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Morphofunctional state of the lungs respiratory part in normotensive and hypertensive rats after combined exposure to intermittent hypoxia and melatonin

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C. Data analysis, statistics / dane analiza i statystyki
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Abstract

Aim. The purpose of this work was to study and compare the combined effect of intermittent normobaric hypoxia (INH) and melatonin on the morphological and biochemical indices of the lungs respiratory part in Wistar and spontaneous hypertensive rats (SHR).

Material and methods. The studies were conducted on 48 young male rats Wistar and SHR lines. The experimental rats were daily exposed to hypoxic gas mixture (12 % oxygen in

nitrogen) in intermittent mode: 15 min deoxygenation / 15 min reoxygenation for 2 hours. Animals also received oral melatonin at a dose of 5 mg / 1 kg of body weight once daily.

Results. The sizes of alveolus were reduced, but their number and placement density were increased in SHR rats after a combined effect of INH and melatonin. The results showed a significant decrease of the interalveolar septum thickness and a tendency to decrease of the hydroxyproline concentration in the lung tissue of the experimental rats, regardless of their line.

Conclusions. The 28-day combined effect of INH and melatonin increase the total alveolar surface area and reduce the amount of connective tissue in the lungs of experimental rats. These changes can improve the efficiency of the intrapulmonary gas exchange processes.

Key words: intermittent normobaric hypoxia; melatonin; lungs.

Introduction

Arterial hypertension is one of the most common diseases that affects 20-30% of the world's adult population [1]. The widespread of the combined respiratory and blood circulatory pathology requires an integrated approach not only to treatment of such patients, but also to improvement of existing methods for early diagnosis and prevention of these diseases. The close functional relationship of the cardiovascular and respiratory systems largely determines the burdensome effect of cardiovascular pathology on the course of pulmonological diseases and vice versa [2]. The development mechanisms of the lungs pathological changes in persons with arterial hypertension have not been sufficiently studied. It was found that in the initial stages of hypertension there are no evident morphological changes, but the signs of impaired respiratory function and oxygen deficiency are often observed. The histomorphological signs of pneumofibrosis and congestion in the pulmonary blood circulation usually appear at the later stages of these disease. In severe cases, pulmonary edema can also develop. Therefore, it is necessary to search for new effective means and methods for prophylaxis and treatment of the pulmonary function disorders in patients with arterial hypertension. Exogenous melatonin and intermittent normobaric hypoxia (INH) are those methods that can improve the lungs functional activity. INH is widely used in clinical practice for treatment and prevention of many cardiovascular, respiratory, endocrine, digestive and immune diseases, as well as to increase the nonspecific resistance and adaptive capabilities of the body [3, 4]. As it is known, melatonin is an important regulator of metabolic, immune and regenerative processes. It is involved in the mechanisms of thermoregulation and aging [5].

The literature data about the effects of INH and melatonin on the morphofunctional state of the lungs are very ambiguous and often contradictory. This may be due to use in experiment the different types and ages of animals, due to differences in the methods and modes of hypoxic mixtures supply (hypo- or normobaric hypoxia), as well as due to differences in doses of melatonin administration, seasonality and duration of experiments etc.

The effects of INH and melatonin on lung functions were carried out mainly in normotensive animals [6, 7]. We have not found the works in which the combined effect of melatonin and INH on the morphofunctional state of the lungs was studied in hypertensive animals or people with arterial hypertension. Therefore, it became interesting to conduct such studies in rats that had constantly high blood pressure.

Purpose of work

The aim of our work was to investigate and compare the morphological and biochemical indicators of the lung status of Wistar and SHR rats after combined exposure to INH and melatonin.

Material and methods

The studies were conducted on 48 normotensive male rats (Wistar line) and spontaneously hypertensive rats (SHR line) in spring. The animals were taken from the vivarium nursery of the Bogomoletz Institute of Physiology, National Academy of Sciences of Ukraine. The age of rats at the end of the experiment was 4 months, weight 270 ± 10 g.

The rats blood pressure was measured in vivarium by a non-invasive method on the caudal artery using a sphygmomanometer (S-2 “SHE” Germany). Spontaneously hypertensive rats were taken in the experiment if they had systolic blood pressure not lower than 145 mm Hg.

