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LONG-TERM FOLLOW-UP ANALYSIS AFTER AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION FOR CHILDREN WITH MULTIPLE SCLEROSIS

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АНАЛИЗ ДОЛГОСРОЧНОГО НАБЛЮДЕНИЯ ПОСЛЕ АУТОЛОГИЧНОЙ ТРАНСПЛАНТАЦИИ ГЕМОПОЭТИЧЕСКИХ СТВОЛОВЫХ КЛЕТОК У ДЕТЕЙ, СТРАДАЮЩИХ РАССЕЯННЫМ СКЛЕРОЗОМ

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Autologous hematopoietic stem cell transplantation (aHSCT) is effective and safe treatment for multiple sclerosis in children. The purpose of this study was to improve treatment outcomes by analyzing long-term follow-up after transplantation and evaluating late effects, as well as studying the immune profile in patients after aHSCT. Sixteen patients were included in the study. All patients included in the study were under 18 years of age. All patients had severe refractory multiple scle-

rosis. Prior to the initiation of aHSCT, all patients received therapy with 1st and 2nd line drugs with no positive effect. Mean improvement on the Expanded Disability Status Scale (EDSS) was 3.1±1.3 during the first 60 days after aHSCT. Long-term remission after stem cell transplantation was achieved in the patients included in the study, with median follow-up period of 48 months (8–93 months). Restoration of neurological functions was confirmed by immunological data: increasing of immune regulation index (CD4+/CD8+) from 1.36±0.84 to 2.2±0.53 as well as CD4+CD25+FoxP3+ T-lymphocytes from 1.38±0.86 % to 3.56±1.53 % (in comparison with the baseline). Late effects were moderate in all patients.

Thus, autologous hematopoietic stem cell transplantation proved to be effective and safe treatment for pediatric patients with severe refractory multiple sclerosis, and the effectiveness of aHSCT was proved by immunological data.

Keywords: autologous hematopoietic stem cell transplantation, multiple sclerosis, immune reconstitution, immunosuppression, children

Аутологичная трансплантация гемопоэтических стволовых клеток (ауто-ТГСК) является эффективным и безопасным методом лечения рассеянного склероза у детей. Целью данного исследования было улучшение результатов терапии путем анализа долгосрочного наблюдения за пациентами после трансплантации и оценки поздних эффектов, а также изучение иммунного профиля у пациентов после ауто-ТГСК. В исследование было включено 16 пациентов в возрасте до 18 лет. У всех пациентов было зафиксировано тяжелое рефрактерное течение рассеянного склероза. До момента начала ауто-ТГСК все пациенты получали терапию препаратами 1-й и 2-й линии без положительного эффекта. Среднее улучшение по расширенной шкале оценки степени инвалидизации (EDSS) в течение первых 60 дней после ауто-ТГСК составило 3,1±1,3 балла. У пациентов, включенных в исследование, удалось добиться длительной ремиссии после трансплантации стволовых клеток – период наблюдения пациентов составил в среднем 48 месяцев (8–93 месяцев). Восстановление неврологических функций подтвердилось иммунологическими данными: наблюдалось увеличение индекса иммунной регуляции (CD4+/CD8+) с 1,36±0,84 до 2,2±0,53, а также количества CD4+CD25+FoxP3+ Т-лимфоцитов с 1,38±0,86 % до 3,56±1,53 %. Поздние эффекты имели умеренный характер у всех пациентов.

Таким образом, аутологичная трансплантация гемопоэтических стволовых клеток оказалась эффективным и безопасным методом лечения детей с тяжелыми рефрактерными формами рассеянного склероза, причем эффективность ауто-ТГСК была доказана иммунологическими данными.

