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EFFECTS OF WARTON'S JELLY HUMANS MESENCHYMAL STEM CELLS TRANSFECTED WITH PLASMID CONTAINING IL-10 GENE TO THE BEHAVIORAL RESPONSE IN RATS WITH EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

ABSTRACT

On the model of experimental analogue of human multiple sclerosis we studied the effects of the mesenchymal stem cells transfected with plasmid vector containing gene IL-10 (MSCs-T) on the functional state of the CNS in rats.

MATERIALS AND METHODS. *The experimental allergic encephalomyelitis (EAE) was induced with spinal cord homogenate of rats with complete Freund's adjuvant. The mesenchymal stem cells (MSCs) were isolated by the explants technique from Wharton's jelly of the human umbilical cord and culture up to two passages. Then the MSCs of second passage were transfected of plasmid vector with gene IL-10 and marker gene of green fluorescent protein. Cell transplantation was performed suboccipitally on the 17th day at a dose of 1 million cells in 100 μl of saline per animal.*

RESULTS. *In the open field test we have established that the use of MSCs-T transfected with gene IL-10 suppressed the vertical locomotor activity and elevated the emotional activity as well as partially corrects horizontal locomotor activity indexes which approach the indexes of intact animals.*

CONCLUSIONS. *The use of MSCs transfected with plasmid vector with gene IL-10 in the rats with induced EAE is more effective method than treatment using non-transfected MSCs. Combined treatment with IL-10 + MSCs in EAE rats is more effective than treatment with transfected MSCs.*

KEYWORDS: *experimental allergic encephalomyelitis, mesenchymal stem cells, interleukin-10, open field test*

The disseminated encephalomyelitis is a forerunner of the disseminated in 30-40 % of cases. Therefore proper effective treatment of this disease is very challenging from the economic, medical and scientific viewpoints. Various therapies are used ranging from immune-correcting, antiviral to cellular [1]. As a model of disseminated encephalomyelitis is the experimental allergic encephalomyelitis (EAE) [2].

In recent time more and more investigations have been conducted to study the mesenchymal stem cells (MSCs) obtained from various sources (bone marrow, lipid tissue etc.) which differ in terms of their availability, effectiveness and safety [3, 4]. The most perspective technique is the MSCs of the Wharton's jelly of the human umbilical cord which have abilities to proliferate *in vitro* [6], to migrate into white matter of spinal cord after intraventricular injections, to transdifferentiate into cells of the ectodermal origin, systemic and local immune-modulatory effects due to rapid increase of regulatory lymphocytes and decrease of the activated antigen-presenting cells.

Notably, the MSCs are able to stimulate synthesis and independently synthesize anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor β (TGF-β.) [7, 8]. These properties of MSCs (relative IL-10 in particular) may be extremely important for correction of many pathological conditions, considering a role that IL-10 plays in preventing development and treatment of the autoimmune conditions and diseases of the CNS [9-11].

In our previous work [12] we showed that the MSCs of the human Wharton's jelly in combination with IL-10 positively influence on the behavioral responses of the experimental animals with EAE. Obtaining and isolation of the necessary amount of IL-10 is not an easy task. Therefore at the Institute of Molecular Microbiology and Genetics of the NAS

of Ukraine we developed the technique of transfection of the gene responsible for synthesis of IL-10 in the human Wharton's jelly MSCs. Here we present the results of our study of influence of the mesenchymal stem cells on the functional parameters of the CNS in rats.

The purpose of the study was to investigate the influence of mesenchymal stem cells from human umbilical cord Wharton's jelly transfected with plasmid vector containing IL-10 gene, on the CNS functioning in the rats with experimental allergic encephalomyelitis.

MATERIALS AND METHODS

The investigations were conducted in the Laboratory of Experimental Neurosurgery of Department of Experimental Neurosurgery and Clinical Pharmacology of the A. P. Romodanov Institute of Neurosurgery NAMS Ukraine involving 53 white outbred mature male rats weighing 200-230 g. They were kept in the Institute vivarium under standard conditions with free access to water and food.

All procedures with experimental animals were performed in accordance with the international rules and norms of the European Communities Council Directives of 24 November 1986, 86/609/EEC and in conformity with the principles of "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes» (Strasburg, 1986) [13] and the Law of Ukraine #3447-IV «Concerning the protection of animals against cruelty» dated 21.02.2006 [14].

