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A pharmacological composition for induction of a reversible torpor-like state and hypothermia in rats

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ABSTRACT

Aims: To initiate a state of artificial torpor we suggested a pharmacological multi-targeting strategy for simulation of the physiological pattern of natural hibernation including a significant reduction in heart rate, respiratory rate, body temperature and oxygen consumption as well as a decline in brain activity known as torpor.
Materials and methods: We have developed a composition which initiates a pharmacologically induced torpor-like state (PITS-composition), made up of eight therapeutic agents, inert gas xenon and lipid emulsion served as a drug vehicle.
Key findings: After a single intravenous injection to rats, PITS-composition causes a rapid decline in heart rate followed by a steady decrease in body temperature from about 38.5 °C to 31.5 °C, at ambient temperature of 22 °C–23 °C. The hypothermic state may continue on average for 16–17 h with the subsequent spontaneous return of heart rate and body temperature to the initial values. In the open field test at torpor the motility, rearing and grooming were suppressed but 4–8 days later they were restored.
Significance: Suspended animation states, including natural hibernation or pharmacologically induced synthetic torpor are of special attention of medicine, since it may improve survival rate after cardiac arrest, brain hemorrhage and ischemia, and during long-term space traveling. The suggested here multi-targeting strategy made possible to develop the pharmacological composition able, after a single intravenous injection, to initiate long, stable and reversible hypothermia and torpor at room temperature. After the torpor, animals were able to spontaneously restore both physiological parameters, and behavioral reactions.

1. Introduction

The torpor state appeared in heterothermic animals during hibernation is characterized by a reduction in metabolic rate, heart rate, body temperature, and rate of blood flow [1], leading to a considerable decrease in the oxygen supply of tissues including the brain. However, animals do not experience hypoxia because the oxygen demand of tissues is low. The blood oxygenation also remains high because of high oxygen affinity of the blood at hibernation [2]. Besides, as a result of the decrease of heat production, the body temperature drops, and during winter in small mammals it may reach 0 °C while the rate of metabolism may slow down to > 90%. On the contrary, the body temperature of large animals like bears decreases only to 30 °C–35 °C while the metabolic rate reaches 20–50% of the initial level [3].

In the course of evolution hibernating mammals acquired elevated resistance to the damaging factors. So, bears show an unprecedented ability to endure brain ischemia that occurs during hibernation [4]. Despite the harsh conditions of wintering, prolonged immobility, reduced functioning of the excretory system, the hibernating bears successfully resist heart and kidney diseases, atherosclerosis, thrombosis, muscular dystrophy, and osteoporosis [3].

Clinical studies also revealed that mild hypothermia may significantly increase the human endurance to damage. The therapeutic efficacy of hypothermia was confirmed by numerous clinical studies [5]. Hypothermia has been widely and successfully used at cardiac arrest or brain ischemia [6,7]. According to ILCOR (International Liaison Committee on Resuscitation) the largest therapeutic potential is expected at 32 °C–36 °C [8]. However, the modern devices developed

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for the physical cooling of a body [9] are not always sufficiently effective, can damage the cardiovascular system, evoke blood electrolyte imbalance, as well as some diseases associated with infection [10,11]. The pharmacologically induced torpor-like state (PITS) can be an effective alternative or extension to physical cooling [12].

In hibernating animals hypothermia is inextricably linked with the state of metabolic depression, called torpor. A similar state such as PITS or synthetic torpor in non-hibernating animals has a great potential of being applied in medicine and implies the establishment of a safe and reversible hypometabolic state that increases resistance to damage and stress [13]. In addition, synthetic hypothermia opens new prospects for the long-lasting space travel to other planets, since it increases the organism resistance to radiation and saves the resources of space ship [13–15].

In the presented study we developed a PITS-composition which affects both central and peripheral systems of metabolic regulation and initiates a torpor-like state in rats, accompanied with spontaneous, prolonged and reversible hypothermia.

2. Materials and methods

Wistar rats (male, age 2–4 months, weight 200–250 g) were delivered from Stolbovaya Breeding Farm (Russian Federation) and kept in standard conditions. Animal care was performed according to the guidelines established by the European Council Directive 2010/63/EU and in accordance with the "Regulations for Studies with Experimental Animals" (Decree of the Russian Ministry of Health of 12 August 1997, No. 755). The protocol was approved by the Commission on Biological Safety and Ethics of the Institute of Cell Biophysics, Russian Academy of Science. The light cycle was 12 h light and 12 h dark. The air temperature was maintained within 21 °C–22 °C. A standard diet and water were provided *ad libitum*. All animals were delivered in the experimental room in no less than two hours before the beginning of the experiment.

2.1. Preparation of PITS-composition

To prepare PITS-composition we used the following substances obtained from Sigma-Aldrich and PMBio: 1. Propranolol hydrochloride, beta-blocker with adrenolytic effect, 5 mg/kg of animal weight; 2. Ivabradine hydrochloride, selective antianginal compound with antiischemic effect, 5 mg/kg of animal weight; 3. Diphenhydramine hydrochloride, H1 histamine receptor antagonist with antiemetic properties, 5 mg/kg of animal weight; 4. Magnesium sulfate, nonspecific inhibitor of NMDA-receptors, 30 mg/kg of animal weight; 5. Propylthiouracil, antithyroid compound, 5 mg/kg of animal weight; 6. Serotonin hydrochloride, serotoninergic compound, 5 mg/kg of animal weight; 7. Reserpine, sympatholytic compound with adrenolytic effect, 1 mg/kg of animal weight; 8. Periciazine, neuroleptic with hypothermic action, 4 mg/kg of animal weight; 9. Lipofundin® MCT/LCT 20%, fat emulsion, 2.0 ml/kg of animal weight. 10. The emulsion was saturated with inert gas, xenon. For the saturation, the vessel was 1/3 filled with PITS-composition. The free volume was filled with xenon under excessive pressure of 0.2 atm and shaking for 30 min at room temperature. Xenon was then replaced by a fresh portion and the procedure was repeated 3 times. The xenon content was measured by mass spectrometry (CH7 Varian, Germany) as the sum of the main peaks of the xenon isotopes. For xenon calibration, the argon content, 0.934%, in the air was used as a standard. The sensitivity of the method was 0.001% by volume. The error was ± 3%. It has been found that the final concentration of xenon was 0.54 ± 0.09 ml/ml (gas/liquid).

