Melatonin protects against oxidative damage in a neonatal rat model of bronchopulmonary dysplasia

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Background: **Oxidative stress plays an important role in the pathogenesis of bronchopulmonary dysplasia (BPD). Melatonin (MT) has direct and indirect free radical detoxifying activity. The present study was to investigate whether treatment with MT would attenuate hyperoxia-induced lung injury and the effect of MT on imbalance of oxidants/antioxidants in the lung of neonatal rats.**

Methods: **BPD was induced by exposure to hyperoxia in neonatal rats (*n*=90). The rats were divided randomly into three groups (*n*=30 each): air-exposed control group, hyperoxia-exposed group, and hyperoxia-exposed MT-treated group. Lung specimens were obtained respectively on day 3, day 7, and day 14 after exposure (*n*=10 each). Activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT), and levels of myeloperoxidase (MPO), nitrite/nitrate, and malondialdehyde (MDA) were assayed. Histopathologic changes were observed in the tissues stained with hematoxylin and eosin and Masson's trichrome stain.**

Results: **Increased levels of MPO, nitrite/nitrate, and MDA in the hyperoxia-exposed rats were significantly reduced by MT (*P*<0.05). Activities of GSH-Px, SOD, and CAT which did not change after exposure to hyperoxia were increased by MT (*P*<0.05). Furthermore, BPD associated histopathological alterations such as reduced total number of alveoli and interstitial fibrosis were obviously abated in the MT-treated group.**

Conclusions: **MT can reverse oxidants/antioxidants imbalance in damaged lung tissue and thus exert a beneficial effect on hyperoxia-induced lung disease in neonatal rats. With regard to humans, there may be a protective effect of MT on BPD.**

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Introduction

Bronchopulmonary dysplasia (BPD) is a chronic pneumopathy most commonly seen in preterm newborn infants requiring prolonged respiratory support. The etiology of BPD is believed to be multifactorial. Probable contributing factors include O_2 toxicity, barotrauma, immaturity, relative surfactant deficiency, and prenatal infection, with complex interactions between the various contributing factors.[1] Pulmonary O_2 toxicity, through the generation of reactive oxygen species (ROS)[2] and reactive nitrogen species (RNS)[3] in excess of antioxidant defenses, is likely to play a central role in the parenchymal injury of BPD.

There is currently no effective preventive therapy for BPD. The putative central role for O_2 toxicity in the development of BPD has led to considerable interest of researchers in developing therapeutic antioxidant approaches to prevention.[4] Melatonin (MT) or N-acetyl-5-metoxy-tryptamine is an indole that is synthesized and secreted from the pineal gland during the night. The fetus receives MT from the mother, but following premature delivery there may be a period of much more prolonged MT deficiency. This deficiency may be harmful because neurohormone has important functions. Blood MT drives circadian rhythms, influences the sleep-wake cycle which changes the physiology from day time to night time in a well co-ordinated manner and aids in the synchronization of circadian rhythmicity in all tissues, which is involved in the development. Recently, it was identified as a powerful direct free radical scavenger and indirect antioxidant.[5] Gitto et al[6] has shown that newborns with propensity to

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develop BPD had reduced inflammatory cytokines in their blood and improved respiratory function when giving MT. However the mechanism underlying MT that may protect BPD is still unclear. Therefore, this study aimed to investigate the protective effect of MT in a rat model of BPD and the effect of MT on the production of oxidative/antioxidative and nitrosiative stress markers, and to test the hypothesis that MT can prevent hyperoxia-induced lung disease via its powerful antioxidant activity.

Methods

Animal models

The hyperoxia-induced rat model of BPD was used.[7] Newborn Wistar rats and fungible mother rats were provided by the Laboratory Animal Department of China Medical University. The animals were divided randomly into three groups (n=30 each): air-exposed control group (group I), hyperoxia-exposed model group (group II), and hyperoxia-exposed MT-treated group (group III). Groups II and III were placed in an oxygen chamber, into which oxygen was continuously delivered (FiO_2=0.85±0.02) (OM-25ME oxygen monitor, USA). CO_2 was kept below 0.5% (Dapex Gas monitor, USA). Temperature and humidity were maintained at 22ºC-25ºC and 60%-70% respectively. Group III received MT (Sigma, St Louis, MO, USA) intraperitoneally at a dose of 4 mg/kg every night at 8PM, and the first dose of MT was given 30 minutes before the hyperoxia exposure. Group I was exposed to air. The chamber was opened for <30 minutes daily to switch dams between air and O_2 environment to protect the dams from oxygen toxicity.

