NEW BIOMEDICAL TECHNOLOGIES

Comparison of the Efficacy of Cell Preparations from Embryonic Ventral Mesencephalon of Various Prenatal Age Transplanted Intrastriatally to Rats with 6-OHDA-Induced Parkinsonism

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Cell preparations of ventral mesencephalon obtained from 8-, 14-, and 16-17-day rat embryos were stereotactically transplanted to homologous rats with 6-hydroxydopamine-induced hemiparkinsonism. Automated analysis of apomorphine-induced motor asymmetry for 3 months after neurotransplantation revealed higher efficacy of cell preparations from 8- and lower from 16-17-day-old embryos. These data correlated with histomorphological findigs, in particular, with the size of grafts, glial reaction, and the number of dopaminergic neurons in the grafts.

Key Words: *rat 6-OHDA-hemiparkinsonism; preparations of embryonic nervous tissue; apomorphine-induced motor asymmetry; dopaminergic neurons*

The efficiency of transplantation of embryonic nervous tissue from ventral mesencephalon (ENT VM) into damaged striatum during Parkinson's disease is determined by survival of specific donor dopaminergic neurons in the recipient tissue [10,11,13]. Low expression of histocompatibility antigens on donor cells is a prerequisite for successful transplantation. Expression of these antigens is minimum at the early stages of gestation. High content of polypotent stem cells differentiating into dopaminergic neurons in recipient brain also plays an important role [12].

It can be hypothesized that transplantation of cell preparations from ENT VM of early embryos for correction of the dopaminergic system will be most effective. At the same time, high survival of transplanted neurons and correction of motor disorders were achieved in rats with experimental parkinsonism after transplantation of ENT VM preparations from 13-, 14-, and 15-day embryos [1,4,7,9]. However, the effects of preparations from earlier embryos (<13 days gestation) were not reported.

Here we comparison the effects of intrastriatal transplantations of homologous ENT VM preparations from embryos of different gestational age (below and over embryonic day 14) to rats with 6-hydroxydopamine (6-OHDA)-induced hemiparkinsonism. In addition, morphological state and the content of dopaminergic neurons in grafts were determined.

MATERIALS AND METHODS

Male Wistar rats (n=15) weighing 270-340 g were used for unilateral lesion of dopaminergic system, 9

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females weighing 190-220 g were used for obtaining embryos of different gestational age. The animals were kept under the conditions of 12-h light-dark cycle with free access to water and food. Body weight was measured periodically. Stereotactic and surgical procedures, brain perfusion, and sacrifice were carried out under ketamine narcosis (80 mg/kg).

For modeling of hemiparkinsonism 4.5 μg 6-OHDA in 7 μl 0.5% ascorbic acid was stereotactically injected into the right ascending mesostriatal tract in 2 points (3.5 μl in each) with the following coordinates: point 1 — AP 4.4, D 1.5, V 7.5; point 2 — AP 4.3, D 1.2, V 7.6. The drug was injected with a microsyringe (Sage-instruments) at the rate of 1 μl/min with 2-min interval between injections.

The efficiency of hemiparkinsonism modeling and the effect of transplantation were evaluated by apomorphine-induced rotation asymmetry (APO-test) recorded on an automatic rotometer. The rats were examined 1, 2, and 3 weeks after hemiparkinsonism modeling and 1, 2, and 3 months after ENT VM transplantation.

The rats were subcutaneously injected with 0.05 mg/kg apomorphine, placed in the rotometer 5 min after injection, and observed for 45 min. The number of rotations per min to each side was recorded.

ENT VM preparations were obtained from ventral mesencephalon of 14- and 16-17-day-old rat embryos [5] immediately before transplantation. In 8-day-old embryos, tissue fragments were isolated from primordial ventral mesencephalon. The age of embryos was counted starting from the morning after night mating. The optimal moment for successful fertilization (estrus) was determined by vaginal content [2]. A total of 48 embryos were used. Immediately before transplantation the cell suspension was centrifuged at 500*g* for 5 min and adjusted to a concentration of 10^6 cells per 7 μl Hanks solution. Cell viability determined by trypan blue exclusion test was above 80%. The cells were transplanted into rat brain no later than 60 min after isolation of ENT. The initial material and ENT preparations were incubated at 4°C.

