transplantation (4). Moreover, increased LVMI in the early posttransplant period was related to increased mortality rate after transplantation (5). Hypertension has a major influence in the development of LVH. Additional factors, such as uremia, hyperparathyroidism, volume overload, high cardiac output state due to anemia, and arteriovenous fistula are also operative in the development of LVH in patients undergoing hemodialysis (6). Although, reduction of LVH has not been a consistent observation (7), it is mostly reported that correction of uremia-related factors and hypertension lead to regression of LVM and normalization of cardiac function, principally during the first year after renal transplantation (8-10). The persistence of hypertension was reported as the most important factor responsible for the failure of further regression of LVMI beyond the second year after renal transplantation (10).

Studies, aimed to investigate the effect of blood pressure (BP) control on the regression of LVMI, were either conducted in the early post-transplant period or study population consisted of the patients with uncontrolled BP (9). There is no prospective report that specifically addressed the effect of better control of HT on LVMI after the first year in renal transplant recipients.

In this study, we aimed to determine whether the improvement in HT control by means of stepped approach with antihypertensive therapy will improve left ventricular structure in renal transplant recipients one year after renal transplantation.

MATERIALS AND METHODS

Study population

Forty-three nondiabetic renal transplant recipients who were followed up at the renal transplant clinic of Marmara University Hospital were candidates for the study. The local ethics committee approved the study protocol and all patients gave written informed consent prior to inclusion to the study. All of the patients underwent echocardiography and ABPM measurement at baseline and at the end of study period. Renal transplant recipients with duration of renal transplantation less than 12 months (n = 5), functional arteriovenous fistula (n = 1), serum creatinine level above 2.0 mg / dl (n = 5), more than 20 % increase in serum creatinine at any time during the preceding 12 months (n = 2) and severe valvular heart disease (n = 1) were excluded from the study. The remaining 29 renal transplant recipients (aged 35 ± 9 years, mean duration of transplantation of 49 ± 32 months) were included in the the study irrespective of their BP levels and LVMI. The duration of renal transplantation was between 13 to 24 months in 10 recipients, between 25 to 36 months in 6, between 37 to 48 months in 3, between 49 to 60 months in 2 and more than 61 months in 8 recipients. The causes of pretransplant renal failure were chronic glomerulonephritis (34 %), hypertensive nephrosclerosis (28 %), chronic pyelonephritis (21 %), and unknown etiology (13 %). Five renal transplant recipients (3 male and 2 female; aged 37 ± 5 years) who had their serum creatinine increased more than 20 % during study period compared to baseline (n = 3), or whose echocardiography was unavailable (n = 2) were excluded from the final analysis at the end of 24 months of follow up.

All renal transplant recipients were on triple immunosuppressive treatment consisting of cyclosporine, prednisolone and azathioprine. The serum level of cyclosporine was between 100 and 200 ng / ml. The dose of prednisolone and azathiprine were 5 to 10 mg / day and 1 mg / kg / day, respectively.

Blood Pressure Measurements

Twenty-four hours ambulatory blood pressure monitoring (ABPM) was performed according to the method described previously (11) at baseline and 24 months after the last modification of antihypertensive drugs. ABPM device (Spacelab 90207; Spacelabs Inc., Redmond, WA, USA) was programmed for 24 hours with readings for every 20 minutes from 07.00 to 23.00 and every 30 minutes from 23.00 to 07.00. Monitors were calibrated against a mercury sphygmomanometer at the beginning of each session. According to the baseline recordings, patients were categorized as "controlled HT" (n = 14) with daytime ABPM below 135/85 mm Hg or "uncontrolled HT" (n = 10) with mean daytime ABPM ≥ 135/85 mm Hg according to current definitions and criteria used by Burt and colleagues (12,13). Renal transplant recipients with "controlled-HT" constituted group A, and patients with "uncontrolled-HT" constituted

group B. Antihypertensive medications were modified according to ABPM measurements at baseline and further revised by ABPM measurements or clinical BP measurements at regular intervals during outpatient visits according to recent guidelines (12). Clinical BP was measured from the same upper extremity using a mercury sphygmomanometer with the patient in a sitting position. After 10 minutes of rest, the averages of three consecutive measurements performed 5 minutes apart were accepted as the final BP value.

Echocardiographic assessment

Two-dimensional guided M-mode echocardiography was performed by standard methods using an ultrasound system (Ultramark 9, Advanced Technology Laboratories, Bothell, WA, USA) with a 2.25-MHz transducer. Left ventricular internal dimension (LVID), interventricular septal thickness (IVST) and posterior wall thickness (PWT) were measured at end-diastole according to the American Society of Echocardiography recommendations (14). Left ventricular mass (LVM) was calculated using the thick-wall prolate-ellipsoidal model with correction based on a necropsy validation study by Devereux et al. (15): $0.832 \times [(LVID + IVST + PWT)^3 - LVID^3] + 0.6$. LVM was considered as an unadjusted variable and normalized for body surface area (BSA) as left ventricular mass index (LVMI). BSA was calculated by using the Du Bois formula: $0.007184 \times (\text{weight [kg]})^{0.425} \times (\text{height [cm]})^{0.725}$.

Laboratory measurements

Serum creatinine was measured using a computerized auto—analyzer (Hitachi 717, Boehringer Mannheim, Germany). Blood cyclosporine-A level was analyzed by FPIA assay using a monoclonal antibody (Abbott, IL, USA).

Statistical analysis

All calculations were done using SPSS computer program. Data were expressed as mean ± SD. Comparisons between groups A and B were performed by using Mann Whitney U test. Comparisons within the groups were done using Wilcoxon signed rank test. Fischer's exact test was used for the analysis of categorical variables. A two-tailed p value less than 0.05 were considered significant.

RESULTS

The clinical characteristics and laboratory data of group-A (controlled HT) and group B (uncontrolled-HT) are presented in Table I. There were no significant differences in serum creatinine, hemoglobin, cyclosporine level, the duration of dialysis previous to transplantation and the duration of renal transplantation between the two groups at baseline. Serum creatinine level increased mildly but significantly in group A at the end of 24th month of follow-up ($p<0.05$).

