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9 EFFICACY OF COMBINED IMMUNOTHERAPY WITH PROPES AND INFLAMAFERTIN FOR SELECTIVE NK AND NKT CELL DEFICIENCY IN CHILDREN WITH AUTISM SPECTRUM DISORDERS ASSOCIATED WITH GENETIC DEFICIENCY OF THE FOLATE CYCLE

JUSTIFICATION

Recent meta-analyses of randomized controlled clinical trials indicate an association between autism spectrum disorders and genetic deficiency of the folate cycle in children [14, 15]. It has been established that genetic deficiency of the folate cycle affects the immune status of children with autism spectrum disorders, forming a kind of immunodeficiency, the basis of which is a decrease in the number and functional activity of natural killer (NK) cells and natural killer T lymphocytes (NKT) [10]. Immunosuppression caused by genetic deficiency of the folate cycle mediates the development of a number of immune-dependent complications that determine the formation of inflammatory encephalopathy in children with autism spectrum disorders, in particular, reactivated opportunistic infections [3, 13], autoimmune reactions against neurons and myelin [4, 5] and systemic inflammation with the phenomenon of hypercytokinemia [12, 16]. Compensation of immunodeficiency induced by genetic deficiency of folate cycle seems to be an attractive prospect for preventing or at least reducing the manifestations of related immune-dependent complications that influence the severity of CNS lesions in children with autistic disorders. However, such therapeutic approaches remain undeveloped and therefore inaccessible to patients. Results of previous small clinical studies indicate the potential benefit of combined immunotherapy with Propes and Inflamafertin to compensate for the deficiency of NK and NKT cells in folate cycle deficiency [1, 2], but these encouraging data need to be verified in larger controlled clinical trials with greater validity of the obtained results. Propes is a biological agent containing alpha and beta defensins, which has a pronounced immunoactivating and lymphoproliferative effect. At the same time, Inflamafertin, which includes alarmins and adrenomedullin, on the contrary, has an anti-inflammatory effect mediated by interleukin 10, which is important in preventing autoimmune complications during drug-induced immune activation. As the accumulated experience of using another highly active immunomodulatory agent – recombinant interleukin 2 – indicates, therapeutic immune activation can cause an undesirable increase in the risk of developing autoimmune complications [7], therefore, the combination of the immunoactivating drug Propes with an anti-inflammatory tolerogenic immunotropic agent seems to be the key to achieving a safe immunomodulatory therapeutic effect.

The aim of the research: to study the effectiveness of combined immunotherapy with Propes and Inflamafertin for NK- and NKT-cell deficiency in children with autism spectrum disorders associated with genetic deficiency of the folate cycle.

Materials and methods. This single-center prospective controlled non-randomized clinical study included 225 children aged 2 to 9 years suffering from autism spectrum disorders associated with genetic deficiency of the folate cycle. These patients constituted the study group (SG). The diagnosis of autism spectrum disorders was made by psychiatrists of regional hospitals or specialized departments according to the criteria of DSM-IV-TR (Diagnostic and Statistical Manual of mental disorders) and ICD-10 (The International

Statistical Classification of Diseases and Related Health Problems). The basis for including the patient in this trial was the presence of written parental consent for the child's participation in the study (Protocol No. 128 dated December 23, 2019 of the Bioethics Commission of the Bogomolets National Medical University).

To verify the genetic deficiency of the folate cycle, the nucleotide substitutions in the folate cycle genes were determined: MTHFR 677 C > T, MTHFR 1298 A > C, MTRR 66 A > G and MTR 2756 A > G in various combinations in the homozygous and heterozygous state by restriction PCR. Such children were diagnosed with persistent hyperhomocysteinemia – serum homocysteine concentration above 5.2 μmol/l, which is a biomarker of folate cycle deficiency. The number of NK and NKT cells in the blood was measured using laser flow cytometry (Epics XI cytometer, USA) using the indirect immunofluorescence method using monoclonal antibodies to CD lymphocyte markers (triple label; Beckman Coulter reagents, USA). NK cells were understood as a subpopulation of lymphocytes with the CD3–CD16 + CD56 + phenotype, and NKT cells were understood as a subpopulation of lymphocytes with the CD3 + CD16 + CD56 + phenotype. Immune status studies were performed monthly for 5 consecutive months both during the 3-month course of immunotherapy and for the next 2 months after the completion of immunotherapeutic interventions.

