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THE ROLE OF GENETIC DETERMINANT IN THE DEVELOPMENT OF SEVERE PERINATAL ASPHYXIA

The frequency of GSTT1 and GSTM1 gene deletion polymorphism was determined in a case-control study of full-term Ukrainian newborns including patients with perinatal asphyxia. Multiplex polymerase chain reaction was used for genotyping 245 full-term newborns. The investigated full-term newborns with perinatal asphyxia were subdivided in the subgroups depending of severity of perinatal asphyxia and neonatal outcome. No significant differences in allele frequencies of homozygous null genotypes of GSTT1 and GSTM1 gene were detected among newborns with moderate perinatal asphyxia and healthy control. However, association with the development of severe perinatal asphyxia was detected for the deletion polymorphism in GSTT1 gene and the combination of the GSTT1 absent/GSTM1 absent in the newborns. The study shows that severe perinatal asphyxia may develop in the consequence of genetic predisposition to this condition as compare with moderate.

Introduction. Perinatal asphyxia (PA) is often associated with adverse neurological outcomes including the development of multiorgan injuries and may result in neurological injury with long-term disabilities, later disorder with behavioral consequences (cerebral palsy, mental retardation, hearing or visual impairment, and attention deficit hyperactivity disorder) [1–4]. Brain injury in the neonates remains a significant social and health problem, especially with the existence of an unfavorable neurological prognosis [5]. PA occurs approximately in 4 of 1000 term births and more frequently among preterm delivery neonates. The neonatal mortalities are higher for the neonates with PA, 23 % of neonatal mortalities world wide is connected with the condition in the neonates. PA is causing more then 8.5 % child deaths [2, 5]. PA is a heterogeneous group with different burden of clinical symptoms with expected adverse outcome [3, 6–8].

A number of studies focused on the pathological changes in the newborns with asphyxia, a few have been concerned with genetic differences which predispose to this disorder development [9–12]. One of the pathogenic changes demonstrated in asphyxia development is decompensate oxidative stress which causes the metabolic reactions that lead to primary and secondary dysfunction of many organs and systems. This may explain the polyorganic effects of decompensate oxidative stress in patients with asphyxia [2, 4].

Cells produce free radicals and reactive oxygen species (ROS) as one part of physiological metabolic processes. Biological systems at cellular level interact with external environmental factors, which determine the increase of ROS level. Antioxidant enzymes (AOEs) may protect the cells from ROS-mediated injury. However in addition, oxidative stress is physiological protection against unfavorable exogenous and endogenous factors [12–14].

Glutathione transferases (GSTs, EC2.5.1.18) are part of an important enzymatic system of the cellular mechanism of detoxification that protects cells against reactive oxygen metabolites due to the conjugation of glutathione with electrophilic compounds. Recent results show that different metabolites of endogenous molecules may also be substrates for GSTs [13–18].

GSTs are a superfamily of enzymes consisting in humans of α, β, π, μ and θ families with sequence similarity and shared properties for reaction of gluthatione with reactive substrates. These GSTs

are mainly found in the cytoplasm of the cell and catalytically active as dimeric proteins. They occur in most instances in multiple forms [15–17].

The homozygous presence (presence in both alleles) of deletion polymorphism in *GSTT1* gene and *GSTM1* gene is defined as null genotype for these genes, with lack of enzyme activities [15, 16]. Many studies found that genetic variation in GSTs may predispose to the development of diseases in consequence of oxidative stress damage. The association of the *GSTT1* deletion and *GSTM1* deletion gene polymorphisms has been reported in numerous investigations with higher risk of diseases development or higher individual susceptibility to diseases [16–19].

Embryonic and fetal development is shown to be dependent of genetic determined variability in GSTs and other AOEs including mother tissues, placenta and embryos or fetuses tissues. The risk of intrauterine damage in embryos and fetus during early ontogenesis is higher for individuals with genetically determined lack of enzyme or lower level of their activity. Genetic variants of *GSTT1* and *GSTM1* have been shown a role in the abnormal development of fetuses, neonates and children, especially with influence of unfavorable factors [20–27].

The intrauterine condition of intracellular fetal AOEs may influence the perinatal capacity of the antioxidant defense in the neonates and predispose to the development of perinatal pathologies and pathological states such as PA.

The newborns with severe PA need special treatment immediately after labour though they do not show any distinct symptoms of severe damage of the brain and other organs [28]. No significant prognostic biochemical or genetic markers of brain injury exist today for the newborns in the perinatal period [29, 30]. Therefore, it is necessary to investigate the genetic differences in the development risk of PA.

