Effect of iloprost on adrenal medullary grafts in central nervous system transplantation and apoptosis

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ABSTRACT

Objective: To investigate whether iloprost, a stable analog of prostacyclin, is useful for the preservation of neural grafts in transplantation surgery.

Methods: This study was conducted at the Microsurgery Laboratory of the Department of Neurosurgery, Faculty of Medicine, Ankara University, Ankara, Turkey in 2003. The animals (rabbits) were divided into 3 groups. In group I, autografts taken from the adrenal medulla were stored in 0.9% sodium chloride (NaCl) solution for 45 minutes before transplantation. In group II, autografts taken from the adrenal medulla were stored in iloprost solution (50 ng/ml) for 45 minutes before transplantation. Graft transplantation was not performed in the third group.

Results: In group I, the grafts partially preserved their viability. In group II, the large adrenal medullary cells had evident euchromatin nuclei fused with neurons, and there was an increase in vascularization.

Conclusions: Three weeks after transplantation surgery, it was determined that iloprost maintained the viability of the graft tissue and probably prevented apoptosis, and facilitated the integration of the graft tissue into the host brain.

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Adrenal medullary grafts have been used in various animal models of Parkinson's disease, in an effort to improve the motor symptoms associated with damage to the nigrostriatal pathway.[1,2] However, autografts of adrenal medullary tissue in the caudate nucleus of patients with advanced Parkinson's disease have been performed on a limited basis without permanent reversal of the parkinsonian symptoms.[3,4] This raises important questions about survival of neural grafts in experiments. Prostacyclin is a powerful vasodilator, and a potent inhibitor of platelet aggregation.[5] Recently, prostacyclin and its analogs were shown to have cytoprotective activity.[6-8] In this study, we investigated the effects of iloprost, a stable analog of prostacyclin,[9] on transplanted adrenal medullary grafts using an experimental model.

Methods. This study was conducted at the Microsurgery Laboratory of the Department of Neurosurgery, Faculty of Medicine, Ankara University, Ankara, Turkey in 2003. Light and electron microscopy examinations were performed at the Department of Histology, Faculty of Medicine, Ankara University. All experimental protocols were approved by the Ankara University Animals Ethics Committee. Fifty male New Zealand white rabbits, each weighing between 2.5-3.0 kg, were used in this study. The rabbits were divided into 3 experimental groups. First group: This group consisted of 15 rabbits, to which the adrenal medullary grafts incubated in 0.9% sodium chloride (NaCl) solution at +4°C for 45 minutes were transplanted. For the 15 rabbits in this group, autologous adrenal medullary grafts taken from the left suprarenal gland were placed at the right caudate nucleus in the right hemisphere. Second group: In this group, a total of 15 rabbits were used, to which the adrenal medullary grafts incubated in 50 ng/ml iloprost (Schering Aktiengesellschaff, Berlin, Germany) solution at +4°C for 45 minutes were transplanted. For the 15 rabbits in this group, autologous adrenal medullary grafts taken from the left suprarenal gland were placed at the right nucleus caudate in the right hemisphere. Third group: This group consisted of 20 rabbits, and was divided into 2 subgroups. Each subgroup consisted of 10 rabbits. The medullary grafts taken from the left suprarenal gland of the first 10 rabbits were incubated in 0.9% NaCl solution at +4°C for 45 minutes, while the adrenal

medullary grafts taken from the left suprarenal gland of the remaining 10 rabbits were incubated in 50 ng/ml iloprost solution at +4°C for 45 minutes. At the end of this period, graft transplantation was not performed, and the viability measurements of both subgroups were determined, and light and electron microscopic evaluations were performed. The animals tolerated all procedures, and standard diet and water was given to all rabbits. Because the cells started to show degeneration after 45 minutes in our laboratory conditions, our Pharmacology and Histology units recommended 45 minutes for the optimal incubation time for the adrenal medullary graft in iloprost solution. The rabbits in the first and second groups were allowed to live in the Animal Experiments Research Laboratory of the Faculty of Medicine, Ankara University for a period of 3 weeks. At the end of this period, they were sacrificed under deep anesthesia (30 mg/kg) of thiopental sodium (Pental Sodyum Enj®, I. E. Ulagay, Istanbul, Turkey), and their whole brains were extracted at the level of the pons. The region of transplantation was divided into 3, and viability measurements, and light and electron microscopic examinations were performed.

