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Original Research

Preclinical Study of the Neuroprotective Effects of Pharmacological Agent-Modulators of HSP70 After Intrauterine Hypoxia

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Abstract

Background: Chronic intrauterine hypoxia (IUH) is a cause not only of early postnatal mortality but also of delayed central nervous system (CNS) impairments in offspring, including increased risks of acute cerebrovascular disorders, epilepsy, and neurodegenerative diseases. The above necessitates the urgent development of targeted neuroprotective strategies for newborns following IUH. The aim of the present study was to evaluate the neuroprotective effects of the 70 kDa heat shock protein (HSP70) modulators (angiolin, thiotriazolin, cerebrocurin, tamoxifen, glutaredoxin, and heat shock factor 1 (HSF-1)), as well as reference drugs, based on their impact on molecular markers of endothelial dysfunction (vascular endothelial growth factor A (VEGF-A), endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), nitrotyrosine), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and caspase-8 in the brains of offspring after IUH at 1 and 2 months of age. **Methods:** Chronic hemic IUH was modeled in pregnant rats by daily administration of sodium nitrite (50 mg/kg, intraperitoneally) on days 16–21 of pregnancy. The male offspring were treated with the study drugs for 30 days starting on P1. Brain homogenates of offspring at P30 and P60 were analyzed using enzyme-linked immunosorbent assay (ELISA) for VEGF-A, eNOS, iNOS, nitrotyrosine, BDNF, NGF, and caspase-8. **Results:** IUH caused persistent endothelial dysfunction, characterized by decreased VEGF and eNOS levels, and nitrosative stress, associated with increased iNOS and nitrotyrosine levels. These changes were accompanied by decreased neurotrophin levels, including BDNF and NGF, and increased caspase-8 levels. Course administration of angiolin, cerebrocurin, HSF-1, thiotriazolin, and glutaredoxin improved the eNOS/iNOS balance, increased VEGF levels, and decreased nitrotyrosine levels, with the effect persisting until P60. Cerebrocurin and angiolin demonstrated the strongest recovery of neurotrophic factors (normalization or exceeding normal BDNF/NGF levels by P60) and effectively suppressed caspase-8 to near-normal levels. A positive correlation was established between BDNF, NGF, and HSP70 levels. **Conclusions:** Pharmacological modulators of HSP70 provide effective multimodal neuroprotection due to endothelioprotective and neurotrophic effects. Angiolin and Cerebrocurin demonstrate significant therapeutic potential, offering a promising strategy for mitigating the long-term neurological sequelae of IUH.

Keywords: intrauterine hypoxia; neuroprotection; cerebrocurin; angiolin; BDNF; NGF; VEGF; eNOS; iNOS; caspase

1. Introduction

Intrauterine hypoxia (IUH) is one of the most common and pathogenetically significant adverse factors of intrauterine development, determining the formation of long-term structural and functional disorders in the offspring [1,2,3]. Oxygen deficiency during critical periods of intrauterine development disrupts the maturation of multiple organ systems, particularly the vascular and nervous systems, leading to persistent structural and functional impairments that extend into postnatal life [4,5]. Hypoxia during pregnancy may induce endothelial dysfunction of cerebral vessels, which can increase the risk of cerebrovascular diseases, including ischemic and hemorrhagic stroke, vascular dementia, cerebral small vessel disease, and intracranial aneurysms. In addition, it can impair cerebral blood flow regulation, promote thrombogenesis, enhance inflammation, damage the blood–brain barrier, and lower the threshold dose for endogenous and exogenous neurotoxins [6,7,8,9,10]. It has been established that endothelial dysfunction of cerebral blood vessels is manifested by reduced nitric oxide (NO) bioavailability due to oxidative stress and depletion of reduced thiols, as well as by

disruption of the interaction between vascular endothelial growth factor (VEGF) and various nitric oxide synthase (NOS) isoforms [11,12,13]. Nerve growth factors and neurotrophic factors are involved in the mechanisms of endogenous neuroprotection under IUH. However, chronic and severe IUH may overload these systems, leading to depletion of their activity and resulting in potential long-term neurological consequences, such as developmental delay, increased risk of stroke, schizophrenia, and intellectual disability [1,14,15,16,17].

These findings open new perspectives for post-hypoxic neuroprotection aimed at modulating specific components of endogenous neuro- and cytoprotective mechanisms. In recent years, intensive research has focused on the role of the 70 kDa heat shock protein (HSP70) in the regulation of endogenous neuroprotection under conditions of IUH [18,19,20]. Heat shock proteins function as intracellular chaperones, playing a critical role in cellular stress protection by restoring damaged proteins and ensuring proper folding, stabilization, and transport of newly synthesized proteins. In addition, HSP70 is involved in the regulation of gene expression [21,22,23,24,25]. Experimental models of IUH and acute ischemia have demonstrated a direct relationship between HSP70 concentration levels, the severity of neurological deficits, and survival rates. Our own studies have provided convincing evidence of the neuroprotective effects of pharmacological modulation of HSP70 at different stages of postnatal life in offspring exposed to IUH [26,27,28,29]. Based on preliminary screening of neuroprotective efficacy, the following HSP70-modulating agents were selected for the present study: angiolín ((S)-2,6-diaminohexanoic acid 3-methyl-1,2,4-triazolyl-5-thioacetate), thiotriazolin (morpholinium thiazotate), tamoxifen, glutaredoxin, and heat shock factor 1 (HSF-1), a transcription factor regulating HSP70 expression.

Endogenous HSP70 promotes neuronal survival during hypoxic and ischemic injury primarily by inhibiting caspase-dependent pathways involved in the initiation of neuronal apoptosis [25,30,31]. Evidence also indicates a positive association between increased HSP70 concentrations and enhanced expression of neurotrophic factors following brain injury [32,33,34]. Post-IUH endothelial dysfunction develops against the background of hypoxia-inducible factor-1α (HIF-1α) deficiency (a factor that activates endothelial nitric oxide synthase (eNOS) expression via serine phosphorylation), reduced VEGF-B expression, nitrosative stress, depletion of the glutathione system, decreased NO bioavailability, and suppression of gene transcription by cytotoxic NO metabolites. These processes ultimately lead to HSP70 deficiency and impairment of endogenous cytoprotective mechanisms [35,36,37]. Suppression of VEGF-B expression results in mitochondrial dysfunction, metabolic imbalances, and an increased risk of vascular pathology in target organs [38]. Endothelial dysfunction of cerebral small vessels is associated with neurotrophic factor deficiency, exacerbating cognitive impairment and increasing the risk of stroke [7,39,40]. Under hypoxic conditions, sufficient HSP70 concentrations ensure that brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) can fulfill their complex roles in enhancing neuronal survival and neuroplasticity [27,41,42]. However, to date, there are no data regarding the effects of HSP70 modulators on the expression of nerve growth factors, neurotrophic factors, endothelial dysfunction, and apoptosis initiation following IUH. Conducting such studies would significantly expand the current understanding of the neuroprotective mechanisms of HSP70 modulators.

The aim of the present study was to evaluate the neuroprotective effects of HSP70 modulators (angiolin, thiotriazolin, cerebrocurin, tamoxifen, glutaredoxin, and HSF-1), as well as reference drugs, based on their impact on molecular markers of endothelial dysfunction (VEGF-A, eNOS, inducible nitric oxide synthase (iNOS), nitrotyrosine), BDNF, NGF, and caspase-8 in the brains of offspring after IUH at 1 and 2 months of age.

2. Materials and Methods

2.1 Experimental Animals and Housing Conditions

The experimental study was conducted using 50 female and 10 male outbred white rats weighing 220–240 g and aged 4.5 months, obtained from the vivarium of the State Enterprise Institute of Pharmacology and Toxicology of the National Academy of Medical Sciences of Ukraine (Kyiv, Ukraine). Prior to inclusion in the experiment, all animals underwent a clinical examination performed by a certified veterinarian to confirm their health status. Animals were subsequently subjected to a 10-day quarantine period and then randomly allocated to experimental groups according to the assigned treatment regimen.

Animals were housed in polycarbonate cages (550 × 320 × 180 mm) with galvanized steel lids (660 × 370 × 140 mm) and glass drinking bottles. No more than five animals were housed per cage. Each cage was labeled with the study identification number, species, sex, animal numbers, and treatment dose. Cages were arranged on racks according to dosage levels and cage numbers. Throughout the experiment, standardized environmental conditions were maintained, including a temperature of 20–24 °C, relative humidity of 30–70%, and a 12 h light/12 h dark photoperiod. Animals had ad libitum access to standard laboratory chow (Phoenix, Kharkiv, Ukraine) and drinking water purified by reverse osmosis and sterilized by ultraviolet irradiation. Autoclaved alder wood shavings (Alnus glutinosa) were used as bedding material.

2.2 Model of Chronic IUH

IUH was induced using a well-established model of chronic hemic hypoxia based on sodium nitrite (1:100, S2252, Sigma-Aldrich Chemie GmbH, Steinheim, Ger-

many) administration during late gestation. On gestational day 16 through day 21, pregnant females were injected daily with sodium nitrite 50 mg/kg intraperitoneally (IP) (n = 45). Pregnant females used as controls (n = 5) were injected with the same amount of physiological saline in the same manner.

2.3 Postnatal Treatment and Experimental Groups

Immediately after birth, offspring from each litter were randomized, labeled, and administered the respective pharmacological agents or physiological saline starting from P1. Until P30, pups were housed with their dams, after which they were separated and placed into individual cages according to the experimental design, treatment type, and sex. To exclude potential sex-related variability, only male offspring were included in further analysis; ten male rats from different litters were randomly selected for each experimental group.