SG children (n = 225) received approved combination immunotherapy due to NK and/or NKT cell deficiency. Propes was administered at a dose of 2 ml i/m every other day at night for 3 consecutive months (45 injections). Accordingly, Inflamafertin was administered at a dose of 2 ml IM every other day at night for 3 consecutive months, alternating with Propes (45 injections).

The control group (CG) consisted of 51 children of similar age and gender distribution, suffering from autism spectrum disorders associated with genetic deficiency of the folate cycle, but did not receive immunotherapeutic interventions to compensate for NK and NKT cell deficiency. These children underwent only currently recommended educational and developmental programs in specialized centers for patients with special needs.

For statistical analysis of the obtained information, structural and comparative analysis methods were used. In order to establish the reliability of the differences in the results, the Student's T-test was used with the calculation of the confidence probability coefficient p (parametric criterion) and the number of signs Z according to Urbach (non-parametric criterion). To study the relationship between the appointment of immunotherapy and the dynamics of the studied indicators of immune status, Pearson's chi-square (χ^2) was calculated with the definition of the Yates correction. To determine the strength of the detected relationships, the φ criterion, Pearson's correlation coefficient (C) and its normalized value (C') were additionally calculated. To verify the obtained data, the calculation of the odds ratio (OR) and 95 % confidence interval (95 % CI) were used. The information was processed using the Microsoft Excel computer program.

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Results and their discussion. Compensation of the immunodeficiency induced by GDFC seems to be an attractive prospect for preventing or at least reducing the manifestations of related immune-dependent complications that affect the severity of CNS damage in children with ASD. However, such therapeutic approaches remain undeveloped at present, and therefore are not available to patients. Earlier, Tucker A. N. et al. in an experimental study showed a positive effect of therapy with a thymus extract preparation in specific disorders of bone marrow hematopoiesis and immunosuppression associated with folate deficiency,

which drew attention to the potential therapeutic properties of peptide immunomodulatory agents in GDFC. The results of previous small clinical studies indicate the potential benefit of combined immunotherapy with Propes and Inflamafertin to compensate for the deficiency of NK and NKT cells in folate cycle deficiency [1, 2], but these encouraging data need to be verified in larger controlled clinical trials with greater validity of the results obtained. Propes is a biological agent containing alpha and beta defensins, which has a pronounced immunoactivating and lymphoproliferative effect. At the same time, Inflamafertin, which includes alarmins and adrenomedullin, on the contrary, has an anti-inflammatory effect mediated by interleukin 10, which is important in preventing autoimmune complications in drug-induced immune activation. As the accumulated experience of using another highly active immunomodulatory agent – recombinant interleukin 2 – indicates, therapeutic immune activation can cause an undesirable increase in the risk of developing autoimmune complications [7], therefore, the combination of the immunoactivating drug Propes with an anti-inflammatory tolerogenic immunotropic agent seems to be the key to achieving a safe immunomodulatory therapeutic effect.

The results of the structural analysis in the observation groups indicate that the number of NK cells reached the lower limit of normal in 39 of 53 patients (74 % of cases) with a baseline deficiency of these lymphocytes, and the average number of NK cells in the blood in SG increased almost twice during the 3-month course of immunotherapy, but returned to almost the baseline level within 2 months after the withdrawal of immunotherapeutic agents. In contrast, the number of NKT cells normalized in 78 of 87 patients (89 % of cases) with a baseline deficiency of these cells, and the average number of NKT cells in the blood in SG increased during the course of immunotherapy by at least half and continued to increase steadily during the 2 months after the withdrawal of the approved immunotropic drugs, increasing almost twice at the end of the observation period.

A significant difference in the mean numbers of NKT cells in the blood in the observation groups occurred in the period from 2 to 5 months of the study ($p<0,05$; $Z<Z_{0,05}$), but not after the first month of treatment, persisting for at least the next 2 months after the withdrawal of the tested immunotropic agents (**Fig. 9.1**). The data of comparative and variance analyses indicate a significant difference in the average numbers of NK cells in the blood in SG and CG during the period of 1–3 months of immunotherapy ($p<0,05$; $Z<Z_{0,05}$), but not after the withdrawal of immunotherapeutic drugs (**Fig. 9.2**).