EAE was induced using rat's spinal cord homogenate with complete Freund's adjuvant (*Sigma*, USA) according to standard protocol [15] with a change in the ratio of components of encephalitogeneic mixture (the ratio of adjuvant to brain tissue 1.6:1), allowing to receive chronic recurrent non-severe relapsing EAE [16]. Chronic relapsing form of EAE allows more detailed study of the influence of factors on the course of the demyelinating process, and avoids mortality of experimental animals which, according to some researchers, in acute EAE reaches 30-60 % [17]. Encephalitogeneic mixture was administered in rat's limb pads according to [16, 17] at a rate of 50 mg of encephalitogeneic mixture per animal.

Experimental groups of animals are presented in **Table 1**. The rats with EAE were treated with intravenous or suboccipital injection of MSCs and IL-10 (in various combinations) and MSCs-T. Earlier we revealed that maximum clinical manifestations in animals with EAE [16] were seen on 17th day after induction of EAE. Therefore we allocated a group of animals, in which on the 10th day we injected IL-10 intravenously for anti-inflammatory effect. At the peak of clinical manifestations on the 16th day the animals were injected with MSCs or MSCs-T expecting to assess anti-inflammatory effects and ability of MSCs and MSCs-T to prevent demyelination processes and accelerate remyelination of the CNS. Human recombinant IL-10 was received from the Institute of Molecular Biology and Genetics NAS of Ukraine.

MSCs were isolated by the method of explants from Wharton's jelly of human umbilical cord. The umbilical cords, obtained after informed consent from healthy women during normal deliveries, were provided by the Kyiv municipal maternity hospital No. 5. Isolation, identification, culture and assessment of viability of the MSCs were performed in accordance with the methodology described in our previous publication [12]. The material was treated no later than 5 hours after the birth. The umbilical cord was washed of the blood, placed in *DMEM* medium with antibiotics 1000 U/ml penicillin and 1 mg/ml streptomycin and antimycotics Amphotericin 50 U/ml (*Kievmedpreparat*, Ukraine) for 30 minutes. The Wharton's jelly was separated, minced with scissors and placed in 75 cm^2 culture vial (*Grenier Bio-One*, Austria) with a-MEM medium, 2 mM L-glutamine (*BioWest*, Spain), antibiotics 100 U/ml benzyl penicillin (*Arterium*, Ukraine), 100 μg/ml streptomycin (Arterium, Ukraine) and 10 % fetal bovine serum (*HyClone*, USA). Cultivation was carried out in standard conditions in a CO_2 incubator (37 °C, 5 % CO_2, humidified atmosphere). Reaching a monolayer with 70 % confluency cells were passed using 0.02 % Versen solution and 0.25 % trypsin in 1:1 ratio. Cells of passage 2 were used for injection into the test animals.

To assess the culture we used inverted microscope DMIL (*Leica*, Germany). FACS-analysis of MSCs surface markers CD105, CD90, CD73 (BD, USA) was performed on the BD FACSAria sorter using the program BD FACSDiva v. 2.6.2 software in the division of cellular and tissue technologies of the Institute of Genetic and Regenerative Medicine NAMS of Ukraine. More than 95 % of cells of passage 2 were positive by the mentioned markers.

MSCs of passage 2 were transfected by the plasmid containing cDNA variant of IL-10 gene [18] and marker gene of green fluorescent protein (GFP) under constitutive promoter of early cytomegalovirus (CMV) genes. Transfection was performed using TurboFect transfection reagent (*Termo Scientific*, USA) according to the firm manufacturer's protocol. Twenty-four hours after the beginning of transfection procedure the MSCs-T were detached from the substrate using trypsin solution and EDTA, was resuspended in the phosphate buffer in the volume of 100 μl [12] and injected subocipitally to experimental animals in the amount of 1 million per animal. The parallel sample was analyzed using the flow cytometry on the BD FACSAria sorter (BD, USA) to assess the percentage of transfected cells for GFP marker. This value was 20 %.

