ORIGINAL ARTICLE

CONTENTS

Preliminary study of the influence of maternal and neonatal NOS3 (rs1799983), IL1B (rs1143634) genes variants and their intergenic interaction on the development of hypoxic-ischemic encephalopathy in newborns in the context of treatment planning

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ABSTRACT

Aim: To determine the influence of maternal and neonatal variants of the eNOS (G894T, rs1799983) and IL1B (C3953T, rs1143634) genes and their intergenic interactions on the development of HIE in newborns.

Materials and Methods: The study included a cohort of 105 newborns and their 99 mothers. Determination of variants of the genes eNOS (G894T, rs1799983) and IL1B (C3953T, rs1143634) was carried out for the patients of study groups.

Results: The frequency of detection of the 894TT genotype by the eNOS gene was increased in newborns with severe asphyxia (p=0.018) and in their mothers (p=0,0057). Further analysis of intergenic interactions, performed in mother-child pairs, revealed an increased frequency of the neonatal 894GG (eNOS)/ maternal 3953C (IL-1B) genotype combination in the comparison group versus the group of newborns with HIE (p=0.007).

Conclusions: The significance of the intergenic maternal combination of 894GG/3953CT genotypes for the eNOS and IL1B genes and the intergenic combination of neonatal 894GG (eNOS)/maternal 3953CT (IL-1B) genotypes in the development of HIE in newborns has been proven. Associations of maternal and neonatal 894TT genotypes for the eNOS gene with the development of severe asphyxia, bradycardia, and respiratory failure were found in newborns with HIE.

KEY WORDS: neonates, hypoxic-ischemic encephalopathy, NOS3 (rs1799983), IL1B (rs1143634), intergenic interaction

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INTRODUCTION

Hypoxic-ischemic encephalopathy (HIE) is a disease with heterogeneous manifestations, most often diagnosed in the neonatal period, and is one of the main causes of severe long-term neurological deficit. According to the data, HIE is one of the main causes of disability in children, and is the leading cause of death in children under the age of 5 [1].

The main mechanisms of neuronal tissue damage are: hypoxia, decreased perfusion with subsequent decrease in cerebral perfusion pressure, inflammation and oxidative stress [2]. It is claimed in the results of some research that cerebral perfusion pressure in newborns almost does not depend on arterial blood pressure (BP), since the external work of the heart in infants is mainly isometric [3]. In critical conditions, there is a violation of autoregulation, while brain perfusion becomes completely dependent on systemic hemodynamics [4].

Currently, it is known that endothelium regulates vascular tone through the release of vasodilator and vasoconstrictor factors and modulates the contractile activity of smooth muscle cells. Nitric oxide (NO) belongs to endothelial dilatation factors. Nitric oxide prevents adhesion and aggregation of platelets, adhesion of monocytes, protects the vascular wall and prevents remodeling of vessels in various pathological conditions [5]. NOS (NO synthase), which catalyzes the biosynthesis of NO, is found in endothelial cells, astrocytes, and neurons. There are three isoforms of NOS: nNOS (neuronal NOS), which regulates synaptogenesis and remodeling and is Ca2+ dependent; eNOS (endothelial NOS), which regulates vascular tone, especially vasodilation, and is also

dependent on Ca2+; iNOS (inducible NOS), which is present in macrophages and astrocytes and is Ca2+ independent.

It has been established that disruption of cerebral blood flow during perinatal HIE is associated with NO activity [6]. The role of NO in the pathogenesis of ischemic brain damage is dual: protective and detrimental, depending on the NOS isoform and the cell type that produces NO. Under oxidative stress, NO produced by nNOS leads to neuronal death, causing mitochondrial damage, energy loss, and subsequent disruption of calcium homeostasis [6]. The endothelial form of NOS has a neuroprotective function, which is realized through NO-mediated enhancement of brain perfusion [7].

In neonates with HIE, the variability of the *eNOS* gene (synonymous name – *NOS3*), which encodes endothelial NOS, can affect both the activity of the enzyme and the formation of NO, which can lead to different clinical outcomes depending on the severity of perinatal HIE. Despite the key role of endothelial dysfunction in the pathogenesis of cerebral blood circulation disorders, there is almost no information about the nature of changes in the vasoregulatory systems of newborns with HIE and potentially possible intergenic interactions.

It should be noted that the concentration of NO can change under the influence of cytokines [8]. In particular, under conditions of hypoxia and inflammation, the cytokine interleukin-1 beta (IL-1β) activates iNOS, which dramatically increases NO production and, accordingly, potentiates brain damage after ischemic perinatal asphyxia [9]. In an in vitro study, it was shown that stimulation of IL-1β production reduced the level of *eNOS* expression in human aortic endothelial cells [10].