The following experimental groups from the offspring were formed: (1) Intact group - offspring of normoxic females injected with physiological saline (5 µL/g, IP); (2) IUH control group - offspring after IUH and injected with physiological saline (5 µL/g, IP); (3) IUH + Angiolin - 50 mg/kg, IP; (4) IUH + Piracetam - 500 mg/kg, IP; (5) IUH + Tamoxifen - 0.1 mg/kg, intranasal administration; (6) IUH + Thiotriazolin - 50 mg/kg, IP; (7) IUH + Nikomex - 100 mg/kg, IP; (8) IUH + Cerebrocurin - 150 µL/kg (equivalent to 0.3 mg/kg of active neuropeptides), IP; (9) IUH + L-arginine - 200 mg/kg, IP; (10) IUH + Glutaredoxin - 200 µg/kg, IP; (11) IUH + HSF-1 - 50 mg/kg, IP; (12) IUH + Mildronate - 50 mg/kg, IP.

The power analysis was conducted A priori based on the results of the pilot study. The significance level α was set at 0.05, and the Type II error rate β at 0.2. Thus, it was assumed that the power of the test, 1 – β, would be 0.8.

The iNOS level, ng/mL, was taken as the primary endpoint in the sample size calculations, and the minimum clinically significant effect Δ was defined as its change after treatment with inter-individual variability σ.

The sample size was calculated using the formula:

$$n \approx 2\frac{(z_{1-\frac{\alpha}{2}} + z_{1-\beta})^2\sigma^2}{\Delta^2}$$

where $z_{1-\frac{\alpha}{2}} = 1.96$ — the critical value for a significance level of 0.05;

$z_{1-\beta} = 0.84$ — the critical value for a power of 1 – β = 0.8.

$$n \approx 2\frac{(1.96 + 0.84)^2 0.29^2}{0.05^2} \approx 10$$

To test the power of the obtained effect from the Post hoc study with a sample size of n = 10, Cohen's d was calculated using the following formula:

$$d = \frac{M_1 - M_2}{S_{pooled}}$$

where $M_1 - M_2$ — the difference between the means, and S_{pooled} — the pooled standard deviation.

A Cohen's d value of 1.99 indicates a large effect size, and thus a sufficiently large sample size.

2.4 Pharmacological Agents

In this study, we used following pharmacological agents:

Modulators of HSP70 system: thiotriazolin (morpholinium thiazotate, 2.5% injectable solution, C01E B23, Arterium Corporation, Kyiv, Ukraine), tamoxifen (tablets, L02BA01, Orion Corporation, Espoo, Finland), angiolin ((S)-2,6-diaminohexanoic acid 3-methyl-1,2,4-triazolyl-5-thioacetate), glutaredoxin (ABS2053, Sigma-Aldrich, St. Louis, MO, USA), HSF-1 (SRP5188, Sigma-Aldrich), and cerebrocurin (injectable solution containing neuropeptides, S-100 proteins, reelin, nerve growth factor (NGF; ≥2 mg/mL), and amino acids; N06B X26, NPO "NIR", Kyiv, Ukraine). Tamoxifen tablet was used to prepare an extemporaneous intranasal gel (1 mg/mL) at the Department of Pharmaceutical Technology of Zaporizhzhia State Medical and Pharmaceutical University. Angiolin was synthesized at the Scientific and Technological Complex "Institute of Monocrystals" of the National Academy of Sciences of Ukraine (Series 2451117, Kharkiv, Ukraine).

Comparator drugs: L-arginine (42% injectable solution, B05X B01, Tivortin, Yuria-Pharm, Kyiv, Ukraine). nikomex (2-ethyl-6-methyl-3-hydroxypyridine succinate, injectable solution 50 mg/mL, MD2056, Lekhim-Kharkiv, Ukraine), mildronate (2-(2-carboxyethyl)-1,1,1-trimethylhydrazinium, 10% injectable solution, C01EB22, Grindeks, Riga, Latvia), and piracetam (200 mg/mL, N06BX03, Farmak, Kyiv, Ukraine).

The dosage was based on previous experimental studies or published literature [28,42,43,44,45,46,47].

Precise weighing of substances and chemical compounds was performed using BT-500 torsion balances (Kyiv Medical Equipment Plant Medaparatura, Kyiv, Ukraine). Electronic micropipettes (Brand, Wertheim, Germany), and chemical glass ware for analytical purposes were used to dilute solutions. Animals were weighed using AUX 220 electronic scales (Shimadzu Corporation, Kyoto, Japan) before every drug administration. For IP administration, the following equipment were used: a 10 µL microsyringe (model 701N, 26G, length 51 mm, outer diameter 0.47 mm; Hamilton, VWR International, UK, cat. No.549-1135), a 1 mL Air-Titele Sterile 27G syringe with a 13 mm needle and 30G×1/2" needles (Becton Dickinson, Spain, cat. No.305106).

The drugs were injected in the first days after birth at a temperature of 38–40 °C, maintained using electric heating pads for premature babies. Prior to procedures, dams were temporarily placed in separate cages containing shavings from their home cages. To reduce stress levels in the animals, these shavings were also rubbed on the researcher's gloves. To avoid stressing the females and to prevent possible litter rejection, injections were performed in a separate room. Before administering the drug, the rats were placed on their backs on a pre-warmed paper towel, to which sawdust from the cage was also added. This stimulated reflex urination when turning over and moving the limbs. For the IP injection, the rats were carefully held by the abdomen between the thumb and forefinger.

2.5 Anesthesia and Euthanasia

Animals were euthanized on postnatal days 30 (P30) and 60 (P60) under general anesthesia induced with thiopental sodium (40 mg/kg, IP). Thiopental sodium lyophilizate (0.5 g vials; Kyivmedpreparat LLC, Kyiv, Ukraine, Cod# N01AF03) was dissolved in physiological solution to make a 4% solution. This solution was administered using an insulin syringe at a dose of 0.1 mL per 100 g of body weight. Then animals for molecular and histomorphological studies are euthanized by cervical dislocation.

2.6 Collection and Processing of Biological Samples

Blood was quickly removed from the brain, and the meninges were separated. The fragments under investigation were placed in liquid nitrogen and ground to a powdery state. Then, the brain samples were homogenized at a temperature of +2 °C in a 10-fold volume of medium containing sucrose (250 mmol), Tris-HCl buffer (20 mmol), and EDTA (1 mmol) at a pH of 7.4. 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 first subjecting it to preliminary centrifugation for 7 minutes at 1000 g, and then subjecting it to a second centrifugation for 20 minutes at 17,000 g. This process was performed to remove large cell fragments. The supernatant was collected and stored at –80 °C [45].

For morphological analysis brain tissue was fixed in Bouin's solution for 24 h, routinely processed, and embedded in paraffin. Serial frontal sections (5 µm) were prepared at the level of the postcentral gyrus, including the somatosensory cortex, hippocampal cortex, choroid, choroid plexus, and branches of the central cerebral and ophthalmic arteries. To assess the morphofunctional state of endothelial cells in capillaries of cortical layers IV–V and in cerebral vessels, deparaffinized sections were stained by Einarson's gallocyanin–chromalum method for specific RNA visualization. Nuclear area, mean nuclear diameter, nuclear RNA concentration, and endothelial nuclear density were quantified. Proliferating endothelial cells were detected by BrdU immunofluorescence. Sections were deparaffinized, rehydrated, treated with 2 N HCl, neutralized, digested with 0.1% trypsin, and incubated with mouse monoclonal anti-BrdU antibodies (1:500, B8434, clone BU-33, Sigma-Aldrich), followed by FITC-conjugated secondary antibodies (1:100, sheep anti-mouse $F(ab')_2$ IgG, F2266, Sigma-Aldrich).

VEGF expression was assessed using a similar immunofluorescence protocol with mouse IgG1 anti-VEGF antibodies (1:500, MAB1665, clone CH-10, Chemicon, Temecula, CA, USA,). VEGF concentration was expressed in optical density units (ODU) as the logarithm of the ratio of specific fluorescence intensity to background extracellular fluorescence. Microscopic analysis was performed using an Axioskop microscope (Zeiss, Jena, Germany). Fluorescent images were acquired with a Zeiss high-emission filter set and a Fluar 40×/1.30 oil-immersion objective, captured using an 8-bit CCD camera COHU-4922 (Cohu, San Diego, CA, USA), and analyzed automatically using the VIDAS-386 image analysis system (Kontron Elektronik, Eching, Germany) with custom macros (VIDAS-2.5 environment). The results of the morphological analysis are presented for the lead compound (angiolin) and the reference drug (piracetam).

For transmission electron microscopy, tissue blocks up to 1 × 1 × 1 mm were excised from the frontal-lobe sensorimotor cortex and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4 °C. Samples were post-fixed in 1% osmium tetroxide, dehydrated, stained en bloc with 2.5% uranyl acetate, embedded in Epon-812 resin, and polymerized sequentially at 36 °C and 56 °C. Ultrathin sections (55–65 nm) were prepared using a PowerTome ultramicrotome (RMC Boeckeler, Tucson, AZ, USA), contrasted with Reynolds' lead citrate, and examined with a PEM-100-01 transmission electron microscope (Selmi, Sumy, Ukraine) operated at 65 kV.

2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of the examined analytes was determined in the cytosolic fraction of rat brain homogenate using a solid-phase ELISA in accordance with the manufacturers' instructions: nitrotyrosine (Hycult Biotech, Uden, The Netherlands; HK501-02), inducible nitric oxide synthase (iNOS) (MyBioSource, San Diego, CA, USA, MBS023874), endothelial nitric oxide synthase (eNOS) (Cloud-Clone Corporation, Wuhan, China; #PAA868Ra01), caspase-8 (Caspase-8 Activity Assay Kit, Abcam, Cambridge, UK; ab219915), BDNF (Rat BDNF ELISA kit, Abcam; ab213899), NGF (Rat beta NGF ELISA kit, Abcam; ab193736), and VEGF (Rat VEGFA ELISA kit, Abbexa Ltd., Cambridge, UK; abx050233).