Transplantation of ENT VM was performed on day 28 after injection of 6-OHDA. The recipients (3 groups, 5 rats per group) received cell preparations from 8-, 14-, and 16-17-day embryos (groups ENT VM E8, ENT VM E14, and ENT VM E16-17, respectively). The suspension of ENT VM was injected at a rate of 1 μl/min into the dorsolateral striatum (stereotactic coordinates: Ap 1.0, L 3.0, V 4.5-4.1) on the side of damage. The cells were resuspended immediately before injection.

In 14 rats, the grafts were examined histomorphologically and immunocytochemically after the end of the experiment, *i. e.* 3 months after neurotransplantation.

In each experimental group, rat brain was perfused via the ascending aorta with 200 ml phosphate buffered saline (PBS) and 400 ml 4% neutral paraformaldehyde in PBS, isolated, and placed in paraformaldehyde of the same concentration. Brain specimens were incubated in 20% sucrose and serial sections were prepared on a freezing microtome. General histomorphological analysis was performed on 20-μ sections stained by cresyl violet (Merck) according to the Nissl method under a light microscope. A total of 8 coronary sections at the level of the striatum and 2-4 sections at the level of the substantia nigra from each rat were examined.

Brain sections were analyzed under a Leica DML microscope. Computer visualization was performed using Leica DC Viever and Leica IM 50 software. The area of grafts on sections was expressed in relative units, 1 rel. unit corresponded to 1000 pixels (at 150 pixel/inch resolution, Adobe Photoshop 5.5). Correlation analysis and comparison of the sum of graft areas on serial sections from 3 experimental groups was carried out.

Dopaminergic neurons were identified by immunoperoxidase staining for tyrosine hydroxylase (TH) on 40-μ brain sections using anti-TH monoclonal antibodies (Sigma) and Vectastain ABC kit (Vector Laboratories) according to manufacturer's protocol. Primary anti-TH antibodies and secondary biotinylated antibodies were diluted 1:2000 and 1:100, respectively. Immunoperoxidase staining was visualized with H_2O_2 and diaminobenzidine substrate mixture. A total of 7 serial sections of the striatum and 2-4 sections of substantia nigra from each rat were examined. The presence of TH-positive neurons in the grafts was evaluated on Nissl stained sections.

The data were processed using Student's *t* test and Pearson correlation coefficient.

RESULTS

No significant changes in the general state of animals were observed throughout the experiment. Minor changes in body weight can be explained by general reaction to surgery. Body weight decreased 1 week after substantia nigra lesion and/or ENT VM transplantation, while 2-3 weeks after ENT VM transplantation the dynamics of body weight did not differ from that in control animals, which allowed to compare the examined parameters in the experimental groups.

In the APO-test, the maximum decrease in rotation rate was observed in ENT VM E8 group. Three months after transplantation rotation rate in this group

