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# ИНФОРМАТИВНОСТЬ КЛЮЧЕВЫХ МОЛЕКУЛЯРНЫХ МАРКЕРОВ ПРИ МОДЕЛИРОВАНИИ БРОНХОЛЕГОЧНОЙ ДИСПЛАЗИИ

© Елена Александровна Лозовская, Анна Александровна Костарева, Наталья Александровна Петрова

Национальный медицинский исследовательский центр им. В.А. Алмазова. 197341, Санкт-Петербург, ул. Аккуратова, 2

**Контактная информация:** Анна Александровна Костарева — д.м.н., директор Института молекулярной биологии и генетики, доцент кафедры внутренних болезней Института медицинского образования. E-mail: akostareva@hotmail.com ORCID ID: 0000-0002-9349-6257

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**Резюме.** Успехи современной медицины позволяют выхаживать глубоко недоношенных детей, но на фоне жизненно важной искусственной вентиляции легких (ИВЛ) часто наблюдается развитие «новой» бронхолегочной дисплазии (БЛД). Поскольку БЛД до сих пор остается актуальной проблемой в педиатрии, необходима разработка новых подходов к моделированию данной патологии для более тщательного изучения патогенеза заболевания и поиска новых эффективных методов терапии. Цель нашего исследования — показать возможность моделирования БЛД на крысах, используя 14-дневную гипероксию. В течение этого срока опытная группа новорожденных животных содержалась в герметичной камере с постоянной подачей 80% кислорода, контрольная группа — при нормальных условиях. По истечении этого времени проведено измерение массы тела животных, морфометрия легочной ткани, оценка экспрессии *tnf-a, vegf.* Протокол исследования одобрен комиссией по контролю содержания и использования лабораторных животных ФГБУ «НМИЦ им. В.А. Алмазова». В результате эксперимента опытная группа показала существенное снижение набора массы тела животными, упрощение альвеоляризации, провоспалительную реакцию легочной ткани и снижение васкулогенеза, что соответствует фенотипу «новой» БЛД. Следовательно, данный подход к моделированию дисплазии может обеспечить потребности в части изучения патогенеза бронхолегочной дисплазии и исследования новых терапевтических агентов.

**Ключевые слова:** бронхолегочная дисплазия; животная модель; гипероксия.

# INFORMATIVE VALUE OF KEY MOLECULAR AND HISTOLOGICAL MARKERS IN THE MODELING OF BRONCHOPULMONARY DYSPLASIA

© Elena A. Lozovskaia, Anna A. Kostareva, Natalia A. Petrova

V.A. Almazov National Medical Research Center. Akkuratova str., 2, Saint Petersburg, Russian Federation, 197341

**Contact information:** Anna A. Kostareva — Doctor of Medical Sciences, Director of the Institute of Molecular Biology and Genetics, Associate Professor of the Department of Internal Medicine of the Institute of Medical Education. E-mail: akostareva@hotmail.com ORCID ID: 0000-0002-9349-6257

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**Abstract.** Advances in modern medicine make it possible to nurse very premature babies, but, due to vital mechanical ventilation, the development of a “new” bronchopulmonary dysplasia is often observed. BPD is still an urgent problem in pediatrics, it is necessary to develop approaches to modeling this problem for a more thorough study of the pathogenesis of diseases and the search for new effective methods of therapy. The aim of our study is to show the possibility of modeling bronchopulmonary dysplasia in rats using 14-day hyperoxia. During this period, the experimental group

of newborn animals was kept in a sealed chamber with a constant supply of 80% oxygen, the control group under normal conditions. After this time, an animal weight measurement, morphometry of the lung tissue, expression of *tnf-a, vegf* were carried out. The study protocol was approved by the commission for the control of the maintenance and use of laboratory animals Almazov NMRC. As a result of the experiment, the experimental group showed a significant reduction in animals weight, simplification of alveolarization, pro-inflammatory inflammation of the lung tissue and a decrease in vasculogenesis, which corresponds to the «new» BLD phenotype. Therefore, the use of this rat model of hyperoxia-induced BPD may fulfill the need for experimental models used in research of the BPD pathogenesis and evaluation of potential therapeutic agents.