2.2. The procedure of injection in rats

The experiments were conducted on rats catheterized in the jugular vein. Animals were able to freely move in a system with swivels (DiLab 100,014, USA). Jugular catheterization was performed as it was suggested elsewhere [16]. One day before the experiment, 100 μl of heparin (50 IU/ml) was injected into the catheter. 24 h after catheterization, animals with smooth catheter throughput, absence of clotting, and with no signs of postoperative complications, such as fiver, lethargy, refusal of food, ptosis, were selected for the experiment. 1 ml of pharmaceutical composition or vehicle (physiological solution) was injected into the catheter in accordance with protocol of the experiment. After injection of drugs, the catheter was filled with 100 μl of heparin (50 IU/ml) to flush the system and prevent clotting in the catheter.

2.3. Measurement of physiological parameters in rats

Blood oxygenation and heart rate were measured with MouseOx (Starr Life Sciences, USA). Core temperature was measured by rectal probe RET-2 (Physitemp, USA, ± 0,1°C). The obtained curves were analyzed with build-in sigmoidal biphasic function suggested by Origin Pro (OriginLab Corporation, USA):

$$x = T_{min} + \frac{(T_{max\,1} - T_{min})}{1 + 10^{((x - x_{0_1}) * h_1)}} + \frac{(T_{max\,2} - T_{min})}{1 + 10^{((x_{0_2} - x) * h_2)}} \quad (1)$$

where:

T_{max1} is the temperature of the first top asymptote.
T_{max2} is temperature of the second top asymptote.
T_{min} is temperature of the bottom asymptote.
x_{0_1} is the first median.
x_{0_2} is the second median.
h_1 is the first slope.
h_2 is the second slope.

2.4. Measurement of oxygen consumption

The measurement of oxygen consumption was performed with a MM-100 metabolic monitor system (CWE Inc., USA). Animals were housed in the chamber of 7.5 l. The rate of air flow was 1500 ml/min. The monitoring was carried out at room temperature in the specified time intervals including 3 min for the measurement.

2.5. ^{1}H NMR spectroscopy

^{1}H NMR spectra were obtained with the NMR Bruker AVANCE III 600 MHz spectrometer (Germany) with Z gradient. The sample temperature was 294 K. The studied concentrations of compounds were similar to those applied in the experiments on animals.

2.6. Assessment of behavioral responses in the open field test

The animals were placed for 30 min at room temperature in the specially designed gray plastic cubes 40 × 40 × 40 cm (RPC Open Science, Ltd., Russian Federation). The area of the field was 0.16 m^2, illumination – 200 lx. Animal behavior was recorded with the GigE Vision digital video camera. The animal activities were analyzed using EthoVision XT, Ver. 11.0 software (Noldus Information Technology, the Netherlands), as well as RealTimer, Ver. 1.21 (RPC Open Science, Ltd., Russian Federation).

2.7. Measurement of biogenic amines in the brain

For measuring biogenic amines, animals were decapitated. After decapitation, the brain was isolated, placed in a small volume of physiological solution (0.87% NaCl, pH 7.4) and kept on ice. The brain samples were homogenized in phosphate-buffered saline (PBS, pH 7.4; 100 mg/ml), and the obtained cell suspension was subjected to two cycles of freeze-thawing. The sample tubes were then centrifuged for 5 min at 300*g*, 4 °C, and the cell-free supernatants were harvested and immediately used for analysis.

For the measurements, the following ELISA kits were used: Rat Melatonin (MT) ELISA Kit; Rat 5-Hydroxytryptamine, (5HT) ELISA Kit; Rat Dopamine (DA) ELISA Kit; Rat Noradrenaline/Norepinephrine (NA/NE) ELISA Kit. All kits were from CUSABIO, China. The subsequent manipulations were carried out in accordance with the analysis protocols that were included in the kits. The optical density was measured at 450 nm with a Titertek Multiscan MCC/340 plate spectrophotometer (Flow Laboratories, Finland).

2.8. Statistical analysis

Statistical analyses was performed using GraphPad Prism 7 software (GraphPad Software Inc., USA), two tailed, nonparametric, unpaired, Mann-Whitney *U* test was used to compare independent measurements. Nonparametric Wilcoxon signed-rank test was applied for series of dependent measurements. Differences were considered significant if $*p < 0.05$, $**p < 0.01$. Values were presented as mean ± SEM.

3. Results

3.1. Initiation of hypothermia

Normally, the body temperature of rats was close to 38 °C. After injection of PITS-composition, a long-term decline in both heart rate and body temperature was observed (Fig. 1).

Sigmoidal biphasic function was fitted to body temperature (Fig. 1B) or heart rate data to determine parameters presented in Table 1. It has been found that after injection of PITS-composition the heart rate decreased rapidly, thereby the median of the heart rate

