Colchicine Protects against Hyperoxic Lung Injury in Neonatal Rats

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Key Words

Bronchopulmonary dysplasia · Colchicine · Tumor necrosis factor- α · Superoxide dismutase · Glutathione peroxidase · Hyperoxia

Abstract

Background: Bronchopulmonary dysplasia (BPD) is characterized by inflammation, fibrosis and mucosal necrosis, which leads to emphysematous coalescence of alveoli. **Objective:** We tested whether prophylaxis with colchicine, an anti-inflammatory, antioxidant and antifibrotic drug, would decrease the severity of lung injury in an animal model of BPD. **Methods:** Twenty-five rat pups were divided into three groups: control (n = 8), hyperoxia (n = 7), and hyperoxia + colchicine (n = 10). The hyperoxia groups were exposed to >95% oxygen from day 1 to 10 of life. On day 10, the animals were sacrificed and the lungs were processed for histology and biochemical analysis. Lung morphology was assessed by the mean linear intercept (MLI), a measure of alveolar size. The degree of lung inflammation and antioxidant capacity were assessed by quantifying lung homogenate tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), malondialde-

hyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) levels. **Results:** Colchicine significantly decreased lung damage as determined by the MLI in the hyperoxia groups ($p < 0.01$). The median level of lung MDA was significantly higher in the hyperoxia group compared with the control group ($p < 0.05$) and the colchicine-treated group ($p < 0.05$). Lung homogenate SOD and GSH-Px activities in the colchicine-treated group were significantly higher than in the hyperoxia group ($p < 0.05$). Furthermore, colchicine-treated pups had lower lung homogenate TNF- α and IL-1 β levels compared with the hyperoxia group ($p < 0.05$). **Conclusions:** Colchicine has favorable effects on alveolarization as well as inflammation and oxidative stress markers in an animal model of BPD.

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Introduction

Bronchopulmonary dysplasia (BPD) is a significant cause of morbidity and mortality in premature infants, occurring in 30% of those with ≤ 28 weeks of gestation [1]. Clinical studies have identified numerous risk factors

for the development of BPD in premature infants, including hyperoxia, ventilator-induced pulmonary injury and antenatal infection [2, 3]. The multifactorial etiology of BPD and limited efficacy of current approaches drive the search for new therapies.

Groneck and Speer [4] demonstrated the importance of inflammation in the pathogenesis of BPD by showing that inflammatory mediators are released in response to direct pulmonary stressors or toxins, including oxidants, free radicals, hypoxia, infection, volutrauma and alveolar shear stress. Subsequent physiological events leading to BPD appear to be mediated through downstream effects of pro-inflammatory cytokines, chemokines and proteinases [5]. High levels of pro-inflammatory cytokines such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) have been found in the bronchoalveolar lavage fluid in experimental models of BPD [6, 7].

Colchicine, a unique anti-inflammatory, antifibrotic and antioxidant drug, has traditionally been used for the management of several inflammatory conditions such as familial Mediterranean fever, gout, idiopathic pulmonary fibrosis and Behçet's disease. Colchicine modulates the production of cytokines, decreases the TNF- α driven response by down-regulating TNF- α receptors and blocks IL-1 β activation. Besides inhibiting several leukocyte functions, such as rolling, adhesion, random mobility, phagocytosis, and cytokine secretion [8], it also inhibits phospholipase activation and decreases chemotactic responses of neutrophils to leukotriene B₄ and IL-8. In addition to its anti-inflammatory effects, colchicine also inhibits lipid peroxidation and stabilizes membranes [9].

As colchicine has been used in several inflammatory disorders, we hypothesized that colchicine prophylaxis would decrease the severity of lung injury in our rat model of BPD. The following parameters were studied: lung histology, anti-inflammatory markers (IL-1 β and TNF- α), malondialdehyde (MDA), an oxidative stress marker, and antioxidant enzyme, i.e. superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in lung homogenate.

Materials and Methods

Animal Model and Treatment

This experimental study was approved by the Experimental Animal Ethics Committee at Ankara Training and Research Hospital (Ankara, Turkey), and the National Institute of Health Guide (Washington, D.C., USA) for the Care and Use of Laboratory Animals was followed.

