Conference Paper

Oncoprotective Effect of Short-Term Hypoxic Training in Model of Ehrlich Ascites Carcinoma in Mice

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Abstract

The search for non-drug therapies to enhance antitumor protection is one of the most urgent tasks of pathophysiology and medicine. We evaluated the impact of a course of interval hypobaric hypoxia (0.306 kg/cm2) on the development of Ehrlich ascites carcinoma (EAC) in mice. In the first series of experiments, the mice were subjected to hypoxic training over 10 days, and in the second series the hypoxic training was provided daily for 3 weeks, except for weekends. The tumor volume was analyzed using a caliper on day 16 (the terminal period). The weight of the mice was measured on day 0, and the final net weight was measured on day 22. The rate of cancer development in mice exposed to hypoxic training within the first 10 days decreased by 58%. Exposure to 3 weeks of hypoxic training showed no significant antitumor effect. Upon completion of the hypoxic training, the rate of tumor development increased, and at the end of the study, no significant differences in survival were found. Further research is needed to determine the optimal antitumor effect and duration of the course of interval hypobaric training.

Keywords: Ehrlich ascites carcinoma (EAC), mice, hypobaric hypoxia, antitumor protection, leukocytes, leukogram

1. Introduction

To date, the problem of prevention and treatment of cancer remains one of the fundamental tasks, despite the existing achievements of modern medicine and pathophysiology. Hence, the possibility of using non-drug therapies optimizing organism's-own functionality with minimal of side effects is a very relevant issue.

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The use of pharmacotherapy for the treatment of cancer so far has certain success; there are targeted drugs that have a directional effect and can prevent the further spread of tumor growth factors [1]

There are studies confirming that a significant role in the spread of cancer in the body is assigned to the individual (genetic) predisposition of the species [2].

At the same time, resistance to the tumor development is mainly attributed to the effectiveness of the antitumor immune protection formed by the genotype. In this regard, it is important to search for physiological methods to strengthen the factors of antitumor protection. Using an adaptation to hypoxia to stimulate the production of nitric oxide (NO)—a protection factor, synthesized by macrophages, thereby reducing the susceptibility of experimental mice to the development of cancer, is one of such methods [3]

Immunity is not only a system protecting a living organism from external and internal damaging factors, but also a set of mechanisms that can preserve its genetic identity as a whole. Accordingly, the functional capabilities of the immune system largely determine the level of the organism's resistance, its adaptive potential. The blood system and immunocompetent blood cells in particular are one of the leading components of antitumor protection against rapidly developing cancer. However, the ability of white blood cells to implement protective antitumor functions may largely depend on the initial level of resistance of the body [4-5]

At the same time, a malignant tumor can alter the normal functioning of various cells, tissues, and organs. In particular, it affects the implementation of protective functions by the cells of the immune system. The strength of the impact may correlate to the area of the tumor surface and, accordingly, to the duration of its presence within a body. In this regard, it is of great interest to determine the extent to which immunocompetent cells are affected by the tumor under the conditions of non-drug maintenance of antitumor immune defense of the body, and, in particular, what quantitative and qualitative changes occur in their composition [6].

The hypoxic factor effects are associated with physiological changes of various intensity occurring in the body. The latter are associated with the mobilization of various functional systems that determine the overall physical capacity and normal functioning of the organism. Compensatory mechanisms of adaptation to hypoxia, which are the basis for improving the functional capabilities of the body, are characterized by quantitative and qualitative changes at different levels of organization and determine the degree of resistance to the effect [7]

In previously published works, it is stated that drug-free strategies of correcting borderline functional states have a significant advantage over the pharmacotherapy use. Among the advantages of non-drug methods we can note the "purity" (physiological nature) of compensatory and adaptive processes induced in the body, their prolonged duration and strength. Hence, the use of natural, physiological methods as preventive and curative measures contribute to the activation of compensatory mechanisms and increase the body's resistance to negative factors, expand its adaptive capabilities and increase the effectiveness of its functional ability [8-9].