**Key words:** bronchopulmonary dysplasia; animal model; hyperoxia.

## INTRODUCTION

Bronchopulmonary dysplasia (BPD) is a chronic multifactorial condition of morphologically immature lungs that develops in newborns, mainly in extremely preterm infants. BPD was first described by Northway et al. in 1967 as a chronic pulmonary disorder developing in premature babies on mechanical ventilation. This pathological process is associated with a high mortality rate because of intense inflammation, necrotizing bronchiolitis, alveolar metaplasia, active fibroblast proliferation followed by the development of fibrosis and scarring of the lung parenchyma, and subsequent respiratory failure [3, 17, 21]. This condition is known as “classic” or “old” BPD. Over the past three decades, the care and treatment of premature infants have changed significantly. Nowadays, in the era of wide use of glucocorticosteroids, surfactants, and less invasive mechanical ventilation modes, the pathomorphological patterns of the “classic” BPD are rarely seen, and the survival rate has significantly increased. A “new” BPD is mainly associated with delayed alveolar formation: less number of alveoli and a significant decrease in the alveolar surface area along with substantial damage to the vascular network, inflammation and mild fibrosis [4, 7, 11, 20]. Despite the therapeutic success, the average incidence of BPD in premature infants is still as high as 35% [3], and the patients have a high risk of mortality, long-term hospitalization, chronic respiratory and cardiovascular diseases, failure to thrive, and neurodevelopmental delay. Up to 50% of pediatric patients with BPD are repeatedly admitted to hospital within the first two years of their life. School-age children are prone to more severe and frequent acute respiratory tract infections. They have a higher risk of asthma-like symptoms, wheezing, increased airway reactivity, exercise-induced dyspnea, and reduced lung diffusion capacity. Adult patients may subsequently develop the chronic obstructive pulmonary disease. Approximately 25% of infants with severe BPD develop pulmonary arterial hypertension (PAH) manifesting in respiratory failure that results in chronic hypoxia, hypercapnia, and acidosis [20, 27]. Despite the high prevalence of BPD, no new medications have been approved for the treatment of this disease over the past two decades. To date, vitamin A, caffeine, and corticosteroids in the postnatal period are the only medications that can decrease the incidence of BPD [21, 25]. Since BPD remains a relevant issue, new approaches and new methods of treatment should be developed.

Intrauterine lung development is a highly organized process that ultimately ensures normal gas exchange between the airways and blood vessels. The limit of fetal viability corresponds to the transition between the canalicular and the saccular stage of lung development (22–24 gestation week) since the saccular stage is characterized by the formation of primitive terminal airspaces, connective tissue thinning, and the beginning of the surfactant production, which is a positive factor for future gas exchange [23, 27]. Infants born so early demonstrate hypoxemic respiratory failure due to insufficient maturity of lung parenchyma and microvessels, the deficiency of surfactant production, and often require mechanical ventilation. At the same time, they are at a higher risk of oxidative stress because of the immaturity of their antioxidant defense system, deficiency of antioxidant enzymes and other bioantioxidants. Therefore, the fetus is adapted to the hypoxic environment (4% $O_2$), and even normal atmospheric oxygen concentrations of 21% can lead to hyperoxic lung injury and BPD development [9, 15, 31]. Morphologically immature pulmonary epithelium does not provide effective mucociliary clearance, thus also contributing to the development of inflammatory reactions and poor prognosis of BPD [29, 30].

Excessive oxygen concentration caused by mechanical ventilation can give rise to the formation of reactive oxygen species (ROS), free radicals with high oxidizing ability. ROS damage membranes, structural proteins, and nucleic acids, thus resulting in cell death and tissue injury, and a subsequent pro-inflammatory progression. There is an imbalance of pro-inflammatory cytokines and growth factors. Tracheal aspirates of preterm infants with BPD on mechanical ventilation demonstrated high concentrations of IL-1, IL-6, cathepsin D, TNFa, monocyte chemotactic factors, and macrophage inflammatory proteins [24]. In response to an increase in the concentration of pro-inflammatory cytokines, adhesion molecules and selectins are activated, contributing to the infiltration of the lungs by inflammatory cells [22, 26].