On the other hand, the pro-inflammatory cytokine IL-1β itself plays an important role in the development of various pathological, in particular, neurological, conditions of newborn children. Thus, there is convincing evidence that excessive production of IL-1β is a leading component of the development of inflammation and subsequent brain damage in newborns with encephalopathy [11]. Increased levels of IL-1β were observed in newborns with asphyxia, which was accompanied by impaired cerebral metabolism and subsequent developmental delay [12, 13].

Considering the importance of eNOS and IL-1β in the development of HIE, the study of gene variants encoding these proteins and affecting their functional state is extremely relevant. Studying these gene variants and their interactions will help to better understand the mechanisms underlying the disease and may provide new opportunities for developing treatment strategies.

AIM

Therefore, the aim of this study was to determine the influence of maternal and neonatal variants of the *eNOS* (G894T, rs1799983), *IL1B* (C3953T, rs1143634) genes and their intergenic interactions on the development of hypoxic-ischemic encephalopathy in newborns.

MATERIALS AND METHODS

The study was conducted in 2022-2023 in the neonatal intensive care unit. The study included a cohort of 105 newborns with a gestational age of 25 to 42 weeks and their 99 mothers.

The main group of the study included 45 newborns (including 4 twins), hospitalized in the intensive care unit after birth with a diagnosis of «hypoxic-ischemic encephalopathy of newborn» (P91.6 according to ICD-10), aged from 1 day to 28 days of life, according to the severity of the condition, as well as 40 of their mothers (one mother refused to participate in the study).

The comparison group consisted of 60 randomly selected otherwise healthy newborns, without HIE (including 1 twin) and their 59 mothers.

Exclusion criteria of the study were: the presence of congenital malformations, genetic diseases, and parents' refusal to participate in the study.

The study was conducted in accordance with the Declaration of Helsinki. The permission of the ethical committee was obtained for the study. The parents gave their informed consent to participate in the study.

The primary registration forms of the Ministry of Health of Ukraine No. 003/o "Medical records of a hospital patient" were used as medical documentation, and the corresponding individual study cards were formed in accordance with study design. The study was conducted within the framework of the initiative research of the Department of Pediatrics No. 1 with Neonatology "To develop clinical and laboratory criteria, methods for predicting and preventing metabolic disorders in young children", No. 0120U102856.

MOLECULAR GENETIC ANALYSIS

The research was carried out using the buccal epithelium as a biological material. Material was collected using disposable sterile brushes and stored and transported in tubes with the preservative «DNA/RNA Shield» (Zymo Research, USA). DNA was isolated using a commercial kit «Quick-DNA Mini Prep Plus Kit» (Zymo Research, USA). Determination of variants of the genes *eNOS* (G894T, rs1799983) and *IL1B* (C3953T, rs1143634) was carried out by the method of polymerase chain reaction with subsequent analysis of polymorphism of the

Table 1. Basic clinical characteristics of newborns and their mothers

Basic clinical indicators of newborns		Research groups		p
		Main group (n=45)	Comparison group (n=60)	
Gestational age, weeks		32,8±4,5	39,0±1,5	<0,0001
Birth weight, g		2164,9±1034,8	3328,9±470,4	<0,0001
Body length, cm		44,3±7,4	51,2±2,8	<0,0001
Gender	Female, n (%)	17 (37,8%)	29 (48,3%)	0,32
	Male, n (%)	28 (62,2%)	31 (51,7%)	
Apgar scale, 1st minute,scores		6 [5; 7]	8 [7; 9]	<0,0001
Apgar scale, 5th minute, scores		7 [6; 8]	9 [8; 9]	<0,0001
Basic clinical indicators of mothers		**Main group (n=40)**	**Comparison group (n=59)**	
Average age, years		27,3±5,5	28,5±5,3	0,34
Obesity, n (%)		9 (23,1%)	7 (11,9%)	0,17
Cardio-vascular diseases, n (%)		6 (15,0%)	7 (11,9%)	0,76
Thyroid gland diseases, n (%)		4 (10,0%)	4 (6,8%)	0,71
Kidneys diseases, n (%)		8 (20,0%)	4 (6,8%)	0,06
Preeclampsia, n (%)		15 (37,5%)	9 (15,3%)	0,016
TORCH-infections, n (%)		17 (42,5%)	4 (6,8%)	<0,0001
Infectious diseases, n (%)		11 (27,5%)	-	
Miscarriage threat, n (%)		18 (45%)	-	
Fetoplacental insufficiency, n (%)		13 (32,5%)	9 (15,3%)	0,052
Polyhydramnios, n (%)		10 (25,0%)	3 (5,1%)	0,0058

length of restriction fragments [14, 15]. Amplification was carried out using the DreamTaq Green PCR Master Mix kit (Thermo Scientific, USA) and primers (Metabion, Germany), restriction – using specific restriction endonucleases (Thermo Scientific, USA).

