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Homogenous Fetal Dopaminergic Cell Transplantation in Rat Striatum by Cell Suspension Methods

**Sang Sup Chung[1], Sun Ho Kim[1], Woo Ick Yang[2], In Joon Choi[2]
Won Young Lee[1], Jae Gon Moon[1], Hyun Sun Park[1], Hyung Sik Shin[1]
Dong Suk Kim[1] and Young Min Ahn[1]**

The transplantation of dopaminegic neurons in the brain has been attempted in experimental animals and humans as the new treatment modality of Parkinson's disease. Before the trial of dopaminergic neuronal transplantation in human, the authors proceeded with the animal experiment of fetal dopaminergic cell transplantation in a rat Parkinson's disease model. The aims of this experiment were to confirm the availability of fetal mesencephalic cells as the donor, to compare the viability of cells according to different cell manipulation methods, and to follow up the functional recovery in the transplanted Parkinson's disease model. As a result, the authors concluded that the simple enzyme digestion method had a better cell survival rate than the multiple enzyme digestion method. Also, the transplanted mesencephalic cells could not only survive in the host animal but also promote functional recovery.

Key Words: Parkinson's disease, fetal mesencephalic dopaminergic neuron, transplantation, cell supension

Parkinson's disease is a well-known degenerative disease which results in the depletion of dopamine-producing neurons in the substantia nigra. Medical treatment with L-dopa has contributed enormously to the treatment of Parkinson's disease in the last several decades. But this drug cannot prevent the further progression of the disease, and the dosage requirement of the drug gradually increases. Also, its side effects may pose significant problems, especially in long-term therapy. The traditional surgical treatment, stereotactic thalamotomy, is quite helpful for the relief of tremor and rigidity and could diminish the drug requirement and the side effects of L-dopa. But, as with the medical treatment, it cannot eliminated the progression of the disease and is not so effective in bilateral symptoms (Matsumoto *et al.* 1984).

With the development of organ transplantation techniques, the possibility of neuronal tissue transplantation has been spotlighted in human degenerative diseases, such as Parkinson's disease and Alzheimer's disease.

The milestone research of neuronal transplantation was performed by Olson *et al.* (1970) who grafted neural tissue to the anterior eye chamber in rats. After that, Backlund *et al.* (1985) performed the first adrenal medullary transplantation in humans, but their results were discouraging. With such a clinical trial, many researchers (Olson and Seiger 1972; Dunnett *et al.* 1984; Nadaud *et al.* 1984; Olson *et al.* 1985; Rose *et al.* 1985) reported fascinating results of fetal mesencephalic cell transplantation in experiment animals to promote the

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Department of Neurosurgery[1], Department of Pathology[2], Department of Microbiology[3], Yonsei University College of Medicine, Seoul, Korea
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Address reprint reguests to Dr. S S Chung, Department of Neurosurgery, Yonsei University College of Medicine, C.P.O Box 8044, Seoul, Korea, 120-752

functional recovery of degeneration of neural tissues. Also, Hitchcock (1989) presented the impressive surgical result of mesencephalic cell transplantation in humans.

But, to this day, there have been no guidelines for fetal mesencephalic cell transplantation in humans, (such as optimal fetal gestational age, cell manipulation method and maximum cell preservation time). So, the authors attempted this experiment to find out the optimal method of neuronal transplantation.

MATERIALS AND METHODS

Animals

Forty young male rats of the Sprague-Dawley strain were used in this experiment. They were 200~250 g at the time of 6-OHDA (6-Hydroxydopamine, Sigma Co.) lesion making. From two weeks before the experiment, they were housed in cages of five rats with food and water available in a 12-hour day and night cycle. Donor tissue was obtained from 13~15 day-old fetuses of the same inbred strain.

6-OHDA lesion formation

All animals were given an unilateral right 6-OHDA injection into the substantia nigra at the beginning of the experiment. The rats were anesthetized by an intraperitoneal (IP) injection of thiopental (30 mg/kg) and the head was fixed on an animal stereotactic frame (Narishige Co.). Eight μg of 6-OHDA was dissolved in 4 μl ascorbic acid solution (0.2 mg ascorbic acid per ml of normal saline) and injected stereotactically over 10 min with a 10 μl Hamilton syringe. Target coordinates were 5.3 mm behind the bregma, 2.5 mm right lateral from the midline, and 7.5 mm inferior from the dura with the incisor bar set 2 mm below the intra-auraline, according to the rat brain atlas (Waston and Paxinos 1984). After the 6-OHDA injection, the needle was kept in place for 10 more min. to prevent regurgiation of the durg.