We hypothesized that the use of interval hypobaric training in inter-line F1 male mice hybrids can strengthen antitumor immunity, thereby increasing the resistance of experimental animals to the cancer development, localize the area of tumor growth and prolong the life of cancer-bearing mice. Hence, we needed to conduct an experimental test of this assumption on the example of a highly malignant type of tumor—Ehrlich ascites carcinoma (EAC). The following questions were addressed:

1. To determine the content of white blood cells in the blood of mice after exposure to hypobaric hypoxia and draw up the leukocyte formula.

2. To evaluate the effect of hypobaric hypoxia on the dynamics of changes in tumor volume and body weight of the tumor-injected animals within the framework of a phenomenological model of tumor development.

The study is aimed at the evaluation of the response of leukocytic fraction of blood in mice with Ehrlich ascites carcinoma (EAC), determination of tumor volume, and investigation of the dynamics of body weight of the animals in the experiment

Experimental studies aimed at evaluating the biological effects of hypobaric hypoxia were conducted on adult male F1 mice weighting 33-35.05 $\pm$3.25 g. Animals were maintained under standard vivarium conditions on a full diet corresponding to the daily nutritional standards for this type of animal, with a standard daily light-dark cycle. Animals were divided into groups by random sampling. The variation in body weight of animals in each group was no more than 10%. At all-time intervals and in each experimental group (control / experiment) at least 10 animals were used [10-11]

Tumor model. Investigations were carried out using transplantable murine Erlich carcinoma. The strain was sustained in the form of ascites carcinoma in inter-line F1 male mice hybrids. For the experiment, Ehrlich carcinoma was transplanted to male mice by subcutaneous injection of 2×105 tumor cells in 0.2 ml of suspension into the upper back area, where the hair was previously depilated.

Design of the experiment. Subcutaneous tumor transplantation and hypobaric training. On day 0, male hybrid mice were subcutaneously injected with 0.2 ml of Ehrlich

ascites carcinoma cells (2 x 105 cells) in the upper back region. Mice with a diffuse solid Ehrlich tumor with volume of ~ 100 mm3 were randomly assigned into two groups: 1) group C—control mice with diffuse solid tumors receiving no treatment of any kind (C; n = 10) 2) mice of the EXP—experimental group with diffuse solid tumors subjected to the daily 60 minutes-long hypobaric hypoxia training (height 3000 m) (EXP, n = 10). Each experiment involved also an intact group of mice without a tumor (n = 8) to establish a baseline for all parameters considered in this study. Hypobaric hypoxia in the experimental chamber was created using a vacuum pump and was p = −0.306 kg/cm2, which corresponds to 3000 m above sea level. Animals of the EXP group were subjected to hypoxia training from the 1st day after the injection of EAC cells, training was repeated daily for the first 10 days of the experiment—in the first series; and for 3 weeks, except weekends—in the second series of the experiment.

1.1. Analysis of the tumor volume.

The tumor volume was measured using a caliper on day 16 (terminal period) after the injection of Ehrlich ascites carcinoma cells. The collected data were applied to calculate the tumor volume using the following formula: tumor Volume (mm3) = 0.52 AB2, where A is the minor axis and B is the main axis.

1.2. Changes in body weight.

Animals with diffuse solid Ehrlich carcinoma who were subjected to hypobaric hypoxia training and the control group of animals were observed for changes in body weight: (initial body weight was measured on day 0, and final body weight was measured on day 22). The body weight gain was defined as the difference between the initial body weight and the final body weight.

1.3. Modeling of interval hypobaric hypoxia.

The animals of the control groups were maintained under normal atmospheric pressure. Animals of the experimental groups were exposed to interval hypoxia using a special chamber. The rate of compression and decompression was 0.5 kPa/min.

