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In Vivo Evaluation of a Polyethylene Glycol-Based Cryoprotectant during Cold Stress in a Rat Model

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ABSTRACT

Cold stress is an environmental factor that impacts the viability of animals and humans. This study aimed to determine the effectiveness of a cryoprotectant based on polyethylene glycol in reducing cold stress in laboratory rats. For the experiment, 30 outbred Wistar rats (5 weeks) with an average body weight of 55.1 ± 5.3 g were used. Three groups of animals were formed (10 rats per group). The first group served as a positive control, kept at a room temperature of +18 – +20 °C and received 0.1 ml of 0.9% NaCl solution. The second group, the negative control, was kept in critically low temperatures (+2–+4°C) and administered 0.1 ml of 0.9% NaCl solution per experimental animal. Rats of the third group were subjected to cold stress and received 0.1 ml of the experimental preparation. Cold stress in laboratory rats was created using a cooling thermostat. Observation for 14 days included monitoring the dynamics of changes in the live weight of animals (before the start of the experiment and on days 7 and 14 of the observation) as well as biochemical and haematological blood indicators. Fecal samples were collected from the rectum to determine the qualitative and quantitative state of the intestinal microbiota. The survival level of animals that received the experimental drug within fourteen days was 80.0%, compared to only 40% in the untreated group. When using the experimental drug in laboratory animals, an increase in body weight was noted. The number of full-fledged *Escherichia coli* in rats that received the drug was 3.4 times higher than the indicator of the group of animals that was kept at a critically low temperature without the drug. The prolonged low temperature in control rats had a negative effect on the animal's body as evidenced by increased leukocyte counts and ALT levels, as well as decreased ALT/AST ratio and total bilirubin. The use of an experimental polyethylene glycol-based preparation had a positive effect on the weight of rats, blood parameters, and intestinal microbiota of rats under cold stress.

Keywords: Biochemistry, Blood morphology, Cold stress, Cryoprotectant, Intestinal microbiota, Rat

INTRODUCTION

Cold stress occurs when the ambient temperature falls below optimal levels. Symptoms of cold stress include loss of coordination, difficulty maintaining balance, slowed heart rate and breathing, loss of consciousness, and, in extreme cases, death. Environmental temperature is an important abiotic factor that influences the adaptation processes of animals and determines the composition and structure of their communities. In addition, temperature changes affect the composition and function of the gut microbiota, an important regulator of host physiological processes. These effects can have significant consequences for the ability of populations to adapt to climate changes (Berg et al., 2016; Bestion et al., 2017). Studies conducted on various animal taxa, such as chordates, arthropods, and Mollusca, confirm stable relationships between temperature, community composition, and functional characteristics of the gut microbiota (Barbian et al., 2015; Carey and Assadi-Porter, 2017; Hammer et al., 2019). The intestinal microbiota of many animals is closely linked to the metabolic, immune, and neuroendocrine systems of the host organism, ensuring their mutual interaction and regulation (Kau et al., 2011; Chevalier et al., 2015). For instance, in mammals and insects, germ-free (axenic) organisms of some species exhibit a number of phenotypic differences compared to those with gut microbiota (Neufeld et al., 2010; Ridley et al., 2012; Li et al., 2019). Experiments transplanting gut microbiota into axenic organisms have demonstrated that changes in microbiota composition can lead to phenotypic changes in hosts (Faith et al., 2014; Gould et al., 2018; Fontaine et al., 2018). Researchers suggest that changes in host phenotype caused by gut microbiota contribute to the evolution of host populations (Chevalier et al., 2015; Rudman et al., 2019) and species (Moeller et al., 2019). The presence of certain microorganisms in the gut is believed to play a key role in maintaining the host's fitness to its environment (Moeller et al., 2019).