Ключевые слова: аутологичная трансплантация гемопоэтических стволовых клеток, рассеянный склероз, иммунная реконституция, иммунносупрессия, дети

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aHSCT – autologous hematopoietic stem cell transplantation
CP – cyclophosphamide
DMDs – disease modifying drugs
EBMT – European Society for Blood and Marrow Transplantation
EDSS – Expanded Disability Status Scale
IRI – immunoregulatory index (CD4+/CD8+)
MRI – magnetic resonance imaging
MS – multiple sclerosis

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system characterized by two interconnected pathological processes: multifocal auto-inflammation, which leads to demyelination of nerve fibers, and neurodegeneration, which leads to irreversible damage to axons and death of neurons [1]. In the pathogenesis of the disease lies a violation of suppressor immune mechanisms, namely: the body has a decreased activity of CD4+CD25+FoxP3+ T-lymphocytes which normally suppress proliferation of cytotoxic T-lymphocytes, inhibit the activity of B-lymphocytes, natural killer cells and macrophagocytes [2–4]. In connection with such an immune regulatory disorder, some T-lymphocytes turn against myelin antigens of the central nervous system and optic nerves (they become autoagressive) and trigger an autoimmune response, that causes demyelination, and further axonal degeneration occurs [5–7].

Today, when pulse therapy with glucocorticosteroids, intravenous administration of human immunoglobulin, plasmapheresis, using DMDs (disease modifying drugs) are ineffective or impossible, an alternative way of treatment of multiple sclerosis is an autologous hematopoietic stem cells transplantation (aHSCT) [5, 8–10].

The principle of aHSCT is based on an immune reconstitution (‘reboot’ of the immune system): firstly, having mobilized a certain number of hemopoietic stem cells from a body by way of conditioning in the form of high-dose chemotherapy or serotherapy, a state of hematopoietic aplasia is induced (most of lymphocytes, including sensitized to autoantigens, is destroyed both in the bone marrow and in the peripheral blood). This enables to arrest inflammatory process and, what is important for the further course of the disease, autoagressive immunological memory system is depleted [11]. Afterwards, the patients are injected with mobilized hemopoietic stem cells, which contributes to profound regeneration and the renewal of the immune system, i.e., immune reconstitution takes place (immune reboot) [12]. Mechanisms of immune reconstitution vary: 1) transplanted cells (or, more precisely, the cytokines secreted by them) induce the production of new T-lymphocytes in the thymus (the regeneration of CD4+ T-lymphocytes (T-helpers) occurs most fully) [13]; 2) a decrease in the secretion of proinflammatory cytokines [14]; 3) deple-

tion of the pool of mucosal-associated invariant T-cells (MAIT) in the peripheral blood; 4) a decrease in the number of microRNAs of those microorganisms that are possibly associated with the development of multiple sclerosis (miR-155, miR-142-3p, miR-16); 5) increased expression by lymphocytes of various checkpoints and regulatory molecules (PD-1, CTLA-4, GITR, TGF-b1127), etc. [15]. As a result, a new promising treatment method takes shape that can help save children with malignant forms of multiple sclerosis that are refractory to 1st and 2nd drug lines [16].

The aim of the study of our multidisciplinary team was to examine aHSCT effectiveness in children with multiple sclerosis, to analyze immunological profiles of patients after transplantation and to evaluate late therapeutic effects.

Material and Methods. The study included 16 patients with multiple sclerosis aged 15–17 years old. The median age of patients at study entry was 16.38±0.7 years old. The sample of 16 patients included 11 girls (68.8 %) and 5 boys (31.2 %). The duration of the disease before aHSCT was 21.7±5.4 months. The age of the disease onset was 12.2±1.9 years old.

Patients included in the study were: 1) under the age of eighteen years; 2) who signed informed voluntary consent to participate in the study (provided that the child was at least fifteen years of age); 3) with a verified diagnosis of multiple sclerosis; 4) who had two or more relapses of multiple sclerosis within the past year; 5) who had an EDSS score above 1.5.

If a patient 1) expressed a desire to no longer participate in the study, 2) could not perform all the procedures assigned to the study group, 3) did not comply with the conditions set forth in the study protocol, 4) responded to the T-lymphocyte injection with urgent adverse reactions, the patient was excluded from the study.