Timed pregnant Wistar rats were kept in a 12-hour dark/light cycle and fed a standard rat chow (Special Diet Services, Witham, Essex, UK) ad libitum. Breeding pairs were allowed access for 1 h on the day when female rats showed very specific sexual behaviors: lordosis, hopping and air-flapping. Pups delivered spontaneously at full gestation and only pups that delivered during a specified 24-hour period were used. All pups were left with their dams to breast feed freely. Experimental treatments began on postnatal day 1 and lasted through postnatal day 10, with the date of birth being day 1.

Twenty-five rat pups were divided into three groups. One group of pups (n = 8) was kept in room air and served as controls (control group). The second group of pups (n = 7) was exposed to hyperoxia (hyperoxia group) and the third group of pups (n = 10) pups was exposed to hyperoxia and treated with colchicine (hyperoxia + colchicine group). The pups exposed to hyperoxia were maintained in transparent Plexiglas chambers in which the oxygen concentration (>95%) was monitored continuously with an oxygen sensor; humidity was maintained above 80%, and CO₂ was removed by soda lime absorption. The oxygen concentration was kept at >95% using a flow of 2.5 liters/min. The control group was maintained in a similar chamber. Weight, evidence of disease and mortality were checked daily.

All pups were weighed each morning and received daily intraperitoneal injections of either colchicine or placebo (normal saline) from day 1 of life to day 10. Pups in the hyperoxia + colchicine group were injected with colchicine (Sigma Chemical Co., St. Louis, Mo., USA) at a dose of 1 mg/kg body weight whereas pups in the control and hyperoxia groups received equal volumes of normal saline administered in the same way.

Nursing mothers were rotated between litters exposed to room air and hyperoxia every 24 h to prevent damage to their lungs. Injections were done 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 10, the animals from each group were killed by intraperitoneal injection of pentobarbital sodium (200 mg/kg).

Tissue Preparation

To avoid postmortem fibrin deposition in the lungs, heparin (100 units; Mustafa Nevzat, Istanbul, Turkey) was injected intraperitoneally. After 5 min, the pups were exsanguinated by transection of the abdominal blood vessels. The thoracic cavity was opened and the right lung was removed, snap-frozen in liquid nitrogen and stored at -80°C until use for biochemical investigations. For histological studies, the trachea was cannulated (Bio-flow 0.6 mm intravenous catheter; Vygon, Veenendaal, the Netherlands), and the left lung was fixed in situ via the tracheal cannula with buffered formaldehyde (4% paraformaldehyde in PBS; pH 7.4) at 25 cm H₂O (2.4 kPa) pressure for 5 min. Then the left lung was removed, fixed in formaldehyde for 24 h at 4°C and 24 h at room temperature. Paraffin blocks were prepared with sections from each lobe.

Morphometric Analysis of Alveolar Size

Four-micrometer sections of the paraffin blocks were stained with hematoxylin and eosin. Ten random, nonoverlapping fields were examined with an ocular grid for each animal and the mean linear intercept (MLI), a measure of alveolar size, was calculated using a previously described technique [10, 11]. The MLI was de-

Table 1. Histopathological and biochemical evaluations in each group

	Control (n = 8)	Hyperoxia (n = 7)	Hyperoxia + colchicine (n = 10)
MLI, μm	42.5 (38.1–51.8)	52.9 (48–67.1) ^a	46.8 (39.2–52.5) ^b
MDA, nmol/g protein	2.8 (1.7–3.8)	10.7 (8.7–13.7) ^a	3.5 (1.7–5.3) ^b
SOD, U/g protein	80.2 (68.1–93.3)	45.9 (26.9–51.6) ^a	65.2 (44.8–74.6) ^{a, b}
GSH-Px, U/g protein	11.3 (7.3–13.5)	6.4 (3.3–8.8) ^a	8.6 (7.1–11.1) ^b
TNF- α , pg/mg protein	1.6 (0.7–2.3)	4.9 (4.1–6.8) ^a	2.2 (1.2–3.6) ^b
IL-1 β , pg/mg protein	2.1 (1.1–2.8)	9.6 (8.2–13.8) ^a	4 (1.1–5.9) ^b

^a Significantly different from control group. ^b Significantly different from hyperoxia group.

terminated by the number of alveolar intercepts of a single horizontal line using the formula $Lm = (N \times L)/m$, where Lm is the MLI, N is the number of fields evaluated, L is the length of the line, and m is the sum of all the intercepts [10].