Anesthesia. Anesthesia was induced with an intramuscular injection of ketamine hydrochloride (Ketolar®, Pfizer, Luleburgaz, Turkey) in a dose of 10mg/kg of body weight and an intramuscular injection of xylazine hydrochloride (Rompun®, Bayer, Istanbul, Turkey) in a dose of 0.5 mg/kg of body weight. Ketamine hydrochloride (0.5 mg/kg) was administered intramuscularly when necessary to maintain anesthesia.

Surgical technique. The same surgical procedure was carried out carefully in all steps of the experiment. Stage 1: In the first stage of the operation, the rabbits were positioned in the lateral decubitus position on their right sides. The area under the last rib line was shaved. Skin and subcutaneous layers were incised by a mildly curvilinear incision. Abdominal musculature was dissected and the peritoneum was opened following the incision. We viewed the kidney on the lateral side, intestines on the front side, the vertebral column on the deep medial side, and the aorta and vena cava inferior on the front side of the vertebral column, in the abdominal cavity. The suprarenal gland, with a pale yellow color and a diameter of 0.7 x 0.8 cm, was located immediately inferolaterally to the origin of the renal artery from the aorta. All of the applications were performed according to sterile surgical techniques. A piece of gland with an average volume of 8 mm^3 was taken. After ensuring hemostasis, the wound was closed in anatomical layers. The adrenal medullary grafts were kept in the 0.9% NaCl solution at +4°C or in the iloprost solution at +4°C for 45 minutes. Stage 2: In the second stage, the rabbits were secured in the prone position, keeping the head neutral and between the 2 front extremities, and the vertex at the top. The scalp incision was performed by a right vertical fronto-parietal incision. Peeling with a periosteal dissector, the right coronal suture and its surroundings were exposed. At this point, a surgical microscope (Carl Zeiss, OPMI-PRIMO 307953) was placed in the operation field. Using a high-speed surgical drill, craniectomy was performed extending from the immediate front of the coronal suture on the right, and backwards for a distance of 0.5 x 0.8 mm, exposing the dura. With a bipolar and micro aspirator, a cortex incision of 0.4 mm was made. Preserving an approximate angle of 30 degrees with front-back vertical plane, the gap was deepened medially. Protecting the vascular structures, the caudate nucleus was accessed. After opening a cavity with an aspirator, the adrenal medullary grafts that had been kept in either 0.9% NaCl solution or 50 ng/ml iloprost solution at +4°C for 45 minutes were placed in the cavity using a microscope. Hemostasis was ensured, and the wound was closed in anatomic layers.

Light microscopy examination methods. The pieces taken for light microscopy were fixated with Bouin and 20% formaldehyde solutions. The cross-sections of 5 microns prepared after the paraffin blocking were subjected to histological staining. The stains applied were as follows: hematoxylin-eosin, toluidine blue-azure II. All preparations were examined using a Nikon 234027 (Labophot, Japan) microscope.

Electron microscopy examination method. Grafts and surrounding brain tissues were divided into 1 mm^3 pieces and were kept in 1% isotonic solution + osmic acid in the refrigerator for 1 hour. They were then kept in 1% uranyl acetate prepared with isotonic. After 15 minutes in the isotonic, they were then subjected to acetone series. They were kept in vestopal w 1, 2 and 3 for 1 hour each, and were placed in capsules with vestopal w 4. To ensure their hardening, they were kept in a 60º stove for polymerization for 24 hours. Thin cross-sections were performed with C. Reichter, Austria OmU3 and examined with JEOL (100 B) electron microscope using a "transmission electron microscopy" method.