ABPM data are presented in Table II. Daytime, nighttime and 24-h ABPM measurements were significantly higher in group B compared to group A at baseline. 24-h SBP decreased from 143 ± 12 mm Hg to 126 ± 8 mm Hg ($p < 0.01$) and 24-h DBP decreased from 88 ± 6 mm Hg to 77 ± 7 mm Hg ($p < 0.01$) in group B at the end of follow-up period (Table II). However, 24-SBP and daytime SBP values were still higher in group-B compared to respective values in group A at 24th month. Finally, only 3 patients (30%) had uncontrolled HT according to daytime ABPM values (135/87 mm Hg, 137/77 mm Hg and 135/93 mm Hg, respectively) after 24 months in group B. All of the patients in group A had controlled BP at the end of study period. Number of antihypertensives prescribed in group B increased significantly from 1.5 ± 0.5 at baseline to 2.6 ± 0.5 after 24 months ($p < 0.01$), whereas the number of antihypertensives did not change in group A. The number of patients receiving angiotensin converting enzyme inhibitors (ACEI) were significantly higher in group B than in group A at the end of study period (29% vs. 80%, $p<0.05$) (Table I). There was a significant difference in hemoglobin levels at the end of 24 months between the two groups (12.0 ± 1.7 g/dl vs. 13.9 ± 1.7 g/dl, $p<0.05$).

While the change in LVID index and IVST index remained insignificant in group-A, PWT index (from 4.8 ± 1.1 mm / m^2 to 6.0 ± 0.9 mm / m^2, $P = 0.009$) increased significantly after two years (Table III). As a result, LVMI increased significantly from 90 ± 21 g / m^2 to 107 ± 26 g / m^2 in group A ($p=0.041$). LVID index, IVST index, PWT index did not change in group B. Therefore, LVMI did not change in group B (113 ± 34 g / m^2 vs 112 ± 29 g / m^2) after two years.

Table I: Clinical characteristics and biochemistry of controlled HT (A) and uncontrolled HT (B) groups of renal transplant recipients at baseline and after 24 months.

	Group A (n = 14)		Group B (n = 10)	
	Baseline	24th month	Baseline	24th month
Age (years)	36 ± 9		35 ± 9	
Gender (male / female, n)	8/6		8/2	
Duration of dialysis before Tx (mo)	20 ± 20		24 ± 22	
Duration of renal Tx (mo)	47 ± 27		55 ± 31	
Serum creatinine (mg / dl)	1.32 ± 0.31	1.51 ± 0.47*	1.25 ± 0.33	1.34 ± 0.39
Hemoglobin (g / dl)	12.6 ± 2.0	12.0 ± 1.7	13.5 ± 1.3	13.9 ± 1.7†
Blood cyclosporine-A level (ng / ml)	159 ± 39	163 ± 38	157 ± 25	147 ± 31

Values are expressed as mean ± SD *p< 0.05 vs baseline in group A, †p<0.05 vs group A at 24th month.

Tx: transplantation.

Table II: ABPM and antihypertensive drug data of controlled HT (A) and uncontrolled HT (B) groups of renal transplant recipients at baseline and after 24 months.

	Group A (n = 14)		Group B (n = 10)	
	Baseline	24th month	Baseline	24th month
Uncontrolled HT (%)	0	0	100	30
24-hour SBP (mm Hg)	118 ± 7	118 ± 8	143 ± 12*	126 ± 8†‡
24-hour DBP (mm Hg)	78 ± 5	77 ± 4	88 ± 6*	77 ± 8†
Daytime SBP (mm Hg)	121 ± 8	122 ± 7	143 ± 11*	128 ± 7†
Daytime DBP (mm Hg)	80 ± 5	79 ± 4	89 ± 4§	80 ± 7†
Nighttime SBP (mm Hg)	112 ± 8	114 ± 10	144 ± 20*	121 ± 12†
Nighttime DBP (mm Hg)	72 ± 4	74 ± 6	86 ± 11§	72 ± 10†
Antihypertensive drugs (n)	1.5 ± 0.8	1.5 ± 0.8	1.6 ± 0.8	2.6 ± 0.8&¶
• ACEI	4/14	4/14	1/10	8/10†‡
• Calcium channel blockers	14/14	14/14	10/10	10/10
• α- receptor blockers	2/14	2/14	2/10	5/10‡
• β-receptor blockers	1/14	1/14	2/10	2/10
• Diuretic	0/14	0/14	0/10	0/10
• Others	0/14	0/14	1/10	1/10

Values are expressed as mean ± SD

*p<0.001 vs group A at baseline, †p<0.01 vs baseline, ‡p<0.05 vs group A at 24th month, §p<0.005 vs group A at baseline,

&p<0.0001 vs baseline, ¶p<0.01 vs group A at 24th month,

ABPM: Ambulatory blood pressure monitorization; SBP: Systolic blood pressure; DBP: Diastolic blood pressure,

ACEI: Angiotensin converting enzyme inhibitor.

Table III: Echocardiography of Controlled HT (A) and uncontrolled HT (B) groups of renal transplant recipients at baseline and after 24 months.

	Group A (n = 14)		Group B (n = 10)	
	Baseline	24th month	Baseline	24th month
LVID index (mm / m^2)	28.5 ± 3.2	27.6 ± 3.3	26.3 ± 3.2	25.7 ± 3.3
IVST index (mm / m^2)	5.3 ± 1.2	5.9 ± 0.6	6.4 ± 0.9*	6.0 ± 0.6
PWT index (mm / m^2)	4.8 ± 1.0	6.0 ± 0.9†	5.7 ± 1.0‡	5.9 ± 1.1
LVMI (g / m^2)	90 ± 21	107 ± 26¶	113 ± 34	112 ± 29

Values are expressed as mean ± SD

*p=0.01 vs group A at baseline, †p=0.009 vs baseline, ‡p<0.05 vs group A at baseline, ¶p<0.05 vs baseline.

LVID: Left ventricular internal diameter; IVST: Interventricular septal thickness; PWT: Posterior wall thickness.

DISCUSSION

This study demonstrated that better control of HT did not result in regression of LVMI in long term renal transplant recipients with "uncontrolled-HT" (group B) and maintenance of BP control in the group with "controlled-HT" (group A) did not prevent progression of LVMI after 24 months of follow-up.

Previous studies demonstrated that control of HT resulted in improvement of LVMI LVH in renal transplant recipients in the early posttransplant period (8,9). Rigatto et al. reported that regression of LVMI was an ongoing process during the first 2 years of transplantation and reached a nadir in the third and fourth posttranplant years (10). In contrast to this study, de Lima et al. reported that LVMI continued to regress from 172 g/m^2 to 136 g/m^2 during 40 months of follow-up (16). However, in this study, first echocardiography was performed 12 months after renal transplantation, and the second echocardiography was performed almost 2 years after the first one. This study was quite similar to design of our study in that they also included patients with duration of transplantation more than 12 months. However, in our study, we intended to control HT in the recipients with uncontrolled HT and maintain controlled BP in the recipients with controlled HT. ABPM were done for all recipients at baseline and at the end of 2 years to determine the level of control of HT. ABPM is accepted as the method more closely associated with LVMI compared to clinical measurements following renal transplantation (17, 18).

