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Research Article

# Evaluation of the neuroprotective effects of synthetic erythropoietin derivatives in a mouse model of mild and moderate hypoxic-ischemic encephalopathy

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## Abstract

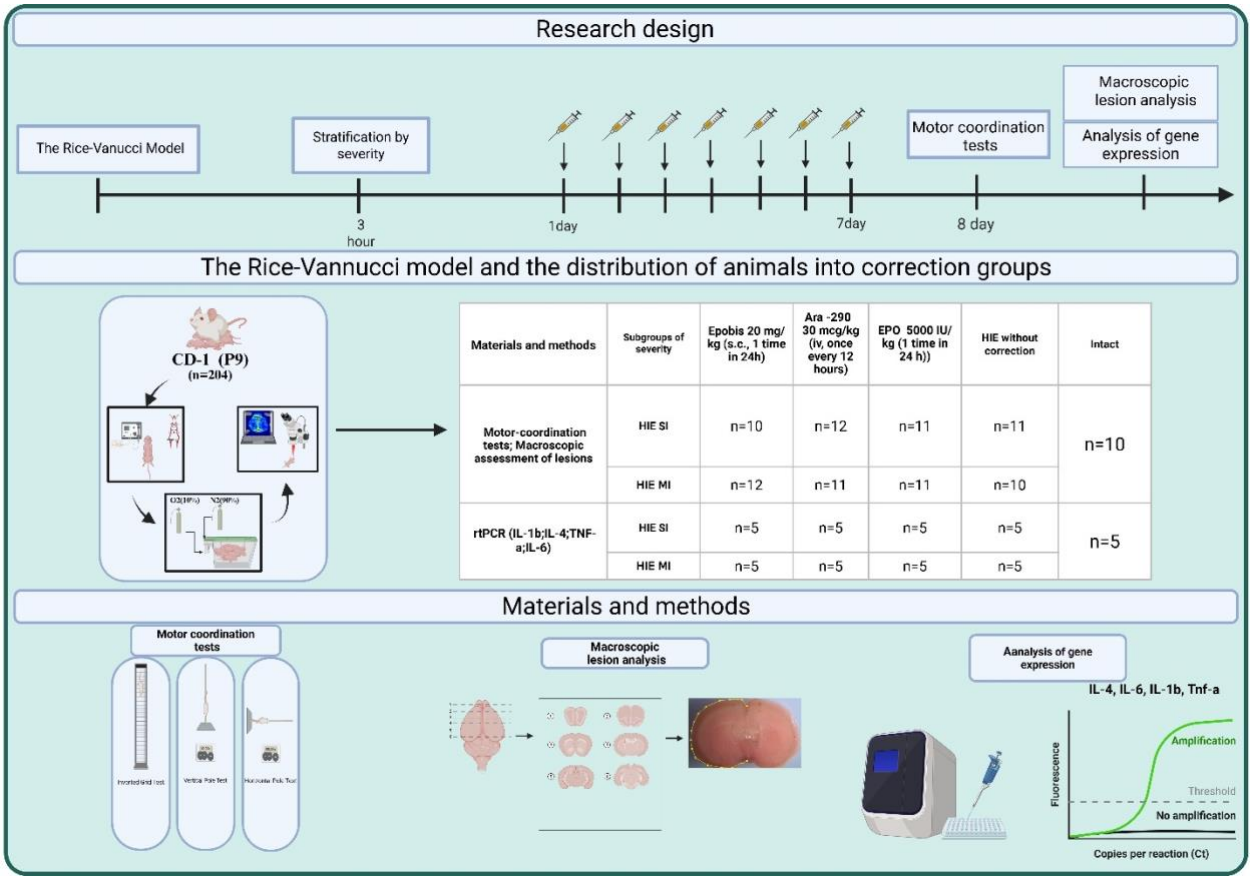
**Introduction:** Hypoxic-ischemic encephalopathy (HIE) is a leading cause of neonatal brain injury and long-term neurodevelopmental impairment, and current therapies only partially prevent adverse outcomes. Although recombinant erythropoietin is neuroprotective via EPOR–CD131 (βcR), its use is limited by hematopoietic side effects, motivating evaluation of non-hematopoietic peptide analogs such as Ara-290 and Epobis in HIE.

**Materials and Methods:** Neonatal hypoxia-ischemia was induced in 9-day-old CD-1 mice (n = 204) using a modified Rice–Vannucci model, and animals were stratified into mild and moderate severity groups 3 hours later by laser speckle contrast imaging (RFLSI-ZW) before assignment to treatment. EPO (5000 IU/kg) and Epobis (20 mg/kg) were administered subcutaneously every 24 hours and Ara-290 (30 μg/kg) intraperitoneally every 12 hours for 7 days, after which motor-coordination performance, macroscopic lesion volume, and expression of IL-4, IL-1b, IL-6, and TNF-α were assessed.

**Results:** In both mild and moderate HIE, Epobis provided the most consistent neuroprotection, improving motor performance and neurological outcomes and being the only treatment to significantly reduce macroscopic lesion volume. EPO produced moderate functional benefits, whereas Ara-290 showed a less stable efficacy profile, with limited or absent effects in several behavioral and morphological endpoints.

**Conclusion:** In mild HIE, Epobis showed the strongest neuroprotection, improving motor-coordination performance, reducing neurological deficits, and producing the greatest reduction in macroscopic lesion volume, whereas EPO and Ara-290 had only moderate effects. In moderate HIE, Epobis and EPO most consistently improved motor outcomes, but only Epobis significantly reduced lesion volume and neurological symptom severity and produced the most pronounced immunomodulation.