The low thermal tolerance of metazoans, compared to unicellular eukaryotes and bacteria, is attributed to a complex systemic process. Aerobic processes in animals are the first to be affected by low and high temperatures, which is associated with impaired circulation and ventilation (Zare et al., 2018; Zhang et al., 2019). Oxygen levels in body fluids may decrease, reflecting either

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increased oxygen demand at high temperatures or inadequate mitochondrial aerobic capacity at low temperatures. Aerobic capacity decreases at temperatures beyond the thermal optimum and ceases at extreme temperatures when the transition to anaerobic mitochondrial metabolism occurs. Changes in mitochondrial density, along with molecular and membrane changes, play a key role in maintaining aerobic capacity and adapting to changes (Smits et al., 2017; Orkin et al., 2019). Oxygen delivery capacity is sufficient to meet all aerobic capabilities only within the thermal optimum. Beyond this range, survival is possible only through time-limited aerobic metabolism, and later through anaerobic metabolism, molecular protection by heat shock proteins, and antioxidant activity. In the hierarchy of causes and effects, the progressive reduction in oxygen availability at extreme temperatures can lead to increased oxidative stress and protein denaturation. Thus, limitations in the efficiency of the oxygen delivery system at a complex level of organization define the limits of thermal stability, which can lead to disruption of molecular functions (Pörtner, 2001; Zhu et al., 2019). Some adaptations, such as the synthesis of low-molecular-weight cryoprotectants that have more specific mechanisms of action, provide direct stabilizing effects on membranes and proteins. The mechanisms used by animals offer insights and alternative approaches that can be effectively applied in the cryopreservation of cells and tissues, a critical requirement in reproductive technologies (Horváthová et al., 2019; Carnaghi et al., 2021; Kolchyk et al., 2024).

Low temperatures adversely affect physical health, causing both general hypothermia and local damage. Among local manifestations, the peripheral areas of the human body are especially vulnerable - feet, hands, and ears. This can lead to cold injuries such as frostbite and trench foot (Heil et al., 2016; Zaneveld et al., 2017; Kokornaczyk, 2021). Preventing and treating such injuries allows one to maintain combat effectiveness and complete the assigned mission during difficult conditions (Sullivan-Kwantes et al., 2021; Plavina, 2023). The ongoing full-scale invasion of Ukraine by Russia has further highlighted the importance of research in this area. Searching for cryoprotectants to prevent and treat hypothermia in humans and animals is a pressing issue. Unfortunately, there is limited data on the effects of polyethylene glycol on cold stress in different animal species. A review of the literature revealed that polyethylene glycol is used in medicine and animal husbandry without adverse effects (Mansoori and Modirsanei, 2011; Lyseng-Williamson, 2018; Wang et al., 2023).

The present study aimed to evaluate the effectiveness of a cryoprotectant based on polyethylene glycol for the correction of cold stress in laboratory animals (rats) under low-temperature conditions (+2–4 °C).

MATERIALS AND METHODS

Ethical approval

The experiment was approved by the Commission for Bioethical Expertise of the Dnipro State Agrarian and Economic University. Experimental studies on laboratory rats were conducted in compliance with the principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (WorldLII, 1986).

Study design

The study aimed at investigating the effectiveness of an experimental preparation based on polyethylene glycol as a cryoprotectant for the treatment and prevention of hypothermia in animals. The research was carried out in the laboratory of the Department of Animal Infectious Diseases and the Biosafety Center of the Dnipro State Agrarian and Economic University, Ukraine. In the vivarium (36 m^2) where the animals were housed, the temperature was maintained at 23°C, humidity at 22%, and lighting was controlled on a 12-hour light/dark cycle (lights on at 07:30). Laboratory rats were kept in cages with 350 cm^2 of floor space per animal. They were fed CARE+ Rat super-premium complete food (energy value 3000 kcal/kg, protein 14%, Beaphar, the Netherlands), and tap water was provided *ad libitum*.

For the experiment, 30 male outbred Wistar rats (age 5 weeks) with an average body weight of 55.1 ± 5.3 g were used. The animals were obtained from the central vivarium of the Dnipropetrovsk Regional State Laboratory of the State Service of Ukraine for Food Safety and Consumer Protection, Ukraine. Before the experiment, laboratory animals were trained for about five days. To do so, the rats were placed in heat-resistant glass boxes and received food rewards from the experimenter for 10-20 minutes per day. During this time, the temperature of the animals was measured five times using a rectal thermometer. The thermometer was lubricated with petroleum jelly and inserted 2 cm from the anal sphincter. This criterion was considered fulfilled if two standard deviations from the average initial temperature (37.5 ± 0.5°C) were determined when the first two measurements were within 0.3-0.5°C of each other (Klune et al., 2020).

