Calcitonin Gene-Related Peptide Ameliorates Hyperoxia-Induced Lung Injury in Neonatal Rats

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Therapies with prolonged exposure to high-concentration oxygen are common in the treatment of critical pulmonary and cardiac conditions in newborns. However, prolonged exposure to hyperoxia could result in lung damages and developmental disorders manifested as acute lung injury and bronchopulmonary dysplasia, respectively. Calcitonin gene-related peptide (CGRP) has been shown to have a broad regulatory effect on the respiratory system. In this study, we explored the protective effects of CGRP on the hyperoxia-induced lung damage. Newborn Sprague-Dawley rats were randomly divided into three groups: normoxia, hyperoxia, and hyperoxia with CGRP. Hyperoxia groups were exposed to 95% oxygen for 14 days and treated once every other day with saline or CGRP. Hyperoxia exposure reduced the survival rate to 73%, when compared with the 93% survival rate observed in the normoxia group. The survival rate was improved to 84% with CGRP treatment. Treatment with CGRP under hyperoxia significantly alleviated the hyperoxia-induced lung histomorphological changes and the increases in leukocyte counts and total protein levels in bronchoalveolar lavage fluid that reflect the pulmonary microvasular damages. CGRP treatment also restored the decreased activity of superoxide dismutase, while it decreased the increased level of malondialdehyde in the lung tissues. Importantly, CGRP treatment significantly decreased the magnitude of the hyperoxia-mediated increase in the expression levels of tumor necrosis factor-α mRNA and transforming growth factor-β 1 protein. In conclusion, the hyperoxia-induced acute lung injury is associated with both oxidative stress and inflammatory responses, and CGRP may ameliorate the hyperoxia-induced lung injury by down-regulating these processes.

Keywords: acute lung injury; calcitonin gene-related peptide; hyperoxia; newborn; rat


Therapies that utilize prolonged exposure to high-concentration oxygen are common in the treatment of critical pulmonary and cardiac conditions in newborns. High levels of oxygen enhance the partial pressure of oxygen available to sensitive tissues. However, overexposure may result in damage to developing lung tissues. The response to prolonged exposure to elevated oxygen concentrations (≥ 50%) differs significantly between mature adult and developing neonatal lung tissues (Bhandari et al. 2006; Gore et al. 2010). In extremely low-birth-weight infants, prolonged exposure to elevated oxygen levels is especially likely to cause lung damage and other developmental disorders, such as hyperoxia-induced acute lung injury (ALI) and subsequent bronchopulmonary dysplasia (BPD) (Bhandari 2008). Though recent improvements in neonatal respiratory management have reduced the occurrence of these conditions, hyperoxia-induced ALI and BPD continue to be notable causes of premature infant mortality and morbidity (Kinsella et al. 2006). Despite the apparent risks, few effective preventive and therapeutic options are available. This oversight is likely due to poor availability of alternative treatments and poor clinician education regarding the risks of hyperoxia-induced lung injury.

Though commonly observed in clinical practice, the physiological mechanism of hyperoxia-induced ALI and BPD in neonatal patients has not been clearly defined. Pro-inflammatory cytokines, however, are suspected to play a central role in the occurrence of these conditions (Lindsay et al. 2000; Yi et al. 2004). Numerous studies have also shown that oxygen radicals, generated primarily in response to hyperoxia, contribute to the development of hyperoxia-induced ALI, thus, implicating the presence of excessive reactive oxygen species (ROS) in the pathogenesis of ALI (Papaiahgari et al. 2004; Cho et al. 2006; Gore et al. 2010). The development of future treatments will require detailed investigation of therapeutic modality effectiveness in new-
born patients, including assessment of cytokine network imbalances corrections, inhibition of inflammatory cells, regulation of inflammatory factor release, and elevation of antioxidant capacity (Gordo-Vidal et al. 2010).