For the diagnosis of multiple sclerosis, the researchers used the clinical criteria for pediatric multiple sclerosis formulated by the International Pediatric MS Study Group as well as the criteria of McDonald W. I. et al. (2001), refined by Polman C. H. et al. (2010 г.). The Expanded Disability Status Scale (EDSS) was used to assess the severity of patients, with a score of 0 on the scale corresponding to no evidence of disease (normal in neurological status) and a score of 10 corresponding to death due to multiple sclerosis. The median EDSS score at the time patients were included in the study (before stem cell mobilization) was 4.7±1.3. All patients had a severe refractory course of multiple sclerosis. All patients were treated with high-dose glucocorticosteroids, plasmapheresis, interferon beta-1b, mitoxantrone with no positive effect.

Autotransplantation was performed with CD34+ hematopoietic stem cells. To isolate CD34+ cells, the patient was first injected intravenously with 2000 mg of cyclophosphamide (CP) per 1 m^2 body surface area. The infusion lasted one hour. Before and during the cyclophosphamide infusion, the researchers also used uromitaxan as an intravenous infusion (the dose of the drug was 180 % of the CP dose). Seven days later the patients were injected with granulocyte colony-stimulating factor filgrastim to stimulate hematopoiesis. Then at least 2×10^6 CD34+ cells per kilogram of body weight were extracted from the patient's body by hardware apheresis. The isolated cells were cryopreserved until day 0.

After stem cell mobilization, the researchers proceeded to conditioning. At this stage, 160 mg of ATG (antithymocyte immunoglobulin) were used as serotherapy for each kilogram of patient weight; 200 mg of cyclophosphamide for each kilogram of patient weight was administered as chemotherapy. Doses of ATG and cyclophosphamide were divided into four identical doses administered on days –2, –1, +1, +2 and on days –5, –4, –3, –2, respectively. CD34+ hematopoietic stem cells were transplanted after conditioning, on day 0. From the fifth day after cell reinfusion, filgrastim was administered at a dose of 5 μg/kg to eliminate hematopoiesis aplasia as soon as possible. The use of antithymocyte immunoglobulin also after T-lymphocyte reinfusion significantly reduced the likelihood of autoimmune response from the transplanted cells.

The therapy was carried out in accordance with the protocol «Autologous transplantation of hematopoietic stem cells in children with refractory forms of multiple sclerosis», approved at a meeting of the Academic Council of the N. I. Dmitry Rogachev on January 20, 2011 (approved by the local ethics committee on December 10, 2010).

The efficacy of aHSCT was determined by several key indicators: 1) stabilization of neurological status; 2) absence of negative progression according to the results of ongoing MRI of the brain and spinal cord; 3) approaching to normal the qualitative and quantitative composition of lymphocytes; 4) insignificance of developing complications (including in the late posttransplantation period).

Direct and lateral projection MRI scans of the spinal cord and brain were performed in T1- and T2-mode in SE and FSE sequences for each patient using a «Signa Horizont» tomograph. Also before and two weeks after transplantation, all patients underwent peripheral blood immunophenotyping using a FACS Calibur flow cytometer with assessment of the quantitative and qualitative subpopulation composition of lymphocytes, including determination of CD4+CD25+FoxP3+ T-lymphocytes and analysis of the CD4+/CD8+ immunoregulatory index (IRI).

The efficacy of aHSCT in all patients was evaluated taking into account how timely high-dose therapy was started. The subjects were divided into 2 groups. Patients who underwent therapy within the first six months to a year after the refractory course of multiple sclerosis was established were assigned to group 1 (timely initiation of high-dose therapy). Patients whose therapy was started one year after the establishment of MS refractory course were included in group 2 (untimely initiation of high-dose therapy).

Patients № 2, 4, 6, 7, 10, 11, 12, 13, 14, 15, 16 (eleven patients in total) were assigned to the first group. Patients № 1, 3, 5, 8, 9 (five patients in total) were assigned to the second group. For the first group, the duration of immunosuppressive therapy from determination of disease refractory to the start of conditioning was recorded as 3.0±0.3 months. For the second group, the duration of immunosuppressive therapy was 12.0±2.3 months (p=0,0007).

