

Evaluation of Antioxidants in Discrete Regions of Brain after the Transplantation of Human Amniotic Epithelial Cells in 2,4,5-Trihydroxyphenylethylamine-lesioned Wistar Albino Rats

Abstract

Background and Aim: Parkinson's disease (PD) a neurodegenerative disorder for which no preventive or long term effective treatment strategies are available. Epidemiological studies have failed to identify specific environmental, dietary or lifestyle factors for PD. However oxidative stress in the substantia nigra (SN) and Corpus striatum is the most broadly accepted hypothesis for the etiopathology of PD. Many experiments state the notion that augmentation of neurotrophic factors and glial cell-derived neurotrophic factors, could prevent or halt the progress of neurodegeneration in PD. **Material and Methods:** The present study was designed to assess the motor behaviour with apomorphine injection and level of enzymatic and non-enzymatic antioxidants after transplantation of Human Amniotic Epithelial (HAE) cells in 2,4,5 trihydroxyphenylethylamine (6-OHDA) lesioned striatum in rats. **Results:** Human Amniotic Epithelial (HAE) cells ameliorated 6-OHDA induced changes in rotational behaviour and modulated the antioxidants. 6-OHDA induced neurotoxicity by the generation of free radicals was pronounced by indication of increased Lipid peroxidation (LPO) levels and decrease in antioxidants level. The present result suggest that increased free radicals and the decrease in the antioxidant defence system possibly lead to structural and functional alterations in membrane-related events and play significant role of the 6-OHDA induced neurotoxicity. In HAE cells transplanted animals the level of LPO was significantly reduced in striatum (36%) and moderately reduced (20%) in midbrain of the 6-OHDA lesioned animals. **Conclusion:** These alterations were found to be recovered after the HAE cells graft during long term. The 6-OHDA induced neurotoxicity by the generation of free radicals was pronounced by indication of the increased LPO level, decrease in antioxidants and alteration in the dopamine and its metabolites.

Keywords: 2, 4, 5-trihydroxyphenylethylamine lesion, corpus striatum, human amniotic epithelial cells, oxidative stress, Parkinson's disease

Introduction

Dopamine-replacing agents such as the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) have been widely used in the treatment of Parkinson's disease (PD) for more than 30 years.^[1] However, long-term dopamine replacement therapy is associated with many disabling side effects, most notably abnormal involuntary movements.^[2] While L-DOPA is the most common treatment for the relief of symptoms in PD, other treatment regimens have incorporated various direct-acting dopamine receptor agonists. Unfortunately, the actions of all these agents are compromised as they also elicit dyskinesia when given even with low doses of L-DOPA.

Radical stress, caused by the production of reactive oxygen species (ROS), is thought to be a critical factor in the modest neuronal degeneration that occurs with aging. Superoxide radicals, hydrogen peroxide (H₂O₂), and hydroxyl radicals are oxygen-centered reactive species that have been implicated in several neurotoxic disorders.^[3] They are produced by many normal biochemical reactions, but their concentrations are kept in a harmless range by potent protective mechanisms. Intra-peritoneal administration of liver growth factor partially protected the dopamine neurons from 2,4,5-trihydroxyphenylethylamine (6-OHDA) neurotoxicity in the substantia nigra and reduced motor deficits in rats.^[4]

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Materials and Methods

Animals

The adult Wistar albino rats weighing around 180–220 g were housed in pairs and were maintained at constant temperature and humidity (21°C–26°C) and 12:12 h light/dark cycle. Animals were allowed *ad libitum* access to food and water when not undergoing behavioral tests and surgery. The experiments were conducted in accordance with the standard procedures of the Institutional Animal Ethical Committee (IAEC). The project approval number is IAEC No 01/008/03.

Experimental groups

The animals were divided randomly into four groups; each group contains six rats. Group I: control; Group II: sham control in which 0.2% ascorbic acid in 0.9% saline was injected; Group III: lesioned in which 0.2% ascorbic acid in 0.9% saline with 6-OHDA was injected; and Group IV: 6-OHDA-lesioned and human amniotic epithelial (HAE) cells transplanted.

Stereotaxic coordinates

Before the lesion was induced, the animals were anaesthetized with Pentothal sodium (i.p., 40 mg/kg of body weight). Incisor bars were set at 3.3 and 1.0. The rats were immobilized in a stereotaxic frame in the flat skull position, and midsagittal skin incision was made on the scalp for 2 cm length to expose the skull. An infusion set was prepared, consisting of a sterilized 26-gauge stainless steel cannula and hypodermic tube, which, in turn, was connected to a 10 microliter Hamilton microsyringe (P/N: 80300/00 Hamilton Bonaduz AG, CH-7402, Switzerland).

The following stereotaxic coordinates were used to target the dorsolateral part of the striatum: AP = 0.2 mm, ML = 3.2 mm, and DV = 4.5 mm from the bregma. Another target was the dorsomedial part of striatum, reached by the following coordinates of AP = 1.1 mm, ML = 2.4 mm, and DV = 3.5 mm. These targets of lesions were reached using the stereotaxic atlas of Paxinos and Watson.^[5] After making the AP and ML coordinates, a hole was made on the skull with dental drill. Then, the cannula was placed through holes drilled in the skull and DV coordinates were used to target the area of striatum from the dura mater.

