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Assessment of the condition of conditioned reflex activity in the process of liquid respiration

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Introduction. Liquid respiration is the ability of mammalian lungs to receive oxygen dissolved in a liquid for respiration and release carbon dioxide into it. A promising field of application is the provision of marine operations. For the use of liquid respiration during deep-sea operations, the technology must ensure the normal functioning of all organs and systems of the body, prevent decompression sickness, and allow conscious activity.

The study aims to assess the safety of conditioned reflex activity during independent liquid respiration in laboratory animals in normobaric conditions.

Materials and methods. We performed the study on male Syrian hamsters aged four months, weighing 120-140 g. Researchers have developed a stand with an eight-level maze with a lock. We immersed the labyrinth in an aquarium. The scientists carried out the study in two stages. At the first stage, we have developed in animals a conditioned reflex of actively avoiding drowning in conditions of breathing air. The researchers placed the animal on the lower level, then immersed the maze in an aquarium filled with water at a speed that only the animal's head was above the water level. The threat of drowning prompted the animal to search for a passage to a higher level. The training was three times a day for ten days. At the second stage, scientists studied the influence of various conditions of liquid respiration on the state of the conditioned reflex activity of animals. We used two respiratory fluids — perfluorohexane (PFH) and perfluorodecalin (PFD) in three temperature regimes: 22.0, 27.0, and 32.0°C. The researchers filled the aquarium with two oxygenated respiratory fluids of the required temperature instead of water. We have entirely immersed the maze with the animal fixed at the lower level in the aquarium. After switching to liquid respiration, scientists removed the animal from fixation. From the moment of removal from fixation, the countdown of the passage of the maze began. First, we assessed the condition of conditioned reflex activity by the number of animals in the group that successfully passed the maze and the average time of its passage.

Results. Animals on liquid respiration in perfluorohexane successfully passed the labyrinth in all temperature conditions. The average transit time at 22.0°C was 323±94 s; 27.0°C — 45±12 s; 32.0°C — 147±101 s. Animals on liquid respiration in perfluorodecalin successfully passed the labyrinth at a temperature of 27.0°C; the average passage time is 131±79 s; at a temperature of 32.0°C, 20% of animals successfully passed the labyrinth, the average time is 32.5 s; at a temperature of 22.0°C, none of the animals passed the maze.

Conclusions. *Conditioned reflex activity during independent liquid respiration in small laboratory animals in normobaric conditions persists and depends on the physico-chemical properties and temperature of the respiratory fluid.*

Ethics. We conducted studies involving laboratory animals in compliance with the necessary regulations (the Helsinki Declaration of 2000 on Humane Treatment of Animals and the "Rules for carrying out work using experimental animals" (Order of the Ministry of Health of the USSR No. 755 of 12.08.1977)). The Ethics Committee of the Izmerov Research Institute of Occupational Health approved the study protocol.

Keywords: *liquid respiration; conditioned reflex activity; physiology of behavior; maze for animals; forced swimming; hypothermia*

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Contribution:

Tonshin A.A., Bonitenko E.Yu., Kotskiyi M.A., Makarov A.F. — the concept and design of the study;
Kovaleva A.S., Makarov A.F. — computed tomography;
Kotskiyi M.A., Makarov A.F. — autopsy;
Rodchenkova P.V., Lapshinova B.O., Blintcova N.V., Kovaleva A.S. — data collection and processing;
Tonshin A.A., Bonitenko E.Yu. — writing the text;
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Introduction. Liquid respiration (LR) — the ability of mammalian lungs to receive oxygen dissolved in liquid for respiration and release carbon dioxide into it.

For the first time, J.A. Kilstra and co-authors conducted experiments on liquid respiration on mice in 1962 [1]. Researchers used isotonic salt buffer solutions saturated with oxygen at a pressure of 7.0 excess atmospheres as a respiratory fluid (RF).

At this pressure, the dissolved oxygen content in water is equivalent to its concentration in the air under normobaric conditions. The authors managed to select such experimental conditions under which the survival time of mice on the RF in hyperbaric reached 18 hours, however, without the possibility of a reverse transition to gas respiration at atmospheric pressure.