The researchers created a computer database to store and further process the data obtained during the treatment of aHSCT patients. In the analysis performed to characterize the distribution of the data, the Kolmogorov – Smirnov and Shapiro – Wilk tests were used. Data are presented as the number of observations in the group, arithmetic mean, and standard deviation. Categorical data are presented as percentages.

The choice of criterion for testing the statistical significance of differences between the analyzed indicators was based on the nature of the data distribution. When comparing two groups, in the case of related samples we used Student's paired t-test and/or its nonparametric analogue – Wilcoxon's test, in the case of unrelated

samples we used Student's unpaired t-test and/or its nonparametric analogue – Mann – Whitney test. The statistical significance of differences in the number of compared groups greater than two was assessed by repeated measures analysis of variance and/or Friedman's rank test for repeated measures, followed by the use of post hoc criteria.

All tests were bilateral; differences between compared groups were considered statistically significant at $p<0.05$; statistical power of the criteria was at least 0.80. Statistical analysis was performed using software: SPSS 13.0 (SPSS: An IBM Company, USA).

Neurologists, hematologists, radiologists and laboratory diagnosticians were involved in hematopoietic stem cell autotransplantation. The same doctors monitored the patients' condition before and after transplantation.

Results and Discussion. The data of all patients who received aHSCT treatment were included in the analysis. The results of transplantation in group 1 and group 2 were analyzed. Patient data are shown in Figure 1.

Patients after aHSCT			
Group 1 (in-time)		Group 2 (late)	
Patient	Decreasing of EDSS	Patient	Decreasing of EDSS
2	5,5	1	2,5
4	3	3	2
6	2	5	2
7	2	8	1
10	3	9	1
11	2		
12	2		
13	2		
14	2		
15	2		
16	2		

Fig. 1. Decreasing of EDSS in group 1 (n=11) and group 2 (n=5)

The neurological status of patients in the first group improved in the days following aHSCT (reduction of EDSS in the first group averaged 2.5±1.02 points in the first sixty days after transplantation). In the second group, the EDSS improvement was only 1.7±0.6 points ($p=0.01$). Such results could be explained by the timely use of antithymocyte immunoglobulin and cyclophosphamide in the first group. Subsequently, the improvement in EDSS averaged 0.2±0.02 points for both groups. This means that precisely conditioning with the use of immunosuppressive drugs is responsible for the pronounced effect of hematopoietic stem cell autotransplantation. The most pronounced reduction in EDSS in the first sixty days in both groups was considered to be 5.5 points, while the average EDSS improvement over the same period was 3.1±1.3 points. Figure 2 shows the clinical assessment of the patients' health status according to the extended disability scale in the dynamics before and after aHSCT. Patients were followed up for an average of 48 months (8–93 months).

Today, the state of remission is observed in 12 of 16 patients (75 %). Four patients (№ 3, 4, 5, 6) belonging to both the first and the second groups had relapses of multiple sclerosis. No errors in aHSCT were detected in these patients. The relapse that occurred five years after autotransplantation in patient 5, for example, was related to stress, while in patient 4, multiple sclerosis reappeared with a significant increase in the number of cytotoxic CD8+ T-lymphocytes and, accordingly, a decrease in the immunoregulatory index.

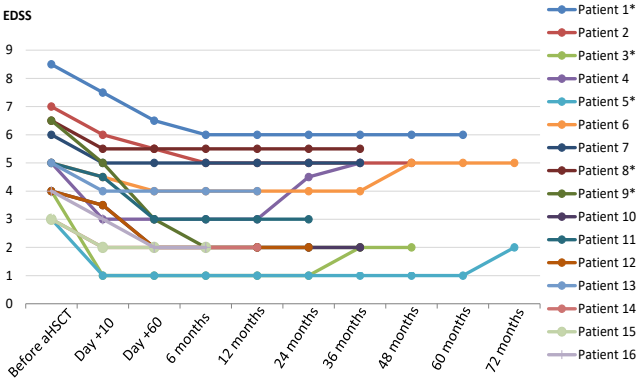

Fig. 2. Clinical assessment of patient progression (n=16) on the EDSS depending on the day relative to aHSCT. Patients in group 2 are marked with *