The increase in LVMI in our renal transplant recipients with controlled HT at baseline was due to increase in the wall thickness of left ventricle. Our findings indicate that control of HT may be a weak factor to influence the course of LVMI in long term renal transplant recipients. The progression in LVMI in this group was probably secondary to significant worsening of renal function and anemia. However, increased use of ACEI and preservation of renal function and hemoglobin level may be responsible from the maintenance of LVMI in patients with "uncontrolled HT". In our study, the decrease in hemoglobin values in group A might explain the increment in LVMI in group A (19).

In this study, we used ABPM measurement for all recipients at baseline and at the end of 2 years. ABPM is accepted as the method more closely associated with LVMI compared to clinical measurements following renal transplantation (17).

A major limitation in this study is that, groups were small to reach a sufficient power to evaluate independent effects of other factors such as decrease in renal function and anemia and the use of ACEI on the structure of left ventricle at long term.

In conclusion, control of HT did not result in regression of LVMI in long term renal transplant recipients. Worsening of renal function and anemia are probably more important risk factors for the maintanence of LVH at long term in renal transplant recipients. Increased use of ACEI would probably effect the left ventricular structure in those patients. Long-term follow-up studies in a large cohort of renal transplant population should be performed in order to investigate whether control of these factors and increased use of ACEI could lead to regression of LVH and have a beneficial effects on cardiovascular outcome.

REFERENCES

1. *Lindholm A, Albrechtsen D, Frodin L, Tufveson G, Persson NH, Lundgren G. Ischemic heart disease-major cause of death and graft loss after renal transplantation in Scandinavia. Transplantation 1995; 60: 451-457.*
2. *Rigatto C, Parfrey P, Foley R, Negrijn C, Tribula C, Jeffery J. Congestive heart failure in renal transplant recipients: risk factors, outcomes, and relationship with ischemic heart disease. J Am Soc Nephrol 2002; 13: 1084-1090.*
3. *Silberberg JS, Barre PE, Prichard SS, Snidermann AD. Impact of left ventricular hypertrophy on survival of end-stage renal disease. Kidney Int 1989; 36: 286-290.*
4. *McGregor E, Jardine AG, Murray LS, et al. Pre-operative echocardiographic abnormalities and adverse outcome following renal transplantation. Nephrol Dial Transplant 1998; 13: 1499-1505.*
5. *McGregor E, Stewart G, Rodger RS, Jardine AG. Early echocardiographic changes and*

survival following renal transplantation. Nephrol Dial Transplant 2000; 15: 93-98.

6. Foley RN, Parfrey PS, Harnett JD, Kent GM, Murray DC, Barre PE. Impact of hypertension on cardiomyopathy, morbidity and mortality in end stage renal disease. Kidney Int 1996; 49: 1379-1385.
7. Huting J. Course of left ventricular hypertrophy and function in end-stage renal disease after renal transplantation. Am J Cardiol 1992; 70: 1481-1484.
8. Parfrey PS, Harnett JD, Foley RN, et al. Impact of renal transplantation on uremic cardiomyopathy. Transplantation 1995; 60: 908-914.
9. Midtvedt K, Ihlen H, Hartmann A, et al. Reduction of left ventricular mass by lisinopril and nifedipine in hypertensive renal transplant recipients: a prospective randomised double-blind study. Transplantation 2001; 72: 107-111.
10. Rigatto C, Foley RN, Kent GM, Guttmann R, Parfrey PS. Long-term changes in left ventricular hypertrophy after renal transplantation. Transplantation 2000; 70: 570-575.
11. Koc M, Toprak A, Ozener IC, et al. QT dispersion in renal transplant recipients. Nephron 2002; 91: 250-254.
12. The Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure. Arch of Int Med, 1997; 157: 2413-2448.
13. Burt VL, Cutler JA, Higgins M,, et al. Trends in the prevalence, awareness, treatment, and control of hypertension in the adult US population. Data from the health examination surveys, 1960 to 1991. Hypertension 1995; 26: 60-69.
14. Sahn DJ, DeMaria A, Kisslo J, Weyman A. Recommendations regarding quantitation in M-mode echocardiography: results of a survey of echocardiographic measurements. Circulation 1978; 58: 1072-1083.
15. Devereux RB, Alonso DR, Lutas EM, et al. Echocardiographic assessment of left ventricular hypertrophy: comparison to necropsy findings. Am J Cardiol 1986; 57: 450-458.
16. De Lima JJ, Vieira ML, Viviani LF, et al. Long-term impact of renal transplantation on carotid artery properties and on ventricular hypertrophy in end-stage renal failure patients. Nephrol Dial Transplant 2002: 17: 645-651.
17. Fernandez-Vega F, Tejada F, Baltar J, Laures A, Gomez E, Alvarez J. Ambulatory blood pressure after renal transplantation. Nephrol Dial Transplant 2001; 16 (Suppl 1): 110-113.
18. Toprak A, Koc M, Tezcan H, Ozener IC, Oktay A, Akoglu E. Nighttime blood pressure load is associated with higher left ventricular mass index in renal transplant recipients. J Hum Hypertens 2003; 17: 239-244.
19. Foley RN, Parfrey PS, Kent GM, Harnett JD, Murray DC, Barre PE. Long-term evolution of cardiomyopathy in dialysis patients. Kidney Int 1998: 54: 1720-1725.

ALTERATIONS IN THE KINETIC ACTIVITY OF AROMATIC-L-AMINO ACID DECARBOXYLASE AND PRELIMINARY 2-DE INVESTIGATION OF THE BRAINS IN A 6-OHDA INDUCED PARKINSON'S DISEASE RAT MODEL

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ABSTRACT

Objective: The aim of this study was to isolate and purify the aromatic-L-amino acid decarboxylase (AADC,EC 4.1.1.28) enzyme rats from Parkinson's Disease (PD) induced and the healthy control group rat brains and compare the alterations in the kinetic activities of the isolated enzyme. The protein spots displaying on the 2-DE patterns of the diseased and the healthy control group crude rat brain homogenates were evaluated.

Medhods: In this study, the Parkinson's Disease model was induced by injecting 6-hydroxydopamine into the brains of the rats. The PD model formation was successful in two rats out of three.

Results: The AADC decarboxylase was isolated and partially purified by DEAE-Sephacel ion exchange chromatography from the brains of PD induced and healthy control animals to compare the kinetic activity of the enzyme. The kinetic activity of the enzyme was reduced 70% in the PD group compared to controls.
In order to determine and correlate the alterations with PD, and the distribution of the proteins displayed by the crude brain homogenates of the diseased and the healthy control group both were investigated. Polyacrylamide gel electrophoresis (PAGE) of the crude brain homogenates under the native and denaturizing conditions displayed matching bands for both of the groups, while two dimensional electrophoresis (2-DE) patterns of the crude brain homogenates of the diseased and the control group displayed considerable differences.