Rotation behavior test

To determine which animals had been successfully denervated, rotation behavior test were conducted with an injection of a low dosage of apomorphine (Sigma Co., 0.25 mg/kg S. C.). The initial rotation behavior test was performed at four weeks after the 6-OHDA lesion formation, and it was repeated again nine weeks after. the rotation behavior tests were conducted by the method of Ungerstedt and Arbuthrott (1970) in hemispheric bowls.

Rotation was counted every 10 minutes during a 40 minute period. Full turns in each direction were counted separately. Only animlas showing over 80 contralateral turns to the lesion side/40 minutes were used for further transplantation procedures.

Preparation of donor

Mesencephalic donor tissue was obtained from 8 pregnant Sprague-Dawley rats of 13~16 days gestation (crown rump length 10~13 mm). The pregnant rats were anesthetized with thiopental (30 mg/1 kg IP) and a cesarean section was performed. Under the surgical microscope, the meninges and cartilage of each fetus were completely peeled off and the ventral mesencephalic tissue was obtained from the mesencephalic flexure in a petri dish containing amniotic fluid.

The resected mesencephalic tissue, approximately 1 mm^3 in size, was collected in a small petri dish containing EBSS (Earle's Balanced Salt Solution, Sigma Co.) at room temperature. About 10~13 pieces of mesencephalic tissue could be obtained from one pregnant rat. Until all fetuses had been removed, the mother was kept anesthetized.

Preparation of cell suspensions

In the preparation of cell suspensions, the authors proceeded with two different methods in order to compare the difference of cell viability between the multiple enzyme digestion method described by Bjorklund *et al.* (1983) and the simple trypsin digestion method popularly used in cell culture.

Multiple enzyme digestion method

The dissected mesencephalic tissue from one pregnant rat was transferred to a microtest tube (350 μl capacity) contained 200~250 μl of trypsin (Sigma type III, 0.025%)-EBSS. The tissue was incubated for 40 min at 37℃. After the incubation, the trypsin was washed off by replacing the fluid with the same amount of EBSS 2 times and EBSS containing 0.004%

DNase (Sigm type I) and 0.0125% trypsin inhibitor (Sigma Co.) 2 times. Finally, the fluid was replaced with EBSS so that the tissue was dissociated into a cell suspension in a total volume of 100㎕.

Simple enzyme digestion method

All steps were identical with the multiple enzyme digestion method but the procedure of washing was done with only EBSS solution. DNase and trypsin inhibitor were not used.

Comparison of cell viability

Immediately after finishing the cell suspension preparation, small amounts (10㎕) of cell suspension were obtained with a micropipette and dropped on the hemocytometer. A droplet of 4% trypan blue was added on the cell suspension. Under high magnification (260x), cell viability was ovserved and the total and viable cell number were separately counted.

Transplantation of fetal mesencephalic cells

Twelve weeks after the lesion formation, fetal mesencephalic cell suspension was stereotactically transplanted into the right caudate nucleus of selected recipient rats which had had a positive response in the rotation behavior test. The head of the rat was fixed on the stereotactic frame and its target coordinates were selected at 1 mm anterior from the the bregma, 2.5 mm right lateral from the midline, and 6 mm inferior from the dura. After making a small burr hole with an electric drill, 10㎕ of cell suspension were slowly injected with a Hamilton syringe into the target. After a 10 min delay, the syringe was removed.

Post-transplantation rotation behavior test

Two months after transplantation, a rotation behavior test was conducted by the same method which was described earlier.

Immunohistochemical analysis

Three months after the transplantation, all rats were sacrificed and their brains were fixed in formalin and embedded in paraffin. Section (4㎛ thick) were deparaffinized with xylene (20 minutes), hydrated with graded ethanol (5 minutes) and phosphate-buffered saline (PBS, pH 7.5) and then treated with 0.3% hydrogen peroxidase for 60 minutes to block the nonspecific endogenous peroxidase activity. The sections were incubated sequentially in a moist chamber and rinsed with PBS between each incubation. Incubation was performed with normal goat serum at room temperature for 30 minutes, with rabbit anti-bovine tyrosine hydroxylase (East-Acres Co.) diluted 1:400 in PBS overnight at 4℃, with goat anti-rabbit immunoglobulins at room temperature for 30 minutes and with peroxidase rabbit antiperoxidase at room temperature for 30 minutes.