Biochemical Analysis

Lung homogenate TNF- α and IL-1 β concentrations were measured in duplicate with a commercially available enzyme-linked immunosorbent assay kit (BioSource Europe SA, Nivelles, Belgium) according to the manufacturer's instructions.

After addition of phosphate buffer (pH 7.4), the frozen tissues were homogenized on an ice cube using a homogenizer (Heidolph Diax 900; Heidolph Elektro GmbH, Kelheim, Germany). The supernatant was used for the entire assay. Initially, the protein content of tissue homogenates was measured as described by Lowry [12] with bovine serum albumin as the standard.

The lipid peroxidation level was measured with the thiobarbituric acid reaction according to the method of Ohkawa et al. [13]. SOD activity was assayed using the nitroblue tetrazolium method of Sun et al. [14]. GSH-Px activity was measured using the method described by Paglia and Valentine [15]. MDA, SOD, GSH-Px activities are expressed as units per gram protein.

Statistical Analysis

Statistical analyses were carried out using SPSS (SPSS for Windows, Version 18.0). Data are expressed as medians and ranges. Three-way comparisons among groups were analyzed using the Kruskal-Wallis test. Post-hoc comparisons among groups with significant values were evaluated with the Bonferroni-corrected Mann-Whitney U tests. Statistical significance was defined as $p < 0.05$.

Results

The median weights of pups were similar in the three groups at birth, i.e. control 5.2 (4.8–5.8) g, hyperoxia 5.1 (4.8–6) g, hyperoxia + colchicine 5 (4.7–6) g. Median weight gain in the hyperoxia group [11.9 (9.5–13.2) g] was significantly lower than in the control [17.8 (16.6–

18.4) g] and hyperoxia + colchicine [16.9 (14–17.8) g] groups ($p < 0.05$). None of the pups died during the study period.

The MLI, MDA, TNF- α , IL-1 β levels, and SOD and GSH-Px activities are shown in table 1. Histologic evaluation of the three groups demonstrated that the MLI was significantly increased in the hyperoxia group versus the control group ($p = 0.009$). The colchicine treatment resulted in less emphysematous injury, as demonstrated by a decreased MLI in the hyperoxia + colchicine group compared with the hyperoxia group ($p = 0.006$). Figure 1 shows histologic sections from all groups, the hyperoxia group having enlarged alveoli.

The median level of lung MDA was significantly higher in the hyperoxia group compared with the control ($p < 0.05$) and the hyperoxia + colchicine groups ($p < 0.05$), suggesting the presence of increased lipid peroxidation in the hyperoxia group. Lung homogenate median SOD and GSH-Px activities in the hyperoxia + colchicine group were significantly higher than in the hyperoxia group ($p = 0.006$ and $p = 0.04$, respectively). Hyperoxia increased the level of TNF- α and IL-1 β in lung homogenates compared with controls ($p < 0.05$), and treatment with colchicine reduced the increases in TNF- α and IL-1 β more than 2 fold ($p < 0.05$) (table 1).

Discussion

This study is the first to describe the effects of colchicine in an animal model of BPD. We demonstrated a significant improvement in weight gain, less lung injury as measured by MLI, decreased inflammatory cytokines (IL-1 β , TNF- α) and oxidative stress marker (MDA) and increased antioxidant enzyme activities (SOD, GSH-Px)

Fig. 1. Lung morphology of the control (a), hyperoxia (b) and hyperoxia + colchicine (c) rat pups. Note that colchicine administration has reduced the prominent increase in alveolar diameter in the hyperoxia group. Hematoxylin and eosin. Original magnification $\times 20$.

when hyperoxic neonatal rats were prophylactically treated with colchicine.

The development of BPD is characterized by an initial acute inflammatory component followed by variable degrees of lung fibrosis and failure of alveolar septation, both of which ultimately impair the development of the immature lung. Central to this inflammatory response is the upregulation of several proinflammatory and chemotactic cytokines [16]. TNF- α is a central mediator of the inflammatory response. High levels of TNF- α may promote chronic inflammation by overwhelming counter-regulatory mechanisms and may lead to the development of BPD whereas low levels of TNF- α may decrease the risk and/or severity of BPD [17]. Similarly, IL-1 β is a pro-inflammatory cytokine that contributes to the pathogenesis of chronic inflammatory diseases such as BPD [7, 18].