2.8 Statistical Analysis

Statistical analyses were performed using Statistica for Windows 6.0 (StatSoft Inc., Tulsa, OK, USA), SPSS

Table 1. Concentration of VEGF, nitrotyrosine, eNOS, and iNOS in the cytosolic fraction of brain homogenate of rats after IUH and its pharmacological correction.

Experimental groups, n = 10	VEGF (pg/mL)		Nitrotyrosine (pg/mL)		iNOS, (ng/mL)		eNOS, (ng/mL)	
	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days
Intact	1213.33 ±99.80	1207.88 ±99.04	3.47 ±0.32	3.48 ±0.31	3.40 ±0.28	3.43 ±0.28	9.24 ±0.73	11.22 ±0.87
Control (IUH)	728.56 ±59.57[1]	714.54 ±58.46[1]	18.47 ±1.54[1]	14.26 ±1.20[1]	14.60 ±1.24[1]	10.78 ±0.91[1]	4.09 ±0.30[1]	5.31 ±0.25[1]
IUH + Angiolin	1254.55 ±102.48[*]	1273.94 ±104.12[*]	6.35 ±0.57[1,*]	5.37 ±0.47[1,*]	4.68 ±0.38[1,*]	4.11 ±0.34[*]	9.10 ±0.31[*]	15.30 ±1.14[1,*]
IUH + Piracetam	984.71 ±80.85[1,*]	998.06 ±81.54[1,*]	14.76 ±1.28[1]	12.42 ±1.072[1]	11.39 ±0.38[1]	10.45 ±0.87[1]	4.42 ±0.51[1]	5.21 ±0.29[1]
IUH + Thiotriazolin	1255.15 ±102.62[*]	1218.79 ±99.92[*]	10.19 ±0.87[1,*]	8.39 ±0.92[1,*]	5.94 ±0.49[1,*]	4.21 ±0.35[*]	8.11 ±0.24[*]	11.67 ±0.83[*]
IUH + Nikomex	899.33 ±73.81[1,*]	923.11 ±75.39[1,*]	14.16 ±1.20[1]	9.40 ±0.77[1,*]	7.64 ±0.63[1,*]	7.24 ±0.60[1,*]	5.73 ±0.20[1,*]	7.58 ±0.34[1,*]
IUH + Cerebrocurin	990.23 ±82.02[1,*]	1244.24 ±101.77[*]	6.88 ±0.57[1,*]	4.62 ±0.42[1,*]	5.79 ±0.50[1,*]	4.56 ±0.38[*]	8.69 ±0.52[*]	11.20 ±1.30[*]
IUH + Tamoxifen	852.17 ±69.63[1,*]	866.62 ±70.92[1,*]	16.92 ±1.41[1]	13.56 ±1.14[1]	11.52 ±0.94[1]	9.70 ±0.80[1]	4.78 ±0.39[1]	5.72 ±0.58[1]
IUH + L-arginine	883.18 ±72.16[1,*]	899.33 ±73.66[1,*]	16.88 ±1.39[1]	14.09 ±3.17[1]	9.43 ±0.77[1]	8.35 ±0.68[1]	8.79 ±0.40[*]	12.48 ±1.09[*]
IUH + Glutaredoxin	861.52 ±70.43[1,*]	918.02 ±74.98[1,*]	8.54 ±0.93[1,*]	6.37 ±0.52[1,*]	7.35 ±0.60[1,*]	5.65 ±0.47[1,*]	7.61 ±0.28[1,*]	11.71 ±1.22[*]
IUH + HSF-1	912.49 ±74.61[1,*]	986.41 ±74.98[1,*]	9.79 ±0.94[1,*]	5.63 ±0.49[1,*]	5.78 ±0.48[1,*]	4.76 ±0.40[1,*]	7.90 ±0.42[1,*]	12.30 ±1.12[*]
IUH + Mildronate	782.94 ±64.07[1]	719.64 ±58.91[1]	17.22 ±1.42[1]	10.32 ±0.88[1]	9.45 ±0.78[1]	9.77 ±0.80[1]	5.89 ±0.23[1,*]	6.67 ±0.34[1]

Notes: [1] $p \leq 0.05$ compared with the intact group; *$p \leq 0.05$ compared to the IUH control group. VEGF, vascular endothelial growth factor; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; IUH, intrauterine hypoxia; HSF-1, heat shock factor 1.

16.0 (SPSS Inc., Chicago, IL, USA), and Microsoft Excel (Microsoft 365, Microsoft Corporation, Redmond, WA, USA). Data that were normally distributed were presented in tables as the mean and standard error of the mean (M ± m). The normality of distributions was evaluated using the Shapiro–Wilk test. Outliers were defined as values exceeding three standard deviations (3σ) from the group mean. One-way analysis of variance was used to assess the effect of the studied substances on molecular parameters. Paired comparisons were performed using a Student's *t*-test for normally distributed data. To additionally verify the robustness of the obtained results, nonparametric analyses were performed using the Kruskal–Wallis H test and the median test. Differences were considered statistically significant at $p < 0.05$.

3. Results

Modeling of IUH resulted in significant changes in the concentrations of studied proteins in the cytosolic fraction of the brain, which may characterize the development of endothelial dysfunction in 1- and 2-month-old animals after IUH (Tables 1,2).

Thus, in the brains of rats after IUH on P30 and P60, a more than twofold decrease in eNOS levels was observed compared with healthy animals of the corresponding age groups. At the same time, animals after IUH demonstrated a marked increase in iNOS: 4.3-fold in 1-month-old rats and more than 3-fold in 2-month-old rats relative to intact animals (Table 1). These results are consistent with our previous study, which demonstrated increased iNOS mRNA expression and decreased eNOS mRNA expression in the CA1 region of the hippocampus compared with healthy 1- and 2-month-old animals [46].

Apparently, a reduction in eNOS-derived NO production occurs, whereas the NO generated through iNOS activation is converted into peroxynitrite. Evidence of oxidative transformation of NO is provided by the increase in nitrotyrosine concentration by 5.3-fold in 1-month-old and by 4.0-fold in 2-month-old animals compared with healthy controls (Table 1). The obtained results indicate significant disturbances in the nitrergic system of the rat brain after IUH, including altered NOS expression profiles, decreased NO bioavailability, and activation of nitrosative stress. In addition, we found that IUH, accompanied by

Table 2. Statistical analysis of significant differences in VEGF, nitrotyrosine, eNOS, and iNOS levels in rats following IUH treated with Angiolin compared with those treated with other pharmacological agents (p).

Experimental groups, n = 10	VEGF		Nitrotyrosine		iNOS		eNOS	
	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days
IUH + Piracetam	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
IUH + Thiotriazolin	0.99	0.24	<0.01	<0.01	<0.01	0.53	<0.01	<0.01
IUH + Nikomex	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
IUH + Cerebrocurin	0.059	0.53	0.0522	<0.01	<0.01	<0.01	0.046	<0.01
IUH + Tamoxifen	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
IUH + L-arginine	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.069	<0.01
IUH + Glutaredoxin	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
IUH + HSF-1	<0.01	<0.01	<0.01	0.24	<0.01	<0.01	<0.01	<0.01
IUH + Mildronate	<0.01	<0.015	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

NO system dysregulation, resulted in a significant reduction in VEGF concentration by approximately 40% at 1 and 2 months of life in experimental animals. A 30-day postnatal course of HSP70 modulators administered immediately after birth (with the exception of tamoxifen) exerted varying degrees of positive effects on the expression of iNOS, eNOS, VEGF, and nitrotyrosine in the cytosolic fraction of the brain of 1- and 2-month-old rats after IUH. The results indicate significant disturbances in the nitroxidergic system of the rat brain after IUH, including a change in NOS expression character, decreased NO bioavailability, and nitrosative stress activation. The most pronounced normalization of these parameters toward intact values was observed with angiolin, cerebrocurin, HSF-1, thiotriazolin, and glutaredoxin, indicating their capacity to mitigate endothelial dysfunction. Specifically, by the end of treatment, eNOS expression increased more than twofold with angiolin, thiotriazolin, and cerebrocurin; by 85% with glutaredoxin; and by 95% with HSF-1. VEGF levels increased by 72% following angiolin and thiotriazolin administration, by 36% with cerebrocurin, and by 25% and 18% with HSF-1 and glutaredoxin, respectively, compared with control values ($p \leq 0.05$).

A significant decrease in nitrotyrosine levels was observed in these groups compared with the control ($p \leq 0.05$): almost threefold after angiolin, 2.7-fold after cerebrocurin, twofold after HSF-1 and glutaredoxin, and by 45% in the thiotriazolin-treated group compared with controls ($p \leq 0.05$). Comparator drugs, such as nikomex and mildronate, with the exception of L-arginine, demonstrated a moderate corrective effect, whereas piracetam had virtually no influence on the expression of iNOS, eNOS, VEGF, or nitrotyrosine. It was also noted that the pharmacological effects of HSP70 modulators and reference drugs persisted after cessation of treatment. Thus, one month after completion of the treatment course with angiolin, cerebrocurin, thiotriazolin, HSF-1, and glutaredoxin, eNOS expression remained elevated by two-fold or greater (threefold in the angiolin group) and by 43%, 26%, and 8% following treatment with nikomex, mildronate, and tamoxifen, respectively, compared with control values ($p \leq 0.05$). In parallel, nitrotyrosine levels decreased by 2.6-fold (angiolin), threefold (cerebrocurin), 2.2-fold (glutaredoxin), 2.5-fold (HSF-1), and by 41% (thiotriazolin) relative to the control ($p \leq 0.05$).

