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Posted Date: 4 November 2024

doi: 10.20944/preprints202411.0122.v1

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Success of Nitric Oxide System Modulators in Pharmacocorrection of Some Indicators of Endothelial Dysfunction After Intrauterine Hypoxia

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Abstract: Prenatal hypoxia plays a crucial role in programming long-term cardiovascular dysfunction through mechanisms like endothelial dysfunction and nitric oxide system impairment, highlighting the potential of therapeutic agents, such as thiotriazoline, angiolin, mildronate, and L-arginine, for mitigating these adverse effects. Methods. Levels of sEPCR, Tie2 tyrosine kinase, VEGF-B, SOD1/Cu-Zn SOD, GPX4, and GPX1 were measured in the heart's cytosolic homogenate using ELISA. Results. Modeling prenatal hypoxia (PH) resulted in significant alterations in the concentrations of proteins linked to endothelial function and oxidative stress in the heart cytosol of experimental animals, including an increase in sEPCR and reductions in Tie-2, VEGF-B, Cu/ZnSOD, GPX4, and GPX1 levels. Postnatal administration of nitric oxide modulators (L-arginine, thiotriazoline, angiolin, and mildronate) demonstrated differential efficacy in normalizing these proteins. Notably, angiolin produced the most substantial therapeutic effect, restoring Tie2, VEGF-B, and antioxidant enzyme levels to near-normal levels. Our results highlight the efficacy of Angiolin and Thiotriazoline in restoring endothelial function and antioxidant enzyme levels in the cardiovascular system following prenatal hypoxia, supporting their potential as early postnatal interventions to prevent long-term cardiovascular dysfunction.

Keywords: prenatal hypoxia; cardioprotective; Angiolin; L-arginine; Thiotriazoline; Mildronate; cEPCR; Tie-2; VEGF-B; Cu/ZnSOD; GPX1; GPX4

1. Introduction

The global progression of cardiovascular disease is influenced by a myriad of factors, including lifestyle choices, dietary habits, unhealthy behaviors, and socioeconomic shocks. Notably, suboptimal intrauterine conditions also play a critical role. Numerous studies have demonstrated that cardiovascular dysfunction in adulthood can be programmed during pregnancy, particularly as a result of poor maternal nutrition, alcohol consumption, substance abuse, chemical exposure, and stress. Among the various complications associated with pregnancies worldwide, fetal hypoxia

 doi:10.20944/preprints202411.0122.v1

emerges as one of the most prevalent issues, significantly impacting long-term cardiovascular health [1,2,3].

Prenatal hypoxia is associated with asymmetric fetal growth restriction, leading to hypertrophic growth of the heart and aorta, altered cardiac function, and sympathetic hyperinnervation of peripheral resistive arteries in newborns. In adulthood, the effects of prenatal hypoxia extend to an increased risk of hypertension, coronary heart disease, ischemic heart disease, heart failure, and metabolic syndrome, as well as heightened susceptibility to ischemic injury. These findings underscore the critical importance of addressing hypoxic conditions during pregnancy to mitigate long-term cardiovascular risks [4,5].

The presence of endothelial dysfunction mechanisms was revealed in the pathology of the cardiovascular system after prenatal hypoxia. Clinical manifestations of functional state disturbance and cardiovascular system maladaptation after prenatal hypoxia directly correlated with signs of endothelial dysfunction (changes in endothelin-1, NO, VEGF production, circulating desquamated endotheliocytes) both in newborns and at older ages [6,7,8].

Nitrogen monoxide system disorders are attributed a certain role in the formation of endothelial dysfunction and cardiovascular pathology, including after prenatal hypoxia. Studies have revealed that prenatal hypoxia can change both production and bioavailability of NO. During prenatal hypoxia, increased concentrations of superoxydradicals and other reactive oxygen species can lead to oxidative modification of NO and convert it to peroxynitrite, which negatively affects fetal organs [5,9,10].

Hypoxia decreases endothelial nitric oxide synthase (eNOS) expression and can alter its enzymatic activity through various posttranslational modifications. In conditions of hypoxia coupled with L-arginine deficiency, eNOS may generate superoxyradicals instead of NO. Such abnormalities in eNOS function are thought to be a major cause of endothelial dysfunction observed in cardiovascular disease [8,11].

Given that prenatal hypoxia exerts both immediate and long-term effects on cardiovascular development, it is essential to explore novel molecular and biochemical markers that reflect the hypoxic impact on the cardiovascular system. Additionally, the development of therapeutic agents targeting these effects is critical. Positive modulators of NO synthesis have attracted the attention of researchers as means of cardio- and endothelioprotection after prenatal hypoxia [121314]. We observed promising experimental outcomes with the use of nitric oxide (NO) modulators, including L-arginine, thiotriazoline, angiolin, and myodronate, following instances of prenatal hypoxia [12,15]. These agents are also described in other studies, which show that cytoprotective, antioxidative and anti-ischemic effects of these agents are associated with a positive effect on the nitric oxide monoxide system - influence on the synthesis, bioavailability or transport of this messenger.