Calcitonin gene-related peptide (CGRP), a ubiquitously distributed neuropeptide in the central and peripheral nervous system, is primarily secreted by the dense network of slow-conducting unmyelinated sensory C-fibers found in peripheral nerves (Sann et al. 1998). CGRP can commonly be found among neuroendocrine cells, blood vessels, and other tissues (Okajima et al. 2006). CGRP is a neuropeptide with 37 amino acids in length, which exerts broad regulatory effects throughout the body, especially in the cardiovascular and respiratory system (Dakhama et al. 2004). In lung tissue, CGRP has been reported to play a role in immunomodulation, vasodilatation, bronchial protection, proliferation of epithelial and endothelial cells, and regulation of airway responsiveness (Dakhama et al. 2004; Kawanami et al. 2009). In addition, it has been associated with the pathogenesis of a variety of respiratory diseases (Dakhama et al. 2004). The presence and function of CGRP in alveolar epithelial cells have been the topic of many recently published research studies (Kawanami et al. 2009; Fu et al. 2010). Of particular interest, several modern studies have highlighted the significant differences between lung tissue responses in neonatal and adult patients (Shimosegawa et al. 1991; Li et al. 2004; Wang et al. 2005; Kawanami et al. 2009). Previous in vitro studies carried out by the authors have demonstrated that CGRP may also have the ability to promote the proliferation of alveolar epithelial cells type II (AECII) (Fu et al. 2008). CGRP has been shown to play a protective role in the prevention of lung injury, primarily due to its antioxidant properties. When AECIs are exposed to high-oxygen-tension environments where oxygen radicals may be common, CGRP can promote the activation of antioxidantase (Fu et al. 2008). In hyperoxia-induced ALI, especially in neonatal patients, whether CGRP has a positive effect in restraining inflammation, enhancing antioxidation, and promoting restoration has not been previously addressed. Thus, the current study explores the relationship between the in vivo effects of exogenous CGRP and elevated oxygen levels in hyperoxia-induced ALI using neonatal Sprague-Dawley rats.

Materials and Methods

Animal model and treatment

All animal procedures conducted in the course of the present study were carried out with both home office and local ethical committee approval. The animals received care according to the “Guide for the Care and Use of Laboratory Animals.” This study also adhered to both the institutional and National Institutes of Health (NIH) guidelines for laboratory animal care.

Timed-pregnant Sprague-Dawley rats (provided by the Experimental Animal Center of Chongqing Medical University, Chongqing, China) were housed in individual cages with free access to water and laboratory food, and the rat pups were delivered sponta-

neously. Neonatal rats with equal body weight were nested on soft-wood shavings and distributed into individual Plexiglas chambers in litters of approximately 10 individuals. The chambers were equipped with a flow-through system for controlled delivery of either room air or medical oxygen. Food and water for the mother rats were made available ad libitum, and lighting was provided on a 12-h light-dark cycle. The room and chamber temperatures were maintained at 22-24°C.

The experiment began within the first 6 h subsequent to delivery of the neonatal subjects and continued until postnatal day 14 (P14). Rat pups were mixed and randomly divided into three groups using a random digits table: normoxia group, hyperoxia group, and hyperoxia with CGRP treatment (HC) group. There were no statistical differences in the age, sex, and weight among the three groups. The oxygen concentration of room air was maintained at 21 ± 2%, which was provided for the normoxia group. For hyperoxia and HC groups, oxygen intervention was provided wherein 6 L/min of medical oxygen was allowed to flow through the chambers under continuous monitoring, maintaining oxygen concentrations at 95 ± 2%. Carbon dioxide concentrations were maintained at 0.3%, and the relative humidity was maintained at 60-80%. To avoid maternal oxygen toxicity, the mother rats were rotated daily between hyperoxic and room air chambers. Drug intervention was provided wherein normoxia and hyperoxia rat pups received normal saline for 14 days (0.1 ml, once every other day) by intraperitoneal microinjection, and HC rat pups were treated for 14 days (once every other day) with CGRP (50 μg/kg, 0.1 ml, AnaSpec, Inc. USA) through intraperitoneal microinjection. The dose of 50 μg/kg of CGRP was found to be the best treatment in preliminary experiments. The death rate of newborn rats was the lowest at this dosage, and the microscopic histological features of the lung tissues were not changed under the normoxia condition but improved most significantly in hyperoxia treatment neonates. Our pilot experiments have shown that treatment with CGRP (50 μg/kg) caused no noticeable changes in the lung architecture under normoxia, indicating that the dose of CGRP exerted no toxic effect on the neonatal rats’ lungs (data not shown). Survival and body weight of rat pups in each group were recorded daily throughout the course of the study.