Preparation of specimens

Pups from each group were killed on days 3, 7 and 14 after experiment and a tracheal cannula was placed. An abdominal incision was made, and the diaphragm was punctured carefully to collapse the lungs. The left lungs were inflation-fixed via a tracheal cannula using 10% neutral formaldehyde solution for morphological observation. The pulmonary artery was cannulated and the left atrial appendage was clipped. The right lungs were gently perfused with 10 ml of 0.9% saline to remove blood, and then put in Eppendorf tubes and stored in a freezer at -80ºC for biochemical analyses.

Biochemical analyses

Lung tissues were homogenized in a four-volume of ice-cold Tris-Hcl buffer (pH 7.4) using a homogenizer after cutting of the lung into small pieces (for 1 minute at 10 000 rpm). Tissue malondialdehyde (MDA) and nitric oxide (NO) levels were determined in the homogenates, which were later centrifuged at 5000 g for 60 minutes to remove debris. Clear upper supernatant fluid was taken and assayed for activities of catalase (CAT), glutathione peroxidase (GSH-Px), and myeloperoxidase (MPO). After the supernatant solution was extracted with an equal volume of ethanol/chloroform mixture (5/3, v/v) and centrifuged at 5000 g for 60 minutes, the upper ethanol phase was taken and used in the superoxide dismutase (SOD) and protein assays. The protein measurement was analyzed in homogenates, supernatant and extracted samples. All indices were determined using a colorimetric assay (Calbiochem, La Jolla, CA, USA) as described previously.[8] All samples were assayed in triplicate.

BPD associated histopathological alterations

The tissues were paraffin sectioned (4 μm) and later stained with hematoxylin and eosin (HE) after deparaffinization. Ten HE stained sections of each time point in each rat were randomly selected. Five fields for each section were examined under a light microscope (× 200) to observe histological changes, estimate radical alveolar counts (RACs)[9] and calculate the mean value. RAC refers to the number of alveoli transected by a perpendicular line drawn from the center of the most peripheral bronchiole (respiratory bronchiole which is not completely covered by epithelium) to the pleura or the nearest interlobular septum. This is an important index to evaluate the stage of lung development.[9]

Collagen was stained with Masson's trichrome stain. Paired slide sets were stained, photographed, and photoprocessed together to allow direct comparisons between photomicrographs.

Statistical analysis

Data were expressed as mean±SEM. Statistical analysis was made by analysis of variance (ANOVA) followed by appropriate post hoc tests including multiple comparison test (LSD). All analyses were made using the SPSS 13.0 statistical software package, and the probability value of <0.05 was considered statistically significant.

Results

Effects of MT on hyperoxia-induced oxidative damage in the lung

Hyperoxia exposure resulted in a significant rise in MDA content of lung tissue which is an index for lipid peroxidation when compared with the control group. In the MT treated group, the MDA content was significantly decreased (Fig. 1A).

As NO measurement is difficult in biological

specimens, tissue nitrite/nitrate contents were estimated as an index of NO production. Hyperoxia exposure produced a significant increase in lung tissue nitrite/nitrate contents when compared with that of the control group. MT significantly prevented the increase of the lung tissue nitrite/nitrate contents (Fig. 1B). MPO activity, a marker of inflammatory cell especially neutrophils influx into tissue, was significantly increased in rats with hyperoxia exposure alone as compared to the control group. The rise in the lung tissue MPO activity produced by hyperoxia was prevented by MT (Fig. 1C).

Effects of MT on hyperoxia-induced antioxidants in the lung

The depletion in GSH-Px, SOD and CAT in the tissue reflects indirectly the generation of free radicals. Hyperoxia exposure could not increase the activities of GSH-Px, SOD and CAT significantly (though activities of these antioxidant enzymes were increased in data in the hyperoxia-exposure group and the control group, no statistical differences were found between the two groups), while MT treatment corrected this deficiency and increased activities of these enzymes significantly (Table 1).