Motor activity in animals persisted for the first 6 hours and was manifested by "a reaction to shaking the capsule and tapping on its walls".

After ensuring the transition from liquid respiration (LR) to gas respiration [2, 3], scientists confirmed the hypothesis about the ability of liquid respiration to prevent the development of decompression sickness [4, 5].

Scientists carried out these studies of the possibility of using LR to ensure deep-sea operations until 1985 [6–9]. There were no later publications in the open press.

All published works devoted to the study of the peculiarities of the functioning of individual physiological systems of the body during LR in hyperbaric conditions and scientists carried out experiments on animals in states of fixation, muscle relaxation, or limited space.

Thus, modern knowledge about the possibility of conscious activity in LR conditions is limited to the data obtained in the very first published work on LR [1]. However, for the use of LR during deep-sea operations, this technology must ensure the normal functioning of all organs and systems of the body, prevent decompression sickness, and allow conscious activity.

The study aims to assess the safety of conditioned reflex activity during spontaneous LR in small laboratory animals in a normobaric condition.

Materials and methods. We performed the study on mature male Syrian hamsters obtained from LLC "Krolinfo" aged 4 months, weighing 120–140 g. Before starting the study, we kept the animals in quarantine for 14 days and kept them under the requirements of GOST 33215-2014[1] and GOST 33215-2014[2] on a standard water and food ration in a separate room.

The research methodology based on the preliminary development of a conditioned reflex of active avoidance in small laboratory animals, followed by its confirmation under conditions of LR immersion in oxygenated water in a normobaric conditions.

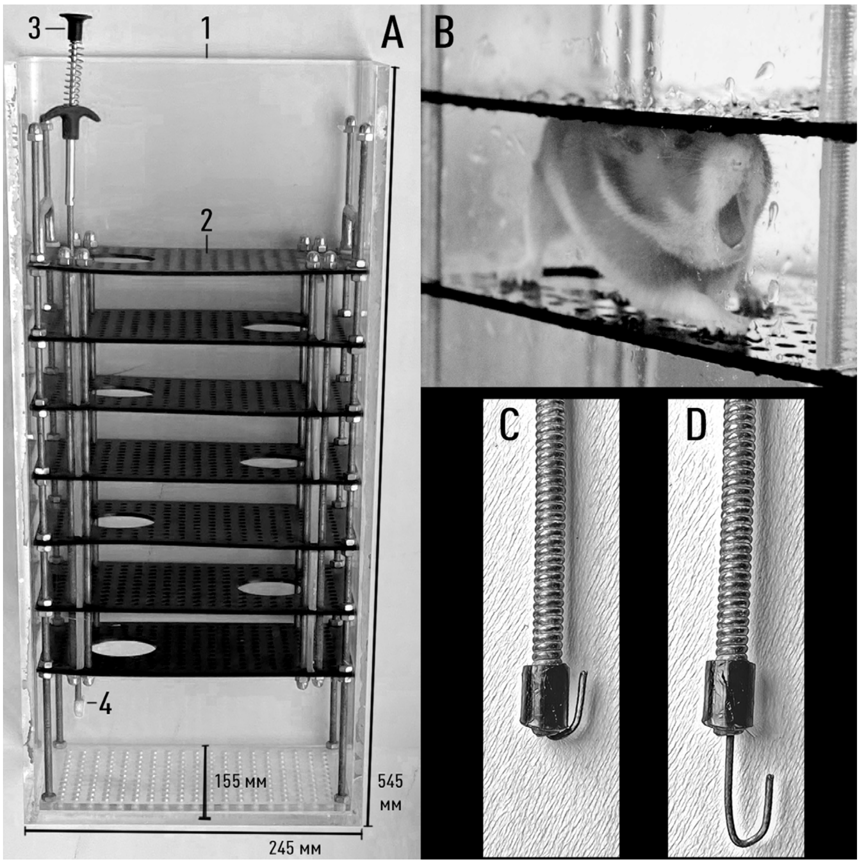


Fig. 1. General view of the stand. A — stand: 1 — aquarium; 2 — maze plate with perforation; 3 — lock button; 4 — animal lock clip; B — a hamster in a maze on liquid respiration (LR); C — closed lock; D — open latch.

