

Yanko Roman, Levashov Mikhail, Chaka Elena, Litovka Irina, Safonov Sergey. Seasonal features of the combined effects of intermittent normobaric hypoxia and melatonin on the thyroid gland morphofunctional state. *Journal of Education, Health and Sport*. 2020;10(4):186-198. eISSN 2391-8306. DOI <http://dx.doi.org/10.12775/JEHS.2020.10.04.021>  
<https://apcz.umk.pl/czasopisma/index.php/JEHS/article/view/JEHS.2020.10.04.021>  
<https://zenodo.org/record/3770209>

The journal has had 5 points in Ministry of Science and Higher Education parametric evaluation. § 8. 2) and § 12. 1. 2) 22.02.2019.  
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The authors declare that there is no conflict of interests regarding the publication of this paper.

Received: 17.02.2020. Revised: 20.04.2020. Accepted: 27.04.2020.

## Seasonal features of the combined effects of intermittent normobaric hypoxia and melatonin on the thyroid gland morphofunctional state

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- B. Data collection entry / zebranie danych
- C. Data analysis, statistics / dane analiza i statystyki
- D. Data interpretation / interpretacja danych
- E. Preparation of manuscript / przygotowanie artykułu
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### Abstract

The **aim** of this work is to compare the effect of the combination influence of dosed intermittent normobaric hypoxia and melatonin in different seasons of the year on the morphofunctional state of the rats' thyroid gland.

**Material and methods.** The study was carried out on 48 male rats of the Wistar line. The hypoxic gas mixture (12% O<sub>2</sub> and 88% N<sub>2</sub>) was daily given to experimental animals in

intermittent mode: 15 minutes deoxygenation / 15 minutes reoxygenation for 2 hours. Melatonin was administered orally in a dose of 5 mg / kg at 10.00 a.m. The total duration of the experiment was 28 days. Histological preparations were prepared according to a standard methodic. Histomorphometry of the digital images of preparations was carried out using the computer program «Image J».

**Results.** It was shown that the thyroid gland of rats reacted differently to the combined effects of intermittent hypoxia and melatonin at different seasons. So the signs of the gland activity were increased after influence of hypoxia and melatonin in spring. It was evidenced by a smaller area of follicles and colloid, a smaller internal diameter of the follicles and colloid-accumulation index, a greater height of the thyrocytes and follicular-colloid index, a smaller width of the interlobar, interlobular and interfollicular connective tissue interlayers compared to the control. The prolonged exposure to intermittent hypoxia and melatonin in autumn decreased the area and height of the follicular epithelium, increased the internal diameter of the follicles, decreased the follicular-colloidal index and increased the colloid-accumulation index. This indicated a decrease in the activity of the thyroid gland.

**Conclusions.** Thus, in the spring, the morphofunctional activity of the thyroid gland is moderately increased, and in the autumn period, on the contrary, decreases.

**Key words:** intermittent normobaric hypoxia; melatonin; thyroid gland.

## **Introduction**

The appearance of the thyroid gland pathology has been expanding rapidly in the last decade, among young people also. This is caused by insufficient provision of a body with iodine, an impact of unfavorable environmental, climatic factors, stress and smoking etc. Unfortunately, the traditional methods of treatment do not always give a positive effect. Therefore, finding new effective methods for the prevention and treatment of the thyroid gland diseases is an important task.

In recent years, the method of intermittent hypoxic therapy has been widely used in medicine. The short-term strong hypoxic stimuli are used to increase the nonspecific and specific resistance of organism [1, 2]. As well known from the literature, an adaptation to high-altitude hypoxia leads to strengthening the functions of the hypothalamic-pituitary-thyroid system at early stages. This was the reason for using hypoxia for treatment of such thyroid diseases as hypo- and hyperthyroidism, autoimmune thyroiditis [3, 4]. It was shown that the impact of normobaric hypoxia leads to restoration of the gland function, which is manifested by normalization of the thyroid hormones level in blood of patients with

subclinical hypothyroidism [5]. Intermittent hypoxia leads to the structural rearrangement of the thyroid gland, decreasing and normalizes the level of thyroid hormones in animals with experimental hyperthyroidism [6].