STATISTICAL ANALYSIS

Statistical processing of the obtained results was carried out using the IBM SPSS Statistics v27 application program package. Qualitative data are presented as absolute numbers and percentages. Quantitative data are presented as mean±standard deviation (if the data followed a normal distribution) and as median [25th quartile; 75th quartile] (if the data did not follow a normal distribution). Fisher's exact two-tailed test was used to compare qualitative data. When comparing basic clinical characteristics, the distribution of the studied variables was first checked for normality using the Kolmogorov-Smirnov test. Then, depending on the obtained results, one-way ANOVA or the Mann-Whitney test was used to compare the data. The group of newborns (main group and comparison group) was considered the independent factor, and the studied characteristic was considered the dependent variable. In all cases, the differences were considered statistically significant at $p<0.05$.

RESULTS

During the analysis of the basic and clinical indicators of the experimental groups, it was determined that the average GA in the main group was 32.8±4.5, and in the newborns of the comparison group – 39.0±1.5. In addition, mothers of newborns in the main group had a complicated obstetric history (Table I).

Preeclampsia, polyhydramnios, and TORCH-infections were diagnosed significantly more often in mothers of newborns in the main group. In the neonatal period, the newborns of the main group were diagnosed with clinical signs of moderate (17.8%) and severe (8.9%) asphyxia, RDS (60%), respiratory disorders (82.2%), respiratory (33.3%) and heart (24.4%) failure, intraventricular hemorrhages (40%), pulmonary hypertension (82.2%) and bradycardia (13.3%). In the newborns of the main group, non-invasive (95.6%) and invasive (68.9%) lung ventilation, surfactant replacement therapy (68.9%) were conducted.

Table II presents the distribution of genotypes and allele frequencies for the *eNOS* (G894T, rs1799983) and *IL1B* (C3953T, rs1143634) gene variants in mothers of the comparison and main groups.

The analysis of genotype and allele frequencies for the eNOS and IL1B genes did not reveal statistically significant differences between the mothers of the

Table 2. Frequency of genotypes and alleles distribution for the eNOS and IL1B genes among mothers in the study groups

Gene (variant)	Genotype, allele	Mothers of comparison group, n=59	Mothers of main group, n=40	p
eNOS (G894T, rs1799983)	894GG	32 (54,2%)	18 (45,0%)	0,42
	894GT	24 (40,7%)	16 (40,0%)	1,00
	894TT	3 (5,1%)	6 (15,0%)	0,15
	894G	88 (0,75)	52 (0,65)	0,16
	894T	30 (0,25)	28 (0,35)	
IL1B (C3953T, rs1143634)	CC	32 (54,2%)	28 (70,0%)	014
	CT	24 (40,7%)	11 (27,5%)	0,20
	TT	3 (5,1%)	1 (2,5%)	0,65
	C	88 (0,75)	67 (0,84)	0,16
	T	30 (0,25)	13 (0,16)	

Table 3. Frequency of genotypes and alleles distribution for the eNOS and IL1B genes among newborns in the study groups

Gene (variant)	Genotype, allele	Neonates, comparison group, n=60 (%)	Neonates, main group n=45 (%)	p value
eNOS (G894T, rs1799983)	894GG	30 (50,0%)	17 (37,8%)	0,24
	894GT	25 (41,7%)	25 (55,6%)	0,17
	894TT	5 (8,3%)	3 (6,7%)	1,00
	894G	85 (0,71)	59 (0,66)	0,45
	894T	35 (029)	31 (0,34)	
IL1B (C3953T, rs1143634)	3953CC	35 (58,3%)	31 (68,9%)	0,31
	3953CT	20 (33,3%)	12 (26,7%)	0,53
	3953TT	5 (8,3%)	2 (4,4%)	0,70
	3953C	90 (0,75)	74 (0,82)	0,24
	3953T	30 (0,25)	16 (0,18)	

comparison and main groups. These results suggest that these maternal genetic variants, on their own, are not associated with the risk of HIE in neonates.

Table III displays the distribution of genotypes and allele frequencies for the studied *eNOS* and *IL1B* gene variants in neonates from both the comparison and main groups.