IUH induces profound and persistent disturbances in neuroplasticity and activates apoptotic mechanisms in rat offspring. This was confirmed by significant alterations in BDNF, NGF, and caspase-8 levels during the first and second months of postnatal development (Tables 3,4). In the IUH control group at one month of age, BDNF levels decreased by more than 50% compared with intact animals, while NGF levels declined by approximately 45%. These changes were accompanied by an almost threefold increase in caspase-8 concentration, indicating robust activation of the extrinsic (receptor-mediated) apoptotic pathway. By the second month of life, partial age-related compensation of neurotrophic parameters was observed; however, BDNF and NGF levels remained significantly below intact values. At the same time, caspase-8 activity remained elevated and showed a tendency toward further increase, reflecting the prolonged nature of hypoxia-induced apoptotic damage. These findings indicate that IUH disrupts key neurotrophic signaling pathways essential for neuronal survival, synaptic plasticity, and postnatal brain maturation, and that these alterations persist beyond the early postnatal period.

Course administration of HSP70 modulators reduced IUH-induced neurotrophic deficits to varying degrees. Among all tested agents, cerebrocurin exhibited the strongest effect. In animals treated with cerebrocurin, BDNF and NGF concentrations were close to intact values at 1 month of age and exceeded them at 2 months of age.

Angiolin also demonstrated a pronounced corrective effect, significantly increasing BDNF levels by 80% at 1 month of age and NGF levels at both time points by 63% and 42%, respectively, compared with control values ($p \leq 0.05$).

Course administration of thiotriazolin and HSF-1 similarly increased BDNF concentrations (by 60% and 70%, respectively) in one-month-old animals after IUH and elevated NGF levels (by 12% and 39% at one month, and by

Table 3. The content of neurotrophic factors and caspase-8 in brain homogenate of rats after IUH and its pharmacological correction.

Experimental groups, n = 10	BDNF, pg/mL		NGF, pg/mL		Caspase-8, ng/mL	
	30 days	60 days	30 days	60 days	30 days	60 days
Intact	244.27 ±8.73	245.26 ±8.53	312.09 ±18.20	312.57 ±13.82	2.38 ±0.13	2.39 ±0.11
Control (IUH)	110.50 ±9.24[1]	187.42 ±12.14[1]	170.67 ±11.16[1]	211.60 ±18.64[1]	6.74 ±0.45[1]	7.36 ±0.44[1]
IUH + Angiolin	197.15 ±12.23[1,*]	214.45 ±18.00[*]	277.36 ±17.85[1,*]	300.13 ±11.25[*]	3.07 ±0.22[1,*]	3.43 ±0.12[1,*]
IUH + Piracetam	167.65 ±11.13[1,*]	190.57 ±17.02[1]	168.90 ±15.19[1]	211.73 ±15.51[1]	5.56 ±0.34[1]	6.15 ±0.32[1]
IUH + Thiotriazolin	177.33 ±15.80[1,*]	196.68 ±16.11[1]	192.15 ±12.61[1,*]	278.91 ±17.33[*]	4.65 ±0.24[1,*]	4.87 ±0.32[1,*]
IUH + Nikomex	169.43 ±10.89[1,*]	179.50 ±10.23[1]	170.53 ±15.13[1]	261.64 ±21.84[1,*]	5.29 ±0.43[1]	6.15 ±0.57[1]
IUH + Cerebrocurin	217.61 ±16.81[*]	252.96 ±15.69[*]	290.61 ±21.40[*]	333.86 ±18.77[*]	2.88 ±0.19[*]	2.97 ±0.13[*]
IUH + Tamoxifen	155.43 ±14.32[1,*]	177.73 ±9.70[1]	167.27 ±9.12[1]	236.21 ±20.21[1]	4.71 ±0.27[1,*]	5.28 ±0.32[1,*]
IUH + L-arginine	121.73 ±10.21[1]	182.27 ±16.74[1]	170.53 ±13.90[1]	210.92 ±17.14[1]	6.34 ±0.42[1]	6.83 ±0.42[1]
IUH + Glutaredoxin	154.44 ±11.20[1,*]	191.55 ±11.72[1]	180.05 ±12.41[1,*]	277.28 ±22.73[*]	5.46 ±0.43[1]	5.73 ±0.27[1]
IUH + HSF-1	187.40 ±15.18[1,*]	197.47 ±9.78[1]	237.30 ±15.33[1,*]	288.97 ±19.20[*]	4.37 ±0.32[1,*]	4.91 ±0.37[1,*]
IUH + Mildronate	126.19 ±8.25[1]	180.29 ±17.02[1]	169.31 ±11.32[1]	220.03 ±17.71[1]	5.22 ±0.47[1]	5.57 ±0.28[1,*]

Notes: [1] $p \leq 0.05$ compared with the intact group; *$p \leq 0.05$ compared to the IUH control group. BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor.

Table 4. Statistical analysis of significant differences in BDNF, NGF, and Caspase-8 levels in rats following IUH treated with Angiolin compared with those treated with other pharmacological agents (*p*).

Experimental groups, n = 10	BDNF		NGF		Caspase-8	
	30 days	60 days	30 days	60 days	30 days	60 days
IUH + Piracetam	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
IUH + Thiotriazolin	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
IUH + Nikomex	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
IUH + Cerebrocurin	<0.01	<0.01	0.15	<0.01	0.053	<0.01
IUH + Tamoxifen	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
IUH + L-arginine	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
IUH + Glutaredoxin	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
IUH + HSF-1	0.13	<0.01	<0.01	0.13	<0.01	<0.01
IUH + Mildronate	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

32% and 37% at two months, respectively) relative to age-matched control animals ($p \leq 0.05$).

Among the reference drugs (piracetam, nikomex, L-arginine, and mildronate), only nikomex and piracetam demonstrated a short-term effect, significantly increasing BDNF concentrations (by 53% and 52%, respectively) in the brains of P30 animals after IUH compared with age-matched controls ($p \leq 0.05$). At P60, these parameters did not differ from control values. In the nikomex-treated group, an increase in NGF levels by 24% was also observed in two-month-old animals. These findings suggest that only selected pharmacological approaches are capable of effectively restoring neurotrophic balance following IUH.

To assess the effect of the pharmacological agents under study on biomarker levels in rats following IUH, a one-way analysis of variance (ANOVA) was performed.

Since the use of the F-test requires that the data satisfy the assumption of homoscedasticity, the data were log-transformed, followed by a back-transformation. The results of the analysis are presented in Table 5.

Table 5. Results of a one-way analysis of variance (ANOVA) for the tested biomarkers.

Biomarker	F(9, 91)	p
BDNF, pg/mL	2.50	0.015000
NGF, pg/mL	5.05	0.000017
Caspase-8, ng/mL	12.90	0.000000
VEGF, pg/mL	4.90	0.000025
Nitrotyrosine, pg/mL	7.90	0.000000
iNOS, ng/mL	17.60	0.000000
eNOS, ng/mL	14.10	0.000000

For all biomarkers, analysis of variance revealed statistically significant differences between treatment groups ($p < 0.05$). Since the main differences were confirmed, a Post hoc analysis for pairwise comparisons was performed using the Student's t-test. The results for the pharmacological modulation methods are presented in Tables 2,4.

The obtained data allowed us to reject the null hypothesis of equal effects and conclude that different treatment methods lead to qualitatively different results.

To test the null hypothesis, we used the Kruskal-Wallis nonparametric test and the median test. The results are presented in Table 6.

It is well known that nonparametric tests, while robust, are less powerful than parametric tests. As shown in Table 6, the results of the nonparametric analysis fully confirm those of the parametric analysis, indicating statistical significance of the treatment effect for most marker groups.

Correlation analysis between neurotrophic factor concentrations and HSP70 levels, based on our previous study [47], revealed a strong positive association at both experimental time points, with a slight reduction by P60 (Fig. 1).

Assessment of caspase-8 levels revealed significant activation of the extrinsic apoptotic pathway in offspring following IUH. At one month of age, caspase-8 concentrations in the control group exceeded those of intact animals by nearly threefold, with a further increase observed at two months (Table 3). These findings indicate persistent pro-apoptotic signaling and incomplete endogenous compensation after IUH.

Course administration of the investigated agents reduced caspase-8 levels compared to the control animals. Cerebrocurin and angiolin exhibited the most pronounced effects, reducing caspase-8 levels to values approaching those of intact animals at both postnatal time points examined. Thiotriazolin and HSF-1 also provided substantial suppression of caspase-8 activation in the brains of animals at P30 and P60 following IUH, decreasing its levels by more than 30% compared with age-matched controls ($p \leq 0.05$).

In contrast, piracetam, nikomex, glutaredoxin, mildronate, tamoxifen, and L-arginine demonstrated limited efficacy in inhibiting apoptotic signaling, as caspase-8 concentrations in these groups remained significantly elevated relative to the intact group. These results suggest that effective attenuation of IUH-induced apoptosis requires more than nonspecific metabolic support and instead depends on targeted modulation of hypoxia-sensitive molecular pathways.

An examination of the vascular component of the cerebral cortex revealed that, in 2-month-old rats following IUH, there was a decrease in the density of capillary endothelial cell nuclei per unit area of the cerebral cortex cross-section compared to intact animals (Table 7). This reflects processes of capillary occlusion and a decrease in the density of functioning capillaries in the cerebral cortex.

Modeling of IUH was accompanied by a decrease in RNA concentration in the nuclei of capillary endothelial cells from 2-month-old rats compared to intact animals.

Administration of Angiolin had a protective effect on the capillary endothelium, resulting in a significant increase in the density of endothelial cell nuclei per unit area of the cerebral cortex section, nuclear hypertrophy, and an increase in RNA concentration within them in 2-month-old rats (Table 7, Fig. 2). Administration of piracetam at a dose of 500 mg/kg to animals immediately after IUH had no protective effect on the endothelium of cerebral cortical capillaries.