Thus, Thiotriazolin (tiazotic acid) is a scavenger of reactive oxygen and nitrogen forms, protects NO from chemical modification and transformation into peroxynitrite, exhibits cardioprotective, membrane-stabilizing and anti-ischemic properties [16].

Angiolin (3-methyl-1,2,4-triazolyl-5-thioacetate (S)-2,6-diaminohexanoic acid) is a structural analog of thiotriazoline that regulates the concentration of reactive oxygen species (ROS), protects NO from conversion into peroxynitrite, and modulates the expression of eNOS and vascular endothelial growth factor (VEGF) during ischemia and hypoxia. It exhibits antioxidative, neuroprotective, endothelial-protective, cardioprotective, and anti-ischemic properties [17].

Mildronate affects NO synthesis by increasing the level of gamma-butyrobetaine, exhibits cardioprotective, anti-ischemic properties [18].

L-arginine, a precursor of NO synthesis, exhibits membrane-stabilizing, cardioprotective, and anti-ischemic properties [19].

Purpose of the study: to evaluate the effect of NO system modulators with different mechanisms of action (L-arginine, thiotriazoline, angiolin and mildronate) on some molecular markers of endothelial dysfunction in the early postnatal period after intrauterine hypoxia.

 doi:10.20944/preprints202411.0122.v1

2. Materials and Methods

2.1. Animal Characteristics

We employed fifty white female rats and ten males, each weighing between 220 and 240 grams and about half a year old, sourced from the vivarium of the Institute of Pharmacology and Toxicology, National Medical Academy of Ukraine. The rats were kept in typical vivarium settings, which included a 20–25°C temperature range, a 50–55% humidity level, a regular light cycle, and unlimited access to food and water suitable for their species. The "European Applicable Protection of Vertebrate Animals used for Experimental and Scientific Purposes" and the rules governing the collection of animals for biomedical research (Strasbourg, 1986, as revised in 1998) were followed in all manipulations. The Zaporizhzhia State Medical University Commission on Bioethics granted ethical permission for the study (protocol No. 33, dated June 26, 2021).

2.2. Experimental Model

In order to create a model based on nitrite, we caused chronic hypoxia, which significantly alters the histology, morphology, and metabolism of the progeny's heart tissue [20,21]. Adult males were paired with females at a ratio of 2:4, and the first day of pregnancy was determined by the presence of spermatozoa in the vaginal smear. Daily intraperitoneal injections of a sodium nitrite solution at a concentration of 50 mg/kg were used to induce moderate hypoxia between days 16 and 21 of pregnancy [Error! Reference source not found.]. An equivalent volume of physiological saline was g iven to control females. The offspring were separated into the subsequent groups: healthy rats from females undergoing normal pregnancies; a control group of pups that underwent PH and were administered physiological saline (days 1 to 30); 4 experimental groups of hypoxia-exposed pups were treated daily with various drugs from postnatal days 1 to 30. Some of the pups were removed from the experiment on the 30th day, immediately after the completion of pharmacological agent administration, while others were removed 60 days after birth (30 days following treatment). The doses of L-arginine and Mildronate were sourced from open literature. The doses of Thiotriazoline and Angiolin were determined experimentally, and these data are included in the DCT report.

2.3. Rationale for the Chosen Medications and Their Attributes

We chose treatments known to impact the NO system based on experimental evidence:

1. The intact group consisted of rats born from females with basic pregnancies and received a physiological solution.
2. The control group included rats born after experiencing intrauterine hypoxia and also received a physiological solution.
3. Thiotriazoline, also known as morpholinium-3-methyl-1,2,4-triazolyl-5-thioacetic acid (2.5% injection solution, "Arterium", Ukraine), is an antioxidant and metabolitotropic cardioprotector that is injected intraperitoneally at a dose of 50 mg/kg [22].
4. Angiolin, additionally known as [S]-2,6-diaminohexane acid 3-methyl-1,2,4-triazolyl-5-thioacecate (substance, RPA "Farmatron", Ukraine) is an endothelium-protective, anti-ischemic injection given intraperitoneally at a dose of 50 mg/kg [24].
5. L-arginine (42% injection solution in vial, Tivortin, Yuria-pharm, Ukraine), an NO precursor; to decrease ischemia-related nitroxidergic system disruptions, is given intraperitoneally at a dose of 200 mg/kg [25].
6. As a metabolitotropic drug, mildronate (2-(2-carboxyethyl)-1,1,1-trimethylhydrazinium) (10% injectable solution in ampoules, Grindex (Latvia)) is injected intraperitoneally at a dose of 100 mg/kg [26].

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2.4. Anaesthesia

On days 30 and 60 of the trial, rats were put to sleep using thiopental anesthesia (40 mg/kg). For additional research, blood samples were taken from the celiac artery.

