

Behavioral and Histological Observations after the Human Amniotic Epithelial Cells Transplantation in the 6-Hydroxydopamine Induced Parkinsonism Disease Model in Wistar Albino Rats

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Research Article

Abstract: Several toxin-induced animals' models simulate the motor deficits occurring in PD. Among them, the unilateral 6-hydroxydopamine (6-OHDA) model is frequently used in rats and has the advantage of presenting side-biased motor impairments. The studies on impairment and improvement of the motor and sensory motor behaviors after the retrograde degeneration with the HAE cells implantation are less or unavailable. In the present work we have studied the amelioration of motor and sensory motor behaviors after the implantation of human amniotic epithelial (HAE) cells in 6-hydroxydopamine (6-OHDA) lesioned rodent model of PD induced rats. The Stereotaxic injection of saline alone (without 6-OHDA) in the striatum could not produce any detectable effects on the behaviors and histological observations. This study demonstrates that substantial and stable improvement of behaviors after the HAE transplantation in unilateral 6-OHDA-induced lesions can be established in rodent model of PD disease, and that these can be functionally assessed using several different behavioral tests. This would suggest that the HAE cells may be a suitable donor tissue to alleviate various degenerative diseases in animal model before the clinical trial in human who are suffering from the various degenerative diseases. The present study indicates that the transplantation of HAE cells could be a viable therapeutic strategy to slow down the progressive degeneration of striatal DA neurons in the animal model of PD. Thus the neurological degenerative disease can be treated by the grafting the HAE cells and can be attempted for faster and sustained recovery of functional loss in the human clinical trials.

Keywords: Parkinson's disease; 6-OHDA lesion, Human amniotic epithelial cells, DiI; Apomorphine induced rotations; stride length, rotarod.

1. Introduction

Parkinson's disease (PD) is characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) with the appearance of Lewy bodies and the subsequent degeneration of the nigro-striatal pathway leading to a loss of striatal dopamine (DA) content [1]. Several centers have reported that uncontrolled open-label trials of human fetal nigral tissue transplantation in PD. These studies have demonstrated the transplants placed in the DA denervated striatum can provide a significant, but not complete, clinical benefits in PD patients [2]. In spite of the recognized quantitative loss of nigrostriatal neurons and their DA content, the exact etiology and process of PD neurodegeneration remain undetermined. Both the genetic and the environmental factors have been implicated as likely causes of PD [3, 4 and 5]. The nigrostriatal damage caused by neurotoxins used in animal models of PD, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone or 6-hydroxydopamine (6-OHDA), has been associated with intense glial activation, as well as lymphocyte infiltration [6,7,8]. Thus, immunity-related processes may represent key players in the cascade events leading to neuronal degeneration and the idea that modulating this response may yield neuroprotection is currently attracting considerable interest [9]. The partial dopamine depletion by local injection of 6-OHDA in striatum has been shown to be a good model of early and

moderate stages of PD [10]. These studies have noted dose-dependent deficits in striatal dopaminergic projection and corresponding behavioral abnormalities. In *in-vitro* studies on human amniotic epithelial (HAE) cells support that the expression of markers for the differentiation of progenitor cells for liver, pancreas, cardiomyocytes and neural tissues [11]. Recent studies aimed at defining the stem cell-like characteristics of HAE cells show that these cells express the surface markers normally associated with embryonic stem cells e.g. SSEA-3, SSEA-4, (stage-specific embryonic antigen) TRA (tumor rejection antigen)-1-60, and TRA- 1-81. These cells also express pluripotent stem cell for the specific transcription factors such as Oct-4 and Nanog [11, 12]. Niknejad *et al.*, have reported that the serum-free condition decreased the viability of HAE cells but increased the rate of neural markers expression [13]. Hsieh *et al.*, have studied the decreased walking speed and stride length after the 6-OHDA induced hemiparkinsonism rats [14]. In our laboratory we have established the transplantation of HAE cells in the spinal cord injury and the recovery of the motor functions [15]. Therefore we had focused to study the muscular coordination in Rotarod, Stride length (SL) and correlated with the histology after 6-OHDA lesion and HAE cells transplantation in the striatum of rat model of PD. The placental tissue, which is normally discarded, may be a use full source of cells for transplantation and regenerative medicine.

