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Targeting mitochondrial dysfunction with HSP70 modulators for neuroprotection in experimental traumatic brain injury combined with acoustic barotrauma

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Introduction: Combat-related traumatic brain injury (TBI) resulting from blast munitions (grenades, mortars, mines, bombs, artillery shells, etc.) has been a primary cause of military casualties over the past two decades, accounting for over 42% of post-evacuation combat deaths. Given the rising incidence of servicemen sustaining TBI combined with blast-induced neurotrauma (acoubarotrauma), optimizing pharmacological neuroprotection through positive modulators of heat shock protein 70 (HSP_{70}) represents an urgent medical priority.

Aim: To evaluate the neuroprotective efficacy of tamoxifen, melatonin, glutamine, Angiolin ([(S)-2,6-diaminohexanoic acid 3-methyl-1,2,4-triazolyl-5-thioacetate]), and HSF-1 (heat shock factor 1) by assessing their effects on mitochondrial dysfunction and energy metabolism in an experimental model of TBI combined with blast injury.

Materials and methods: Combined TBI and blast injury was induced using a pressurized CO_2 stream via a gas-cylinder pneumatic device in 170 anesthetized male Wistar rats aged 3 months (weighing 230–240 g; n = 10 sham-operated). Melatonin (5 mg/kg), tamoxifen (1 mg/kg), glutamine (25 mg/kg), and piracetam (500 mg/kg) were administered intragastrically; HSF-1 (200 μL/kg) was injected intraperitoneally; and Angiolin gel (50 mg/kg) was delivered intranasally for 4 consecutive days immediately post-TBI. Animal mortality and neurological deficits were assessed. In blood serum, as well as cerebral cytosolic and mitochondrial fractions, levels of matrix metalloproteinase-9 (MMP-9), neuron-specific enolase (NSE), HSP70, ATP, ADP, AMP, malate, pyruvate, lactate, NAD+-malate dehydrogenase (NAD+-MDH) activity, mitochondrial permeability transition pore (mPTP) opening rate, and mitochondrial inner

membrane potential ($\Delta\Psi m$) were determined. Ultrastructural changes in the hippocampal CA1 subfield were examined via transmission electron microscopy.
Results: On day 4 post-TBI, untreated control animals exhibited severe neurological deficits, increased mortality, elevated circulating NSE ($p < 0.05$) and MMP-9 ($p < 0.05$), and a cytosolic/mitochondrial HSP_{70} deficit ($p < 0.05$). These changes were accompanied by secondary mitochondrial dysfunction: decreased ATP and ADP alongside increased AMP ($p < 0.05$), elevated lactate with decreased pyruvate and malate ($p < 0.05$), suppressed NAD+-MDH activity ($p < 0.05$), loss of $\Delta\Psi m$ ($p < 0.05$), accelerated mPTP opening ($p < 0.05$), and an increased count of structurally compromised mitochondria ($p < 0.05$). Treatment with the investigated agents upregulated HSP_{70} expression, normalized energy metabolism, mitigated mitochondrial dysfunction, and provided overall neuroprotection. Comparative efficacy ranking demonstrated the following order: piracetam < glutamine < tamoxifen < melatonin < HSF-1 < Angiolin.
Conclusion: These findings substantiate the neuroprotective potential of HSP_{70} modulators in experimental combined TBI and blast injury. Preclinical development of Angiolin gel and HSF-1 is experimentally justified as a promising neuroprotective strategy for blast-induced neurotrauma.

KEYWORDS
Angiolin, blast injury (acoubarotrauma), heat shock protein 70 (HSP_{70}), malate-aspartate shuttle, mitochondrial dysfunction, neuroprotection, traumatic brain injury (TBI)

1 Introduction

Combat-related traumatic brain injury (TBI) has been a leading cause of military casualties, accounting for 20%–25% of combat injuries in U.S. military conflicts and accounting for over 42% of combat deaths recorded after evacuation to the hospital. More recent epidemiological data from the current conflicts in Iraq and Afghanistan indicate that up to 30% of combat injuries involve the head and neck region and that the vast majority (over 80%) of these casualties are the result of blast. Explosive devices (i.e., improvised explosive devices (IEDs), grenades, mortars, mines, bombs, etc.) account for 76% of casualties among U.S. military personnel (McLaughlin, 2013; Shear and Tortella, 2013; McKee and Robinson, 2014). Due to ongoing combat operations in Ukraine and the increasing incidence of combined TBI and blast trauma among military personnel, which leads to the loss of combat capability of the personnel of the Armed Forces of Ukraine, optimization of approaches to drug neuroprotection is relevant (Shvets et al., 2020; Chernenko, 2025). The above facts, as well as the variety of clinical manifestations and severity of TBI, necessitate the development of strategies of scientifically substantiated approaches to its pathogenetic treatment, based on the study of various aspects of its pathogenesis. In the pathogenesis of TBI, an important role is played by secondary damage to brain tissue, the central place among which is occupied by neurohypoxia and cerebral ischemia, as a result of which a cascade of molecular and biochemical reactions is launched, leading to the development of functional and morphological disorders of brain structures (Freire et al., 2023; de Macedo Filho et al., 2024)). One of the main mechanisms of damage development in tissues and organs after TBI is mitochondrial dysfunction, which has a multifaceted effect on intracellular processes: it contributes to the development of energy deficiency, oxidative stress, and activates the mechanisms of apoptosis, mitoptosis, and ferroptosis (Luan et al., 2023). In this regard, mitochondria are considered as one of the promising targets for neuroprotection in the development of technologies for protecting the brain in TBI, combined with acoubarotrauma by influencing the target links of mitochondrial dysfunction (ROS-dependent damage to the mitochondrial inner membrane protein (ATP/ADP antiporter), oxidative damage to membrane proteins, deficiency of intramitochondrial antioxidants, deficiency of intramitochondrial heat shock protein 70 kDa (HSP_{70}), oxidative damage to the lipid layer of mitochondrial membranes) with various pharmacological agents (Cheng et al., 2012; Belenichev et al., 2025b; Himic et al., 2026). Neuroprotection in combat TBI is aimed at preventing and mitigating the consequences that arise after the primary action by correcting secondary brain damage using supportive therapy and interventions. Modern medicine has a wide range of drugs for the treatment and prevention of the consequences of TBI, but not all drugs meet modern requirements. Thus, neuroprotective drugs used in the treatment of strokes and providing some therapeutic success have proven ineffective in combat-related brain injury. Many of them have a number of side effects that limit their use in these conditions (Gu et al., 2017). Therefore, the search and development of new highly effective, safe means of pharmacological brain protection, characterized by polytropy of pharmacodynamic effects, aimed at the simultaneous correction of different links in the pathogenesis of TBI is one of the priority areas of development of fundamental

and clinical medicine (Shear and Tortella, 2013; Buccilli et al., 2024).

Currently, the role of endogenous neuroprotection and its intermediate – HSP_{70} in the regulation of neuronal survival mechanisms in conditions of cerebral ischemia is being considered. HSP_{70} performs a chaperone function and takes part in the regulation of growth, development, transfer of genetic material, signal transduction, and cell death. The neuroprotective properties of HSP_{70} have been confirmed in various models of ischemic damage *in vitro* and *in vivo*. The neuroprotective effect of HSP_{70} in conditions of ischemia is explained by its direct antiapoptotic, antioxidant activity, and effect on the thiol-disulfide system of the brain (Yenari, 2013; Belenichev et al., 2023; Su and Zheng, 2025). There are works devoted to the study of the positive effect of HSP_{70} on the energy metabolism of the brain in conditions of cerebral ischemia, which is realized due to the direct mitoprotective effect, as well as by increasing ATP production in compensatory cytosolic-mitochondrial shunts. It has been established that the level of HSP_{70} expression in the brain determines the severity of neurological disorders in modeling CNS pathology in laboratory animals (Wang et al., 2012; Chauhan and Sharma, 2024).

Thus, the development of new approaches to neuroprotection of TBI combined with acoubarotrauma by using or creating agents – positive modulators of HSP_{70} is an important task of modern pharmacology. Of particular interest in this regard are agents with high neuroprotective potential – tamoxifen, melatonin, glutamine, Angiolin ([(S)-2,6-diaminohexanoic acid 3-methyl-1,2,4-triazolyl-5-thioacetate) and heat shock factor-1 (HSF-1) (Belenichev, 2014). Angiolin, an anti-ischemic and endothelioprotective agent, increases the expression of HSP_{70} in the mitochondria and cytosol of the brain and myocardium during acute and chronic ischemia, as well as in cases of alcohol-related disease. Furthermore, in response to ischemia, it normalizes the expression of c-fos and VEGF (Belenichev, 2014). Tamoxifen, when administered in low doses, acts as an estrogen receptor agonist and is capable of increasing HSP_{70} concentrations in the mitochondria and cytosol of the brain and myocardium during acute ischemia (Pavlov and Belenichev, 2014). Glutamine mitigates ischemic brain injury and promotes functional recovery via HSP70, which points to its potential as a neuroprotective agent (Luo et al., 2019). Melatonin is also of interest for research into its efficacy in cases of traumatic brain injury (TBI), as it exerts a neuroprotective effect linked to the increased expression of HSP_{70} (Jung et al., 2022) HSF-1 is a potent modulator of endogenous neuroprotection, and the activation of the chaperone HSP_{70} serves as its primary molecular mechanism for protecting nerve cells during cerebral ischemia and neurodegenerative pathology. It is of potential interest as a neuroprotective agent for TBI (Kim et al., 2020; Zatsepina et al., 2021) The data presented here indicates the relevance of this study. All this justifies the assessment of the neuroprotective properties of Angiolin, tamoxifen, glutamine, HSF-1, and melatonin in experimental TBI conditions and the possible discovery of previously unknown HSP_{70}-dependent mechanisms aimed at correcting mitochondrial dysfunction in the implementation of this neuroprotective effect.

The aim of the study: To evaluate the neuroprotective effect of tamoxifen, melatonin, glutamine, angiolin and HSF-1 via the influence of the drug on the indices of mitochondrial dysfunction and energy metabolism in the conditions of modeling of TBI combined with acoubarotrauma.