Bronchoalveolar lavage

Rat pups, totaling about eight animals from each group, were killed on postnatal day 14 (P14). Under deep pentobarbital anesthesia (50 mg/kg, intraperitoneal injection), a midline incision was made through the sternum. Both lungs were slowly lavaged five times via a tracheal cannula with 0.5 ml of normal saline containing 1 mM EDTA. Lavage volumes were recorded and deemed satisfactory if more than 70% of the initial volume was recovered. Leukocyte counts were measured with a hemocytometer. The fluid was then centrifuged at 450 × g for 10 min before the supernatant was separated from the cells. The total protein concentration was detected in the supernatant by the method of Lowry, according to the manufacturer’s instructions.

Tissue preparation

At P14, the rats were killed after administering anesthesia, and the chests were opened. Blood sample (20 μl) was collected with capillary tubing using the cardiac puncture method for leukocyte count. Then, the right main bronchus was ligatured, and the left lung was perfused with 4% paraformaldehyde for 10 min under a constant
airway pressure of 10 cm H₂O, which was maintained via a tracheal catheter. Then, the whole-lung tissue was subsequently extracted for further analysis (Husari et al. 2006). The sampled left lung tissues were excised and fixed by overnight immersion in 4% paraformaldehyde in sodium perborate at 4°C. The specimens were dehydrated in a graded ethanol series and embedded in paraffin. The sampled right lung tissues were resectioned after perfusion with ice-cold sodium perborate, snap-frozen in liquid nitrogen, and stored at −80°C for further biochemical analyses.

**Histological examination**

For general morphology analysis, 5-µm-thick sections were cut from the paraffin blocks, preserving the left lung tissues, and stained with hematoxylin and eosin (HE). Under light microscopy, magnified digital images were captured using an Olympus BX40 microscope (Olympus Optical, Tokyo, Japan). The Murakami technique, which helps in histological assessment of features, such as edema, congestion, hemorrhage, infiltration of inflammatory cells, and cell proliferation, was employed to grade the degree of lung tissue injury (Murakami et al. 2002). Each of these features was graded according to the following scale: 0, absent and appears normal; 1, light or non-significant damage; 2, moderate damage; 3, strong damage that may interfere with function; and 4, intense damage that may highly impair or eliminate function. A total score was calculated for each subject. To standardize the analyses and eliminate bias, lung sections were taken from central areas of the superior lobe of the left lung tissue, and morphometric analysis of each section was carried out in a blind fashion by two independent technicians.

**Malondialdehyde analysis**

As an indicator of protein oxidation and lipid peroxidation, malondialdehyde (MDA) was detected in homogenized lung tissues using MDA assay kit (Nanjing Jiancheng Biological Engineering Research Institute, Nanjing, China). MDA was measured using the thiobarbituric acid method according to the protocol of the manufacturer. This technique measures the degradation product of lipid peroxidation, which condenses with penthiobarbital and leads to a red product with measurable absorbance using a UV spectrophotometer.

**Superoxide dismutase analysis**

After homogenization of the lung tissues, the activity of the antioxidant enzyme superoxide dismutase (SOD) was measured simultaneously by absorption spectrometry using the Superoxide Dismutase Assay (SOD) kit (Nanjing Jiancheng Biological Engineering Research Institute, Nanjing, China). SOD was measured using the xanthine oxidase method according to the manufacturer’s instructions. According to this method, superoxide anion radicals lead to the oxidation of hydroxylamine, resulting in a purple nitrite compound measurable by spectrophotometric analysis, with a maximum absorbptive length of 568 nm. SOD activity leads to the reduction of the nitrite compound, thus, allowing for a measurably lower absorbance that correlates with SOD presence.

**Detection of tumor necrosis factor-α, interleukin-6, and aCGRP mRNA**

Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) mRNAs were detected by real-time quantitative polymerase chain reaction (RT-qPCR). Lung tissue samples were ground into a powder in the presence of liquid nitrogen, and gene expressions were subse- }

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Statistical Analysis

All the samples were selected randomly using a random digits table. Biochemical experiments were carried out for at least three independent times to ensure the precision of the experimental data, with each data point representative of the mean of at least three parallel samples. The resultant data were expressed as mean ± SEM. The differences between the groups were evaluated by one-way ANOVA, followed by Fisher's least significant difference (LSD) post hoc test and unpaired Student’s t-test, as needed. All the data were analyzed using SPSS statistical software (16.0 for Windows; IBM SPSS, Inc., Chicago, USA). A P value of < 0.05 was considered to be statistically significant.