The number of leukocytes in patients quickly reached the value of 1 thousand/μl and more, which may indicate a rapid integration of the leukocytic growth into the patient's body, averaging 12±1.9 days. Aplasia of hematopoiesis was thus not prolonged, which is very important to prevent the development of various significant complications. Being represented by febrile neutropenia without signs of infection (n=11) (culture negative fever; there was no Uthoff phenomenon – worsening of neurological symptoms due to decreased conduction in demyelinated nerve fibers against the background of increased temperature [17]), cystitis (n=1) and reactivation of cytomegalovirus infection (n=1) within 100 days after the transplantation, complications were not life threatening for patients. The data are presented in Table 1.

Table 1

Clinical characteristics of patients before and after aHSCT (n=16)

Patients with multiple sclerosis	Age at aHSCT	EDSS at aHSCT	EDSS after 6 months after aHSCT	Complications	ANC >500
Patient 1	17	8.5	6.0	Febrile neutropenia	+11
Patient 2	17	9.0	3.5	No	+12
Patient 3	17	7.0	5.0	Febrile neutropenia	+10
Patient 4	16	4.0	1.0	No	+12
Patient 5	17	5.0	3.0	Febrile neutropenia	+10
Patient 6	16	3.0	1.0	Febrile neutropenia	+11
Patient 7	15	5.0	3.0	No	+10
Patient 8	15	6.0	5.0	Febrile neutropenia	+13
Patient 9	17	6.5	5.5	Febrile neutropenia	+12
Patient 10	16	5.0	2.0	No	+13
Patient 11	17	4.0	2.0	Febrile neutropenia, cystitis	+10
Patient 12	16	5.0	3.0	Febrile neutropenia	+9
Patient 13	16	4.0	2.0	Febrile neutropenia, CMV reactivation	+10
Patient 14	16	3.0	2.0	Febrile neutropenia	+11
Patient 15	17	3.0	2.0	No	+10
Patient 16	17	4.0	2.0	Febrile neutropenia	+10

Before autotransplantation, the analysis of magnetic resonance imaging of the brain in all subjects in the T2-mode revealed foci of increased magnetic resonance signal. Pathological foci with clear boundaries and homogeneous structure were mainly located in the large hemispheres, in the corpus callosum, around the ventricles, and in the cerebellum. The foci ranged in diameter from 3 to 19 mm and were round or irregular in shape. According to the increase of ventricular indices as well as diffuse pronounced dilation of the cerebral convexital subarachnoid spaces, cerebral atrophy was detected in most observations (in 12 patients).

Magnetic resonance imaging of the spinal cord in ten patients in T2-mode revealed contrast-accumulating circular lesions. In the sagittal view, pathological foci were detected in one or two (but not more) spinal cord segments. In the remaining six patients, heterogeneity of the spinal cord structure was detected, which was well visualized in its atrophy.

Each of the study subjects underwent magnetic resonance imaging of the brain and spinal cord again after autotransplantation at one, three, and six months. Absolute disappearance of pathological foci was not found, but it is worth noting that during remission, some narrowing of the lesion areas was still observed.

The efficacy of the treatment was proved immunologically by analyzing the quantitative and qualitative composition of lymphocytes in each subject (Table 2). In comparison with the data before autotransplantation, the researchers found a significant (p=0.004) decrease in the number of CD8+ T-lymphocytes. In this regard, the immunoregulatory index (CD4+/CD8+) also changed – increased from 1.36±0.84 to 2.2±0.53. The increase in IRI was one of the most important indicators of decreased autoimmune aggression after transplantation. An increase in the suppressor subpopulation of CD4+CD25+FoxP3+ T-lymphocytes from 1.38±0.86 % to 3.56±1.53 % turned out to be no less important; the data were statistically significant (p=0.0001).