2. Material and Methods

2.1. Animals

Adult male Wistar albino rats weighing 180 to 220gm of body weight at the beginning of the experiment were used. They were housed in pairs and allowed for 7 to 10 days to acclimatize to the animal care facility before the behavioral tests and surgery. They were maintained in a room at constant temperature and humidity (21°C to 26°C) and 12h light and 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.

2.2 Experimental Groups

S. No	Group	Experiments	No of Animals
1	Group I	Control	12
2	Group II	Sham Control (Ascorbic saline)	12
3	Group III	6-OHDA lesioned	12
4	Group IV	6-OHDA lesioned and HAE cells transplanted	12

2.3 Stereotaxic co-ordinates

The animals were anaesthetized by intra-peritoneal administration of Pentothal sodium at the dose of 40 mg/kg body weight. The rats were immobilized in a stereotaxic frame in the flat skull position and mid-sagittal skin incision was made on the scalp for 2cm length to expose the skull. An infusion set was prepared, consisting of a sterilized 26gauge stainless steel cannula and hypodermic tube, which in turn, was connected to a 10 microlitre Hamilton micro syringe (P/N: 80300/00 Hamilton Bonaduz AG, CH-7402, Switzerzlerland). The stereotaxic coordinates used to place the cannula were AP = 0.2mm, ML=3.2mm, DV = 4.5mm from the Bregma. With these co-ordinates the dorsolateral part of the striatum was targeted. Another target was the dorso-medial part of striatum, reached by the co-ordinates of AP = 1.1mm, ML = 2.4mm and DV =3.5mm. These targets of lesions were reached using the stereotaxic atlas of Paxinos and Watson[16]. After making the co-ordinates, a hole was made on the skull with dental drill. The cannula was placed through holes drilled in the skull and advanced so that the internalized tip was located within the striatum.

2.4. Drug and dosage

The 6-OHDA is a selective catecholaminergic neurotoxin widely used to investigate the pathogenesis and progression of PD. The specificity of 6-OHDA neurotoxicity has been associated with its uptake and accumulation by transport mechanism specific for catecholaminergic neurons. One mg of 6-OHDA was dissolved in 0.5ml of Ascorbate saline. Just 30 min before the 6-OHDA injection the desipramine hydrochloride (25mg/kg/b/wt/i.p) was injected. This partial striatal lesion induces a moderate and more progressive dopamine depletion compared with the more severe and rapid lesion induced by a single injection to the medial forebrain bundle [17].

2.5. Isolation of HAE cells

The human placenta was obtained from an uncomplicated elective caesarean section after obtaining the consent of the patient admitted at Andhra Mahila Saba Hospital, Chennai, INDIA. Isolation of HAE cells was followed the method of Sankar and Muthusamy[15]. Briefly, after separation from the placenta the connective tissue was completely removed by scraping with the cotton, membrane was then treated with 0.125% trypsin (Hi-media) three times each for 20min. The HAE cells, obtained after second and third treatment, were cultured in Minimum Essential Medium MEM (AT 006) or RPMI 1640 (AT 028) (Hi media) medium supplemented with 10% fetal calf serum under a humidified atmosphere of 5% CO₂ in air at 37°C.

2.5.1. Labeling of HAE cell with DiI.

After 5 to 7 days of culture, the cell suspension was mechanically dissociated into single cell suspension using phosphate buffered saline (PBS). Before grafting, aliquot of the cell suspension was assessed for viability and concentration by trypan blue (T6146- Sigma-aldrich) exclusion method. The viability of the cell just before grafting was more than 85%. The cultured HAE cells were labeled with fluorescent marker 1, Iodotadeyl 3, 3, 3' 3' tetramethylindocarbocyanin per chlorate (DiI) (Molecular probe, dissolved in 100% methanol) and incubated for 30 min just prior to the transplantation in lesioned striatum.