The incubated sections were stained with a freshly prepared soluation of 2.2% amino-ethyl carbazole (AEC) and 0.5% hydrogen peroxidase in acetate buffer for five minutes. After being rinsed in distilled water, the sections were counterstained with Mayer's hematoxylin, then dehydrated, mounted and examined with a light microscope. As a negative control, the primary antisera were replaced by the preimmune rabbit sera (Domesick *et al.* 1983; Pearson *et al.* 1983).

Statistical analysis

Data are expressed as mean±S.D. The data are analysed with the paired t-test and p-value less than 0.05 were considered to be significant.

RESULTS

Rotation Behavior test

Four weeks after lesion formation, twenty-one rats met the selection criteria (80 contralateral turns/40 min) in the roatation behavior test with apomorphine. The rats showed a maximum contralateral rotation (28.6±3.28 turns, Mean±S.D.) at 20 minutes after apomorphine injection. The same rats received the rotation behavior test again at nine weeks after lesion formation. At this time, the maximum contralateral rotation could be observed in the same time range (45.24±3.39 turns), and the total number of contralateral turns were markedly increased in comparison with the rotation behavior test of four weeks ($p<0.03$) (Fig. 1).

Cell viability

Fetuses obtained from eight pregnant rats were studied for the cell viability test (multiple

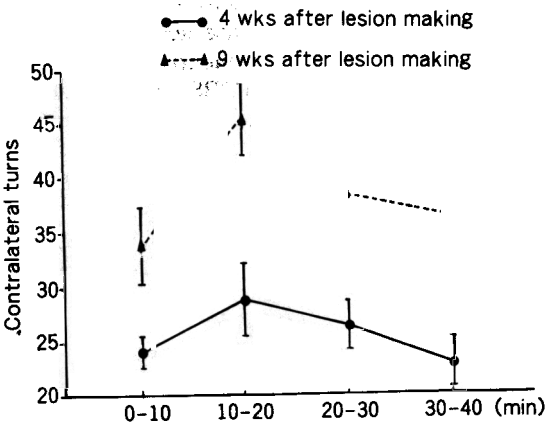

Fig. 1. *Rotation Behavior test with apomorphine (n= 21). 4 weeks, 9 weeks after 6-OHDA lesion making (p<0.03).*

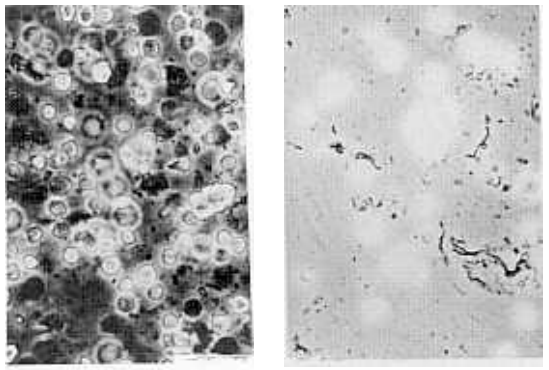

Fig. 2. *Result of cell viability test (4% trypan blue stain, 260×). Left: with the simple enzyme digestion method, more than 85% of cells had round halos in maximum cell population field. Right: with the multiple enzyme digestion method, a few cells had round halos and scattered celldebris were found.*

enzyme digestion method: four rats, simple enzyme digestion method: four rats). Viable cells were counted at the five maximum cell population fields under high magnification (260x) from the cell suspension of each pregnant rat separately. As a result, cells treated by the simple enzyme digestion method had a better cell survival rate (85.0±5.3%) than those of the multiple enzyme digestion method (40.0±2.3%) ($p<0.03$) (Fig. 2).