Viability measurements. The pieces taken for viability measurements were stained with hematoxylin-eosin, and the viability rates in cell populations were determined by calculating the number of living cells per hundred cells in the cross-sections.

Statistical analysis. Statistical analysis was performed using the t-test variance analysis and significance was assumed when *p*-value was less than 0.05. The statistical analysis was carried out by means of statistical package SPSS 10.0 for windows.

Results. ***Histological examinations of adrenal medulla grafts not transplanted (third group).*** a) Light microscopy findings. In the light microscope

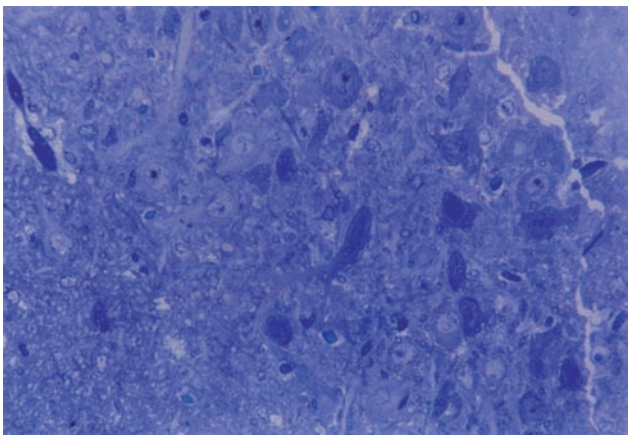

Figure 1 - The cross-sections taken 3 weeks after transplantation of the grafts incubated in 0.9% NaCl. The cells partially preserved their viability, edema is present, their nuclei are eccentrically positioned, chromatolysis has started, and Nissl bodies have disappeared (toluidine blue, azure II X 250).

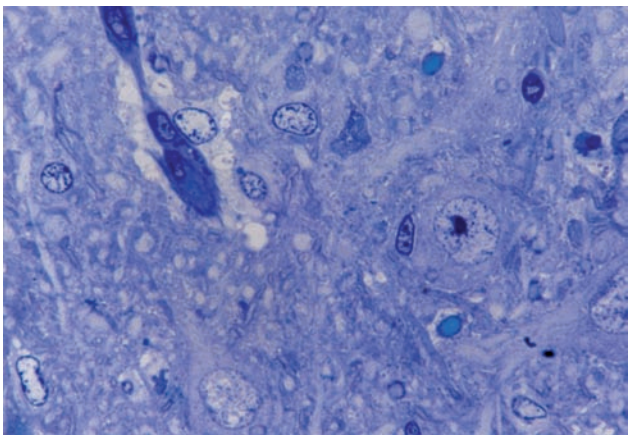

Figure 2 - The cross-sections taken 3 weeks after transplantation of the grafts incubated in iloprost solution. There is an increase in vascularization. Moreover, the satellite cells accompanying the adrenal medulla originated chromaffin cells can be seen (toluidine blue, azure II X 250).