Conclusion: The results of this study confirm the power of 2-DE-PAGE technique of the proteome analysis. Currently only the proteome analysis enables the identification of disease correlated proteins.

Key Words: Proteome, Parkinson Disease, AADC, 2-DE

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INTRODUCTION

Parkinson's disease (PD) is a progressive neurological disorder that results from the degeneration of neurons in an area of the brain known as substantia nigra. These neurons produce an important chemical, dopamine, a chemical messenger responsible for transmitting signals between the substantia and the next "messenger center" of the brain corpus striatum (1). Aromatic-L-Amino acid decarboxylase (AADC, EC 4.1.1.28) catalyzes the synthesis of dopamine and serotonin found in the nervous system (2).

Although the enzyme is known as DOPA decarboxylase, since it shows the best known catalytic activity on DOPA, immunological (2) and biochemical studies (3) have shown that this enzyme is also responsible for the decarboxylation of 5-hydoxy tryptophane. However, AADC is found in the nerves synthesizing and storing monoamine neurotransmitters. AADC is important in neurodegenerative disorders such as Parkinson's disease. Although tyrosine hydroxylase is the rate-limiting enzyme in the synthesis of neurotransmitters, some clinical findings revealed that, even though the amount of precursors of neurotransmitters is sufficient or ten times more than normal, some people displayed symptoms similar to Parkinson's disease (4), due to lack of enzymes in the synthesis of neurotransmitters such as dopamine and serotonin. Life quality is clinically reduced in Parkinson's patients.

Up-to-date immunological and biochemical studies have been carried out with relation to AADC. Although many hypotheses regarding the pathogenesis of PD have been proposed-including genetic factors, immunological abnormality, endogenous and exogenous toxins, mitochondrial dysfunctions and oxidative stress-the specific etiology of PD remains unclear (5). There is not enough data on the cause of the disease. The human genome is almost elucidated but the transformations that cause the disease have not yet been revealed. Gene series are identified in genome studies but since the functional molecules are the proteins in the body, these transformations could only be recognized and related to the diseases through the gene expression. Post-translational modifications are important in understanding the pathological situations. These post translational modifications can be determined only by proteome analysis.

In this study, a toxin specific to dopamine nerve cells 6-hydoxydopamine (6-OHDA) was used to induce PD. AADC which was isolated from the brains of the PD-induced and healthy control rats, was partially purified to compare the alterations in kinetic activities. In order to identify the differences between enzyme preparations obtained from the PD-induced rat brains by injecting 6-OHDA and enzyme preparations obtained from the healthy rat brains, the two dimensional polyacrylamide gel electrophoresis (2-DE) method, which was the heart of proteome analysis, was performed.

MATERIAL AND METHOD

6-OHDA, apomorphine.HCl, 2,4,6 trinitro benzene sulfonic acid , leupeptin, pepstatin, phenyl-methyl sulfonyl fluoride (PMSF), tris, bromphenol blue, *bis*-acrylamide, TEMED were purchased from Sigma,Chemicals,Co., St Louis, MO, ascorbic acid, potassium phosphate, benzene UVasol, SDS, glycerol were purchased from Merck, Darmstadt, Germany, acrylamide, urea, Dithiothreitol (DTT) and ampholytes; pH 2-4 and 3-10 were purchased from Fluka. Ketamine.HCl and chlorpromazine were purchased from a local drug company.

6-Hydroxydopamine (6-OHDA) Induced Rat Model of PD

All procedures were carried out according to the guidelines of Institutional Animal Care and Use Committee of Marmara University the animals were housed in a temperature-controlled room (20±3°C) with a 12 hr light/dark cycle and fed on a standard diet and water *ad libitum*. Six male Sprague-Dawley rats (~280 g) were used in this study. Three rats were labeled as the control group and the others were labeled as Parkinson's group. On the experiment day, the rats were deeply anaesthetized intraperitonally with ketamine.HCl (100 mg/kg) and chlorpromazine (0.5 mg/kg) and placed in a stereotaxic frame (Stoelting Model 51600). 6-OHDA used to induce PD in rats using surgery in the rat, 6-OHDA is delivered directly at a number

of different points along the nigrostriatal pathway. Two unilateral micro injections of 4 μL of 6-OHDA (2 μg/μL of the active compound in 0.8% ascorbic acid solution, injection rate 0.5 μL/min (6) were performed slowly according to the coordinates from the Rat Brain Atlas of Paxinos and Watson (7). The stereotaxic coordinates were 4.8 mm posterior, 2.2 mm lateral from the bregma and 7.8 mm ventral from the surface of the skull. The control group received only saline microinjections (6,8). The cannula was left in place for an additional 2 min before slow retraction (8). Uptake of 6-OHDA directly into the substantia nigra lead to the death of dopamine nerve cells resulting in damage similar to that in PD.

Behavioral Test

After the lesion formation, apomorphine (0.5 mg/kg i.p.) was injected into the rats on 7^{th} and 14^{th} days. Upon the injection of apomorphine, the rotational behavior of the rats was followed in a plastic cage for thirty minutes (7-9).

Removal of Brains

After the behavioral test, the animals in the control group and the rats revealing rotational behavior were decapitated under deep ether anaesthetizia. The brains were removed and placed at -20°C.

Purification

All procedures were carried out at 4°C. All the buffers used contained 0.1.mM dithiothreitol and 0.1 mM EDTA.

Step 1: Extraction: Upon removal of the brains, ~ 5 g brains were immediately minced with a knife and homogenized in a homogenizer with 0.5 volume of 10 mM potassium phosphate buffer, pH 7.8 containing 1 mM leupeptin, 0.1 mM pepstatin, 0.5 mM PMSF and 2 mM EDTA. The homogenate was then centrifuged for 40 min at 12,000xg in a Sigma 3K30 centrifuge followed by an additional centrifugation of the supernatant at 100,000xg for 1 hour (10). A part of supernatants was kept for electrophoresis at -70°C.

Protein Determination

Protein concentration was assessed according to the Lowry method (11) using bovine serum albumin as a standard. Column elutes were monitored by measuring absorbance at 280 nm.$\varepsilon^{1\%}_{1cm}$ 1.4 M^{-1} (12).

Enzyme Assay

AADC activity was measured by the method of Sherald *et.al.* (13), according to the modification of Charteris and John (14). The standard reaction mixture contained, in a final volume of 250 μL, 100 μL of 0.1 M Potassium Phosphate Buffer (pH 6.8) and 25 μL of 5 mM L-DOPA and 5 μL of enzyme . The reaction was incubated for 5 min at 25°C and then stopped by heating at 100°C for 1 min. Benzene (1.5 mL) and 2,4,6 trinitro benzene sulfonic acid (TNBS) (1 mL of a 4.3 mM solution in 0.1 M Potassium Phosphate Buffer, pH 7.5) were added, and the trinitrophenylamine derivative formed was measured in the benzene layer at 340 nm with a Shimadzu UV 1601 spectrophotometer using 12,400 $M^{-1}cm^{-1}$ as the molar absorption coefficient for trinitrophenyl dopamine. One unit of enzymatic activity (U) is defined as the amount of protein which catalyzes the production of 1 nmol amine/min.