Results

Body weight gain

Body weight was significantly lower in the hyperoxia group (22.4 ± 1.42 g), when compared with that observed in the normoxia group (32.8 ± 1.61 g) at P14 (P < 0.05). This decrease in body weight gain observed in the hyperoxia group was significantly improved with CGRP treatment (26 ± 1.26 g) (P < 0.05) (Fig. 1).

Survival rate and leukocyte counts in blood

Elevated oxygen exposure significantly reduced the survival rate of rat pups, resulting in only 73% survival at the end of the experiment (P14), when compared with the 93% survival rate observed in the normoxia group. This survival rate was improved to 84% with CGRP treatment (HC). Kaplan-Meier survival analysis showed a statistically significant difference in the cumulative probability of survival among normoxia, hyperoxia, and HC groups (P < 0.05, Breslow test) (Fig. 2). When compared with the normoxia group, the leukocyte counts in blood were significantly increased by high oxygen concentrations (hyperoxia and HC groups) (P < 0.05), but no significant changes in leukocyte counts were observed in hyperoxia cases where CGRP treatment was applied (HC) when compared with hyperoxia group at P14 (Fig. 3).

Leukocyte counts and total protein levels in bronchoalveolar lavage fluid

Total leukocyte counts and protein levels in BAL fluid were greatly elevated in pups after 14-day exposures to 95% oxygen (hyperoxia) [leukocyte: (450 ± 61.4) × 10^{-7}/L; protein: 235 ± 43.4 mg/L] when compared with air-exposed animals (normoxia) [leukocyte: (43 ± 12.2) × 10^{-7}/L; protein: 25 ± 4.5 mg/L] (P < 0.05). In the HC group, the increases in leukocyte counts were significantly reduced [HC, (210 ± 32.3) × 10^{-7}/L] (P < 0.05), and elevated protein level was also markedly decreased (HC, 145 ± 23.7 mg/L), parallel to the effect on BAL leukocyte counts (Fig. 4). These results show that CGRP treatment can effectively limit hyperoxia-induced pulmonary leukocyte and protein leakage in neonatal rats.

Lung histopathology

HE-stained images under light microscopy revealed that tissues exposed to hyperoxic conditions when compared with those of the normoxia group, exhibited edema-like formations, neutrophils filtration, enlarged alveoli, fewer secondary septa, increased interstitial inflammatory cell recruitment, and high levels of cell proliferation at P14. The degree of these histopathological changes in lung tissues seemed to be attenuated with CGRP treatment (HC). Histopathological scoring was significantly higher in the hyperoxia group when compared with the normoxia group (P < 0.05); however, the HC group exhibited significantly lower score than the hyperoxia group (P < 0.05), indicating reductions in cellular and tissue damages that may impair the normal functions of these structures. Both photomicrographs and scoring, completed according to the previously
described standard, demonstrated the existence of variations between the experimental groups further described in Fig. 5.

Lung malondialdehyde level
The level of MDA in the lung tissue was increased by approximately two-fold under conditions of high oxygen concentrations (21.27 ± 1.92 nmol/mg protein) when compared with normal oxygen levels (11.86 ± 1.63 nmol/mg protein) (P < 0.05). CGRP treatment significantly reduced the MDA levels (14.38 ± 1.85 nmol/mg protein) when compared with the hyperoxia group (P < 0.05) (Fig. 6A).

Lung superoxide dismutase activity
Under the high-concentration oxygen conditions, the activity of SOD was markedly decreased (52.8 ± 3.88 U/mg protein; P < 0.05) when compared with the SOD activity observed under normal oxygen conditions (normoxia, 84.21 ± 7.49 U/mg protein). Treatment with CGRP (HC) significantly enhanced the SOD activity (71.43 ± 6.86 U/mg protein) when compared with that observed in subjects subjected to hyperoxia (P < 0.05) (Fig. 6B).
The expression levels of TNF-α, IL-6, and CGRP mRNAs in lung tissues