2.5. Biological Material Preparation

The heart was washed with a 1:10 dilution of cooled 0.15 M KCl solution, kept at 4°C. After removing excess fat, connective tissue, blood vessels, and clots, the heart was rinsed with a 1:10 dilution of 0.15 M KCl solution at 4°C. Utilizing a WT500 torsion balance (manufactured in Moscow, Russia), 100 milligrams of heart tissue were meticulously weighed after being previously ground into a fine powder using liquid nitrogen. Next, 10.0 mL of a medium kept at 2 ◦C was thoroughly mixed with the pulverized tissue. The concentration of the following ingredients in millimoles per liter (mmol/L) was 7.4 pH-adjusted: 250 mmol/L of sucrose, 20 mmol/L of Tris-HCl buffer, and 1 mmol/L of EDTA. Large cell fragments were then extracted from the homogenate by pre-centrifuging it in a Sigma 3-30k chilled centrifuge (Osterode am Harz, Germany) for 7 minutes at 1000× g at +4 ◦C. The resultant supernatant was carefully collected and put through a second centrifugation process using the identical Sigma 3-30k refrigerated centrifuge (Germany) for 20 min at 17,000× g at +4 ◦C. Following this procedure, the supernatant was collected and refrigerated at -80°C. Subsequent to resuspension, the thick mitochondrial precipitate was employed for additional research.

2.6. Immunoenzymatic Assay

The soluble endothelial protein C receptor (sEPCR) was measured in the cytosolic homogenate of the heart using a solid-phase enzyme-linked immunosorbent assay (ELISA) sandwich method. The assay was performed with the Rat soluble endothelial protein C receptor (sEPCR) ELISA Kit, Catalog #MBS265381 from MyBioSource, Inc. (USA), following the provided instructions.

The Tie2 tyrosine kinase was also determined in the cytosolic homogenate of the heart using a solid-phase ELISA sandwich method. The assay was conducted with the Rat Tie2 (Rat Tek Tyrosine Kinase, Endothelial ELISA Kit), Catalog #MBS036226 from MyBioSource, Inc. (USA), in accordance with the instructions.

Vascular Endothelial Growth Factor B (VEGF-B) was determined in the cytosol of heart homogenate using a solid-phase sandwich ELISA method, Rat Vascular Endothelial Growth Factor B (VEGF-B) ELISA Kit, Catalog # MBS269676 MyBioSource, Inc. (USA), according to the instructions.

SOD1/Cu-Zn SOD was determined in the cytosol of heart homogenate using a solid-phase sandwich ELISA method, Rat Superoxide dismutase [Cu-Zn] ELISA Kit, Catalog # MBS761294 MyBioSource, Inc. (USA), according to the instructions.

Glutathione peroxidase 4 (phospholipid hydroperoxidase) (GPX4) was determined in the cytosol of heart homogenate using a solid-phase sandwich ELISA method, Rat Phospholipid hydroperoxide glutathione peroxidase, mitochondrial, GPX4 ELISA Kit, Catalog # MBS934198 MyBioSource, Inc. (USA), according to the instructions.

Glutathione Peroxidase 1 (GPX1) was determined in the cytosol of heart homogenate using a solid-phase sandwich ELISA method, Rat Glutathione Peroxidase 1 ELISA Kit, Catalog # MBS3809062 MyBioSource, Inc. (USA), according to the instructions. All studies were conducted using a plate enzyme immunoassay analyzer (SIRIO S, Italy).

2.7. Statistical Analysis:

Experimental data were statistically analyzed using “StatisticaR for Windows 6.0” (StatSoft Inc., Tulsa, OK, USA, AXXR712D833214FAN5), “SPSS16.0”, and “Microsoft Office Excel 2010” software. Prior to statistical tests, we checked the results for normality (Shapiro–Wilk and Kolmogorov–Smirnov tests). In the normal distribution, intergroup differences were considered statistically significant based on the parametric Student’s t-test. If the distribution was not normal, the comparative analysis was conducted using the non-parametric Mann–Whitney U-test. To compare independent variables in more than two selections, we applied ANOVA dispersion analysis for the

 doi:10.20944/preprints202411.0122.v1

normal distribution and the Kruskal–Wallis test for the non-normal distribution. To analyze correlations between parameters, we used correlation analysis based on the Pearson or Spearman correlation coefficient. For all types of analysis, the differences were considered statistically significant at $p < 0.05$ (95%).

3. Results

Prenatal hypoxia (PH) modeling leads to changes in the concentration of various proteins in the heart cytosol of experimental animals, which may indicate the development of endothelial dysfunction (Tables 1 and 2). We observed a significant increase in the concentration of the soluble form of the endothelial protein C receptor (sEPCR), rising by 1.92 times at 1 month of life and by 2.14 times at 2 months of life. Additionally, we found a significant decrease in the tyrosine kinase receptor Tie-2, by 42.3% at 1 month of life and by 37.9% at 2 months. The concentration of vascular endothelial growth factor B (VEGF-B) was also significantly reduced after PH, decreasing by 28.1% and 35.2% at 1 and 2 months, respectively, in experimental animals. Furthermore, we identified a reduction in the expression of antioxidant enzymes, which play a crucial role in limiting the damaging effects of oxidative stress intermediates—such as superoxide radicals, hydroperoxides, and lipid peroxides. In the cytosol of rat hearts after PH, a significant decrease in the concentration of the Cu/Zn-dependent isoform of superoxide dismutase (Cu/ZnSOD) by 27.6% (at 1 month of life) and by 31.6% (at 2 months of life) was observed. A reduction in the concentration of glutathione peroxidase 4 (phospholipid hydroperoxidase) (GPX4) was also found at 1 and 2 months of life, by 49.5% and 47.8%, respectively. The concentration of glutathione peroxidase 1 (GPX1) also decreased, by 51.2% at 1 month of life and by 54.3% at 2 months.