Step 2 DEAE-Sephacel Ion-Exchange Chromatography: The final supernatant was loaded on a DEAE-Sephacel Column (0.5x3 cm) previously equilibrated with 10 mM Potassium Phosphate Buffer, pH 7.8. The column was then washed with 20 mM Potassium Phosphate Buffer, pH 7.8 until the absorbance at 280 nm of the effluent was 0.1 or less. The enzyme was eluted with 50 mM Potassium Phosphate Buffer, pH 7.8. 1 mL fractions were collected and those with the highest AADC activity were pooled (15) and PAGE under native conditions was performed. After DEAE-Sephacel ion-exchange chromatography, total enzyme activity and kinetic activity were assayed and K_M, V_{max}, K_{cat}, K_{cat}/K_M values were calculated. Statistical analysis was performed using Student's t-test.

Electrophoresis of Crude Samples

PAGE Electrophoresis of crude samples was carried out under native conditions by Davis PAGE (16) and denaturing conditions according to Laemmli SDS-PAGE (17) .

Two dimensional electrophoresis (18) of crude extracts was performed, where isoelectric focusing was performed in first dimension and Laemmli SDS-PAGE (16) was performed in second dimension.

2-DE-PAGE

Preparation of Protein Samples

First, the volume of probe (Y) in microliters was determined. The numerical value of this volume was denoted factor Y. Using this factor, the addition of urea, DTT and ampholytes was calculated.

2-DE was performed essentially as reported by Jungblut (18). Just prior to the first dimension of 2-DE, lysis buffer that contained 9 M urea, 70 mM DTT and 40% ampholyte pH 2-4 were added to the sample.

2-DE was performed with a Biorad Tube Cell Isoelectric Focusing (IEF) system for the first dimension, and SDS 12,5% polyacrylamide gels for the second dimension. SDS-PAGE was run in a Schleicher&Schuell Profile System (Schleicher&Schuell, Germany).

The IEF was performed at 20°C with the following voltage program: 100 V for 75 min, 200 V for 75 min, 400 V for 75 min, 600 V for 75 min, 800 V for 10 min, 1000 V for 5 min. Prior to the second dimensional gel separation, the IEF gels were equilibrated for 10 min with gentle shaking in 5 mL of equilibration solution (125 mM Tris/phosphate (pH 6.8), 40 % (w/v) glycerol, 65 mM DTT, 3% (w/v) SDS, brom phenol blue (trace)).

The second-dimensional SDS-PAGE with a 12.5% running gel was carried out without a stacking gel. After placing the IEF gel rod on top of the second dimensional polyacrylamid gel, electrophoresis was performed by increasing the voltage in a stepwise manner, according to the following voltage program: 35 V for 5 min, 55 V for 10 min, 100 V for 15 min, 150 V for 60 min. The Power supply was purchased from Apelex (Apelex PS 9009 TX), France.

RESULTS

PD model was induced by 6-OHDA. The model was tested by intraperitonal injections of apomorphine.HCl. The behavioral test results are given in Table I.

Protein concentrations of the crude enzyme extracts obtained from healthy and diseased rat brains and DEAE-Sephacel Ion-Exchange Chromatography Fractions are summarized in Table II.

The AADC was partially purified by DEAE-Sephacel Ion-Exchange Column Chromatography in both cases. DEAE-Sephacel Ion-Exchange Column Chromatography profile related to control and diseased groups is displayed in Fig. 1.

Total enzyme activity and specific enzyme activity of crude extract and DEAE-Sephacel Ion-Exchange Chromatography elute obtained from control and PD-induced rat brains are summarized in Table III.

The kinetic data of enzyme activities were calculated from Lineweaver-Burk (19) curves (Figs. 2 and 3). The kinetic data are summarized in Table IV.

Davis-PAGE electrophoresis patterns related to Parkinsonian rats and control rat groups after DEAE-Sephacel Ion-Exchange Chromatography are displayed in Fig. 4, also the results of PAGE of the crude brain homogenates under native and denaturizing conditions are displayed in Fig.5.

Table I: Results of Behavioral Test

Rats	7th Day	14th Day
1	110 Rotation (Counterclockwise)	130 Rotation (Counterclockwise)
2	10 Rotation (Counterclockwise)	—
3	4 Rotation (Counterclockwise)	60 Rotation (Counterclockwise)

Table II: Protein Concentrations of Both Groups

Samples	Protein (mg/dL)
Crude extract from Control group	330
Crude extract from Parkinson's Rats	420
DEAE Sephacel Column Elution of Control group (Fr.17-19)	30
DEAE Sephacel Column Elution of Parkinson Induced Rats (Fr.20-23)	38

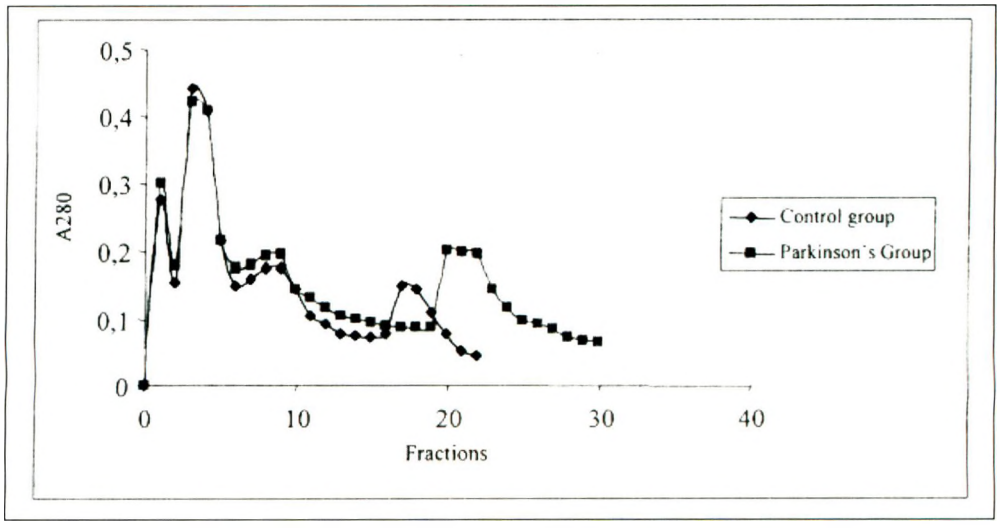


Fig.1: **Diethyl amino ethyl** (DEAE)-Sephacel ion exchange profile of the enzyme (fr 17-19 for control group and fr 20-23 for Parkinson's group).