The animals were randomly assigned to three groups (10 rats each). The first group, which was a positive control (I, K+) kept at a room temperature of +18- +20 °C, received 0.1 ml of 0.9% NaCl solution. The second group was a negative control (II, K-) kept in critically low temperatures (+2-+4°C). They received 0.1 ml of 0.9% NaCl solution per experimental animal. Rats of the third group (III, experimental drug) were under cold stress and received experimental preparation of one concentration (2 drops [0.1 ml] per experimental animal) via forced oral administration. Cold stress in laboratory rats was created using a cooling thermostat (TSO-80 MIKROmed, 2020, Ukraine). To determine the dynamics of rat mass in experimental research models, mathematical calculations of linear dynamics were used, which take into account the rate of mass gain or loss depending on time (Brygadyrenko et al., 2019).

The experimental preparation

The experimental preparation consisted of the following components included ionol - 25.0 g/l, dimethyl sulfoxide - 37.5 g/l, polyethylene glycol PEG 400 - 230.0 g/l, PEG 1500 - 540.0 g/l in the form of a soluble drug with a gel-like consistency. This composition of the experimental drug has already been successfully used in previous studies (Zazharskyi et al., 2024a).

Morpho-biochemical analyses

Observation for a period of 14 days included monitoring changes in the live weight of animals (before the start of the experiment, and on 7 and 14 days of observation), as well as the biochemical and haematological indicators of blood. Blood samples (up to 0.5 ml) were collected from the tail vein in Eppendorf microtubes during decapitation for biochemical and morphological analyses. The number of erythrocytes and leukocytes in stabilized rat blood was determined using an automatic hematology analyzer (BC-2800Vet, Mindray, China). To calculate the leukogram, blood smears were prepared according to the Pappenheim method. The biochemical indicators of blood were examined by photometric and spectrophotometric methods with photometersMicrolab-200, (International Microlab, Shenzhen, China, 2021) and Vitalab Eclipse (Merck, the Netherlands, 2011). Analyses were performed using the respective software after setting the reaction with appropriate diagnostic test kits from Lachema (Erba Lachema, Karásek, the Czech Republic, 2021).

Study of microbiota

At the end of the experiment, animals that survived were euthanized via carbon dioxide inhalation (Qin and Meng, 2006). Fecal samples were collected from the rectum after the death of the animal to determine the qualitative and quantitative composition of the intestinal microbiota, taking into account the parietal microflora and the microflora of the intestinal lumen. Fecal samples (1 g) were collected in a sterile container by cutting the rectum following the rules of asepsis, after which serial dilutions in physiological solution (1:9) to 10^{-11} were carried out (Kaminska, 2015).

From each dilution tube, 1 ml of suspension was taken with a sterile pipette and introduced into selective nutrient media (bifidum medium [Himedia, India]), lactobact agar, enterococcus agar, Endo agar, bismuth sulfite agar, Wilson-Blair agar, Byrd-Parker agar, and Saburo agar (Himedia, India). It was then rubbed on the surface with a sterile spatula. Most representatives of the intestinal microflora (cocci, enterobacteria) are able to grow on nutrient media for 24 hours, but some microorganisms (staphylococci, enterococci, bifidobacteria, clostridia, yeast-like fungi) are cultured for 48 hours and sometimes even 72 hours. Cultivation was carried out for 24-72 hours at 24-43 °C (mesophilic aerobic and facultative anaerobic microorganisms were cultivated at a temperature of 37 °C, yeast - 24-28-30 °C, hemolytic Escherichia coli - 42-44 °C). Anaerobic bacilli were isolated using GENbox anaer bags (Biomerieux, France). Anaerobic conditions were monitored using the Anaer indicator (Biomerieux, France). The number of viable microorganisms was counted in Petri dishes and expressed in CFU/g (colony-forming units in 1 g of intestinal contents). Morphological signs and tinctorial properties of the selected microorganisms were studied after staining smears according to the Gram and Romanovsky-Giemsa methods under the immersion microscope system MICROmedXS-3330 (Ukraine). Differentiation was carried out by studying their biochemical properties on Hiss media with various sugars, Olkenytskyi, Christensen, Simmons, and malonate agar (Pharmaktiv, Ukraine). Identification was carried out taking into account their biological properties according to Bergey's identifier of bacteria.

Statistical analysis

The collected data were compared using ANOVA with the Statistica 6.0 package (StatSoft Inc., USA). Data are presented as mean ± standard deviation ($x \pm SD$). Differences between values in the groups were determined using the Tukey test, where the differences were considered significant at $p < 0.05$ (subject to the Bonferroni Amendment).

