

Conditioning Regimens of Periodic Normobaric Hypoxia Do not Have Intrinsic Effects on the Behavior of Rats

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The development of preventive and therapeutic methods based on the principles of hypoxic conditioning is a pressing issue for experimental research, given the high efficacy of such interventions in the prevention and correction of a wide range of pathological conditions. We have previously developed hypoxic conditioning regimens using moderate periodic normobaric hypoxia (PNH) created by inhaling gas mixtures with reduced oxygen content. The aim of the present study was to investigate the behavioral effects of the PNH regimens developed in our work and applied independently rather than as conditioning for pathological states in rats. No significant differences in behavioral measures of rats subjected to PNH as compared with control animals were found in the open field, elevated plus maze, or novel object recognition tests. Thus, the PNH regimens used here do not have intrinsic effects on behavior and can be used for conditioning without the risk of side effects.

Keywords: periodic normobaric hypoxia, conditioning, OXYTERRA device, hypoxia/hyperoxia Funding.

Conditioning – exposure to short but intense episodes of extreme factors in a mode that causes urgent mobilization of the body's adaptive resources, leading to an increase in the body's resistance – is a promising method of physical therapy [Rybnikova and Samoilov, 2015; Burtscher et al., 2024; Simonenko et al., 2025]. Experiments have shown that as well as increasing resistance, conditioning has a wide range of therapeutic neuroprotective effects, correcting the formation of neurological and psychoemotional disorders [Zenko and Rybnikova, 2020]. Ischemia, i.e., short-term restriction of blood flow to an organ or body part (ischemic or remote ischemic conditioning) by occluding their supply vessels, is primarily used for conditioning [Murry et al., 1986; Zhao et al., 2006; Baranova and Zenko, 2018]. We have developed non-invasive hypoxic conditioning methods based on the use of hypobaric or normobaric hypoxia [Zenko and Rybnikova, 2020; Zenko et al., 2023]. These methods are more effective because the entire body is exposed, and they have greater translational potential because they are easier to dose and monitor, and they have fewer

contraindications than ischemia. Our studies using a model of hypobaric hypoxia produced in a pressure chamber have thoroughly investigated the mechanisms of the neuroprotective effects of hypoxic conditioning and have identified the key role of the rapid induction of hypoxia-inducible factor-1 (HIF-1) and the multilevel modification of the regulation of the main hormonal adaptation system, i.e., the hypothalamic-pituitary-adrenocortical axis (HPAS) [Rybnikova and Samoilov, 2015; Zenko and Rybnikova, 2020]. The mechanisms of hypoxic conditioning are fundamentally different from those of hypoxic training or mountain acclimation used in spa medicine, whose actions are based on the gradual restructuring of the mechanisms of oxygen transport and utilization [Barbashova, 1960; Rybnikova et al., 2019].

In recent years, our studies on rats have shown that, in addition to hypobaric hypoxia, periodic normobaric hypoxia (PNH) created by inhaling gas mixtures with reduced oxygen content at normal atmospheric pressure can also be successfully used for conditioning [Zenko et al., 2023], and high efficacy was found when hypoxic episodes were alternated not with normoxia, but with mild hyperoxia (30% oxygen in the breathing mix). Methods of normobaric hypoxic/hyperoxic conditioning have great translational potential,

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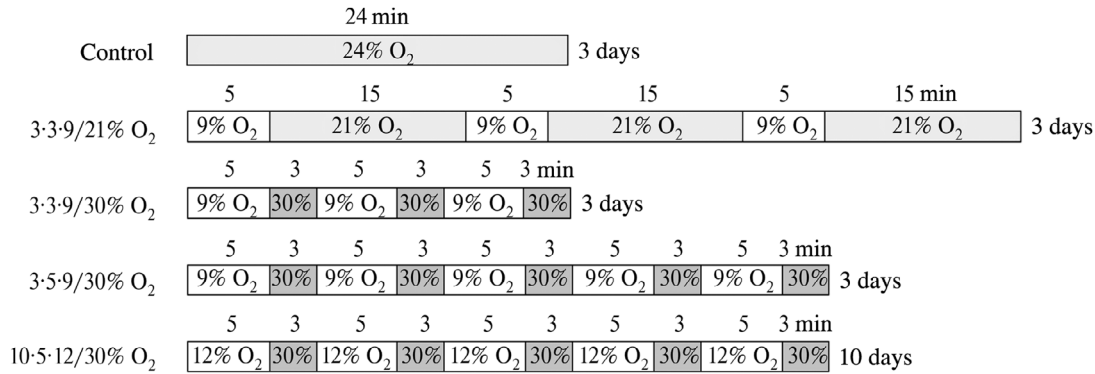


Fig. 1. PNH regimens studied here. See text for explanation.

though their implementation requires, first, investigation of the possible side effects of its actions in normal conditions. This was the aim of the present study, which addressed the behavioral effects of four modes of moderate periodic normobaric hypoxia previously developed by us for conditioning or training in order to prevent and correct anxious-depressive and diabetic pathologies in rats.