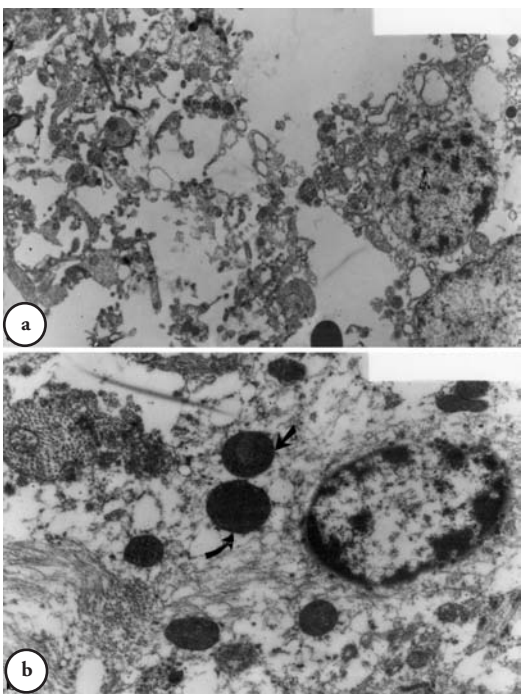

Figure 3 - **a**) Evident extracellular edema is seen in the sample taken from the area, in which the grafts kept in 0.9% NaCl solution were transplanted (X 4800). **b**) The nucleus membrane of the chromaffin-cell-like cell cannot be observed and the cells display degeneration findings. Black arrow: Large degenerated granules (X 10,000).

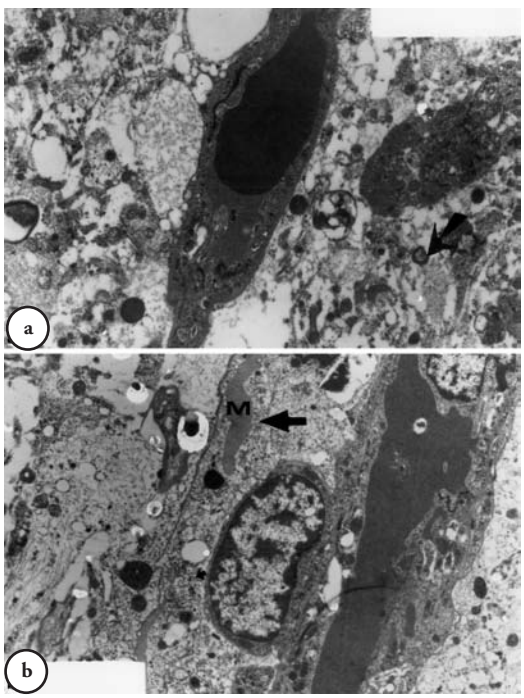

Figure 4 - **a**) In the sample taken from the area in which the grafts kept in the iloprost solution were transplanted, there is a little apparent extracellular edema and the myelin is preserved (black arrow) (X 4800). **b**) Well preserved chromaffin cells around the capillary, long mitochondrion (M) and small granules (black arrow) are seen (X 4800).

examination of the grafts taken from the surrenal gland and kept in 0.9% NaCl solution before transplantation, it was observed that the intracellular components had decreased, the graft cells were partly viable and there was edema around the capillaries. In the light microscope examination of the grafts kept in 50 ng/ml iloprost solution, it was observed that both the extensions and the components of the cells were better preserved and the cells better integrated to the tissue around the capillaries. b) Electron microscopy findings. In the examinations performed using an electron microscope, it was found that there was a decrease in the organelles of the cells of the grafts kept in 0.9% NaCl solution, and the cells displayed degeneration findings. Conversely, for the grafts cells incubated in the iloprost solution, the cells and nuclei were better preserved except for minimal edema. c) Viability measurements. The viability rates in cell populations were determined by ascertaining the number of living cells per hundred cells in the cross-sections. No significant difference was found between the 2 groups concerning the viability rates, $p>0.05$.