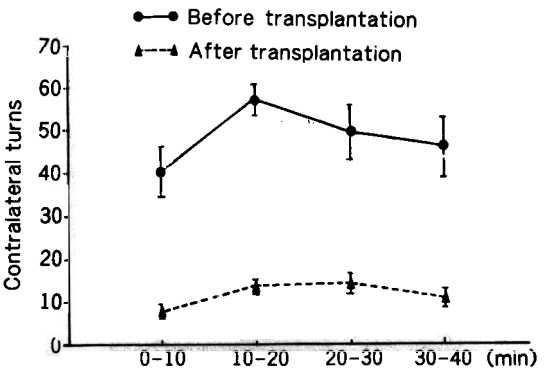

Fig. 3. *Rotation behavior test with apomorphine (n= 11). Successful transplantation group (p< 0.03).*

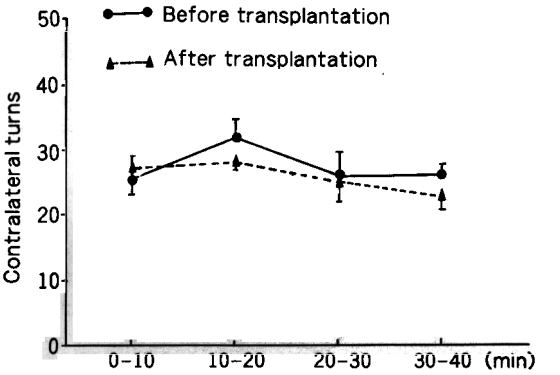

Fig. 4. *Rotation behavior test with apomorphine (n=4). Failed transplantation group.*

Thus, in the actual trasnplanation procedure, the simple enzyme digestion method was used in 15 successfully denervated rats and the multiple enzyme digestion method was used in two rats. Four rats died during the experiments.

Rotation behavior test after transplantation

Eleven rats which showed a marked reduction (over 70%) of contralateral rotation were grouped as the successful transplantation group (Fig. 3) and this had a statistical significance according to the paired T test ($p<0.03$). Four rats (two rats transplanted by the multiple enzyme digestion method) which showed a

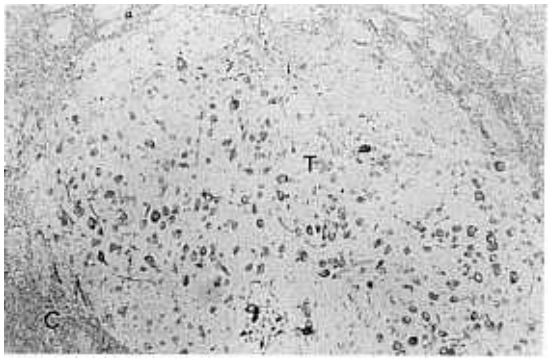

Fig. 5. Transplanted cell group (T) were easily identified in the host caudate nucleus (C). TH immunoreactivity could be observed in the transplants (100×).

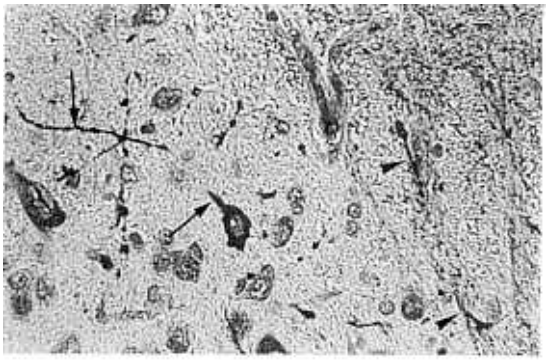

Fig. 6. TH immunoreactive axons and dendrites were found (arrow) inside of the graft. Extended outgrowth of TH immunoreactive fibers were found at the margin between the transplants and host caudate (arrowhead) (400×).

minimal reduction or increment (83~104%) of contralateral rotation were grouped as failed (Fig. 4). Two rats which were transplanted by the simple enzyme digestion method died during the experiment.

Immunohistochemical analysis

In all rats (n=21) which showed marked contralateral rotation to 6-OHDA injection, tyrosine hydroxylase (TH) immunoreactive neurons could not be found in the substantia nigra ipsilateral to the 6-OHDA injection. In the sections with transplanted fetal mesencephalic cells, the transplanted cell group were easily identified in the host caudate nucleus, and TH immunoreactivity could be observed (Fig. 5) in 11 rats which showed marked reduction of contralateral rotation after transplantation. Almost all of the TH immunoreactive axons and dendrites were found inside the graft, and an extended outgrowth of TH immunoreactive fibers which crossed over the margin between the transplanted cells and host caudate was identified (Fig. 6). But, these findings were not observed in the rats which were classified in the failed group.