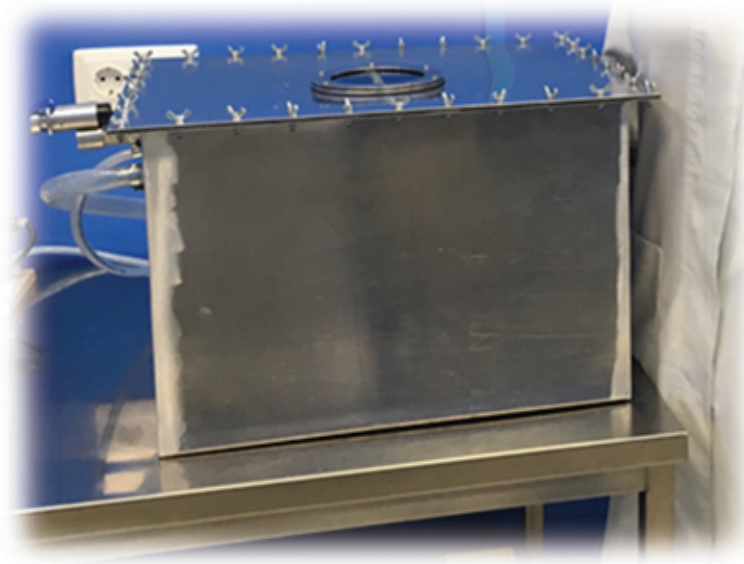To create a low pressure, a hypobaric pressure chamber designed for laboratory animals was used (Fig. 01).

Figure 1: A picture of a hypobaric pressure chamber, used for creating a low pressure

The underpressure in the chamber was created using a vacuum pump and was −0.306 kg/cm2, which corresponds to 3000 m above the sea level.

The hypobaric pressure chamber has a hermetically sealed lid, a shut-off valve and two pipes: for connection to a vacuum pump and a vacuum gauge. Before the experiment the cage with the animals (not shown at Fig.01) was placed inside the pressure chamber. The pressure chamber was closed with a sealed lid; then a vacuum pump was turned on to create underpressure conditions. The pressure in the chamber was lowered stepwise: the first stage = height of 1000 m equivalent with underpressure of −0.1 kg/cm2, lasting 180 seconds; the second stage = 2000 m with underpressure of −0.21 kg/cm2 and the same duration; and the last stage = 3000 m height, which corresponds to the underpressure of −0.306 kg/cm2. The level of underpressure was measured by the vacuum gauge. At the end of the hypobaric session, the pressure in the chamber was returned to normal, also stepwise, but in a reverse order, using a shut-off valve.

Blood smears were prepared according to standard procedures. Staining of the prepared smears was carried out using May-Grunwald method, followed by final straining using Romanovsky-Gimze method. White blood cells were counted manually in a counting chamber with a Goryaev grid. The leukocyte formula was derived manually, using 11-key leukocyte counters and a ligh microscope (magnification x 1000) with an immersion lens [14].

Statistical analysis. The blood elements (white blood cells) count and other measured parameters are given as Mean ± standard error of mean (M±m). The data were analyzed

using descriptive statistics for a normal distribution. Normality was checked using the Liliefors test. Groups were compared using the Student's T-test. The differences were considered significant at the p < 0.05 level.

2. Methods and Equipment

Experimental studies aimed at evaluating the biological effects of hypobaric hypoxia were conducted on adult male F1 mice weighting 33-35.05 $\pm$3.25 g. Animals were maintained under standard vivarium conditions on a full diet corresponding to the daily nutritional standards for this type of animal, with a standard daily light-dark cycle. Animals were divided into groups by random sampling. The variation in body weight of animals in each group was no more than 10%. At all-time intervals and in each experimental group (control / experiment) at least 10 animals were used [10-11]

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3. Results

The tumor volume. Figure 2 shows that the use of hypobaric hypoxic training in tumor-injected mice led to a deceleration of tumor development, which became statistically significant as early as day 16 after the start of the therapy. The percentage of differences in tumor volume in mice in the experimental group (1436 ± 145) mm3 and in the control group (without hypoxic training) (3416 ± 144) mm3 reached 57.96% ($p < 0.01$) (Fig. 02). Such tumor volumes ratio indicates a significant tumor growth regression in mice from the experimental group compared to the control group.
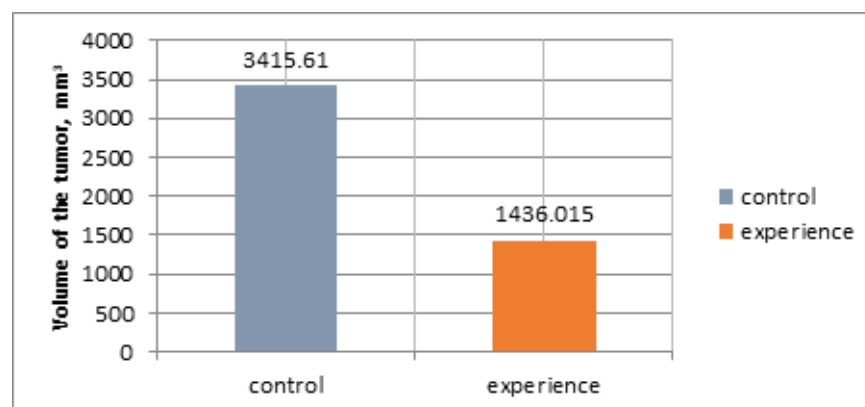