Table 1. System parameters of cytosolic fraction in 1-month-old rats after prenatal hypoxia and treatment.

Experimental Groups	cEPCR, pg/ml	Tie-2, pg/ml	VEGF-B, pg/ml	Cu/ZnSOD, pg/ml	GPX1, pg/ml	GPX4, pg/ml
Intact (Rats born from rats with normal pregnancies) (n = 10)	22.5 ± 0.411	17.7 ± 0.348	44.7 ± 1.012	87.7 ± 1.802	43.3 ± 1.044	67.8 ± 1.676
PH (Rats with prenatal hypoxia) (control) (n = 10)	43.2 ± 1.360[1]	10.2 ± 0.275[1]	32.1 ± 1.012[1]	63.5 ± 1.360[1]	21.1 ± 0.538[1]	34.2 ± 0.537[1]
PH +L-arginine (n = 10)	38.0 ± 0.854[1]*	14.2 ± 0.348[1]*	34.7 ± 1.486[1]	62.7 ± 1.739[1]	22.8 ± 0.696[1]	38.3 ± 1.328[1]*
PH + Thiotriazoline (n = 10)	33.5 ± 1.012[1]*	12.7 ± 0.316[1]*	36.8 ± 1.170[1]*	77.8 ± 1.961[1]*	38.8 ± 0.696[1]*	57.7 ± 0.949[1]*
PH + Angiolin (n = 10)	28.2 ± 0.538[1]*	16.4 ± 0.380[1]*	47.8 ± 0.885[1]*[1]	79.7 ± 1.676[1]*	40.7 ± 1.012*	62.8 ± 1.803*
PH + Meldonium (n = 10)	40.5 ± 2.119[1]	11.0 ± 0.231[1]*	31.1 ± 1.170[1]	65.2 ± 1.961[1]	22.7 ± 0.348[1]	37.3 ± 0.601[1]*

Notes: [1]—$p \le 0.05$ in relation to the intact group of animals; *—$p \le 0.05$ in relation to the control group of animals.

Table 2. Cytosolic fraction parameters in 1-month-old rats after prenatal hypoxia and treatment.

Experimental Groups	cEPCR, pg/ml	Tie-2, pg/ml	VEGF-B, pg/ml	Cu/ZnSOD, pg/ml	GPX1, pg/ml	GPX4, pg/ml
Intact (Rats born from rats with normal pregnancies) (n = 10)	21.2 ± 0.348	18.2 ± 0.253	48.8 ± 1.012	91.9 ± 2.308	46.4 ± 0.664	72.4 ± 1.676

 doi:10.20944/preprints202411.0122.v1

PH (Rats with prenatal hypoxia) (control) (n = 10)	45.4 ± 0.727[1]	11.3 ± 0.221[1]	31.6 ± 0.696[1]	62.8 ± 1.581[1]	21.2 ± 0.949[1]	37.8 ± 0.569[1]
PH +L-arginine (n = 10)	35.2 ± 0.537[1]*	15.2 ± 0.348[1]*	32.7 ± 0.854[1]	66.7 ± 1.328[1]	24.3 ± 0.569[1]*	39.4 ± 0.443[1]
PH + Thiotriazoline (n = 10)	32.2 ± 0.569[1]*	15.7 ± 0.243[1]*	37.8 ± 1.075[1]*	78.7 ± 1.992[1]*	42.6 ± 0.791[1]*	68.7 ± 1.360[1]*
PH + Angiolin (n = 10)	21.2 ± 0.632*	18.4 ± 0.379*	52.8 ± 1.202*[1]	88.7 ± 2.625*	48.8 ± 1.075*	77.8 ± 1.834[1]*
PH + Meldonium (n = 10)	44.9 ± 1.676[1]	10.4 ± 0.127[1]*	34.7 ± 0.601[1]*	64.4 ± 1.391[1]	27.4 ± 0.601[1]*	42.5 ± 1.518[1]*

Notes: [1]—$p \le 0.05$ in relation to the intact group of animals; *—$p \le 0.05$ in relation to the control group of animals.