Several cell types are involved in the pulmonary inflammatory response. Macrophages are one of the key cells. Hyperoxic exposure results in the macrophage polarization towards M1 (CD68-positive cells), with M2 (CD163-positive cells) phenotype suppression [1]. M1 macrophages make a key contribution to the development of an inflammatory response by synthesizing a wide range of pro-inflammatory cytokines, while M2 macrophages have anti-inflammatory spectrum. Neutrophils attracted to the site of inflammation by chemokines degranulate with the

release of proteases. Dysregulation of the extracellular matrix is one of the key pathways driving BPD pathogenesis. Matrix remodeling disrupts the normal development of alveolar structures and the microcirculatory bed and is involved in major signaling cascades such as VEGF, HIF, and TGFb. Neutrophil elastase also activates the expression of IL-8, IL-1, IL-33 in the pulmonary epithelial cells, causes metaplasia of goblet cells as well as damage to the ciliary structures [29]. There are assumptions about the involvement of mast cells and T-lymphocytes in the pathogenesis, but their role is not completely clear [10, 14, 19].

In the presence of inflammation and matrix remodeling, intercellular communications are disrupted, and since complex paracrine interactions form the basis of the normal formation of the pulmonary parenchyma and microcirculatory bed, the impairment of further alveolarization and the development of the vascular bed occurs resulting in BPD [24]. Since infant lung tissue samples with lung dysplasia are virtually unavailable, we owe the progress in understanding of the molecular and histological patterns of the BPD pathogenesis to the use of animal models [23].

So far, there is no unique standardized animal model of BPD that fully ensures the translational relevance. The histological lung specimens obtained from infants who had died from BPD showed impairment of alveolarization, the vascular bed development, as well as inflammation [8], therefore, an animal model should also demonstrate similar alterations. Different approaches are used to simulate the BPD-like changes in model animal. The most common model that is based on the effects of oxygen, is the hyperoxia model [2, 4, 12, 28].

The aim of our study is to identify key and, most importantly, easy-to-detect morphological and molecular markers to confirm the formation of bronchopulmonary dysplasia in an animal model for the potential search for new therapeutic agents.

## MATERIALS AND METHODS

Based on our objectives and needs, we selected a rat as our model animal. Rodents have an advantage over other animals in terms of modeling BPD since mice and rats are born during the saccular stage of lung development, just like premature infants. This stage in rodents starts during the intrauterine period and ends by the fifth day of postnatal development. It should be noted that birth during the saccular stage is typical for rodents, therefore, newborn animals are already capable of normal gas exchange with a sufficient amount of surfactant and active antioxidant systems, which is not the case in premature humans [6, 13, 16]. Other significant advantages of the rodent model are a short-term gestational period and numerous offspring, allowing to perform experiments within a short amount of time and receive statistically significant results [5, 18].

To induce bronchopulmonary dysplasia, newborn Wistar rats were placed in a hermetically sealed controlled environment chamber. A chamber with a built-in oxygen concentration controller produced by Vetfactory (Russia) was used. Animals were kept at a relative humidity 30–60% (measuring step 1%), temperature 22–24 °C (measuring step 1 °C). The oxygen delivery system was set to maintain 80% oxygen concentration. Nursing females were changed daily in order to reduce the negative effect of hyperoxia on adult animals, and rats were given ad libitum access to food and water. Control animals were kept in normoxia. Each group comprised of eight newborn rats from the same litter. Animals were randomly assigned to the experimental groups. After 14 days, the animals were euthanized, and histological examination and molecular genetic analysis of their lungs were performed. Hematoxylin-eosin staining and CD68+light immunohistochemistry (reaction with DAB — diaminobenzidine) were used for histological examination of the lung tissue. Lung samples for molecular genetic analysis were snap frozen immediately upon receipt and stored at –80 °C. Frozen samples were homogenized with a TissueLyzer (QIAGEN) for 10 minutes at 45 Hz in Extract RNA reagent (Evrogen). The quality and quantity of obtained RNA was estimated using the NanoDrop 3300 SpectroPhotometer (Thermo Fisher Scientific) and agarose gel electrophoresis. cDNA was transcribed from RNA using Random (dN) 10-primer (Evrogen) and MMLV RT kit (Evrogen) primers according to the recommendations of the manufacturer. The resulting cDNA was subjected to qPCR using a 7500 Real-Time PCR System (7500 Software v2.3, Life Technologies Ltd, Paisley, UK). The primer sequences used for qPCR to study expression were as follows TNFα (F: ATGGGCTCCCTCTCATCAGT, R: GCTTGGTGGTTTGCTACGAC), VEGFa (F: GCAGCGACAAGGCAGACTAT, R: TGGCACGATTTAAGAGGGGA) Gapdh (F: CCAGTATGACTCTACCCACG, R: CATTTGATGTTAGCGGGATCTC). QPCR was performed for 40 cycles. Data analysis was conducted using the 2-ΔΔCT method; relative gene expression was normalized on the GAPDH gene expression level. Statistical analysis was carried out using GraphPad Prism Software (version 9.3.1), the results were considered significant if the p-value was less than 0.05 ($p < 0.05$).