## Graphical Abstract




## Keywords

hypoxic-ischemic encephalopathy, motor-coordination functions, Epobis, Ara-290, erythropoietin, laser speckle contrast imaging

## Introduction

Hypoxic-ischemic encephalopathy (HIE) is a brain injury that develops within the first hours of life as a result of the combined effects of hypoxia, ischemia, and impaired metabolic regulation of brain tissues, and is characterized by a spectrum of neurological disorders of varying severity as outcomes of the disease. HIE remains one of the leading causes of postnatal central nervous system injury and subsequent neuropsychological developmental disorders in children. According to contemporary clinical observations, hypoxic-ischemic brain injury is diagnosed in 1.5–3 per 1000 term newborns and up to 60 per 1000 preterm newborns. Despite progress in intensive care, including hypothermia as the treatment of choice, and the development of neuroprotective methods, existing approaches provide only partial reduction of neurological sequelae, and there are few effective pharmacological agents with proven neuroprotective activity (Walas et al. 2020; Arnautovic et al. 2024; Glass et al. 2024; Dolan and Wintermark 2025). Recombinant erythropoietin has demonstrated neuroprotective effects in the treatment of HIE, acting via the innate repair receptor (IRR), which is a heterocomplex of EPOR and CD131 (βcR) (Juul et al. 1999; Wang et al. 2016; Anusornvongchai et al. 2018; Schneider Gasser et al. 2019; Simon et al. 2019). Activation of IRR initiates cyto-, angio-, and neuroprotective pathways; however, the clinical use of high doses of EPO is limited by the risk of thrombosis due to activation of the homodimeric EPOR receptor located in hematopoietic tissues and by pharmacokinetic features of molecular penetration across the blood–brain barrier (Wu et al. 2022). Promising compounds with confirmed neuroprotective effects include synthetic erythropoietin analogs that lack hematopoietic activity while retaining its tissue-protective properties. Two of these are Ara-290 and Epobis. A number of preclinical studies related to the correction of ischemic stroke and autoimmune encephalitis in mice and rats have demonstrated

neuroprotective effects of these compounds; however, their efficacy in the correction of HIE has not been previously studied (Pankratova et al. 2012; Dmytriyeva et al. 2016; Wang et al. 2024).

**Research objective**: to evaluate the neuroprotective effects of EPOBIS and Ara-290 in the early remodeling phase in a mouse model of mild and moderate hypoxic-ischemic encephalopathy.

# Materials and Methods

### Study design

To form experimental groups, CD-1 animals were mated at a male-to-female ratio of 1:3. On the following day, the presence of a copulatory plug was recorded, and the date of visualization was taken as the first day of pregnancy. After 20–21 days, offspring were obtained in a total number of 204 animals. After birth, litters were mixed and fostered to lactating females with no more than 8 pups per female, maintaining an equal number of animals per foster dam. Modeling neonatal fetal hypoxia-ischemia (HIE) using a Rice–Vannucci modification was performed at 9 days of age. Three hours after pathology modeling, animals were stratified into mild and moderate HIE severity groups as previously described, using the RFLSI-ZW system (RWD Life Science, China), followed by allocation of animals to treatment groups (Table 1). The first injection of the studied drugs was administered on the day of pathology modeling after group assignment. EPO and Epobis were administered once every 24 hours subcutaneously, while Ara-290 was administered every 12 hours intraperitoneally for 7 days. Seven days after the therapy, the effect of pharmacological correction on changes in motor-coordination functions was evaluated, followed by assessment of changes in macroscopic lesion volume and analysis of gene expression levels of IL-4, IL-1b, IL-6, and TNF-α.

**Table 1.** Experimental groups and pharmacological interventions

| | | Number of animals in the experimental treatment groups | | | | |
|---|---|---|---|---|---|---|
| **Materials and Methods** | **Group designation** | **EPO 5000 ME/kg** | **Epobis 20 mg/kg** | **Ara-290 30 µg/kg** | **Control** | **Intact** |
| Motor-coordination tests; assessment of macroscopic injury | Slight injury HIE | n = 11 | n = 10 | n = 12 | n = 11 | n = 10 |
| | Moderate injury HIE | n = 11 | n = 12 | n = 11 | n = 10 | |
| Neuroinflammation gene expression analysis | Slight injury HIE | n = 5 | n = 5 | n = 5 | n = 5 | n = 5 |
| | Moderate injury HIE | n = 5 | n = 5 | n = 5 | n = 5 | |

### Animals

HIE modeling was performed in 204 CD-1 mice aged 9 days. The study was approved by the Institutional Committee for Control of Housing and Use of Laboratory Animals of Belgorod State National Research University (BelSU) (Approval No. 01-10i/25 of October 1, 2025). Mice were housed in an SPF animal facility at BelSU under a 12-hour light cycle at 22–26 °C, with ad libitum access to water and standard chow. All procedures were conducted in accordance with the European Convention ETS No. 170 and Directive 2010/63/EU.

### HIE modeling and stratification of animals by severity

Neonatal hypoxia-ischemia was induced using a modified Rice–Vannucci protocol in CD-1 mice at 9 days of age (n = 204) (Sheldon et al. 2018). Briefly, the left common carotid artery was coagulated; pups were then returned to the dams for 90 minutes, after which they were placed in a chamber with reduced oxygen content for 50 minutes (10% $O_2$; 90% $N_2$). Three hours after hypoxia, animals were stratified according to the degree of reduction in perfusion blood flow in the lesion area using laser speckle contrast imaging of cerebral blood flow (RFLSI-ZW, RWD Life Science, China), as previously described (Pokrovskii et al. 2026).

### Drugs and doses

EPO, erythropoietin (epoetin beta; PHARMAPARK, Pharmstandard-UfaVITA, Russia), was administered at a dose of 5000 IU/kg. Short-chain peptide analogs of erythropoietin were used: Ara-290 (Russian Peptide, Russia) at 30 µg/kg and Epobis (Russian Peptide, Russia) at 20 mg/kg. The doses of the studied compounds were selected based on preclinical studies of these compounds in models of ischemic stroke and autoimmune encephalomyelitis (Wang et al. 2024; Dmytriyeva et al. 2016).

### Assessment of motor and coordination functions

Motor and coordination functions were assessed 7 days after HIE modeling using the "Inverted grid", "Vertical pole", and "Horizontal pole" tests with modifications reflecting motor and cognitive behavioral impairments in juvenile mice, as previously described (Pokrovsky et al. 2025).

### Macroscopic assessment of injury

Macroscopic assessment of injury was performed 7 days after pathology modeling. After decapitation, the brain was incubated for 40 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma Aldrich, USA) at 37 °C, followed by addition of 10% formalin solution. After 24 hours, 1-mm-thick coronal sections were prepared using a Mouse/40-75g/Coronal/1mm/Stainless steel brain matrix (RWD Life Science, China). Sections were photographed using a stereo microscope equipped with a camera (BUCSF-830CC, China). The areas of the injured and contralateral hemispheres were quantified using QuPath 0.4.3.. To compare the extent of macroscopic injury between groups, the lesion index was calculated as the difference between the total tissue areas of the injured and contralateral hemispheres. The analysis was performed on a series of 1-mm-thick coronal sections: seven sections per animal obtained 7 days after HIE induction.