2 Materials and methods

2.1 Experimental animals and housing conditions

Experimental studies were conducted on 3-month-old male Wistar white rats (Rattus norvegicus) weighing 230–240 g. The animals were obtained from the vivarium of the Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of Ukraine. A total of 170 animals participated in the experiment. Upon receipt, each animal was examined by a qualified veterinarian to determine its health status. The animals were housed in polycarbonate cages measuring 550 × 320 × 180 mm with galvanized steel lids measuring 660 × 370 × 140 mm and glass drinking bowls. Each cage contained 5 rats. Each cage was labeled with the study number, species, sex, animal numbers, pharmacological agent, and its dose. The cages were arranged on racks according to the dose levels and cage numbers indicated on the labels. The animal housing room was maintained at a temperature of 20–24 °C, humidity of 30%–70%, and a 12-h light/12-h dark cycle. All rats were fed ad libitum a standard laboratory diet supplied by Phoenix, Ukraine. Water from the city tap (reverse osmosis and UV sterilization) was available ad libitum. Autoclaved alder (Alnus glutinosa) sawdust was used as bedding. Upon receipt, the animals were weighed, numbered, temporarily observed using a brilliant green solution, and placed in cages (5 individuals per cage) for acclimatization for 7 days. Before the experiment, animals that met inclusion criteria (healthy appearance, food and water consumption, and motor activity within normal limits) were randomly assigned to groups of 20 each. These animals were sequentially marked using a fucorcin solution. Animals that did not meet the criteria (those who were sick, had previous or concomitant illnesses, such as depressive behavior, or those who became agitated under certain stressors) were excluded from the experiment during quarantine. Data obtained during the experiment were recorded directly in the experimental data log in the animal room. This log also contained the dated signatures of the researchers conducting the study and observing the animals. All noticeable signs of poor health and behavioral deviations were recorded daily between 10:00 and 12:00 during the experiment, and a death check was conducted at 17:00.

2.2 Bioethics

The studies were conducted in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Scientific Purposes (Council of Europe, 1986), as well as national bioethics standards and regulations. The bioethical aspects of the study protocol were approved by the Bioethics Committee of the ZDMPhU (Protocol No. 32 of February 21, 2026) in accordance with the Methodological Guidelines "Bioethical Review of Preclinical and Other Scientific Research

Conducted on Animals" (2006). All animal procedures complied with ARRIVE 2.0 guidelines.

2.3 Model of TBI combined with blast acoubarotrauma

Experimental TBI combined with blast acoubarotrauma, caused by a pressurized carbon dioxide flow, was simulated using an original model and a Baikal MP-654K gas-cylinder pneumatic pistol (Izhevsk, Russia, certificate No. ROSS RU MZh03.B02518CO2) (12 g) under pressure (Crosman, USA, serial no. 456739) (Semenenko, 2024). The constancy of the CO_2 pressure at the muzzle was monitored by preliminary recording the flight speed of a spherical steel ball (Crosman, USA, serial number 03675482) with a caliber of 4.5 mm and a mass of 0.3 g at a distance of 1 cm from the inner muzzle opening using an induction muzzle chronometer. It was found that when using carbon dioxide cylinders (t_{air} = 19 °C, P_{atm} = 720-755 mm Hg) of the same serial number 03675482, when firing the next 10 shots at 5-min intervals after the first 5 test shots, the flight speed of the ball was constant, without reliable fluctuations, and amounted to 110–115 m/s. With these velocity characteristics, the carbon dioxide pressure at the muzzle was uniformly constant at 7.4–7.5 J, allowing for the reproduction of brain injury under identical conditions in all series using a CO_2 cylinder, with no more than 10 experimental shots. To ensure a unified approach to creating severe TBI, the range of action of the damaging agent (CO_2 under pressure) was kept close to each other. Rats anesthetized with sodium thiopental (40 mg/kg) underwent right-sided osteoplastic craniotomy intraperitoneally in the projection of the middle cerebral artery, with a hole diameter of 5 mm^2. After fixing the rat in a prone position with its head down, the shot was fired, the bone fragment on the periosteum, along with the aponeurosis, was returned to its place, and the wound was sutured layer by layer. In order to thoroughly evaluate the magnitude of the neuroprotective effect of pharmacological agents and to establish possible mechanisms of this effect, only severe TBI was subsequently used, which was achieved under conditions where the opening of the bolt carrier of the air pistol was pressed closely to the center of the trepanation hole.

2.4 Experimental groups

The test drugs (tamoxifen, melatonin, HSF-1, glutamine, Angiolin) and the reference drug piracetam were administered daily for 4 days (the acute period of brain injury). A separate group of sham-operated animals was formed; they underwent all stages of the surgical procedure, but without the pistol shot.

Animals were randomized into experimental groups:

1. Sham-operated animals (SO) (n = 10);
2. Laboratory animals with TBI (control) (n = 40);
3. Laboratory animals with tamoxifen (1 mg/kg) (Pavlov and Belenichev, 2014) (n = 20);
4. Laboratory animals with TBI with the administration of melatonin (5 mg/kg) (Belenichev, 2014) (n = 20);
5. Laboratory animals with TBI treated with HSF-1 (200 μl/kg, or 50 mg/kg (Belenichev and Bila, 2017) (n = 20);
6. Laboratory animals with TBI treated with glutamine (25 mg/kg) (Belenichev, 2014) (n = 20);
7. Laboratory animals with TBI administered Angiolin (50 mg/kg) (Belenichev et al., 2025a) (n = 20);
8. Laboratory animals with TBI administered the reference drug piracetam (500 mg/kg) (Severyukhin et al., 2021) (n = 20).

A priori sample size calculation was performed based on the results of a pilot study using the primary efficacy endpoint–the neurodestruction marker NSE. To ensure a statistical power of $1-\beta = 0.80$ at an alpha error level of $\alpha = 0.05$ two-tailed test), the formula for comparing the means of two independent samples was used:

$$n = 2 \times \left(\frac{Z_{1-\alpha/2} + Z_{1-\beta}}{d}\right)^2$$

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where $Z_{1-\alpha/2} = 1.96$ (the critical value of the standard normal distribution for $\alpha = 0.05$),$Z_{1-\beta} = 0.84$ (the value corresponding to the statistical power 80%), and d is Cohen's effect size (the expected clinically meaningful difference between means divided by the pooled standard deviation). Based on pilot data, the minimum expected effect size was $d \approx 1.0$. Substituting these values into the formula determined the minimum required sample size:

$$n = 2 \times \left(\frac{1.96 + 0.84}{1.0}\right)^2 \approx 15.68$$

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Given the high expected mortality in the control group during TBI modeling (up to $60-65\%$), the size of the experimental groups was initially increased to $N = 20$ animals. Effect sizes (Cohen's d). $d = \frac{|M_1 - M_2|}{S_{pooled}}$ with 95% confidence intervals provided.

During the experiment, blinding was applied at the stages of behavioral testing using the P. McGraw scale, as well as the collection and analysis of biological material. Animal testing according to the P. McGraw scale was performed sequentially across different experimental groups, and the investigator conducting the tests was blinded to the group allocation of the animals. Biological samples were assigned sequential numbers starting from 1 and upwards. The research teams performing biochemical and ELISA analyses, as well as transmission electron microscopy (image capture and counting), were unaware of the experimental group to which the biological samples belonged. Access to the decoding key was restricted exclusively to the principal investigator.

Pharmacological agents–melatonin, tamoxifen, glutamine, and piracetam–were administered intragastrically using an atraumatic metal tube in 1% starch mucus. HSF-1 was administered intraperitoneally. To enhance neuroavailability, Poly-L-arginine hydrochloride (Sigma-Aldrich, Cat# 26982-20-7) was used at an HSK-1-to-PL ratio of 5:1. Angiolin in the form of a 1% nasal gel was administered intranasally into both nostrils using a 1 ml Becton Dickinson (BD) syringe without a needle and 14G × 1/2" needles with an atramatic tip (Becton Dickinson, Spain, Cat. No. 309659, Kruuse, Denmark, Cat. No. 120755). The dosed volume of gel is

injected to a depth of 2–3 mm into the rat's nostril, allowing the rat to inhale it, drop by drop every 2–3 s. The gel is administered with the rat in a supine position with an intranasal grasp (pulling the scalp back closer to the nose) in the experimenter's hands and its neck held parallel to the floor.

2.5 Pharmacological agents and drugs

In this study, we used the following pharmacological drugs: tamoxifen (tablets, Cod# L02BA01, Orion Corporation, Espoo, Finland), angiolin (substance (S)-2,6-diaminohexanoic acid 3-methyl-1,2,4-triazolyl-5-thioacetate) (Scientific and Technological Complex "Institute of Single Crystals" of the National Academy of Sciences of Ukraine (batch 2451117, Kharkiv, Ukraine), composition of the 1% nasal gel (per 100 g): Angiolin – 1.0 g; Sodium carboxymethyl cellulose (Na-CMC) – 1.5 g; D-panthenol – 1.0 g; Tween-80 – 1.2 g; benzalkonium chloride – 0.02 g; purified water – up to 100 g.Nasal administration of the neuroprotector Angiolin represents an innovative drug delivery method directly into the brain via the nasal cavity, circumventing the blood-brain barrier, which significantly enhances neuroavailability and minimizes the loss of the active substance (Belenichev et al., 2024b), HSF-1 (recombinant, expressed in E. coli, SRP5188, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany, UNSPSC Code# 12352200), melatonin (capsules, Now Foods, USA, Cod# 348), glutamine (substance, Merck KGaA, Darmstadt, Germany) Code#12352209), piracetam (tablets, N06BX03, Farmak, Kyiv, Ukraine). 1% Angiolin nasal gel was prepared in the laboratory of the Department of Medicine Technology at ZSMU. Pharmaceutical-grade excipients were used, obtained from SINBIAS Research and Production Company LLC and Istok-Plus LLC, Ukraine. The composition of the excipients in Angiolin nasal gel was substantiated based on previous studies (Belenichev et al., 2024a). Precise weighing of substances and chemical compounds was performed using a WT-500 torsion balance (Kyiv Medical Equipment Plant "Medaparatura", Kyiv, Ukraine). Electronic micropipettes (Brand, Wertheim, Germany) and analytical-grade chemical glassware were used to dilute and prepare solutions and solutions based on 1% starch mucus. Animals were weighed using an AUX 220 electronic scale (Shimadzu Corporation, Kyoto, Japan) before each drug administration.

2.6 Pharmacological methods

Every day for 4 days, the animals were assessed for neurological deficits using the C. P. McGraw stroke index scale with our modification (McGraw, 1977; Belenichev et al., 2024b). The severity of the condition was recorded using a score: mild – up to 3 points, moderate – from 3 to 7 points, and severe – greater than 7 points. Animal mortality was calculated after recovery from anesthesia in each group and presented as a percentage of the group and in absolute terms.

2.7 Anesthesia and euthanasia

Animals were euthanized on day 4 of the experiment under general anesthesia induced by sodium thiopental (40 mg/kg, intraperitoneally). Sodium thiopental lyophilisate (0.5 g vials; Kyivmedpreparat LLC, Kyiv, Ukraine, code No. N01AF03) was dissolved in saline to obtain a 4% solution. This solution was administered using an insulin syringe at a dose of 0.1 ml per 100 g of body weight. Animals were then sacrificed by cervical spine transection for biochemical, molecular, and ultrastructural studies.