Behavioral responses of rats of groups 1, 2, 3 and 4 and 6 on days 12, 15 and 24 after EAE induction were studied by open field test three times, and animals from group 5 (intact) – once. The first and second tests were performed in experimental animals to study the degree of EAE development. The third test was performed on the 7th day after suboccipital injection of MSCs (or MSCs-T) to study effects of treatment on the behavioral responses of animals after induction with EAE.

Adaptive behavior of animals was tested using the open field test for 10 minutes (for the first 5 minutes, for the second 5 minutes and for 10 minutes of the study – the total index) in terms of horizontal (crossing of the central and peripheral squares) and vertical locomotor activity (standing up on back paws – vertical rack), exploratory activity (looking into the holes), emotional activity (grooming and defecation). Using the software on the study of the following behavioral responses of animals we registered: latent period **(LP)**, number of episodes for the first 5 minutes **(n1)**, number of episodes for the second 5 minutes **(n2)**, the total number of episodes for 10-minute observation **(ns)**, duration of episodes for the first 5 minutes **(T1)**, duration of episodes for the second 5 minutes **(T2)**, the total duration of episodes within **(Ts)**, average duration of individual episode for the first 5 minutes **(t1)**, average duration of individual episode for the second 5 minutes **(t2)** and average duration of individual episode for 10 minutes of observation **(ts)**.

Table 1. Experimental groups.

GROUP	NUMBER OF ANIMALS	TREATMENT
1	8	Control group, EAE w/o treatment
2	8	Injection of MSCs suboccipitally (1•10^6 cells) on day 17
3	8	Injection of IL-10 intravenously (1 μg/ml) on day 10 and IL-10 suboccipitally (1 μg in 100 μl) on day 17
4	7	Injection of IL-10 intravenously (1 μg/ml) on day 10 and IL-10 (1 μg) + MSCs (1•10^6 cells) suboccipitally on day 17
5	12	Intact animals
6	10	Injection of MSCs-T suboccipitally (1•10^6 cells) on day 17

Table 2. Comparison of behavioral responses in rats with induced EAE treated with MSCT and other treatment (M ± m)