### Gene expression analysis

Dissection of brain structures from the injured hemisphere was performed after completion of therapy on ice immediately after decapitation of the animal under injectable anesthesia. All tissues were immediately placed into tubes containing lysis buffer from the RNeasy Mini Kit (250) RNA extraction kit (Qiagen, Germany) kept on ice, where they underwent ultrasonic homogenization. Subsequent RNA isolation was performed according to the manufacturer's instructions. Purified RNA solutions were analyzed spectrophotometrically using a NanoDrop OneC instrument (Thermo Scientific, USA) and diluted to a uniform concentration of 100 ng/µL.

Reverse transcription was performed using the MMLV RT reagent kit (Evrogen, Russia) according to the manufacturer's instructions with addition of 1 µg of RNA from the analyzed sample. The synthesized cDNA was used as a template in real-time PCR using the 5X qPCRmix-HS SYBR reagent kit (Evrogen, Russia). Gene-specific primers to the coding DNA sequences of IL-1b, IL-6, IL-4, and Tnf-α were used, along with primers specific to the cDNA of the reference mouse gene Gapdh (Table 2).

**Table 2**. List of nucleotide primer sequences for quantitative analysis of gene expression by real-time PCR

| List of the studied genes | Sequences | | |
|---|---|---|---|
| | Forward sequence | Reverse sequence | Primer annealing temperature |
| IL-1b | TTG ACG GAC CCC AAA AGA TG | AGG ACA GCC CAG GTC AAA G | 61°C |
| IL-6 | CCA CGG CCT TCC CTA CTT C | TTG GGA GTG GTA TCC TCT GTG A | 61°C |
| IL-4 | CCC ACC TGC TTC TCT GAC TAC A | CAG CGC TAT CCA GGA ACC A | 61°C |
| Tnf-α | TCC AGG CGG TGC CTA TGT | GCC CCT GCC ACA AGC A | 61°C |
| Gapdh | ATG ACC ACA GTC CAT GCC ATC | GAG CTT CCC G TTC AGC TCTG | 61°C |

Relative normalized quantitative assessment of expression was performed using the 2-ΔCt method, where ΔCt = Ct(target gene) − Ct(reference gene).

### Statistical data processing

Statistical processing of the results and graphical visualization of the parameters obtained were performed using GraphPad Prism Software 8.0 (GraphPad Software Inc., USA). Numerical data in the text are presented as mean ± standard deviation (M ± SD). The distribution of numerical data was assessed using the Shapiro–Wilk test. In the case of normally distributed data, analysis of variance was performed using Welch ANOVA followed by between-group comparisons with the Games–Howell post hoc test. In the case of non-normal data distribution, analysis of variance was performed using the Kruskal–Wallis test followed by between-group comparisons with Dunn's post hoc test.

# Results

### Assessment of changes in motor-coordination functions and macroscopic lesion volume after pharmacological correction of mild HIE

Analysis of the time animals remained on the inverted grid in mild HIE treated with erythropoietin derivatives showed that in the untreated group this value was 14.36 ± 2.91 s, which was almost twofold lower than in the intact group (29.4 ± 4.48 s). Correction of mild HIE with Epobis at a dose of 20 mg/kg increased the value to 20.5 ± 3.6 s, which was 30% higher than in the control group. In the EPO group, the inverted grid holding time was 17 ± 3.23 s, and in the Ara-290 group it was 18.00 ± 4.59 s, which was 18.4% and 25.3% higher than in the control group, respectively. The Games–Howell post hoc analysis revealed that the Epobis group significantly exceeded the mild HIE group ($p = 0.0004$) and differed from the EPO group ($p = 0.0389$). At the same time, all groups differed significantly from the intact group ($p < 0.0001$) (Fig. 1).

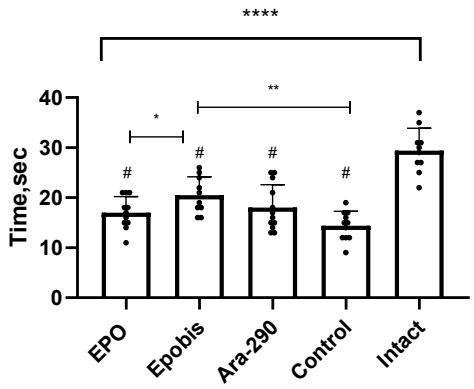



**Figure 1**. Effect of EPO (5000 IU/kg), Epobis (20 mg/kg), and Ara-290 (30 μg/kg) on holding time in the inverted grid test during correction of mild HIE 7 days after pathology modeling. ***Note:*** # – comparison with the intact group; explanations are provided in the text.

When analyzing the data on the time to complete the horizontal pole test and the accompanying assessment of neurological signs during correction of mild HIE, the following results were obtained.

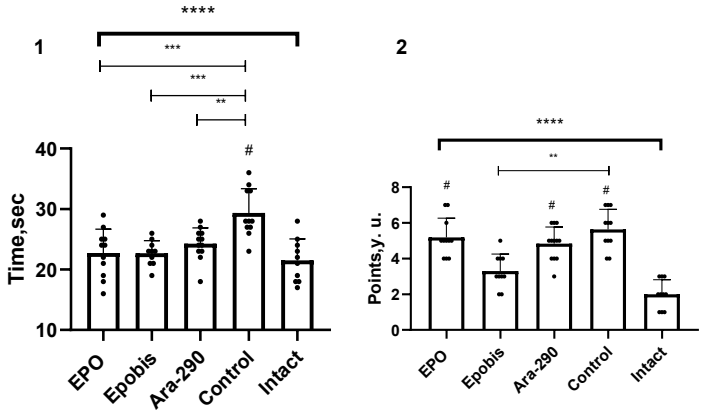



**Figure 2**. Effect of EPO (5000 IU/kg), Epobis (20 mg/kg), and Ara-290 (30 μg/kg) on the time to complete the horizontal pole test and changes in accompanying neurological signs during correction of mild HIE 7 days after pathology modeling. ***Note:*** **1** – time to complete the horizontal pole test; **2** – change in accompanying neurological signs. # – comparison with the intact group; explanations are provided in the text.