Methods. Experiments were carried out in the morning on 30 male Wistar rats aged about two months and weighing 220–250 g obtained from the Collective Use Center “Biocollection of the Institute of Physiology of the Russian Academy of Sciences for the Study of Integrative Mechanisms of the Activity of the Nervous and Visceral Systems.” Rats kept in standard conditions (alternating 12/12 h light/dark, six rats in each cage of size 15 × 35 × 50 cm, with free access to water and food, temperature ~21°C) were randomly divided into five groups each of six animals without additional randomization, forming a control group and four experimental groups. The study was not blinded; sample size was not calculated.

Periodic hypoxic effects. Experiments used the OXYTERRA apparatus (Cellgym-Rus OXYTERRA LLC, Russia), which generates hypoxic, hyperoxic, and normoxic gas breathing mixes in the required modes. The modification of the apparatus developed specially by the equipment manufacturer for working with laboratory rodents supplies gas mixtures to a semi-hermetic cage measuring 15 × 35 × 50 cm, divided by lattice partitions into individual compartments for the rats.

Schemes for regimens of periodic normobaric hypoxia (PNH) are presented in Fig. 1. Rats were exposed to PNH in the following regimens: 3 × 3 × 9/21% O₂ – sessions consisted of three cycles per day of 5 min in 9% O₂ and 15 min normoxia in 21% O₂, carried out daily for three days; 3 × 3 × 9/30% O₂ – similarly, but reoxygenation using 3 min hyperoxia in 30% O₂; 3 × 5 × 9/30% O₂ – daily sessions consisted of five cycles of hypoxia/hyperoxia; 10 × 5 × 12/30% O₂ – sessions of five cycles of hypoxia/hyperoxia daily for 10 days. Previous studies indicated that all four regimens exerted protective effects in models of rat pathology, though the regimen using three-day intermittent hy-

poxia/hyperoxia (3 × 3 × 9/30% O₂) demonstrated the highest efficacy in most cases [Zenko et al., 2023].

Behavioral tests. The effects of PNH were studied in the open field (OF) and elevated plus maze (EPM) behavioral tests, as well as in the novel object recognition (NOR) cognitive test.

The OF test, which is a classic method for studying the behavior of rodents in new, stressful conditions, assesses motor activity and exploratory behavior, as well as anxiety and emotionality in rats [Hall, 1934; 1936]. Testing was conducted on day 3 after the final PNH session in a square apparatus of area 1 m² divided into 36 sectors, with a light source overhead in the center of the apparatus (illumination intensity approximately 150 lx). The test animal was placed in the center of the open field and its activity was recorded for 5 min. During the experiment, the latent period (of excursions from the center), the number of squares crossed in the various zones of the apparatus, and the durations of grooming, vertical rearing, and immobilization were recorded, though defecation and urination were not recorded due to their lower specificity.

Animals’ anxiety levels were assessed using the elevated plus maze behavioral test (arm length 50 cm, width 12 cm) [Pellow et al., 1985] on day 4 after the final exposure to PNH. The EPM apparatus was a maze elevated 1 m above the floor with two open illuminated (stressful) and two closed (shaded and essentially protected) arms. Rats were placed in the center of the apparatus and the numbers of visits to the arms and the center of the central compartment and the durations of time spent in all these zones were recorded for 5 min [Pellow et al., 1985; Walf and Frye, 2007].

