistochemical scoring. The degree of positive staining was evaluated by semiquantitative scoring on a scale of 1 to 4 for intensity and for distribution.[10]

5.1. Biochemical analyses

Lung tissues were homogenized at 4°C in 50 mM phosphate buffer solution (pH: 7.4, 1/10 g/mL) containing 0.2 μM phenylmethanesulfonyl fluoride, 1 mM EDTA, and 1 μM leupeptin. Homogenates were centrifuged at 10,000 *g* for 5 minutes. Clear upper supernatant fluid was obtained and assayed for biochemical analyses including GSH, SOD, NO, TNF-α, and NFκB. Lung tissue SOD activity was measured by the method described by Sun et al,[11] and NO levels were measured using a modified version of the Griess reaction, a method by Navarro-Gonzalves et al.[12] The tissue levels of GSH, TNF-α, and NFκB were measured by commercial enzyme-linked immunosorbent assay kits (Bender Medsystems GmbH, Campus Vienna Biocenter, Vienna, Austria). The results of GSH, SOD, NO, TNF-α and NFκB were expressed as micrograms, nanograms, micromoles, and picograms per gram of wet tissue, respectively.

5.2. Statistical analysis

For statistical analysis, the results were subjected to nonparametric tests (Kruskal-Wallis test, Mann-Whitney *U* test) using the Statistical Package for Social Sciences for Windows (Version 10.0; SPSS Inc., Chicago, IL, USA), as appropriate. All values are expressed as median, minimum-maximum. A *p* value less than 0.05 was considered significant.

6. Results

There was no statistically significant difference between the groups in terms of median weight (10.6 g in Group 1, 11.3 g in Group 2, 10.4 g in Group 3, and 10.2 g in Group 4) before the experiment ($p > 0.05$). Eight animals died during the study, four in Group 3 and four in Group 4. However, these animals' lung tissues were not included the study. Sixty-two percent of neonatal rats exposed to hyperoxia survived, with most deaths occurring between 7 and 10 days of life. Prolonged neonatal exposure to hyperoxia adversely affected growth. At the end of the study, the median weights of rats in the hyperoxia-exposed groups (12.4 g in Group 3 and 13.8 g in Group 4) were significantly lower than those in the air-exposed groups (29.1 g in Group 1 and 25.5 g in Group 2) ($p < 0.001$). Although the median weight of rats in Group 4 was higher than that of the rats in Group 3, this result was not statistically significant.

As shown in Table 1, exposure to hyperoxia resulted in a significant increase in mean ASA and fibrosis when compared with the air-exposed groups ($p < 0.05$). In the hyperoxia-exposed groups, although resveratrol treatment resulted in lower mean ASA and fibrosis when compared with saline, these results were not statistically significant ($p = 0.074$).

6.1. SMA immunostaining

The lung SMA scores in the study groups demonstrated a marked increase in smooth muscle content in the hyperoxia-exposed animals ($p < 0.05$, Figure 1). In addition, the results demonstrated that resveratrol treatment significantly decreased the SMA scores when compared with hyperoxia-exposed + saline animals ($p < 0.05$). The SMA scores of the groups are shown in Table 1.

6.2. Biochemical effects of hyperoxia and effects of resveratrol

In this study, there was no statistically significant difference for the activities of antioxidant status including SOD and GSH between the air-exposed and hyperoxia-exposed

Table 1 The histopathologic effects of resveratrol on hyperoxia-induced lung injury

	Alveolar surface area score Median (min–max)	Fibrosis score Median (min–max)	SMA score Median (min–max)
Group 1 ($n = 10$)	1 (0–1)	0 (0–1)	1 (1–2)
Group 2 ($n = 11$)	1 (0–1)	0 (0–1)	1 (1–2)
Group 3 ($n = 6$)	2 (1–3)*	2 (1–3)*	9 (4–16)‡
Group 4 ($n = 7$)	1 (1–2)†	1 (1–2)†	4 (2–9)†

* Group 3 > Group 1 and Group 2 ($p < 0.05$).
† Group 4 > Group 1 and Group 2 ($p < 0.05$).
‡ Group 3 > Group 1, Group 2 and Group 4 ($p < 0.05$).