Throughout the experiment, the rats were in standardized conditions with a standard diet and natural light conditions. The animals were divided into 4 groups: I and III – control rats of the Wistar and SHR line respectively, II and IV – normotensive and hypertensive animals, which were subjected to combined exposure to INH and melatonin. Experimental rats were daily exposed to hypoxic gas mixture in special sealed chamber. Hypoxic gas mixture (12% oxygen in nitrogen) was supplied into this chamber from the membrane gas separator element in the intermittent mode: 15 min deoxygenation / 15 min reoxygenation for 2 hours. The rats were in cages and breathed atmospheric air at all the remaining time of day (22 hours). Exogenous melatonin (Unipharm Inc., USA) was orally administered to experimental animals at 10.00 in the morning at a dose of 5 mg / kg once a day. The total duration of the experiment was 28 days.

The rats were removed from the experiment by decapitation under ether narcosis. All research protocols corresponded to the provisions of the Council of Europe Convention on Bioethics (1997), the Helsinki Declaration of the World Medical Association (1996), the European Convention for the Protection of Vertebrates, which are used for experimental and other scientific purposes (Strasbourg, 1985), the general ethical principles of animal experiments, adopted by the First National Congress of Ukraine on Bioethics (2001), as well as a committee with biomedical ethics of the A. A. Bogomoletz Institute of Physiology, National Academy of Sciences of Ukraine.

Histological preparations of lung tissue were prepared according to a standard procedure: fixed in Buen's liquid, dehydrated in spirits of increasing concentration and dioxane and poured into paraffin. The obtained preparations were used for morphological and morphometric studies. The sections were stained with Bemer's hematoxylin and eosin, and for the detection of connective tissue elements – by the Van Gyzon and Mason method [8]. Microscopic preparations were photographed on a microscope "Nicon Eclipse E100" (Japan) using a digital camera. The morphometry of the preparations digital images was performed using the computer program "Image J".

On the lung tissue histological sections were measured: the mean diameter of the alveolar lumen, the depth and area of the alveolus, the alveolus entrance width, the number of alveolus were counted and the density of their placement per unit area were determined, the thickness of the interalveolar septum, the diameter of the respiratory bronchioles, alveolar courses and sacs were measured. The ratio of the alveolar entrance width to its depth and the ratio of the respiratory bronchioles diameter, alveolar courses and sacs to the double depth of the alveolus were determined [9].

The concentration of total hydroxyproline in the lung tissue was determined photometrically by oxidation of hydroxyproline with chloramine T. The lipids concentration in lung tissue was determined by the phosphor-valeric method using a standard set of reagents from the firm «Filisit Diagnostika».

Data analysis

Statistical processing was carried out using variation statistics methods using the computer program Statistica 6.0. The normal distribution of digital arrays was verified using the Pearson criterion. When the distribution was normal, the Student's t-test was used to estimate the difference in the reliability of the difference between the control and experimental groups. Differences were considered significant at $p < 0.05$.

Results

The lungs respiratory part (LRP) is represented by respiratory bronchioles (RB), alveolar courses (AC), alveolar sacs (AS) and alveolus. Because of the difficulty in identifying structural differences between AC and AS in histological sections, as well as the differences between peripheral bronchioles and AC, they are generally considered to be one group (Figure) [9].