Inflammation has an important role in the etiopathogenesis of BPD [19]. The anti-inflammatory effect of colchicine in BPD may be due to its inhibitory effects on anti-TNF- α and IL-1 β activation. It is known that colchicine downregulates TNF- α receptors and TNF- α driven responses in macrophages, and suppresses IL-1 β processing and release [8, 20].

Oxygen causes tissue injury through formation of highly reactive and destructive radicals, such as hydroxyl radicals, and through peroxidation of membrane lipids [21, 22]. Oxidative stress plays a major role in the development of BPD; therefore, antioxidant therapies are thought to improve lung morphology. The immaturity of the intracellular enzymatic antioxidant defense – which includes SOD, catalase and GSH-Px – makes premature infants highly susceptible to oxidative injury [23]. Reiter et al. [24] proposed that impairment of antioxidant defense

mechanisms could permit enhanced free radical-induced tissue damage.

There has been only scant information on antifibrotic medications with regard to antioxidant and anti-inflammatory effects. Almaria et al. [25] tested whether pentoxifylline prophylaxis had a protective effect on hyperoxia-induced lung injury in an experimental study. They demonstrated that treatment with pentoxifylline significantly decreased lung edema and macrophage infiltration. Furthermore, as in our study, pentoxifylline treatment increased lung antioxidant enzyme activities, including SOD, GSH-Px and catalase.

We observed a significant increase in lung MDA level in the hyperoxia group, suggesting increased lipid peroxidation. Measurement of lipid peroxidation is a practical and safe method to evaluate the factors causing cellular injury and tissue MDA content; MDA, the last product of lipid breakdown caused by oxidative stress, is considered to be a good indicator of free radical-induced lipid peroxidation. Colchicine inhibits lipid peroxidation and stabilizes membranes [9], and prophylaxis by colchicine might have prevented an increase in lung MDA levels in our study. Our results demonstrated the protective role of colchicine in BPD development through its inhibitory action on oxygen-derived free radicals.

MLI is a commonly used measure of alveolar development and is inversely proportional to alveolarization [6, 26]. Larger MLI values correspond to greater disruption of normal alveolar growth. Colchicine administration resulted in less emphysematous injury, as demonstrated by a decreased MLI in the hyperoxia + colchicine group compared with the hyperoxia group. Our findings suggest that the antioxidant and anti-inflammatory activities of col-

chicine might have a favorable effect on the process of ongoing alveolarization in newborn rats. Limitations of the animal model, such as too much oxygen, absence of respiratory support, timing and dosages of drugs may be irrelevant to the real clinical course of BPD in the contemporary concept and strategy of lung protection.

Conclusions

Our report is the first to demonstrate the beneficial effects of colchicine on alveolarization, lung inflammation and oxidative stress in neonatal rats with hyperoxia-

induced lung injury. This study emphasizes the potential of colchicine as an anti-inflammatory and antioxidant agent in the prophylaxis of BPD in premature infants. However, further studies are needed to elucidate the mechanism underlying the beneficial effect of colchicine therapy on BPD and to clarify possible side effects of colchicine on other systems and organs.

Disclosure Statement

The authors have no conflict of interest to disclose.