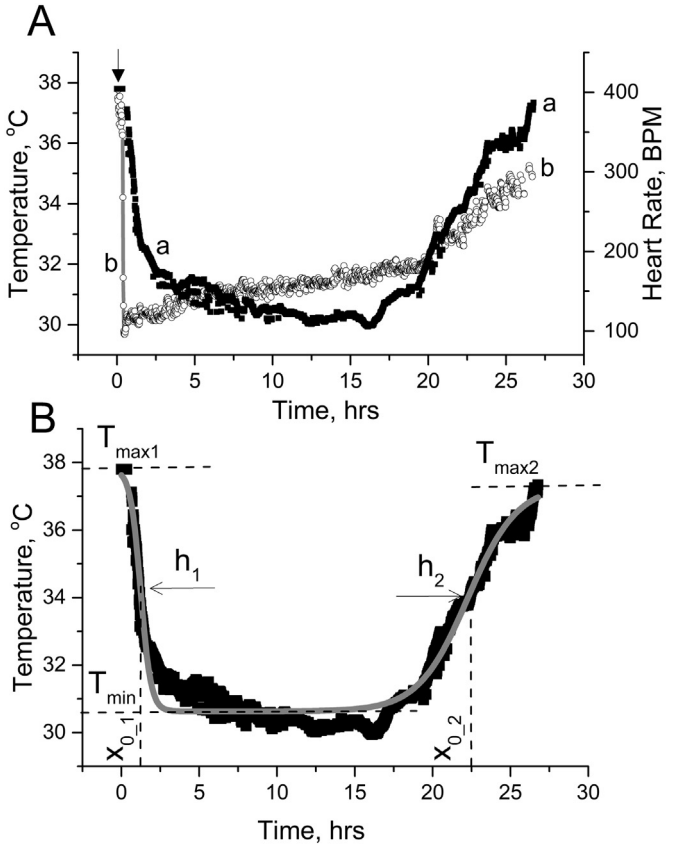

Fig. 1. A – Body temperature (a) and heart rate, beats per minute, BPM (b) in rats after injection of PITS-composition (PC8 + Lip + Xe). The injection implemented at zero time point is indicated by an arrow. B – Sigmoidal biphasic function (gray), according to Eq. (1) (Section 2.3), fits to the body temperature data (black). Throughout the experiment, animals were kept at 21 °C–22 °C.

Table 1
Changes in temperature and heat rate parameters obtained using data fitting technique, as it is shown in Fig. 1B.

Abbreviations	Definitions	Values	Units
Temperature curves			
T_{min}	Bottom asymptote	31.42 ± 1.08	°C
T_{max1}	First top asymptote	38.24 ± 0.34	°C
$(T_{max1}) - (T_{min})$	Fall of temperature	6.82	°C
T_{max2}	Second top asymptote	37.79 ± 0.38	°C
x_{0_1}	First median	1.04 ± 0.31	h
x_{0_2}	Second median	17.80 ± 3.60	h
$(x_{0\_2}) - (x_{0\_1})$	Time of hypothermia	16.76	h
h_1	First slope	1.30 ± 0.21	–
h_2	Second slope	0.41 ± 0.13	–
R^2	COD[a]	0.94 ± 0.017	–
Heart rate curves (the initial part)			
T_{min}	bottom asymptote	126.73 ± 33.22	bpm
T_{max1}	first top asymptote	401.74 ± 36.71	bpm
h_1	first slope	22.43 ± 13.53	–
R^2	COD[a]	0.97 ± 0.04	–

Note:
[a] COD – the coefficient of determination.

reduction was achieved for less than two minutes (x_{0_1} = 0.03 h or 1.8 min). Reduction in the heart rate caused a slow decrease in body temperature from 38 °C to 31 °C which corresponded to the temperature fall of about 7 °C. The median of temperature decrease was achieved in about one hour (x_{0_1} = 1.04 h). A significant difference in the decrease of heat rate and temperature is evidenced by the 15-fold greater of the first slope of heat rate (h_1 = 22.43) compared to the first slope of temperature (h_1 = 1.30). After a sharp decline, the heart rate began to recover slowly, while the body temperature remained low. The total time of cooling, as estimated by the time interval between the medians, was $[(x_{0\_2}) - (x_{0\_1})] \approx 17$ h. Recovery of the initial body temperature took much more time than the time of temperature decrease, since the h_1 coefficient (h_1 = 1.30 ± 0.21) was 3-fold as much compared to the h_2 coefficient (h_2 = 0.41 ± 0.13) As mentioned above, the temperature growth was preceded by a slow rise of heart rate, but at the moment of temperature recovery to the initial values ($A_{max2} \approx 37.8$°C), the recovery of heart rate was not completed.

The PITS-composition was obtained by careful selection of components. The absence of any compound in the composition led to a significant decrease in its efficacy, as evidenced by a decline of surface under curve (SUV) of temperature changes (Fig. 2). The greatest effect was observed in experiments without neuroleptic periciazine. Probably, it could be explained by the fact that in the absence of this neuroleptic the animals retained their motility and were able to actively resist cooling and thus did not enter the torpor-like state.

The injection of PITS-composition initiated not only a decrease in body temperature, and but also oxygen consumption (Fig. 3), which indicated the reduction of energy production by the organism. It is noteworthy that changes of blood oxygenation were insignificant. Within the first hour after the injection, there was a slight fall of the blood oxygenation level, but later it not only returned to normal, but often exceeded the original level by 1% to 3%. This may indicate that in spite of the significant decrease in the heart rate the animals did not experience hypoxia because at the decreased body temperature tissues consumed less oxygen.

3.2. Drug interactions with particles of Lipofundin. The NMR study

As it was mentioned above, PITS-composition includes pharmacological substances (PC8) and fatty emulsion Lipofundin, which contains spherical drops of soy oil, of about 300 nm in diameter, stabilized with soy lecithin as an emulsifier. Many of the medications used in our study are characterized by high lipophilicity, since the value LogP > 1