The obtained data indicate the ability of the applied combined immunotherapy to increase the number of NK and NKT cells in the blood in children with autism spectrum disorders associated with genetic deficiency of the folate cycle, normalizing their immune status. However, the response patterns of different lymphocyte subpopulations to the tested immunotherapeutic agents differ from each other. Thus, NK cells respond to immunotherapy faster and more intensively, but the achieved effect is short-lived and lasts only against the background of the applied immunotherapy, while the number of NKT cells in the blood increases more slowly with a 1-month delay, but a prolonged positive effect is achieved, since the gradual increase in the number of these lymphocytes in the blood persists even during the first 2 months after the withdrawal of immunotropic drugs.

To test the association between combined immunotherapy and normalization of the number of NK and NKT cells in the blood, we conducted a Pearson chi-square (χ^2), chi-square with Yates' correction, and chi-square with likelihood correction. These data would allow us to determine whether the immunotherapeutic interventions used were the cause of the changes in the immune status of SG patients. It was assumed that

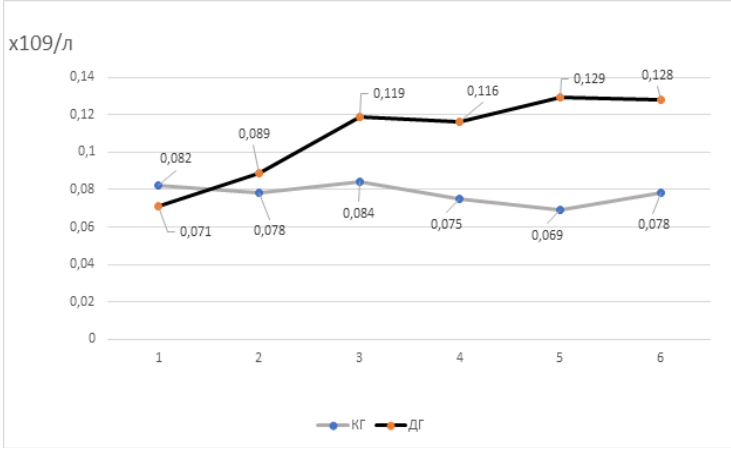

Fig. 9.1. Dynamics of the number of NKT cells in the blood in the observation groups during the clinical study

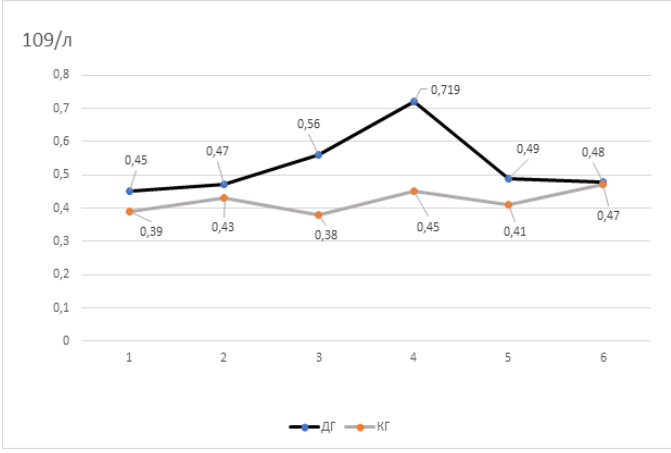

Fig. 9.2. Dynamics of the number of NK cells in the blood in the observation groups during the clinical study

the number of NKT cells was restored in 78 of 87 SG patients with an initial deficiency of these lymphocytes and only in 5 of 32 CG patients with a deficiency of these cells before the start of the study, and the number of NK cells reached normal in 39 of 53 patients with a corresponding deficiency in SG and only in 3 of 18 people with an initial deficiency in CG. The results obtained are presented in **Table 9.1**.

● **Table 9.1.** Evaluation of Pearson's chi-square (χ^2) and other indicators of association between immunotherapy administration and normalization of immune status indicators in SG patients (n = 225)

Indicator	NK cells		NKT cells	
	value	probability	value	probability
Pearson's chi-square	18,016	< 0,001	60,65	< 0,001
chi-square with Yates' correction	15,737	< 0,001	57,307	< 0,001
chi-square with likelihood correction	18,613	< 0,001	60,282	< 0,001

The results obtained (**Table 9.1**) indicate a connection between the implementation of immunotherapy and the achievement of normalization of impaired immune status indicators – the number of NK and NKT cells in the blood – in children with autism spectrum disorders associated with genetic deficiency of the folate cycle. This indicates that the prescribed immunotropic drugs were the most likely cause of the positive changes in the immune status of SG patients.