Drug and dosage

The 6-OHDA is a selective catecholaminergic neurotoxin that has been widely used to investigate the pathogenesis and progression of PD. 1 mg of 6-OHDA was dissolved in 0.5 ml of ascorbate saline (0.02 g of ascorbic acid in 10 ml of sterile normal saline). Just 30 min before the 6-OHDA injection, the desipramine HCl (i.p., 25 mg/kg body weight) was injected.

Mechanism of 2, 4, 5-trihydroxyphenylethylamine

In the CNS, the 6-OHDA produces marked alteration of both dopaminergic and noradrenergic neurons after injection into the parenchyma of the brain or into the brain cavities. The primary action is related to the direct destructive action of 6-OHDA on the neurons within 48 h after the treatment. When critical intraneural concentration of 6-OHDA or its metabolites is attained, destructive processes begin and cellular enzymes and energy-producing cytochromes or related elements of the respiratory transport chain could be destroyed.^[6]

Isolation of human amniotic epithelial cells

The human placenta was obtained from an uncomplicated elective cesarean section after obtaining the consent of the patient admitted at Andhra Mahila Saba Hospital, Adayar, Chennai-20. Isolation of HAE was followed according the method of Sakurgawa *et al.*^[7] Briefly, after separation from the placenta, the connective tissue was completely removed by scraping with cotton; membrane was then treated with 0.125% trypsin (Hi-media) three times each for 20 min. The HAE cells obtained after second and third treatment were cultured in Minimum Essential Medium (AT 006) or RPMI 1640 (AT 028) (HiMedia) medium supplemented with 10% fetal calf serum under a humidified atmosphere of 5% CO₂ in air at 37°C. The cells were counted using a hemocytometer with the trypan blue exclusion method before transplanting into the lesioned striatum.

Labeling of human amniotic epithelial cells with DiI (lipophilic membrane stain)

After 5-7 days of culture, the cultured HAE cell suspension was mechanically dissociated into single cell suspension using phosphate-buffered saline. Before grafting, an aliquot of the cell suspension was assessed for viability and concentration by trypan blue exclusion method. The viability of the cells was 85% just before the transplantation. The cultured HAE cells were labeled with fluorescent marker, 1-1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) and the cultured cell suspension was incubated for 30 min just prior to the transplantation into the lesioned striatum.

Human amniotic cell transplantation

About 5-10 µl of cell suspension (2×10^4 cells/µl) was stereotaxically injected into the denervated striatum of rats using a 10 µl Hamilton microsyringe fitted with a steel cannula. The cultured HAE cells were transplanted using following coordinates: AP = 0.2, ML = 2.7, DV = 5.5 and 2. AP = 1.1, ML = 2.7, and DV = 5.5.^[5] The injection was made at the rate of 1 µl/min. Immediately after the HAE cell transplantation, the cyclophosphamide was given at the dose of 5 mg/kg body weight for 3 days to prevent the graft versus host rejection.

Apomorphine-induced rotation

The apomorphine-induced rotation was carried out 10–12 days after the 6-OHDA lesions. 0.05 mg/kg/body weight of apomorphine hydrochloride (a 4393, Sigma) was injected through subcutaneous route in the neck region. We followed the basic principles of rotational behaviour study described by Olsson *et al.*,^[8] Since we could not design the 'automatic' rat rotometer; we simplified the observation on rotational behavior. Each animal was placed in a glass cylinder measuring 30 cm height and 22 cm diameter, and the number of rotation of the animals in cylinder was counted for a period 50 min.

Biochemical assay

Enzymic and nonenzymic antioxidants

Assay of superoxide dismutase

The superoxide dismutase (SOD) activity was estimated based on the method described by Marklund and Marklund.^[9] Briefly, to make the assay mixture, 1 ml of pyrogallol (0.4 mM) air-saturated in Tris-HCl buffer (0.1 M, pH 8.2), ethylenediaminetetraacetic acid (EDTA) (1 mM), and diethylenetriaminepentaacetic acid (DETPA2 mM) were taken in separate test tube. To this mixture, 0.2 ml homogenate and 0.8 ml of water were added. The rate of pyrogallol autoxidation was taken from the increase in absorbance at 420 nm at 0, 1, and 3 min against the blank. The blank containing 0.2 ml of distilled water was also treated in the same way against a buffer blank.

Assay of catalase

The activity of catalase (CAT) was assayed by the method of Sinha.^[10] 1 ml of triplicates of tissue homogenate triplicates was taken in separate tubes and 5 ml of phosphate buffer (0.01 M, pH 7.0). 4 ml of H₂O₂ (0.2 M) was added to these tubes to initiate reaction. The reaction was arrested immediately by adding 2.0 ml of dichromate-acetic acid reagent (5% solution of potassium dichromate in acetic acid [1:3]) at 0, 30, and 60 s intervals. The blank was prepared by addition of 1 ml of buffer and 2.0 ml of dichromate acetic acid. The test and blank tubes were then heated for 10 min in a boiling water bath to develop green color. After cooling to room temperature, the intensity was measured at 570 nm against the blank.