The values for time to complete the horizontal pole test were: EPO 22.73 ± 3.95 s; Epobis 22.70 ± 2.06 s; Ara-290 24.25 ± 2.63 s; HIE 29.4 ± 4.1 s; intact group 21.50 ± 3.57 s. Relative to the HIE group, the values were lower by 19.1%, 19.2%, 13.7%, and 23.5% for EPO, Epobis, Ara-290, and the intact group, respectively. Welch ANOVA revealed statistically significant differences among the five groups; however, the magnitude of the differences was moderate ($p =$

0.01). Post hoc comparisons using the Games–Howell test showed that the EPO, Epobis, and Ara-290 groups differed significantly from the HIE group ($p = 0.0485$, $p = 0.0178$, and $p = 0.01$, respectively) (Fig. 2-1). In addition, the untreated group differed significantly from the intact group ($p = 0.0098$). When analyzing neurological signs in the Dana test, no statistically significant differences were observed between the treatment groups and the HIE group, with mean values of 4.7 ± 0.6 in the EPO group, 3.6 ± 0.9 in the Epobis group, 4.8 ± 0.9 in the Ara-290 group, 4.8 ± 1 in the HIE group, and 1.7 ± 0.6 in the intact group; however, a statistically significant difference was found when comparing the EPO and Epobis groups (Fig. 2-2).

During correction of mild HIE, the descent time on the vertical pole increased in the studied groups compared with the control group (12.64 ± 3.36) by 36.6% in the EPO group (17.2 ± 4.2 s), by 42.4% in the Epobis group (18.00 ± 3.3 s), and by 28.5% in the Ara-290 group (15.08 ± 2.07 s). In the Games–Howell test, a statistically significant difference was recorded for the Epobis and EPO groups compared with the control group (Fig. 3-1).

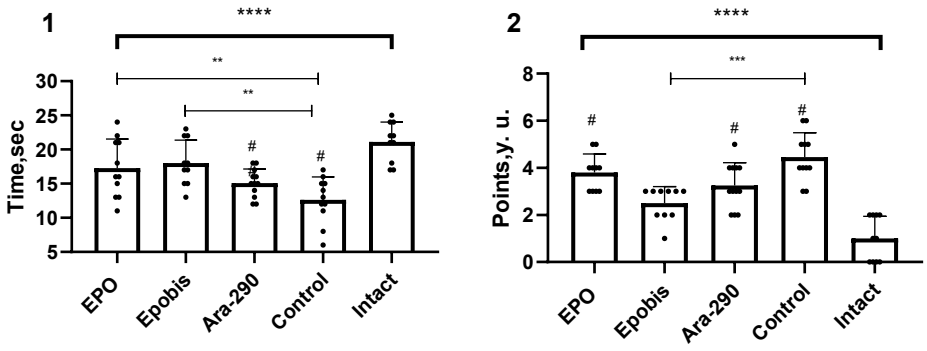



**Figure 3**. Effect of EPO (5000 IU/kg), Epobis (20 mg/kg), and Ara-290 (30 μg/kg) on descent time in the vertical pole test and changes in accompanying neurological signs during correction of mild HIE 7 days after pathology modeling. ***Note*:** 1 – descent time in the vertical pole test; 2 – change in accompanying neurological signs. # – comparison with the intact group; explanations are provided in the text.

A similar pattern was observed in the analysis of accompanying neurological impairments, which decreased by 14.7% in the EPO group (3.8 ± 0.7), by 43.9% in the Epobis group (2.5 ± 0.7), and by 27.1% in the Ara-290 group (3.2 ± 0.9) compared with the untreated HIE group. Dunn's post hoc analysis revealed a statistically significant difference between the Epobis and HIE groups ($p = 0.007$) and no statistically significant differences relative to the intact group, indicating a high magnitude of effect that was not observed in the mild HIE correction groups treated with EPO and Ara-290 (Fig. 3-2).

In mild HIE, the lesion volume in the untreated group was 3644 ± 374.7 px. In the treatment groups, a reduction in the volume of injured tissue was observed by 32.7% in the EPO group (2454 ± 272.5 px), by 54.2% in the Epobis group (1733 ± 291.8 px), and by 35% in the Ara-290 group (2369 ± 271.1 px) compared with the control group values.

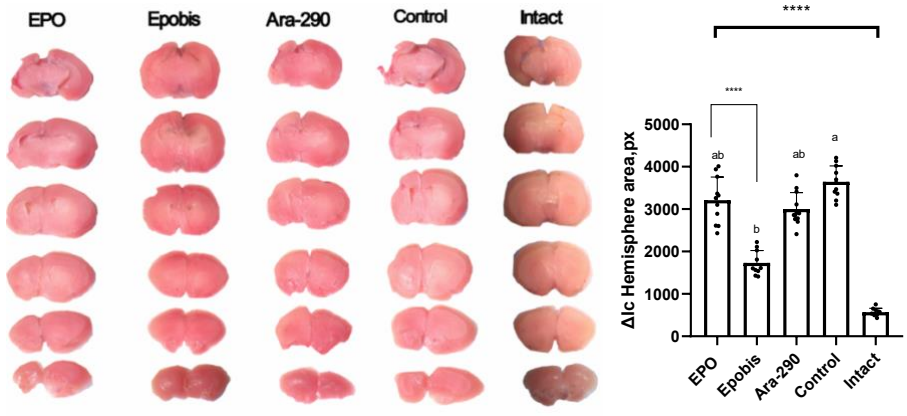



**Figure 4**. Effect of EPO (5000 IU/kg), Epobis (20 mg/kg), and Ara-290 (30 μg/kg) on the volume of macroscopic brain tissue injury during correction of mild HIE in the phase of reperfusion-inflammatory changes, 7 days after pathology modeling. ***Note*:** # – comparison with the intact group; @ – comparison with the untreated group; explanation is provided in the text.

The Games–Howell post hoc analysis revealed statistically significant between-group differences. All HIE correction groups differed significantly from the control group ($p < 0.0001$) and the intact group ($p < 0.0001$). A statistically significant difference in the efficacy of HIE correction with Epobis compared with EPO and Ara-290 was recorded ($p = 0.001$); however, no statistically significant difference was found between EPO and Ara-290, indicating identical therapeutic effects of EPO and Ara-290 and a more pronounced efficacy of Epobis in correcting mild HIE (Fig. 4).