2.8 Collection and processing of biological samples

Blood for research was collected from the cervical aorta. Blood samples were centrifuged using an Eppendorf 5804R centrifuge at +4 °C with a speed of 1500 rpm for 20 min. Blood was quickly collected from the brain, and the meninges were separated. The fragments to be analyzed were placed in liquid nitrogen and ground to a powder. Then, an accurately weighed sample of brain tissue ground in liquid nitrogen, weighed on a WT-500 torsion balance (Kyiv Medical Equipment Plant "Medaparatura", Kyiv, Ukraine), was homogenized at a temperature of +2 °C in a 10-fold volume of a medium containing sucrose (250 mmol), Tris-HCl buffer (20 mmol) and EDTA (1 mmol) at pH 7.4. The cytosolic and mitochondrial fractions were separated by differential centrifugation at a temperature of +4 °C using a Sigma 3-30k refrigerated centrifuge (Sigma Laborzentrifugen GmbH, Germany, cat. No. SIGMA_22007 EAN: 4064343399104). The fraction was purified by pre-centrifugation for 7 min at 1000 g, and then subjecting it to a second centrifugation for 20 min at 17,000 g. This process was carried out to remove large cell fragments. The supernatant was collected and stored at –80 °C. The dense mitochondrial pellet obtained after resuspension was used for further studies. To conduct enzyme-linked immunosorbent assay and biochemical analysis of the mitochondria, they were disrupted with the non-ionic detergent Triton X-100. For this purpose, 0.2 ml of a 0.1% Triton X-100 solution prepared in 0.2 M Tris-HCl buffer, pH 7.4, was added to the mitochondrial pellet. The pellet was suspended in a Potter-Elvehjem microhomogenizer (DWK Life Sciences GmbH, Germany). The samples were left on ice for 30 min. And then centrifuged for 20 min at 24,000 g using the same refrigerated centrifuge Sigma 3-30k (Germany).

2.9 Enzyme-linked immunosorbent assays

Matrix metalloproteinase-9 (MMP-9) concentrations were determined by enzyme-linked immunosorbent assay in serum using the Rat Total MMP-9 ELISA Kit, Catalog # RMP900 (Biotechne, USA), according to the instructions. MMP-9 is an important molecular marker and a contributor to the development of secondary brain injury following traumatic brain injury (TBI). Its concentration increases immediately after trauma and correlates inversely with Glasgow Coma Scale (GCS) scores, while the dynamics of MMP-9 help assess the risk of complications and vascular instability, and predict subsequent neurological outcomes (Liu et al., 2014).

NSE concentrations were determined by enzyme-linked immunosorbent assay in serum using the Neuron-Specific Enolase, NSE (NSE) ELISA Kit for Rat, Catalog # MBS161801

(MyBioSource.Inc, USA), according to the instructions. NSE is an intracellular enzyme (γ-enolase isoform) found predominantly in neurons, which serves as a laboratory biomarker for neural tissue damage in TBI. The concentration of NSE in blood and cerebrospinal fluid correlates with TBI severity, and its dynamics help predict subsequent neurological deficits and increased risks of mortality following TBI (Behforouz et al., 2025).

Heat shock protein 70 kDa (HSP_{70}) concentrations were determined by enzyme-linked immunosorbent assay in the cytosolic and mitochondrial fractions of brain homogenate using the Rat HSP_{70} ELISA Kit, Catalog # A7766 (Antibodies.com, UK). All studies were performed using a plate enzyme immunoassay analyzer (SIRIO S, Italy).

2.10 Biochemical research methods

Biochemical analyses were performed according to the methods described by Belenichev et al. (2024b). The concentration of ATP, ADP, and AMP in the mitochondrial fraction was determined by thin-layer chromatography using a dioxane-isopropanol-water-ammonia (4:2:4:1) solvent system as the mobile phase, followed by quantitative determination by spectrophotometry at a wavelength of 260 nm. Pyruvate concentration was determined in the cytosol fraction using the Zohl-Lamprecht method, which is based on the NADH-dependent reduction of pyruvate to lactate under the action of lactate dehydrogenase. Measurements were made at a wavelength of 340 nm based on the decrease in the amount of NADH. Malate concentration was determined in the cytosolic fraction using the Hochorst method. The principle of the method lies in the conversion of malate to oxaloacetic acid under the action of malate dehydrogenase in a hydrazine-glycine buffer with parallel equimolar reduction of NAD+ to NADH. Spectrophotometric determination of the NADH increase was performed at a wavelength of 340 nm. Lactate concentration was determined in the cytosolic fraction using the Hochorst method using lactate dehydrogenase, which converts lactate to pyruvate in the presence of NAD+ in a hydrazine-glycine buffer medium. NADH formation is equimolar to the amount of oxidized lactate. Spectrophotometric measurement of NADH was performed at a wavelength of 340 nm. NAD-dependent malate dehydrogenase activity was determined in the mitochondrial fraction by changing the rate of NAD reduction in the incubation medium at saturated concentrations of substrates and cofactors. Measurements were made immediately after the addition of 0.1 ml of the sample (E1) and after 3 min (E2) at a wavelength of 340 nm.

Rate of mitochondrial permeability transition pore (mPTP) opening: A mitochondrial suspension (1 mg protein per sample) is added to the incubation medium (70 mM sucrose, 5 mM HEPES, 70 mM KCl, 0.5–1 mM KH2PO4, pH 7.4). The rate of mPTP opening was determined at λ = 540 nm and 25 °C under constant stirring for 25 min by recording the optical density difference of the mitochondrial suspension ($Å_2$-$Å_1$). A substantial absolute value of ΔE serves as a classical kinetic marker of pore opening (mPTP) and the concomitant matrix swelling characteristic of mitochondrial dysfunction. This wavelength is chosen because, at 540 nm, there is no significant absorbance by cytochromes, phospholipids, proteins, or respiratory complexes, thereby enabling the isolated registration of changes solely in mitochondrial geometry and volume while minimizing artifacts from redox state fluctuations.

The membrane potential (MP) (Ψ) of the mitochondrial charge is measured in the presence of safranin O. Measurement procedure: The potential generated on the inner mitochondrial membrane was recorded spectrophotometrically in dual-wavelength mode (511–533 nm) with safranin O as a potential-dependent probe (18 μM). The measurements were performed in a 10 × 10 mm glass cuvette with a working volume of 2 ml. The measurements were performed in a sodium medium. The sodium medium contained: 0.62 mM NaCl; 40 mM Caps (3-[cyclohexylamino]-1-propanesulfonic acid) -NaOH (pH = 10). The protonophore uncoupler FCCP (p-trifluoromethoxyphenylhydrazone) and the antiporter monensin were used for potential dissipation. The level of total protein in the samples was determined by the Lowry method using a kit from Bio-Rad Laboratories, Inc, Hong Kong. The measurements were performed on an Eppendorf BioSpectometer (USA) and an Agilent Fluorescence-spectrophotometer (USA).

2.11 Electron microscopy

For transmission electron microscopy, brain tissue (hippocampal CA_1 area) was fixed with 3% glutaraldehyde in buffer, pH 7.4, for 2 h at 40 °C. The tissue was then post-fixed with 1% osmium tetroxide in buffer for 1.5 hours and dehydrated in successive alcohol solutions (70% alcohol saturated with uranyl acetate). The tissue samples were embedded in Epon-812 epoxy resin, diluted with acetone by 3, 2, and 1.5 times, respectively, each time, allowing the specimens to soak in the resin overnight at room temperature. The final step was to place the specimens in undiluted resin at 37 °C overnight and then polymerize the Epon at 60 °C until a solid glassy state was achieved. Serial ultrathin sections were prepared on an LKB-III ultramicrotome and stained with Reynolds lead. The resulting preparations were examined and photographed under a TEM 100-01 transmission electron microscope (Selmi, Ukraine). To quantify mitochondria with damage, the electron microscopic images were scanned, and the total number of mitochondria and the number of damaged mitochondria were counted using a SAI-01 computerized ultrastructural image analyzer (Selmi, Ukraine). For each specimen, 3 ultrathin sections were prepared. When counting the number of mitochondria, 20 fields of view were analyzed. The proportion of mitochondria with damage was calculated as the ratio of the number of damaged mitochondria to the total number of mitochondria in the image. Signs of mitochondrial damage were considered to be: mitochondrial swelling (increased volume, clearing of the matrix); destruction or dissolution of cristae (complete or partial disappearance, fragmentation, or chaotic arrangement of cristae); vacuolization of the matrix (appearance of intramitochondrial vacuoles or membrane ruptures); loss of membrane integrity (rupture of the outer and/or inner mitochondrial membrane); and the appearance of megamitochondria. Non-overlapping images containing at least 80 mitochondria were selected for counting and scanning. Mitochondria were counted on a computer using Adobe Photoshop 7.

2.12 Statistical analysis

Statistical analysis of the results was performed using Statistica software (version 10.0, IBM Corp., Armonk, NY, USA) and Microsoft Excel (Microsoft 365, Microsoft Corp., Redmond, WA, USA). The normality of the distribution of quantitative variables was assessed using the Shapiro–Wilk test. Data with a normal distribution are presented as the mean (M) and standard deviation (SD, σ). Outliers were defined as values exceeding three standard deviations (3σ) from the group mean and were excluded from further analysis. The statistical significance of differences between two independent groups was evaluated using Student's two-sample *t*-test with Bonferroni correction. For multiple comparisons of means across experimental groups, one-way analysis of variance (ANOVA) was performed, followed by Tukey's *post-hoc* test for pairwise comparisons. For multiple comparisons of nonparametric data, Fisher's test with Bonferroni correction was applied. The critical level of significance (p) for testing statistical hypotheses was set at < 0.05. The significance level α was set at 0.05, and the statistical power (1-β) was 0.80 (β = 0.2).

3 Results

As a result of the TBI simulation, significant neurological deficits were observed in all experimental groups on the first day after animals emerged from anesthesia. In the sham-operated (SO) group, minor neurological changes were observed, but these were temporary; no mortality was observed in this group throughout the experiment. In the control group, neurological deficit increased to 10.7 points on day 4, with a 35% survival rate on day 4 of the experiment (Table 1). Administration of the study drugs reduced the severity and frequency of neurological symptoms. Angiolin and HSF-1 demonstrated the greatest activity; their administration increased the survival rate of animals after TBI to 90% in both groups and reduced neurological impairment by 7.7 and 7 points, respectively ($p < 0.05$) compared to the untreated group (control). Following administration of melatonin, tamoxifen, and glutamine to animals with TBI, their neurological symptoms decreased by 6.53–3.23 points ($p < 0.05$) by day 4 relative to control values. The test samples and comparison drug ranked in terms of mortality reduction as follows: piracetam – glutamine – tamoxifen – melatonin – HSF 1 and Angiolin (Table 1).