The protocol of the present study was approved at a meeting of the commission for the control of the maintenance and use of laboratory animals (IACUC Almazov NMRC) on March 29, 2019.

## RESULTS

The average weight of the study group animals at the time of euthanasia was 9% less than the average weight of the control animals (Figure 1). Histological examination of the lung specimens was performed to assess the average area of the lung tissue per field of view. In the study group, this area was 54.2 ± 3.2 vs 68.4 ± 2.2 in the control group (Figure 2). The lungs of the study group animals demonstrated a less complex alveolar structure. CD68-positive cells occupied 25 ± 6 and 3 ± 2 of the field of view in the study group and control group specimens, respectively (Figure 3). Molecular genetic analysis demonstrated increased expression of tumor necrosis factor-alpha (TNFα; a proinflammatory factor), as well as reduced expression of the vascular endothelial growth factor (VEGF) in the study group, indicating limited vascularization (Figure 4).

These data allowed making a conclusion that following 14-day exposure to hyperoxia, the study group animals developed the phenotype of BLD.

## CONCLUSION

Despite the fact that BPD is a multifactorial disorder, the key element of its pathogenesis is hyperoxia-induced lung tissue damage during mechanical ventilation of immature lungs. Therefore, hyperoxia-induced BPD may be considered an optimal animal model. Our study has demonstrated that 14-day exposure to hyperoxia (80% oxygen concentration) associated with: 1. Less complex alveolar structure; 2. Increase CD68-positive cells; 3. Increase expression of TNFα; 4. Decrease expression VEGF. So, these criteria can be used as indicators of developed BPD in an animal model and this approach

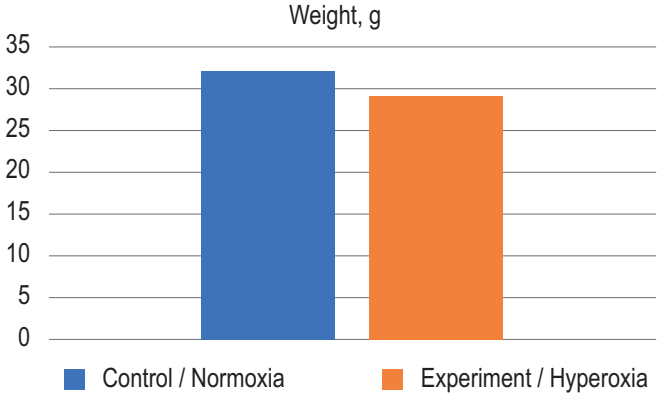


**Fig. 1.** **Mean body weight of animals on the 14th day of their postnatal life. The mean weight was 32.1 g and 29.4 g in the study group and the control group, respectively, indicating 9% weight reduction**