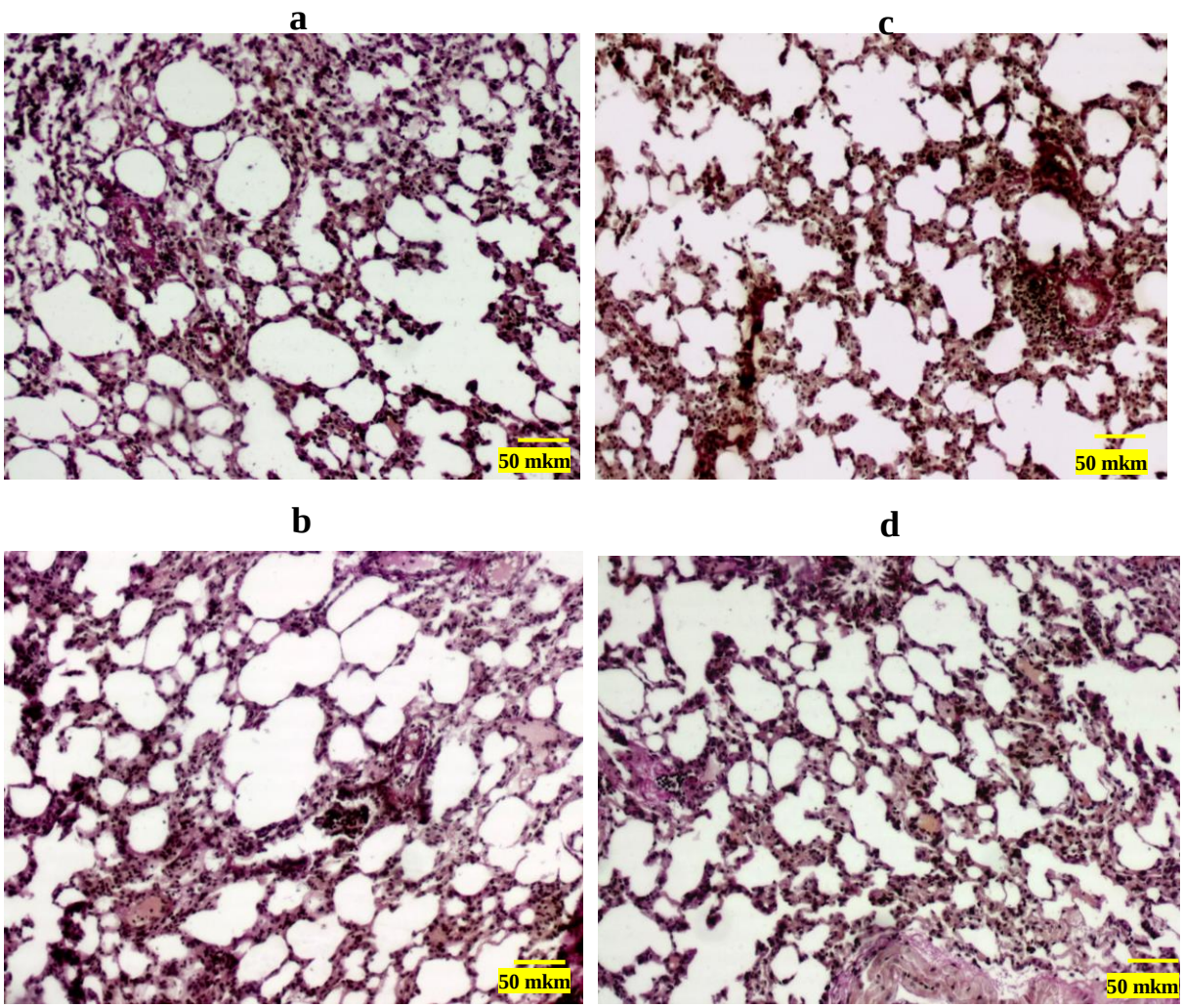

Figure: Microphotograph of the lungs respiratory part in control animals (a – Wistar line, b – SHR line) and rats treated with intermittent hypoxia and melatonin (c – Wistar line, d – SHR line). Van Gieson color. x200.

Morphological differences in LRP between control rats of different lines were revealed. The SHR had a reliably larger cross-sectional area of the alveolus (by 26%), a smaller number of alveolus and a lower density of their placement per unit area (by 27%), a larger lumen diameter of RB, AC and AS (by 14%) compared with Wistar rats (Table 1).

Table 1. Morphometric indices of the state of the lungs respiratory part in control and experimental groups (M ± m: n = 12)

Index	Wistar line		SHR line	
	Control	Hypoxia+ Melatonin	Control	Hypoxia+ Melatonin
Mean diameter of alveolus lumen, μm	23.6±0.6	22.4±0.5	24.7±0.7	22.6±0.4
Depth of alveolus, μm	21.7±0.1	22.5±0.6	22.0±0.9	24.1±0.6
Width of the entrance to the alveolus, μm	14.6±0.2	13.2±0.3*	14.4±0.7	13.9±0.3
Cross-sectional area of alveolus, μm^2	671.5±18.3	709.3±20.7	844.9±20.2**	756.2±15.4*
The number of alveolus (0.37 mm^2), pcs	73.1±1.6	67.2±2.1	53.1±1.6**	62.2±2.1*
The density of the alveolus, pcs. / mm^2	197.6±6.2	181.6±5.3	143.5±2.3**	168.1±3.4*
Diameter of lumen of respiratory bronchioles, alveolar courses and alveolar sacs, μm	72.5±2.3	74.5±2.1	82.7±1.6**	69.1±2.4*
Thickness of interalveolar septum, μm	5.1±0.2	4.1±0.2*	4.6±0.4	3.5±0.1*
The ratio of the width of the entrance to the alveolus to its depth	0.67±0.02	0.59±0.02*	0.65±0.02	0.58±0.02*
The ratio of the diameter of the respiratory bronchioles, alveolar courses and alveolar sacs to the double depth of the alveolus	1.67±0.03	1.66±0.05	1.88±0.05**	1.43±0.06*

Note: here and in Table 2 * - $p < 0.05$ – significant differences in comparison with the control. ** - $p < 0.05$ – significant differences compared to the control Wistar line.