The levels of VEGF binding and the nature of proliferative activity in the capillary endothelium were studied to characterize the revascularization processes in greater detail.

It was found that hypoxic brain injury led to a significant decrease in VEGF binding to the vascular endothelium of the cortical capillary network, while the administration of Angiolin had a positive effect on this process, as evidenced by an increase in the concentration of VEGF. IUH inhibited the proliferative activity of the vascular endothelium of cerebral cortical capillaries in the postnatal period, as evidenced by a decrease in the density of proliferating cells per 1 mm^2 of the cortex. Administration of Angiolin significantly increased the proliferative activity of the vascular endothelium, as evidenced by a significant increase in the density of proliferating endothelial cells, as well as the area and diameter of the nuclei of proliferating cells, compared to the group of untreated animals.

The data obtained indicate that Angiolin, either through its interaction with VEGF or independently, significantly increases the proliferative activity of the endothelium in the capillaries of the cerebral cortex, thereby promoting effective revascularization of hypoxia-damaged brain tissue.

Table 6. Results of the nonparametric Kruskal-Wallis test and the median test for the tested biomarkers.

Experimental groups, n = 10	Kruskal-Wallis H test, H (9, N = 100)		Median test	
	F(9, 91)	p	χ^2	p
BDNF, pg/mL	14.8	0.090	4.8	0.850
NGF, pg/mL	31.2	0.003	20.0	0.020
Caspase-8, ng/mL	56.4	0.001	31.2	0.003
VEGF, pg/mL	28.2	0.009	15.2	0.008
Nitrotyrosine, pg/mL	52.6	0.001	53.6	0.001
iNOS, ng/mL	68.3	0.001	68.0	0.001
eNOS, ng/mL	65.6	0.001	59.2	0.001

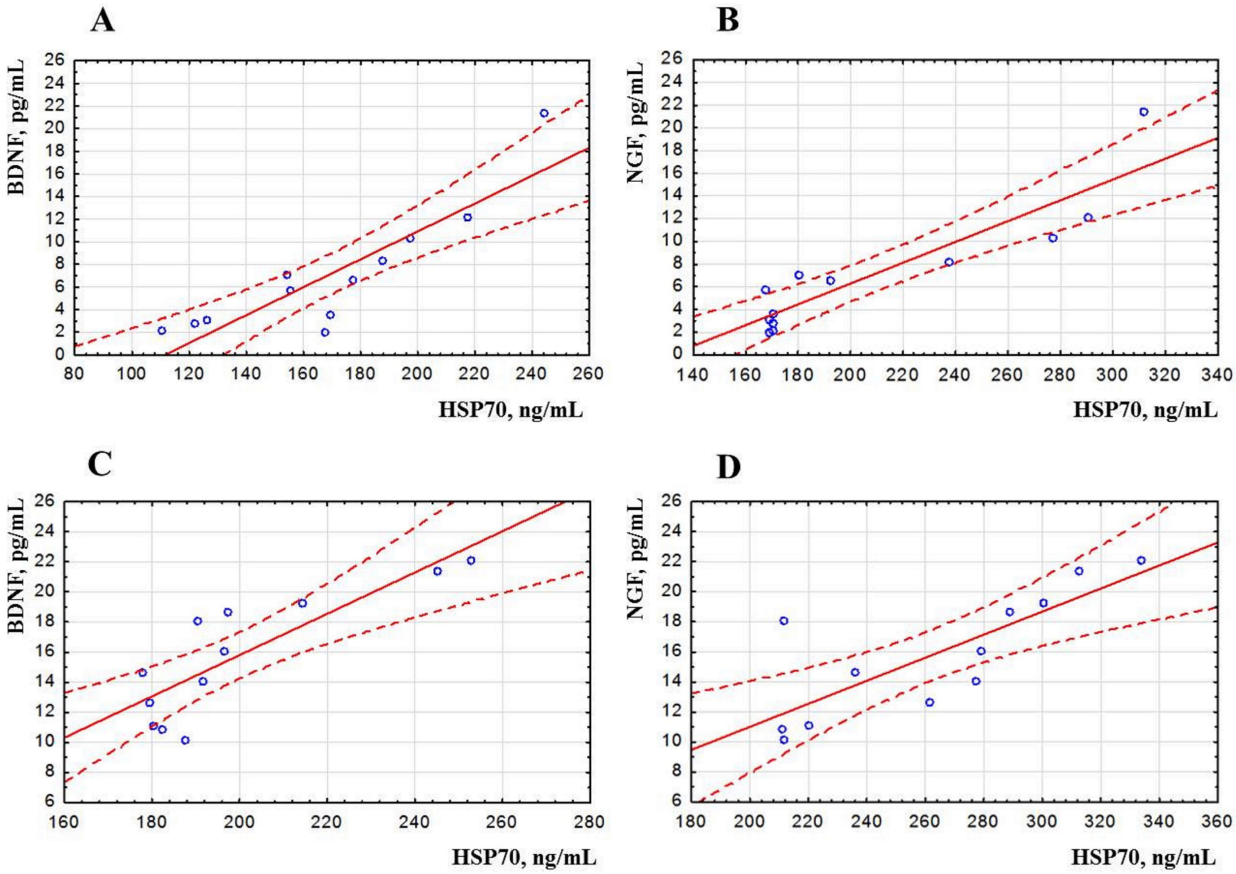

Fig. 1. Correlation analysis between neurotrophic factors content and HSP70 levels in rat brain homogenate after IUH. P30: (A) BDNF/HSP70 r = 0.88 (p = 0.0003) (n = 120), (B) NGF/HSP70 r = 0.91 (p = 0.0001) (n = 120); P60: (C) BDNF/HSP70 r = 0.83 (p = 0.001) (n = 120), (D) NGF/HSP70 r = 0.80 (p = 0.0024) (n = 120). HSP70, 70 kDa heat shock protein.

4. Discussion

It is well established that modeling IUH through the administration of sodium nitrite to pregnant rats induces hemoglobin-dependent hypoxia due to the formation of methemoglobin. This condition is associated with tissue hypoxia resulting from the disruption of oxidative phosphorylation processes in mitochondria. The consequent reduction in maternal blood oxygenation impairs uteroplacental circulation, thereby creating hypoxic conditions for the developing fetus. Administration of sodium nitrite at a dose of 50 mg/kg induces moderate hypoxia in pregnant females and leads to hypoxic damage in fetal target organs [48,49].

Our previous studies have demonstrated that this IUH model results in persistent disturbances in energy metabolism, deficiency of macroergic compounds, impairment of the GABAergic system in the brain, deterioration of the morphofunctional characteristics of CA1 hippocampal neurons, and weakening of intrinsic neuro- and cytoprotective mechanisms mediated by HSP70 and HIF-1α. Ultimately, these alterations give rise to sustained cognitive, mnemonic, behavioral, and emotional impairments [29,37,43]. The findings of the present study further confirm that chronic IUH contributes to the development of endothelial dysfunction in cerebral vessels, occurring in par-

Table 7. Morphological characteristics of endothelial cells in the capillaries of the IV–V layers of the cerebral cortex in 2-month-old rats after IUH and pharmacological correction.

Indicator	Experimental groups, n = 10			
	Intact	Control (IUH)	IUH + Angiolin	IUH + Piracetam
Nucleus density per 1 mm^2 of cortex	892 ± 15	610 ± 12	842 ± 15^{*,1}	657 ± 15^{1}
Nuclear area, μm^2	8.88 ± 0.08	8.32 ± 0.05	8.44 ± 0,03*	9.11 ± 0.05*
Nuclear diameter, μm	2.78 ± 0.01	3.08 ± 0.01	2.92 ± 0,01*	3.11 ± 0.01^{1}
RNA concentration in nuclei, ODU	0.311 ± 0.002	0.211 ± 0.001	0.310 ± 0.002^{*,1}	0.283 ± 0.001*
Density of proliferating endothelial cells per 1 mm² of the cortex	781 ± 27	488 ± 23	678 ± 22^{*,1}	487 ± 21^{1}
The area of the nuclei of proliferating endothelial cells, μm²	6.72 ± 0.11	7.32 ± 0.10	8.56 ± 0.07^{*,1}	7.45 ± 0.06^{1}
Nuclear diameter of proliferating endothelial cells, μm	2.83 ± 0.01	2.88 ± 0.02	3.22 ± 0.01^{*,1}	2.91 ± 0.02
Concentration of endothelial growth factor, ODU	0.85 ± 0.01	0.72 ± 0.01	0.97 ± 0.02^{*,1}	0.76 ± 0.02

Notes: ^{1} $p \leq 0.05$ compared with to the intact group; $^{*}p \leq 0.05$ compared to the IUH control group. ODU, optical density units.