Assay of glutathione peroxidase

The activity of glutathione peroxidase (GPx) was assayed by the method of Rotruck *et al.*^[11] 0.1 ml of triplicate samples triplicate was mixed with 0.4 ml of phosphate buffer, 0.2 ml each of EDTA (0.8 mM), sodium azide (10 mM), and H₂O₂ (2.5 mM) and was incubated at 37°C. Subsequently, 0.2 ml of GSH (3 mM) was added to all these tubes and the reaction was arrested by the addition of (trichloroacetic acid [TCA], 10%) at 0, 1.5, and 3 min intervals. The tubes were then centrifuged at

2000 rpm and 1.0 ml of the supernatant was transferred to fresh tubes. 1 ml of blank (distilled water) and the GSH standard (stock: 200 µg/ml) at a concentration range of 5–20 µg were also taken in separate tubes. To all the above tubes, 4ml of disodium hydrogen phosphate (0.3 M) and 0.5ml of DTNB -5-5'-dithio bis 2 nitrobenzoic acid substrate (DTNB - 0.6mM in 1% trisodium citrate) were added. The colour developed was read at 420nm immediately against the blank.

Estimation of reduced glutathione

The reduced glutathione GSH level was measured by the method of Moron *et al.*^[12] One ml of homogenate was precipitated with 1 ml of TCA 10% and the precipitate was removed by centrifugation. To 0.5ml of supernatant, 2ml of DTNB [0.6 mM 5,5'-dithio bis (2-nitrobenzoic acid) in 0.2 M sodium phosphate buffer] was added and the total volume was prepared up to 3ml with phosphate buffer (0.2M, pH 8.0). The absorbance was read at 412 nm.

Estimation of ascorbic acid (Vitamin C)

The level of ascorbic acid was estimated by the method of Omaye *et al.*^[13] To 0.5 ml of homogenate, 0.5 ml of water and 1 ml of TCA-5% were added, mixed thoroughly, and centrifuged. To 1 ml of the supernatant, 0.2 ml of DTC reagent (3 g of 2,4-dinitrophenyl hydrazine, 0.4 g of thiourea, and 0.05 g of copper sulfate in 100 ml of 9N sulfuric acid) was added and incubated at 37°C for 3 h. After incubation, 1.5 ml of sulfuric acid (65%) was added, mixed well, and the solutions were allowed to stand at room temperature for another 30 min. The yellowish orange color developed was measured at 520 nm against blank.

Estimation of Vitamin E (α-tocopherol)

The level of Vitamin E (α-tocopherol) was estimated by the method of Desai.^[14] To 1 ml of tissue homogenate, α-tocopherol acetate (1 mg/ml) as a standard and blank (distilled water) was taken separately in centrifuge tubes, and 1 ml of ethanol was added and thoroughly mixed. Then, 3 ml of petroleum ether was added, shaking rapidly, and centrifuged. 2 ml of supernatant was taken and evaporated to dryness. To this, 0.2 ml of bathophenanthroline (0.2% 4,7-diphenyl-1,10-phenanthroline in ethanol) was added. The assay mixture was protected from light. Then added 0.2 ml of ferric chloride (0.001 M in ethanol) followed by 0.2 ml of Phosphoric acid (0.001 M in ethanol) and the total volume was made up to 3 ml with ethanol. The color formed was read at 530 nm.

Estimation of protein

Protein was estimated by the method of Lowry *et al.*^[15] The alkaline copper reagent was prepared by mixing 50 ml of solution A (2% sodium carbonate in 0.1 N NaOH) and solution B (0.5% copper sulfate in 1% sodium potassium tartrate). 0.1 ml of tissue homogenate and standard bovine

serum albumin taken at a concentration ranging from 10 to 40 μg was made up to 1.0 ml with distilled water. To each of tubes, 4.5 ml of alkaline copper reagent was added and kept at room temperature for 10 min. Subsequently, 0.5 ml of Folin and Ciocalteu's phenol reagent (1:2 dilution) was added and allowed to stand at room temperature. The color developed was read after 20 min at 640 nm against blank.

Lipid peroxidation assay

The activity of lipid peroxidation (LPO) was estimated as described by Ohkawa *et al.*^[16] 0.2 ml of tissue homogenate, reagent blank (0.8ml of distilled water) and malondialdehyde standard (concentration range of 3–12 nmoles) were mixed with 0.2 ml of Sodium dodecyl sulfate (8.1%), 1.5 ml of acetic acid (20%, pH 3.5) and 1.5 ml of thiobarbituric acid (0.8%). This mixture was made up to 4 ml with distilled water and heated in boiling water bath at 95°C for 60 min. After cooling in tap water, 1 ml of distilled water and 5 ml of n-butanol/pyridine mixture (15:1 v/v) were added. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured.

Results

Biochemical observation

Apomorphine-induced rotations

In the present experiment, the sham-lesioned animals (Group II) did not show any abnormal rotation after the apomorphine injection in all the duration of rotational behavior test. In Group III animals, there was a significant increase in contralateral rotation (1st week 26.60 folds; 2nd week 27.04 folds; 3rd week 25.75 folds; and 4th week 25.72 folds) when compared with the nonlesioned control animals in all the period. The Group IV animals showed decrease in contralateral rotation by 96% in the 1st week, 97% in the 2nd week, 98% in 3rd week, and 97% in 4th week when compared with the Group III animals [Table 1]. In contrast to Group III, reduced asymmetric rotation in the Group IV animal would depend on placement and interaction of the HAE cell graft within the denervated striatum.