**Assessment of changes in motor-coordination functions and macroscopic lesion volume after pharmacological correction of moderate HIE**

In moderate HIE, the holding time in the untreated group decreased to 10.2 ± 2.35 s. Pharmacological correction led to a marked increase in holding time on the inverted grid. Relative to the HIE group, the parameter was higher by 39.9% in the EPO group (14.27 ± 1.74 s, $p = 0.0028$), by 52.7% in the Epobis group (15.58 ± 2.31 s, $p = 0.0003$), and by 23% in the Ara-290 group (12.55 ± 2.91 s), and was statistically significant in the moderate HIE correction groups EPO ($p = 0.0028$) and Epobis ($p = 0.0003$) in multiple comparisons using the Games–Howell test (Fig. 5).

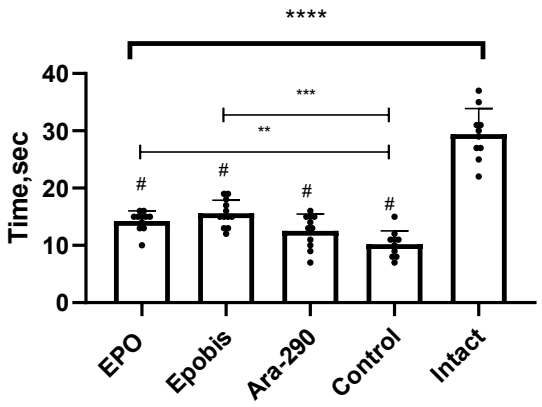



**Figure 5**. Effect of EPO (5000 IU/kg), Epobis (20 mg/kg), and Ara-290 (30 μg/kg) on holding time in the inverted grid test during correction of moderate HIE 7 days after pathology modeling. ***Note*:** # – comparison with the intact group; explanations are provided in the text.

When analyzing the time to complete the horizontal pole test and the accompanying assessment of neurological signs during correction of moderate HIE, the following results were obtained:

The time to complete the horizontal pole test was: EPO 33.5 ± 3.6 s, Epobis 31.8 ± 3.5 s, Ara-290 35.9 ± 4.2 s, HIE 40.7 ± 4.0 s, intact group 21.5 ± 3.5 s. Relative to the moderate HIE group, the values were lower by 17.6%, 21.8%, 11.8%, and 47.2% for EPO, Epobis, Ara-290, and the intact group, respectively. Welch ANOVA revealed pronounced statistically significant differences among the five groups ($p < 0.0001$). According to the Games–Howell post hoc comparison, the EPO and Epobis groups differed significantly from the HIE group ($p = 0.0023$ and $p = 0.0002$, respectively) (Fig. 6-1).

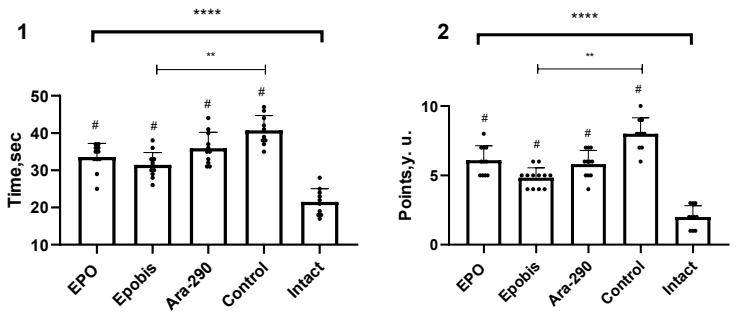



**Figure 6.** Effect of EPO (5000 IU/kg), Epobis (20 mg/kg), and Ara-290 (30 μg/kg) on the time to complete the horizontal pole test and changes in accompanying neurological signs during correction of moderate HIE 7 days after pathology modeling. ***Note:*** **1** – time to complete the horizontal pole test; **2** – change in accompanying neurological signs; # – comparison with the intact group; explanations are provided in the text.

When analyzing changes in neurological signs in the horizontal pole test during correction of moderate HIE, the following mean values were obtained: EPO 6 ± 1; Epobis 4.8 ± 0.7; Ara-290 5.8 ± 0.9; HIE 8 ± 1.5; intact group 1.7 ± 0.6. Relative to the HIE group, these values were 23.9%, 39.6%, 27.3%, and 78.8% lower in the EPO, Epobis, Ara-290, and intact groups, respectively. Multiple comparisons using Dunn's test revealed statistically significant differences between the Epobis and HIE groups ($p < 0.0001$) (Fig. 6-2).

When analyzing the time to complete the vertical pole test and the accompanying assessment of neurological signs during correction of moderate HIE, the following results were obtained:

Descent time on the vertical pole increased in the studied groups compared with the control group (7.8 ± 3.9 s) by 65.5% in the EPO group (12.9 ± 2.2 s) ($p = 0.0052$), by 76.3% in the Epobis group (18.00 ± 3.3 s) ($p = 0.0006$), and by 25.9% (15.08 ± 2.07 s) in the Ara-290 group. In the Games–Howell test, a statistically significant difference was recorded between all studied groups and the intact group ($p < 0.0001$) (Fig. 7-1).

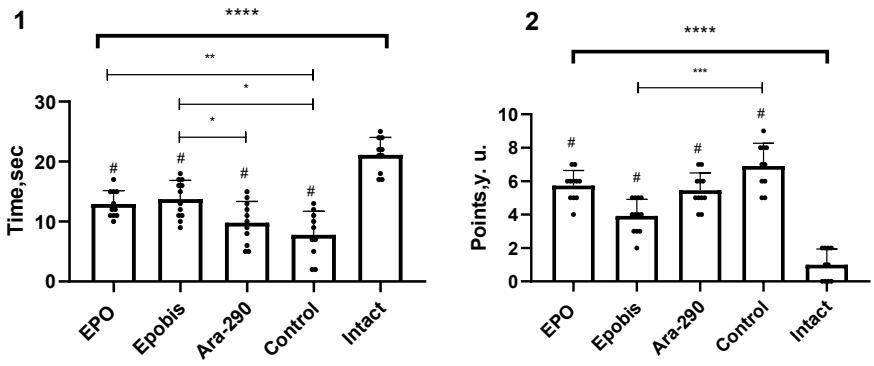



**Figure 7.** Effect of EPO (5000 IU/kg), Epobis (20 mg/kg), and Ara-290 (30 μg/kg) on descent time in the vertical pole test and changes in accompanying neurological signs during correction of moderate HIE 7 days after pathology modeling. ***Note*:** 1 – descent time in the vertical pole test; 2 – change in accompanying neurological signs. # – comparison with the intact group; explanations are provided in the text.