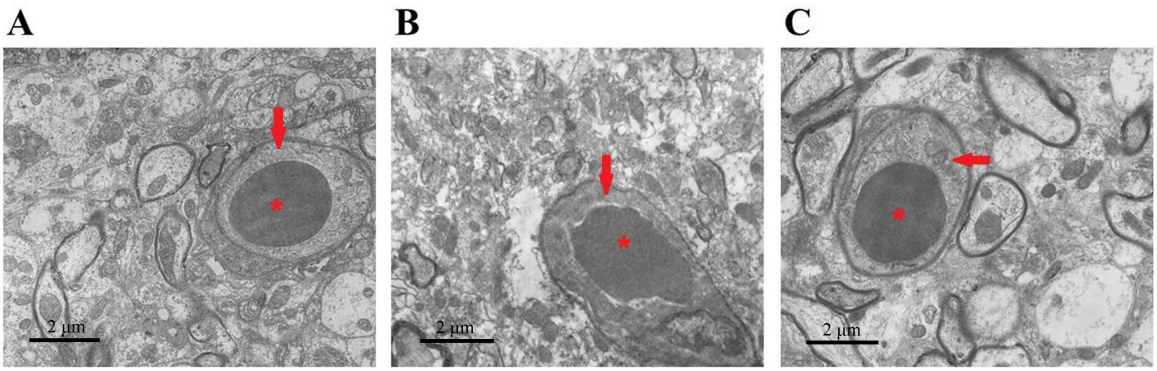

Fig. 2. Cross-section of cerebral cortex capillaries in 2-month-old rats. (A) intact group; (B) after IUH (control); (C) after IUH and 30 days of Angiolin administration (50 mg/kg). The red arrows indicate endothelial cell, * indicate erythrocyte. Scale bar, 2 μm. TEM ×8000. TEM, transmission electron microscopy.

allel with dysregulation of neurotrophic signaling and activation of apoptotic processes in one- and two-month-old offspring.

The observed changes are multifactorial and include impaired NO production and transport, leading to increased nitrosative stress, as well as the activation of NO-dependent and oxidative mechanisms underlying neuroplasticity impairment and the caspase-dependent apoptotic cascade. These results are consistent with current concepts describing hypoxic brain injury, including intrauterine forms, as a multistage pathological process. Our previous work identified significant disturbances in NO metabolism in the brains of rats following IUH, including an imbalance between eNOS and iNOS mRNA expression in the CA1 region of the hippocampus. These alterations were accompanied by oxidative modification of NO and its conversion to peroxynitrite, as evidenced by increased accumulation of nitrotyrosine in the brains of offspring [37,46]. This indicates a reduced adaptive capacity of neural tissue to oxygen deficiency and impaired NO-mediated regulation of vascular tone, ultimately leading to endothelial dysfunction in the post-hypoxic period, as well as activation of NO-dependent mechanisms of neurotoxicity and neurodegeneration.

The development of endothelial dysfunction following IUH is closely associated with HIF-1α deficiency, a key regulator that promotes eNOS expression through serine phosphorylation, as well as increased nitrosative stress. These processes are accompanied by decreased expression of HSP70, suppression of immediate early genes such as *c-fos*, depletion of intracellular reduced glutathione (GSH) reserves, impairment of GSH-dependent enzymatic systems, and accumulation of cytotoxic concentrations of reactive species [40,50,51,52,53].

Reduced HIF-1α expression after IUH may lead to suppression of HIF-1–regulated genes, including erythropoietin, VEGF, and glucose transporter 1 (GLUT-1). The development of cerebral endothelial dysfunction and decreased neuroplasticity observed after IUH may also be associated with HSP70 deficiency. HSP70 is a critical component of endogenous neuroprotection, acting through the attenuation of oxidative and nitrosative stress, reduction of glutamate excitotoxicity, mitigation of oxygen and glucose deprivation, stabilization of the HIF-1α protein, and regulation of gene expression involved in proliferation, apoptosis, and repair of oxidatively damaged proteins, including eNOS [12,17,26,44,54,55,56].

Experimental data obtained in murine models indicate that systemic knockout of genes encoding VEGF or eNOS disrupts vascular development and extraembryonic angiogenesis, resulting in impaired maternal–fetal transport of nutrients and oxygen and, ultimately, embryonic lethality [57]. These findings are consistent with our results demonstrating decreased VEGF and eNOS levels in the cytosolic fraction of brain tissue from one- and two-month-old rats following IUH.

Beyond alterations in neurotrophic regulation, an essential component of cerebral adaptation to oxygen deficiency involves the activation of angiogenic processes, with VEGF serving as a principal regulator. Although stimulation of the HIF-1α–VEGF signaling pathway is generally aimed at increasing microvascular density and improving oxygen delivery to neural tissue, this response may become dysregulated and maladaptive under conditions of IUH [58,59]. Aberrant or spatially heterogeneous VEGF expression can disrupt the formation of the neurovascular unit, increase blood–brain barrier permeability, and promote perivascular edema, thereby exacerbating hypoxic brain injury [60,61]. One of the significant consequences of IUH observed in the present study was persistent inhibition of angiogenesis, as evidenced by a significant decrease in VEGF levels in brain homogenates at P30 and P60. VEGF is a central regulator of vascular growth and remodeling, as well as an important neuroprotective factor that supports neuronal survival, neurogenesis, and neurovascular interactions. A sustained reduction in VEGF levels following IUH indicates disruption of adaptive mechanisms within the cerebral microcirculatory system and may predispose to chronic hypoperfusion and increased metabolic vulnerability of neural tissue [62,63].

VEGF exhibits a functionally interdependent relationship with the NO signaling system, and the effectiveness of angiogenesis largely depends on eNOS activity. A major contributor to neuronal injury after IUH is the emergence of oxidative–nitrosative stress driven primarily by induction of iNOS. The increased expression of iNOS in the neurons, astrocytes, and microglia of rat offspring's brains leads to the prolonged and uncontrolled production of nitric oxide. In hypoxic states and in the presence of mitochondrial dysfunction nitric oxide then reacts with superoxide anion to form the highly reactive molecule, peroxynitrite [64,65,66,67]. Peroxynitrite possesses pronounced cytotoxic properties and induces nitration of protein tyrosine residues, manifested by accumulation of nitrotyrosine, which is regarded as a reliable marker of nitrosative stress. Elevated nitrotyrosine levels in brain structures of rats after IUH indicate profound impairment of enzymatic activity, damage to components of the mitochondrial respiratory chain, alterations of cytoskeletal proteins, and disruption of receptor systems. These processes lead to energy deficiency, exacerbation of neuroinflammation, and reduced neuronal resistance to damaging factors [68,69,70,71].

In the present study, offspring subjected to IUH exhibited marked activation of iNOS, indicative of the establishment of a pathological NO-dependent pathway. Excessive iNOS expression was associated with uncontrolled NO production and its rapid conversion to peroxynitrite, a central effector of nitrosative cellular injury. The substantial elevation of iNOS observed in the IUH control group, which persisted at later time points, suggests prolonged activation of inflammatory and oxidative processes in the brain following IUH exposure [72,73,74].

Changes in nitrotyrosine levels were correlated with iNOS expression, and alterations in its cytosolic concentration reflect disruption of the balance between the generation and utilization of reactive nitrogen species. The decrease in nitrotyrosine observed in two-month-old animals after IUH likely does not indicate attenuation of nitrosative stress per se, but rather depletion of available protein substrates for nitration or a shift of NO metabolism toward alternative cytotoxic pathways. This interpretation is consistent with the concept of "dysfunctional NO," whereby NO availability does not correspond to its physiological or protective efficacy [12,75,76,77].

Endothelial dysfunction in cerebral vessels significantly impairs neuroplasticity—the brain's capacity for structural and functional reorganization—by promoting neuroinflammation, oxidative stress, and reduced cerebral blood flow. It also compromises blood–brain barrier integrity and decreases the availability of key regulatory factors, including neurotrophins. These changes result in neuronal damage, cognitive impairment, and an increased risk of neurodegenerative disorders. Consequently, a vicious cycle is established in which primary vascular dysfunction disrupts neural tissue integrity and limits its adaptive and reparative capacity [78,79,80,81].

IUH initiates a cascade of molecular and cellular alterations that may not be immediately apparent at the morphological level but form the basis for long-term functional impairments in postnatal life. It simultaneously activates adaptive compensatory responses and maladaptive injury processes, with the overall outcome determined by their dynamic balance and temporal coordination. One of the key consequences of IUH is impaired neurotrophic support of the developing brain, primarily due to dysregulation of BDNF and NGF expression. BDNF is a central regulator of neuronal survival, differentiation, dendritic growth, and synaptic plasticity, particularly in brain regions critical for cognitive function, such as the hippocampus and cerebral cortex [82,83].

Experimental study [17] in rats have demonstrated that IUH leads to decreased BDNF levels and disruption of signaling via the TrkB receptor during early postnatal development. These changes result in reduced neuronal plasticity, activation of apoptotic pathways, and the development of learning and memory deficits. The balance between mature BDNF and its precursor, proBDNF, is also

critical; a shift toward proBDNF promotes activation of the p75NTR receptor and triggers pro-apoptotic signaling pathways [84,85,86,87].

Similar alterations have been described for NGF, which plays an essential role in the development of cholinergic neurons in the basal forebrain, regulation of neuroimmune interactions, and maintenance of neural network integrity. Changes in NGF expression following IUH in rats are associated with impaired autonomic regulation, increased anxiety, and reduced adaptive capacity of the nervous system [88,89,90].

An important pathogenetic component of post-hypoxic alterations is a marked disruption in the synthesis of neurotrophic factors. The significant decrease in BDNF and NGF levels observed in offspring after IUH confirms that hypoxic exposure during critical developmental periods interferes with the establishment of neurotrophin-dependent signaling cascades responsible for neuronal survival, differentiation, and synaptic plasticity. Although partial age-related compensation of these parameters was observed at P60, complete normalization did not occur, indicating persistent "reprogramming" of neurotrophic regulation.

Nitrosative stress is closely integrated with mechanisms of programmed cell death, with caspase-8 representing one of the key mediators of this interaction. Caspase-8 is an initiator caspase of the extrinsic, receptor-mediated apoptotic pathway, which is activated by proinflammatory cytokines and damage-associated signals characteristic of hypoxic injury [91,92]. In the brains of rat offspring following IUH, increased caspase-8 activity indicates activation of a cascade of apoptotic processes leading to neuronal and glial cell death, impaired myelination, and the development of persistent structural abnormalities. Caspase-8 also plays a critical role in regulating the switch between apoptosis and necroptosis. Under conditions of severe hypoxic and inflammatory stress, functional insufficiency of caspase-8 may trigger necrotic pathways, accompanied by exacerbated inflammatory responses and secondary damage to brain tissue [93,94].