On the contrary, studies conducted under conditions of natural hypoxia in mountains showed that the prolonged effect of hypoxia (60 days) reduced the functional activity of thyroid gland against a background of a general decrease in metabolism [7].

Melatonin, the main hormone of the epiphysis, is a regulator of diurnal rhythms, metabolic, immune and regenerative processes. It participates in mechanisms of thermoregulation and aging. Melatonin preparations are increasingly used in clinical practice. It is known that the activating effect of melatonin on metabolism is realized via other endocrine glands, mainly via thyroid hormones [8]. However, the influence of melatonin on thyroid function is investigated poorly.

Information from the specialized literature regarding the influence of intermittent hypoxia and melatonin on the thyroid gland is very ambiguous and often contradictory. This may happen due to differences in the modes of hypoxic gas mixtures supply, different dosages of melatonin administration, age aspect, seasonality and duration of experiments etc. Therefore, the study of the combined effect of these two factors on the morphofunctional activity of the gland is of great interest.

### **Purpose of work**

The aim of this work is to compare the effect of the combination influence of dosed intermittent normobaric hypoxia and melatonin in different seasons of the year on the morphofunctional state of the young rats' thyroid gland.

### **Material and methods**

The study was carried out on 48 male rats of the Wistar line. 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  $270 \pm 10$  g. Rats were held in unified conditions and the standard diet. The animals were divided into 4 groups: I and III – control rats in spring (March-April) and autumn (October-November), respectively; II and IV – experimental rats in the same seasons of the year, respectively. Experimental rats were daily exposed to combined effects of hypoxic gas mixture and melatonin. The rats were placed in a sealed chamber for hypoxic session. A 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). The experimental animals also received exogenous melatonin ("Unipharm Inc., USA") at the dose of 5 mg / kg body weight at 10.00 orally on a daily basis. 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 thyroid gland tissue were prepared according to a standard procedure: fixed in Buerger'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. Microscopic preparations were photographed on a microscope "Nikon" (Japan) using a digital camera. The morphometry of the preparations digital images was performed using the computer program "Image J". The following parameters were measured on histological sections of the thyroid gland: the cross-sectional area of follicles, the colloid and follicular epithelium, the effective, external and internal diameter of the follicles, the height of the follicular epithelium and the width of the interlobar, interlobular and interfollicular connective tissue interlayers, the number of thyrocytes in the follicle. Based on the results obtained, a follicular-colloidal index and an colloid-accumulation index were calculated [9, 10].

### **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 certain morphological differences were revealed in the thyroid gland of control rats depending on the season. Thus the thyroid gland in autumn had a significantly lower area of the follicles and colloid by 13% and 32%, respectively, a smaller inner diameter of follicles by 26%, a higher height of thyrocytes by 35%, a higher follicular-colloidal index by 56% and a smaller colloid-accumulation index by 45% in comparison with the same parameters of the thyroid gland of control animals in spring. The condition of not only the parenchymal elements, but also the state of the interlobar, interlobular and interfollicular connective tissue were evaluated. It was established that the thyroid gland had a smaller thickness of the interlobar and interfollicular connective tissue interlayers in autumn by 27% and 17% ( $p < 0.05$ ) respectively, compared with spring (Table).

**Table.** Morphometric indices of the functional state of the thyroid gland of control and experimental rats in spring and autumn ( $M \pm m$ ;  $n = 12$ )