Differences in morphometric indicators of the LRP state between control and experimental rats of different lines were also revealed. After combined exposure to INH and melatonin the lungs of Wistar rats had a significantly smaller width of the alveolus entrance (by 10%) and the thickness of the interalveolar septum (by 20%). The ratio of the alveolus entrance width to its depth was smaller (by 12%) compared with control (Table 1).

More significant changes in the structure of LRP were observed in experimental SHR rats. They revealed a significant smaller area of the alveolus – by 10%, the lumen diameter of the RB, AC and AS – by 16%, a smaller thickness of the interalveolar septum – by 24%, a smaller ratio of the alveolus entrance width to its depth – by 11% and the ratio of the diameter

of RB, AC and AS to the doubled depth of the alveolus by 24% compared with the control. The decrease in the size of the alveolus was offset by a significant increase in their number and distribution density by 17% (Table 1). Increasing of the alveolar surface total area can be considered as a factor promotes greater efficiency of the intrapulmonary gas exchange.

The results of the connective tissue specific staining showed no significant differences in the color intensity and the collagen fibers amount between the control and experimental rats of both lines. The largest number of collagen fibers were located around the RB and blood vessels. There were significantly fewer in the interalveolar septum (Figure).

Collagen is known to be the main structural component of connective tissue. Its characteristic feature is a high content of hydroxyproline (12-14%). This allowed to consider hydroxyproline as a specific marker of collagen. Its concentration can be judged by the rate of collagen catabolism. The determination of the hydroxyproline concentration is often used to assess the state of connective tissue in various organs [10]. In our studies the concentration of hydroxyproline in the lung tissue of experimental rats had a distinct tendency to decrease (Table 2).

Table 2. Hydroxyproline and lipids concentration in the lung tissue of control and experimental groups (M ± m; n = 12)

Index	Wistar line		SHR line	
	Control	Hypoxia+ Melatonin	Control	Hypoxia+ Melatonin
Concentration of hydroxyproline in the lungs, μg / mg	5.69±0.66	5.30±0.46	4.49±0.66**	4.24±0.53
Concentration of lipids in the lungs, mg / g	16.8±0.5	21.6±0.2*	17.6±0.9	16.0±0.9

The relatively high lipid content is one of the important characteristic of the lung tissue compared to other tissues. Lipids, phospholipids, which are synthesized in lung tissue, are used mainly for the surfactants synthesis [11]. Surfactants, in the form of a bi-cellular layer, are located in the alveolus at the boundary with the air and regulate the surface tension with a change in their volume. It was revealed that after combined exposure to INH and melatonin, the lipid concentration in the lungs tissue of Wistar rats significantly increased by 29%. The concentration of lipids in the lungs of experimental SHR rats on the contrary, tended to decrease compared to the control (Table 2).

Discussion

The genetically determined variety of morphological and biochemical characters is one of the important factors determining the wide variability of the individual adaptive reactions of the body and its resistance to various environmental factors. Rats of different lines are often used in experiments for studying genetically determined features of the body sensitivity to oxygen deficiency etc. Wistar and SHR line rats most frequently used for these purposes. In addition SHR rats are an adequate experimental model for studying the mechanisms of arterial hypertension formation and means of its correction. Normotensive Wistar rats are most often used in such studies as a control [12]. That's why these two lines of rats were selected for our research.

The results of our studies indicated the presence of pronounced morphofunctional differences in the LRP of normo- and hypertensive rats. The alveolar area, the lumen diameter of RB, AC, AS and the lipid concentration were greater in the lungs of control SHR rats. But the thickness of interalveolar septum and the hydroxyproline concentration, on the contrary, were lower than in Wistar rats. Such differences give reason to speak about more air fullness and lower of connective tissue content in the lungs of SHR rats compared to control Wistar rats. Obviously, these morphological and functional differences in LRP are genetically determined features of SHR, which must be taken into account when conducting experimental studies on these animals.

It was found that the 28-day combined effect of INH and melatonin altered the morphofunctional state of the lungs in both normotensive and hypertensive rats. These changes are more evident in SHR rats. The combined effect of INH and melatonin reduced the size of the alveolus, but their total number and density per unit area were increased. Such changes increased the total area of the alveolar surface, which contributed to increasing the efficiency of the intrapulmonary gas exchange processes.

The interalveolar septum consisted of epithelial layers of alveolus, subepithelial basal membranes, a network of capillaries, as well as elastic, reticular and collagen fibers [13]. After combined exposure to INH and melatonin, the thickness of the interalveolar septum in rats of both lines significantly decreased. This may be due to decrease in the thickness of the connective tissue fibers in the interalveolar septum. Such changes contribute to increasing of the alveolar ventilation and intrapulmonary gas exchange efficiency.