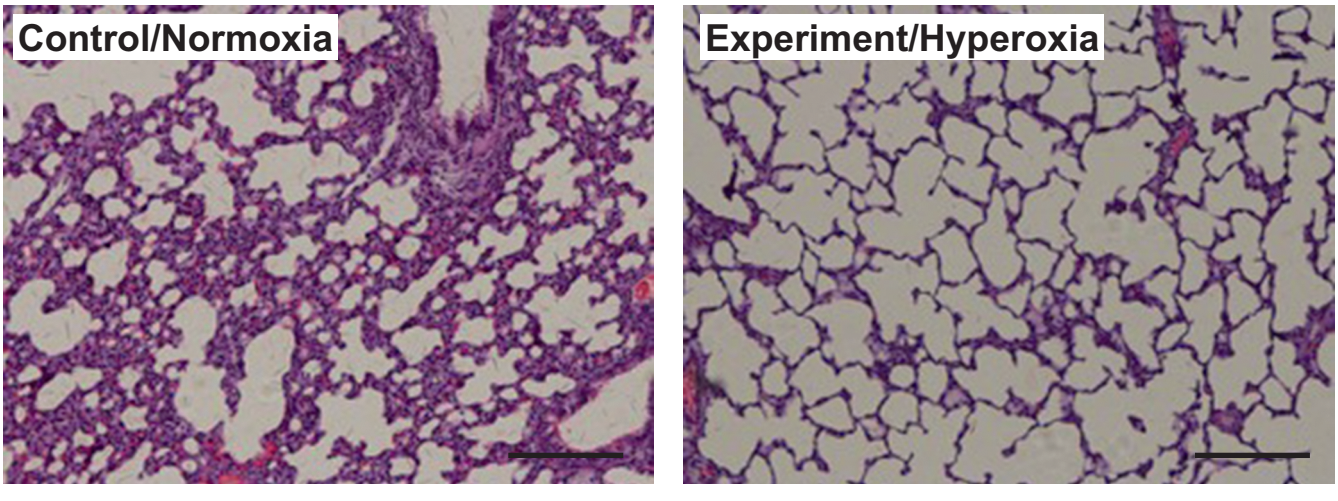


**Fig. 2.** **Histological examination, hematoxylin-eosin, × 10 magnification. The sections obtained from the study group animals demonstrated less complex alveolar structure and a significant decrease in the mean area occupied by the lung tissue (by 14% compared with the specimens obtained from the control group animals). Scale bars indicate 200 μm**