Course administration of drugs that are modulators of the nitric oxide system for 30 days immediately after birth leads to varying degrees of normalization in the expression of these proteins (sEPCR, Tie-2, VEGF-B, Cu/ZnSOD, GPX) (Tables 1-2). The administration of L-arginine resulted in a significant reduction in sEPCR by 12.0% immediately after discontinuation of the drug and by 22.4% one month after the end of L-arginine treatment, indicating a lasting effect. L-arginine administration significantly increased the concentration of Tie-2 by 1.4 times in the cytosol of experimental animals both immediately after discontinuation of the drug and one month after the end of the treatment course. However, L-arginine administration did not affect the concentration of VEGF-B and Cu/ZnSOD in the heart cytosol of experimental animals.

The introduction of L-arginine led to a significant increase in GPX4 expression immediately after administration, while GPX1 expression increased a month after the course ended. Thiotriazoline significantly reduced cEPCR levels in the cytosol of the hearts of rats after PH at both observation periods (1 and 2 months of life of experimental animals) by 22.4% and 29.0%, respectively. The course administration of thiotriazoline resulted in a significant increase in Tie-2 by 24.5% and 39.0% for the respective observation periods (1 and 2 months after PH). Additionally, thiotriazoline led to a significant increase in VEGF-B by 14.6% and 19.6% for the respective observation periods. Thiotriazoline significantly elevated the expression of antioxidant enzymes in the cytosol of the myocardium of experimental animals—Cu/ZnSOD by 22.5% and 25.3%, GPX1 by 83.8% and 200%, GPX4 by 68.7% and 87.7% for the respective observation periods after the drug administration. As can be seen, thiotriazoline has a greater impact on GPX1, which is consistent with its previously established antioxidant properties.

It is noteworthy that the measured indicators in the group of animals with PH receiving Thiotriazoline did not significantly differ from those in the group of animals born after a normally progressing pregnancy. Angiolin demonstrated the most pronounced therapeutic effect among all the studied agents (tables 1-2). Specifically, Angiolin significantly reduced cEPCR levels in the cytosol of the hearts of rats after PH at both observation periods—immediately after the course of administration and one month after its cessation (1 and 2 months of the experimental animals' life)—by 34.7% and 53.3%, respectively.

It is important to note that cEPCR levels in the cytosol of the myocardium of 2-month-old animals after PH receiving Angiolin were comparable to those of animals born after a physiologically normal pregnancy. The course administration of Angiolin led to a significant increase in Tie-2 by 60.7% and 62.8% for the respective observation periods (1 and 2 months after PH). CEPCR indices in myocardial cytosol of 2-month-old animals after PH receiving Angiolin were at the level of animals born after physiologically normal pregnancy.

The administration of Angiolin also led to a significant increase in VEGF-B by 48.9% and 67.0% for the respective observation periods. It is important to note that the concentration of VEGF-B in the cytosol of the myocardium of 1- and 2-month-old rats after PH was significantly higher than that of age-matched animals born after a physiologically normal pregnancy. The use of Angiolin also

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resulted in an increase in the expression of antioxidant enzymes—Cu/ZnSOD by 25.5% and 41.2%, GPX1 by 92.8% and 130.1%, and GPX4 by 83.6% and 105.8% for the respective observation periods after the drug administration—indicating a significant antioxidant mechanism of action for the drug.

Mildronate, when administered in a course after PH, had the least pronounced effect compared to the other studied drugs (tables 1-2). We observed a significant change in the group receiving Mildronate compared to the control group in the levels of Tie-2 and GPX4 immediately after a one-month course of administration, as well as a significant change compared to the control group in the levels of VEGF-B, Tie-2, GPX1, and GPX4 one month after the course of the drug.

4. Discussion

The modeling of PH through the introduction of sodium nitrite to pregnant females leads to hemic hypoxia due to the formation of methemoglobin. This hypoxia is accompanied by tissue hypoxia caused by the uncoupling of oxidation and phosphorylation processes. The disruption of blood oxygen transport in pregnant female rats results in impaired uteroplacental blood flow and oxygen starvation of the fetus or embryo [22]. Administration of sodium nitrite at a dose of 50 mg/kg leads to hypoxia of medium severity in adult individuals, according to the criteria proposed by N.F. Ivanitskaya [27].

The modeling of PH leads to the development of postnatal heart defects. In both newborns and adult animals, our model allows for the assessment of the physiological development of offspring and the effectiveness of experimental cardioprotective therapy following prenatal hypoxia (PH). The administration of sodium nitrite to pregnant rats results in increased methemoglobin levels [28], specifically, hypoxic damage to the target organs of the fetus. Our previous studies have shown that modeling chronic hypoxia with sodium nitrite leads to persistent ECG abnormalities, reduced myocardial contractility, and sinus node dysfunction [29], focal dystrophy, as confirmed by an increase in the concentration of a highly sensitive marker of myocardial remodeling and the risk of heart failure, ST2 [15].

We also revealed a significant impairment of myocardial nitriergic system in rats after prenatal hypoxia (PH) - an imbalance in the ratio of eNOS/iNOS expression on the background of NO deficiency and increased nitrotyrosine levels [12]. This suggests impaired cardiac tolerance to ischemia/reperfusion, damage to endothelial-dependent mechanisms of vasodilation/vasoconstriction, and may further contribute to the development of endothelial dysfunction following intrauterine hypoxia. Endothelial dysfunction after prenatal hypoxia develops against a background of HIF-1α deficiency (a factor that activates eNOS expression through serine residue phosphorylation) and nitrosative stress, which also leads to HSP70 deficiency, glutathione system depletion, reduced NO bioavailability, and suppression of gene transcription by cytotoxic NO products [12,15,30].