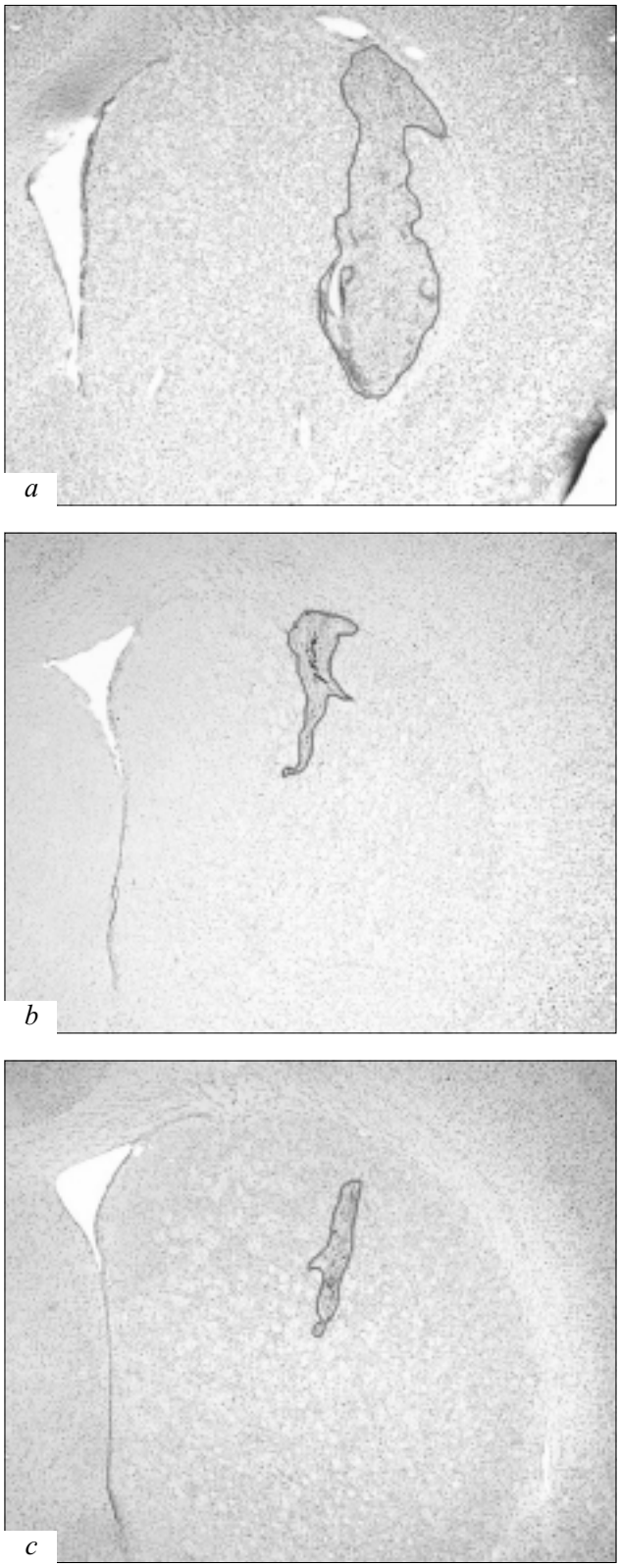

Fig. 1. Morphology of the grafts (encircled areas) of embryonic ventral mesencephalon from rat embryos on gestational days 8 (*a*), 14 (*b*), and 16-17 (*c*) in the striatum of rats with 6-OHDA-induced hemiparkinsonism 3 months after neurotransplantation. Cresyl violet staining, ×25.

decreased to 31%, while in ENT VM E14 and ENT VM E16-17 groups it decreased to 48 and 76% of the initial level, respectively. In ENT VM E16-17 group, parameters of the APO-test surpassed the initial values during the first month after neurotransplantation. At the same time, positive effect of ENT VM E8 preparations on motor asymmetry surpassed that in ENT VM E14 group as soon as 1 month after neurotransplantation.

Histomorphological studies revealed the absence of typical polygonal neurons including TH-positive neurons in substantia nigra pars compacta in 6-OHDA-damaged areas in all rat groups: there were areas occupied by microglia.

Three months after neurotransplantation the maximum size of grafts was found in ENT VM E8 group rats (Fig. 1). The total area of graft in ENT VM E8 group was 452±73 rel. units *vs.* 38±6 ($p<0.001$) and 54±14 rel. units ($p=0.002$) in ENT VM E14 and ENT VM E16-17 groups, respectively. Moreover, histomorphological analysis revealed less pronounced glial reaction in ENT VM E8 group compared to ENT VM E14 rats characterized by the presence of continuous or discontinuous glial scar around the graft and along the needle track and accumulation glial cells in the graft. In ENT VM E16-17 group, practically no neurons were found in the graft. We observed accumulation of glial cell along the needle track most pronounced in the parietal cortex and corpus callosum, where fibrillar formations and deformed structures were seen (gliomesodermal reaction).