Thus, the purpose of this study was to evaluate the influence of *GSTT1* and *GSTM1* genes deletion polymorphism on the development risk of PA with neurological complications in full-term newborns.

Materials and methods. *Study population*. In the case-control study 135 full-term newborns with PA and 110 clinical healthy full-term newborns were involved. The newborns with PA were subdivided into two groups depending of the value according to Apgar scale and neurological disorders during the first several days after birth: newborns with severe PA ($n = 50$), newborns with moderate PA ($n = 85$). 110 clinically healthy full-term newborns formed a control group. The 135 full-term newborns with moderate and severe asphyxia were treated in the division of intense therapy in the maternity hospitals in 2006–2007 years.

The diagnosis was performed according to World Human Organization (WHO) recommendation ICD-10 (http://www.who.int/classifications/apps/icd/icd10online/), version 2007. The inclusion criteria were clinical symptoms of PA and gestational age of 38–40 weeks. The exclusion criteria were congenital defects, intrauterine infection, gestational age less then 38 weeks, weight less then 2500 g. The newborns of the three groups were not significantly different regarding anthropometric indexes and gestational age. Standard general and laboratory methods of investigation were performed in the newborns. The study was according to the declaration of Helsinki and was approved by the local Medical Ethical Committee of National Medical Academy of Postgraduate Education named after P.L. Shupyk.

Genetic analyses. Peripheral blood samples of 2.7 ml were obtained in Monovettes containing EDTA («Sarstedt», Germany). Genomic DNA was isolated from the blood samples using DNK sorb B kit («AmpliSens», Russia). The *GSTT1* and *GSTM1* gene polymorphism was determined in the investigated newborns using primers previously described by Arand et al. [31].

The multiplex PCR was performed in a total volume of 25 μl containing 150 ng of human DNA, 5 μL 5 × PCR buffer, 1.5 mM $MgSO_4$, 200 μM of each dNTP, 20 pM of each primer and 1 unit of *Taq* DNA polymerase («AmpliSense», Russia). The PCR protocol was performed as described earlier through the initial denaturation at 94 °C for 2 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 64 °C, 1 min at 72 °C, with an ensuing 5-min extension at 72 °C in a thermocycler Applied Biosystems 2700 («Perkin Elmer», USA). Fragments were separated by electrophoresis in a 1.5 % agarose gel. The results of electrophoresis were subsequently visualized by UV detection. A characteristic multiplex PCR for the presence or absence of *GSTT1* and *GSTM1* genes in examined newborn patients with PA and healthy newborns are presented in Figure.

This method do not discriminate heterozygous null individuals (+/–) from homozygous individuals with wild type alleles of *GSTT1* (+/+) or *GSTM1* (+/+). The addition of the internal albumin amplification control allowed the unequivocal discrimination between samples from double null individuals (–/–) and samples that failed to amplify because of a low amount of starting DNA or the presence of interfering impurities.

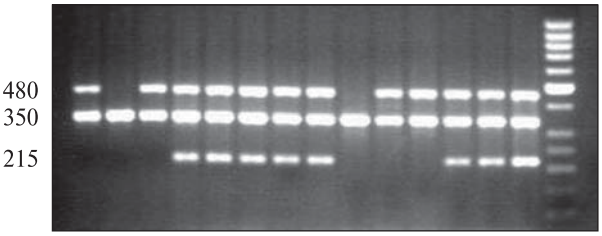
Statistical analyses. The genotyping results and the data obtained from collected maternal questionnaires, past and neonatal case histories were analyzed using following statistical methods. Differences in comparative groups were assessed by Yates corrected χ^2 and Fisher analyses (the Yates corrected Chi-square test and Fisher-test in electron version Microsoft Excel Table). $P < 0.05$ was considered to be statistically significant.

Results. We observed no differences in anthropometric indexes and gestational age for the newborns of the three groups, see Table 1.

Perinatal and maternal risk factors for the PA development were analysed in all investigated groups. This analyzes included mother's diseases, complicated obstetric and gynecological past histories, course of current and preceding pregnancies, labor (results not shown). No significant difference were found among newborns with PA and healthy controls in the maternal and perinatal risk factors frequencies.

The frequency of *GSTT1* null genotype was significantly increased in newborns with severe PA as compared with healthy controls ($\chi^2 = 23{,}72$, $p = 0{,}0001$) and newborns with moderate PA ($\chi^2 = 8{,}68$, $p = 0{,}003$), see Table 2. No significant differences were detected in the frequency of *GSTT1* null genotype between newborns with moderate PA and healthy newborns ($\chi^2 = 3{,}28$, $p = 0{,}07$). No significant difference was detected in the frequencies of *GSTM1* null genotype between the newborns of all investigated groups.