ACTIVITY	INDEX	INTACT, n=12	EAE, n=8	EAE+MSCS, n=8	EAE+ IL-10 + MSCS, n=4	EAE+ MSCS-T, n=10
Horizontal activity in central squares	Lp	1.50 ± 0.19	1.16 ± 0.07	6.32 ± 3.12	2.19 ± 0.69	1.52 ± 0.17
	n1	7.25 ± 1.21	14.38 ± 3.83	8.25 ± 1.60	14.75 ± 7.36	8.80 ± 2.14
	n2	6.17 ± 1.20	5.50 ± 1.95	4.00 ± 1.41	27.50 ± 17.88	5.80 ± 1.57
	ns	13.42 ± 1.90	19.88 ± 4.98	12.25 ± 2.33	42.25 ± 25.10	14.60 ± 3.14
Horizontal activity in peripheral squares	Lp	8.07 ± 1.16	9.59 ± 6.05	11.58 ± 4.13	10.45 ± 3.63	4.66 ± 0.39**
	n1	67.75 ± 5.40	60.88 ± 10.54	50.00 ± 10.29	75.50 ± 24.94	60.80 ± 9.50
	n2	50.25 ± 4.04	29.00 ± 7.49	24.63 ± 5.11	54.25 ± 18.10	46.00 ± 6.64**
	ns	118.00 ± 7.31	89.88 ± 14.96	74.63 ± 12.94	129.75 ± 39.80	106.80 ± 10.22
Vertical activity	Lp	44.74 ± 10.13	42.10 ± 12.15	86.15 ± 46.05	104.53 ± 45.75	78.53 ± 35.51
	n1	14.58 ± 1.76	16.63 ± 2.31	12.63 ± 2.99	13.50 ± 4.86	9.90 ± 1.99*
	n2	16.75 ± 1.44	10.63 ± 2.50	11.50 ± 2.65	12.50 ± 2.33	12.10 ± 2.34
	ns	31.33 ± 2.74	27.25 ± 4.42	24.13 ± 5.11	26.00 ± 5.28	22.00 ± 4.03
	T1	41.34 ± 5.38	42.53 ± 6.75	35.51 ± 9.57	34.89 ± 12.63	25.68 ± 5.57
	T2	51.88 ± 5.13	37.96 ± 10.02	34.67 ± 7.71	40.25 ± 8.17	37.60 ± 7.49
	Ts	93.22 ± 8.83	80.48 ± 15.89	70.18 ± 15.60	75.13 ± 15.19	63.38 ± 12.45
	t1	2.83 ± 0.21	2.51 ± 0.21	2.41 ± 0.37	2.57 ± 0.40	2.28 ± 0.28
	t2	3.17 ± 0.22	3.40 ± 0.31	3.20 ± 0.27	3.19 ± 0.30	3.12 ± 0.21
	ts	2.99 ± 0.12	2.84 ± 0.22	3.02 ± 0.21	2.91 ± 0.36	2.84 ± 0.12
Emotional response (grooming)	Lp	102.23 ± 13.21	60.62 ± 11.72	68.56 ± 24.00	57.39 ± 8.47	53.43 ± 8.73
	n1	3.67 ± 0.74	4.00 ± 0.42	4.63 ± 0.73	5.00 ± 1.22	4.00 ± 0.37
	n2	3.42 ± 0.62	4.00 ± 1.00	5.75 ± 0.80	4.75 ± 1.75	3.10 ± 0.74**
	ns	7.08 ± 0.99	8.00 ± 0.91	10.38 ± 1.16	9.75 ± 2.17	7.10 ± 0.50**
	T1	15.54 ± 2.53	39.99 ± 7.09	40.08 ± 9.76	33.40 ± 9.39	56.68 ± 13.35
	T2	25.45 ± 6.73	32.63 ± 11.52	88.44 ± 14.34	47.51 ± 20.30	43.81 ± 9.94**
	Ts	40.99 ± 8.08	72.62 ± 9.98	128.51 ± 16.60	80.91 ± 27.34	100.48 ± 6.28*
	t1	4.92 ± 1.00	10.22 ± 1.59	10.96 ± 3.56	6.54 ± 1.05	13.91 ± 3.03
	t2	10.41 ± 3.20	7.66 ± 2.46	16.58 ± 3.23	9.40 ± 2.60	13.54 ± 3.66
	ts	7.08 ± 1.82	9.20 ± 1.08	14.11 ± 3.24	7.50 ± 1.42	15.16 ± 1.87* / ***
Emotional response (boluses)	n	0 ± 0	0.63 ± 0.50	0 ± 0	0 ± 0	1.50 ± 0.81
Willingness to explore (holes)	Lp	202.41 ± 47.44	479.81 ± 65.74	317.20 ± 58.90	432.99 ± 129.60	517.08 ± 58.48**
	n1	1.83 ± 0.39	0.25 ± 0.16	1.00 ± 0.38	0.50 ± 0.50	0.10 ± 0.10**
	n2	1.67 ± 0.53	0.38 ± 0.18	0.50 ± 0.27	1.75 ± 1.44	0.60 ± 0.34
	ns	3.50 ± 0.68	0.63 ± 0.18	1.50 ± 0.53	2.25 ± 1.31	0.70 ± 0.33
	T1	4.91 ± 1.08	0.41 ± 0.30	1.57 ± 0.70	1.19 ± 1.19	0.06 ± 0.06**
	T2	4.19 ± 1.30	0.59 ± 0.31	0.69 ± 0.40	5.61 ± 5.00	2.05 ± 1.46
	Ts	9.09 ± 1.67	1.00 ± 0.34	2.26 ± 0.95	6.81 ± 4.68	2.11 ± 1.45
	t1	2.01 ± 0.39	0.41 ± 0.30	0.91 ± 0.32	0.60 ± 0.60	0.06 ± 0.06**
	t2	1.86 ± 0.49	0.59 ± 0.31	0.51 ± 0.27	1.34 ± 0.83	0.85 ± 0.51
	ts	2.80 ± 0.30	1.00 ± 0.34	1.21 ± 0.27	1.93 ± 0.72	0.90 ± 0.50

*Notes: LP – latent period; n1 – number of episodes for the first 5 min; n2 – number of episodes for the second 5 min; ns – total number of episodes; T1 – duration of episodes for the first 5 min; T2 –duration of episodes for the second 5 min; Ts – total duration of episodes; t1 – average duration of individual episode for the first 5 min; t2 – average duration of individual episode for the second 5 min; ts – average duration of individual episode for 10 minutes of observation; * – probable differences in the compared groups EAE and EAE + MSCsT, p<0.05; ** – significant differences in the compared groups EAE + MSCs and EAE+MSCsT, p<0.05; *** – significant differences in compared EAE + IL-10 + MSCs and EAE + MSCsT, p<0.05.*

Statistical analysis was performed using Microsoft Excel 2010 and Statistica v.6.1 software. A statistically significant difference was evaluated using nonparametric U Mann-Whitney test. A statistically significant difference was considered when $p < 0.05$.