Collectively, the experimental findings indicate that IUH initiates a complex, multilevel pathogenic process in the brain, including activation of NO-dependent mechanisms of endothelial dysfunction, induction of nitrosative stress, downregulation of neurotrophins such as BDNF and NGF, and activation of caspase-dependent apoptosis. The observed increase in caspase-8 activity in offspring after IUH suggests predominance of the extrinsic apoptotic pathway. Of particular importance is the progressive elevation of caspase-8 levels during later postnatal stages, indicating that apoptotic processes are not only initiated during intrauterine injury but also persist during subsequent brain development, potentially limiting functional maturation [95,96].

Disruption of the balance between endogenous neuroprotective mechanisms and neurodestructive processes during critical periods of ontogenesis creates a structural and functional basis for long-term neurological, cognitive, and behavioral impairments. This underscores the importance of further research aimed at identifying effective neuroprotective strategies following IUH. Course administration of HSP70 modulators and comparator agents—including nootropics, antioxidants, and metabolic regulators—demonstrated the feasibility of correcting most of the identified disturbances; however, their therapeutic efficacy varied considerably depending on their mechanisms of action.

The most pronounced integrative corrective effects were observed with angiolin and cerebrocurin. Angiolin confirmed its previously described endothelium-protective activity, associated with modulation of VEGF expression in cerebral vessels and normalization of the eNOS/iNOS ratio under ischemic conditions, as well as with normalization of molecular markers of endothelial dysfunction (VEGF-B, soluble endothelial protein C receptor (sEPCR), Tie-2) in cardiac vessels following IUH [37].

In our studies [19,28,37,42,43], angiolin demonstrated pronounced effects on HSP70-mediated endogenous neuroprotection, exhibiting significant antihypoxic, neuroprotective, and cardioprotective properties mediated by antioxidant activity (scavenging of reactive oxygen species and NO), modulation of gene expression, and regulation of ROS/GSH-dependent pathways of HSP70 expression (Fig. 3). In addition, angiolin enhances metabolic adaptability by activating compensatory cytosolic and mitochondrial energy shuttles, potentiates GABAergic neurotransmission through increased receptor affinity, and attenuates excitotoxicity [26,97,98].

The combination of endothelial-protective, neuroprotective, antioxidant, and metabolically adaptive effects positions angiolin as a promising candidate for the pathophysiologically grounded treatment of cerebrovascular disorders following IUH.

Cerebrocurin proved to be the most effective agent in correcting the identified molecular disturbances associated with endothelial dysfunction and impaired neuroplasticity. Our previous studies [28,42,47] have demonstrated that cerebrocurin exerts neuroprotective effects by protecting neurons from lactate acidosis–induced injury, suppressing free radical generation, enhancing neuronal survival, and preventing neuronal death under hypoxic–ischemic conditions. In addition, it attenuates neurotoxicity mediated by glutamate and other excitatory amino acids, exhibits pronounced antioxidant activity, and improves functional neuromodulation. These effects are accompanied by improvements in cognitive function, memory processes, and information retrieval, as well as stimulation of mental activity and modulation of behavioral responses. Cerebrocurin also stimulates neurotrophic activity, and enhances interactions

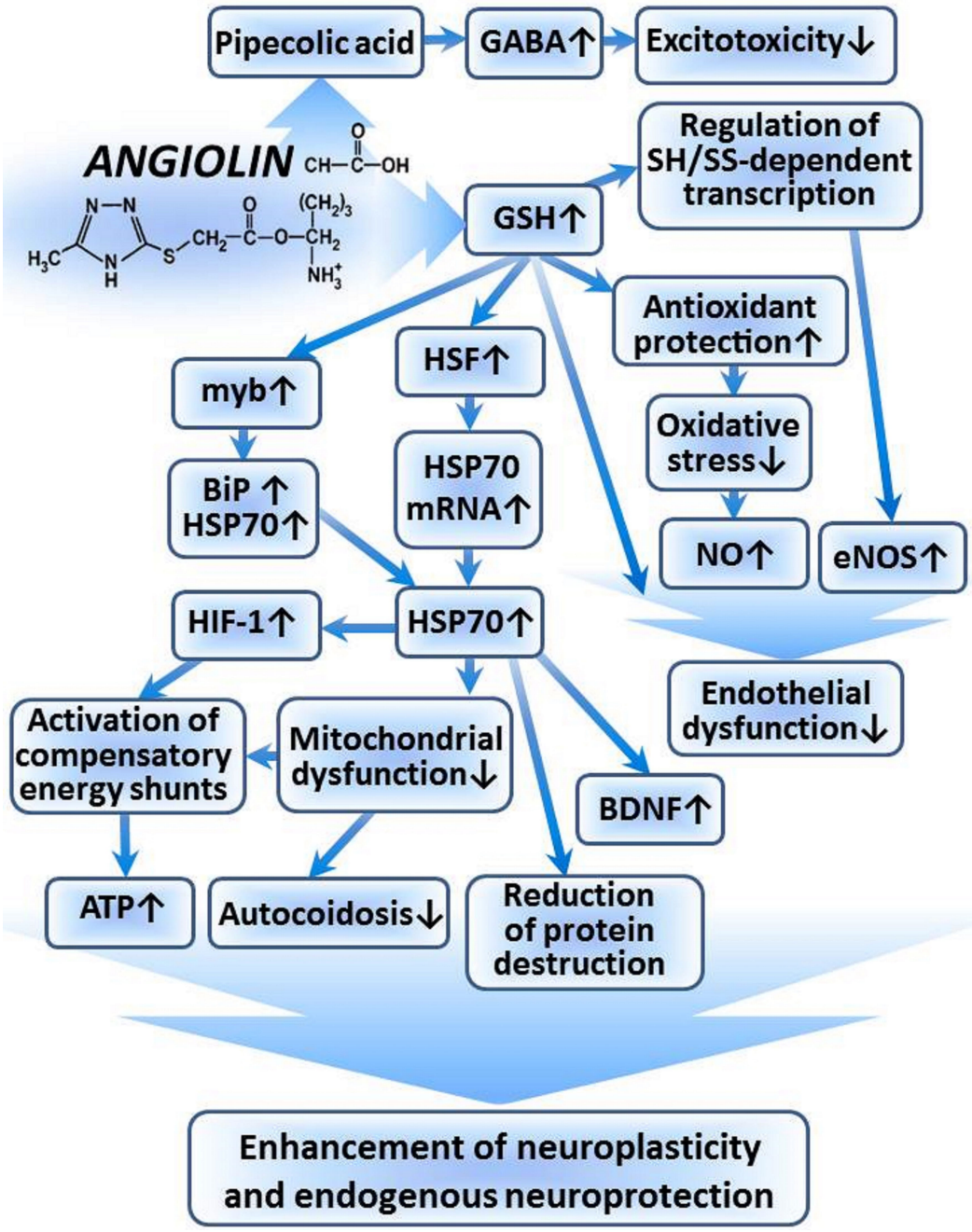

Fig. 3. Hypothetical mechanism of action of Angiolin. ↑ indicates an increase in level/concentration, and ↓ indicates a decrease in level/concentration. Angiolin is an antioxidant, specifically a superoxide radical and NO scavenger. Angiolin is capable of restoring the thiol–disulfide balance and increasing the levels of cytosolic and mitochondrial glutathione due to the presence of 3-methyl-1,2,4-triazolyl-5-thioacetate in its structure. Glutathione enhances the nuclear translocation of HSF1 and increases the HSP70 concentration by upregulating *HSP70* mRNA. GSH can induce HSP expression at the transcript level by activating its promoters. Thus, high concentrations of glutathione increase the expression of myb, which activates the promoters of the *HSP* gene (BiP HSP70B) and increases the expression of HSP70 and its dependent mechanisms of endogenous neuroprotection. In addition, glutathione increases the stability of *HSP70* mRNA by increasing the level of antioxidant protection against oxidative stress. The possibility of post-translational modification of HSP by S-glutathionylation is also not excluded. An increase in HSP70 concentration can activate the signaling pathway of BDNF expression. Thus, HSP70 may promote BDNF expression by inducing the activity of cAMP response element-binding protein (CREB). L-lysine in the Angiolin molecule is converted to pipecolic acid, which increases GABA affinity and reduces excitotoxicity. As a result, the administration of Angiolin leads to a reduction in mitochondrial damage, activation of compensatory energy shunts, interruption of various pathways initiating apoptosis, nitrosative stress, and damage to proteins that play a role as signaling molecules, receptors, and ion channels.