RESULTS

During the experiment, the number of dead rats in groups II and III was compared during the period of 7 and 14 days of observation (Table 1). In the experimental drug group (III), two animals died during the first seven days, while in the group of animals that were in critically low-temperature conditions without treatment (II), the death of four animals was detected in the first week of observation, and two more animals died in the following week.

The dynamics of rat weight during the observation period indicated the inhibition of animals under the influence of cold stress (Table 2). After 7 and 14 days of the experiment, the weight of rats in group II (cold maintenance without treatment) lagged behind the indicators of the comfort group (I) by 26.0% ($p < 0.05$) and 24.0% ($p < 0.05$), respectively. In the experimental drug group (III), the weight lag was 15.5% and 6.2%. However, a positive dynamic weight gain was noted in animals that were administered the experimental drug.

Cold stress over 14 days in laboratory rats influenced the morpho-biochemical indicators of blood, as presented in Table 3. A decrease in the level of globulin in animals of group II by 6.8% was revealed, which may be a result of cold stress. In contrast, when the experimental drug was used (group III), the level of urea increased by 38.6% and 34.0% compared to animals of the control groups I and II ($p < 0.05$). It was found that the creatinine level in the group exposed to the experimental drug was within the comfort group of 50.2 μmol/L. In group II (K-), creatinine was 16.1% higher than in the control group (K+), which may indicate acute renal failure. However, a sharp decrease in the level of total bilirubin in animals in the experimental group II by 9.6 times is noticed compared to rats of the control group I ($p < 0.05$).

It was established that the experimental drug does not have a negative effect on the level of AST or ALT. These indicators are within the limits of the comfort group (I, K+). There was an increase in the ALT level (252.3 U/L; $p < 0.05$) with a decrease in the ALT/AST ratio (1.0 unit; $p < 0.05$) in animals of group K- compared to comfort group K+. An increase in amylase activity was observed in animals of group III (experimental drug) by 10.2% compared to the comfort group. Notably, glucose levels were elevated in Groups II and III by 3.9-fold and 2.1-fold, respectively, compared to Group I ($p < 0.05$). The authors of the current study consider the positive dynamics of lowering the cholesterol level in animals of group III (experimental drug) to groups K+ and K- by 40.7% ($p < 0.05$) and 23.8%, respectively. An increase in the number of leukocytes in animals of the K- K-group was found in comparison with comfort (I) and group III by 3.6 and 2.2 ($p < 0.05$) due to an increase in the level of neutrophils with segmented nuclei by 50.0% and 22.4%, respectively.

It was established that the number of obligate and facultative microorganisms of genera *Bifidobacterium* and *Lactobacillus* during exposure to cold stress in both of the groups of animals with and without the drug decreased by half or more compared to control animals that were kept under comfortable conditions, but no statistical difference was detected (Table 4).

The total number of full-fledged *Escherichia coli* in rats exposed to cold conditions decreased by 19.7 times compared to those kept in comfortable conditions ($p < 0.05$). Despite the fact that there was a decrease in the total number of full-fledged *Escherichia coli* (by 5.9 times) in the group of animals that were injected with the drug and kept in the cold, compared to the group that was in comfortable conditions ($p < 0.05$), this indicator was 3.4 times higher ($p < 0.05$) than the rate in animals that were kept in cold conditions without the drug.

The number of lactose-negative *Escherichia coli* when rats were kept in cold conditions increased 6.2 times compared to animals under comfortable conditions ($p < 0.05$) and 2.3 times compared to animals receiving the drug ($p < 0.05$). During the experiment, bacteria of the genus *Citrobacter* were not detected in any group. As for other representatives of the intestinal microbiota of rats, the number of *Enterobacter spp., Proteus spp.*, and fungi of the genus *Candida* decreased under the influence of cold in both experimental groups, but no statistical difference between the indicators was found ($p > 0.05$). On the contrary, the number of *Escherichia coli* with altered enzymatic properties and lactose-negative *E. coli*, *Enterococcus spp.*, *Clostridium spp.*, *Klebsiella spp*., as well as *Staphylococcus epidermidis*, and *Staphylococcus aureus*, increased during the experiment in cold conditions, yet no statistical difference between the indicators was detected ($p > 0.05$).