RESULTS AND DISCUSSION

At the third open field test the group 6 rats (24th day after induction of EAE and one week following suboccipital injection of MSCs-T) in the comparison with group 1 (24th day after EAE induction without treatment), we detected probable significant decline of the vertical locomotor activity (decrease of the number of vertical stands – **n1**) and an increased emotional activity – increase of total and medium duration of episodes of grooming Ts and ts **(Table 2)**. There was a tendency seen in the horizontal locomotor activity: decrease of the crossings of central squares and increase of the crossings of peripheral squares, as well as decrease of exploratory activity for the first 5 minutes and its increase for the second 5 minutes.

At the third open field test the rats of group 1 (24 hours after induction of EAE and one week following sub-occipital injection of MSCs-T) in comparison with group 2 (24 days after induction of EAE and one week following suboccipital injection of MSCs) showed probable significant increase of horizontal locomotor activity (at reduced latent period **LP** – the number of crossings of peripheral squares increased – **n2**), decline of emotional activity (reduced number of grooming episodes – **n2**, ns and reduced duration of episodes of grooming – **T2**) and decline of exploratory activity (at increased latent period **LP** the exploratory activity looking-into-holes – **n1**), average duration of episodes – **T1**, **t1**. Also, there was a tendency towards a decrease of the vertical locomotor activity.

In the rats of group 6 (24th day after EAE induction and one week after suboccipital injection of MSCs-T) in the comparison with group 4 animals (24th day after EAE induction and two weeks following intravenous injection of IL-10 and one week following suboccipital injection of IL-10 and MSCs) in the third open field test we observed the statistically significant increase of emotional activity (average duration of grooming episodes – **ts**). There was also a tendency towards the decrease of horizontal and vertical locomotor activities.

Various methods of treatment (MSCs, IL-10 and IL-10 in combination with MSCs) in the rats with induced EAE in the open field study of behavioral reactions were described in our previous paper [12], where we found that treatment of EAE animals only with IL-10 is less effective than that with MSCs and IL-10 + MSCs. Therefore in the present study we compared effects of MSCs transfected of plasmid vector containing IL-10 gene with two methods of treatment namely treatment with MSCs and combined treatment (IL-10 + MSCs).

Application of MSCs-T in the rats with induced EAE in the open field test significant reduces vertical locomotor activity and increases emotional activity as well as partially corrects horizontal locomotor activity indexes which approach the indexes of intact animals **(Table 2)**. In the comparison of two methods (MSCs-T and MSCs) of treatment of the rats with induced EAE we have found that MSCs-T significant reduces emotional and exploratory activities of animals and positively influences on the horizontal locomotor activity indexes showing probable increase to the indexes of intact rats **(Table 2)**. The obtained results have shown that the method with MSCs-T versus MSCs is more effective. Comparison of two methods (MSCs-T and combined IL-10 + MSCs in the rats with induced EAE has shown that MSCs-T significant increased the emotional activity indexes. When using combined treatment IL-10 + MSCs of the rats with induced EAE versus intact animals group no significant differences between indexes of behavioral responses were found. However, application of this method contributed to correction of vertical locomotor activity as well as exploratory and partially emotional activities bringing them close to the indexes of intact rats **(Table 2)**. Therefore we find the combined method of treatment IL-10 + MSCs more effective than treatment with MSCs-T.

CONCLUSIONS

1. ***Treatment with MSCs-T of the rats with induced EAE decreases vertical locomotor activity and increases emotional activity.***
2. ***Treatment using MSCs-T versus MSCs in the rats with induced EAE is more effective.***
3. ***Combined treatment with IL-10 + MSCs in rats following EAE induction is more effective than treatment with MSCsT.***

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