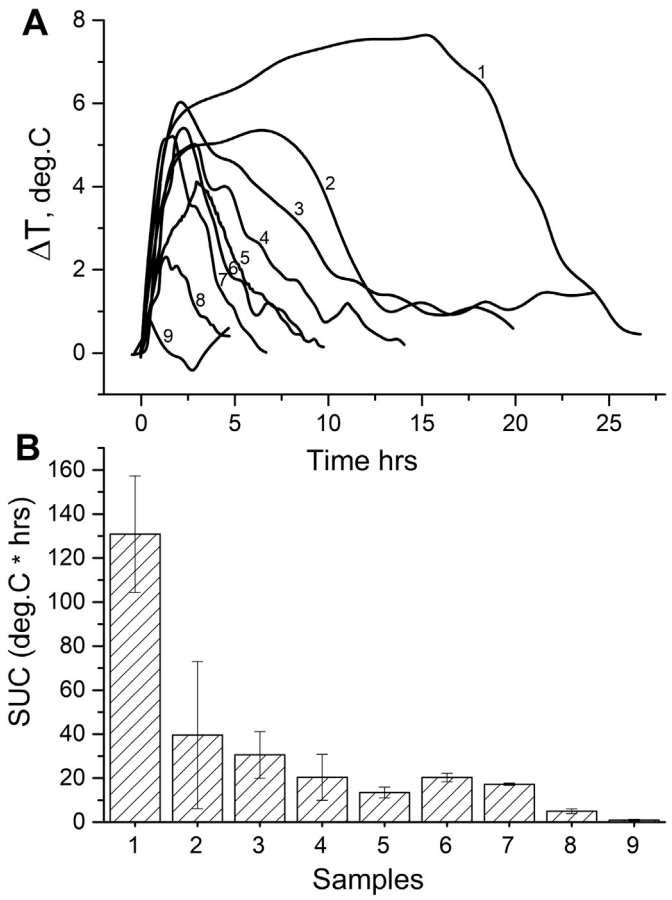

Fig. 2. A – Changes of body temperature after injection of the full PITS-composition (1), as well as of PITS-composition minus one of the following compounds: propranolol (2), diphenhydramine (3), Lipofundin saturated with xenon (4), magnesium (5), serotonin (6), reserpine (7), propylthiouracil (7), periciazine (8). Curves are presented after the mathematical transformation: $\Delta T = T_{(t)_*}(-1) + T_{max1}$. The injection was carried out at zero time. B – Surface under curve (SUC) of body temperature. Data are represented as Mean ± S.E.M., $n = 3$–5. Samples 2–9 are significantly different from sample 1 according to Mann-Whitney nonparametric U test at $^*p < 0.05$. Experiments were conducted under conditions identical to those presented in Fig. 1.

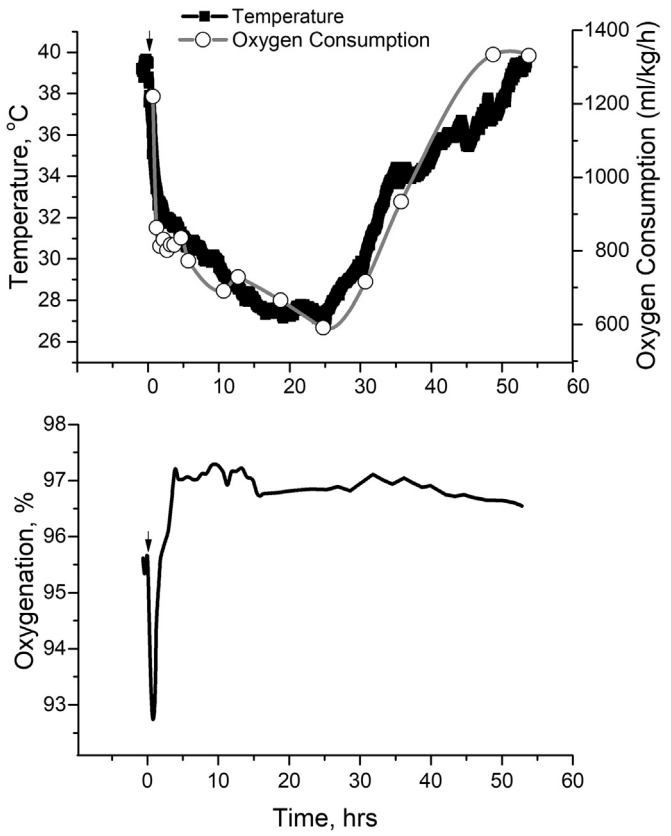

Fig. 3. Changes in the body temperature, oxygen consumption (top panel) and the level of blood oxygenation (bottom panel) after injection of PITS-composition. The injection was performed at zero time (indicated by arrows). Experiments were conducted under conditions identical to those presented in Fig. 1.

Table 2
Lipophilicity and the chemical shift in the presence of Lipofundin determined with ^{1}H NMR for compounds of PC8 (magnesium is not presented).

Pharm. compounds	CLogP	ExpLogP	Δ(δ), ppm
Serotonin	0.755999	0.21**	0.008
Diphenhydramine	2.799999	–	0.011
Propylthiouracil	0.971000	0.76**	0.030
Ivabradine	3.265999	–	−0.037
Propranolol	2.753395	3.3*	0.076
Periciazine	3.435500	3.52**	–
Reserpine	4.547334	3.2*	–

Note: Calculated data of lipophilicity (logarithm of the compound distribution in the octanol-water system) obtained with ChemBio3DUltra14.0 (CLogP). Experimental values of lipophilicity presented by *DrugBank or **Chemistry Dashboard (ExpLogP). The maximal chemical shifts in the aromatic parts of the corresponding ^{1}H-NMR spectra (Δ(δ), ppm).

(Table 2), which suggests the ability of these compounds to preferably dissolve in the lipid particles of the emulsion. To confirm the interaction between pharmaceuticals and particles of the emulsion we used ^{1}H NMR.

It is known that the location of guest molecules in lipid membranes can be determined by chemical shift changes in the ^{1}H NMR spectra [17–19]. The magnitude of the change in the chemical shift of each signal of the drug depends on the depth of immersion of the corresponding part of the molecule in the lipid bilayer. Thus, comparing the maximum values of changes in the chemical shifts of the signals of each of the drugs presented in Table 2, one can estimate their degree of affinity to the emulsion particles.