For both genes, the analysis revealed no statistically significant differences in the distribution of genotypes and alleles between neonates in the comparison group and the main group. These findings suggest that, in this cohort, the genotypes and alleles of the studied variants of the *eNOS* and *IL1B* genes alone do not significantly contribute to the genetic predisposition to HIE in neonates.

After that, we evaluated the effect of *eNOS* and *IL1B* gene variants on the course of the neonatal period in newborns of the main group, the risk of developing neonatal syndromes, and the need for medical interventions.

An association of the G894T variant of the *eNOS gene* (both neonatal and maternal) with the risk of developing severe asphyxia in newborns was found. The frequency of detection of the 894TT genotype by the *eNOS* gene was increased in newborns with severe asphyxia (50.0% vs. 2.4%: p=0.018) and in their mothers (75.0% vs. 7.5%: p=0, 0057).

An association between G894T variant of the *eNOS* gene (both in newborns and in mothers) and an increased risk of neonatal bradycardia were also found. Thus, the frequency of 894TT genotype was higher in newborns with bradycardia (33.3% vs. 2.6%: p=0.043) and in their mothers (66.7% vs. 5.5%: p=0.0015).

Among mothers of newborns with HIE, who were diagnosed with respiratory failure in the early neonatal period, an increased frequency of the 894TT genotype for the G894T variant of the *eNOS* gene was found, in particular, it was observed in 33.3% compared to 3.4% of cases (p=0.013) in mothers of comparison group, whose children didn't experience respiratory failure. In newborns with respiratory failure, a significant increase in the frequency of the 894TT genotype was also observed (20.0% vs. 0.0%: p=0.032).

In the study of intergenic interactions, no influence of neonatal combinations of genotypes on the

development of HIE in newborns was found, but an increase in the frequency of the maternal combination of genotypes 894GG/3953C for the *eNOS* and *IL1B* genes was detected in the comparison group, in contrast to the mothers of the main group (27.1% vs. 2.5%: p=0.001), that is, this maternal combination of genotypes can reduce the risk of developing HIE in newborns.

Further analysis of intergenic interactions, performed in mother-child pairs, revealed an increased frequency of the neonatal 894GG (*eNOS*)/maternal 3953C (*IL-1B*) genotypes combination in the comparison group versus the group of newborns with HIE (27.1% vs. 5.0%: p=0.007), i.e. they showed a protective effect against the development of HIE.

DISCUSSION

As a result of the research, no associations of the *eNOS* and *IL1B* genes variants with the development of HIE were found, instead, associations of maternal and neonatal genotypes 894TT for the *eNOS* gene with the development of severe asphyxia, bradycardia and respiratory failure in newborns with hypoxic-ischemic encephalopathy were detected.

The G894T variant (rs1799983) in exon 7 of the *eNOS* gene is one of the most common and causes the substitution of glutamine for asparagine at position 298 of the eNOS protein sequence. This substitution is conservative, but the presence of this variant has nevertheless been shown to lead to the generation of protein products with different susceptibilities to cleavage, suggesting that G894T has a functional effect on the eNOS protein [16]. The results of a meta-analysis have shown that the G894T variant affects the production of nitric oxide, in particular, it was determined that carriers of the T allele had lower levels of NO (which is equivalent to lower levels of nitrates/nitrites) [17]. Research by Sofowora et al. indicates that the clinical significance of this genetic variant can be manifested only in the presence of endothelial dysfunction [18].

That is why the G894T variant of the *eNOS* gene has been actively studied in various pathological conditions in newborn children. In particular, it was established that the frequency of the T allele can be a risk factor for pulmonary hypertension in newborns with congenital heart defects [19]. The association of the G894T variant with the risk of development and severity of respiratory distress syndrome in preterm infants was also shown [20]. In a study by Szpecht et al. it was found that premature newborns with the GT genotype had a 3.4 times higher risk of intraventricular hemorrhage [21]. In our study, we got the associations with other pathological conditions in neonates with HIE, but they most fully characterize endothelial dysfunction that occurred in the early neonatal period and increased neurological damage, occurred in the antenatal and intranatal periods. It is worth mentioning that the 894TT genotype, in turn, is associated with low levels of NO, which is critically important for the normal development and functioning of the vascular system of both the fetus and the newborn.

Clinical studies and preclinical animal models have shown dramatic increases in systemic levels of cytokines, particularly IL-1β, as a result of perinatal brain injury caused by severe asphyxia [22]. It should be noted that activity of IL-1β and its biological functions are mediated by variants of the *IL1B* gene. In particular, the variant C3953T (rs1143634) in exon 5 of the *IL1B* gene is one of the most studied. Although this variant is synonymous (or "silent", that means, it does not lead to an amino acid substitution in the protein sequence), in vitro it was demonstrated that individuals with the TT genotype secreted significantly higher levels of IL-1β than individuals with the CT and CC genotypes [23].