Analysis of accompanying neurological impairments revealed a decrease of the parameter by 39% in the EPO group (5.182 ± 0.874), by 53.9% in the Epobis group (3.9 ± 0.9), and by 27.3% in the Ara-290 group (5.818 ± 1.1) compared with the untreated HIE group (8.5 ± 1). Dunn's post hoc analysis recorded a statistically significant difference between the Epobis, EPO, and HIE groups ($p = 0.0001$). Statistically significant differences in neurological symptom scores were found in the EPO and Ara-290 groups compared with the intact group (Fig. 7-2).

In moderate HIE, the lesion volume in the untreated group was 6266 ± 766.1 px. In the treatment groups, a reduction in the volume of injured tissue was observed by 13.5% in the EPO group (5417 ± 339 px), by 22.2% in the Epobis group (4877 ± 300 px), and by 8.9% in the Ara-290 group (5710 ± 336.2 px) compared with the control group.

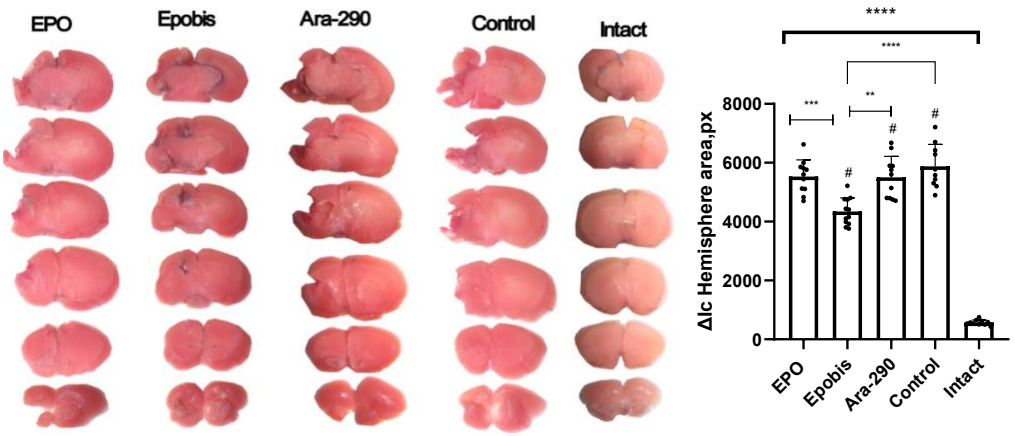



**Figure 8**. Effect of EPO (5000 IU/kg), Epobis (20 mg/kg), and Ara-290 (30 μg/kg) on the volume of macroscopic brain tissue injury during correction of moderate HIE in the phase of reperfusion-inflammatory changes, 7 days after pathology modeling. ***Note*:** # – comparison with the intact group; explanation is provided in the text.

In the Games–Howell post hoc analysis, statistically significant between-group differences were identified. All HIE correction groups differed significantly from the intact group ($p < 0.0001$). Epobis therapy significantly reduced the volume of tissue injury compared with the untreated group ($p = 0.001$), in contrast to the values recorded for the Ara-290 and EPO groups, while significantly exceeding their efficacy. Specifically, Epobis differed from Ara-290 ($p = 0.001$) and from EPO ($p = 0.0001$) (Fig. 8).

**Gene expression analysis after completion of therapy in the mild and moderate HIE groups**

In mild HIE, the expression levels of IL1-β, IL-4, IL-6, and TNF-α reliably indicated a persistent inflammatory tone in the absence of therapy and pronounced anti-inflammatory efficacy in the treatment groups. In the HIE group, IL-6 (3.10 ± 0.47) and TNF-α (2.64 ± 0.83) expression was higher than in the intact group by 47% ($p = 0.0093$) and twofold ($p < 0.0001$), respectively, confirming the presence of an ongoing inflammatory process 7 days after modeling mild HIE. IL-4 expression (2.56 ± 0.60) in the HIE group was lower than in the intact group ($p = 0.0019$), indicating insufficiency of the anti-inflammatory reparative component in the sub-delayed period.

**Table 3**. Effect of EPO (5000 IU/kg), Epobis (20 mg/kg), and Ara-290 (30 μg/kg) on quantitative changes in expression of neuroinflammation marker genes in the reperfusion-inflammation phase during correction of mild HIE

| Expression of the studied gene | EPO | Epobis | Ara-290 | Control | Intact |
|---|---|---|---|---|---|
| IL1-β | 0.32 ± 0.03 | 0.37 ± 0.04 | 0.41 ± 0.11 | 0.52 ± 0.15 | 0.13 ± 0.04 |
| IL-4 | 4.2 ± 0.6 b d e | 9.96 ± 1.2 a b c e | 5.27 ± 0.11 a b c d | 2.56 ± 0.3 a c d e | 3.77 ± 0.9 b d e |
| IL-6 | 1.89 ± 0.5 b d | 1.03 ± 0.3 a b c e | 2.08 ± 0.45 b d | 3.1 ± 0.21 a c d e | 2.1 ± 0.4 b d |
| TNF-α | 1.1 ± 0.3 b | 1.25 ± 0.25 b | 1.72 ± 0.39 b | 2.64 ± 0.37 a c d e | 1.22 ± 0.23 b |

***Note*:** Numerical data are presented as mean ± standard deviation; a – significant difference vs the intact group; b – significant difference vs the HIE group; c – significant difference vs the EPO group; d – significant difference vs the Epobis group; e – significant difference vs the Ara-290 group.

At the same time, no significant differences vs the intact group were recorded for IL1-β expression, which is most likely due to a reduced pathophysiological role of this marker at the given time points. Against the background of therapy with EPO, Epobis, and Ara-290, a statistically significant reduction in IL-6 relative to the untreated group was observed by 39% ($p = 0.0004$), 66.8% ($p < 0.0001$), and 32.9% ($p = 0.0039$), respectively, as well as a statistically significant reduction in TNF-α relative to mild HIE by 58.3% ($p < 0.0001$), 52.7% ($p < 0.0001$), and 34.8% ($p = 0.02$), respectively, indicating decreased transcription of the studied pro-inflammatory genes after correction of mild HIE with recombinant EPO and its peptide analogs. In parallel, a statistically significant increase in IL-4 was noted in the EPO, Epobis, and Ara-290 groups relative to the HIE group by 64.1% ($p < 0.0001$), threefold ($p < 0.0001$), and twofold ($p < 0.0001$), respectively; notably, Epobis demonstrated the most pronounced induction of IL-4, as it exceeded EPO ($p < 0.0001$) and Ara-290 ($p < 0.0001$) for this parameter.