A one-way analysis of variance (ANOVA) revealed a significant effect of treatment on the severity of neurological deficit F(6, 105) = 3,4, $p < 0.05$. Subsequent analysis using Tukey's post-hoc test (Table 1) showed that the neurological deficit score in the TBI group following experimental therapy with Angiolin (M = 10.75), (SD = 0.35) was significantly lower than in the untreated TBI control group (M = 10.75) баллы, (SD = 1.2); ($p < 0.05$), as well as compared to the experimental groups receiving piracetam, glutamine, melatonin, and tamoxifen (Table 1). The difference between the Angiolin and HSF-1 treatment groups was not statistically significant ($p = 0.342$).

1. The greatest protective effect was observed with the TBI + HSF 1 and TBI + Angiolin regimens. The risk ratio (RR) for both groups was 0.15, indicating an 85% reduction in the risk of animal mortality compared to the TBI control group.

2. Melatonin (RR = 0.31; 69% risk reduction) and tamoxifen (RR = 0.38; 62% risk reduction) also demonstrated high, statistically significant efficacy.

3. For the TBI + glutamine group, the result was borderline: the odds ratio (OR = 0.29 [0.09; 0.89]) suggests a significant effect, yet the confidence interval for the risk ratio includes one ([0.28; 1.02]).

4. In this series of experiments, the use of piracetam yielded the lowest protective effect (RR = 0.62), which, given the current sample size, was not statistically significant (both intervals include 1.0).

An analysis of animal survival rates across the comparison groups, using Fisher's test with a Bonferroni correction for multiple comparisons, confirmed the following trend in the efficacy of the pharmacological agents: piracetam < glutamine < tamoxifen < melatonin < HSF < Angiolin.

TABLE 1 Effect of the studied pharmacological agents on the neurological status (according to the McGraw scale) and mortality of rats on the 4th day after TBI, (M ± m).

Groups/Parameters	Mean P. McGraw scale score (Day 4) [95% CI]	Survivors on Day 4/Total animals	Survival rate on Day 4 (%) [95% CI]
Sham-operated (SO), $n = 10$	0.2 ± 0.1 [0,.13;0.7]	10/10	100
CTBI (control), $n = 14$	10.75 ± 1.2[1] [10.06;11.44]	14/40	35[1] [10;60]
CTBI + tamoxifen, $n = 15$	5.32 ± 0.7[1*#] [4.93; 5.71]	15/20	75* [53;97]
CTBI + melatonin, $n = 16$	4.22 ± 0.4[1*#] [4.01;4.43]	16/20	80*# [60;99]
CTBI + HSF 1, $n = 17$	3.75 ± 0.37[1*#] [3.56;3.94]	18/20	90*# [75;103]
CTBI + Angiolin, $n = 18$	3.00 ± 0.35[1*#] [2.83;3.17]	18/20	90*# [76;104]
CTBI + glutamine, $n = 13$	7.52 ± 0.54[1*] [7.19;7.84]	13/20	65* [39;91]
CTBI + piracetam, $n = 12$	7.8 ± 0.32[1*] [7.59;8.00]	12/20	60* [32;88]

[1] $p < 0.05$ compared to the SO group, * $p < 0.05$ compared to the control group, # $p < 0.05$ compared to the group of animals with TBI that received piracetam.

TABLE 2 Exact *p*-values and effect sizes with confidence intervals.

Groups/Parameters	Survivors on Day 4/Total animals	RR [95% CI]	OR [95% CI]	*p* (Fisher's exact test)
Sham-operated (SO), *n* = 10	10/10	–	–	–
CTBI (control), *n* = 14	14/40	–	–	–
CTBI + tamoxifen, *n* = 15	15/20	0.38 [0.17; 0.85]	0.18 [0.05; 0.60]	0.006
CTBI + melatonin, *n* = 16	16/20	0.31 [0.12; 0.76]	0.14 [0.04; 0.48]	0.002
CTBI + HSF 1, *n* = 17	18/20	0.15 [0.04; 0.58]	0.06 [0.01; 0.30]	<0.001
CTBI + Angiolin, *n* = 18	18/20	0.15 [0.04; 0.58]	0.06 [0.01; 0.30]	<0.001
CTBI + glutamine, *n* = 13	13/20	0.54 [0.28; 1.02]	0.29 [0.09; 0.89]	0.053
CTBI + piracetam, *n* = 12	12/20	0.62 [0.34; 1.10]	0.36 [0.12; 1.09]	0.097

For the comparisons–Angiolin vs. control, Angiolin vs. piracetam, HSF vs. control, HSF vs. piracetam, melatonin vs. control, melatonin vs. piracetam, tamoxifen vs. control, and tamoxifen vs. piracetam–the Bonferroni-adjusted *p*-values were statistically significant ($p < 0.05$) (Table 1).

Thus, the obtained data confirm the ability of HSP_{70} system modulators to reduce the manifestations of neurological impairment and mortality in animals after TBI.

On the 4th day after TBI, in the blood of animals of the control group, an increase in the concentration of molecular markers of neurodestruction characteristic of TBI, intracerebral hemorrhage – MMP-9 by 77 times and NSE by 36 times was found compared to the indicators of the sham-operated animals ($p < 0.05$). Which indicates the development of significant destruction of neurons and the prevalence of the necrotic type of their death. The administration of the studied pharmacological agents to animals after injury led to a decrease in the concentration of these molecular markers in the blood at varying degrees of severity, which indicated a possible neuroprotective effect (Table 3). Thus, the most pronounced effect was shown by Angiolin and HSF 1, after the introduction of which, in the blood of rats with TBI, the concentration of MMP-9 and NSE was reduced compared to the control indicators ($p < 0.05$) by 71.1%; 64.4% and 57.1%; 53.4%, respectively. Administration of melatonin, tamoxifen, and glutamine also resulted in a significant decrease in MMP-9 and NSE in the blood of rats with TBI compared to the control in the range of 37.3%–15%. Administration of piracetam had a significant effect only on NSE (-20.3%). In terms of reduction in the concentration of MMP-9 and NSE in the blood of rats with TBI, the test samples and comparison drug were ranked as follows: piracetam – glutamine – tamoxifen – melatonin – HSF 1 and Angiolin (Tables 1–3). Biochemical studies of the cytosol and mitochondria of brain homogenates from experimental animals on the 4th day after TBI revealed a 4.3-fold ($p < 0.05$) decrease in membrane potential (Ψ) in the control group, while mitochondrial pore opening (mPTP) increased 5.29-fold ($p < 0.05$) relative to the sham-operated group (Table 4). These data confirm damage to the mitochondrial apparatus of brain cells and the possible development of mitochondrial dysfunction.

TABLE 3 Effect of the studied pharmacological agents on the concentration of molecular markers of neurodestruction in the blood of rats on the 4th day after TBI, (M ± m).

Experimental groups	MMP-9, ng/ml	NSE, ng/ml
Sham-operated (SO), *n* = 10	0.10 ± 0.002	0.31 ± 0.02
CTBI (control), *n* = 14	7.72 ± 0.57^{1}	11.8 ± 1.2^{1}
CTBI + tamoxifen, *n* = 15	$5.25 \pm 0.52^{1*\#}$	$7.4 \pm 0.54^{1*\#}$
CTBI + melatonin, *n* = 16	$3.31 \pm 0.22^{1*\#}$	$5.5 \pm 0.34^{1*\#}$
CTBI + HSF 1, *n* = 17	$2.23 \pm 0.31^{1*\#}$	$4.2 \pm 0.15^{1*\#}$
CTBI + Angiolin, *n* = 18	$3.97 \pm 0.42^{1*\#}$	$6.7 \pm 0.52^{1*\#}$
CTBI + glutamine, *n* = 13	$6.58 \pm 0.37^{1*}$	$8.8 \pm 0.42^{1*}$
CTBI + piracetam, *n* = 12	7.12 ± 0.42^{1}	$9.4 \pm 0.32^{1*}$

$^{1}p < 0.05$ compared to the SO group. $^{*}p < 0.05$ compared to the control group. $^{\#}p < 0.05$ compared to the group of animals with TBI that received piracetam.

A course of pharmacological agents had a positive effect on the functional parameters of mitochondrial cells isolated from the brains of animals. Angiolin, HSF-1, and melatonin had the most pronounced mitoprotective effect, inhibiting mitochondrial pore opening by 77% and 70% ($p < 0.05$) against the background of an increase in the membrane potential of mitochondria by 3.7; 3.3 and 3.3 times ($p < 0.05$) relative to the control group (Table 4). Other pharmacological agents - tamoxifen and glutamine also significantly ($p < 0.05$) reduced the opening of mitochondrial mPTP (by 55.5% and 44.5%), which indicates a significant suppression of the development of mitochondrial dysfunction. At the same time, a significant ($p < 0.05$) increase in the charge of mitochondrial membranes was observed (tamoxifen by 2.8 and glutamine by 2 times, respectively) (Table 4). Piracetam significantly increased only the membrane potential of mitochondria by 1.8 times ($p < 0.05$) relative to the control group (Table 4). Also, on the 4th day after TBI, a significant decrease in the level of macroegic phosphates in the brain mitochondria–ATP and ADP–was observed in the control group relative to the sham-operated group by 60.5% ($p < 0.05$) and 47.3% ($p < 0.05$), respectively. At the same time, the level of AMP in the mitochondria increased by 2.4 ($p < 0.05$) (Table 4), indicating progressive energy deficiency and intensification of damage, since AMP can act as a prooxidant.

Following the administration of pharmacological agents, an increase in macroerg levels was observed in the mitochondria of rats after TBI 4 days after experimental therapy. The most effective effects were demonstrated by Angiolin and HSF-1, which increased ATP concentrations by 116.7% and 94.7% ($p < 0.05$) and ADP by 76.7% and 66.7% ($p < 0.05$), respectively, while AMP levels decreased by 55.2% and 55.2% ($p < 0.05$) compared to the

TABLE 4 Assessment of adenine nucleotide content and mitochondrial functional activity in the rat brain on day 4 post-TBI and following administration of pharmacological agents (M ± m).