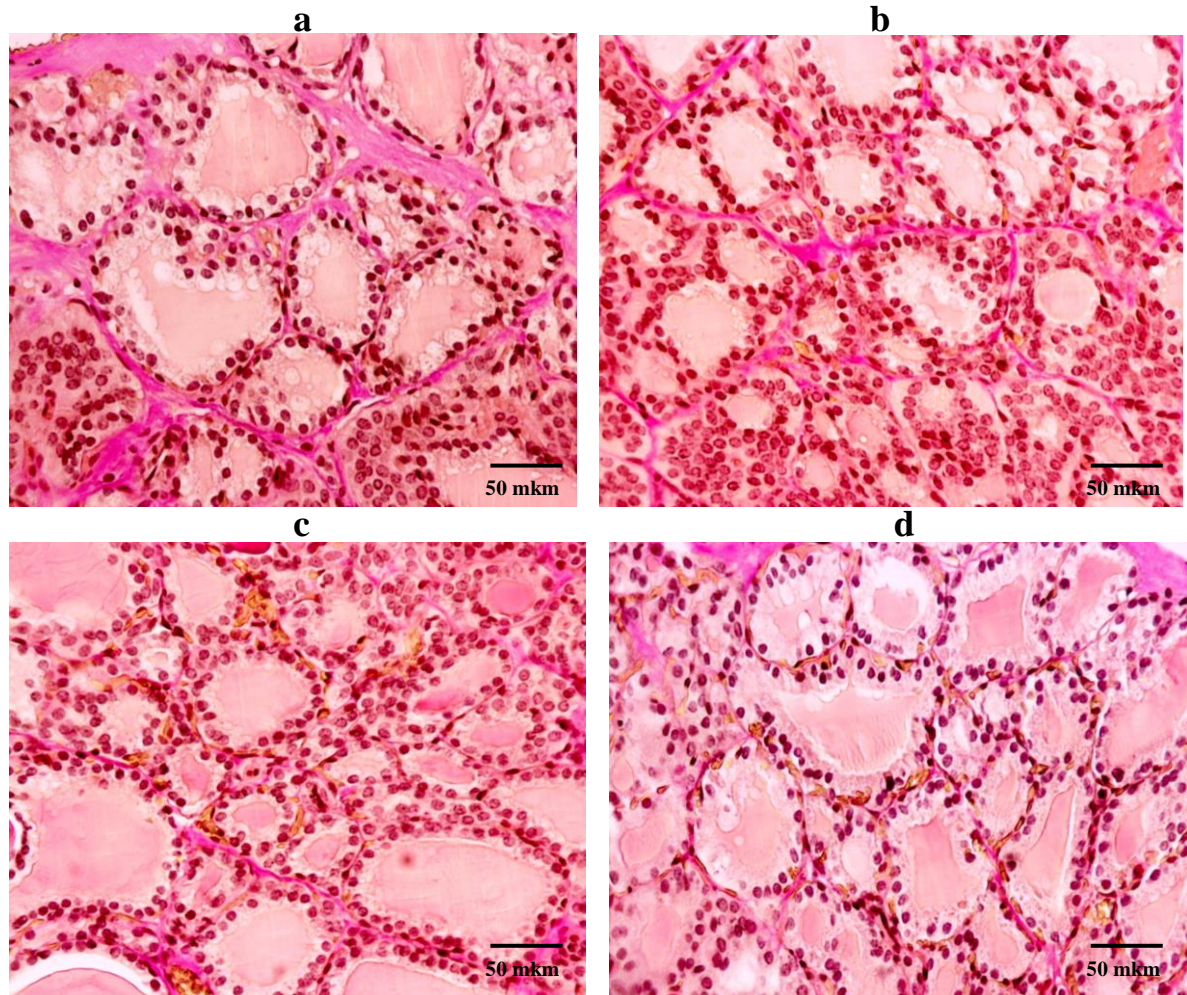
Index	Spring		Autumn	
	Control	Hypoxia + Melatonin	Control	Hypoxia + Melatonin
Area, $\mu\text{m}^2$ :				
follicle	3283 $\pm$ 100	2871 $\pm$ 104*	2859 $\pm$ 99**	2561 $\pm$ 104
colloid	1618 $\pm$ 125	1375 $\pm$ 94*	1105 $\pm$ 104**	1107 $\pm$ 80
follicular epithelium	1665 $\pm$ 96	1496 $\pm$ 61	1754 $\pm$ 92	1454 $\pm$ 60*
Diameter of the follicle, $\mu\text{m}$				
external	63.9 $\pm$ 3.0	61.0 $\pm$ 1.6	57.9 $\pm$ 1.8	57.4 $\pm$ 2.4
internal	47.0 $\pm$ 1.2	42.4 $\pm$ 1.2*	34.9 $\pm$ 1.8**	38.0 $\pm$ 1.4
effective	72.4 $\pm$ 3.9	70.3 $\pm$ 1.6	69.4 $\pm$ 2.2	67.1 $\pm$ 1.8
Height of thyrocytes, $\mu\text{m}$	8.5 $\pm$ 0.2	9.3 $\pm$ 0.1	11.5 $\pm$ 0.7**	9.7 $\pm$ 0.3*
Number of thyrocytes in the follicle, units	20.4 $\pm$ 0.6	20.9 $\pm$ 0.9	19.2 $\pm$ 0.5	20.3 $\pm$ 0.6
Follicular-colloidal index	1.02 $\pm$ 0.12	1.09 $\pm$ 0.09	1.59 $\pm$ 0.12**	1.31 $\pm$ 0.08*
Colloid-accumulation index	2.78 $\pm$ 0.22	2.28 $\pm$ 0.07*	1.52 $\pm$ 0.20**	1.96 $\pm$ 0.11*
Width of connective tissue interlayers, $\mu\text{m}$				
interlobar	40.1 $\pm$ 1.5	33.6 $\pm$ 2.0*	29.3 $\pm$ 2.9**	35.8 $\pm$ 2.8*
interlobular	12.8 $\pm$ 0.1	11.3 $\pm$ 0.1*	12.6 $\pm$ 0.5	12.3 $\pm$ 0.7
interfollicular	2.10 $\pm$ 0.07	1.90 $\pm$ 0.03*	1.75 $\pm$ 0.08**	1.37 $\pm$ 0.09*