We developed a stand for studying conditioned reflex activity in both gas and liquid environments. The stand consists of an 8-level structure (maze) in a transparent tank — aquarium ***(Fig. 1)***. Except for the first one, the levels consist of opaque rectangular perforated (for free penetration of gas and liquid) acrylic plates measuring 225×135 mm with one hole with a diameter of 50 mm for the passage of the animal. We fixed the levels on four metal studs located at the corners. The height of the 1st level was 100, 2–8 — 45 mm. The holes arrange for the passage of the animal in staggered order.

We carried out the holding of the animal at the starting position of the lower level using a remote lock with a button. The retainer was a modified collet clamp for fixing a laboratory animal by the base of the tail. Scientists have performed a study in two stages.

At the first stage, animals developed a conditioned reflex of actively avoiding drowning in conditions of breathing air. Next, we filled the aquarium with water at room temperature (20.0–22.0°C). Next, the researchers immerse the maze entirely in the aquarium, and the upper-level platform is underwater. Next, scientists fixed the animal at the starting position of the lower level, after which the maze smoothly plunges into the aquarium. Finally, we selected a diving speed at which the animal's head was above the water level.

The threat of drowning prompted the animal released from the retainer to move to a higher level. After the complete passage of the maze, we dried the animal with a hairdryer and placed it in a cage. The researchers trained three times a day for ten days. We assessed the degree of fitness of the animal by the shortest time of passage of the maze, recorded on the 10th day. Scientists formed six experimental groups according to the results of the fitness assessment.

At the second stage, we studied the influence of various LR conditions on the condition of the conditioned reflex activity of animals. The scientists used two respiratory fluids (RF): perfluorohexane (PFH) and perfluorodecalin (PFD). They differ significantly in their physico-chemical properties ***(Table 1)*** in three temperature regimes of 22.0, 27.0, and 32.0°C.

At the same time, the saturation of O_2, both respiratory liquids were the same and amounted to ~90.0 vol%. We presented immediate conditions of the LR for all experimental groups in ***Table 2***.

When evaluating conditioned reflex activity in LR conditions, we filled the aquarium with oxygenated water of the required temperature instead of water.

Researchers carried out the oxygenation of RF by bubbling using a Nidek Mark 5 Nuvo Lite concentrator (USA) for an hour with an oxygen consumption of three-liter/min.

The oxygen concentration in RF we determined using the oxygen meter Aktakom ATT-3010 (Russia) with the function of a thermometer.

Researchers measured a rectal temperature in animals before testing. During the study, a maze with a fixed animal we wholly immersed in an aquarium.

[1] GOST 33215-2014 Guidelines for the maintenance and care of laboratory animals. Rules of equipment of premises and organization of procedures.

[2] GOST 33216-2014 Guidelines for the maintenance and care of laboratory animals. Rules for the maintenance and care of laboratory rodents and rabbits.

Table 1

Basic physico-chemical properties of respiratory liquid (RL)

Indicators	Perfluorohexane	Perfluorodecalin
CAS	355-42-0	306-94-5
Gross formula	C6F14	C10F18
Manufacturer	LLL "PKF SpetcNefteproduct"	JSC "HaloPolimer"
Content of the main substance, %	99,9	>95
Kinematic viscosity at 25.0°C, mm^2/s	0.38	2.9
Density at 25.0°C, g/cm^3	1.67	1.95
Melting point, °C	–82.25	–10.0
Boiling point, °C	56.6	142
Oxygen solubility at 25.0°C, ml of gas/100 ml of liquid	62	51
Solubility of carbon dioxide at 25.0°C, ml of gas/100 ml of liquid	241	232

After switching to the LR, as evidenced by deep breathing movements, visually determined by trickles of liquid with a refractive index change due to heating in the lungs coming out of the nose and mouth, we removed the animal from fixation (30 seconds after immersion in RF). The moment of removal from focus was the beginning of the countdown of the passage of the maze.