Groups	ATP μmol/g tissue	ADP μmol/g tissue	AMP μmol/g tissue	mPTP opening, Δ E 540 nm, u.o.	Membrane charge, safranin –O, Ψ
Sham-operated (SO), $n = 10$	2.89 ± 0.07	0.57 ± 0.02	0.12 ± 0.01	0.051 ± 0.002	0.26 ± 0.011
CTBI (control), $n = 14$	1.14 ± 0.05[1]	0.30 ± 0.02[1]	0.29 ± 0.01[1]	0.27 ± 0.02[1]	0.06 ± 0.002[1]
CTBI + tamoxifen, $n = 15$	1.68 ± 0.06[1*#]	0.39 ± 0.03[1*]	0.16 ± 0.01[*1]	0.12 ± 0.02[1*#]	0.17 ± 0.008[*1]
CTBI + melatonin, $n = 16$	1.87 ± 0.05[1*#]	0.43 ± 0.01[1*#]	0.14 ± 0.01[*]	0.072 ± 0.003[1*#]	0.20 ± 0.01[*#]
CTBI + HSF 1, $n = 17$	2.22 ± 0.08[*#]	0.50 ± 0.03[*#]	0.13 ± 0.007[*#]	0.062 ± 0.003[*1#]	0.20 ± 0.007[*#]
CTBI + Angiolin, $n = 18$	2.47 ± 0.07[*#]	0.53 ± 0.02[*#]	0.13 ± 0.01[*#]	0.062 ± 0.001[1*#]	0.22 ± 0.01[*#]
CTBI + glutamine, $n = 13$	1.65 ± 0.05[1*]	0.38 ± 0.0123[1*]	0.16 ± 0.008[*1]	0.15 ± 0.007[1*#]	0.13 ± 0.007[*1]
CTBI + piracetam, $n = 12$	1.48 ± 0.07[1*]	0.37 ± 0.01[1*]	0.16 ± 0.009[*1]	0.25 ± 0.02[1]	0.11 ± 0.006[*1]

[1] $p < 0.05$ compared to the SO group. [*] $p < 0.05$ compared to the control group. [#] $p < 0.05$ compared to the group of animals with TBI that received piracetam.

control group (Table 3). Other pharmacological agents also had a positive effect on energy metabolism in the brain and significantly ($p < 0.05$) increased the ATP-ADP level: tamoxifen by 47.3%–30% ($p < 0.05$), melatonin by 64.0%–43.3% ($p < 0.05$), glutamine by 44.7%–26.6% ($p < 0.05$). Piracetam increased the ATP-ADP level by 29.8%–23.3% ($p < 0.05$). In parallel, all pharmacological agents significantly ($p < 0.05$) decreased the AMP level: tamoxifen by 44.8%, melatonin by 51.2%, glutamine by 44.8% and piracetam by 44.8%. Simulated TBI disrupts key components of energy metabolism, including carbohydrate metabolism. For example, on the fourth day after TBI, a significant increase in lactate levels by 152.9% ($p < 0.05$) was recorded, accompanied by a decrease in pyruvate and malate levels by 59.2% ($p < 0.05$) and 56.2% ($p < 0.05$), respectively. These data indicate the final transition of cells to anaerobic respiration and, as a result, the accumulation of underoxidized lactate, a decrease in pyruvate levels, and the possible development of lactic acidosis. Meanwhile, in the control group, a sharp drop in mitochondrial NAD-MDH activity by 65.8% ($p < 0.05$) was observed on day 4 post-TBI (Table 5), indicating suppression of mitochondrial transport and energy-producing functions and, possibly, suppression of the compensatory malate-aspartate shuttle mechanism. Administration of pharmacological modulators led to the normalization of key components of energy metabolism–inhibition of low-productivity anaerobic glycolysis and ATP production through the activation of more efficient systems, particularly the malate-aspartate shunt. The most active in this regard were Angiolin and HSF-1, which led to a decrease in the concentration of lactate in the cytoplasm of the brain homogenate of animals after TBI by 56.4 and 51.4% ($p < 0.05$), an increase in pyruvate by 127.2 and 113.6% ($p < 0.05$), an increase in malate by 142.8 and 114.2% ($p < 0.05$) against the background of an increase in the activity of mitochondrial NAD-MDH by 329.8% and 185.9% ($p < 0.05$) compared to the control group values. The introduction of melatonin and tamoxifen also had a reliable effect on these studied parameters – a decrease in the lactate level by 39.3 and 29.2% ($p < 0.05$), an increase in the pyruvate level by 68.2 and 50% ($p < 0.05$), an increase in the malate level by 71.4 and 64.2% ($p < 0.05$) against the background of an increase in mitochondrial NAD-MDH by 140.3 and 107.0% ($p < 0.05$ relative to the control group. The introduction of glutamine significantly ($p < 0.05$) increased the content of only malate. The reference drug piracetam, on the contrary, provoked lactic acidosis, as evidenced by an increase in the lactate content ($p < 0.05$) relative to the indicator of the control group and the sham-operated group ($p < 0.05$). Increased activity mNAD-MDH in the mitochondria of the brain of animals after TBI, who received various pharmacological agents, occurred against the background of an increase in their malate level, which indicates the activation of the compensatory malate-aspartate shuttle mechanism under the influence of these drugs.

Changes in energy metabolism in the brain of animals following simulated TBI were identified, with a decrease in HSP_{70} concentrations in the cytosol and mitochondria of brain neurons. In the control group, on day 4 of ischemia, a decrease in HSP_{70} concentrations was observed in both the cytosolic and mitochondrial fractions: by 64.7% ($p < 0.05$) and 67.6% ($p < 0.05$), respectively, relative to the sham-operated group (Table 6).

Administration of the studied pharmacological agents had a positive effect on HSP_{70} concentrations in the cytosol and mitochondria of the animal brain on day 4 post-TBI, to varying degrees. Thus, HSF-1 and Angiolin took the leading position: an increase in the concentration of HSP_{70} was observed by 3.8 and 3.37 times (cytosol) ($p < 0.05$) and by 2.51 and 3 times (mitochondria) ($p < 0.05$) compared to the control group indicators. It should also be noted that in the groups of animals with TBI receiving HSF-1 and Angiolin, the concentration of HSP_{70} in the cytosol was higher ($p < 0.05$) than the values of sham-operated animals. Administration of tamoxifen to animals after TBI increased the level of HSP_{70} by 2.3 times (cytosol) and 1.7 times (mitochondria) compared to the control group. Following melatonin administration to animals after TBI, cytosolic HSP_{70} concentrations increased by 1.58-fold ($p < 0.05$) and mitochondrial HSP_{70} concentrations by 1.3-fold ($p < 0.05$) relative to the control group. A course of glutamine administration to animals after TBI significantly ($p < 0.05$) increased the level of HSP_{70} in the cytosolic fraction by 1.26-fold ($p < 0.05$) relative to the control group (Table 5). Piracetam administration had no significant effect on HSP_{70} levels.

Thus, we can conclude that all the studied drugs have positive modulating activity on the HSP_{70} system in the brain after TBI. The increase in HSP_{70} levels under the influence of these pharmacological agents not only improves energy metabolism but also, possibly, exerts direct mitoprotective activity.

Electron microscopy of the CA_1 region of the hippocampus in rats after TBI on the fourth day of the experiment revealed

TABLE 5 Some parameters of carbohydrate-energy metabolism in the rat brain on the 4th day after TBI and during the administration of pharmacological agents (M ± m).

Groups	Pyruvate, μmol/g tissue	Lactate, μmol/g tissue	Malate, μmol/g tissue	NAD-MDH, μmol/g tissue/ /minute
Sham-operated (SO), $n = 10$	0.54 ± 0.021	2.72 ± 0.15	0.32 ± 0.01	1.67 ± 0.08
CTBI (control), $n = 14$	0.22 ± 0.011[1]	6.88 ± 0.22[1]	0.14 ± 0.005[1]	0.57 ± 0.03[1]
CTBI + tamoxifen, $n = 15$	0.33 ± 0.011*[1]	4.87 ± 0.20*[1#]	0.23 ± 0.01*[1#]	1.18 ± 0.103*[1#]
CTBI + melatonin, $n = 16$	0.37 ± 0.022*[1]	4.17 ± 0.12*[1#]	0.24 ± 0.02*[1#]	1.37 ± 0.08 *[1#]
CTBI + HSF 1, $n = 17$	0.47 ± 0.011*[1#]	3.34 ± 0.20*[1#]	0.30 ± 0.01*[#]	1.63 ± 0.07*[#]
CTBI + Angiolin, $n = 18$	0.50 ± 0.009*[1#]	3.00 ± 0.17*[#]	0.34 ± 0.02*[#]	2.45 ± 0.08*[1#]
CTBI + glutamine, $n = 13$	0.24 ± 0.032[1]	5.89 ± 0.07[1#]	0.20 ± 0.03*[1]	0.87 ± 0.02*[1#]
CTBI + piracetam, $n = 12$	0.27 ± 0.012[1]*	8.24 ± 0.33*[1]	0.17 ± 0.007[1]	0.60 ± 0.02[1]

[1]$p < 0.05$ compared to the SO group, *$p < 0.05$ compared to the control group #$p < 0.05$ compared to the group of animals with TBI that received piracetam.

TABLE 6 HSP_{70} protein concentration in the cytosolic and mitochondrial fractions of rat brain on day 4 after TBI and during administration of pharmacological agents (M ± m).

Group	HSP_{70}, cytosolic fraction, ng/ml	HSP_{70}, mitochondrial fraction, ng/ml
Sham-operated (SO), $n = 10$	17.4 ± 0.55	8.87 ± 0.52
CTBI (control), $n = 14$	6.14 ± 0.10[1]	2.87 ± 0.12[1]
CTBI + tamoxifen, $n = 15$	14.6 ± 0.43[1*#]	4.88 ± 0.21[1*#]
CTBI + melatonin, $n = 16$	9.73 ± 0.37[1*#]	3.77 ± 0.14[1*]
CTBI + HSF 1, $n = 17$	23.5 ± 1.12[1*#]	7.22 ± 0.32[1*#]
CTBI + Angiolin, $n = 18$	20.7 ± 2.00[1*#]	8.78 ± 0.22*[#]
CTBI + glutamine, $n = 13$	7.81 ± 0.16[1*]	3.00 ± 0.19[1]
CTBI + piracetam, $n = 12$	6.84 ± 0.43[1]	2.81 ± 0.32[1]

[1]$p < 0.05$ compared to the SO group. *$p < 0.05$ compared to the control group. #$p < 0.05$ compared to the group of animals with TBI that received piracetam.

significant ultrastructural abnormalities in neurons (Figures 1, 2). Some neurons showed signs of necrosis, while most neurons had nuclei with signs of severely limited functional activity and a fragmented nucleolemma. The perikaryon showed vacuolar-lytic damage to organelles. The mitochondrial structure in neurons of rats in the control group with moderate injury was disrupted by a vacuolar-lytic pattern. Most mitochondria were enlarged, had a fragmented outer membrane, destroyed cristae, and an electron-transparent matrix. Giant mitochondria were also observed due to severe matrix edema. Micromitochondria with a heteromorphic structure were characteristically present. The disturbance of the ultrastructure of neuronal mitochondria was manifested by a significant increase in the total proportion of damaged neurons in the overall mitochondrial structure, which was characterized by an increase in the absolute number of damaged mitochondria by more than 12 times compared to the intact group of animals (Table 6). The administration of pharmacological agents to rats after TBI for 4 days led to an improvement in the ultrastructure of neurons in the CA_1 zone of the hippocampus to varying degrees. The most pronounced protective effect was demonstrated by Angiolin and HSF-1 (Table 6). Thus, the subacute administration of Angiolin gel and HSF-1 for 4 days immediately after the TBI model led to an improvement in the ultrastructure of neurons in the CA_1 zone of the hippocampus. The disturbances in the ultrastructure of neurons in these groups of animals were less pronounced and more diverse in nature compared to the control group. In addition to isolated neurons with signs of necrobiotic changes and a large number of neurons with signs of relative adaptation, neurons with signs of apoptosis are also observed. These are primarily the initial stages of apoptotic processes developing according to the mitochondrial type. These cells exhibit coarsely dispersed nucleoplasmic damage with partially preserved nuclear activity. Lysosomes are few. In the perikaryon, there is moderate disorganization of the granular reticulum with swelling of the cisternae and the formation of vacuole-like cisternae. Mitochondria have areas of defragmentation of the outer membranes, partially destroyed cristae, and uneven electron density of the matrix. Mitochondria with preserved cristae structure are also observed, especially in the group receiving Angiolin (Figure 3). In these experimental groups, the majority of neurons exhibit moderate perikaryon damage of the vacuolar type and contain nuclei with active nucleoplasm. The nucleolemma is stable throughout, without fragmentation. Chromatolysis, swelling, and vacuolization of the lamellar apparatus cisterns and endoplasmic reticulum are not evident in the hippocampus of rats from these groups.