Thus, in mild HIE, therapy not only reduced pro-inflammatory activity but also shifted the cytokine profile toward enhanced anti-inflammatory signaling, most clearly with Epobis. IL1-β expression in mild HIE did not show statistically significant differences between groups, which indicates either the absence of a sustained transcriptional contribution of IL1-β at the selected time point or its high variability and temporal limitation of the peak at earlier time points (Table 3, Fig. 9-1).

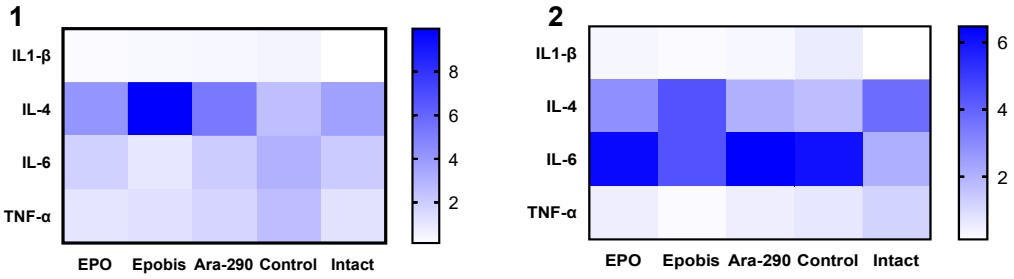



**Figure 9.** Effect of EPO (5000 IU/kg), Epobis (20 mg/kg), and Ara-290 (30 μg/kg) on quantitative changes in expression of neuroinflammation marker genes in the phase of reperfusion-inflammation changes during correction of mild and moderate HIE. ***Note*: 1** – illustration of quantitative changes in gene expression during correction of mild HIE; **2** – illustration of quantitative changes in gene expression during correction of moderate HIE presented as a heat map.

Analysis of the expression of pro-inflammatory marker genes in brain tissues within the lesion area in animals with moderate HIE severity 7 days after modeling revealed a more pronounced neuroinflammatory expression pattern of the studied genes, differing in magnitude from that in mild severity. IL-6 expression in the untreated group was statistically significantly higher than in the intact group by twofold ($p < 0.0001$); however, when comparing expression levels of this gene in the treatment groups with moderate HIE, significant reductions were established only in the Epobis group ($p < 0.0001$), in which it was reduced by 1.5-fold.

**Table 4**. Effect of EPO (5000 IU/kg), Epobis (20 mg/kg), and Ara-290 (30 μg/kg) on quantitative changes in expression of neuroinflammation marker genes in the phase of reperfusion-inflammatory changes during correction of moderate HIE

| Expression of the studied gene | EPO | Epobis | Ara-290 | Control | Intact |
|---|---|---|---|---|---|
| IL1-β | 0.35 ± 0.20 | 0.25 ± 0.09 | 0.31 ± 0.50 | 0.60 ± 0.08 | 0.13 ± 0.04 |
| IL-4 | 2.89 ± 0.37 b d | 4.43 ± 0.90 b c e | 2.07 ± 0.41 a d | 1.73 ± 0.11 a c d | 3.77 ± 0.90 b e |
| IL-6 | 6.25 ± 1.10 a d | 4.42 ± 0.45 a b c e | 6.47 ± 1.10 a d | 6.05 ± 1.50 a d | 2.10 ± 0.40 b c d e |
| TNF-α | 0.53 ± 0.09 | 0.23 ± 0.04 | 0.55 ± 0.13 | 0.72 ± 0.12 | 1.22 ± 0.23 |

***Note*:** Numerical data are presented as mean ± standard deviation; a – significant difference vs the intact group; b – significant difference vs the HIE group; c – significant difference vs the EPO group; d – significant difference vs the Epobis group; e – significant difference vs the Ara-290 group.

This indicates persistence of an IL-6-associated inflammatory circuit in the sub-delayed period under more severe injury. For IL-4, the HIE group maintained a statistically significant decrease relative to the intact group and was lower by 54.7% ($p < 0.0001$). At the same time, Epobis and EPO demonstrated a statistically significant increase in IL-4 relative to HIE by 1.5-fold ($p < 0.0001$) and by 67.7% ($p < 0.0001$), respectively, and Epobis was significantly higher than EPO ($p < 0.0001$) and Ara-290 ($p = 0.001$), indicating a more pronounced ability of Epobis to induce the anti-inflammatory component of the response during correction of moderate HIE. In the Ara-290 group, IL-4 was statistically significantly lower than in the intact group and did not differ from HIE, which is consistent with the absence of restoration of the IL-4-dependent reparative component within this treatment in moderate severity (Table 4, Fig. 9-2).

TNF-α and IL1-β expression in moderate severity did not show statistically significant differences between groups, which is consistent with the fact that at the selected time point these transcripts are not the main discriminators of the therapeutic response in moderate severity, or their dynamics are shifted to earlier windows after injury.

# Discussion

According to current concepts of the course of HIE, the pathological process is conventionally divided into three phases: 0–6 h (acute energy failure), 6–72 h (secondary inflammatory phase), and the remodeling phase (from 72 h to several weeks) (Chakkarapani et al. 2025; Chan et al. 2025; Konrad et al. 2025; Yang et al. 2025). In clinical practice, therapy should be initiated no later than 6 hours in the form of therapeutic hypothermia, the neuroprotective effect of which consists in reducing cerebral metabolism, which leads to a decrease in the intensity of early excitotoxicity, mitochondrial dysfunction, and production of reactive oxygen species; however, real-world organization of care and variability in the time to diagnosis result in some patients not entering the optimal time window, which subsequently leads to cognitive deficits of varying severity (Arnautovic et al. 2024). In this study, therapeutic interventions were initiated 4–5 hours after HIE modeling, with prior stratification of animals by severity of the pathological process over 7 days, which corresponds to correction of the secondary inflammatory phase with transition to the early remodeling phase. The secondary phase is considered, is considered crucial for delayed injuries, including oxidative stress and membrane damage, which leads to activation of neuroinflammatory cascades, blood-brain barrier dysfunction, and apoptosis. Accordingly, pharmacological strategies should be phase-specific.