Features of the emulsion effect on ^{1}H NMR spectra of pharmaceutical preparations are shown by the example of diphenhydramine (Fig. 4). Here we present aromatic parts of spectra in which it is easier to analyze spectral differences. In the presence of Lipofundin, some diphenhydramine signals in the aromatic part of the spectrum (7.30–7.45 ppm) shifted towards the higher field. The change in the value of the chemical shift shows the nature of the binding of diphenhydramine with emulsion particles (Fig. 4B). The chemical shift of proton signals at position 1 has not changed, indicating its presence in the aqueous phase, but not in the hydrophobic part of the emulsion. The proton signal at position 2 moves to a high field at 0.006 ppm, suggesting the interaction of this fragment with the aliphatic tails of lipid molecules of emulsion particles. The proton signal at position 2 moves to a high field at 0.006 ppm, showing the interaction of this fragment with the aliphatic tails of lipid molecules of emulsion particles. The greatest displacement to the high field (0.011 ppm) is observed for protons at position 3. This part of the molecule penetrates most deeply into the hydrophobic region of the emulsion particles and is present there for longer time. Therefore, it can be concluded that the binding of diphenhydramine molecules occurs in a hydrophobic layer near to the surface of Lipofundin particles.

Similarly, we analyzed changes in the chemical shifts of ^{1}H NMR signals of all PC8 compounds except for magnesia.

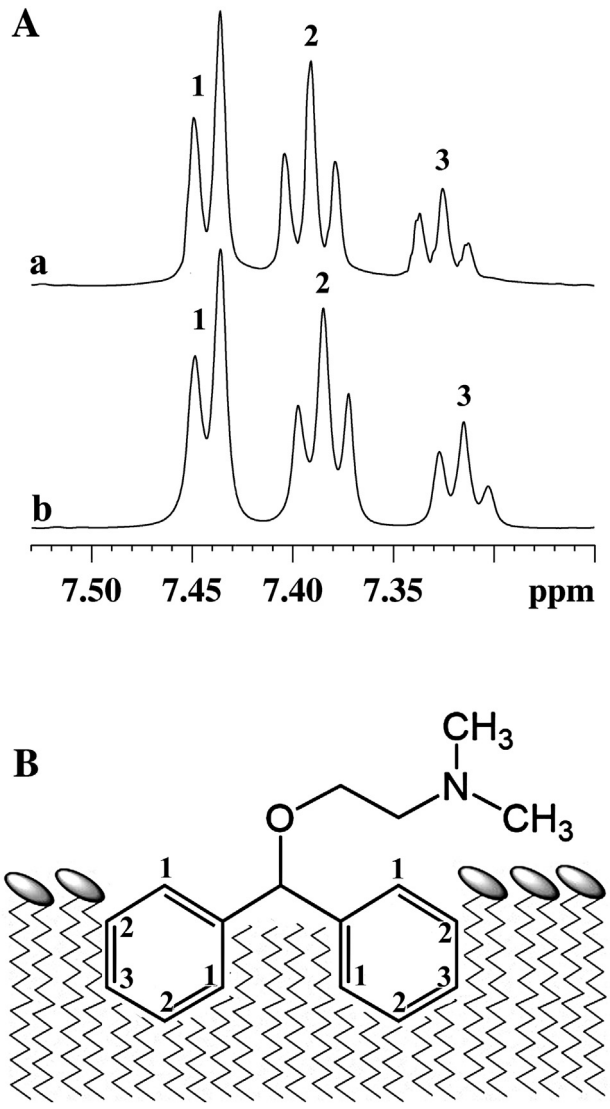

Fig. 4. A – The aromatic portion of ^{1}H NMR spectrum of diphenhydramine in the aqueous environment (a), and after addition of Lipofundin (b). The final concentration of Lipofundin was 10%. B –Interaction of diphenhydramine with lecithin shell of Lipofundin particles. The numerical assignment of ^{1}H NMR spectra of diphenhydramine to the moieties of diphenhydramine molecule was obtained from the database https://www.drugbank.ca

According to the ^{1}H-NMR spectroscopy (Table 2):

- Serotonin molecules have the lowest affinity for the lipophilic part of Lipofundin particles.
- Molecules of propylthiouracil are localized predominantly in the lipophilic part of the emulsion particles.
- α-Naphthol radical of propranolol is characterized by the greater affinity to the hydrophobic portion of Lipofundin particles.
- Periciazine affinity for the emulsion particles can be estimated only indirectly. In the presence of Lipofundin, the ^{1}H NMR signals of periciazine are broadened, so that they cannot be registered. This indicates the penetration of the molecule into the hydrophobic core of the emulsion particles and strong binding to the lipid inside.
- The negative shift of the ivabradine signal implies the interaction of the drug with the hydrophilic surface of the Lipofundin particles, while the absolute value of the shift supposes the strong binding of ivabradine to emulsion particles.
- As to interaction of reserpine with the emulsion particles it is impossible to make any conclusion based on ^{1}H-NMR spectroscopy data because in the aqueous environment reserpine produced molecular associates and the proton signals of this compound were highly broadened and not available for registration.

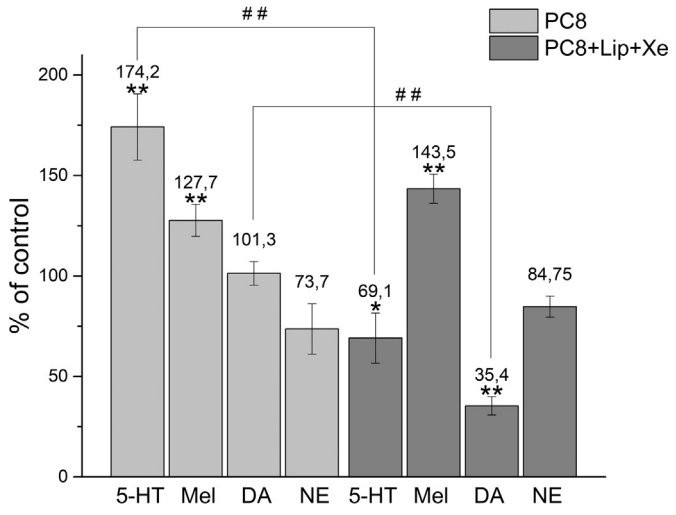

Fig. 5. Changes in biogenic amines levels in the brain observed 2 h after the injection of PC8 or PITS-composition (PC8 + Lip + Xe). The abbreviations used: serotonin (5-HT), melatonin (Mel), dopamine (DA), norepinephrine (NE). The levels of the compounds in the brain of intact animals are taken as 100%. Data are presented as Mean ± S.E.M. Asterisks above the individual data sets indicate the levels of statistical significance compared to the control group of animals: $*p < 0.05$, $**p < 0.01$. Number signs above the square brackets mean the significance of statistical comparison between two individual data sets: $\#p < 0.05$, $\#\#p < 0.01$. Statistical analyses were performed with Mann-Whitney *U* test.