We also note that in the CA_1 hippocampus of rats treated with Angiolin and HSF-1 after TBI, mitochondria showed no signs of outer membrane disruption, moderate cristolysis, and an electron-clear matrix. Giant mitochondria were not detected. Some mitochondria had relatively stable cristae, were small in size, and had a moderately dense matrix. The number of micromitochondria did not differ significantly from the control group; however, they

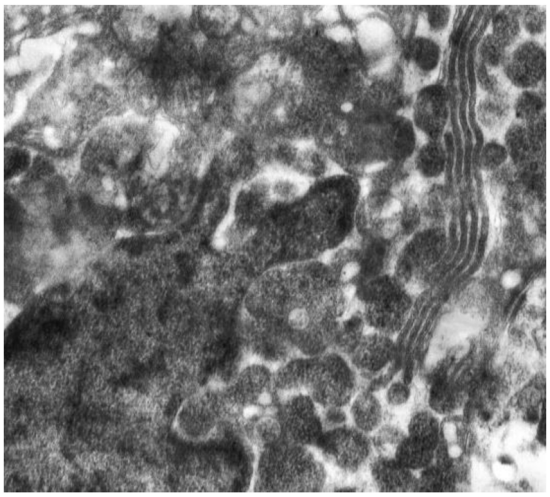

FIGURE 1
Sham-operated animals. Rat hippocampal neurocyte fragment. Mitochondria with cristae and matrix of varying density. Numerous elements of the rough endoplasmic reticulum. Developed Golgi complex. Electron micrograph (EM). ×25,000.

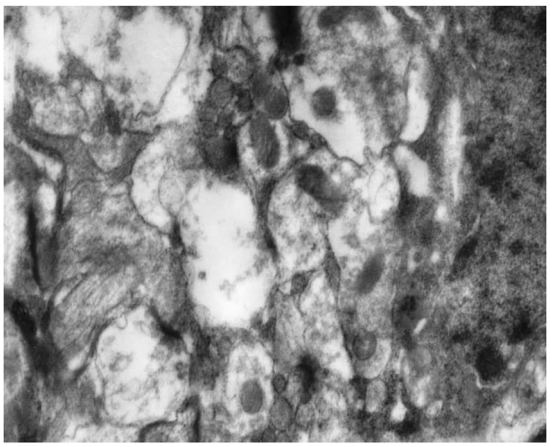

FIGURE 2
Animals with TBI, untreated for 4 days (control). Rat hippocampal neurocyte fragment. Vacuolar-lytic damage to the perikaryon. Massive mitochondrial cristolysis. Electron-dense inclusions within numerous micromitochondria. Destruction of the rough endoplasmic reticulum membranes. EM. ×30,000.

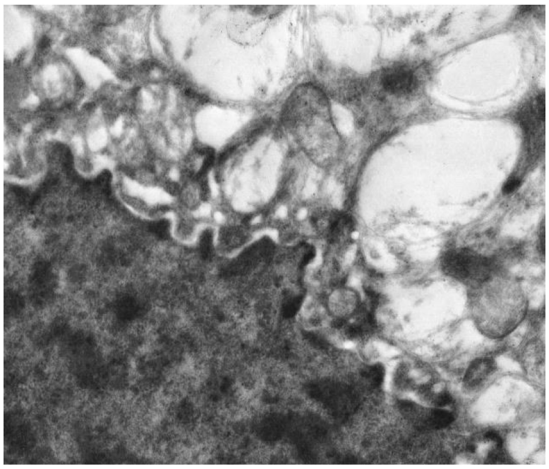

FIGURE 3
Animals with TBI treated with Angiolin nasal gel (50 mg/kg) for 4 days. Rat hippocampal neurocyte fragment. Mitochondrial apoptosis in the induction phase. Destabilization of mitochondrial membranes. Partial cristolysis and uneven electron density of the matrix. EM. ×30,000.

all contained stable outer membranes and cristae, a homogeneous matrix without electron-dense inclusions. In rats treated with tamoxifen and melatonin after TBI, a similar mitigation of ultrastructural damage of hippocampal CA_1 neurons was also observed, but it was less pronounced than in the groups treated with Angiolin and HSF-1.

We also recorded a decrease in the total number of damaged mitochondria in the brain of animals receiving experimental therapy (Table 7). Thus, the administration of Angiolin to rats immediately after TBI led to a decrease in the number of damaged mitochondria by more than 51.9% ($p < 0.05$), HSF-1 by 45.1% ($p < 0.05$), melatonin by 15.4% ($p < 0.05$), tamoxifen by 24.0% ($p < 0.05$), and glutamine by 9.68% ($p < 0.05$) relative to animals in the control group. The ultrastructure of neurons in the CA1 region of the hippocampus in animals treated with piracetam after TBI did not differ from that in the untreated group (Table 7). Thus, our results demonstrate significant mitoprotective, anti-ischemic, and metabolic-correcting effects of pharmacological agents acting as positive modulators of HSP70 after TBI combined with acoubarotrauma, ultimately resulting in a neuroprotective effect through reduced mortality and neurological deficits. In terms of their impact on the biochemical, molecular, and ultrastructural indicators of mitochondrial dysfunction and neurodestruction–as well as on animal survival and neurological impairment following the induction of TBI–tamoxifen, melatonin, and, in particular, HSF and Angiolin, significantly outperform the reference drug, the neurometabolic cerebroprotective agent piracetam.

4 Discussion

Our findings indicate that modeling TBI combined with blast acoubarotrauma via a blank shot triggers a cascade of molecular and biochemical mechanisms of neurodestruction characteristic of post-ischemic brain damage, leading to oxygen deprivation accompanied by impairments in tissue respiration, biological substrate oxidation, and mitochondrial energy production (Cao et al., 2026; Moallem et al., 2026). Our results are consistent with other studies that have shown that disturbances in brain energy metabolism were observed as early as 1 h after injury, which worsened after 3 h, and the peak of mitochondrial dysfunction was observed at approximately 12–24 h, which provides an opportunity for effective intervention in mitochondrial dysfunction (Gilmer et al., 2009; Freire et al., 2023). Energy deficiency and damaged mitochondria are the main causes of neuronal damage after brain injury combined with blast acoubarotrauma (Agoston, 2017).

The resulting cascade of molecular and biochemical reactions leads to significant neurodestruction and elevated marker molecules in the blood. NSE has high diagnostic potential as a

TABLE 7 Mitochondrial status in the CA_1 hippocampus of rats on day 4 after TBI and after administration of pharmacological agents (M ± m).

Groups	Number of damaged mitochondria (absolute value)	Percentage of damaged mitochondria, %
Sham-operated (SO), $n = 10$	6.2 ± 0.57	8.2%
CTBI (control), $n = 14$	75.4 ± 3.4^{1}	96.5%
CTBI + tamoxifen, $n = 15$	$57.3 \pm 3.2^{*1\#}$	53.7%
CTBI + melatonin, $n = 16$	$63.8 \pm 4.2^{*1}$	60.4%
CTBI + HSF 1, $n = 17$	$41.2 \pm 2.8^{1*\#}$	48.8%
CTBI + Angiolin, $n = 18$	$36.2 \pm 3.5^{*1\#}$	46.4%
CTBI + glutamine, $n = 13$	$68.1 \pm 3.0^{1*}$	92.3%
CTBI + piracetam, $n = 12$	76.2 ± 4.7^{1}	96.0%

$^{1}p < 0.05$ compared to the SO group. $^{*}p < 0.05$ compared to the control group. $^{\#}p < 0.05$ compared to the group of animals with TBI that received piracetam.

long-term prognostic biomarker and therapeutic indicator in neurological intensive care for traumatic brain injury, ischemic stroke, and hemorrhagic stroke (Ghaith et al., 2022). Matrix metalloproteinase-9 was also measured as a molecular marker. Increased expression of this enzyme after traumatic brain injury and hemorrhagic stroke contributes to increased permeability of the blood-brain barrier and is associated with mortality. In patients and experimental animals with TBI and hemorrhagic stroke, MMP-9 expression correlated with mortality (Jia et al., 2010; de Lima et al., 2020). Biochemical, immunoenzymatic, and ultrastructural studies of the brain in experimental animals reveal the development of secondary mitochondrial dysfunction after modeling TBI combined with acoubarotrauma. Mitochondria play a critical role in maintaining cellular vital activity, as well as possessing a high destructive potential. Impairment of any mitochondrial function–energetic, thanatogenic, or activation of their free radical production–can cause the development of functional and morphological abnormalities in brain tissue after TBI. TBI combined with acoubarotrauma (pressure damage caused by a blast) and mitochondrial dysfunction leads to severe disruption of cellular energy metabolism, oxidative stress, and mitochondrial dysfunction (Xiong et al., 2013; Chauhan et al., 2025; Laye and Hubbard, 2026). The blast wave from barotrauma exacerbates the physical impact on the brain by disrupting cell membranes, and secondary damage cascades lead to the cessation of mitochondrial energy production, causing progressive cell death. Mitochondrial dysfunction, caused by excessive Ca^{2+} uptake by mitochondria via the voltage-dependent uniporter, is a key event in the development of severe excitotoxicity, which leads to depolarization of the inner mitochondrial membrane, ROS generation, ATP depletion, and increased mitochondrial membrane permeability, leading to apoptosis or necrosis. In the pathogenesis of traumatic brain injury, where death or survival are key factors, mitochondrial dysfunction may play a major role (Cheng et al., 2012; Hiebert et al., 2015; Salvagno et al., 2024). Under conditions of lactic acidosis, hyperproduction of excitotoxic amino acids, decreased normal accumulation of Ca2+ by mitochondria, and targeted damage to mitochondrial membranes by ROS, giant mitochondrial pores open and apoptosis is induced (Belenichev et al., 2024c; Feofilaktova et al., 2025). The mitochondrion, and in particular its membranes, appears to be the key link linking the various apoptotic programs. Increased permeability of the mitochondrial pore and the subsequent release of death effectors lead to cell death. Several models of mitochondrial membrane pore formation exist: direct pore formation through existing pores, ex novo pore formation, and a transition in mitochondrial membrane permeability (a process of sudden increase in inner membrane permeability). The second model is realized through either direct oxidation of ROS or nitrosation of the thiol groups of the cysteine-dependent region of the mitochondrial inner membrane protein (ATP/ADP antiporter) by reactive NO derivatives, converting it into a permeable nonspecific channel–a pore. The opening of the pore leads to serious cellular consequences, such as necrosis. The charge of the inner mitochondrial membrane, necessary for the production of ATP from ADP, is lost. The result is rapid mitochondrial dysfunction and excessive ROS production, ultimately leading to necroptosis. Furthermore, rapid pore occlusion by ROS or NO leads to significant mitochondrial swelling and potentially complete rupture of the outer membrane and the massive release of death effectors (Khan et al., 2022; Percio et al., 2024; Carraro et al., 2026; Mihaylova et al., 2026). Thus, the secondary mitochondrial dysfunction that develops after TBI combined with blast acoubarotrauma is currently considered as a promising pharmacological target (Belenichev et al., 2025b).