Enzymic and nonenzymic antioxidants

For the maintenance of normal function, cells have developed several antioxidant defense mechanisms. The accumulated evidence both *in vitro* and *in vivo* suggest that the toxicity of 6-OHDA involved the generation of ROS, since 6-OHDA could auto-oxidize to form semiquinone and superoxide anion (O_2^-), which could subsequently be converted to the more cytotoxic hydroxyl radical ($\bullet\text{OH}$) through interaction with H_2O_2 .

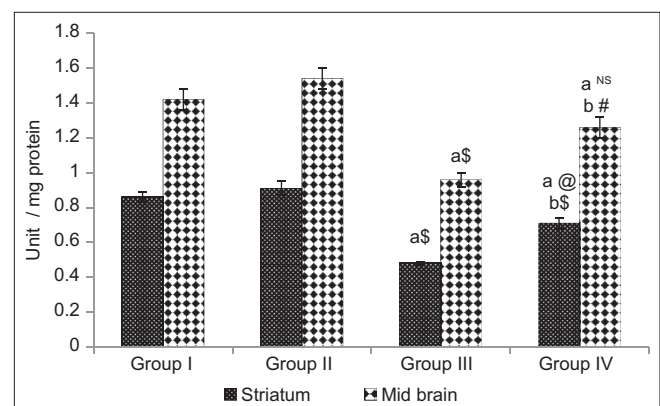
Superoxide dismutase

The mean value of SOD in the striatum of normal rats was 0.86 ± 0.03 units/mg protein. In 6-OHDA-lesioned

rats after 150 days was 0.48 ± 0.01 units/mg protein and in lesioned and transplanted animals after 150 days was 0.71 ± 0.03 units/mg protein. The level of SOD in striatum was decreased (44%) in 6-OHDA lesioned animals when compared with non lesioned normal animals. However in HAE cells implanted animals SOD was significantly increased (48%) compared with the lesioned animals. The mean value of SOD in the midbrain of normal rats was 1.42 ± 0.06 units/mg protein. In 6-OHDA lesioned rats after 150 days was 0.96 ± 0.04 units/mg protein, and in lesioned and transplanted animals after 150 days was 1.26 ± 0.06 units/mg protein. In midbrain the level of SOD is slightly reduced (32%) in 6-OHDA lesioned animals when compared to the control animals, whereas in HAE cells grafted animals SOD level moderately increased (31%) over the lesioned animals [Graph 1].

Catalase

The mean value of CAT in striatum of normal rats was 2.30 ± 0.11 μmoles of H_2O_2 consumed/min/mg protein in the striatum. The CAT was also reduced in the 6-OHDA lesioned animals. This was because of the increase in LPO due to the production of free radicals in the brain regions. The mean value of CAT normal mid brain was 3.48 ± 0.16 μmoles of H_2O_2 consumed/min/mg protein. In this study the level of CAT was reduced both in the striatum to 31% (Mean = 1.59 ± 0.06 μmoles of H_2O_2 consumed/min/mg protein) and in midbrain 24 % (mean = 2.64 ± 0.12 μmole of H_2O_2 consumed/min/mg protein) of the 6-OHDA lesioned animals when compared to the non lesioned animals. However in the HAE cells transplanted animals, CAT increased in striatum (32%) (Mean 2.10 ± 0.11 μmoles of H_2O_2 consumed/min/mg protein) and midbrain (22%) (Mean 3.23 ± 0.14 μmole of H_2O_2 consumed/min/

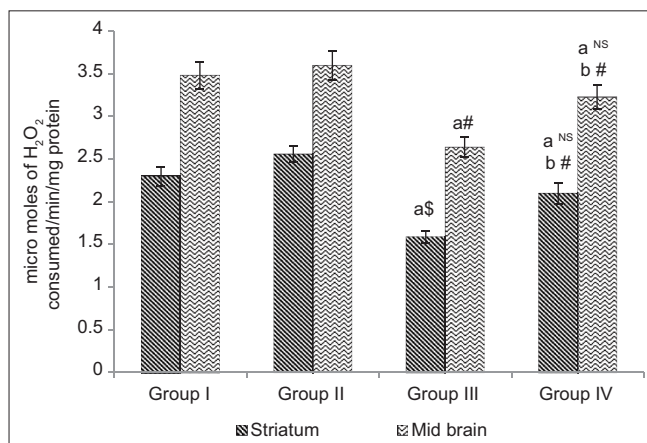


Graph 1: Superoxide dismutase activity in striatum and midbrain of 2,4,5-trihydroxylphenylethylamine lesioned and human amniotic epithelial cells-transplanted animals. Superoxide dismutase activity is expressed as units/mg protein (1 U = amount of enzyme that inhibits the auto-oxidation of pyrogallol by 50%). The columns represent mean, and error bars are denoted standard error of mean. Group I: Control; Group II: Sham control; Group III: 2,4,5-trihydroxylphenylethylamine lesioned; Group IV: 2,4,5-trihydroxylphenylethylamine Lesioned and human amniotic epithelial cells transplanted. a- comparison between Group I vs. III & IV; b- comparison between Group III vs. IV; NS- not significant; P \leq 0.001- $\$$; P \leq 0.01- $\#$; P \leq 0.05- $\text{\textcircled{a}}$

mg protein), when compared with the 6-OHDA lesioned animals [Graph 2].