The number of TH-positive neurons significantly differed in the studied groups. In ENT VM E8 grafts, TH-positive neurons were revealed in 5 rats (Fig. 2) at least in one section (1-14 neurons per section), in ENT VM E14 group these cells were found in 2 of 5 rats (1-6 TH-positive neurons per section). No TH-positive neurons were found in ENT VM E16-17 grafts.

TABLE 1. Parameters of APO-Test after Transplantation of ENT VM Preparations from Embryos of Different Gestational Age into the Striatum of Rats with 6-OHDA-Induced Hemiparkinsonism (*M*±*m, n*=5)

Time of examination	ENT VM E8	ENT VM E14	ENT VM E16-17
Before transplantation	9.28±1.41	10.18±2.11	10.84±1.22
After transplantation, month			
1	4.62±1.37	7.16±1.04^{+}	12.06±1.63*
2	3.68±1.36	6.76±2.01	10.78±1.81*
3	3.34±1.08	4.88±1.25	7.94±1.48*

Note. $p<0.05$: *compared to ENT VM E8 or $^{+}$ENT VM E16-17 groups.

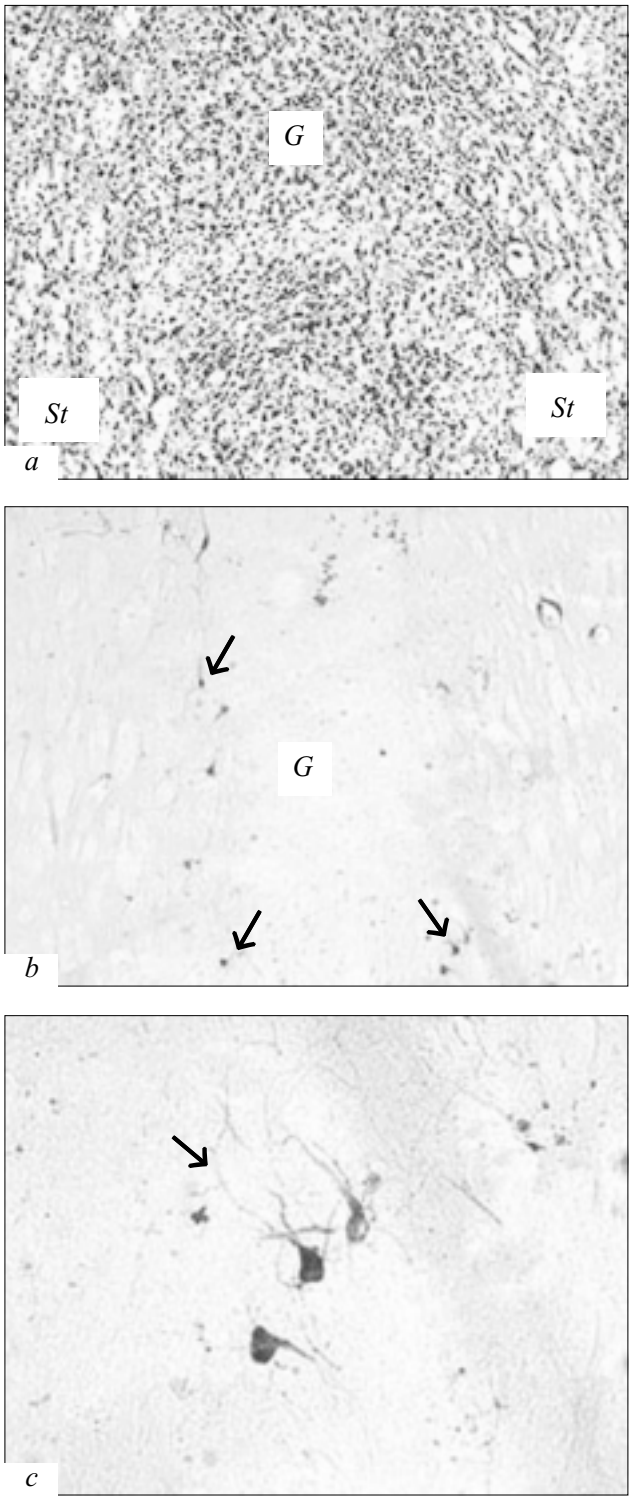

Fig. 2. Morphology and immunomorphology of intrastriatal graft (*G*) 3 months after homolateral transplantation of ventral mesencephalon preparation from 8-day embryos into the striatum (*St*) of rats with 6-OHDA-induced hemiparkinsonism. Nissl staining (*a*) and immunoperoxidase staining for tyrosine hydroxylase (TH), ×100 (*a, b*), ×400 (*c*).

Thus, comparative histomorphological analysis at the end of the experiment (3 months after neurotransplantation) showed the best condition of ENT VM E8 grafts. These grafts contained significant number of embryonic neurons (including dopaminergic neurons visualized by TH immunoperoxidase staining) and were characterized by weak glial reaction in the striatum. The size of ENT VM E8 grafts 10-fold surpassed that of ENT VM E14 and ENT VM E16-17 grafts. ENT VM E16-17 grafts contained mainly microglia. These data agree with the results of APO-test, a specific functional indicator of the state of the dopaminergic system. Comparison of parameters of the APO-test in ENT VM E8 group and combined ENT VM E14+ ENT VM E16-17 group confirmed higher dopaminergic activity of ENT VM E8 preparations (Fig. 3). The analysis revealed a negative correlation between the total area of grafts and parameters of the APO-test in all experimental groups (r=-0.75).