When stopped for more than 10 seconds, researchers stimulated the animal by touching a steel stud through the perforations of the plates. If the animal did not respond to stimulation, scientists considered that the animal failed, and they stopped the experiment.

A group of researchers assessed the condition of conditioned reflex activity by the number of animals in the group who successfully passed the maze and the average time of its passage. After completion of testing, we repeatedly measured rectal temperature in animals.

In the case of the animals' non-fulfillment of the experimental program, we conducted an additional study to determine the reasons that led to such a result. Considering the fact that with an independent liquid respiration (LR), lung damage may develop, we euthanized the animals after removing them from the stand and carried out computed tomography and pathoanatomic examination.

Researchers carried out the study on a Hi-Speed CT/e spiral computed tomograph of General Electric (USA) in compliance with the norms, rules, and hygienic standards in the field of radiation safety (according to SanPiN 2.6.1.1192-03). We conducted scanning in a spiral mode with a slice thickness of 2 mm and a Pitch of 1.8 in the pulmonary and soft-tissue methods. We evaluated the structure of the organs of the thoracic cavity of animals and the filling of the lungs of animals respiratory fluid (RF) by obtaining cross-sectional X-ray images with the construction of multiplanar

Table 2

Division of animals into groups depending on the conditions of liquid respiration during testing

Animal group No.	Respiratory fluid	Characteristics of respiratory fluid			
		O_2 saturation, vol%	Temperature, °C		
			22.0	27.0	32.0
I.	Perfluorohexane	90	+	–	–
II.			–	+	–
III.			–	–	+
IV.	Perfluorodecalin		+	–	–
V.			–	+	–
VI.				–	+

reconstructions using the Maxaon postprocessing image processing program.

Researchers observed the tested animals for seven days after the LR and assessed the animals' overall condition in dynamics and survival.

The Ethics Committee of Izmerov Research Institute of Occupational Health approved the protocol of the study.

We have performed statistical processing of the results using the Statistica 6.0 software package for Windows. Researchers carried out the calculation of the average values of the recorded indicators by generally accepted statistical methods. Scientists used a normal distribution of indicators in experimental groups and equality of variances parametric statistics methods (comparing hands using the Student's t-test for related and unrelated samples). To compare the average values and establish the statistical significance of the differences with the control in the absence of signs of normal distribution, we performed statistical processing using nonparametric tests (Wilcoxon for related and Mann–Whitney for unrelated samples).

Results. According to the results of the first stage, we trained the animals by developing a conditioned reflex of active avoidance of drowning in conditions of breathing air. Researchers formed experimental groups. We represent the average time of passage of the maze by animals in groups in ***figure 2***.

During the second stage of the study, we found that in the groups breathing PFH, all animals successfully passed the maze regardless of the temperature RF ***(Table 3)***. However, when living PFD, the best result we obtained in the fifth group at a temperature of RF 27.0°C, in which 100% of the animals passed the test. In turn, 20% of animals

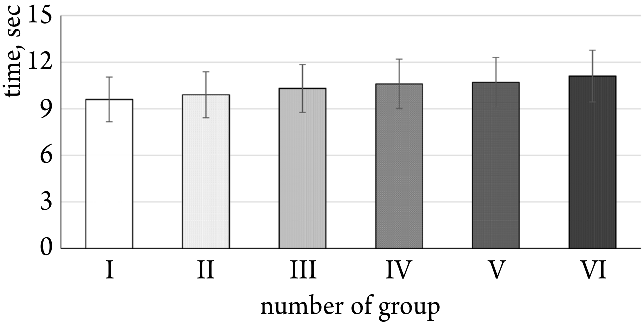


Fig. 2. The average time of passage of the maze by animals in groups.