Our research findings also confirm that this model of PH leads to pathological changes in the cardiovascular system of newborns and the development of endothelial dysfunction. EPCR levels increase on endothelial cells during post-ischemic neovascularization. It is important to note that the exogenous addition of NO significantly enhanced the formation of endothelial angiogenic sprouts from aortic rings and primary endothelial cells isolated from mice with a PAR1 mutation. Thus, maintaining NO bioavailability during angiogenic processes is a primary function of EPCR-PAR1 endothelial signaling [31,32].

The release of EPCR from the endothelium often leads to the formation of the soluble form of EPCR (sEPCR). It was found that SS mice had higher levels of soluble EPCR (sEPCR) in plasma compared to their AA counterparts. The endothelial protein C receptor (EPCR) plays a crucial role in the anticoagulant and anti-inflammatory effects of the protein C pathway, whereas its soluble form (sEPCR) exhibits opposing properties. High levels of sEPCR in plasma and tissues have been observed in individuals with the A3 haplotype of the PROCR gene, the EPCR gene. Elevated levels of sEPCR in plasma have also recently been reported in women with preeclampsia (PE), a multisystem syndrome involving inflammation, endothelial dysfunction, and thrombosis [33].

 doi:10.20944/preprints202411.0122.v1

Tie2 plays an important role in providing barrier protection to prevent excessive vascular permeability and maintains an antithrombotic surface to improve blood circulation. It remains activated throughout the healthy vascular system of an adult due to the continuous secretion of angiopoietin-1 from perivascular cells and platelets, promoting endothelial stability by inhibiting the inflammatory NF-κB [34]. In animal models simulating critical illness, Tie2 levels in organs are temporarily reduced. The functional consequences of these reduced Tie2 levels for microvascular endothelial behavior are associated with increased microvascular inflammation [35]. It has been shown that mice with null Tie-2 exhibit severe vascular damage and cardiac abnormalities, leading to embryonic lethality, as Tie-2 is essential for supporting the development and stabilization of fetoplacental vessels and regulating NO production [36]. Data have been obtained demonstrating the potential of activating Tie2 with a pharmacological agent, leading to a reduction in the thromboinflammatory state of the endothelium in COVID-19 [34]. VEGF-B is a powerful survival factor for various cell types, inhibiting apoptosis by suppressing the expression of apoptosis-related proteins and genes and is crucial for the survival of blood vessels; however, it does not induce blood vessel growth. Pharmacological modulation of VEGF-B results in a strong cytoprotective and anti-apoptotic effect without triggering general angiogenic activity [37]. The heart expresses a high level of VEGF-B, which exerts a strong anti-apoptotic effect on cardiomyocytes by suppressing the expression of pro-apoptotic genes (BMF, BAD, BID, BAX, CASP9, DCN, TP53INP1, TNF). VEGF-B induces several antioxidant genes (GPX1, GPX4, SOD-1, SOD-2, etc.) and suppresses genes responsible for oxidative stress. VEGF-B reduces endothelial cholesterol content by inhibiting the recirculation of low-density lipoprotein receptors, influences uptake, and increases the utilization of fatty acids by the myocardium for energy production [38].

Preclinical studies have demonstrated the therapeutic potential of VEGF-B in revascularizing ischemic myocardium by modulating endothelial cell proliferation and migration [39]. It has been established that VEGF-B primarily interacts with Flt-1 (vascular endothelial growth factor receptor) and sFlt-1 (soluble vascular endothelial growth factor receptor-2) and inhibits vascular endothelial dysfunction in preeclampsia. Administration of a recombinant VEGF-B preparation to rodents with experimental preeclampsia restored the angiogenic environment in plasma, normalized blood pressure, and reduced the severity of ischemia [40]. The reduced expression of the main antioxidant antioxidant phkrments, which we have found in the previously detected increase of nitrotyrosine in the myocardium of 1 and 2 month old rats following PH [12], shows a significant activation of oxidative stress after PH. Oxidative stress in the fetal heart and vasculature underlies the mechanism by which prenatal hypoxia programs cardiovascular pathology and endothelial dysfunction later in life [4]. Our present study and previously published results are not contradicted by other investigators who have shown that PH contributed to aortic thickening with enhanced nitrotyrosine staining and increased expression of cardiac HSP70, as well as marked impairment of NO-dependent relaxation in arteries and increased myocardial contractility with sympathetic dominance [41].