Table 1. Survival of rats during the cold stress administered with cryoprotectant based on polyethylene glycol (n=10)

Observation period (day)	Number of live animals / (%) in groups		
	I*	II*	III*
1	10/100.0	10/100.0	10/100.0
7	10/100.0	6/60.0	8/80.0
14	10/100.0	4/40.0	8/80.0

* I: The first group, positive control (I, K+), was in comfortable conditions at a room temperature of +18 – +20 °C and received 0.1 ml of 0.9% NaCl solution per rat; II: The second group, negative control (II, K-), was in critically low temperatures (+2–+4°C) and received 0.1 ml of 0.9% NaCl solution per rat; III: The third group (III experimental drug) was critically in low temperatures (+2.0-+4.0°C) and were administered the experimental preparation.

Table 2. Dynamics of rat weight (g ± SD) in groups during the observation period (14 days) administered with cryoprotectant based on polyethylene glycol

Groups / Observation period (day)	I*	II*	III*
1	54.2 ± 4.7	55.1 ± 5.3	55.7 ± 4.2
7	66.9 ± 4.1^{a}	49.5 ± 3.7^{b}	56.5 ± 5.3^{ab}
14	76.4 ± 4.7^{a}	58.0 ± 4.2^{b}	71.1 ± 6.5^{ab}

Note: ^{ab} Different letters indicate selections that significantly ($p < 0.05$) within the row differ from each other. * I – The first group, positive control (I, K+), was in comfortable conditions at a room temperature of +18 – +20 °C and received 0.1 ml of 0.9% NaCl solution per rat; II – The second group, negative control (II, K-), was in critically low temperatures (+2–+4°C), receiving 0.1 ml of 0.9% NaCl solution per rat; III – The third group (III experimental drug) was critically in low temperatures (+2.0-+4.0°C), administered with the experimental preparation.

Table 3. Changes in blood biochemical parameters (mean ± SD) of rats at a critically low temperature during 14 days of the experiment (administering a cryoprotectant based on polyethylene glycol)

Parameters	Groups		
	I*	II*	III*
Total protein, (g/L)	67.2 ± 7.4	63.1 ± 6.9	66.2 ± 8.7
Albumins, (g/L)	29.1 ± 3.4	28.6 ± 2.7	30.2 ± 3.2
Globulins, (g/L)	38.2 ± 3.9	35.6 ± 3.7	36.1 ± 4.2
The albumin-globulin ratio, (units)	0.8 ± 0.1	0.8 ± 0.2	0.8 ± 0.1
Urea, (mmol/L)	8.8 ± 0.2^{a}	9.1 ± 0.4^{a}	12.2 ± 0.3^{b}
Blood urea nitrogen, (mg/100 g)	16.8 ± 2.3	17.4 ± 1.6	23.3 ± 2.5
Creatinine, (μmol/L)	50.3 ± 6.4	58.4 ± 5.9	50.2 ± 5.1
Aspartate aminotransferase (AST), (U/L)	171.3 ± 22.7	240.1 ± 19.8	256.6 ± 23.6
Alanine aminotransferase (ALT), (U/L)	68.1 ± 5.7^{a}	252.3 ± 21.9^{b}	97.4 ± 8.7^{a}
De Ritis Index (AST/ALT), (units)	2.5 ± 0.1^{a}	1.0 ± 0.1^{b}	2.6 ± 0.2^{a}
Alkaline phosphatase, (U/L)	305.6 ± 29.8^{a}	236.0 ± 31.7^{a}	181.4 ± 23.4^{b}
Alpha amylase, (U/L)	594.3 ± 48.6	624.5 ± 59.1	655.0 ± 57.3
Total bilirubin, (μmol/L)	10.6 ± 1.2^{a}	1.1 ± 0.2^{b}	7.4 ± 1.4^{a}
Glucose, (mmol/L)	2.1 ± 0.4^{a}	8.2 ± 1.3^{b}	4.4 ± 0.5^{c}
Calcium, (mmol/L)	2.3 ± 0.1	2.0 ± 0.2	2.5 ± 0.2
Phosphorus, (mmol/L)	4.3 ± 0.5	4.6 ± 0.3	3.9 ± 0.6
Ca/P, (units)	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.1
Cholesterol, (mmol/L)	2.7 ± 0.1^{a}	2.1 ± 0.2^{ab}	1.6 ± 0.1^{b}
Gamma-glutamyl transferase (GGT), (U/L)	9.2 ± 1.8	6.3 ± 0.9	9.1 ± 1.2
Hemoglobin, (g/L)	106.4 ± 12.4	127.1 ± 24.2	128.7 ± 21.7
Hematocrit, (%)	23.5 ± 3.3	30.8 ± 4.6	28.2 ± 2.9
Erythrocytes, (10^{12}/L)	3.1 ± 0.5	3.7 ± 0.5	3.7 ± 0.2
MCV (mean corpuscular volume), (10^{-15} L)	75.8 ± 8.2	83.2 ± 9.3	76.2 ± 8.6
MCH (mean corpuscular haemoglobin), (10^{-12} g)	34.9 ± 4.8	34.3 ± 7.4	34.5 ± 5.1
MCHC (mean corpuscular haemoglobin concentration), (%)	45.1 ± 4.7	41.2 ± 3.8	45.9 ± 6.4
Color indicator, (units)	1.03 ± 0.02	1.03 ± 0.01	1.04 ± 0.01
Erythrocyte sedimentation rate (ESR), (mm/h)	1.1 ± 0.1	6.0 ± 0.2	1.0 ± 0.2
Platelets, (10^{9}/L)	366.4 ± 42.5	401.6 ± 37.7	454.2 ± 41.8
Leukocytes, (10^{9}/L)	3.2 ± 0.4^{a}	11.4 ± 2.2^{b}	5.3 ± 0.7^{a}
Leukocyte formula, (%):			
Basophils	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Eosinophils	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Lymphocytes	74.3 ± 5.4	64.1 ± 7.9	72.6 ± 8.4
Monocytes	9.2 ± 1.3	10.4 ± 2.2	7.7 ± 2.1
Myelocytes	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Neutrophils:			
– young	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
– stick-core	1.0 ± 0.1	2.2 ± 0.3	1.2 ± 0.2
– segmented-nucleus	16.4 ± 2.1	24.6 ± 3.7	20.1 ± 2.9