Figure 2: Mean tumor volumes in mice from the experimental and control groups measured on day 16 (terminal period).

Body Weight. Table 1 shows the effect of hypobaric hypoxic training on animal weight, which was studied at the end of the experiment on day 22. Weight gain in mice in the control group was 7.43 ± 0.38 g. Respectively, the weight gain in mice in the hypoxia

group was 8.60 ± 0.45 g, which represents statistically significant changes (p < 0.05). The percentage difference was 13.95% (Tab. 01).

TABLE 1: Dynamics of body weight of mice with a tumor

Parameter	Experiment	Control
Initial body weight (g) day 0 (Mean ± standard error of mean)	35.05 ± 3.25	32.7 ± 5.07
Final body weight (g) day 22 (Mean ± standard error of mean)	43.65 ± 3.57	40.13 ± 6.8
Body weight gain (g)	+ 8.60	+ 7.43

Effect of hypobaric hypoxia on white blood cells in mice with a tumor.

In the first experiment, on the fifth day the total white blood cell count in the control group of mice was 7.09·109/L, in the experimental group it was at the same level and was equal to 7.21·109/L. On the tenth day, there was a significant increase in the number of white blood cells in the control group up to 16.59·109/L, against 11.67·109/L in the experimental group (Fig. 03).
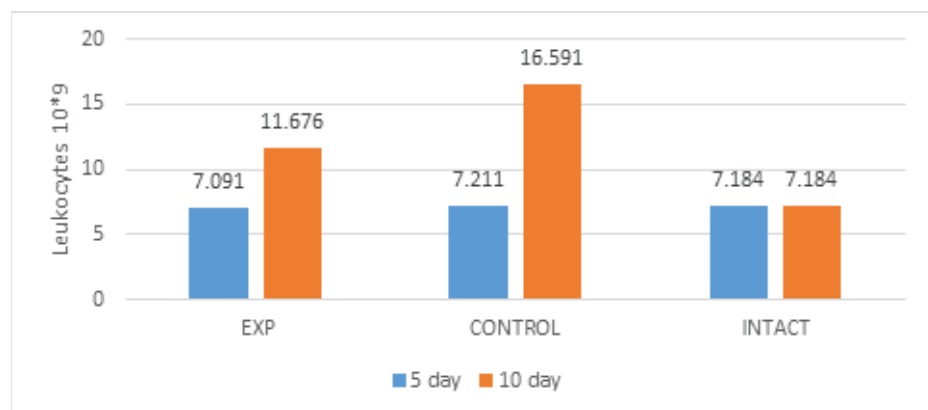

Figure 3: Dynamics of white blood cell levels in control and experimental groups of mice, first experiment.

Thus, we observe a significant increase in the number of white blood cells; it was almost 2 times greater in the animals of the control group, compared to the mice of the experimental group. The content of white blood cells in the intact group of mice remained unchanged and was equal to 7.18·109/L (Fig. 03).