References

- 1 Jobe AH, Bancalari E: Bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 2001; 163:1723–1729.
- 2 Ozdemir R, Erdevi O, Dizdar EA, Oguz SS, Uras N, Saygan S, Karabulut E, Dilmen U: Clarithromycin in preventing bronchopulmonary dysplasia in *Ureaplasma urealyticum*-positive preterm infants. *Pediatrics* 2011;128:e1496–e1501.
- 3 Keyburn B, Martin RJ, Prakash YS, MacFarlane PM: Mechanisms of injury to the preterm lung and airway: implications for long-term pulmonary outcome. *Neonatology* 2012;101:345–352.
- 4 Groneck P, Speer CP: Inflammatory mediators and bronchopulmonary dysplasia. *Arch Dis Child Fetal Neonatal Ed* 1995;73:1–3.
- 5 Van Marter LJ: Epidemiology of bronchopulmonary dysplasia. *Semin Fetal Neonatal Med* 2009;14:358–366.
- 6 Polglase GR, Dalton RG, Nitsos I, Knox CL, Pillow JJ, Jobe AH, Moss TJ, Newnham JP, Kallapur SG: Pulmonary vascular and alveolar development in preterm lambs chronically colonized with *Ureaplasma parvum*. *Am J Physiol Lung Cell Mol Physiol* 2010; 299:232–241.
- 7 Viscardi RM, Atamas SP, Luzina IG, Hasday JD, He JR, Sime PJ, Coalson JJ, Yoder BA: Antenatal *Ureaplasma urealyticum* respiratory tract infection stimulates proinflammatory, profibrotic responses in the preterm baboon lung. *Pediatr Res* 2006;60:141–146.
- 8 Terkeltaub R: Colchicine update: 2008. *Semin Arthritis Rheum* 2009;38:411–419.
- 9 Das D, Pemberton PW, Burrows PC, Gordon C, Smith A, McMahon RF, Warnes TW: Antioxidant properties of colchicine in acute carbon tetrachloride induced rat liver injury and its role in the resolution of established cirrhosis. *Biochim Biophys Acta* 2000;1502: 351–362.
- 10 Dunnill MS: Quantitative methods in the study of pulmonary pathology. *Thorax* 1962; 17:320–328.
- 11 Ballard HO, Bernard P, Qualls J, Everson W, Shook LA: Azithromycin protects against hyperoxic lung injury in neonatal rats. *J Invest Med* 2007;55:299–305.
- 12 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–275.
- 13 Ohkawa H, Ohishi N, Yagi K: Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979;95:351–358.
- 14 Sun Y, Oberley LW, Li Y: A simple method for clinical assay of superoxide dismutase. *Clin Chem* 1988;34:497–500.
- 15 Paglia DE, Valentine WN: Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* 1967;70:158–169.
- 16 Wright CJ, Kirpalani H: Targeting inflammation to prevent bronchopulmonary dysplasia: can new insights be translated into therapies? *Pediatrics* 2011;128:111–126.
- 17 Kazzi SN, Kim UO, Quasney MW, Buhimschi I: Polymorphism of tumor necrosis factor-alpha and risk and severity of bronchopulmonary dysplasia among very low birth weight infants. *Pediatrics* 2004;114:e243–e248.
- 18 Merritt TA, Deming DD, Boynton BR: The 'new' bronchopulmonary dysplasia: challenges and commentary. *Semin Fetal Neonatal Med* 2009;14:345–357.
- 19 Speer CP: Chorioamnionitis, postnatal factors and proinflammatory response in the pathogenetic sequence of bronchopulmonary dysplasia. *Neonatology* 2009;95:353–361.
- 20 Ben-Chetrit E, Bergmann S, Sood R: Mechanism of the anti-inflammatory effect of colchicine in rheumatic diseases: a possible new outlook through microarray analysis. *Rheumatology* 2006;45:274–282.
- 21 Saugstad OD, Sejersted Y, Solberg R, Wollen EJ, Bjørås M: Oxygenation of the newborn: a molecular approach. *Neonatology* 2012;101: 315–325.
- 22 Saugstad OD, Aune D: In search of the optimal oxygen saturation for extremely low birth weight infants: a systematic review and meta-analysis. *Neonatology* 2011;100:1–8.
- 23 Thomas W, Speer CP: Nonventilatory strategies for prevention and treatment of bronchopulmonary dysplasia – what is the evidence? *Neonatology* 2008;94:150–159.
- 24 Reiter RJ, Tan DX, Osuna C, Gitto E: Actions of melatonin in the reduction of oxidative stress. A review. *J Biomed Sci* 2000;7:444–458.
- 25 Almario B, Wu S, Peng J, Alapati D, Chen S, Sosenko IR: Pentoxifylline and prevention of hyperoxia-induced lung-injury in neonatal rats. *Pediatr Res* 2012;71:583–589.
- 26 Margraf LR, Tomashefski JF Jr, Bruce MC, Dahms BB: Morphometric analysis of the lung in bronchopulmonary dysplasia. *Am Rev Respir Dis* 1991;143:391–400.