Table 3

Maze trial pass time results for each group of animals at liquid respiration

Animal group No.	Number of animals that have passed the maze	Average time of passage of the maze, sec.	Температура ректальная, °C		Body temperature difference before and after testing, °C
			before testing	after testing	
I.	10	323±94	32,9±0,2	25.8±1,0	7.1
II.	10	45±12*		29.0±0,2	3.9
III.	10	147±101		32.1±0,2	0.8
IV.	0	—		—	—
V.	10#	131±79		27.7±0,5	5.2
VI.	2	38		32.5	0.4

Note: * — the difference with group I is significant ($p<0.05$); # — the difference with group II is significant ($p<0.05$).

passed the test in group VI, and in IV, this indicator was equal to 0. When the animals failed to pass the maze, they first stopped, but when stimulated, they continued to move.

However, after a series of stimulations, the animals refused to continue performing the test. At the same time, they continued to respond to stimulus, trying to dodge the touch of the hairpin. Thus, there were no cases of respiratory arrest during the experiment.

In the groups in which the LR we conducted using PFH, the shortest passage time of the maze was at a temperature of RF 27.0°C (group II).

It was 7.18 and 3.27 times less than in groups I and III, respectively. When using PFD, the shortest time was in group VI with a temperature of 32.0°C, which was 3.45 times less than in group V.

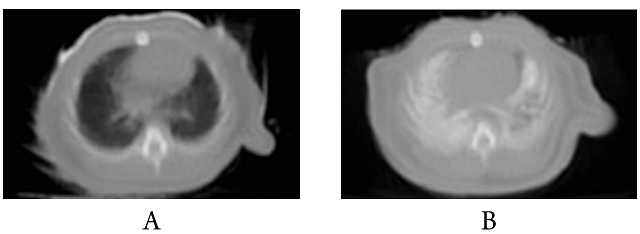
In all groups, we watched a significant increase in the passage time of the last levels of the maze compared with the first. In group III, with a PFH temperature of 32.0°C, the animals either passed the maze quickly or slowly. In turn, in group VI, animals with a PFD temperature of 32.0°C animals either passed the maze soon or did not pass it at all.

In all groups, after passing the maze, we observed a decrease in rectal body temperature, and the degree of reduction was proportional to the body temperature and the duration of testing.

When studying computed tomograms of the chest, the researchers found that the lung parenchyma was almost filled with respiratory fluid in all animals that failed the test ***(Figure 3)***.

During a macroscopic examination of the lungs in all animals that did not pass the test, the lungs were full-blooded; we observed venous stagnation and isolated areas of preservation of airiness. There are no cases of perfluorocarbonothorax.

When assessing the outcomes of the LR, we found that when using PFH, the death of laboratory animals



A B

Fig. 3. Animal lungs computed tomography scan (A — normal reference; B — after liquid respiration).

was 60–70%. While no adverse effects in the PFD groups ***(Table 4)***.

Researchers recorded the death of animals after the LR PFG either early (during the first hour after testing) or late (at the end of the first — beginning of the second day).

There were no significant differences in the structure of adverse outcomes depending on the temperature regime of the LR PFH.

The death of animals, both in the early and late periods after the LR, was due to one reason — the development of acute respiratory and cardiovascular insufficiency.

We observed the phenomena of acute respiratory failure in animals that died in the early stages after switching to gas respiration, rapidly progressed causing an unfavorable outcome within the first hour after the LR. At a late date, death developed against the background of rapidly progressive respiratory failure that occurred after a long latent period at the end of the first — beginning of the second day after the LR.

Discussion. In order to carry out this study, it was necessary to create equipment that allows the study of conditioned reflex activity on small laboratory animals with independent liquid respiration in conditions of immersion of respiratory fluid, and the choice of the conditioned reflex itself. However, based on the analysis of existing approaches to the assessment of conditioned reflex activity, the conditioned reflex of active avoidance we have chosen for this study [10]. To do this, in a gaseous environment, we have developed a conditioned reflex of active avoidance of drowning, which, when drowning in respiratory fluid, encourages animals to pass a maze to complete the test (to eliminate the threat).