We analyzed the leukograms in mice on the first day, after a session of hypobaric hypoxia. Previously, it was found that in mice, in particular, in F1 hybrids, the leukogram profile is lymphocytic. But under the conditions of simulated hypoxic exposure the mice of the experimental group showed a leucogram shift to the left. In animals of the control group there were no changes in the leukocyte formula, and the ratio of all types of leukocytes remained within the normative values.
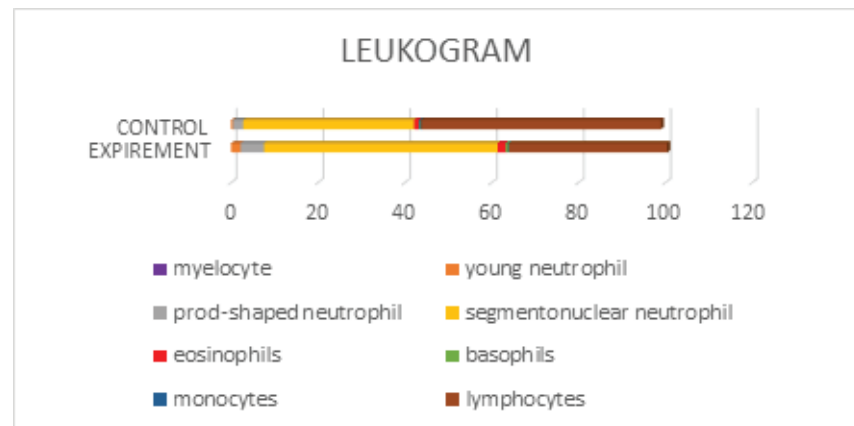
Figure 4: Leukocyte formula of the experimental (EXP) and control (C) groups of mice, day 1 of hypoxic training.

Previously, it was found that the occurrence and development of cancer contributes to an increase in the content of neutrophilic white blood cells. The mechanism of tumor-induced neutrophilia has not been fully studied yet. However, the production of polypeptide cytokines belonging to the group of granulocyte-macrophage colony-stimulating factors (colony stimulating factor 2 (granulocyte-macrophage), GM-CSF) by tumor cells may be related to the mechanisms of activation of neutrophil secretion by the central hematopoietic organ. Besides, such cytokines as interleukin-1 and interleukin-6, synthesized, similarly, by cancerous tumors, most likely contribute to an increase in the content of neutrophils in the blood. The described neutrophilia can be a "bad" indicator for certain types of cancer: lung cancer, renal carcinoma, and melanoma. In this regard, an increase in neutrophils quantity in some types of tumors is a reason for negative prognosis of the disease. Since in most cases an increased pool of neutrophilic leukocytes in peripheral blood is associated with the inflammatory process induced by an infectious disease or tissues integrity damage, the increase in white blood cells number in the area of the tumor focus is seen as a confirmation of the statement about the significance of inflammation in the process of tumor genesis and the increased expansion of the area of "infection" caused by it [15].

At the same time, contrary to the results reported in most studies, including those we mentioned earlier, an increase in the content of neutrophils in the peripheral blood due to their enhanced elimination from the central organ of hematopoiesis (neutrophilia) can not be considered a negative sign regarding the disease development in all cases. So, for the diagnosis of gastric cancer, the increase in neutrophilic leukocytes is quite contrary interpreted as a favorable prognosis for the course of the disease. This observation may indicate that neutrophils in certain situations can control the dynamics of cancer development. Previously, their "killer" function towards cancer cells was detected both

in vivo and in vitro. At the same time, a feature of neutrophils of tumor-bearing animals was discovered, which involved an enhanced cytotoxic effect on certain types of tumor cells. Neutrophils obtained from human blood can directly destroy certain types of cancer cells by cytotoxic means. Thus, the influence of neutrophils on the processes of growth and development of the tumor and on the course of the disease has an alternative effect, and therefore further in-depth research is needed to determine the cause of the alternative action [14]
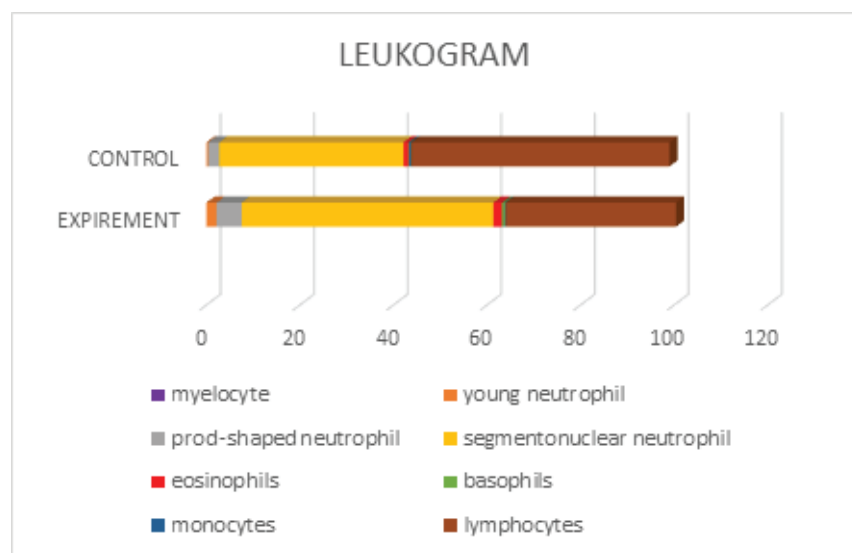