Note: [abc] Different superscript letters indicate significant differences ($p < 0.05$) within each row. * I: The first group, positive control (I, K+), was in comfortable conditions at a room temperature of +18 – +20 °C, and received 0.1 ml of 0.9% NaCl solution per rat; II: The second group, negative control (II, K-), was in critically low temperatures (+2–+4°C), received 0.1 ml of 0.9% NaCl solution per rat; III: The third group (III experimental drug) was critically in low temperatures (+2.0-+4.0°C) with the use of experimental preparation.

Table 4. Qualitative and quantitative composition of the intestinal microbiota (lg 10 CFU/gram of feces) in groups of rats under cold stress (Mean ± SD) during 14 days of experiment administered with a cryoprotectant based on polyethylene glycol

Gut microbiota	I*	II*	III*
Bifidobacterium spp.	7.81 ± 7.34	3.20 ± 7.26	7.39 ± 7.28
Lactobacillus spp.	7.66 ± 7.34	4.40 ± 7.32	7.42 ± 7.32
E. coli (normal enzymatic properties strains)	7.65 ± 7.11[a]	6.35 ± 6.02[b]	6.88 ± 6.18[c]
E. coli (weakly fermenting strains)	2.90 ± 2.00	8.42 ± 4.28	4.88 ± 4.62
E. coli (lactose-negative strains)	2.41 ± 2.05[a]	7.45 ± 2.49[b]	2.73 ± 2.34[a]
Enterococcus spp.	4.64 ± 4.36	2.85 ± 6.51	6.0 ± 5.76
Enterobacter spp.	3.49 ± 3.17	5.93 ± 2.48	3.18 ± 2.68
Citrobacter spp.	0 ± 0	0 ± 0	0 ± 0
Klebsiella spp.	3.83 ± 3.23	3.92 ± 3.92	4.09 ± 3.73
Proteus spp.	3.45 ± 3.26	3.33 ± 3.02	3.35 ± 3.14
Staphylococcus epidermidis	3.40 ± 2.19	4.16 ± 3.19	2.97 ± 2.49
Staphylococcus aureus	2.85 ± 3.27	3.10 ± 3.59	4.15 ± 3.94
Clostridium spp.	4.39 ± 1.90	3.31 ± 2.89	3.45 ± 3.22
Candida spp.	2.30 ± 4.28	4.24 ± 3.58	4.08 ± 3.76

Note: [abc] Different superscript letters indicate significant differences ($p < 0.05$) within each row. * I: The first group, positive control (I, K+), was in comfortable conditions at a room temperature of +18 – +20 °C, and received 0.1 ml of 0.9% NaCl solution per rat; II: The second group, negative control (II, K-), was in critically low temperatures (+2–+4°C), received 0.1 ml of 0.9% NaCl solution per rat; III: The third group (III experimental drug) was critically in low temperatures (+2.0-+4.0°C), administered the experimental preparation.