Erythropoietin derivatives used in this study are considered promising candidates for HIE therapy because they reproduce the tissue-protective effects of recombinant erythropoietin, which is approved for clinical use; however, its application is limited due to severe hematopoietic complications, which is unlikely in the case of potential correction with its derivatives because their mechanism of action is associated with the heterodimeric EPOR-CD131 receptor rather than the homodimeric EPOR receptor located in hematopoietic tissues. Ara-290 is a short 11-amino-acid peptide aimed at activating the tissue-protective receptor circuit associated with CD131. Epobis is a peptide fragment of erythropoietin that mimics a portion of the binding site (amino acid residues 36–56). Substantial differences in their pharmacokinetics compared with

recombinant EPO and with each other are fundamental for interpreting neuroprotective effects. Recombinant EPO is characterized by plasma elimination within several hours, with complete clearance approximately 8.8 hours after administration (Lissy et al. 2011). However, as a large glycoprotein molecule, it has limited penetration into the CNS under an intact BBB. After a hypoxic-ischemic episode, BBB permeability may increase within the first hours and exhibit a phase-dependent pattern against a background of neuroinflammation and neurovascular unit dysfunction, which explains the appearance of endogenous EPO in the cerebrospinal fluid after 2–3 hours but allows for limited direct central action in the earliest minutes after injury (Fong et al. 2024). For Epobis, a longer systemic exposure up to 24 hours and detection in cerebrospinal fluid as early as 2 hours even with an undamaged BBB have been described, which is attributed to its tetrameric structure (Dmytriyeva et al. 2016). These pharmacokinetic characteristics potentially increase the likelihood of influencing secondary injury cascades and may be more relevant for realizing tissue-protective effects in the subacute and delayed periods (Arnautovic et al. 2024). In contrast, Ara-290 has an extremely short systemic exposure and may be eliminated within several minutes. At the same time, for agonists of the tissue-protective receptor circuit, a mechanism is discussed in which short-term exposure initiates long-lasting intracellular programs; therefore, the effect may be determined by the duration of the signaling response after receptor activation, including in regions with early barrier dysfunction (Collino et al. 2015). Thus, the known pharmacokinetic properties of the studied compounds justify their use in the phase of secondary inflammation and early remodeling.

The use of LSCI-based stratification increases the translational value of the study. Objectification of baseline severity makes it possible to evaluate the neuroprotective properties of the drugs at an initially controlled level of injury prior to therapeutic interventions and to interpret differences in morphological, behavioral, and molecular outcomes within fixed time windows more accurately.

Pharmacological correction of HIE in the early remodeling phase with the erythropoietin derivatives Epobis and Ara-290 was generally accompanied by a neuroprotective effect, manifested as improved motor-coordination functions, reduced severity of neurological symptoms, and decreased macroscopic lesion volume compared with untreated HIE groups. The magnitude and persistence of the effect depended on the severity of injury and the drug selected.

In mild HIE, Epobis showed the greatest advantage according to the combination of morphological and behavioral criteria. Its use was associated with a more pronounced restoration of motor-coordination functions, in particular an increase in holding time on the inverted grid compared with the control group and superiority over EPO, a reduction in accompanying neurological symptoms in the vertical pole test reaching values comparable to the intact group, and the most pronounced decrease in macroscopic injury volume. In comparison, EPO and Ara-290 demonstrated moderate, overall comparable efficacy; however, across most key outcomes they were inferior to Epobis, indicating a more comprehensive mode of action of Epobis in mild injury. Additionally, it should be noted that in the horizontal pole test, statistically confirmed improvement was recorded for EPO and Epobis, whereas for Ara-290 no pronounced effect was detected in this test, which limits interpretation of its motor efficacy within this behavioral domain.

In moderate HIE, therapy also improved motor-coordination parameters and reduced neurological deficit relative to the untreated control; however, the advantage of the agents was distributed differently. The most robust statistically confirmed effects in motor tests were shown by Epobis and EPO, providing an increase in holding time on the inverted grid and a reduction in the time to complete the horizontal pole. At the same time, Epobis was distinguished by a more pronounced reduction in neurological symptomatology and represented the only correction option for which a statistically significant decrease in macroscopic lesion volume was confirmed, with superiority over EPO and Ara-290. In moderate severity, Ara-290 exhibited a less stable efficacy profile, which was manifested by a smaller number of significant differences across a range of functional and morphological parameters.

Concurrently, in mild HIE, therapy not only reduced pro-inflammatory activity but also shifted the cytokine configuration toward enhanced anti-inflammatory signaling, most clearly with Epobis. IL1-β expression in mild severity did not demonstrate statistically significant differences between groups, indicating either the absence of a sustained transcriptional contribution of IL1-β at the selected time point or its high variability and temporal limitation of its peak at earlier time points. In moderate HIE, a more pronounced neuroinflammatory response persisted. In the untreated group, IL-6 expression remained substantially elevated. A statistically significant reduction in IL-6 expression compared with the untreated group was identified only with Epobis. IL-4 expression remained reduced in the absence of therapy; against the background of Epobis and EPO, an increase was observed, with Epobis demonstrating a significant advantage over EPO and Ara-290, whereas Ara-290 did not provide restoration of IL-4. TNF-α and IL-1b expression in moderate HIE did not differ statistically significantly between groups.

# Conclusion

Thus, in the mild HIE model, Epobis demonstrated the greatest neuroprotective effect, providing a more pronounced restoration of motor-coordination functions, reduction of accompanying neurological deficit during testing, and maximal decrease in macroscopic injury volume, whereas EPO and Ara-290 exerted a moderate effect and were inferior to Epobis in most parameters. In moderate HIE, the most reproducible improvements in motor function were observed with Epobis and EPO compared with Ara-290. Importantly, a statistically significant reduction in macroscopic lesion volume and a more pronounced attenuation of neurological symptomatology compared with EPO and Ara-290 were confirmed only for Epobis. At the molecular level, Epobis provided the most pronounced immunomodulation, producing an anti-inflammatory shift in mild HIE and, in moderate HIE, significantly reducing IL-6 while simultaneously inducing IL-4 most effectively.

# Additional Information

### Conflict of interest

The authors declare the absence of a conflict of interests.

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### Ethics statement

The study was approved by the Animal Ethics Committee of Belgorod State National Research University (BelSU), approval No. №01-10/25 dated 1 October 2025.

### Data availability

All of the data that support the findings of this study are available in the main text.

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