The observed changes in energy metabolism in the animal brain and the development of mitochondrial dysfunction occurred against the background of decreased HSP_{70} concentrations in both the cytosolic and mitochondrial fractions of the brain after TBI. This finding confirms our previous studies, which demonstrated parallelism in changes in malate levels and mitochondrial NAD-MDH activity, cytoplasmic cytoplasmic aspartate aminotransferase, and HSP_{70} and HIF-1a levels. Mitochondrial energy production during acute cerebral ischemia depends on the functioning of the malate-aspartate shunt, which is activated and controlled by HSP70 (McKenna et al., 2006; Volobouева et al., 2010; Belenichev et al., 2012, 2023; Rae et al., 2025). Many years of study of molecular and biochemical mechanisms of ischemic neurodestruction have allowed us to identify mechanisms of endogenous neuroprotection aimed at increasing the resistance of neurons to ischemia. A special role in the implementation of these mechanisms is played by heat shock proteins HSP_{70}, which exhibit antiapoptotic and cytoprotective properties (Turturici et al., 2011; Tregub et al., 2024).

The main functions of HSP_{70} are associated with their chaperone activity, which ensures the formation of secondary and/or tertiary structure of protein chains. HSP_{70} also participates in the mechanisms of repair or degradation of damaged and

denatured proteins. The HSP_{70} group belongs to stress-sensitive proteins, the level of the latter increases sharply under conditions of ischemic, thermal, oxidative damage, etc. Under stress conditions, HSP_{70} exhibits a direct anti-apoptotic and neuroprotective effect, which has been confirmed by a number of experimental studies on various *in vitro* and *in vivo* models (Singh et al., 2025; Wentink et al., 2026). Some researchers have suggested that HSP_{70} may counteract so-called mitoptosis by promoting protein transport and folding within the mitochondria and limiting the damaging effects of free radicals on the mitochondrial membrane. Furthermore, due to HSP_{70}'s ability to extend the lifespan of HIF, compensatory mitochondrial-cytosolic energy production shunts are activated (Havalová et al., 2021; Ferretti et al., 2024; Singh et al., 2025). Therefore, positive modulation of the HSP_{70} system to reduce mitochondrial dysfunction is a promising concept for neuroprotection that can improve the effectiveness of treating TBI combined with blast acoubarotrauma, especially during combat.

The studied pharmacological agents, by positively influencing HSP_{70} expression, had a beneficial effect on brain energy metabolism and also exerted a direct mitoprotective effect after TBI. Thus, the studied pharmacological agents, by increasing HSP_{70} concentrations in the cytosol and, especially, in the mitochondria, enhanced mitochondrial energy production and reduced energy deficit after TBI. The high level of HSP_{70} under the influence of the studied pharmacological agents stimulates the functioning of the alternative malate-aspartate pathway of ATP production, which is to say, the high level of NAD-MDHm against the background of their purpose. Increased expression of HSP_{70} in the cytosol and mitochondria of the brain after TBI under the influence of these pharmacological modulators may possibly prolong the "lifetime" of HIF-1a, and also independently stimulate the expression of mitochondrial NAD-MDH, thereby promoting the activity of the malate-aspartate shuttle mechanism (Semenza, 2011; Gogate et al., 2012) The effects of the studied pharmacological agents that positively modulate HSP_{70}, which we established, to some extent explained their protective effect on the structural integrity and functional activity of mitochondria, as evidenced by an increase in the mitochondrial membrane charge potential and blockade of mitochondrial pore opening. This effect also resulted in a significant reduction in mortality, neurological impairment, and a decrease in blood levels of specific molecular markers of neurodestruction after TBI.

Angiolin's effectiveness is based on its ability to positively influence the activity of anaerobic glycolysis by preserving energy production at the tricarboxylic acid site, influencing the activation of the dicarboxylic acid site, and thereby stabilizing cellular energy metabolism under conditions of ischemic injury. The lysine residue found in the Angiolin molecule combines with methionine in neurons, resulting in the formation of a carnitine molecule. Carnitine acts as a "shuttle" in the formation of acetyl-CoA from fatty acids. Since the processes of fatty acid activation and oxidation are spatially separated, the cell has a mechanism for transporting long-chain fatty acids across the inner mitochondrial membrane into the mitochondrial matrix involving carnitine and a specialized enzymatic system. This system reversibly transfers acyl on the outer side of the membrane from CoA to carnitine, and on the inner side from carnitine to intramitochondrial CoA. It is also known that the enhancement of mitochondrial energy metabolism caused by carnitine can prevent the formation of peroxynitrite and other free radicals under hypoxic conditions. The thiazate residue, part of the Angiolin structure, can activate the conversion of lactate to pyruvate, limit lactic acidosis, and enhance energy production by activating the compensatory malate-aspartate shunt (Figure 4) (Belenichev, 2014; Solovieva et al., 2023).

The mechanism of Angiolin's action in this model of TBI is likely related to its ability to significantly increase HSP70 concentrations in the cytosol and mitochondria of neurons by activating glutathione- and other redox-dependent pathways and HSP70 mRNA expression (Belenichev et al., 2023) and implements HSP70-dependent mechanisms of mitoprotection. HSP70, which "extends" the action of HIF-1α, also independently maintains the expression of mitochondrial NAD-dependent malate dehydrogenase activity, thereby maintaining the activity of the malate-aspartate shuttle mechanism under conditions of energy deficiency. HSP70 protects mitochondria by increasing the expression of intramitochondrial antioxidant enzymes and stabilizing oxidatively damaged macromolecules (Belenichev et al., 2025b). Also, Angiolin itself, due to the peculiarity of the structure of its molecule, is capable of exerting a direct mitoprotective effect by binding NO and inhibiting NO pathways that form mitochondrial dysfunction (Belenichev et al., 2024c), and the ability of the structural fragment of the molecule, thiazate, to convert lactate into pyruvate. The HSP70-dependent and direct mitoprotective effect of Angiolin in this TBI model leads to the preservation of the ultrastructural integrity of brain mitochondria and their functional activity (increased inner membrane charge $\Delta\Psi$, decreased rate of opening of the mPTP permeability transition pore (MPTP), increased concentrations of ATP, ADP, and AMP). A decrease in mitochondrial dysfunction under the influence of Angiolin leads to a decrease in neurodestruction (decreased MMP-9, NSE) and, ultimately, a decrease in mortality and neurological impairment after TBI combined with acoubarotrauma.

Lysine residues in brain tissue are also metabolized into pipecolic acid, which enhances the affinity of the GABA-benzodiazepine receptor complex. This effect of lysine is particularly important in conditions of glutamate excitotoxicity, which occurs during hypoxia and leads to an increase in intracellular Ca^{2+} concentration, activation of NO synthase, and the intensive formation of NO and peroxynitrite ($ONOO^-$), which is the direct cause of cell death. By enhancing GABA receptor affinity, pipecolic acid reduces the hyperexcitability of glutamate receptors and reduces the release of excitatory amino acids (glutamate and aspartate), thereby neutralizing the manifestations of glutamate excitotoxicity (Bernasconi et al., 1986; Hallen et al., 2013).

The significant mitoprotective activity of Angiolin is due to its antioxidant properties, as well as due to the positive modulation of the expression of the endogenous neuroprotector HSP_{70}, which has chaperone activity and plays an important role in maintaining the ultrastructure of neuronal mitochondria, protecting them from oxidative stress and preventing the development of mitoptosis and the formation of mitochondrial dysfunction (Belenichev et al., 2024c, 2025b). Thus, the conducted studies revealed the HSP-mediated mitoprotective properties of the nasal gel Angiolin, which provides experimental justification, after clinical trials, for its use in the complex therapy of TBI combined with acoubarotrauma.

The high efficiency of HSF-1 can be explained by the fact that it is a natural stress-inducible transcription factor of the HSP

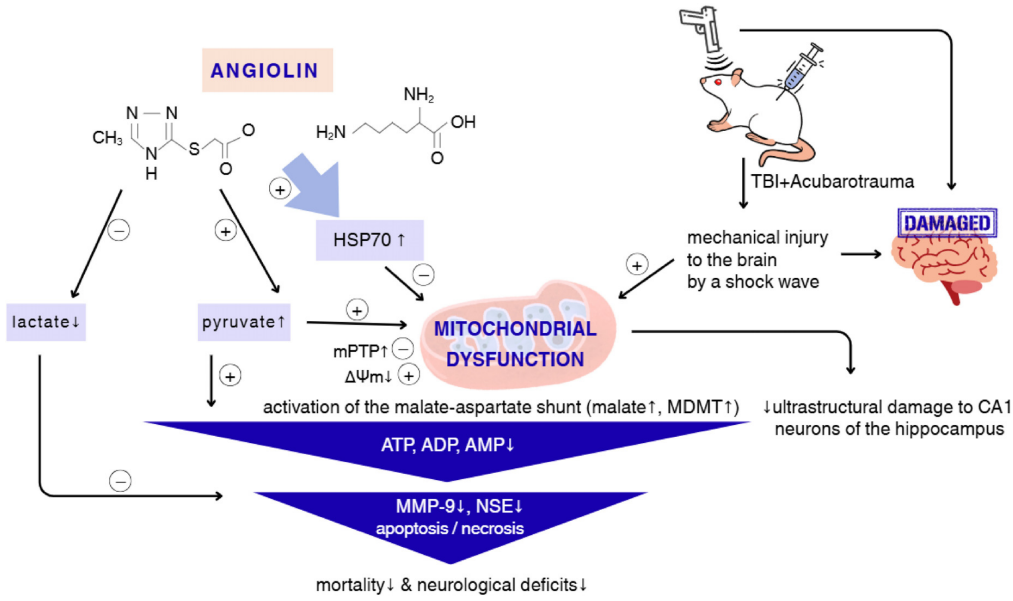

FIGURE 4
Hypothetical mechanism of Angiolin's neuroprotective effect in a model of TBI combined with acoubarotrauma.

family. In the brain, the accumulation of products of oxidative and nitrosative stress results in trimerization of HSF-1 from the remaining inactivated HSP_{70} molecules. The activated HSF-1 complex is transported into the nucleus and, combined with heat shock elements (HSE), initiates the transcription process of the Hsp70 gene. HSF1 is essential for normal neuronal survival, and increased HSF1 expression protects neurons not only from death under the influence of non-proteotoxic stimuli, but also in models where protein misfolding or aggregation was not observed. HSF1 protects neurons *in vitro* from death caused by high concentrations of neurotoxins by stimulating the transcription of genes encoding HSP_{70}. By increasing HSP_{70} concentrations, HSF-1 also exhibits antioxidant activity during cerebral ischemia. Thus, the obtained findings reveal the HSP70-inducing and mitoprotective potential of HSF-1, experimentally supporting its further translational investigation for blast-induced neurotrauma (Qu et al., 2018; Zhang et al., 2025; Belenichev and Bila, 2017). HSF1 is also a key mediator of mitochondrial function and a potential mitoprotector, suggesting that many of the brain protective benefits provided by HSF1 may be due to the maintenance of mitochondrial homeostasis (Labbadia, 2023).