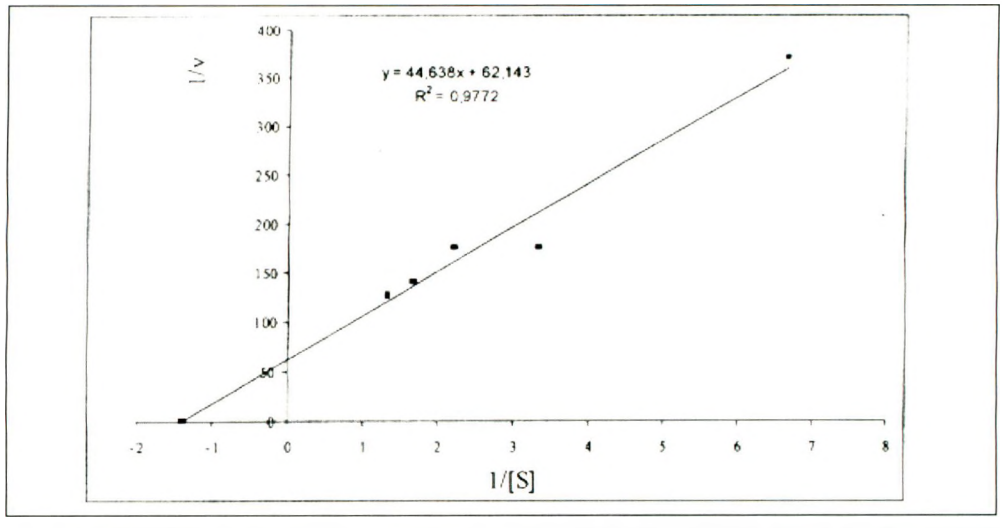


Fig.2: Lineviewer-Burk Curve of **Aromatic-L-Amino Acid Decarboxylase**[AADC (EC 4.1.1.28)] for Control Group.

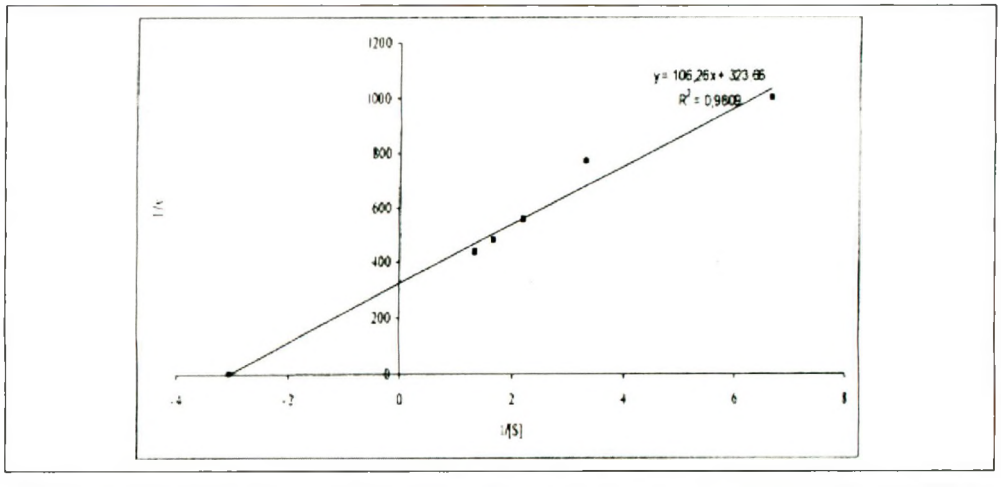


Fig.3: Lineviewer-Burk Curve of **Aromatic-L-Amino Acid Decarboxylase**[AADC (EC 4.1.1.28)] for Parkinson's Group.

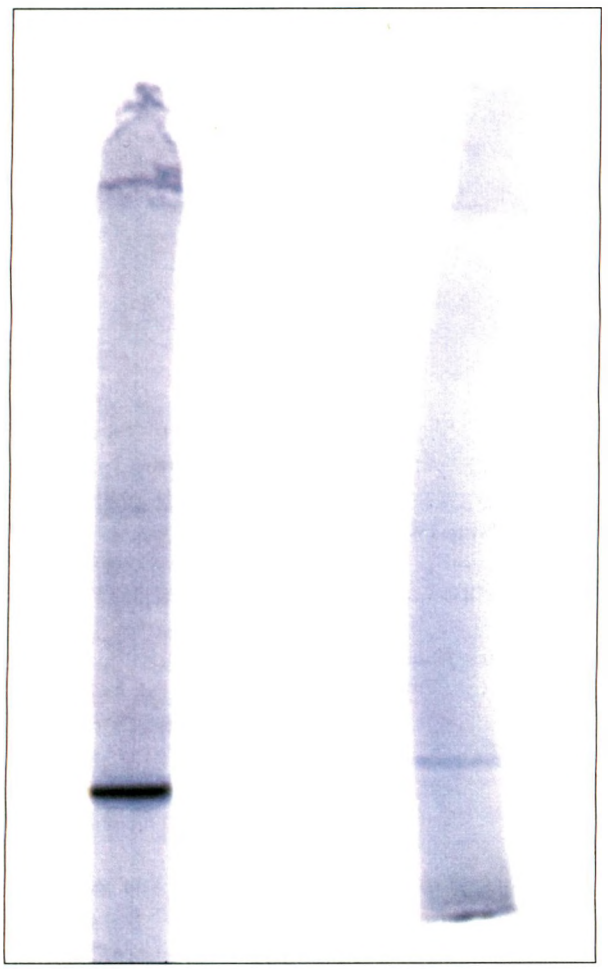
Table III: Total and Specific Activity of Samples

Samples	Total Activity (U)	Specific Activity (U/mg)
Crude Extracts of Control Group	717.74	2.17
DEAE Sephacel Column Fractions of Control Group (Fr 17-19)	217	7.25
Crude Extracts of Parkinson's Group	217.6*	0.52
DEAE Sephacel Column Fractions of Parkinson's Group (Fr 20-23)	80.64	2.12

* Comparison of control group vs. Parkinson's group was statistically significant ($p<0.01$, according to Student's t test).