Histological examination of the grafts 3 weeks after transplantation. The common feature was that the grafts in each group (first and second groups) preserved their viabilities at various degrees, and were integrated partially with the surrounding tissues and displayed neovascularization. a) Light microscopy findings. In the histopathological examination performed 3 weeks after the transplantation of the adrenal medullary grafts kept in 0.9% NaCl solution before transplantation, it was observed that there was a decrease in the living cell population, the cells had partially lost their viability, and there was significant cellular edema (**Figure 1**). In the histopathological examination of the grafts kept in 50 ng/ml iloprost solution, it was observed that the ratio of living cell population was higher, and there was an increase in vascularization (**Figure 2**). b) Electron microscopy findings. In the electron microscopy examination performed 3 weeks after the transplantation of the adrenal medullary grafts kept in 0.9% NaCl solution before transplantation, it was observed that an evident extracellular edema had developed in the area of transplantation, and astrocytic hypertrophy and mononuclear cell infiltration were present around the graft tissue (**Figure 3**). In the electron microscopy of the grafts kept in 50 ng/ml iloprost solution at +4°C for 45 minutes, vascularization was evident, there was less extracellular edema and the myelin was preserved (**Figure 4**). c) Viability measurements. When the living cell ratios of the 2 groups were compared, it was found that the living cell population in the group incubated with iloprost was significantly higher in statistical terms than in the group incubated with the isotonic ($p<0.001$).

Adrenal medullary originated neural (chromaffin) cells preserved their viability at certain rates whether they were kept in 0.9% NaCl or 50 ng/ml iloprost solution at +4°C for 45 minutes, after they were taken from the suprarenal gland. In addition to its anti-aggregating and vasodilator effects, the stable prostacyclin analog iloprost has cytoprotective effects. In particular, it increases the resistance of the cells, particularly against hypoxia and external physical factors. In group I, the grafts incubated in 0.9% NaCl before transplantation partially preserved their viability and it was observed that there was edema, their nuclei positioned eccentrically, chromatolysis had started and Nissl bodies disappeared. In group II, the grafts incubated in iloprost solution before transplantation, the large adrenal medullary cells had evident euchromatin nuclei fused with neurons and there was an increase in vascularization. Also, satellite cells accompanying the adrenal medullary originated chromaffin cells was observed. Finally, when chromaffin cells originating from the adrenal medulla, which were to be transplanted to the central nervous system, were incubated with iloprost, iloprost preserved their viability at higher rates.

Discussion.

The concept of neural transplantation of adrenal medullary grafts has become a popular scientific issue, because of the public interest in the amelioration of degenerative disease of the central nervous system.[2,10] Adrenal medullary autografting to hundreds of patients with Parkinson's disease has been performed worldwide.[1,11] However, grafted chromaffin cells show poor survival in autopsy cases, which may limit the clinical efficacy of this procedure.

Iloprost, a new stable analog of prostacyclin, has highly potent vasodilatory, anti-aggregatory, and cytoprotective effects.[8,12] Topically applied iloprost decreases the vasospasm created by the electrical stimulation or intracisternal autolog blood injection in the dog or rabbit basilar artery.[13,14] Another important pharmacodynamic characteristic of iloprost, though less analyzed, is prevention of the changes attributable to ischemia in mitochondrial functions, observed in rabbit heart preparations.[15] It was shown that in pigs, an iloprost infusion of 0.5 microgram/kg/min performed in situ for 15 minutes and initiated 45 minutes before the renal ischemia, kept the renal vascular resistance at normal limits for 24 hours after the kidney was taken out and ensured normal blood flow and urine discharge immediately after the transplantation.[6] It was reported that the cortical neural grafts taken from the fetus were better preserved with iloprost incubation, and that they preserved their viability at higher rates after they were implanted.[16,17] In our study, it was also determined that adrenal medullary grafts preserved their viability at higher rates with iloprost.

Application of adrenal medullary grafts to human CNS was first performed by Backlund on a Parkinson patient.[10] Adrenal medullary grafts were also used in the cases reported by Madrazo[18] in his first exciting results. In addition to these successful developments, recent laboratory studies have shown that grafts taken from the fetus are superior to adrenal medullary grafts.[16,17,19] The main reason for this is that fetal grafts can live for extended periods, making suitable synapses with surrounding neurons and, most importantly, ensuring more successful clinical results.[19] Bjorklund et al,[20] showed that successful transplantation was possible, if human fetal substantia nigra grafts were taken in humans were taken in the 9th-11th gestational weeks, and they introduced the concept of "transplantation window," in reference to the optimum fetal age for taking grafts.[20] Nevertheless, in clinical applications, the practice of taking grafts from the fetus has a number of unresolved technical and ethical problems due to the reasons and method of abortion.[21] In our study, we determined that rabbit adrenal medullary grafts preserved their viability to a great extent in the iloprost group (94.5%) (standard deviation ±1.779) 3 weeks after transplantation, and they became functional.