3.3. Biogenic amines in the brain

It was found (Fig. 5) that 2 h after the intravenous injection of PC8 a significant increase of melatonin and serotonin (5-HT) was observed in the brain, whereas injection of PITS-composition (PC8 + Lip + Xe) decreased the levels of serotonin and dopamine, although there was a considerable rise in the level of melatonin in both cases. Changes of norepinephrine level were statistically unreliable.

It is known that reserpine, which is part of the injected PITS-composition, can cause a drop in the level of dopamine and serotonin, that leads to motor disorders resembling Parkinson's disease [20]. From the presented data it can be concluded that in PC8 the reserpine action is limited, while in PITS-composition (PC8 + Lip + Xe) the lipid emulsion saturated with Xe facilitates manifestation of reserpine activity. The most remarkable finding is that the level of melatonin increases. It was especially evident in case of injection of PITS-composition, so the decreased serotonin level can be explained by possible partial transformation of serotonin in melatonin [21].

3.4. Behavioral reactions in the open field test

The above changes in biogenic amine levels may lead to a depressive state in animals and have a significant impact on brain functioning. Therefore, the study of dynamics of behavior recovery is very important. The motor activity, as locomotion speed and distance, was assessed in the open field test. Reduction in the motor activity was observed after injection of PITS compositions, and was the most pronounced in the period of maximal cooling (Fig. 6). Two days after injection, the parameters of animal motility returned to the normal and did not show a statistically significant difference from the control. As to behavioral reactions, the number of animal rearing (vertical stand) was very low, this can be interpreted as a restriction of the cognitive response to novelty. The number of grooming episodes also dropped

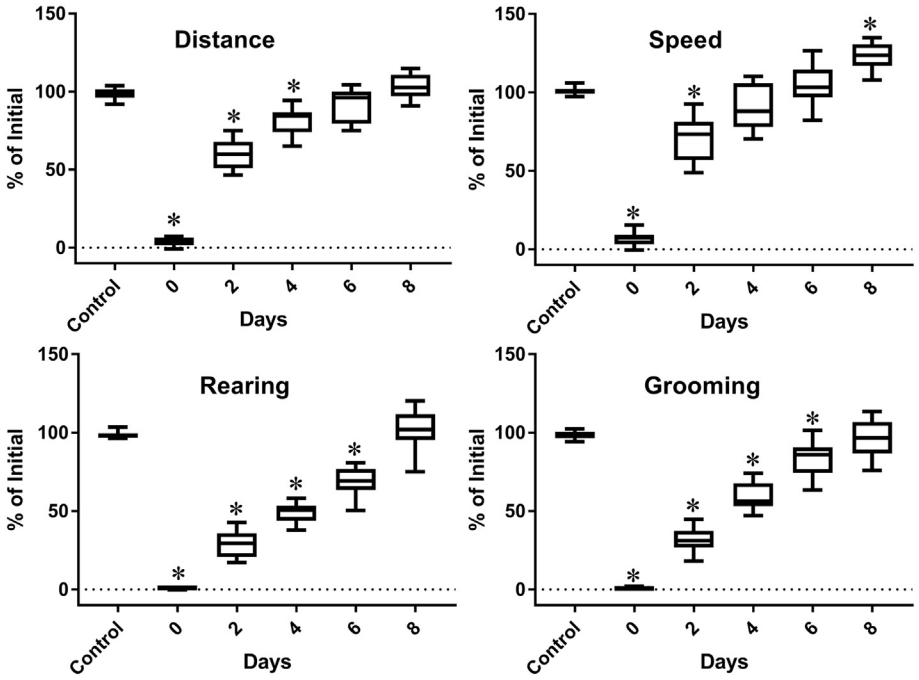

Fig. 6. Behavioral responses of rats ($n = 9$) in the open field test. We analyzed the speed and the distance of animal locomotion (upper panels), as well as the number of grooming and rearing episodes (lower panels). The test was performed before the injection of PITS-composition (Control); at maximal cooling after the injection (0); every two days (2, 4, 6, 8) after the injection. Data are presented as Mean ± SEM. According to Wilcoxon signed-rank test, asterisks indicate the level of statistical significance of differences between medians of the experimental groups in comparison with the initial level taken for 100%, $*p < 0.05$.

sharply at the time of the greatest decrease in body temperature after injection. Restoration of this parameter to the initial level that was statistically indistinguishable from the control was observed only by the eight day.

4. Discussion

The suggested PITS-composition was able to initiate in rats a torpor-like hypometabolic state similar to that observed in naturally hibernating animals. In order to induce this state we targeted different receptors with a constructed set of common medicinal compounds. An additional factor was the inert gas xenon, dissolved in lipid emulsions, known as a neuroprotective agent, effective in combination with hypothermia [22].

When creating PITS-composition, we used the known data on hibernating animals. Accordingly, we supposed to achieve the maximum reduction of metabolism and induce a state of depression in the central nervous system, which is typical of the natural hibernation. For this purpose, we selected both pharmaceuticals able to lower the rate of blood circulation in tissues (due to the lower frequency and force of cardiac contractions and decrease in blood pressure) and suppress metabolism, and compounds capable of reducing locomotor activity. In addition, we included substances supporting the organism at hypothermia. The absence of any of the suggested components significantly reduces the effect of PITS-composition on animals.