Glutathione peroxidase

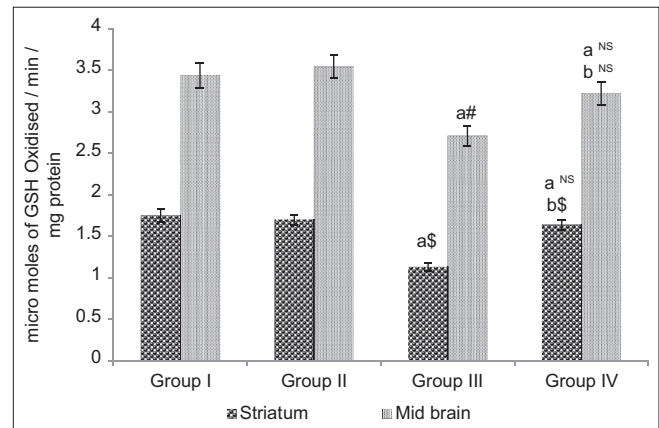
In the normal animals the value of GPx was $1.75 \pm 0.07 \mu$ moles oxidized/min/mg protein in striatum. In striatum of 6-OHDA lesioned animals the level of GPx was decreased (35%) (Mean 1.13 ± 0.04 as μ moles oxidized/min/mg protein), when compared with the control animals. However its level was significantly increased (45%) (Mean 1.64 ± 0.06 μ moles oxidized/min/mg protein), in the striatum of HAE cells grafted animals. In the normal animals the value of GPx was 3.44 ± 0.07 μ moles oxidized/min/mg protein in midbrain. The level of GPx in midbrain of 6-OHDA lesion animals also decreased (21%) (Mean 2.71 ± 0.12 μ moles oxidized/min/mg protein), when compared with the non-lesioned control animals, whereas in the transplanted animals it was slightly increased (19%) (Mean 3.22 ± 0.06 μ moles oxidized/min/mg protein), compared with the lesioned animals [Graph 3].



Graph 2: Catalase activity in striatum and midbrain of 2, 4, 5-trihydroxyphenylethylamine-lesioned and human amniotic epithelial cell-transplanted animals. Catalase activity is expressed in micromoles of H₂O₂ consumed/min/mg protein. The columns represent mean, and error bars indicate standard error; Group I: Control; Group II: Sham control; Group III: 2,4,5-trihydroxyphenylethylamine lesioned; Group IV: 2,4,5-trihydroxyphenylethylamine Lesioned and human amniotic epithelial cells transplanted. a- comparison between Group I vs. III & IV; b- comparison between Group III vs. IV; NS- not significant; P<0.001-\$; P<0.01-#; P<0.05-@

Reduced glutathione

The mean value of GSH was 0.026 ± 0.001 μ moles of GSH oxidized /min/mg protein in striatum and 0.059 ± 0.003 μ moles of GSH oxidized/min/mg protein in the midbrain of normal rats. In the present work the level of reduced GSH was decreased in striatum (35%) (Mean 0.017 ± 0.001 μ moles oxidized /min/mg protein), and in midbrain (24%) (Mean 0.045 ± 0.003 μ moles of GSH oxidized/min/mg protein) of lesioned group when compared with the non lesioned control animals. However in the HAE cells transplanted animals it was observed that GSH levels increased in both the striatum (30%) (Mean 0.022 ± 0.001 μ moles oxidized/min/mg protein), and midbrain (29%) (Mean 0.057 ± 0.003 μ moles of GSH oxidized/min/mg protein), when compared with the 6-OHDA lesioned animals. The GSH could also be tainted due to oxidative stress to dopaminergic neurons in the striatum and midbrain, which is believed to be one of the leading causes of neurodegeneration in PD [Graph 4].



Graph 3: Glutathione peroxidase activity in striatum and midbrain of 2, 4, 5-trihydroxyphenylethylamine-lesioned and human amniotic epithelial cell-transplanted animals. Glutathione peroxidase activity is expressed in micromoles of glutathione (reduced glutathione) oxidized/min/mg protein. The bars represent mean, and error bars denote standard error; Group I: Control; Group II: Sham control; Group III: 2,4,5-trihydroxyphenylethylamine lesioned; Group IV: 2,4,5-trihydroxyphenylethylamine Lesioned and human amniotic epithelial cells transplanted. a- comparison between Group I vs. III & IV; b- comparison between Group III vs. IV; NS- not significant; P<0.001-\$; P<0.01-#; P<0.05-@

Table 1: Rotation for 60 min after apomorphine induced in 2, 4, 5-trihydroxyphenylethylamine lesion and human amniotic epithelial cell transplantation from 1st week to 4th week