Effect of MT on lung injury during hyperoxia exposure

On the 3rd day of the experiment, the immature structure of pulmonary alveoli with a small alveolar lumen and thick alveolar septum was observed in each group. And infiltration of a small amount of inflammatory cells was observed in the alveoli and septum in the hyperoxia-exposed group. In the MT treated group, the number of infiltrated inflammatory cells was decreased. Between 7 and 14 days, alveoli were regular in size in the control group. In the hyperoxia-exposed group, however, the alveolar spaces became large on day 7 along with thickening of alveolar septum, and on day 14, alveolar spaces enlarged more significantly and the number of alveoli decreased, the alveolar diameter increased, and the number of interstitial cells and local pulmonary interstitial fibroblasts increased. In the MT treated group, however, the alveoli were more regular in size and pulmonary interstitial fibrosis was significantly decreased (Fig. 2). No difference was observed in the lung RAC among the three groups within 3 days after birth. On days 7 and 14, however, the lung RAC of the hyperoxia group was significantly lower than that of the control and the MT treated groups (Table 2).

Since lung interstitial fibrosis is another important histopathological alteration of BPD and is closely

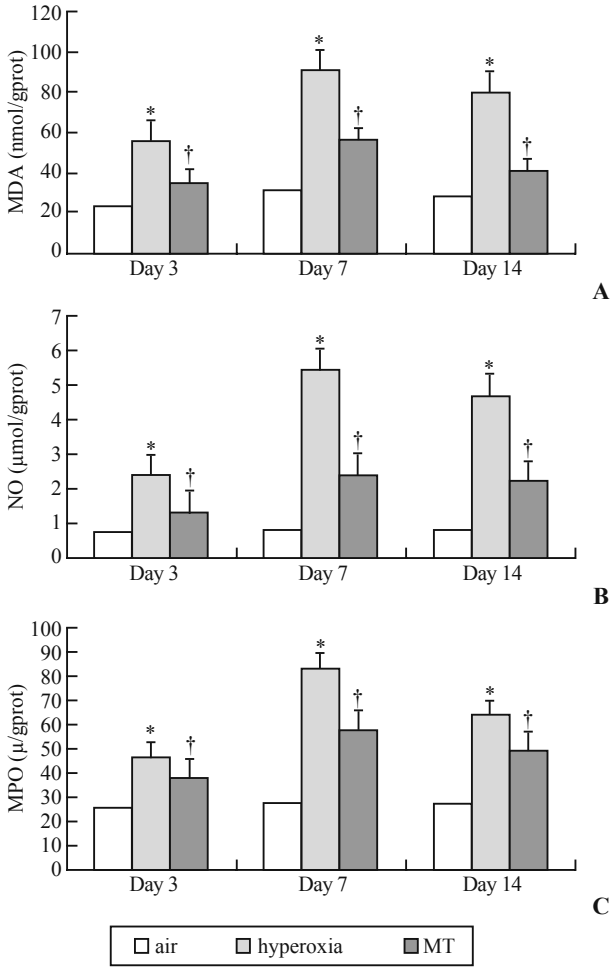

Fig. 1. Effect of MT on hyperoxia-induced changes in the levels of MDA (**A**), NO (**B**) and MPO (**C**). Hyperoxia exposure caused a significant increase of these parameters, and treatment with MT significantly prevented these increases. Data presented as mean±SEM of the three groups. *: $P<0.05$ vs. group I (the air-exposed group). †: $P<0.05$ vs. group II (the hyperoxia-exposed group).

Table 1. The effect of hyperoxia on antioxidant enzymes activities and the effect of MT on these enzymes in the lung tissue of neonatal rats

Groups	Superoxide dismutase (nU/mg tissue)			Glutathione peroxidase (U/mg tissue)			Catalase (U/mg tissue)		
	Day 3	Day 7	Day 14	Day 3	Day 7	Day 14	Day 3	Day 7	Day 14
I	28.93±2.95	68.58±3.32	73.69±2.55	2.58±1.73	3.88±0.89	4.79±1.58	5.98±2.13	8.29±2.94	9.02±2.16
II	36.48±1.53	70.23±2.36	72.82±4.79	2.88±0.52	4.06±1.23	4.98±0.73	6.93±1.94	9.53±2.46	10.36±1.11
III	44.36±1.88*	105.13±8.52*	119.67±10.23*	4.88±1.02*	9.08±0.56*	11.14±1.26*	9.65±1.94*	24.95±2.13*	29.88±2.14*

Data presented as mean±SEM of the three groups. *: $P<0.05$ vs. all other groups.