The “novel object recognition” cognitive test performed during the animals’ training evaluates their object/subject memory over periods of a few minutes to hours. The test was conducted according to the generally accepted methodology in an opaque plastic apparatus (60 × 40 × 40 cm) under shaded conditions [Bertaina Anglade et al., 2006; Antunes and Biala, 2012]. The objects to be recognized were selected in three identical pairs made of different materials (glass, aluminum, painted steel) and had different colors and shapes but were of approximately equal volume and mass

and were too heavy for the animals to move. The objects occupied less than 2% of the area of the test apparatus, allowing the animals to explore them freely. The floor, walls, and objects inside were cleaned with a water–alcohol mixture between trials with each rat to prevent odor interference. During the 4-min training phase (T_0), each rat sequentially examined the first pair of identical objects. All 30 animals examined the first pair of identical objects for a total of more than 15 sec during the training phase and were included in the subsequent test. The T_1 and T_2 test phases (3 min each) took place 2 and 4 h after T_0 respectively, with one of the original objects replaced by a corresponding new one (the “new” objects in T_1 and T_2 were different new objects, so as to maintain the condition of novelty). During testing, the duration of interactions of rats with the new and old objects was recorded. Interactions were defined as the rat’s nose being less than 2 cm from the object while examining or sniffing it, but not while leaning on it. If the total interaction time was ≥ 5 sec, the animal was included in the analysis. Based on this criterion, 27 rats were tested at the 2-h mark and 21 rats at the 4-h mark. The difference in interaction time between the “old” and “new” objects can be used to judge the retention or absence of object memory after training at particular time points, and to evaluate the dynamics of its extinction [Bertaina-Anglade et al., 2006; Antunes and Biala, 2012].

Statistical analysis. The distribution of the parameters analyzed here was tested for normality using the Kolmogorov–Smirnov and Shapiro–Wilk tests, after which the non-parametric Mann–Whitney test was used (Statistica 12.0 software package). Differences between groups were taken as significant at $p \leq 0.05$. Results for experimental subgroups are presented as mean values and standard errors of the mean ($M \pm SEM$).

Results and Discussion. The present study analyzed the behavioral and cognitive effects of four PNH regimens used as conditioning or training interventions in rats which, with the aim of allowing assessment of their intrinsic effects, had not been subjected to pathological influences.

Assessment in the OF test showed that the latent period was significantly longer than that in the control group (Fig. 2, *a*) only in the $3 \times 3 \times 9/30\%$ O_2 group (the most effective conditioning regimen); this was not observed in any of the other groups. According to the classical interpretation of this parameter, an increase in the latent period may reflect an increase in anxiety; however, this interpretation requires that the corresponding changes affect the indexes of motor and exploratory activity. In this case, the horizontal motor activity of rats in the OF did not differ from that in the control group and there were no differences between any groups (Fig. 2, *b*). On the one hand, this may indicate that the effects of the PNH used here had no side effects on the locomotion of the animals (Fig. 2, *b*); on the other hand, it does not allow us to interpret the increase in the latent period of onset of movement as an anxiety reaction. Instead of immobilization in the central starting zone, which would be characteristic of

an increase in the latent period in an animal in an anxious or stressed state, rats of the $3 \times 3 \times 9/30\%$ O_2 group performed orientational sniffing in the starting zone or, later – after the latent period cutoff point – vertical rearing, which may suggest a lesser manifestation of thigmotaxis (preference for the outer zones relative to the center and avoidance of the center), which, conversely, is associated in rodents with exploratory activity and anxiolysis [Hall, 1934; Treit and Fundytus, 1988; Prut and Belzung, 2003]. The absence of an intrinsic effect of moderate PNH on locomotion is also an interesting finding in light of the fact that more severe hypoxia combined with ineffective hypoxic preconditioning regimens produce consistent impairments of horizontal motor activity in rats, as in our previous work [Rybnikova et al., 2022].

The duration of grooming (the sum of “facial” and “overall” grooming), which is often interpreted as a displacement activity (basal “comfort” activity in rats is associated with a low level of grooming; elevated levels of this indicator are observed under stressful conditions), also did not differ in rats of the experimental groups as compared with the control groups or with each other. This indicates that PNH did not have a stressful effect on animals, which is also confirmed by the absence of immobilization in all groups, reflecting low anxiety levels [Hall, 1934, 1936; Prut and Belzung, 2003]. Overall vertical motor activity, specifically the duration and number of rearings in the OF, primarily reflecting the animals’ exploratory activity, also did not differ between groups.

Results from the OF test were consistent with the data obtained in the EPM test. None of the indicators of exploratory activity – the duration and number of excursions to the center and the open arms of the EPM apparatus – showed any difference between the animals of the experimental groups as compared with the controls, indicating low anxiety levels, similar to the level in control animals (Fig. 2, *c*, *d*). Thus, the PNH regimens studied here did not have anxiogenic or anxiolytic effects in animals without pathology, though these treatments have been found to have significant anxiolytic effect in a model of post-traumatic stress disorder (PTSD) [Zenko et al., 2023].