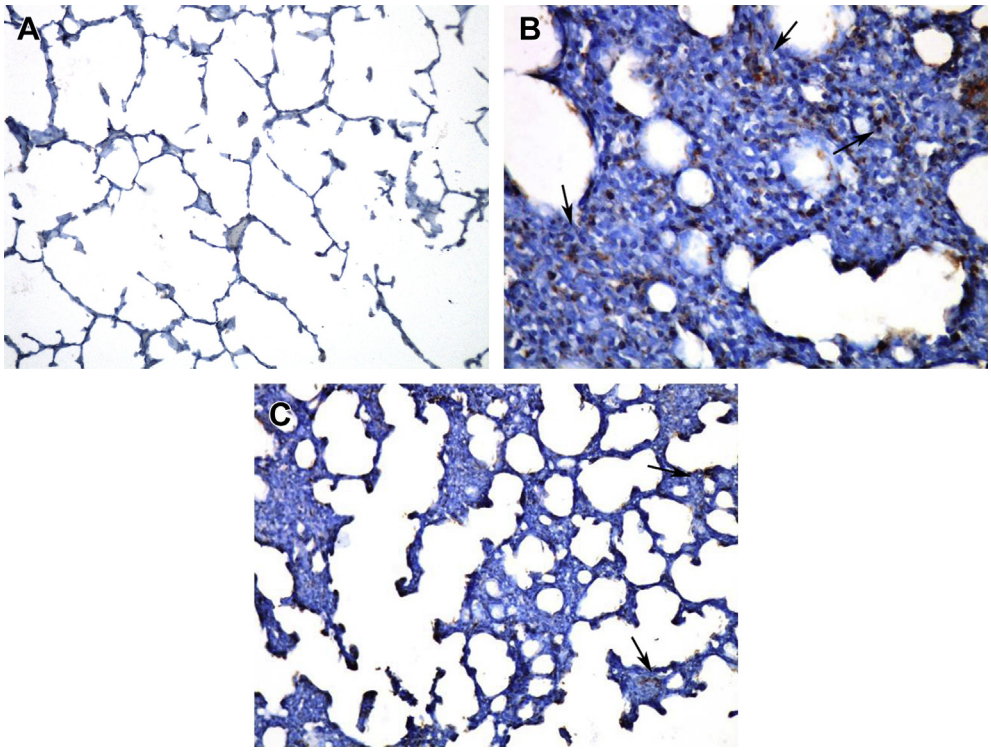

Figure 1 Representative light micrographs showing decreased smooth muscle actin (SMA) immunostaining with resveratrol treatment. (A) Air-exposed + saline group; (B) hyperoxia-exposed + saline group (the arrows are pointing to SMA immunostained areas); (C) hyperoxia-exposed + resveratol-treated group (SMA immunostain, ×10).

groups. However, in the hyperoxia-exposed groups, resveratrol treatment significantly increased these antioxidant activities when compared with saline ($p < 0.05$, Table 2). The lung tissue NO levels were found to be significantly higher in the hyperoxia-exposed group when compared with the air-exposed groups The lung tissue NO levels were found to be significantly higher in the hyperoxia-exposed + saline group when compared with the air-exposed and hyperoxia-exposed + resveratrol groups ($p < 0.05$). In addition, resveratrol treatment significantly reduced the NO levels ($p < 0.05$, Table 2). Although it was found that the hyperoxia-exposed Group 3 had the highest median NFκB and TNF-α levels, these results were not statistically significant ($p > 0.05$). Resveratrol treatment significantly decreased the levels of TNF-α in the hyperoxia groups when compared with the hyperoxia-exposed + saline group ($p < 0.05$). However, this decreased effect of resveratrol was not statistically significant for NFκB ($p > 0.05$, Table 2).

7. Discussion

Despite notable advances in neonatal critical care, BPD remains a major complication, frequently resulting in mortality as well as short-term and long-term morbidities.[13] It is characterized by an arrest in alveolar and vascular lung development, inflammation, and abnormal coagulation and fibrinolysis, resulting in alveolar fibrin deposition and oxidative stress.[14]

The hallmark features of BPD are a decreased number of alveoli, increased variability in alveolar size, and interstitial fibrosis.[15] There is evidence from animal models that exposure of the developing lung to high concentrations of oxygen inhibits alveolus formation and decreases lung surface area.[6,7,16] Similar to other animal studies, our study showed a significant decrease in ASA in rats exposed to hyperoxia. The histopathological changes in severe airway injury and alternating sites of overinflation and fibrosis that used to be seen in older forms of BPD have been replaced by a milder form that is characterized by alveolar and capillary hypoplasia and variable interstitial cellularity and/or fibroproliferation.[1,17] In our study, a median alveolar fibrosis score was absent in all air-exposed animals. However, exposure to hyperoxia resulted in moderate fibrosis in all of the animals, as in previously reported results.[6,7,17] In addition, although we found that resveratrol treatment in neonatal rats exposed to hyperoxia was associated with improved ASA and fibrosis compared with the hyperoxia-exposed + saline group, these results were not statistically significant. However, immunostaining for SMA demonstrated a marked decrease in smooth muscle content in the hyperoxia-exposed + resveratrol group. In contrast, median SMA score was significantly higher in the hyperoxia-exposed + saline group than in the other groups. Therefore, this study showed that resveratrol has a protective effect on hyperoxia-exposed lung injury in histopathological studies. In many different studies, investigators have shown that resveratrol has various pharmacological effects, including anti-inflammatory properties (such as reducing and regulating the release of