It is known that during hibernation there is a significant decrease in the heart rate [1] which may slow down the rate of blood flow and oxygen delivery to tissues, thereby reducing the metabolic rate [2]. Similar changes were observed in our experiments at the initiation of pharmacological torpor, while the blood oxygenation remained high, indicating a decrease in oxygen consumption by tissues in the state of hypometabolism.

Ivabradine as a component of PITS-composition, is selective If-channel inhibitor controlling spontaneous diastolic depolarization in the sinus node. Ivabradine has no effect on myocardial contractility, but reduces the heart rate [23,24]. For a further decrease in the blood flow rate, propranolol was used. Its influence on cardiac function is different as compared to ivabradine. In experiments on rats at weak hypothermia, propranolol is able to reduce the force and increase the refractory period of cardiac contractions [25]. This effect can be explained by the influence of propranolol on β-adrenergic receptors [26,27].

A significant contribution to the development of the torpor-like state can be made by compounds that inhibit the locomotor activity and facilitate a depressive state in animals. Out of these compounds we used periciazine, which enhances the inhibitory effect on the CNS of antihypertensive, analgesic and tranquilizing compounds. Furthermore, periciazine exhibits some hypothermic effect. This agent is pharmacologically similar to chlorpromazine, known for its ability to initiate hypothermia [28], but has more pronounced sedative, antiemetic, anticholinergic and antiserotonergic activities [29].

The sedative effect of periciazine can be amplified by the antihistamine compound diphenhydramine that blocks histamine H1-receptors in cholinergic neurons of basal ganglia, relaxes smooth muscles and provides an analgesic effect [30,31]. Furthermore, it was shown that diphenhydramine is able to exert a protective effect through prevention of excessive vasoconstriction at decreased body temperature and motor activity [32].

Reserpine, the indole alkaloid of vegetable origin, is important for development of the torpor-like state because it is able to inhibit a vesicular monoamine transporter (VMAT2). Reserpine is able to induce depression and dyskinesia. Our analysis of the biogenic amines in the brain shows a decrease in amine levels: serotonin and dopamine, which is a typical picture of reserpine action. It is known that reserpine promotes the release of intracellular amines and their degradation in synaptic space [20,33,34]. All this may reduce motor activity and initiate depressive states in animals [20,35].

The composition also includes the neurotransmitter serotonin (5-HT). Normally, serotonin is synthesized both in the brain, in the raphe nuclei, located in the reticular formation of the medulla oblongata, and on periphery by intestinal enterochromaffin cells. For a long time it was believed that these two pools: the brain serotonin and serotonin of blood, exist independently, because the blood-brain barrier (BBB) is impermeable to serotonin. Only recently there have been found on rats, that serotonin transporter SERT is involved in the exchange of serotonin between blood and brain [36]. Although the effectiveness of such exchange is unknown, it may explain ambiguous influence of serotonin on the blood pressure. Earlier, based on experiments with isolated endothelial cells, serotonin was considered as a vasoconstrictor, contributing to an increase in blood pressure [37]. However, in

experiments on rats, serotonin, after intravenous administration, exerts hypotensive effects [38]. It has been hypothesized that the hypotensive effect is a result of serotonin penetration into the brain through BBB with SERT transporter [36].

In contrast to the above mentioned, we have found that after the intravenous injection of eight pharmaceuticals (PC8) without Lipofundin and Xe, serotonin and melatonin levels in the brain were elevated. When the whole PITS-composition, containing both Lipofundin and Xe (PC8 + Lip + Xe) was injected, the level of serotonin in the brain decreased, while the level of melatonin increased. It is known that serotonin is involved in melatonin synthesis in the pineal gland [21]. We assume that part of serotonin, administered intravenously, may penetrate into the brain through BBB and during the first minutes this can enhance the hypotensive effect induced by drug formulation. However, at the moment of our testing (about 2 h after the injection) serotonin underwent transformation into melatonin. Xenon supposedly accelerates this process. It is known that melatonin is involved in regulation of circadian rhythms, as well as of seasonal changes related to hibernation [39]. The elevated melatonin level in the brain contributes both to sleep [21] and to the development of a torpor state in hibernating animals [40]. Melatonin is also effective during exit from torpor [41,42]. The elevated level of melatonin, the most pronounced in the presence of xenon, can have diverse protective effects on the brain [43], for example, it may contribute to protection of mitochondria against oxidative stress [44].

It is known that tanycytes, the glial cells of hypothalamus enriched with melatonin receptors and involved in transport and metabolism of thyroid hormones, play an important role in regulation of seasonal rhythms of hibernating animals [39]. Thyroid hormones possess the ability to activate metabolic processes and raise the body temperature [45]. Propylthiouracil, present in our pharmacological composition, contributes to reduction in the iodine level in the thyroid gland, has antithyroid action: inhibits thyroid peroxidase and participates in transformation of thyroxine to triiodothyronine. This drug is widely used in medicine, and many endocrinologists prefer to use propylthiouracil for treatment of hyperthyroidism [46–48]. In addition, it should be mentioned that hypothyroidism induced by propylthiouracil is accompanied by mild hypothermia [49–51].

Magnesium cations present in our pharmacological composition are involved in many physiological processes, regulate metabolism, have anticonvulsant, antiarrhythmic, vasodilatory, hypotensive and sedative effects [52–55]. It is known that magnesium sulfate exerts neuroprotective effects in therapeutic hypothermia [56,57]. Magnesium can be regarded as the safest antagonist of NMDA receptors possessing a neuroprotective effect at hypothermia [58]. It was shown that in conjunction with antagonists of NMDA receptor, *e.g.* ketamine [59] or ethyl alcohol [60], magnesium may contribute to the lowering of body temperature. Furthermore, the introduction of magnesium together with sedatives and analgesics is recommended to use when inducing therapeutic hypothermia to diminish the shivering reaction that can prevent the process of cooling [6].