DISCUSSION

Hypothermia is defined as a decrease in body temperature below 35°C (Brodeur et al., 2017). It is widely used for therapeutic purposes, including cardiopulmonary bypass, craniocerebral injuries, organ transplantation, and neonatal encephalopathy. It is classified as either primary (accidental) or secondary. Prolonged exposure of an animal with normal heat production in cold conditions leads to the development of primary hypothermia. Secondary hypothermia occurs when heat production and thermoregulation are disrupted due to diseases, injuries, or exposure to medications (Brodeur et al., 2017). Based on duration, hypothermia can be acute (several hours) or chronic (days or weeks) (Tveita and Sieck, 2022).

Cold stress is a significant environmental factor affecting animal viability and productivity (Hao and Wang, 2017; Worthmann et al., 2017; Zazharska et al., 2024). Scientists believe that long-term exposure of animals to low temperatures is closely associated with pathogens and infectious diseases that can cause harm to animal health and significant economic damage to livestock enterprises (Landin and Bonastre, 2018).

Despite extensive research on cold stress, no data were found on the effects of polyethylene glycol (PEG) under such conditions. Polyethylene glycol (PEG), through its properties as an osmotically active substance, creates osmotic pressure in the intercellular environment, retaining water inside the cells and preventing them from drying out (Mansoori and Modirsanei, 2011; Lyseng-Williamson, 2018; Wang et al., 2023). PEG acts as a membrane stabilizer, maintaining its liquid structure even at low temperatures. PEG is biocompatible and non-toxic, which makes it safe for use in plants, animals, and cell cultures (Barer, 2015).

PEG is a multifunctional polymer that has a wide range of applications, including its use as a food and cosmetic additive and as a carrier in PEGylated therapeutic agents (Ibrahim et al., 2022). It is administered orally to humans (Lyseng-Williamson, 2018). In Japan, for instance, treatment with PEG 3350 was well tolerated in patients with chronic constipation, which resulted in sustained improvements in bowel function (Nakajima et al., 2019). In another study, PEG was fed to chickens without negative effects (Mansoori and Modirsanei, 2011).

The authors of this study suggest that polyethylene glycol may mitigate cold stress due to its physicochemical properties, its role in maintaining the water balance of cells, its ability to preserve membrane structures, and its activation of protective mechanisms. Therefore, the use of the experimental drug in the present study ensured the survival of twice as many animals until the end of the experiment than in the group without the drug. Hematological and biochemical blood parameters are often the main diagnostic criteria (Zazharskyi et al., 2024a). Their fluctuations may indicate adverse effects of substances on organs and systems.

The number of leukocytes in the blood of animals under cold stress increased 3.6 times compared to the group at a comfortable

temperature. However, the use of cryoprotectant based on polyethylene glycol led to an increase in the number of leukocytes by only 1.7 times compared to the control group of rats (K+), where no statistical difference was found. A positive effect on blood components and a wound-healing action have already been reported in previous studies in the treatment of burns in laboratory rats by the experimental drug containing ionol - 25.0 g/l, dimethyl sulfoxide - 37.5 g/l, polyethylene glycol PEG 400 - 230.0 g/l, and PEG 1500 - 540.0 g/l. The protein concentration in the blood of guinea pigs receiving the experimental drug remained within physiological norms. Total bilirubin is a product of heme metabolism, which is part of hemoglobin in red blood cells and is responsible for transporting oxygen to tissues. After the destruction of red blood cells, bilirubin is released and goes to the liver, where it is metabolized and excreted from the body with bile. On the third day of burn treatment using the experimental drug, a significant decrease in bilirubin concentration by 65.2% ($P < 0.01$) was observed, which is probably due to a decrease in the level of intoxication of the body (Zazharskyi et al., 2024a).