Other important factors that affect the results in the central nervous system transplantation are the method chosen for graft collection, preparation, and implantation. It was reported that when the collected graft tissue was stored in a solid fragment without being torn into pieces, and when it was implanted into a cavity created in the brain, re-vascularization was ensured in a short time, and 95% of the cells remained alive.[1,3] It is obvious that while this technique can easily be employed for transplantation to the areas closer to the brain surface, it will be very difficult to employ it in cases of transplantation to deep structures such as the putamen or caudate nucleus in Parkinson cases. Therefore, the tissue was separated mechanically or by using enzymes such as trypsin to prepare cell suspensions. However, it has been stated that neither this method nor the solid fragment-cavitation method was superior to the other.[22] In a study by Hitchcock et al,[23] it was shown that the cell suspensions were less resistant to external factors than the non-fragmented cell groups, and if the cells were injected as in the stereotactic method, the number of living cells decreased as the injector lumen narrowed. Madrazo et al,[18] reported more successful results with their methods in which they implanted the graft without tearing it into pieces and preserved its integrity to a cavity via craniotomy as compared to the stereotactic methods. In our study, we preferred the open method because of the difficulties of application of the stereotactic method in rabbits, and since non-fragmented grafts generally produce more successful results. In our study, using the microsurgical technique, the structures surrounding the nucleus caudate were less traumatized, and furthermore, the frontal horn of the lateral ventricle was accessed to the greatest extent possibly without distorting the ependyma and better nutrition of the grafts was ensured. It was shown that graft cells developed finger-like extensions toward the ventricle in histological examinations.

The effect of iloprost on apoptosis is a recent and controversial issue. Li et al[24] hypothesized that iloprost induces the apoptosis of vascular smooth muscle cells by a cAMP mediated inhibition of extracellular signal-regulated kinase activity. Adderley and Fitzgerald[25] showed that apoptosis in cardiomyocytes following ischemia/reperfusion, a response to administration of an anthracycline, is attenuated by iloprost. Gao and Yamaguchi[26] showed that rat marrow cells cultured in a medium of parathyroid hormone and prostaglandin E2 for 7 days preserved their viability. In our study, it was histopathologically shown that iloprost better protected the cells from hypoxia and other external factors. We believe that these protective effects of iloprost may be attributable to attenuation of apoptosis in chromaffin cells in the adrenal medullary, as in cardiomyocytes. Yet, the mechanisms triggered in this process are still unclear. New studies should be performed to resolve these questions. In future, central nervous system transplantation may be employed for functional reconstructive purposes in many fields, which are not currently perceived.

In conclusion, the positive effects of iloprost on the viability of adrenal medulla transplants can be explained by the various features of this material. Since it is a potent vasodilator, it will increase the circulation within the tissue and facilitate the re-vascularization of the graft. It may be possible that the powerful membrane stabilizing effect of iloprost protects the cells from the effects of lysosomal enzymes in the environment. Free oxygen radicals, which have very harmful effects on the cells, are eliminated by iloprost. Moreover, it should be noted that its effects on calcium channels, which result with prevention of calcium facilitated secretion of excitatory amino acids, play an important role in preserving the viability of the cells.

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ETHICAL CONSENT

All manuscripts reporting the results of experimental investigations involving human subjects should include a statement confirming that informed consent was obtained from each subject or subject's guardian, after receiving approval of the experimental protocol by a local human ethics committee, or institutional review board. When reporting experiments on animals, authors should indicate whether the institutional and national guide for the care and use of laboratory animals was followed.