No significant differences were detected in the frequency of certain variant combinations for two genes in the newborns of the analyzed groups except *GSTT1* absent/*GSTM1* absent combination. We observed significant increase of the frequences in combination of the *GSTT1* absent/*GSTM1* absent ($p < 0{,}001$) in the newborns with severe PA compared to healthy controls. The results of distribution in combined *GSTT1* and *GSTM1* polymorphic variants among newborns with severe or moderate PA, respectively, and healthy controls are shown in Table 3.



The analysis of multiplex PCR products by electrophoresis on an 1.5 % agarose gel. *GSTT1* 480 bp, *GSTM1* 215 bp and internal albumin control 350 bp. Samples: *1, 3, 10, 11 – GSTT1*present/*GSTM1*absent; *2, 9 – GSTT1*absent/*GSTM1*absent; *4–8, 12–14 – GSTT1*present/*GSTM1*present; *15* – DNA Ladder

Table 1

Basic characteristics of the study population of newborns with perinatal asphyxia (PA) compared to healthy controls

Parameter	Severe PA ($n = 50$)	Moderate PA ($n = 85$)	Healthy controls ($n = 110$)
Sex, male/female	26/24	44/41	57/53
Gestational age ± SE	38.1 ± 0.5	38.2 ± 0.4	38.6 ± 0.6
Length, cm ± ± SE	50.1 ± 0.42	50.2 ± 0.43	50.3 ± 0.43
Weight, g ± SE	3312.2 ± 0.52	3215.3 ± 0.53	3270.4 ± 0.55

Table 2

The distribution of polymorphic variants in *GSTT1* and *GSTM1* genes in the newborns, *n* (%)

Genotype	Severe PA ($n = 50$)	Moderate PA ($n = 85$)	Healthy controls ($n = 110$)
GSTT1			
absent*	27 (54)	23 (27)	17 (15)
present**	23 (46)	62 (73)	93 (85)
GSTM1			
absent*	27 (54)	38 (45)	51 (46)
present**	23 (46)	47 (55)	59 (54)

* –/– genotype (deletion polymorphism); ** +/+ and +/–genotypes.

Discussion and conclusions. The frequency of polymorphic variants of many genes shows diversities

Table 3

The distribution of polymorphic variant combinations of *GSTT1* and *GSTM1* genes in the newborns with severe and moderate PA compared to healthy controls, *n* (%)

Polymorphic variants combination	Severe PA ($n = 50$)	Moderate PA ($n = 85$)	Healthy controls ($n = 110$)
*GSTT1*absent/ *GSTM1*absent*	13 (26)	12 (14)	6 (5)
GSTT1 present/ *GSTM1*present**	15 (30)	31 (36)	48 (44)
*GSTT1*present/ *GSTM1*absent	16 (32)	28 (33)	45 (41)
*GSTT1*absent/ *GSTM1*present	6 (12)	14 (16)	11 (10)

* –/– genotype (deletion polymorphism); ** +/+ and +/– genotypes.

in population and ethnicity with inter- and intraethnicity variability. The frequency of *GSTT1* null genotype was reported for the Caucasians with a small degree of no significant differences between 13–26 % (for example, Sweden – 13 %, Germany – 19 %). The same was found for the frequency of *GSTM1* null genotype among the Caucasians with differences between 42–60 %, Sweden – 55 %, Germany – 51 % [32]. Our results in healthy controls had no significant differences in comparison with the other population in the Caucasians: *GSTT1* null genotype – 15 %, *GSTM1* null genotype – 46 %.

This study shows that an important factor for developing severe PA is the presence of *GSTT1* null genotype in combination with *GSTM1* null genotype. We initially studied the prevalence of *GSTT1* and *GSTM1* gene polymorphism in newborns with perinatal pathologies, including perinatal brain damage, respiratory distress syndrome, necrotizing enterocolitis, neonatal jaundice. The prevalence of *GSTT1* deletion polymorphism and its combination with *GSTM1* deletion polymorphism was significantly higher in newborns with perinatal pathologies [33]. These initial studies showed that most of newborns had PA onset before development of mention above neonatal syndrome. In agreement with the initial studies we have focused on perinatal hypoxic state such as PA in full-term newborns. The obtained correlation have demonstratied the influence of genetic diversity on the risk of PA development. The earlier studies found that the newborns and the children with these gene deletions had higher risk of lung immaturity and development disorder depending of impairment factors and genotype of investigated *GSTT1* and *GSTM1* genes [23–27].