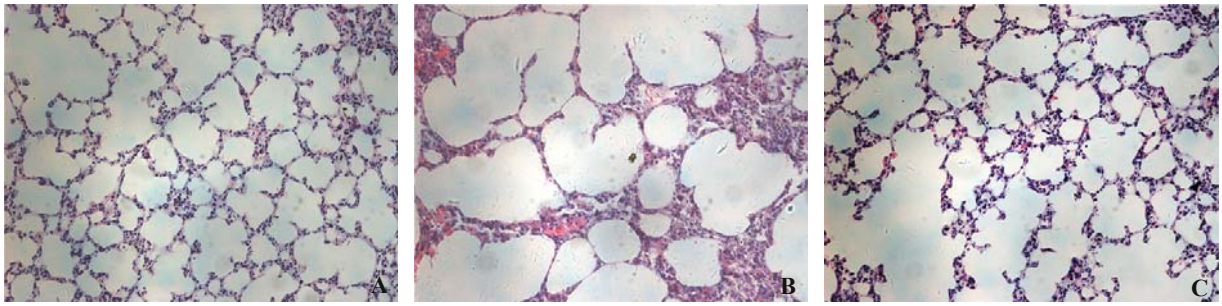

Fig. 2. Lung morphology. These figures are representatives of the lungs from each group on day 14 (HE, original magnification × 200). Alveoli were regular in size in the control group (**A**). Hyperoxia exposure produced enlarged alveolar spaces, decreased numbers of alveoli as well as increased local pulmonary interstitial fibrosis (**B**). A marked prevention of the hyperoxia-induced histological changes was seen in the rat lungs in the MT treated group (**C**).

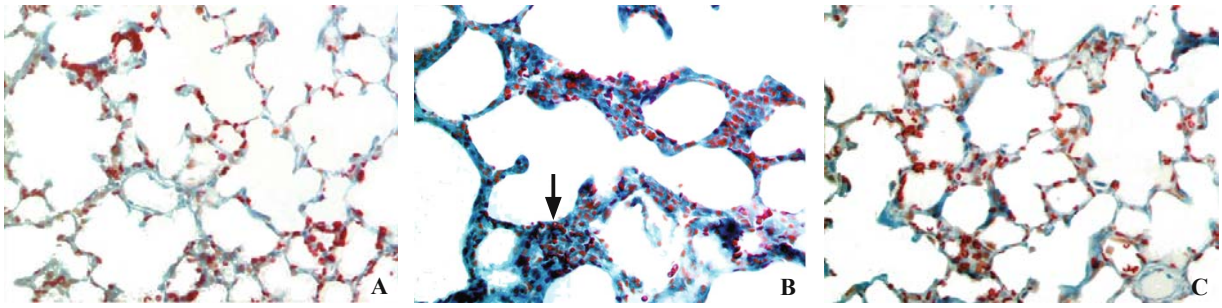

Fig. 3. Collagen staining. These figures showing sections of rat lung stained for collagen (blue staining) on day 14 (Masson's trichrome stain, original magnification × 400). Little collagen fiber staining can be seen in the control group (**A**). Significantly enlarged alveolar spaces and thickened alveolar septa (arrow) were observed in the hyperoxia-exposed group (**B**). MT may be of benefit in preventing lung fibrosis as seen after hyperoxia exposure (**C**).

Table 2. Effect of MT on hyperoxia-induced changes in radical alveolar count of neonatal rats

Groups	Radical alveolar count		
	Day 3	Day 7	Day 14
I	6.17±0.71	7.83±0.54	9.83±0.52
II	6.26±0.86	6.75±0.76*	5.25±0.36*
III	6.23±0.42	7.42±0.63†	7.38±0.44†

Data presented as mean±SEM of the three groups. *: $P<0.05$ vs. group I (the air-exposed control group); †: $P<0.05$ vs. group II (the hyperoxia-exposed group).

related to the prognosis, we evaluated the fibrosis with collagen fiber stained with Masson's trichrome stain. The results demonstrated marked changes in lung collagen content after MT treatment. Hyperoxia exposure was associated with increased staining of collagen fibers, while lung sections from the control and MT treated groups showed less collagen fibers stained in the interstitia on day 14 (Fig. 3).