The object recognition index determined in the NOR test was calculated as the difference between the duration of exploration of the “new” and “old” objects, adjusted for the total duration of interaction with them: $((N - O)/(N + O)) \times 100$ [Bertaina-Anglade et al., 2006]. Again, no significant between-group differences were observed for this indicator (Fig. 3). In contrast to the control group, positive values of this indicator were obtained in some of the hypoxia groups even after 4 h, indicating longer retention of object memory in these animals after training. It seems that this may indicate potential for using these PNH modes in neurology and gerontology.

Conclusions. Thus, the PNH modes studied here had no significant effects on the behavior of rats, indicating the probable absence of side effects when used as conditioning

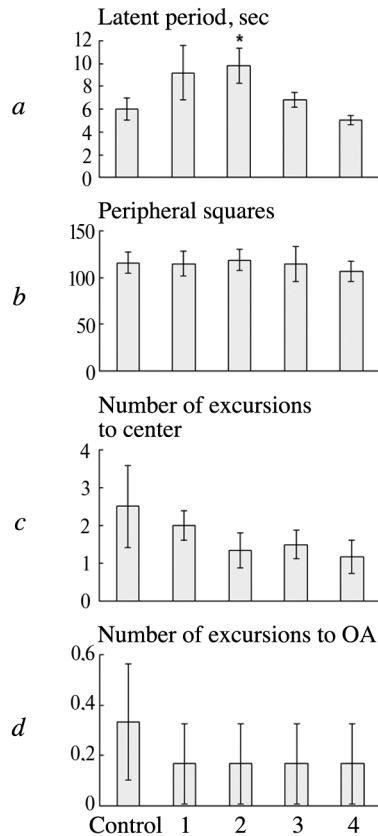


Fig. 2. Results of behavioral testing in the OF and EPM: a) latent period, sec; b) number of peripheral squares crossed; c) number of excursions to the center of the EPM; d) number of excursions into the open arms of the EPM. Control – control group ($n = 6$); 1) the $3 \times 3 \times 9/21\%$ O_2 group ($n = 6$), 2) the $3 \times 3 \times 9/30\%$ O_2 group ($n = 6$); 3) the $3 \times 5 \times 9/30\%$ O_2 group ($n = 6$); 4) the $10 \times 5 \times 12/30\%$ O_2 group ($n = 6$). *Significant difference from control, $p < 0.05$, Mann–Whitney test.

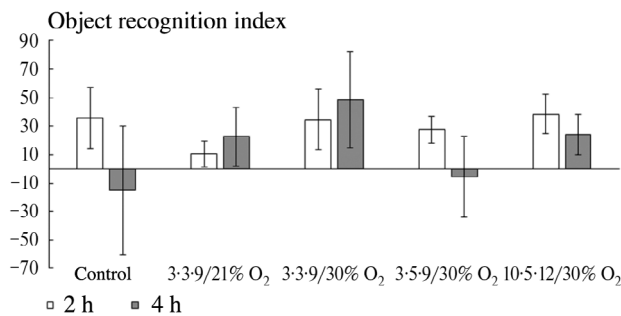


Fig. 3. Object recognition index in the NOR test in rats of different groups at the 2-h (white columns) and 4-h (black columns) time points after initial training.

regimens. Of particular interest is the lack of effect on anxiety levels in rats, as the anxiolytic effect is seen as integral to hypoxic factors of moderate intensity, as in experimental hypoxia and in natural conditions during acclimation to the mid-altitude region [Rybnikova et al., 2019]. The fact that the same regimens have powerful anxiolytic and antidepressant effects on the behavior of rats stressed using models of psychopathologies clearly leads to the conclusion that the

effects of PNH are context-specific, that is, their manifestation depends on the conditions and physiological state of the organism. Furthermore, the results obtained in the present study indicate that, despite the use of 9–12% oxygen, the PNH regimens used in this study are quite mild, at least for rats; however, further preparation of the method for testing on humans requires account to be taken of interspecies differences in resistance to hypoxia. Experiments using PNH in lower primates may provide an appropriate test system for analyzing this issue.

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This study was conducted in accordance with international and Russian ethical standards, and in compliance with the Declaration of Helsinki. The experimental protocol was approved by the Ethics Committee of the Pavlov Institute of Physiology RAS (protocol No. 06/05 dated June 5, 2025).

The authors declare that they have no obvious or potential conflicts of interest related to the publication of this article.

Authors' contributions: Zenko – conduct of experiments, data processing, writing the report; Baranova – conduct of experiments; Rybnikova – concept and supervision of the work, writing and editing the report.

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Primary data can be requested from the authors of the article if required.

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