Several studies demonstrated that GSTs gene expression identifes the sensitivity to chemical compounds from environment in early stages of ontogenesis [13, 22, 23, 26, 27]. The GSTs gene expression was found in investigations in human embryonic and fetal tissues [20, 21]. It was shown that the individuals with deletion variation in *GSTT1* and *GSTM1* genes have higher susceptibility to cellular damage from environmental toxins and oxidant stress-related products [13, 16, 19].

Genetic differences influencing metabolic processes of the fetus are important for prenatal development and the initiation of labour [24, 26]. Becher et al. [34] discussed that brain damage leading to birth asphyxia exists before starts of labour. Genetically influenced functional changes in the cellular antioxidant pathways may occur in newborns with PA and lead to different reactions on the environmental toxicants. Therefore, the problem of abnormalities and severe PA onset is connected, besides increased ROS, also with increased environmental influence and gene-environmental interaction. On the other hand, delivery related malpractice was due to severe PA in one descriptive study in Sweden [6]. Though also, the other perinatal risk factors were considered involved in the PA occurrence in some studies, for example – using of local anaesthetics [7].

The lack of significant distinctions in maternal and perinatal risk factors in our investigation may be caused by low prevalence of these factors among subjects included in this study of newborns or it may be explain that severe PA is really a genetically determined state. The obtained association between the presence of deletion in *GSTT1* gene and the development of severe PA has proved the necessity of determining these and other genetic markers in the development of PA and to estimate the severity of the developing pathological hypoxic state.

The newborns with severe PA require timely started forced treatment. Some efforts of finding prognostic biomarkers focused on the examination of neuron specific enolase (NSE) and S 100 protein concentration [28, 29, 35]. The obtained results were inconsistent. The encouraging results were

obtained in the examination of NSE in cerebrospinal fluid of asphyxiated newborns with correlation to severity. But the serum or whole blood samples are more available in general practice [28].

Majority of studies concerning the analyses of genetic factors in the development of severe PA and neurological disorders observed gene polymorphism in different cytokines. It was demonstrated that apoptosis of nervous cells was stimulated at certain polymorphic variants of cytokines genes [11]. Cytokines are involved in the apoptosis pathways in intrauterine infection and hypoxia [36], but the prediction algorithm must be based on earlier prognosis after labour than appearance of cytokines.

The described genetic reason of cerebral palsy [9] was in one investigation spontanious dominant genetic mutation, that type of mutations usually doesn't prevalence widely, rather it was one case. This interesting finding applied to development of neurological outcome from intrauterine mutation process. It is infrequent occurrence as to cerebral palsy with intrauterine brain damage stimulating birth asphyxia.

Severe PA was associated in our investigation with combined deletion polymorphism in *GSTT1* and *GSTM1* genes. The abnormal function of additional polymorphic variants may intensify greater defects in the antioxidant pathways. The investigated distribution of polymorphic variants in *GSTT1* and *GSTM1* genes among newborns with moderate PA suggests the idea about its heterogeneity stipulated by the obstetric assistance peculiarities.

The obtained results in our study have demonstrated the necessity of further studies of several genes as genetically determined changes of antioxidant defense have a significant influence on the development of severe hypoxia impairments in the perinatal period with the consequence of damaged nervous system.

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РОЛЬ ГЕНЕТИЧЕСКОЙ ДЕТЕРМИНАНТЫ В РАЗВИТИИ ТЯЖЕЛОЙ ПЕРИНАТАЛЬНОЙ АСФИКСИИ

Представлены результаты определения частоты делеционного полиморфизма генов *GSTT1* и *GSTM1* у доношенных новорожденных в Украине. В исследовании, организованном по принципу случай–контроль, были обследованы новорожденные с перинатальной асфиксией и клинически здоровые новорожденные. Для генотипирования 245 доношенных новорожденных проведена мультиплексная полимеразная цепная реакция. Обследованные новорожденные с перинатальной асфиксией были поделены на две группы в зависимости от степени тяжести перинатальной асфиксии и течения неонатального периода. При сравнении частот делеционного полиморфизма исследованных генов у новорожденных с умеренной асфиксией и клинически здоровых новорожденных не было зарегистрировано достоверных различий. Выявлена ассоциация делеционного полиморфизма гена *GSTT1* и комбинации делеционного полиморфизма обоих исследованных генов с развитием тяжелой перинатальной асфиксии у новорожденных. При проведении исследования установлено, что тяжелая перинатальная асфиксия у новорожденных в отличие от умеренной может быть следствием генетической склонности к развитию этого состояния.

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