Note: \* -  $p < 0,05$  the reliability of differences compared to control.

\*\* -  $p < 0,05$  the reliability of differences compared to the control in the spring.

The parenchyma of the thyroid gland of the experimental rats retained its physiological structure after combined effect of intermittent normobaric hypoxia (INH) and melatonin. It was possible to isolate the central and peripheral zones of the thyroid gland parenchyma. From the outside, the thyroid gland was surrounded by the capsule made of

dense fibrous connective tissue from which the connective tissue trabeculae penetrated into the interior of the gland. They carried blood and lymph vessels and nerves. Trabeculae separated the parenchyma of the gland into lobules (Figure).



**Figure:** A microphotograph of the thyroid gland of control (a – spring, c – autumn) and experimental (b – spring, d – autumn) rats: Van Gieson color. x400.

The thyroid gland of the experimental rats, exposed to INH and melatonin in spring, had significantly smaller interlobar, interlobular and interfollicular connective tissue interlayers, respectively, by 16%, 12% and 10% compared to the control. This indicated a decrease in the number of the gland connective tissue elements, which can facilitate the transport of thyroid hormones into the bloodstream. But significantly greater width of interlayers of the interlobar connective tissue (by 22%) and smaller width of interlayers (by 21%) of the interfollicular connective tissue in the gland of the experimental rats in autumn were noted (Table).

The thyroid gland of the experimental animals contained follicles, mainly oval form and different sizes. The colloid of thyroid follicles was moderate density, less often foamy with numerous vacuoles (in spring) or dense (in autumn) after combined effect of INH and melatonin (Figure). It was revealed that the thyroid gland of experimental rats during the spring had a significantly smaller area of follicles and colloid by 13% and 15%, respectively, compared with the control. This may indicate an increase in thyroid activity and increase the excretion of its hormones into the blood. The gland contains predominantly large follicles in a low-active state because of depositing hormones within the follicles and increasing the volume of the colloid [9]. Only the area of the follicular epithelium was decreased by 17% after influence of INH and melatonin in autumn (Table).