Group	1 st week	2 nd week	3 rd week	4 th week
Group I	9.00±1.29	10.50±0.42	11.50±0.95	11.33±1.22
Group II	9.00±0.81	7.50±0.95	6.83±0.40	6.66±0.66
Group III	255.66±18.17 ^{a,***}	305.00±19.76 ^{a,***}	319.33±26.77 ^{a,***}	314.16±20.92 ^{a,***}
Group IV	9.50±1.11 ^{a, NS, b,***}	9.16±0.79 ^{a, NS, b,***}	8.33±0.21 ^{a, NS, b,***}	9.33±0.49 ^{a, NS, b,***}

^aComparison between Group I versus III and IV, ^bComparison between Group III versus IV, ^{***}P<0.001. Values were measured in number of rotation for 60 min after 0.05 mg/kg/sc apomorphine. Each value represents mean±standard error of six rats. Group I: Control, Group II: Sham control, Group III: 6-OHDA lesioned, Group IV: HAE cell transplant. NS: Not significant, 6-OHDA: 2, 4, 5-trihydroxyphenylethylamine, HAE: Human amniotic epithelial

Vitamin C

The vitamin C level was $0.037 \pm 0.001 \mu\text{g}/\text{mg}$ protein in striatum and $0.29 \pm 0.01 \mu\text{g}/\text{mg}$ protein in the midbrain of normal rats. In this experiment the level of vitamin C was decreased in both the striatum (35%) (Mean $0.24 \pm 0.001 \mu\text{g}/\text{mg}$ protein), and the midbrain (41%) (Mean $0.17 \pm 0.001 \mu\text{g}/\text{mg}$ protein) after the 6-OHDA infusion in rats when compared with the control animals. However the level of vitamin C in HAE cells transplanted animals were increased in the striatum (33.3%) (Mean $0.32 \pm 0.01 \mu\text{g}/\text{mg}$ protein) and in the midbrain (47%) (Mean $0.25 \pm 0.001 \mu\text{g}/\text{mg}$ protein) when compared with the 6-OHDA lesioned animals. Neurodegeneration in PD could also be linked to be deficiency of antioxidant components such as folic acid, vitamin A, C and E in the body and this would give rise to progressive dopaminergic degeneration in the PD [Graph 5].

Vitamin E

In this study, the level of Vitamin E in striatum of 6-OHDA-lesioned animals was decreased (31%) (mean = $0.97 \pm 0.05 \mu\text{g}/\text{mg}$ protein), when compared with the control animals (mean = 1.41 ± 0.06). However, in HAE cell-transplanted animals, the level of Vitamin E was increased (29%) (mean = $1.25 \pm 0.05 \mu\text{g}/\text{mg}$ protein), when compared with the 6-OHDA animals. In midbrain also, the level of Vitamin E was decreased (26%) (mean = $1.70 \pm 0.07 \mu\text{g}/\text{mg}$ protein) in lesioned animals when compared to normal animals (mean = $2.29 \pm 0.10 \mu\text{g}/\text{mg}$ protein), whereas in HAE cell-grafted animals, it was

increased (22%) (mean = $2.08 \pm 0.09 \mu\text{g}/\text{mg}$ protein), than the 6-OHDA-lesioned animals. The Vitamin E attenuates the 6-OHDA-induced toxic effects in the corpus striatum of rats [Graph 6].

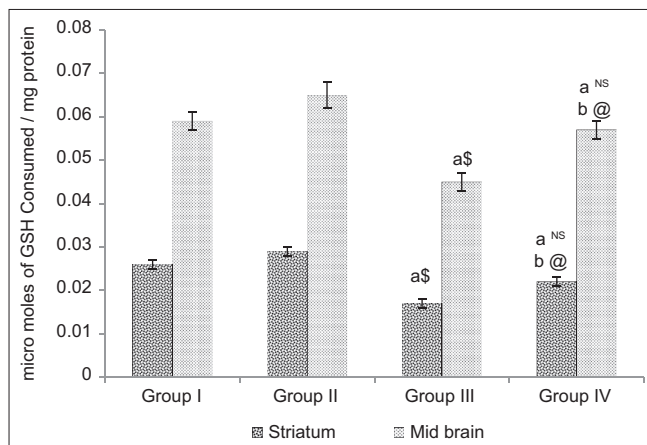
Lipid peroxidase

The mean value of LPO in the striatum of normal rats was 0.89 ± 0.03 micromoles of MDA formed/mg/protein. In 6-OHDA lesioned rats after 150 days were 1.29 ± 0.04 micromoles of MDA formed/mg/protein and in lesion and transplanted animals after 150 days was 0.83 ± 0.03 micromoles of MDA formed/mg/protein. The LPO in the striatum of 6-OHDA lesioned rats was increased (45%) when compared to the control animals. However in HAE cells transplanted animals LPO level was moderately decreased (35%) when compared with 6-OHDA lesioned animals. The mean value of LPO in the midbrain of normal rats was 0.68 ± 0.03 micromoles of MDA formed/mg/protein. In 6-OHDA lesioned rats after 150 days was 0.94 ± 0.04 micromoles of MDA formed/mg/protein, and in lesion and transplanted animals after 150 days was 0.75 ± 0.04 micromoles of MDA formed/mg/protein. In midbrain also the LPO significantly increased in the 6-OHDA lesioned animals (38%) when compared with the control animals, whereas in HAE cells transplanted animals it was reduced (20%) compared to the lesioned animals [Graph 7].