GPX-4 is most important for cellular protection under oxidative stress, directly reducing phospholipid hydroperoxides, even when incorporated into membranes and lipoproteins. GPX-4 can also restore fatty acid hydroperoxide, cholesterol hydroperoxide and thymine hydroperoxide. Plays a key role in protecting cells from oxidative damage by preventing membrane lipid peroxidation. GPX-4 is required to prevent cells from ferroptosis, non-apoptotic cell death resulting from iron-dependent accumulation of lipid reactive oxygen species [42,43]. GPx4 is required to prevent the death of mitochondrial cells by mediating the reduction of cardiolipin hydroperoxides. GPx4 is involved in the direct detoxification of lipid peroxides in the cell membrane and is an inhibitor of ferroptosis induced by lipid peroxidation. The cytosolic isoform of GPx4 plays a key role in inhibiting ferroptosis in somatic cells, while the mitochondrial isoform of GPx4 (mGPx4) may play a role in reducing the risks of mitochondrial dysfunction [44]. It has been discovered for the first time that PH can lead to ferroptosis in human trophoblast cells, which may subsequently cause miscarriage. This underscores the importance of GPX-4 [45]. (GPx-1) is an intracellular antioxidant enzyme that enzymatically reduces H2O2 to H2O to limit its harmful effects, as well as regulates H2O2-dependent signaling mechanisms mediated by growth factors, mitochondrial function, and the maintenance of

 doi:10.20944/preprints202411.0122.v1

normal thiol redox balance. Our findings indicate that the decreased expression of GPx-1 in the hearts of rats after PH may be associated with an excess of cytotoxic forms of NO in the context of high iNOS expression [46], as we found in the previous study [12]. GPx-1 plays an important role in maintaining endothelial function and NO bioavailability [46] GPx-1 deficiency leads to marked vasoconstriction and forms endothelial dysfunction [47].

SODs are generally classified into four groups: manganese SOD (MnSOD), copper-zinc SOD (Cu/ZnSOD), iron SOD (FeSOD) and nickel-SOD (NiSOD). Cu/ZnSOD and MnSOD localize in the cytoplasm, respectively, and serve as the major radical scavenger in the intracellular environment and have attracted much attention because of their physiological function and therapeutic potentials [48]. Our studies showing a low concentration of Cu/ZnSOD in rat cytosol after PH are supported by other studies showing that PH reduces Cu/ZnSOD expression at both transcriptional and posttranslational levels. In addition, PH decreases Cu/ZnSOD activity and may be a cause of subsequent cardiovascular disease [49] and endothelial dysfunction [50]. There is strong evidence of a proven link between reduced activity of antioxidant enzymes and the occurrence of adverse pregnancy outcomes, as oxidative has a deleterious effect on maternal physiology, pregnancy and fetal development, impairing placental function and impairing oxygen and nutrient delivery to the developing fetus and contributing to cardiovascular disorders, in particular cardiomyopathy and endothelial dysfunction [51]. Positive modulators of NO, by increasing physiologic concentrations of this messenger, participate in the mechanisms of S-nitrosation of a cysteine residue and regulate post-translational modification of various proteins, including eNOS [52]. All of this, as well as our previous research [11,12,15] allowed us to justify the use of positive NO modulators in the experimental therapy of cardiovascular complications after PH. The highest activity in this study was demonstrated by Angiolin (S)-2,6-diaminohexanoic acid 3-methyl-1,2,4-triazolyl-5-thioacetate, which has properties of NO scavenger, in which fragments of its chemical structure of the molecule take part. Angiolin can form nitrothiols and increase NO bioavailability. Angiolin normalizes eNOS/iNOS expression. In studies on the model of cerebral ischemia in rats the endothelioprotective activity of Angiolin was also demonstrated, i.e. increase in the density of endotheliocytes of muscle-type vessels and microcirculatory channel, increase in the density of proliferating endotheliocytes, as well as increase in the expression of vascular endothelial growth factor (VEGF) and receptor binding coefficient [11,53]. There is evidence that VEGF enhances the regulation of the enzyme ecNOS and induces a biphasic stimulation of endothelial NO production [54] this suggests a possible VEGF-mediated expression of eNOS under the action of Angiolin. Angiolin can influence on the expression of endotheliotropic factors and antioxidative components through the influence on thiol-disudyphide system by increasing the level of glutathione and regulating posttranslational mechanisms. There is evidence of positive effects on Cu/ZnSOD, GPX1 and GPX4 activity in the cytosol of rat myocardium and brain during cardiac or cerebral ischemia. This may be due to the interruption of NO-dependent mechanisms of suppression of the expression of these enzymes [11,55].

Thiotriazoline, a drug registered in many countries as a methiabolitotropic cardioprotective agent, also exhibits NO scavenger properties, but more moderate effect on eNOS expression in cardiocytes under conditions of myocardial ischemia. Thiotriazoline can increase endothelioprotective properties of L-arginine by increasing NO bioavailability [56,57,58]. Thiotriazoline exhibits antioxidant properties in many studies its ability to reduce the formation of oxidative and nitrosative stress end products, increase the activity of Cu/ZnSOD, GPX1 and GPX4 in the liver, heart and brain of animals with various experimental pathologies has been established [57].