The mRNA expression levels of inflammatory mediators TNF-α and IL-6 were detected with RT-qPCR. Significantly increased mRNA levels for TNF-α and IL-6 were observed in the hyperoxia group (TNF-α: 0.118 ± 0.024; IL-6: 0.098 ± 0.019) when compared with those observed in the normoxia group (TNF-α: 0.021 ± 0.004; IL-6: 0.039 ± 0.005) at P14 (P < 0.05). The hyperoxia-induced increase in TNF-α mRNA expression was signifi-
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Significantly attenuated with CGRP treatment (HC, 0.072 ± 0.009) ($P < 0.05$); however, the attenuation of IL-6 mRNA levels was not statistically significant in the hyperoxia group (0.082 ± 0.009). The mRNA expression levels of endogenous CGRP in the three groups (normoxia: 0.018 ± 0.0034; hyperoxia: 0.024 ± 0.0052; and HC: 0.022 ± 0.0049) were not different at P14 (Fig. 7).

Expression levels of TGF-β1 protein in lung tissues

The expression levels of TGF-β1 protein in each group of neonatal rat lung tissues were examined. TGF-β1 protein levels were significantly higher in animals exposed to hyperoxia (0.39 ± 0.056) than in those exposed to normal oxygen conditions (0.03 ± 0.009) ($P < 0.05$). Importantly, treatment with CGRP significantly decreased the expression level of TGF-β1 protein in animals kept under hyperoxia (HC, 0.12 ± 0.032) ($P < 0.05$) (Fig. 8).

Discussion

The response of neonatal rats to high oxygen exposure levels provides an important milestone for the study of ALI and BPD in neonatal patients (Okajima et al. 2006). Prolonged exposure of newborn rat pups to hyperoxic conditions resulted in the development of lung tissue damage similar to that observed in human infants with hyperoxia-induced ALI and BPD. Although the precise mechanism...
involved in hyperoxia-induced ALI has been poorly described to date, the imbalance of oxidant and antioxidant enzymes as well as altered inflammatory response may play a pivotal role in the pathogenesis of hyperoxia-induced ALI and BPD (Bhandari 2008, 2010). Elevation of total leukocyte counts and protein level in BAL and the increase in the inflammatory mediators TNF-α, IL-6, and TGF-β1 in lung tissue represent the pulmonary response to hyperoxia, stimulating the release of other pro-inflammatory cytokines and oxygen-free radicals. This process plays an important role in the propagation of inflammation by activating neutrophils. TNF-α is especially notable for its involvement in the development of various types of organ failure, which probably occur through similar mechanisms (Okajima 2001). Previous studies have also shown that pretreatment with anti-TNF-α antibodies may significantly attenuate the effects of hyperoxia-induced lung injury (Wherry et al. 1993).

The present study extends upon previous work by demonstrating that after 95% oxygen exposure over the course of 14 days, a number of factors are influenced in neonatal rat pups, including reduced body weight gain, decreased survival rate, and increased total protein level in BAL and leukocyte counts both in blood and BAL. These symptoms are likely to have significant long-term developmental effects, requiring further exploration in subsequent research studies. Lung injury characteristics are manifested as widened interstitial space, disruption of overall lung architecture, enlarged alveoli, alteration of secondary septa, increased inflammatory cell recruitment, abnormal levels of neutrophils filtration, and excess fibrous tissue proliferation. These changes are probably concurrent with alterations in the activity of oxidant and antioxidant enzymes, such as MDA and SOD, although they may also result from increased levels of inflammatory mediators in the lungs, such as TNF-α, IL-6, and TGF-β1. These findings suggest that hyperoxia may induce lung damages through oxygen radicals and inflammatory cytokines.

Numerous studies have shown that CGRP is one of the most potent microvascular vasodilators (Brain et al. 2004; Gherardini et al. 2009; Huang et al. 2011; Smillie et al. 2011). Owing to this property, CGRP has long been considered to be involved in the inflammation response; however, earlier studies have demonstrated that ablation of certain sensory fibers could result in a marked increase in the severity of inflammation (Szallasi et al. 1996). In fact, the