Figure 5: Dynamics of white blood cell levels in control and experimental groups of mice, second experiment

In the second experiment, the study was conducted for 35 days, followed by a natural exit of the animals from the experiment.

On the first day, the animals of the control group showed a tendency to increase the number of white blood cells, while the number of white blood cells in animals of the experimental group, on the contrary, decreased 2 times ($p \leq 0.05$). On the eighth day of the experiment, the content of white blood cells in animals of the control and experimental groups equalized. The last blood collection was made on the 35th day; there was no significant difference between the groups, the level of white blood cells showed a tendency to increase (compared to day 8) [16-17].

4. Discussion

In a study on the effect of hypoxia on the dynamics of the development of ascites carcinoma in mice, it was noted that the use of hypoxic training has an antitumor effect and inhibits the growth of Ehrlich ascites carcinoma cells.

The results obtained can most likely be attributed to the influence of hypoxic training on the functioning of the immune system of tumor-bearing animals. There are studies that confirm the improvement of cellular and humoral immunity under such conditions. The content of various classes of white blood cells increases and the synthesis of immunoglobulins activates [18]

Many researchers suggest that conducting sessions of moderate hypobaric hypoxia contributes to triggering immune response; though, the resistance of experimental animals to infectious diseases remains the same or increases. However, it is believed that the degree and "sign" of developing processes is directly determined by the amount of hypoxic exposure. In previous studies, it was noted that a monthly course of interval hypobaric hypoxia (height 4500 m) contributes to the relief of tuberculosis in white mice and increases the life expectancy of experimental tumor-bearing animals [19]

According to other data, it was noted that a month-long course of preliminary adaptation to hypobaric hypoxia (height 4500 and 7500 m) increases the resistance of the experimental mice to further infection with tuberculosis, due to the launch of mechanisms of non-specific (anti-infectious) resistance. It was also reported that the average life expectancy of tumor-bearing mice is almost twice as high as the specified parameter in control group 3 [20]

We observed such changes in the first series of experiments using a 10-day course of hypoxic training.

However, the longer 3-week course of hypobaric hypoxia leads to a pro-tumor effect and activates the growth of ascites carcinoma cells. It is known that with prolonged hypoxia in animals that adapt to "height" the resistance to bacterial and protozoan infections, on the contrary, significantly decreases. There are reliable data that under conditions of prolonged and deep hyperbaric hypoxia, simulating a height of 5000 – 6000 meters, the sensitivity of experimental animals to streptococcal, staphylococcal, pneumococcal, Salmonella infections, typhoid fever, trypanosomes and Plasmodium malariae, pathogens of tularemia and of gas gangrene increases.

5. Conclusion

Thus, the application of the 30-day course of hypoxic exposure did not increase the antitumor properties of the body, which may be attributed to the insufficient oxygen supply of immunocompetent cells and, consequently, to the inhibition of antibody synthesis; though, the question if a direct correlation between the resistance of animals

to infection and the content of antibodies really exists have not been fully investigated yet.

This mechanism may be directly related to the shortness of the period of tumor growth inhibition, when at a certain point the inhibition is replaced by the reverse growth of ascites carcinoma cells.

Thus, to clarify the mechanisms and the causes of short duration of the inhibitory effect of interval hypoxic training and to establish optimal conditions for antitumor action of the selected exogenous effect on the body, further in-depth research is necessary.

Acknowledgement

Conflict of Interest

The authors have no conflict of interest to declare.

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