Discussion

Despite advances in neonatal critical care including less traumatic ventilation and reduced oxygen concentration, BPD still affects approximately one-third of very low birth weight preterm babies and thus is associated with significant morbidity and mortality. Pathophysiology and pathogenesis of BPD have been substantially understood, and upon recognition of ROS in the pathogenesis of BPD,[10] major efforts have been made to find antioxidants for the prevention or mitigation of BPD, but not all of them have proven beneficial and thus more novel approaches are therefore warranted.[11]

MT that can prevent lung injury and brain damage in many adult animal models[12-17] is also thought to be a good candidate for studies in human newborns at risk for developing lung disease. In the present study, we assessed whether exogenous MT during hyperoxia exposure would attenuate hyperoxia-induced lung injury in neonatal rats. Because the half-life of MT was <1 hour, the product was only effective for 3-4 hours. Therefore continuous nocturnal administration of MT would be ideal. Although data were not shown, the study was dose-response with 1 mg/kg, 4 mg/kg and 10 mg/kg MT.[6,12-17] Nocturnal administration of 4 mg/kg MT seemed to be the most appropriate one, and would prevent BPD associated histopathological alterations such as reduced total number of alveoli and interstitial fibrosis as assessed by semiquantitative morphological indices of RAC and collagen fiber

staining. Furthermore, MT prevented the hyperoxia-induced increases of the MPO, MDA and NO levels and attenuated the hyperoxia-induced depletion of antioxidant enzyme activities in the damaged lung tissue of rats.

The mechanism through which MT may prevent hyperoxia-induced lung injury is likely related to its ability to reduce damage to lung structures in the early stage of the disease processes as a powerful antioxidant. Oxygen toxicity to cells is mediated through ROS and RNS that are generated endogenously by several mechanisms under pathophysiological conditions. The hyperoxia-induced lung injury involves, as an initial event, the generation of oxidant species by direct hyperoxia exposure, and further damage is probably elicited by activated inflammatory cells recruited into the damaged lung induced by hyperoxia exposure. Since MT has a protective effect against ROS[18] and RNS,[19] it could reduce the ROS and RNS production or ROS and RNS-induced progression in the early inflammatory phase. Accumulation of inflammatory cell produces significant damage in BPD and antioxidant agents may protect against tissue damage by reducing neutrophils influx into the tissue.[20] In the present study, the inhibitory effects of MT on the accumulation of leukocytes into the lung manifested by the reduction in MPO activity in lung tissue may contribute to a further protection of the lung from free radical damage produced by leukocytes.

NO-mediated tyrosine nitration of proteins plays a significant role in the pathogenesis of hyperoxia-induced lung disease and increased nitrite levels in the damaged lung tissue induced by hyperoxia exposure in rats.[21] Similarly in the present study, hyperoxia exposure caused an increase in the lung tissue nitrite/nitrate levels, which was significantly inhibited by MT treatment, supporting the role of RNS in the pathogenesis of hyperoxia-induced lung disease and the protective effect of MT against RNS.

Because of the developmental regulation of antioxidant enzymes (AOEs), preterm babies with an immature antioxidant defense system may be exceptionally vulnerable to hyperoxia-induced lung injury, unless they are capable of rapidly mounting an antioxidative response when exposed to hyperoxia. Indeed, the regulation of pulmonary AOEs in hyperoxia differs in animal species and also in different age groups within the same species.[22] In this study, activities of SOD, CAT and GSH-Px were not significantly increased after hyperoxia exposure. Lack of AOEs may be due to developmental regulation or possible inability to upregulate them in response to hyperoxia. Possibly, however, their activities were up-regulated during the acute phase of hyperoxia exposure but exhausted immediately after the rapid and ponderosus production of ROS. These activities were significantly higher in the MT treated group. These findings confirmed the promotional effects of MT on antioxidant enzymes,[23] but we cannot determine whether the observed antioxidant effect of MT is related to its direct free radical scavenging activity of the indole or to its indirect promotion of antioxidant enzyme activities of lung tissue.

A final relevant topic to be discussed is the possibility that the observed effects of MT in this study are directly due to binding of MT to MT receptors in the lung, but not just due to MT functioning as an antioxidant. The up-regulation of MT receptor mRNA in lipopolysaccharide (LPS)-induced lung injury[24] suggests that ligand-receptor binding reaction might be involved in the mechanism through which MT prevent lung injury.

We recognize that oxygen is a life-saving treatment for many diseases. However, if we focus on its oxidant capacity, MT seems to be a novel remedy.[25,26] There is a general agreement that short-term MT therapy is safe in neonates.[27] However, long-term effects of early MT treatment have seldom been investigated. Only one study[17] showed that MT did not affect growth rate and behavior at adulthood. Preclinical studies in developing animals may be mandatory to analyze these possible long-term effects. The present study supports that MT is promising for the treatment of preterm infants with lung disease.

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