To study the strength of the relationship between the implementation of approved immunotherapeutic interventions and the normalization of the studied indicators of the immune status, the values of the φ coefficient, the Pearson correlation coefficient and its normalized value were calculated. This would allow us to assess how effectively Propes and Inflamafertin act on the impaired immune link in children with autism spectrum disorders associated with GDFC. The results obtained are contained in **Table 9.2**.

● **Table 9.2.** Evaluation of the φ criterion and other indicators of the strength of the relationship between immunotherapy and normalization of immune status indicators in SG patients (n = 225)

Indicator	NK cells		NKT cells	
	value	bond strength	value	bond strength
criterion φ	0,504	relatively strong	0,715	strong
Pearson's correlation coefficient (C)	0,450	relatively strong	0,581	relatively strong
normalized value of Pearson's correlation coefficient (C')	0,636	strong	0,822	very strong

As can be seen from the data in **Table 9.2**, there was a predominantly strong or relatively strong relationship between immunotherapy and the achieved changes in immune status, which indicates the high effectiveness of the tested immunotherapeutic agents in SG. NKT cells were somewhat more sensitive to combined immunotherapy than NK lymphocytes, although for both lymphocyte subpopulations convincing data were obtained on a strong relationship between immunotherapy and normalization of their number in the blood.

To verify the data on a strong relationship between the used immunotherapy and normalization of the number of NK and NKT cells in SG patients, the odds ratio (OR), standard error of the odds ratio (S) and 95 %

confidence interval (95 % CI) were calculated. This would avoid errors in assessing the conjugation between the studied processes at the previous stages of statistical analysis. The results obtained are presented in **Table 9.3**.

● **Table 9.3.** Estimation of the odds ratio (OR) and other indicators of the association between immunotherapy and normalization of immune status indicators in SG patients (n = 225)

Indicator	NK cells	NKT cells
odds ratio (OR)	13,929	46,800
standard error of the odds ratio (S)	0,705	0,601
95 % confidence interval (95 % CI)	3,498–55,468	14,415–151,937

As can be seen from the data in **Table 9.3**, the calculation of OR and 95 % CI confirms the previously obtained results on the close relationship between the implementation of the approved immunotherapy and the normalization of the studied indicators of immune status in SG patients. The fact was demonstrated again, identified at the previous stage of statistical analysis of the data, regarding the higher sensitivity of NKT cells compared to NK lymphocytes to combined immunotherapy with Propes and Inflamafertin in SG.

These data indicate the proper immunomodulatory effect of the approved immunotherapeutic strategy in a specific form of immunodeficiency observed in children with autism spectrum disorders associated with genetic deficiency of the folate cycle. Immunodeficiency in children with autism spectrum disorders is most likely responsible for the development of a number of immune-dependent complications that affect both the severity of mental disorders and the level of health in general. In particular, these include abnormally high microbial load on the body [3, 13], persistent immunoinflammatory enterocolitis [6], a tendency to generate allergic manifestations [17], systemic inflammation with hypercytokinemia [12, 16], and autoimmunity against neurons and myelin [4, 5]. Normalization of the impaired immune status is a key to preventing the development of a number of immune-dependent complications in children with autism spectrum disorders, which will contribute to improving their clinical condition and can significantly improve the response to neuroprotective therapeutic strategies [8, 9, 11].

Normalization of the impaired immune status is a key to preventing the development of a number of immune-dependent complications in children with autism spectrum disorders, which will contribute to improving their clinical condition and can significantly improve the response to neuroprotective therapeutic strategies [8, 9, 11].

Conclusions. The results obtained in this controlled non-randomized clinical trial indicate that combined immunotherapy with Propes and Inflamafertin is an effective strategy for the treatment of immunodeficiency caused by genetic deficiency of the folate cycle in children with autism spectrum disorders. These biological immunotropic drugs are able to normalize previously reduced numbers of NK and NKT cells in the blood of this category of patients within a 3-month course of immunotherapy with a more frequent, stronger and more persistent effect on NKT cells compared to NK lymphocytes.

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