Hydroxyproline concentration in the lung tissue of experimental rats had a clearly marked tendency to decrease. This may indicate a violation of the dynamic equilibrium between the destruction and biosynthesis of collagen. Such changes characterize the

predominance of collagen degradation processes and indicate a decrease in the relative mass of connective tissue in the lungs.

Some scientists observed a decrease in the connective tissue proportion and severity of the pulmonary fibrosis after exposure to exogenous melatonin [6]. It was shown that melatonin administration (at a dose of 4 mg / kg) reduced the growth of connective tissue in the lungs with artificially induced fibrosis [14, 15]. Zhou L. et al. found that in mice with chronic bronchial asthma, after exposure to melatonin, the total area of collagen fibrils decreased [16]. It was shown that administration of melatonin (4 mg / kg) to rats prevented the development of pathological changes (perialveolar edema, peribronchial and transvascular infiltration, thickening of the interalveolar septum) after exposure to cyclosporine [17]. It is proved that the intake of melatonin in various doses reduces the severity of inflammatory processes, the degree of epithelium damage, the thickness of the interalveolar septum and prevents the development of bronchopulmonary pathology [18].

A number of studies have shown an increase in collagen and growth factor expression in lung tissue after effect of hypobaric and normobaric hypoxia [19]. Falanga V. et al. showed that the total collagen concentration, which was determined by the content of hydroxyproline, was increased only under conditions of hypobaric hypoxia. But after effect of normobaric hypoxia it remained unchanged [20]. Apparently, the differences in content of oxygen and atmospheric pressure magnitude caused the peculiarities of the lung connective tissue reaction to hypoxic action.

We have previously studied a separate effect of INH and melatonin on the lungs morphofunctional state. It was found that melatonin administration to Wistar rats (to a greater extent) and SHR rats (to a lesser extent) increased the alveolar size and the depth and width of the entrance to alveolus. But the thickness of the interalveolar septum and the hydroxyproline concentration in lungs tissue were decreased. This can contribute to an increase in the total area of the alveolar surface and accelerate of oxygen diffusion through the alveolus-capillary membranes [21]. Positive dynamics on the morphofunctional activity of the rats lungs of different lines was also detected after exposure to INH [7].

Facts regarding the therapeutic effect of melatonin in some lung diseases have been published in the scientific literature [17, 18]. It has been shown that after an intraperitoneal injection of melatonin at a dose of 10 and 15 mg / kg for 23 days, the condition of experimental animals with allergic asthma have been improved significantly [22]. It was noted that exogenous melatonin had a protective effect in case of hyperoxic lung injury [23]. Melatonin at a dose of 10 mg / kg reduced lipid peroxidation in the lungs and protected the

heart, lungs and kidneys from oxidative stress under intermittent hypobaric hypoxia [24]. It has been shown that, during aging, melatonin reduced the degree of damage to alveolocytes mitochondria [25].

Rozova EV. was shown the adaptive changes in the rats LRP under hypoxic conditions. The number of surfactant-synthesizing structures was increased – lamellar bodies (by 40%) and free surfactants (by 75%) [26]. Other authors showed that after long INH sessions undergo adaptive ultrastructural rearrangements in both the epithelial and endothelial components of the pulmonary alveolus, which are manifested by increased metabolic function. The phagocytic activity of pulmonary macrophages was increased, the activity of the surfactant system of the lungs was activated, hyperplasia was manifested, and the volume of osmiophilic lamellar bodies was increased too [27].

Conclusion

The combined effect of intermittent normobaric hypoxia and melatonin within 28 days leads to a decrease in the size of the alveolus in spontaneous hypertensive rats. However, the number of alveolus and their density per unit area increases. An increase in the total area of the alveolar surface helps to improve the efficiency of the processes of intrapulmonary gas exchange. A decrease in the thickness of the interalveolar septum and the concentration of hydroxyproline in the tissue of the lungs of experimental rats indicates a decrease in the number of elements of the connective tissue, which contributes to the improvement of alveolar-capillary gas exchange. An increase in the concentration of total lipids in the lungs of experimental rats may indicate an increase in the phospholipids content of pulmonary surfactant, which prevents the walls of the alveolus from sticking together and improves the airiness of the lungs and increases the efficiency of intrapulmonary gas exchange. The results of the study have not only theoretical, but also practical significance when using hypoxic gas mixtures and melatonin in sanatorium-resort or medical institutions for the treatment of pulmonological diseases.

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