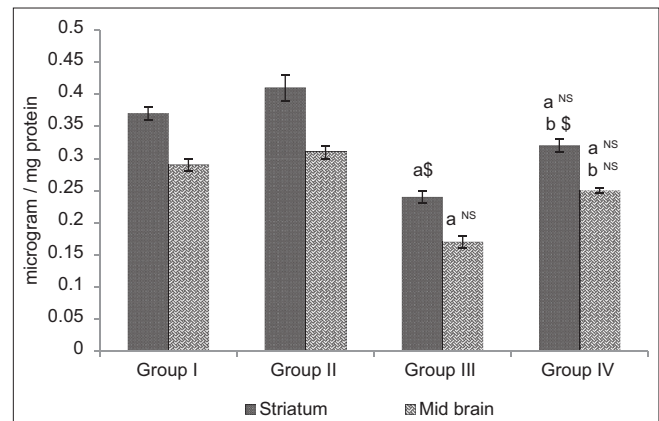
Discussion

Apomorphine-induced rotations

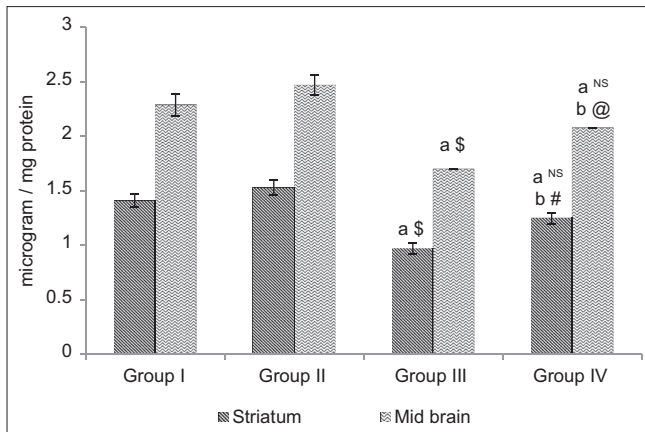
We assessed the motor function using apomorphine-induced rotation test. This test is considered as reliable objective and closely related to the degree of nigrostriatal



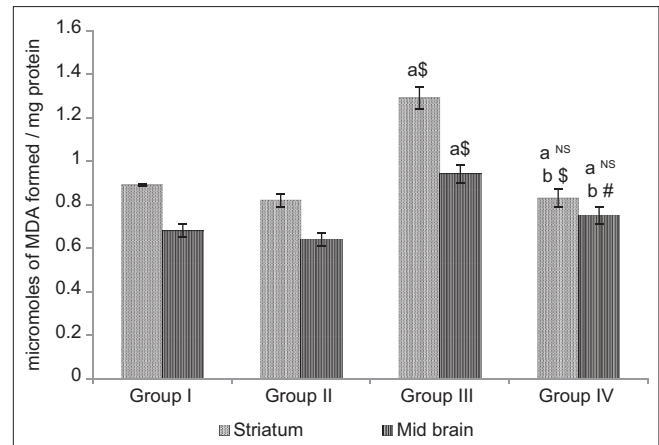
Graph 4: Reduced glutathione activity in striatum and midbrain of 2, 4, 5-trihydroxyphenylethylamine-lesioned and human amniotic epithelial cell-transplanted animals. Exhibits reduced glutathione activity expressed as micromoles of glutathione consumed/mg protein in striatum and midbrain of 2, 4, 5-trihydroxyphenylethylamine-lesioned and human amniotic epithelial cell-transplanted animals. The columns are represented as mean and error bars are denoted standard error of mean. Group I: Control; Group II: Sham control; Group III: 2,4,5-trihydroxylphenylethylamine lesioned; Group IV: 2,4,5-trihydroxylphenylethylamine lesioned and human amniotic epithelial cells transplanted. a- comparison between Group I vs. III & IV; b- comparison between Group III vs. IV; NS- not significant; $P \leq 0.001$ - $\$$; $P \leq 0.01$ - $\#$; $P \leq 0.05$ - $@$



Graph 5: Vitamin C (ascorbic acid) activity in striatum and midbrain of 2, 4, 5-trihydroxyphenylethylamine-lesioned and human amniotic epithelial cell-transplanted animals. Level of vitamin C in striatum and midbrain of 2, 4, 5-trihydroxyphenylethylamine-lesioned and human amniotic epithelial cell-transplanted animals. The values are expressed in microgram/mg protein. Each column represents mean and error bars denote standard error of mean; Group I: Control; Group II: Sham control; Group III: 2,4,5-trihydroxylphenylethylamine lesioned; Group IV: 2,4,5-trihydroxylphenylethylamine lesioned and human amniotic epithelial cells transplanted. a- comparison between Group I vs. III & IV; b- comparison between Group III vs. IV; NS- not significant; $P \leq 0.001$ - $\$$; $P \leq 0.01$ - $\#$; $P \leq 0.05$ - $@$