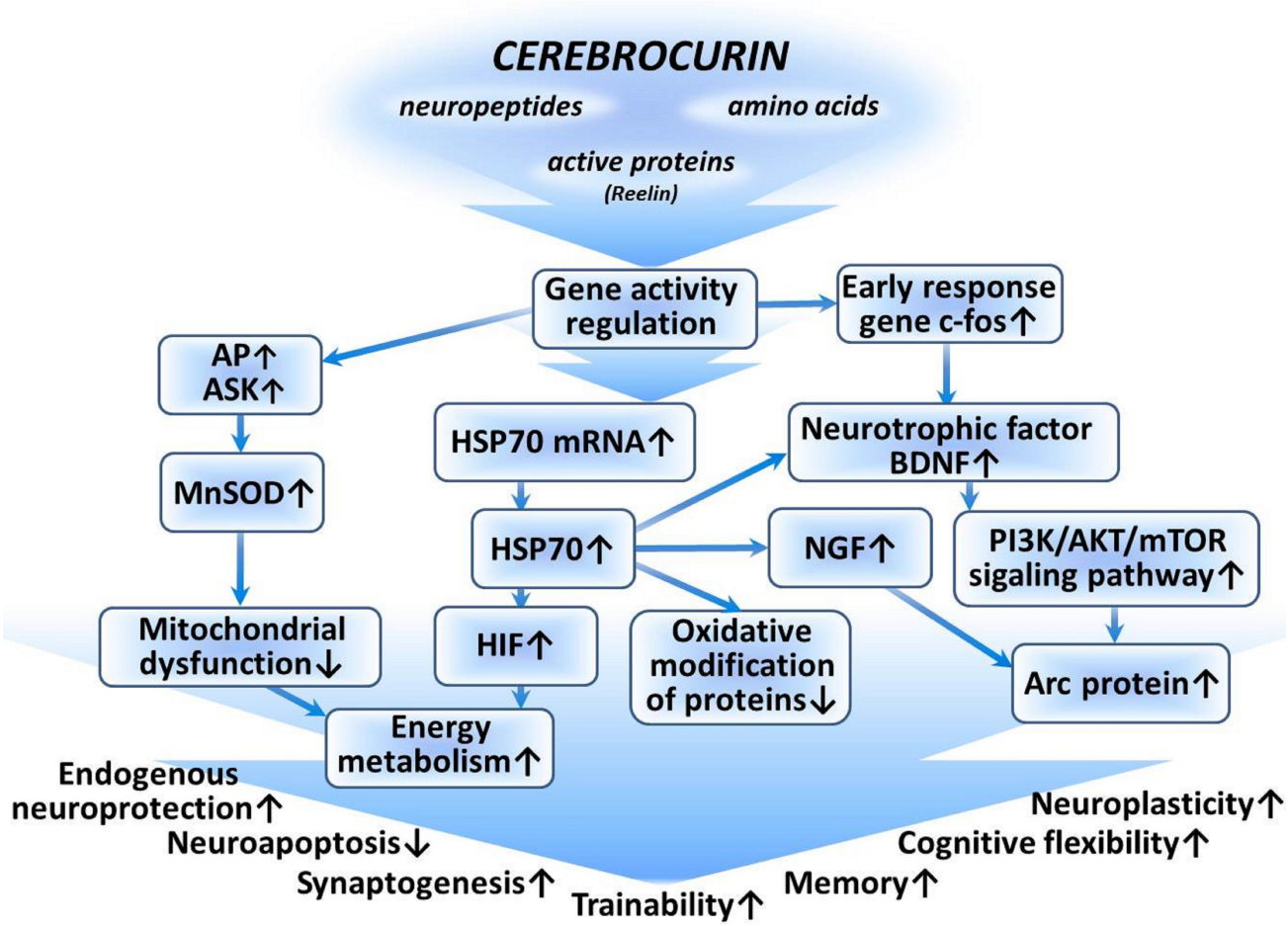

Fig. 4. Hypothetical mechanism of action of Cerebrocurin. ↑ indicates an increase in level/concentration, and ↓ indicates a decrease in level/concentration. Cerebrocurin contains peptides, amino acids, and proteins (reelin) that carry high signaling potential and trigger the regulation of gene activity. Cerebrocurin triggers the expression of HSP70, regulates the activity of early response genes, thereby triggering compensatory and adaptive mechanisms aimed at increasing neuron survival. By increasing HSP70, cerebrocurin prolongs the "life" of HIF-1 and improves ATP production, as well as triggers the BDNF expression pathway. Through the activation of c-fos, cerebrocurin activates the expression of NGF. Cerebrocurin increases BDNF binding to receptors and, with the help of BDNF, enhances the PI3K/AKT/mTOR signaling pathway for Arc protein expression. All this leads to the normalization of long-term memory, enhanced synaptogenesis, pro-neurogenic effects, increased cognitive flexibility, and neuroplasticity.

with neuropeptide and neurotransmitter systems (Fig. 4). Importantly, its neuroprotective effects are observed not only in transient but also in prolonged cerebral ischemia. The neuropeptides and trophic factors contained in cerebrocurin interact with multiple molecular targets, enabling modulation of apoptotic pathways at different stages of the pathological process. This broad spectrum of activity expands adaptive and compensatory responses and provides a basis for effective treatment, as well as for the successful physical, cognitive, and social rehabilitation of children following IUH [26,27,97,99,100,101].

Thiotriazolin, HSF-1, and glutaredoxin demonstrated moderate corrective effects across most of the evaluated parameters, which may be attributed to their antioxidant and stress-protective properties [102,103]. In contrast, tamoxifen, piracetam, L-arginine, mildronate, and nikomex exhibited relatively low therapeutic efficacy, particularly with respect to the regulation of caspase-8 and neurotrophic factor levels. These findings suggest that effective correction of IUH-induced alterations requires targeted modulation of hypoxia-sensitive molecular pathways.

Overall, the results of this study indicate that cerebrovascular disturbances associated with reduced neurotrophic factor expression following IUH are systemic and persistent. Their pharmacological correction may be feasible through interventions targeting key pathogenic mechanisms, including the nitric oxide system, endothelial dysfunction, neurotrophin-dependent signaling pathways, and apoptosis. The data obtained provide an experimental basis for the further development of combined or multimodal

pharmacological strategies aimed at preventing and treating the long-term consequences of IUH, particularly those related to cerebrovascular pathology.

5. Conclusions

Chronic IUH induces persistent and long-lasting cerebrovascular disturbances. Post-hypoxic alterations are characterized by the development of endothelial dysfunction, evidenced by decreased VEGF levels, reduced eNOS expression, increased iNOS expression, and elevated nitrotyrosine concentrations, as well as by morphological signs of impaired capillary network function, including decreased endothelial nuclear density, reduced nuclear RNA concentration, and suppressed endothelial proliferative activity. These changes are accompanied by suppression of neuroplasticity, reflected in reduced BDNF and NGF expression, as well as prolonged activation of apoptosis involving caspase-8.

A 30-day course of pharmacological intervention initiated immediately after birth in rats subjected to IUH, including HSP70 modulators (angiolin, thiotriazolin, cerebrocurin, tamoxifen, glutaredoxin, and HSF-1) and comparator agents (piracetam, nikomex, L-arginine, and mildronate), resulted in varying degrees of correction of the identified abnormalities, both immediately after treatment and 30 days following its cessation. The efficacy of the experimental therapy was determined by the mechanisms of action of the pharmacological agents and their impact on key pathogenetic pathways.

Among the tested compounds, angiolin and cerebrocurin demonstrated the greatest corrective potential. Angiolin exhibited a pronounced endothelium-protective effect by restoring the eNOS/iNOS balance, regulating VEGF expression, restoring endothelial functional activity, and stimulating endothelial proliferative activity. Cerebrocurin showed significant neurotrophic effects.

The obtained results provide experimental support for the potential inclusion of HSP70 modulators, particularly angiolin and cerebrocurin, in neuroprotective therapy following IUH.

6. Study Limitations

Several key limitations should be noted:

6.1 Limited Time Period

Neuroprotective effects were studied within 1 month after the course of treatment with pharmacological drugs, but long-term effects were not studied.

6.2 Statistical Limitations

The study uses n = 10 animals per group, which is relatively small. Although this sample size is likely sufficient to detect significant effects, it may limit the detection of more subtle neuroprotective benefits or side effects.

6.3 Limitations Related to the Mechanism of Action

Incomplete analysis of pathways: The study focused on individual molecular markers (eNOS, iNOS, VEGF, BDNF, NGF, nitrotyrosine), but did not conduct a comprehensive analysis of all potential pathways involved in the formation of endothelial dysfunction (studies on isolated vessels, proliferating endothelial cell test, endothelial cell morphometry).

6.4 Limitations Related to Translational Medicine

6.4.1 Sexual Dimorphism

The experiment used only male offspring and did not take into account possible sex differences in the response to drugs, which may limit the applicability of the obtained results.

6.4.2 Species-Specific Limitations

The use of rats alone limits extrapolation to human conditions without additional testing on other animal species or human cell models.

6.4.3 Age-Related Limitations

The study used adult 1- and 2-month-old rats, but did not examine the effect of these pharmacological agents on the aging brain in animals after IUH, which may have different sensitivity to HSP70 modulators.

7. Future Research Directions

Further research should be oriented to the study of HSP70-dependent mechanisms in cytoprotection and their correlation with the neurovascular and neurotrophic response to IUH. Molecular markers able to predict the resilience or sensitivity to neuropharmacological therapy should be identified to enable a more specific targeting of the pharmacological approach, directing the neuroprotective drugs toward receptors or pathways that can influence the main post-hypoxic disturbances, such as endothelial and neurotrophic dysfunctions or activation of apoptosis. In order to implement rational approaches for the development of new neuroprotective drugs, bench data should be translated to the prognosis-relevant biomarkers.

The importance of combined schemes that include HSP70 modulators and pharmacological agents that modulate the mitochondria functioning or the neuroimmune communication should be highlighted. To increase neuroprotection, all the molecular mechanisms involved in the hypoxic brain damage should be addressed. For this reason, aiming at a combined approach involving drugs that act on several targets will be the most promising strategy.

Availability of Data and Materials

All data of this study are available from the corresponding author on reasonable request.

Author Contributions

IB and OA designed the research study. SO, SL and KY performed literature search. OA, NB and VR performed the research. VR, SL and KY analyzed the data. OA and SO wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experimental procedures were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (1986) and national regulations concerning the use of laboratory animals. The study was approved by the Zaporizhzhia State Medical and Pharmaceutical University Commission on Bioethics (Protocol No. 34, May 12, 2022) for studies involving animals. All animal procedures followed the ARRIVE 2.0 guidelines (**Supplementary Material**).

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/JIN51482.

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