The external and effective diameters of the follicles in the thyroid gland of the experimental rats were at the control level. The internal diameter of the follicles was by 10% less than in control animals after the combined effect of INH and melatonin in spring. On the contrary, in autumn it tended to increase by 9% (Table).

It is known that the form of thyrocytes depends on the functional state of the gland. They are cubic in shape in normofunction, and they become flat in hypofunction and acquire a prismatic shape in hyperfunction. It was revealed that the thyrocytes of the control rats' thyroid gland are predominantly cubic in shape. It was noted that in animals, exposed to combined effects of INH and melatonin in the spring, the thyrocytes have cubic and prismatic forms, with a height by 10% greater than in the control. The thyrocytes of the experimental rats' gland have of cubic and flat forms, with a significantly lower height by 16% compared with the control rats in autumn. The number of thyrocytes in the follicle of the gland in the experimental and control animals was the same and did not depend on the season of the year (Table).

Interfollicular islets of the thyroid gland contain little-differentiated (cambial) cells and are the source of the formation of new follicles. In this connection, the clearly pronounced tendency to increase in the number of interfollicular islets in the experimental rats in the spring may indicate activation of physiological regeneration of the thyroid.

The follicular-colloid index or index of thyroid activity (the ratio of the follicular epithelium area to the area of the colloid) of the experimental rats tended to increase by 7% in spring. On the contrary, in autumn it was less by 18% than in the control (Table). As a rule, the functional activity of the thyroid gland is directly proportional to the relative area of the follicular epithelium and inversely proportional to the content of the colloid. The follicular-

colloidal index decreases with a decrease in the activity of the gland and grows with the activation of the secretory activity of the organ.

The colloid-accumulation index (the ratio of the mean internal diameter of the follicle to the double height of the thyroid epithelium) also had significant differences depending on the season of exposure to INH and melatonin. Thus, this indicator in the experimental rats was less in spring by 18%. On the contrary, in autumn it was significantly higher compared to the control rats by 28% (Table). This fact also indicated that after the combined effect of intermittent hypoxia and melatonin, the synthetic activity of the gland increased in spring and decreased in autumn.

### **Discussion**

The results of our studies indicated the presence of distinctly expressed histomorphometric differences in the thyroid gland of rats depending on the season. The number of connective tissue elements, the area of the follicles and colloid, the inner diameter of the follicles of the control rats thyroid gland were smaller, but the height of the thyrocytes and the follicular-colloid index were higher in autumn than in spring. The data obtained make it possible to conclude, that the functional activity of the thyroid gland was significantly higher in autumn than in the spring. These morphofunctional differences in thyroid gland should be taken into account when conducting experimental studies at different seasons of the year.

It was revealed for the first time that thyroid gland reacted differently to the 28-day combined effect of INH and melatonin at different seasons of the year. The signs of an increase in activity of the gland after prolonged exposure to INH and melatonin were identified in spring. The area of follicles and colloid, the internal diameter of the follicles, the colloid-accumulation index and width of interlobar, interlobular and interfollicular connective tissue interlayers were smaller, but height of the thyrocytes and follicular-colloid index were larger in experimental rats compared to control. The effect of INH and melatonin leads to a decrease in the area and height of the follicular epithelium, to an increase in the internal diameter of follicles, to a decrease in the follicular-colloid index, and to an increase in the colloid-accumulation index in autumn. These facts indicated decrease in gland activity.

We have previously studied the thyroid status of the same age Wistar rats which were under separate influence of INH or melatonin in spring and autumn. A multidirectional effect of this influence on the structure and morphometric parameters of the functional state of the gland in different seasons of the year was revealed. Thus, the 28-day exposure to INH had a



positive effect on the morphofunctional state of the thyroid gland in spring. This was evidenced by an increase in the height of the thyrocytes, and decreased colloid-accumulation index and the width of the interlobar and interfollicular connective tissue interlayers in the gland of experimental animals [11]. The thyroid gland activity of the rats, which received the hypoxic mixture in autumn, on the contrary, was decreased. Changes in the thyroid gland of rats exposed to melatonin did not depend on the season of the year. They were expressed in the increase of the internal diameter and average cross-sectional area of the follicles, the area of the colloid, of the colloid-accumulation index, and the thickness of the connective tissue layers. But the area of the follicular epithelium and the height of thyrocytes and follicular-colloid index were decreased. A small amount of resorption vacuoles appeared in the colloid. The data obtained allowed us to conclude that the introduction of exogenous melatonin both in the spring (to a greater extent) and in the autumn reduced the functional activity of the thyroid gland [12].