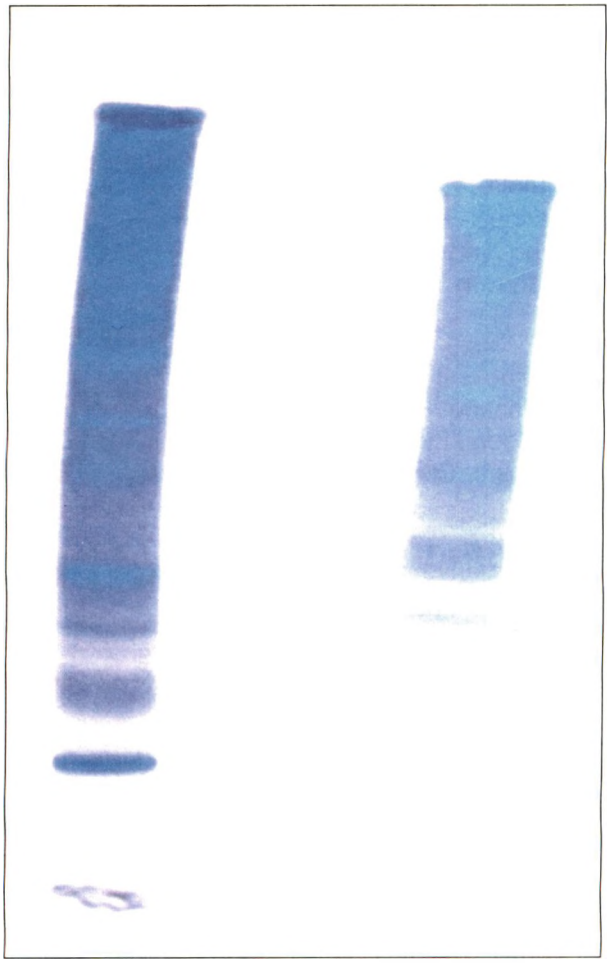
Table IV: Kinetic Data

Samples	K_M(mM)	Vmaks (mMdk^{-1})	K_{cat}(S^{-1})	K_{cat}/K_M ($mM^{-1}S^{-1}$)
Control Group	$7.2x10^{-4}$	$1.6x10^{-5}$	0.44	611.11
Parkinson's Group	$3.3x10^{-4}$	$3.8x10^{-6}$	0.083	251.52



a b

Fig.4: Davis-**Poliacrylamide Gel Electrophoresis** (PAGE) Patterns after **diethyl amino ethyl** (DEAE)-Sephacel Ion-Exchange Chromatography
(a) Control Group, (b) Parkinson's Group.



a b

Fig.5: Davis- **Poliacrylamide Gel Electrophoresis** PAGE Patterns (a) Crude Extracts of Control Group (b)) Crude Extracts of Parkinson's Group.

Laemmli-SDS-PAGE of crude enzyme extracts displayed matching bands and the Laemmli-SDS-PAGE patterns of both groups are shown in Fig. 6.

2-DE-PAGE patterns of control group and diseased group are displayed in Fig. 7.

DISCUSSION

PD model formation was successful in two out of three rats. The animals revealed rotational behaviors in counterclockwise direction, while the other rat showed insufficient rotational behavior on the 7th day and did not show any rotational behavior on the 14th day. So these two animals (number 1 and 3) revealing rotational behaviors were included to the study. The

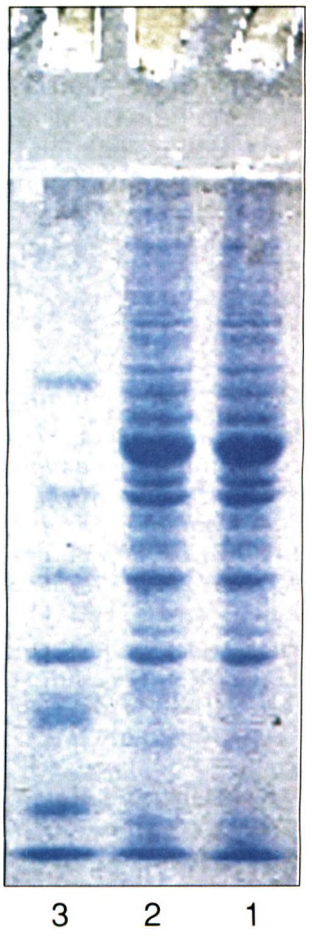
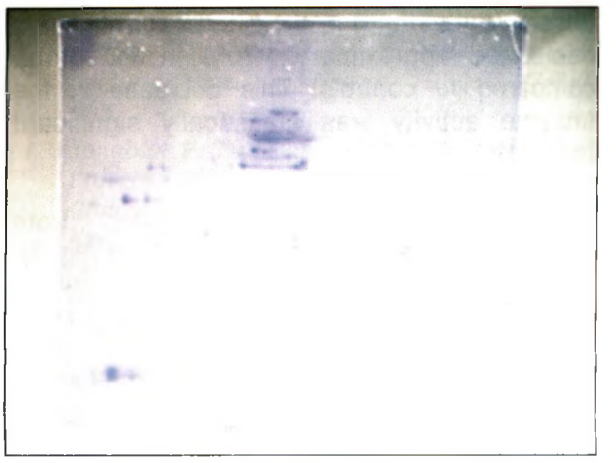
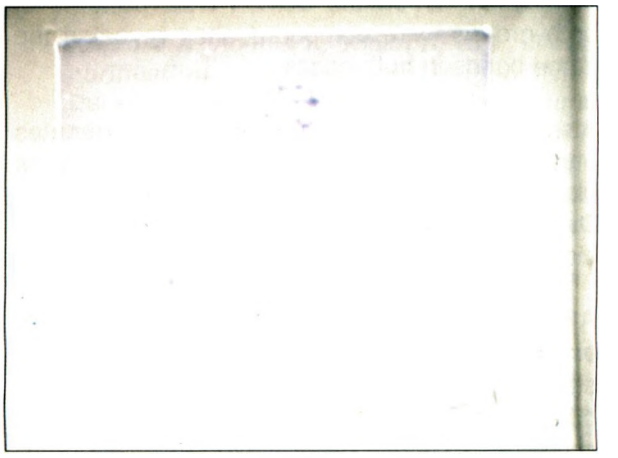


Fig.6: Laemmli-**Sodium Dodecyl Sulfate Poliacrylamide Gel Electrophoresis** (SDS-PAGE) Pattern of Crude Enzyme Extracts.
1.Control Group
2.Parkinson's Group
3.Standards



(a)



(b)

Fig.7: **Two Dimensional Electrophoresis** (2-DE)-PAGE Patterns. (a) Control Group, (b) Parkinson's Group.

behavioral test was carried out for control rats but they did not reveal any rotational behavior. This result was expected for healthy animals.

In this study the material collected from rats was pooled since the protein concentration obtained from one rat brain was insufficient for the 2-DE analysis. In proteomics techniques using animals as material, the collected material is generally pooled (20).