Despite the potential clinical significance of the C3953T variant, no associations with the risk of complications such as sepsis, intraventricular hemorrhage, and bronchopulmonary dysplasia were found in the examined newborns [24-26]. In our study, there was also no significant effect of the C3953T variant of the *IL1B* gene on the risk of developing HIE and other pathological conditions in newborns with HIE. However, during the analysis of intergenic interaction (combinations of both maternal and neonatal genotypes), a decrease in the frequency of the maternal combination of genotypes 894GG/3953C for the *eNOS* and *IL1B* genes in the main group was revealed, i.e. a protective effect with regard to the risk of HIE in newborns (in the presence of the 894GG genotype for *eNOS*, as mentioned, reveal higher levels of NO, and with the heterozygous variant – genotype 3953CT for the *IL1B* gene, the level of the cytokine IL-1β is moderately increased)**.** Considering the fact that high levels of IL-1β cytokine were noted in newborns with various pathological conditions [27], it is possible that, in certain clinical situations, the influence of the maternal factors also takes place here – that is, the vertical transfer of maternal immune cells [28]**.** But the increase in the frequency of distribution of the maternal heterozygous variant 3953CT for the *IL1B* gene, which we found in combinations with both maternal and neonatal 894GG genotypes for the *eNOS* gene in the comparison group, indicates the presence of an underestimated and unstudied protein interaction, when with sufficient production of NO, a certain

increase in the level of pro-inflammatory cytokines can block potential negative effects associated with excessive reactions in this pathogenetic link during the development of pathological conditions.

The maternal and neonatal intergenes interactions, we have studied, open up the new opportunities in the formation of new approaches to neuroprotective treatment. As it is known, arginine is a substrate for eNOS and subsequent NO production. It has been detected that hypoargininemia is quite common among premature babies and there are already positive results regarding arginine therapy [29]. As for the regulation of cytokine levels, trimethylglycine can be a promising substance, as it is able to inhibit the production and release of IL-1β [30]. But taking into account the increase in the frequency of heterozygosity 3953CT for the *IL1B* gene in the comparison group and the potential protective effect against the development of HIE when combined with the 894GG genotype for the *eNOS* gene, we need further large-scale studies. For the use of both arginine and trimethylglycine, it is necessary to know the genotype-mediated dose-dependent effect of these amino acids and the need for other macro- and micronutrients, since the pathogenesis of HIE is multicomponent and it is necessary to avoid unwanted clinical effects during treatment**.** Experimentally, with the use of NG-monomethyl-L-arginine, an inhibitor of NOS, it was confirmed that among cytokines only IL-1β directly affects the formation of NO [31]. Therefore, with the simultaneous use and, accordingly, with the protein interaction of trimethylglycine and arginine, there will be the conditions for sufficient production of NO. Given the increase in arginine intake and NO production, the processes contributing to endothelial dysfunction will be inhibited.

In our opinion, the use of arginine and trimethylglycine in pregnant women and some time after childbirth can be effective in the prevention of HIE, especially in the case of complicated obstetric medical history. Thus, these amino acids will exert their biological effects even at the stage of fetal development, and after birth the child will receive them with mother's milk. But it is also worth strengthening the prevention and treatment of HIE by correcting mitochondrial dysfunction. It should be noted, that it is much easier to choose the dosage of a certain drug or biologically active compound for mothers. Mostly all therapeutic doses are already known and it is possible to track the blood levels of these substances, adjust them with the usage of genotyping, and achieve the optimal genotype-mediated dose-dependent effect. In the nearest future genotyping of mothers and newborns, especially with complicated obstetric anamnesis, can be used for personalized treatment tactics to minimize neurological consequences in newborns with HIE.

CONCLUSIONS

The significance of the intergenic maternal combination of 894GG/3953CT genotypes for the *eNOS* and *IL1B* genes and the intergenic combination of neonatal 894GG (eNOS)/maternal 3953CT (IL-1B) genotypes in the development of HIE in newborns has been proven. Associations of maternal and neonatal 894TT genotypes for the *eNOS* gene with the development of severe asphyxia, bradycardia, and respiratory failure were found in newborns with HIE**.** Further studies of intergenic and interprotein interactions of the axis eNOS – IL1β (arginine – trimethylglycine) are necessary to make a prediction and strategy of neuroprotective personalized treatment of HIE in newborns.

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CONFLICT OF INTEREST
The Authors declare no conflict of interest

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