Regarding tamoxifen, we assume that it interacts with estrogen receptors (ER), resulting in the dissociation of heat shock protein 70 from the latter molecules, which subsequently acts as a cytoprotector (Egeland et al., 2015; Song et al., 2024; Kalabay et al., 2025). At the same time, the level of Hsp70 mRNA expression under the influence of tamoxifen also has a positive dynamics, which indicates the direct participation of the drug in signaling and transcription of HSP_{70} mRNA according to the feedback principle. Thus, as the HSP_{70} protein is used in anti-apoptotic processes, tamoxifen triggers transcription of HSP_{70} mRNA. In addition, our previous studies have established direct genomic protection of tamoxifen in relation to HSP_{70} mRNA (Belenichev, 2014). Tamoxifen is also able to exert a mitoprotective effect, protecting the membrane structures of mitochondria from the effects of ROS and, thereby, maintaining the functional activity of these organelles (Nazarewicz et al., 2007; Pavlov and Belenichev, 2014; Colón and Miranda, 2016).

Melatonin increases HSP_{70} levels under ischemic conditions by activating melatonin receptors MT1 and MT2 (Andrabi et al., 2015; Kleszczyński et al., 2015; Lee and Back, 2024). In addition, the unique structure of the melatonin molecule makes it an effective scavenger of ROS/AFA and prevents total damage to polypeptide bonds, inactivation of enzyme systems, antioxidant links of endogenous protection, including HSP_{70}. Melatonin is also able to exert a cytoprotective effect by supporting the activity of glutathione peroxidase, Cu, Zn- and Mn-superoxide dismutase, and γ-glutamylcysteine ligase (Kołodziejska et al., 2025). At the same time, the hormone has been shown to inhibit a number of pro-oxidant enzymes, such as lipoxygenase and NO synthase, which reduces ROS production under ischemia/reperfusion conditions (Gilad et al., 1998). The positive effect of melatonin on energy metabolism is explained by its ability to prevent damage to aconitate hydrolase and thus maintain the Krebs cycle at the citrate-isocitrate stage (Cipolla-Neto et al., 2014; Zhang et al., 2017).

Glutamine acts as a substrate for the restoration of depleted resources of the thiol-disulfide system, namely, glutathione (Sappington et al., 2016; Cruzat et al., 2018). The level of reduced forms of glutathione restores intracellular redox potential and signaling/activity of transcription factors. Maintaining an optimal ratio of reduced and oxidized forms of glutathione is an important link in maintaining cellular vitality (Mukherjee et al., 2025). Glutathione also prevents damage to the HSP_{70} structure under oxidative stress, supporting its cytoprotective functions (Zhang et al., 2022). Glutamine is an important mitochondrial substrate and exhibits an antioxidant mechanism of mitoprotective action (Ahmad et al., 2001). Our preliminary studies have shown its moderate antioxidant activity in cerebral ischemia (Belenichev and Bila, 2017).

The reference drug piracetam, which is included in some national guidelines for the treatment of TBI, showed relatively low efficacy in our studies in a model of TBI combined with with blast acoubarotrauma. This may be due to its preference for activating anaerobic glycolysis, which in the acute phase of brain injury can lead to a further increase in lactate levels and the risk of acidosis (Domańska-Janik and Zaleska, 1977), no effect on the mechanisms of HSP_{70} protection of cells and their organelles. This is consistent with the work of other authors, who have shown the role of piracetam as an adjuvant in mild trauma or cerebral stroke, for improving cognitive functions of the central nervous system, the therapeutic benefit of which remains limited in severe acute neurotrauma (Ricci et al., 2012; El Sayed et al., 2018).

5 Conclusion

It has been established that the neuroprotective effect of Angiolin, tamoxifen, melatonin, HSF-1, and glutamine is aimed at increasing the concentration of the endogenous neuroprotector, heat shock protein 70 kDa (HSP_{70}), in the brain damaged after TBI combined with blast acoubarotrauma. This enhances the compensatory and adaptive mechanisms of neurons' resistance to ischemia. A course of Angiolin, tamoxifen, melatonin, HSF-1, and glutamine normalizes brain energy metabolism after TBI combined with blast acoubarotrauma by positively modulating HSP_{70}-dependent mechanisms of activation and regulation of the malate-aspartate shuttle. For the first time, it has been established that a course of Angiolin administration to animals after TBI with blast acoubarotrauma, as well as the administration of tamoxifen, melatonin, HSF-1, and glutamine, reduces the number of structurally altered mitochondria in the CA1 hippocampus of rats after TBI and improves the functional properties of mitochondria, indicating a direct mitoprotective effect and, consequently, reduces mortality and the manifestations of neurological disorders. Based on the conducted studies, pharmacological modulation of HSP_{70} expression targeting the correction of mitochondrial dysfunction has been experimentally substantiated as a promising approach to the comprehensive treatment of combat brain contusion. The experimental data support the prospects for further research on HSF-1 with the goal of developing a neuroprotective drug based on it. Intranasal administration of neuroprotectors immediately after combat brain contusion and further preclinical studies of a new dosage form of Angiolin nasal gel have also been experimentally substantiated. Experimentally established mechanisms of action of HSP_{70} modulators can contribute to the creation of a new generation of neuroprotectors that have a targeted effect on key target links of neurodestruction after combat brain injury – mitochondrial dysfunction and energy deficiency.

6 Study limitations

Several key limitations should be noted:

1. Limited time period.

Neuroprotective effects were studied 4 days after injury and pharmacological treatment, but long-term effects were not examined. No assessments of CNS cognitive-mnemonic functions, psycho-emotional behavior, or motor activity were conducted on day 18 or 21 post-injury; likewise, no investigations into the morphofunctional state of the cerebral cortex and hippocampus were performed at these later time points. Furthermore, the baseline status of the endogenous neuroprotection system–evaluated via HSP_{70} concentrations in the animals' blood–was not assessed.

2. Statistical Limitations.

The study used 10 to 20 animals in each group, which is a relatively small sample size. While this sample size is likely sufficient to detect significant effects, it may limit the detection of more subtle neuroprotective benefits or adverse effects.

3 Mechanism-related limitations.

Incomplete pathway analysis: The study focused on individual molecular, ultrastructural, and biochemical markers (MMP-9, NSE, iNOS, ATP, ADP, AMP, malate, pyruvate, lactate, NAD-MDH, mitochondrial ultrastructure) but did not conduct a comprehensive analysis of all potential pathways involved in mitochondrial dysfunction (studies on isolated mitochondria, pore protein expression, pro- and anti-apoptotic factors, hypoxia-induced factors, molecular markers of mitochondrial dysfunction, HSP_{70} mRNA, HIF-1 mRNA, neuronal and glial cell morphometry).

4 Limitations related to translational medicine.

4.1 Sexual dimorphism.

The study used only male offspring and did not account for potential sex differences in drug response, which may limit the applicability of the results.

4.2 Species-specific limitations.

The use of rats alone limits extrapolation to humans without additional studies in other animal species or human cell models.

4.3 Age limitations.

The study used adult rats aged 3 months, but did not examine the effects of these pharmacological agents on brain aging in animals with combined TBI and blast acoubarotrauma, which may have different sensitivities to HSP_{70} modulators.

Future research directions

Further research should focus on the neuroprotective effects of modulating HSP_{70}/HIF-1-dependent mitochondrial protection mechanisms after TBI with combined blast acoubarotrauma. We also plan to identify and validate specific biochemical and molecular markers of secondary mitochondrial dysfunction and determine their correlation with mitochondrial ultrastructural abnormalities and neurological impairments at different time points after TBI with blast acoubarotrauma. Using new information technologies, develop predictive programs that, taking into account the identified cascade mechanisms of neurodestruction after TBI, will algorithmically identify top drug candidates that target the most beneficial receptors or pathways in a given region of the neurodestructive cascade and are capable of mitigating key post-traumatic disturbances, such as edema, calcium overload, endothelial, mitochondrial, and neurotrophic dysfunction, or apoptosis. To implement rational approaches to the development of new neuroprotective drugs, laboratory data must be converted into prognostically significant biomarkers. The potential of a new route for delivering neuroprotective agents immediately after

combat brain contusion–the intranasal, dosed administration of a gel or spray in a convenient, field-use packaging–should also be emphasized. We also emphasize the combined effects of neuroprotectors with different mechanisms of action–those that reduce excitotoxicity, neuronal Ca channel blockers, HSP_{70} modulators, and pharmacological agents that modulate mitochondrial function or neuroimmune communication. Therefore, a stepwise, combined approach to drug therapy for TBI combined with blast acoubarotrauma is of interest.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies were conducted in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Scientific Purposes (Council of Europe, 1986), as well as national bioethics standards and regulations. The bioethical aspects of the study protocol were approved by the Bioethics Committee of the ZDMPhU (Protocol No. 32 of February 21, 2026) in accordance with the Methodological Guidelines "Bioethical Review of Preclinical and Other Scientific Research Conducted on Animals" (2006). All animal procedures complied with ARRIVE 2.0 guidelines. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

IB: Validation, Project administration, Methodology, Formal analysis, Investigation, Writing – review & editing, Writing – original draft. OP: Project administration, Visualization, Writing – original draft, Formal analysis, Validation, Investigation, Data curation, Methodology, Conceptualization, Writing – review & editing. VR: Writing – review & editing, Writing – original draft, Validation, Formal analysis. NB: Formal analysis, Validation, Writing – review & editing. SP: Formal analysis, Writing – review & editing. PP: Writing – review & editing. OK: Writing – review & editing, Validation.

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