PITS-composition also includes lipid emulsion Lipofundin® MCT/LCT saturated with xenon. The main reason for introduction of Lipofundin was its ability to dissolve significant amounts of xenon [61]. When dissolved in emulsion, xenon is able to exert an inhibitory effect on NMDA (*N*-methyl-D-aspartate) receptors of glutamate and thereby exhibit neuroprotective properties [61,62]. Xenon can also reduce the perception of pain [63]. It is known, that hypothermia facilitates the neuroprotective effect of xenon [64]. Furthermore, Lipofundin, known for its ability to reduce toxic effects of local anesthetics [65], psychotropic agents, antidepressants and beta-blockers [66], can be used as an antidote to reduce the systemic toxicity of various substances [67,68], may favor to normalization of systolic pressure [69,70].

An important aspect is interaction of drugs with Lipofundin particles, which depends on their lipophilicity. The lipophilicity, defined as a coefficient of the compound distribution between water and n-octanol, is one of the most important parameters responsible for many aspects of the drug action [71]. Lipophilicity is responsible for such key properties of drugs as ADMET: adsorption, distribution, metabolism, excretion and toxicity [72,73]. Lipid emulsions, capable of absorbing lipophilic drugs, increase their solubility and improve their pharmacodynamics and pharmacokinetics. The particles of emulsion are able to serve as a reservoir and multifunctional platform for delivery of medicinal substances in the body. Due to the slow, controlled release of drugs, their effectiveness grows, toxicity reduces, the drugs are protected from enzymatic degradation and oxidative stress [74–76]. NMR data show that not only xenon but some other compounds of PC8 can interact with the lipid particles due to the elevated lipophilicity of these compounds. The interaction with the emulsion may affect their pharmacokinetics and pharmacodynamics, though this aspect requires further investigation.

The effect of hypothermia on the rodent behavior can be analyzed in the open field test [77]. The open field test is useful for the analysis of depressive state occurred after injection of 1 mg/kg and large doses of reserpine which is present in PC8. The depression can be accompanied by ptosis, catalepsy, hypothermia [35,78] as well as a decrease in motor activity [79].

Reduced metabolism and body temperature after the injection of PITS-composition lead to immobility of animals which was determined in the open field test. Two days after the injection, when body temperature returned to the initial level, the values of the locomotion speed and covering distance reached the level statistically undistinguishable from the original. However, not all of the observed behavioral responses rapidly return to the initial state. Thus, the number of recorded episodes of grooming and rearing is similar to that before the beginning of the experiment only after a week. The observed reduction of the behavioral reactions can be associated with the depression state and low levels of dopamine in the brain. We observed also a decreased level of serotonin in the brain tissue, which may indicate imbalance in biogenic amines. It is necessary to mention the importance of grooming behavior that may characterize not only a desire of the animal to maintain hygiene, but also contributes to thermoregulation and social communication [80].

In natural hibernation research, the idea of the existence of a hibernation inducing trigger (HIT) is prevailing. Attempts to induce a torpor-like state in animals and humans are usually based on searching for HIT agents, antipsychotics, antidepressants, or sedatives, which may target the receptors of dopamine, serotonin, or GABA. In addition, the agents able to reduce the motor activity, suppress mitochondrial respiration, reduce the energy consumption of membrane pumps have been studied [15,28].

For example, encephalin DADLE can serve as a HIT [4], hydrogen sulfide together with hypoxia can initiate hypothermia and a hibernation-like state [81]. Hypothermic activity of nicotine is very significant, but the action lasts for about an hour [82]. The thyroid agent 3-Monoiodothyronamine T1AM switches the central temperature setting to lower values that leads to a controlled decrease in body temperature. However, T1AM exhibits high toxicity and low efficacy, influencing a variety of targets in the body [83]. Quite prolonged hypothermia can be achieved under the combined action of bacterial lipopolysaccharide (LPS) and the serotonin receptor agonist DOI. Within a few hours, the body temperature experienced a sharp drop by 5–6 °C and return to the original values [84]. A similar sharp and rather short-term decrease in body temperature was observed after injection of synthetic agent PD149163 [85], or non-selective inhibitor of nitric oxide synthase L-NAME [86]. Activation of adenosine A1 receptors in the central nervous system induces a state similar to hibernation in rats as a result of suppression of thermogenesis [87].

However, in all the above cases it was not possible to achieve a long (for > 10 h) and stable decrease (with a plateau) in body temperature, in particular at 22 °C. The subject concerning a hypothetical existence of a single chemical trigger capable of activating an evolutionarily

determined sequence of physiological changes leading to the development of a long and stable torpor in natural hibernation is disputable. The results of our experiments show that simultaneous targeting of different receptors can rather efficiently induce a state of artificial torpor in non-hibernating animals as a result of synergistic effect of several pharmacological substances. The advantage of the proposed multitargeting is its flexibility, namely, the possibility of altering PITS-composition for the experiments with different species of animals or for various therapeutic purposes.

In the present study, there are several limitations. In our experiments FITS lasts less than one day. Under cooling the temperature curve experiences deviations from the mean by about 1 °C. We believe that further improvement of the proposed approach should be based on the use of additional injections of the drug with careful monitoring of physiological parameters. This will not only increase the FITS time, but may reduce the temperature deviations during cooling.

In conclusion, the obtained data demonstrate that the pharmacological composition we suggest is able to simulate in rats the physiological pattern of natural hibernation. After a single intravenous injection, the composition induced metabolism depression, a long-lasting, stable, and reversible hypothermia, and torpor at room temperature. After spontaneous exit from the torpor, animals restored the heart rate, body temperature as well as behavioral reactions.

Abbreviations

HIT hibernation inducing trigger
PC8 pharmacological composition of eight compounds
PITS pharmacologically induced torpor-like state
PITS-composition consists of PC8 + Lipofindin® (commercial lipid emulsion) + xenon

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