Most of the studies about the hypoxic gas mixture effects on the thyroid gland were carried out under hypobaric conditions. The literature data indicated that under the mid-mountain conditions the activity of the gland increased, but at high altitudes it was inhibited [13, 14]. The functional activity of the animals' thyroid gland increased in the early periods (3 days) of exposure to intermittent hypoxia. It was manifested by an increase in the level of thyroid hormones and structural changes in gland parenchyma. The gland functional activity stabilized with an increase in time of hypoxic exposure to 15 days and decreased with a further increase in the duration of exposure (30th day). Intermittent exposure of the hypoxic gas mixture to animals with experimental hyperthyroidism at the early stages (3rd day) leads to an increase in the content of thyroid hormones. This correlated with the morphometric parameters and the morphological picture of the thyroid gland. Long-term adaptation of such animals to intermittent hypoxia (30 days) leads to a significant decrease in the content of thyroid hormones. It was confirmed by morphometric indices (an increase in the area of the follicle cross section, the area of the colloid, the colloid-accumulation index, decrease the height of the thyroid epithelium and follicular-colloid index) [6].

Other researchers noted the positive effect of normobaric hypoxic gas mixtures on the functional state of the gland. Thus, Ma Y. et al. found that the effect of dosed hypoxia (5% O<sub>2</sub>) enhanced the synthesis of hypoxia-inducing factor which stimulated the synthesis of thyroid hormones [15]. An increase in the expression of transcription markers of thyroid hormones and an increase in the differentiation of the gland during hypoxia were shown [16].

The oppressive effect of melatonin on thyroid activity has been shown in most publications [17-19]. The age aspect plays an important role when studying the influence of melatonin on the activity of the gland. It was established that the administration of melatonin in both physiological (0.05, 0.5 mg / kg) and pharmacological (2.5, 5, 10 mg / kg) doses had an inhibitory effect on the thyroid hormone activity of young reproductive age animals [20]. While daily injections of melatonin into old rats, even in physiological doses (0.05 and 0.5 mg / kg), on the contrary, stimulated the synthesis and production of thyroid hormones. At the same time, the relative number of thyrocytes with diploid nuclei was increased, which can be regarded as a "rejuvenation" of the thyroid gland [21]. The same positive effect of melatonin on the thyroid gland was noted by other researchers [22, 23].

Analysis of the results of our studies suggests that seasonal differences in the effect of INH and melatonin on the morphofunctional state of the thyroid gland may be associated with the peculiarities of its physiological activity in spring and autumn. In spring, during a period of lesser thyroid activity, the prolonged combined effect of INH and melatonin moderately increases its functional activity. On the other side, in autumn, during a period of higher physiological activity of the thyroid gland, INH and melatonin decreases it. Thus, the use of intermittent normobaric hypoxia and melatonin allows to smooth seasonal differences in thyroid activity. However, the physiological significance of this effect requires further study and refinement.

### **Conclusions**

The results of histomorphological studies indicate the presence of distinctly pronounced seasonal differences in the morphofunctional activity of the thyroid gland in young male rats of the Wistar line. In autumn the activity is generally higher than in spring.

The effect of prolonged combined use of intermittent normobaric hypoxia and melatonin on the activity of the thyroid gland also has seasonal patterns. In the spring, the morphofunctional activity of the thyroid gland is moderately increased, and in the autumn period, on the contrary, decreases.

The obtained data can have not only theoretical value, but also be of some practical interest when using intermittent normobaric hypoxia and melatonin for therapeutic and health purposes in patients with thyroid dysfunction in different seasons of the year.

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