Fig. 8. Expression of TGF-β1 protein in the lung. (A) The TGF-β1 protein levels were measured by Western blot analysis in homogenized lung tissue from rats at P14. Representative data are shown. (B) Arbitrary unit of TGF-β1 abundance in immunoblot are expressed as densitometric ratios of the TGF-β1 over the GADPH in each group. N, normoxia group (n = 8); H, hyperoxia group (n = 8); and HC, hyperoxia with CGRP treatment group (n = 8). The data are the mean ± SEM. *P < 0.05 vs. normoxia group, #P < 0.05 vs. hyperoxia group.
sensory neurons and their peptides may contribute to the maintenance of tissue integrity by regulating the inflammatory response, as suggested by the results of the current study. Consistent with these findings, researchers have noted that CGRP treatment can inhibit the production of TNF-α and other pro-inflammatory cytokines (Millet et al. 2000; Consorni et al. 2011). In addition, as one of the prostaglandins, PG12 can exert anti-inflammatory effects by inhibiting the production of TNF through inhibition of its transcription (Jorres et al. 1997). Despite this effect, CGRP treatment has been demonstrated to increase the endothelial production of prostaglandin I2 (Arai et al. 2003). Other studies have further demonstrated that adrenomedulin, a peptide structurally related to CGRP, can act via both CGRP and adrenomedullin receptors to mediate its effects. Adrenomedulin has also been implicated in ROS generation (Yoshimoto et al. 1998; Dakhama et al. 2004; Rahman et al. 2006; Itoh et al. 2007; Kim et al. 2010). Furthermore, CGRP has been found to play an important role in protecting myocytes, kidney cells, pancreatic B cells, and liver cells from the potentially damaging effects of ROS (She et al. 2003; Kroeger et al. 2009; Song et al. 2009). The results of the current study suggest that CGRP contributes to an overall reduction in tissue injury by attenuating the inflammatory response and limiting oxidizing reactions in a variety of neonatal tissues, predominantly the lung tissues.

Although the endogenous CGRP expression levels were not obviously influenced by both hyperoxia and exogenous CGRP, animals treated with exogenous CGRP over the course of the present study showed significantly improved body weight gain and reduced abnormal lung histopathology when subjected to hyperoxic conditions. CGRP could also inhibit the leukocytes and proteins leaking from capillary to alveolar vessels. These findings demonstrate that the anti-inflammatory effects and protective alveolar development of CGRP, such as attenuation of the hyperoxia-induced increase in MDA and decrease in SOD activity, are markers of anti-lipid peroxidation and antioxidation. Moreover, reduction of TNF-α and TGF-β1 expression levels were observed, indicating that CGRP administration is likely to play a protective role in hyperoxia-induced ALI in newborn rats through inhibition of oxidative stress and inflammatory response in various tissues. Reviews suggest that CGRP receptors are expressed on the cell surface of the bronchial epithelium, alveolar epithelial cells, and vascular endothelial cells (Brouns et al. 2006). When CGRP binds to these receptors, the receptor-coupled G proteins will be activated, which leads to the induction of intracellular cyclic AMP formation (Bhandari et al. 2006). The resultant accumulation of cAMP inhibits the accumulation of nuclear factor kB (NF-kB) complexes in the nucleus by preventing the phosphorylation and degradation of the NF-kB inhibitor (Gore et al. 2010). Inhibition of NF-kB activity would trigger the protective mechanism of CGRP to confine the inflammatory response (Sann et al. 1998). In the present study, the survival rate, however, was probably impacted by leukocyte counts in blood, and the level of IL-6 in the CGRP treatment group decreased insignificantly by an unknown mechanism. These findings suggest that a complex interaction of biochemical mechanisms is involved in the responses to high oxygen levels and mediation of adaptive response to ROS and inflammation.

While the fully mechanistic pathway for CGRP treatment’s ability to attenuate the symptoms of hyperoxia-induced ALI and BPD is not completely understood, current findings suggest that regulated oxidant and antioxidant enzymes coupled with the anti-inflammatory activity of CGRP may play a critical role in protecting tissues from damage in neonatal patients. However, the different distributions of the oxidase, antioxidant, and inflammatory factor, such as MDA, SOD, TNF-α, TGF-β1, and IL-6, in the lung tissue under normoxia, hyperoxia, and hyperoxia with CGRP treatment condition still need further investigation. In the case of hyperoxia-induced ALI and BPD, a satisfactory clinical solution is yet to be developed, necessitating further examination and characterization of the physiological functions of CGRP treatment in hyperoxia-induced ALI and BPD neonatal patients. Further investigation will be necessary to develop highly selective pharmacological agents and other novel strategies, such as CGRP antagonist or CGRP-knockout animal models.

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Conflict of Interest

The authors declare no conflict of interest.

References