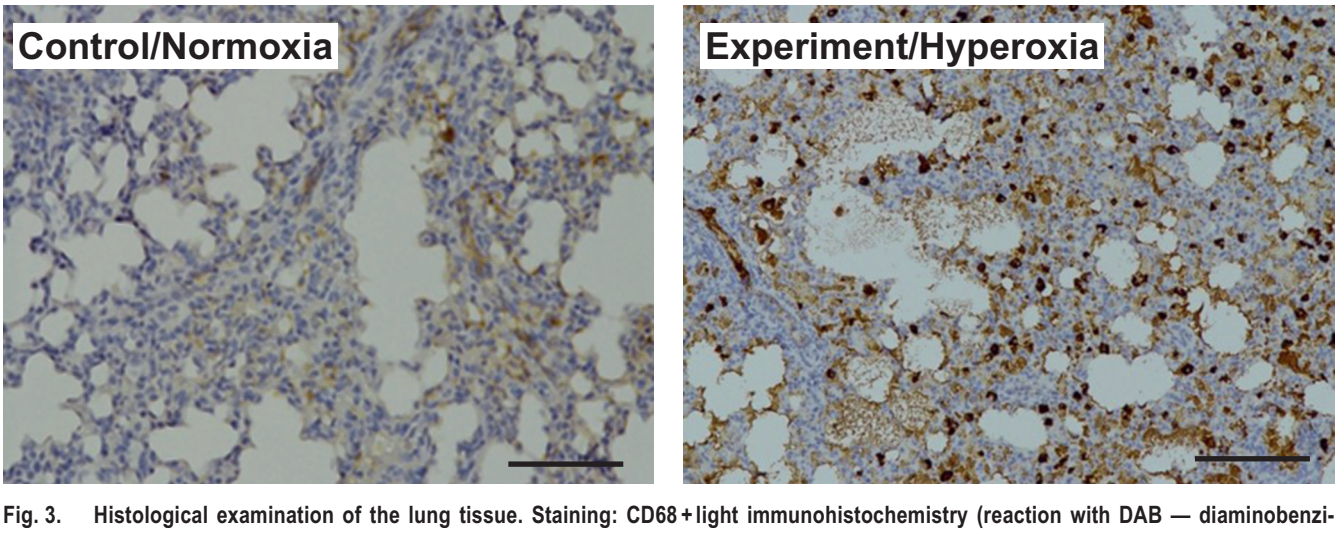


**Fig. 3.** **Histological examination of the lung tissue. Staining: CD68 + light immunohistochemistry (reaction with DAB — diaminobenzidine), × 10. The quantity of CD68-positive cells was 88% higher in the experimental group compared with the control group. Scale bars indicate 200 μm**

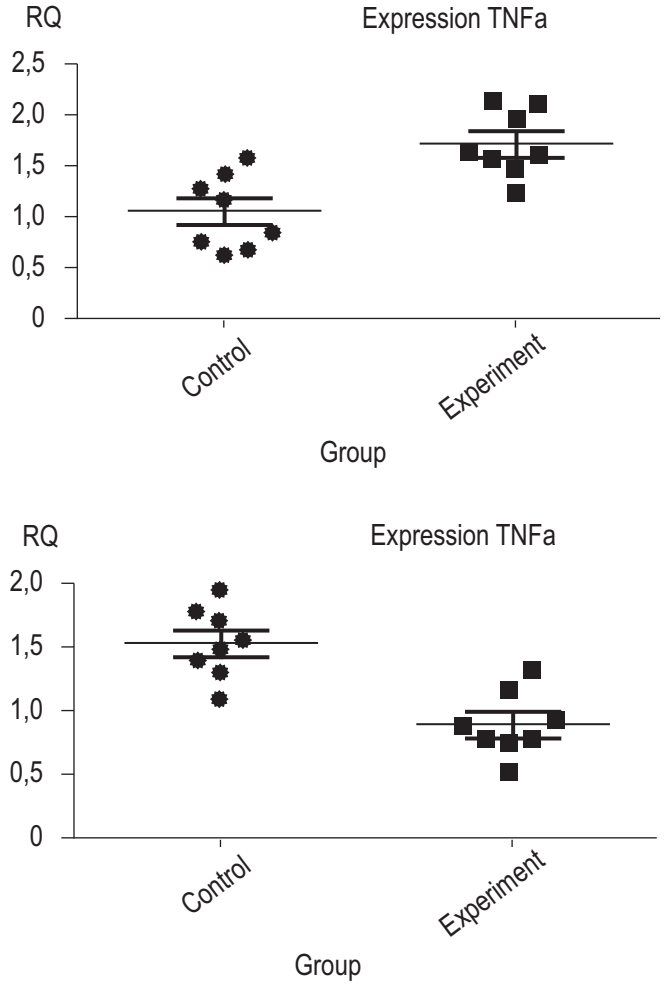


**Fig. 4. Changes in TNFa and VEGF expression levels. The experimental group has a significant increase in TNF-α and a decrease in VEGF expression levels, indicating an active inflammatory reaction and reduced vascularization**

to modeling dysplasia may open the need for a study of the pathogenesis of bronchopulmonary dysplasia and the seach of new therapeutic agents.

## ДОПОЛНИТЕЛЬНАЯ ИНФОРМАЦИЯ

**Вклад авторов.** Все авторы внесли существенный вклад в разработку концепции, проведение исследования и подготовку статьи, прочли и одобрили финальную версию перед публикацией.

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## ADDITIONAL INFORMATION

**Author contribution.** Thereby, all authors made a substantial contribution to the conception of the study, acquisition, analysis, interpretation of data for the work, drafting and revising the article, final approval of the version to be published and agree to be accountable for all aspects of the study.

**Competing interests.** The authors declare that they have no competing interests.

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**Experiments with animals** were carried out in accordance with international rules (Directive 2010/63/EU of the European Parliament and of the Council of the European Union of September 22, 2010 on the protection of animals used for scientific purposes).

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