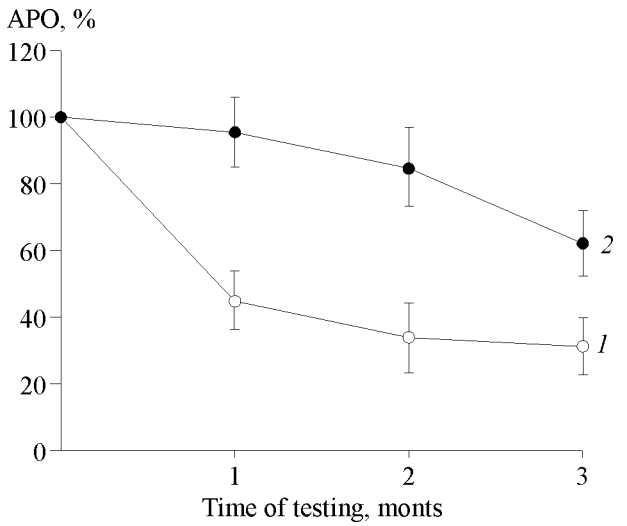

Fig. 3. APO-test. Therapeutic efficacy of intrastriatal transplantation of ventral mesencephalon preparations from embryos on early (8 days, *1*) and late (14 and 16-17 days, *2*) gestational stages to rats with 6-OHDA-induced hemiparkinsonism. Vertical lines: confidence intervals.

It is believed that the therapeutic effect of neurotransplantation in experimental parkinsonism induced by injection of 6-OHDA depends on both dopaminergic activity of the graft and nonspecific stimulation of reparative processes in the damaged striatum [6,14]. This can explain the effect of preparation obtained from late embryos (ENT VM E16-17). In rats receiving these preparations correction of apomorphine-induced asymmetry at the end of the experiment was only 24% of initial motor impairment. It should be noted that, apomorphine-induced motor asymmetry without neurotransplantation persisted at the initial level within 3 months of observation.

Thus, ENT VM preparation from 8-day rat embryos showed higher therapeutic activity after intrastriatal transplantation in rats with 6-OHDA-induced

hemiparkinsonism compared to preparations from 14- and 16-17-day embryos. This contradicts published data indicating that 13-15-day embryos are the optimal source of ventral mesencephalon for neurotransplantation. However, our conclusion appears to be correct because hemiparkinsonism was confirmed by morphological examination of the substantia nigra revealing unilateral lesion in all experimental animals, while the efficacy of neurotransplantation was verified by adequate physiological and immunohistochemical tests.

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