AADC was first purified from the pig kidney, which has the richest enzyme activity, by DEAE - Sephacel ion-exchange chromatography QAE-Sephadex ion-exchange chromatography and Sephadex-G_{100} gel filtration column chromatography methods respectively (15). But purification from other tissues was difficult, it could be possible with advanced techniques (21). In this study, AADC (EC 4.1.1.28) is partially purified by using ultracentrifugation and DEAE-Sephacel ion-exchange chromatography methods, further purifications by chromatographic methods were not performed due to the fact that the protein concentration decreases dramatically after DEAE-Sephacel ion-exchange column chromatography. After the purification by DEAE-Sephacel ion-exchange column chromatography, protein concentrations dramatically decreased in both of the cases (Table II), due to dilution during the chromatography process, hence further purifications could not be done.

In previous studies the enzyme activity obtained from bovine brain was found 612 nmol/min(22) by H.P.L.C., with electrochemical detection. In our study, the total enzyme activity was found 717.74nmol/min by spectrophotometric method. The brain and serum revealed the lowest enzyme activity respectively. In this study we

observed that total and specific activity were decreased approximately 70% in PD group compared to controls. This decrease in the enzyme activity was statistically significant ($p<0.01$).

The kinetic data was calculated from Lineweaver-Burk (22) curves (Fig. 2 and Fig. 3). When the kinetic data of both groups shown in Table IV are compared, a decline in the enzyme efficiency was observed. Thus kinetic assays support the success of the formation of Parkinson's Model.

PAGE was performed in order to follow the purification. When the Davis-PAGE patterns of both groups were compared, they revealed the same bands in both cases.

Also PAGE of the crude brain homogenates under native and denaturizing conditions displayed matching bands for both of the groups. Davis-PAGE of the crude enzyme extracts displayed 14 bands in both groups.

Also Laemmli-SDS-PAGE of crude enzyme extracts displayed matching bands.These situations were expected results since differences between healthy and disease conditions can only be observed in 2-DE-PAGE patterns. Thus the power of 2-DE-PAGE technique in differentiation of proteins in diseased cases was very well understood.

2-DE patterns of the control and the diseased groups showed considerable differences, 13 spots displayed in the control group disappeared completely in the diseased group while 2 spots displayed in the diseased group are absent in the control group. The intensities of some of the protein spots diminished drastically in the Parkinson's induced group but the intensity of a single protein spot appearing in the control group increased considerably in the Parkinson's group.

In conclusion, in this study we showed the protein differentiation of brains from control and Parkinson's disease model by 2-DE technique which is the heart of proteome analysis. In further studies we are going to elucidate these differentiated proteins by advanced complementary techniques.

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REFERENCES

1. *Cutillas B, Ambrosio S, Unzeta M, Neuroprotective effect of the monoamine oxidase inhibitor PF 9601N (N-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine) on rat nigral neurons after 6-hydroxydopamine-striatal lesion. Neurosci Lett 2002:329;165-168.*
2. *Christenson JG, Dairman W, Udenfriend S. On the Identify of DOPA Decarboxylase and 5-Hydroxytryptophan Decarboxylase. Proc Natl Acad Sci U.S.A. 1972;69:343-347.*
3. *Yuwiler A, Geller E, Eiduson S. Studies on 5-Hyroxytryptophan Decarboxylase I.In vitro Inhibition and Substrate Interaction. Arch Biochem Biophys 1959;80:162-173.*
4. *Maller A, Hyland K, Milstein S, Biaggioni I, Butter I.J. Aromatic-L-Amino Acid Decarboxylase deficiency: Clinical features, diagnosis and treatment of a second family. J Child Neurol 1997; 12:349-354.*
5. *Goldman SM, Tanner C. Etiology of Parkinson Disease In: Jankovic J, Tolosa E, eds Parkinson's Disease and Movement Disorders. 3 rd ed. Baltimore: Williams and Wilkins, 1998;133-158.*
6. *Bardo MT, Robinet P.M, Mattingly BA, Margulies JE. Effect of 6-Hydroxydopamine or repeated amphetamine treatment on mesencephalic mRNA levels for AMPA glutamate receptor subunits in the rat. Neurosci Lett 2001;302:133-136.*
7. *Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates, 4th ed. San Diego, California: Academic Press, 1998.*
8. *Kirik D, Rosenblad C, Björklund A. Characterization of behavioral and neurodegenerative changes following partial lesions of the nigrostriatal dopamine system induced by intrastriatal 6-Hydroxydopamine in the rat. Exp Neurol 1998;152:259-277.*

9. May HC, Guilarte TR, Wagner HN, Vogel S. Intrastriatal infusion of Lisuride-A potential treatment for Parkinson's Disease. Neurodegeneration 1994;3:305-313.
10. Voltattorni CB, Dominici P, Moore PS. Modified purification of L-Aromatic Amino Acid Decarboxylase from pig kidney. Protein Expr Purif 1993;4:345-347.
11. Lowry HO, Rosebrough NJ, Farr AL, Randall RJ, Protein Measurement with the Folin Phenol Reagent. J Biol Chem 1951;193:265-275.
12. Johnstone AP. Thorpe R. Production and Assay of the Interleukins. J Immunol Methods 1985;83:1-27.
13. Sherald A, Sparrow CJ. Wright RFT, A spectrophotometric assay for Drosophila Dopa Decarboxylase. Anal Biochem 1973; 56:300-305.
14. Charteris A, John R. An investigation of the assay of Dopamine using Trinitrobenzenesulfonic Acid. Anal Biochem 1975;66:365-371.
15. Voltattorni CB, Guartosio A, Turano C. Aromatic-L-Amino Acid Decarboxylase. Methods Enzymol 1987; 142:179-187.
16. Davis BJ. Method and application to human serum proteins. Ann NY Acad Sci 1964;121:404-427.
17. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680-685.
18. Jungblut P. Two-Dimensional Electrophoresis Protein Structure Analysis, Preparation, Characterization and Microsequencing. In: Kamp, RM, Choli-Papadopoulou T, Wittmann-Liebold B eds. Springer-Verlag, Inc., Berlin, Germany, 1997; 183-214.
20. Eckerskorn C, Jungblut P, Mewes W, Klose J, Lottspeich F, Identification of mouse brain proteins after two dimensional electrophoresis and electroblotting by microsequence analysis and amino acid composition analysis. Electrophoresis 1988; 9:830-838.
21. Ichinose H, Kojima K, Togari A, et al. Simple purification of Aromatic-L-Amino Acid Decarboxylase from human pheochromocytoma. Anal Biochem 1985; 150:408-414.
22. Nishigaki I, Ichinose H, Tamai K, Nagatsu T. Purification of Aromatic-L-Amino Acid Decarboxylase from bovine brain with a monoclonal antybody. Biochem. J 1988; 252:331-335.
19. Lineweaver H, Burk D. The determination of enzyme dissosiation constants. J Amer Chem Soc 1934;56:658-666.