Table 2 The biochemical effects of resveratrol on hyperoxia-induced lung injury

	GSH (μg/g) Median (min–max)	SOD (ng/g) Median (min–max)	NO (mM/g) Median (min–max)	TNF-α (pg/g) Median (min–max)	NFκB (pg/g) Median (min–max)
Group 1 ($n = 10$)	2198.73 (1656.05–2563.68)	293.75 (280.66–307.30)	0.1392 (0.12–0.34)	443.15 (265.60–508.87)	225.16 (139.67–746.85)
Group 2 ($n = 11$)	2207.82 (646.39–2380.02)	296.31 (279.30–309.55)	0.1480 (0.10–0.18)	475.66 (328.11–603.75)	203.75 (87.17–1959.68)
Group 3 ($n = = 6$)	2296.92 (1944.59–2342.37)	271.56 (261.66–280.99)	0.2463† (0.17–0.89)	510.49 (407.77–542.83)	703.89 (170.38–1457.62)
Group 4 ($n = 7$)	2477.10* (2422.16–3225.90)	312.18* (261.47–377.03)	0.1649 (0.12–0.21)	408.67†‡ (312.71–468.79)	390.69 (79.97–446.95)

* Group 4 > Group 3 ($p < 0.05$).
† Group 3 > Group 1, Group 2 and Group 4 ($p < 0.05$).
‡ Group 4 < Group 3 ($p < 0.05$).

inflammatory mediators such as TNF-α, IL-1β, and IL-6), inhibition neutrophil infiltration, reduction in lung tissue collagen content, and the improvement of antioxidant defense mechanisms.[3–5,18,19] In the current study, we also found that resveratrol treatment significantly decreased the lung tissue levels of TNF-α in hyperoxic lung injury.

Pulmonary oxygen toxicity, through the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in excess of antioxidant defenses, is likely to play an essential role in the parenchymal injury of BPD.[1,7,20] NO, either endogenous or exogenous in origin, may function as either a pro-oxidant or antioxidant. Dawis et al demonstrated a strong correlation between the products of NO reactivity and BPD.[20] In several studies, it was reported that a high concentration of NO was produced by inducible NO synthase (iNOS) in inflammation, and the prevention of the expression of iNOS might be an important anti-inflammatory mechanism.[20–22] Tsai et al reported that resveratrol inhibited the induction of iNOS and the activation of NFκB, and reduced NO generation.[21] Thus, the authors concluded that the anti-inflammatory properties of resveratrol might be mediated by inhibition of iNOS expression through downregulation of NFκB. We found that the tissue levels of NFκB in the hyperoxia-exposed + saline group were higher than those of other groups, and that resveratrol treatment reduced NFκB levels; however, these results were not statistically significant. Recently, Pan and colleagues also reported that NO-mediated tyrosine nitration of proteins played an important role in the pathogenesis of hyperoxia-induced lung injury and increased nitrite levels in the damaged lung tissue induced by hyperoxia exposure in rats.[7] In the current study, the NO levels of hyperoxia-exposed + saline group rats were significantly higher than those of the air-exposed groups. Moreover, the NO levels of the hyperoxia-exposed + resveratrol group were significantly lower than those of the hyperoxia-exposed + saline group. Thus, we also showed that NO was a critical mediator of the inflammatory response for the development of hyperoxia-induced lung injury and that resveratrol significantly decreased the lung tissue levels of NO.

The evidence suggests the presence of an oxidant-antioxidant imbalance in lungs that are at risk of BPD.[1] Oxygen may damage the lung cells directly via the generation of ROS or indirectly via the action of the inflammatory cells and inflammatory mediators. These responses, in turn, overwhelm the cellular antioxidant defenses and lead to the accumulation of toxic levels of ROS.[23,24] High concentrations of oxygen also increase the formation of other free radicals, such as NO and peroxynitrite, which harmDNA and other biomolecules.[25] Several studies reported that lung injury induced by sepsis or bleomycin was coupled with GSH depletion, and that resveratrol treatment reversed this GSH depletion.[4,5] However, Pan and colleagues reported that the activities of antioxidant enzymes including SOD, GSH-peroxidase, and catalase did not change after exposure to hyperoxia.[7] In the current study, the activities of GSH and SOD, which did not change after exposure to hyperoxia, were significantly increased by resveratrol treatment. Previous studies have also shown the antioxidant effects of resveratrol via elevation of the antioxidant status such as GSH and SOD.[5,26]

In conclusion, this study showed that NO and oxidative stress play an important role in the etiopathogenesis of hyperoxic lung injury, and that resveratrol is effective in terms of preventing hyperoxic lung injury because of its anti-inflammatory and antioxidant effects.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

This study was supported by Pamukkale University Research Fund (Project no. = 2012TPF005). The authors thank Barbaros Şahin and Pamukkale University Animal Research Laboratory for their help with experimental techniques.

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