Table 2

Immune profile before and after aHSCT for children with multiple sclerosis

Phase Indicator (%)	Before aHSCT (n=16) M±m [min; max]	After aHSCT (n=16) M±m [min; max]	*p*-value
CD3+	73.32±8.32 [64.80; 86.60]	69.76±8.47 [55.10; 77.40]	0.09
CD8+	30.87±6.28 [23.70; 39.10]	20.27±5.19 [16.80; 31.70]	0.004
CD4+	35.47±4.65 [33.20; 42.50]	39.89±6.45 [35.20; 45.50]	0.11
CD4+/CD8+	1.36±0.84 [0.9; 2.40]	2.2±0.53 [1.90; 2.80]	0.009
CD19+	11.59±3.44 [7.20; 19.70]	10.23±3.27 [5.90; 18.40]	0.46
CD16+CD56+	19.48±5.38 [8.80; 27.90]	20.69±8.95 [10.28; 32.40]	0.32
CD4+CD25+Foxp3+	1.38±0.86 [1.0; 1.80]	3.56±1.53 [2.90; 4.70]	0.0001

Despite a wide variety of means and methods used with varying efficacy for the treatment of multiple sclerosis in children, the challenge in treating the disease forms refractory to 1st and 2nd line drugs remain unresolved. An intensive procedure such as autologous transplantation of hematopoietic stem cells preceded by conditioning in the form of high-dose chemotherapy with cyclophosphamide and serotherapy with antithymocyte immunoglobulin helped to achieve significant success in arresting the autoimmune inflammatory process observed in multiple sclerosis. It should be noted that the role of conditioning, using strong immunosuppressive drugs, is extremely important in the early post-transplantation period in the case of extremely severe, life-threatening forms of multiple sclerosis, since conditioning quickly and efficiently (even before the transplanted cells begin to act) helps to arrest inflammation. At the same time, the toxicity of the therapy is low.

The effectiveness of aHSCT was reflected in the gradual recovery of neurological functions, which corresponded to the improvement of the EDSS score, as well as positive changes in neuroimaging (with those studied in the high-dose therapy group demonstrating a more pronounced approach of neurological status to normal than those studied in the untimely start of high-dose therapy group). In addition, obtaining clinical data was accompanied by immune profiling of pediatric patients by determining the qualitative and quantitative composition of lymphocytes. The efficacy of hematopoietic stem cell autotransplantation was proved immunologically by an increase in the immunoregulatory index (CD4+/CD8+) as well as by an increase in the number of CD4+CD25+FoxP3+ T-lymphocytes (T-regulators) whose number is reduced in refractory forms of multiple sclerosis due to the production by autoaggressive Th1-lymphocytes of proinflammatory cytokines, which inhibit the differentiation of T-regulatory cells. The detected correlation between the data of immune profiling of patients and indicators on the extended disability scale proves the hypothesis of immune reconstitution occurring after hematopoietic stem cell autotransplantation.

The widespread use of aHSCT as a method of treating multiple sclerosis in children is limited by insufficient data not only on the effectiveness of this therapy in the late stages after transplantation, but also on the possible development of late complications. In addition, there is very little information about the immune profile of patients, both before and after aHSCT. Our study, using prolonged observation of patients under conditions of multiple sclerosis control by clinical manifestations, data of magnetic resonance imaging of brain and spinal cord, laboratory indices, relying on the activities of multidisciplinary team of doctors, confirmed the effectiveness of hematopoietic stem cell autotransplantation in terms significantly distant from the moment of therapy, as well as the absence of pronounced late complications of the treatment. An important place in the study belongs to the study of the dynamics of the immune profile in pediatric patients with multiple sclerosis against the background of hematopoietic stem cell autotransplantation.

Conclusions. Thus, among the methods of treatment of severe refractory to previous therapy forms of multiple sclerosis in children a new effective and safe method – autologous hematopoietic stem cell transplantation – can take a worthy place. The most important success factor in this case is the timeliness of initiation of therapy. Immune reconstitution («rebooting» of the immune system), which aHSCT is aimed at, effectively and quickly arrests autoimmune inflammation in the central nervous system, preventing the further launch of this pathological immune process, which means protecting the body from potential relapses of multiple sclerosis. In the study, the effectiveness of aHSCT was proved by immunological data obtained as a result of studying the dynamics of the immune profile in patients before and after stem cell transplantation.

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