Keeping animals in a cold environment (group K-) negatively affected many morpho-biochemical parameters of blood (Table 3). A sharp decrease in the level of total bilirubin in animals in experimental group II by 9.6 times may indicate irreversible pathological processes and the transition to chronic renal failure. There was an increase in the ALT level with a decrease in the ALT/AST ratio in animals of group K-, compared to comfort group K+, which is associated with signs of liver disease and myocardial dysfunction. The decrease in Ca loss in the blood of rats in the control group (K-) occurred by 15% compared to the comfort group. Research has documented various substances affecting blood parameters (Zazharskyi et al., 2024b; 2024c). For instance, the use of alcohol tincture of *Aralia elata* in rats leads to a decrease in the levels of creatinine, glucose, urea, cholesterol, bilirubin, and total calcium (Brygadyrenko et al., 2019).

In the present study, the level of total protein increases due to albumins, urea, and urea nitrogen in the blood of rats under cold stress when using an experimental preparation based on polyethylene glycol. After 14 days, alkaline phosphatase levels normalized to those of the comfort group. The level of Erythrocyte sedimentation rate (ESR) corresponds to the animals of the comfort group, with a significantly higher indicator in the K- group (6 times), which indicates the presence of an inflammatory process in the body of intact rats. Long-term use of low temperature in rats of the control group without treatment in the second week of the experiment led to a sharp decrease in the level of Gamma-glutamyl transferase (GGT), the enzyme which is localized in the cells of the liver and biliary tract and plays the role of a catalyst in specific biochemical reactions.

Environmental changes can impact animals by influencing the composition of their gut microbiota. Fluctuations in temperature can reduce the diversity of the microbiome, leading to a loss of key functions and potentially having negative consequences for the health and survival of animals (Fotina et al., 2018; Horváthová et al., 2019; Borovuk and Zazharska, 2022).

Bilan et al. (2019) found that co-exposure to glyphosate and common food additives had a significant impact on the gut microbiota composition of rats without changing the number of *Escherichia coli*, *Bifidobactrium*, and *Lactobacillus spp*. According to the present data, cold stress leads to a sharp decrease in these microorganisms. The number of lactose-negative *Escherichia coli* for the rats kept in cold conditions increased 6.2 times compared to animals under comfortable conditions and 2.3 times compared to animals receiving the drug.

Mixtures of glyphosate and food additives (saccharin and sodium benzoate) were found to allow microorganisms of the genera *Klebsiella*, *Enterobacter*, and *Pseudomonas*, as well as opportunistic yeast-like fungi *Candida* to spread more widely in the intestines of rats (Bilan et al., 2019). Based on the present findings, keeping rats at low temperatures contributes to a wider reproduction of the number of microorganisms of the genera *Klebsiella* and *Staphylococcus*, but to a decrease in the genera *Enterobacter* and *Candida* fungi.

In the present study, fluctuations in the number of probiotic strains of *Bifidobacteri*a and *Lactobacilli* (downward from the reference value of intestinal content after 14 days of the experiment) were observed in rats against the background of drug use. The number of *Escherichia coli* with normal enzymatic properties decreased due to an increase in the number of lactose-negative *Escherichia coli* and *Escherichia coli* with altered enzymatic properties, leading to fluctuations in the number of other *enterobacteria* and anaerobic *clostridia*.

CONCLUSION

The experimental cryoprotectant drug based on polyethylene glycol exhibits a markedly positive general biological effect on laboratory animals, resulting in a markedly higher survival rate of animals compared to the control group. The use of the experimental preparation has a positive effect on the morpho-biochemical parameters of the blood and intestinal microbiota of rats under cold stress. In the blood of rats exposed to cold stress using an experimental drug, the level of total protein increases due to albumin, urea, and urea nitrogen. Moreover, alkaline phosphatase normalizes to the level of the comfort group after 14 days of the experiment. The number of full-fledged *E. coli* in rats that received the drug was 3.4 times higher than in the group of animals kept in the cold without the drug. Future research can explore the effectiveness of polyethylene glycol-based cryoprotectants in combination with *Echinacea* extract during cold stress in laboratory animals.

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Availability of data and materials

The datasets generated during the current study are available from the corresponding author upon reasonable request.

Ethical considerations

Ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been checked by all the authors.

Authors' contributions

Olexyi Zaslavskyi and Ivan Biben conceived and designed the experiment, and Olexandr Sosnickyi and Volodymyr Zazharskyi conducted experiments on animals. Marina Bilan conducted bacteriological studies. Volodymyr Zazharskyi and Nadiia Zazharska analyzed the obtained results, interpreted and wrote the manuscript, and edited and reviewed the article. All authors have read and approved the final draft of the manuscript for publication in the journal.

Conflicts of interests

The authors declare that there is no conflict of interest.

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