DISCUSSION

Burns *et al.* (1983) reported that intravenous administration of N-methyl-4 phenyl-1, 2, 3, 6-tetra-hydropyridine (NMPTP) to rhesus monkeys produced a disorder like Parkinson's disease (akinesia, rigidity, postural tremor, flexed posture), and this method was used for the research of the action mechanism of drugs in the treatment of Parkinson's disease. In 1970 Ungerstedt and Arbuthnott reported the quantitative recording method of rotational behavior in rats which had a 6-OHDA injection in the nigrostriatal dopaminergic system. This model could not mimic the actual symptoms of human Parkinson's disease like the NMPTP lesion of Burns *et al.* but neurochemical changes of the nigrostriatal system similar to those of Parkinson's disease could be reproduced. Therefore, if the motor abnormalities, such as rotation behavior could be signficantly reduced in the dopaminergic cell transplanted Parkinson's disease model, this suggests that functional inputs were provided from grafted tissue.

The catecholamine-containing neuronal tissues from the carotid body, adrenaly medulla and sympathetic ganglion could be used as donor tissue (Marschitz *et al.* 1984; Itakura *et al.* 1988). But the fetal mesencephalic cell has several advantages compared with other dopamine-containing tissues. It may be able to survive better in the anoxic condition than the mature cell and has less surgical trauma during dissection (Bjorklund *et al.* 1983). Also, this immature cell has the capacity for continued neurogenesis after transplantation.

For survival of the grafted neuron in the host brain, vascularization of the grafted cells is the most important factor. To promote cell survival, many authors (Rosenstein and Brightman 1978; Wuerthele *et al.* 1981; Freed 1983) grafted the neuronal cells to a site adjacent to CSF circulation or used the two staged grafting procedure (Dunnet *et al.* 1981; Stromberg *et al.* 1985). In this method, a small suction cavity made at the target region would have a newly developed vascularized pial bed for grafting. But this method is not easily applicable for humans. To overcome these limitations, Bjorklund *et al.* (1983 a, b) developed the cell suspension method which involved the intracerebral injection of a dissociated suspension of mesencephalic cells.

Bjorklund *et al.* (1983a) dissociated the donor tissue to cell suspension by the action of several enzymes. Theoretically, this method has the advantages of achieving a greater specificity of cells for transplantation and not requiring special vascular support or access to CSF space for cell survival because the implanted cell could be in direct contact with the host neutrophil. But it requires at least four steps of cell washing and several enzymes (trypsin, trypsin inhibitor, DNase). Therefore, there was some possibility of cellular damage due to the multiple steps of cell manipulation and the unpredictable action of enzymes. So, the authors used the method modified from the standard cell culture technique and compared this with the Bjoklund method under the idea that minimal manipulation could prevent cell damage.

As a result, the simple enzyme digestion method had a superior cell survival rate than the multiple enzyme digestion method. But this result still requires further experiments for a complete conclusion. As other factors, such as the time interval between dissection and transplantation, temperature of cell storage, etc., were not observed in this study. Also, our cell viability test did not express the actual functional activity of the cell itself.

Recent studies (Bjorklund *et al.* 1983b; Jaeger 1985; Mahalik *et al.* 1985) have provided strong evidence that transplanted mesencephalic tissue could restore the functional input to the denervated nigrostriatal system, and it has been suggested that transplanted neurons could make functional contact with host caudate neurons indirectly. In our experiment, the same result could be observed in the rotational behavior test. In the immunohistochemical analysis, the authors tried to find direct evidence that TH immunoreactive neurons from transplanted mesencephalic cells provide the actual synapses in the host caudate. The 6-OHDA lesion could cause an almost complete destruction of TH immunoreactive cells and fibers in the ipsilateral nigrostriatal system. Therefore, any TH immunoreactive neuron which was found in the ipsilateral caudate should be derived from the grafted neurons.

In our experiment, we were able to determine the outgrowth of TH immunoreactive fibers which penetrated the host caudate. These findings suggested that transplanted mesencephalic cells provide the functional synapses to the 6-OHDA denervated nigrostriatal system by means of an outgrowth of transplatned neuronal fibers and formation of a connection between host and transplanted cells. But we could not find evidence of migration or proliferation of transplanted mesencephalic cells into the host caudate nucleus.

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