Mildronate (3-(2,2,2,2-trimethylhydrazine) propionate) reversibly blocks gamma-butyrobetaine hydroxylase, which catalyzes the conversion of gamma-butyrobetaine into carnitine and thereby significantly inhibits the entry of carnitine, which provides transport of fatty acids across the membrane into the cells of muscle tissue. This effect of mildronate is accompanied by a decrease in carnitine-dependent oxidation of free fatty acids (FFA) and, consequently, leads to activation of glucose oxidation, which is more economical in conditions of ischemia. An important feature of the action of mildronate, distinguishing it from other drugs affecting myocardial metabolism, is the absence of accumulation of underoxidized fatty acids inside mitochondria, increase in NO

 doi:10.20944/preprints202411.0122.v1

production [59]. Our studies have confirmed the antihypoxic activity of mildronate in PH [15]. The endothelioprotective effect of mildronate has not been established by our studies and the present work. Course administration of mildronate to rats after PH resulted in increased expression of various forms of glutathione peroxidase, which is consistent with other studies on its antioxidative activity [11,60].

However, this alone is insufficient to exert a protective effect on the cardiovascular system. In this study, we did not observe a significant positive effect of mildronate on the NO system parameters in the myocardium of animals that underwent PH. L-arginine is a common substrate for NO and polyamines (putrescine, spermine, and spermidine). NO and polyamines play important roles in reproduction, embryogenesis, reducing neonatal mortality, and embryonic angiogenesis. NO regulates gene expression, protein synthesis, and facilitates proliferation, growth, and differentiation of the fetus [61]. Currently, encouraging results have been obtained regarding the use of L-arginine in neonatology as a hypoxic and endotheliotropic agent [13]. A certain positive effect of L-arginine on molecular indices of endothelial dysfunction in the heart of rats after PH has been revealed. Weaker effect of L-arginine in comparison with Angiolin and Thiotriazolin can be explained from the point of view of NO life duration under ischemia and hypoxia accompanied by oxidative stress. "Newborn" NO immediately runs the risk of being 'bitten' by superoxydradical [11,62] and converted into the sinister peroxynitrite [63-65].

Only combinations of L-arginine with SH-group donators or antioxidants can enhance its NO-modulating activity [57].

5. Conclusions

We have obtained convincing results indicating that the modeled PH leads to significant disorders in the cardiovascular system of offspring (1- and 2-month-old rats). In the myocardium of rats that underwent PH, an increase in the marker of endothelial dysfunction—sEPCR—was detected against a background of decreased Tie-2 and VEGF-B, which perform protective functions, alongside antioxidant deficiency reduction as well as Cu/ZnSOD, GPX). Our results experimentally substantiate the necessity for early postnatal cardio- and endothelial protection using NO modulators, considering the role of NO-dependent mechanisms in the pathogenesis of cardiovascular system disorders in newborns after PH. We have shown that only two representatives of this group, Angiolin and Tiotriazoline, are capable of exerting a complete effect on the indicators of endothelial dysfunction after PH (with a decrease in sEPCR against an increase in Tie-2, VEGF-B, and Cu/ZnSOD, GPX), which perform protective functions and antioxidative functions. Based on the conducted research, the feasibility of further preclinical studies of Angiolin as a promising means of cardioprotection after PH has been experimentally justified. Additionally, the results obtained support the potential for conducting further preclinical and clinical studies of Tiotriazoline (as an approved medication) as a treatment for cardiovascular system pathologies following intrauterine hypoxia.

Author Contributions: Conceptualization, I.B., O.P., N.B., V.R.; methodology, O.Y., S.P., O.K., V.O.; software, N.B., V.R., S.P., O.K.; validation, I.B., O.P., O.Y., V.O.; formal analysis, S.P., V.O., O.K., V.R.; investigation, I.B., O.P, O.Y., N.B.; data curation, I.B., O.P., V.R.; N.B.; writing—original draft preparation, I.B., O.P., O.Y., N.B.; writing—review and editing, S.P., O.K., V.O.; visualization, O.P., V.R, O.K., V.O.; supervision, I.B., O.Y., S.P. All authors have read and agreed to the published version of the manuscript.

Funding: Valentyn Oksenych was a recipient of the following grants: the Research Council of Norway Young Talent Investigator grant (#249774); the Liaison Committee for Education, Research, and Innovation in Central Norway (#13477; #38811); the Norwegian Cancer Society (#182355); the Research Council of Norway FRIMEDBIO grants (#270491 and #291217); The Outstanding Academic Fellow Program at NTNU (Stjerneprogrammet 2.0); and Karolinska Institutet Stiftelser och Fonder (#2020-02155).

Institutional Review Board Statement: Zaporizhzhia State Medical University Commission on Bioethics approved the experimental study (protocol No. 33 of June 26. 2021).

Informed Consent Statement: Not applicable.

 doi:10.20944/preprints202411.0122.v1

Data Availability Statement: The manuscript contains the original contributions made during the study, further inquiries can be directed to the corresponding authors.

Conflicts of Interest: The authors declare no conflict of interest.

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 doi:10.20944/preprints202411.0122.v1

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