Table 4

Survival rates of animals after liquid respiration (M±m)

Animal group No.	Number of dead animals		Total number of dead animals	Average time of death	
	on the 1st day	after the 1st day		on the 1st day, minutes	after the 1st day, hours
I.	4	3	7	51±10	22±2
II.	3	4	7	47±8	26±3
III.	4	2	6	49±12	23±3
IV.	0	0	0	0	0
V.	0	0	0	0	0
VI.	0	0	0	0	0

The analysis of the obtained results indicates that the physicochemical properties of the selected RF had a significant impact on the test results.

Considering the latter, the use of PFH was preferable to PFD since, on the one hand, it has a lower viscosity (7.63 times) and density (1.17 times), and on the other, more excellent solubility for oxygen and carbon dioxide. Based on these differences, we assumed that the reason for the better test results in the groups receiving PFH is both a decrease in energy consumption for the LR process itself and a more optimal gas exchange in the lungs, which leads to less pronounced hypoxia.

There is a significant influence of the temperature of the respiratory fluid (RF) on the process of independent liquid respiration (LR) during immersion in the work of J.A. Kylstra and co-authors (1962) [1]. Thus, in the temperature range of 6.0–41.0°C, the survival time of mice in LR conditions varied from 11 to 247 minutes. At the same time, we observe the maximum survival duration at a respiratory fluid temperature of 20°C. A temperature deviation of 1.0–2.0°C leads to a decrease in the latter by 2–3 times. The maximum duration of survival we observed at a temperature of 20.0°C, and a temperature deviation of 1.0–2.0°C led to a decrease in the latter by 2–3 times. We can explain that hypothermia leads to a slowdown in metabolic processes in the body. At a temperature of 20 degrees, there is a speed at which energy consumption is at a minimum level sufficient to maintain life at rest.

In the study, the best testing time we recorded in group II when breathing PFH at a temperature of 27.0°C. An increase in the optimal temperature of RF by 7.0 °C compared to the results obtained by J.A. Kylstra and co-authors (1962) [1] is due to different experimental conditions and registered indicators. The metabolic rate at a temperature of 20.0°C was sufficient for long-term maintenance of life at rest but not optimal for performing heavy physical work. This assumption supported the fact that in the study at 22.0°C, the animals became sluggish and refused to move around the maze while they continued to breathe liquid. In turn, at a temperature of RF 32.0°C, the metabolic rate, on the contrary, turned out to be too high. At the same time, the LR process itself turned out to be the most important, as the key to ensuring vital activity. We use the remaining energy for motor activity. However, this did not apply to animals that quickly passed through the maze, as it was in group VI.

As can be seen from the presented data, we do not link the refusal to pass the test either with an incomplete filling of the lungs by RF or with the development of hydrothorax (perfluorocarbonthorax) in animals.

In turn, the death of animals both in early and late periods during the respiration of PFH associated with the peculiarities of the physicochemical properties of the latter.

Conclusions:

1. The researchers used the developed experimental stand to study the effect of various types of respiratory fluid on the condition of the conditioned reflex activity of small laboratory animals, including in combination with various physical factors.

2. The proposed approach of developing a conditioned reflex of active avoidance in a gaseous environment with subsequent testing in immersion in RF you can use to assess the state of conditioned reflex activity in small laboratory animals with independent LR.

3. Conditioned reflex activity during spontaneous LR in small laboratory animals in normobaric conditions persists.

4. Its physico-chemical properties, as well as the temperature of the latter, have a significant impact on the state of conditioned reflex activity during independent liquid respiration (LR) in immersion in the respiratory fluid (RF).

5. The survival rate of animals after LR directly depends on the physicochemical properties of RF. So, if PFD does not cause severe respiratory failure in Syrian hamsters, leading to adverse outcomes after the LR, then the use of PFH leads to the death of animals in 66.7% of cases. The death of animals when using PFH can occur both in the early stages during the first hour after the LR and in the late at the end of the first or beginning of the second day (after the latent period).

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