Graph 6: Vitamin E activity in striatum and midbrain of 2, 4, 5-trihydroxyphenylethylamine-lesioned and human amniotic epithelial cell-transplanted animals. Level of vitamin E in striatum and midbrain of 2, 4, 5-trihydroxyphenylethylamine-lesioned and human amniotic epithelial cell-transplanted animals. The values are expressed in microgram/mg protein. Each column represents mean and error bars denote standard error of mean; Group I: Control; Group II: Sham control; Group III: 2,4,5-trihydroxyphenylethylamine lesioned; Group IV: 2,4,5-trihydroxyphenylethylamine lesioned and human amniotic epithelial cells transplanted. a- comparison between Group I vs. III & IV; b- comparison between Group III vs. IV; NS- not significant; P<0.001-\$; P<0.01-#; P<0.05-@



Graph 7: Level of lipid peroxidation in striatum and midbrain of 2,4,5-trihydroxyphenylethylamine lesioned and human amniotic epithelial cells-transplanted animals. Exhibits status of lipid peroxidation measured malondialdehyde levels in striatum and midbrain of 2,4,5-trihydroxyphenylethylamine lesioned and human amniotic epithelial cells-transplanted animals. The columns are represented as mean and error bars are denoted standard error of mean. Group I: Control; Group II: Sham control; Group III: 2,4,5-trihydroxyphenylethylamine lesioned; Group IV: 2,4,5-trihydroxyphenylethylamine lesioned and human amniotic epithelial cells transplanted a- comparison between Group I vs III & IV; b- comparison between Group III vs. IV; NS- not significant; P<0.001-\$; P<0.01-#; P<0.05-@

dysfunction, as well as DA depletion. Kumar *et al.*, have demonstrated that the intra-striatal injection of 6-OHDA causes retrograde degeneration of SN dopaminergic neurons, resulting in depletion of the striatal dopamine.^[17] In HAE cell-transplanted animals, restoration of motor function preservation of nigro striatal neuronal pathway is considered to be an important neuroprotective mechanism required for pharmacological and biogenic agents.

Biochemical observation

Antioxidants

The brain is thought to be vulnerable to oxidative damage due to its high oxygen consumption, presence of high levels of polyunsaturated fatty acids, and the nonregenerative nature of neurons.^[18] Auto-oxidation of dopamine and 6-OHDA produces H_2O_2 , which is subsequently converted to hydroxyl radical by Fe^{2+} , causing fragmentation of the lipid or alteration of its chemical structure.^[19] Lipid hydroperoxides inhibit re-cyclation process in the neuronal membranes.^[20] The present results revealed that the LPO level was higher in the 6-OHDA-lesioned animals, which reflects the decrease of phospholipids due to interaction of the 6-OHDA with cell membrane that may enhance LPO. As a result of this process, the level of Vitamin C and Vitamin E was reduced due to the production of free radicals in the damaged dopaminergic neurons in the striatum and midbrain of 6-OHDA-lesioned animals. The present results suggest that increased free radicals and the decrease in the antioxidant defense system possibly lead to structural and functional alterations in membrane-related events and play a significant role in the 6-OHDA-induced neurotoxicity.

The GSH system is responsible for removing free radicals and maintaining protein thiols in their appropriate redox state in the cytosol and mitochondria and is an important protective mechanism for minimizing oxidative stress.^[21] The antioxidant enzymes, such as SOD, Cat, GPx, GR, and GST, are also important mediators that reduce the overload of oxidative stress. Because these enzymes are related to ROS, in the present experiment, the decrease of antioxidant could be due to an increased extent of LPO. The HAE cell-transplanted animals showed moderate increase in both the enzymatic and nonenzymatic enzymes in striatum and midbrain. This finding may suggest that the increase in the level of antioxidants and decrease in the level of LPO are based on enhancement of remaining survival neurons through the factors secreted by the graft in the striatum.

Lipid peroxidation

In the present work, the level of LPO was increased considerably in striatum and midbrain after the 6-OHDA lesion, which supports the previous work after the 6-OHDA lesion. The increase in LPO might be due to either enhanced free radical generation or depletion in antioxidant defense capacity, following 6-OHDA administration.^[22] In HAE cell-transplanted animals, the level of LPO is significantly reduced in striatum (36%) and moderately reduced (20%) in midbrain from the lesioned animals. The neurotransmitters and their metabolites were studied using high-performance liquid chromatography electrochemical detection method and it was published.^[23]

Conclusion

This work involves the behavioral assessment after the transplantation of HAE cells in 6-OHDA-lesioned striatum. The 6-OHDA-induced neurotoxicity by the generation of free radicals was pronounced by indication of the increased LPO level, decreases in antioxidants, and alteration in the dopamine and its metabolites. These alterations were found to be recovered after the HAE cell graft during the long time period. Further molecular studies would be merit to validate the survival and integration of HAE cells with the host striatum. We authenticate that the HAE cells would help alleviate the various degenerative diseases such as PD and Alzheimer's disease as a potential source of stem cell.

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Ethical clearance

The project approval number is IAEC No 01/008/03.

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Conflicts of interest

There are no conflicts of interest.

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