2.5.2. HAE cells transplantation

5 to 10µl of cell suspension (2x10⁴cells/µl) were stereotaxically injected into the denervated striatum of the recipient rats, using a 10 µl Hamilton micro syringe fitted with a steel cannula. The following coordinates were used: 1. AP=0.2, ML=2.7, DV=5.5 and 2. AP=1.1, ML=2.7, DV=5.5 according to the atlas of Paxinos and Watson [16]. The injection was made at the rate of 1µl/min. After the completion of the injection, the cannula was left in situ for another five minutes before being slowly retracted. Immediately after the HAE cells transplantation, the cyclophosphamide at the dose of 5mg/kg/b/wt were given for 3 days to prevent the graft versus host rejection.

2.6. Apomorphine induced rotation

The apomorphine-induced rotation was carried out 10 to 12 days after the 6-OHDA lesions. The apomorphine hydrochloride (A 4393, Sigma) dose was 0.05-mg/kg body weight and injected through subcutaneous route in the neck region. We followed the basic principle of rotational behavior study described by

Ungerstedt [17, 18]and Olsson *et al.*, [19]. 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 30cm height and 22cm diameter and the number of rotation of the animals in cylinder were counted for a period 60 min (Fig. 1A).

2.7. Stride length

After training in wide runway, trained animals were allowed to run on a white sheet pasted on the wide runway. Just before allowing the animal, either forelimb or hindlimb’s paw were dipped in INDIAn ink, so that the footprints could be marked on the white sheet during running. The stride distance of forelimb and hindlimbs were measured in separate tests. The distance between the central pads of two steps of left forelimb or right forelimb were measured as a SL. Similarly the SL of hind limbs was also measured (Fig. 1C) [22].

2.8. Histology

Animals from all the experimental groups at 60th day 120th day of survival were sacrificed for the histological investigation of corpus striatum and SNpc. The animals were anesthetized with over dose of Pentothal sodium (120mg/kg/b/wt/ip) and perfused with normal saline followed by the formal saline for 20min period and immersed in 10% formalin for one week. The brains were removed and fixed for an additional four days in the same fixatives then processed for paraffin techniques. Serial sections were stained using ToluIdine Blue,Cresyl Fast Violet and Silver Nitrate.

2.9. Statistical analysis

The data expressed as Mean ± S.E.M was analyzed by analysis of variance (ANOVA) followed by Turkey test and *p* values < 0.05 were considered statistically significant using SPSS 14.0, USA.

3. Results

Table 1: Rotation for 50 minutes after apomorphine induced in 6-OHDA lesion and HAE cells transplantation from first week to fourth week

Group	1st week	2nd 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***

Values were measured in number of rotation for 50 minutes 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 cells transplant. 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 2: Stride length for left fore limb in wide runway after 6-OHDA lesion and HAE cells transplantation from 7th day to 150th days

Group	7 th day	15 th day	30 th day	60 th day	120 th day	150 th day
Group I	12.12 ± 1.02	13.24 ± 0.77	14.10 ± 0.48	14.65 ± 0.48	14.48 ± 0.47	14.24 ± 0.53
Group II	13.71 ± 0.71	14.34 ± 0.58	14.50 ± 0.72	15.12 ± 0.89	15.18 ± 0.80	14.83 ± 0.46
Group III	8.84 ± 0.50 a*	8.65 ± 0.64 a***	10.93 ± 0.49 a**	11.33 ± 0.057 a*	11.38 ± 0.45 a**	10.44 ± 0.51 a***
Group IV	11.50 ± 0.50 a ^{NS} b ^{NS}	12.58 ± 0.46 a ^{NS} b ^{NS}	13.30 ± 0.53 a ^{NS} b ^{NS}	13.08 ± 0.90 a ^{NS} b ^{NS}	14.75 ± 0.66 a ^{NS} b*	15.27 ± 0.44 a ^{NS} b***

Values were measured stride length in cm; Each value represents mean \pm standard error of six rats; Group I: Control; Group II: Sham control; Group III: 6-OHDA lesioned; Group IV: HAE cells transplant. a- comparison between Group I Vs III & IV; b- comparison between Group III Vs IV; NS- not significant; $P \leq 0.001$ ***; $P \leq 0.01$ ** ; $P \leq 0.05$ *

Table 3: Stride length for left hind limb in wide runway after 6-OHDA lesion and HAE cells transplantation from 7th day to 150th days

Group	7 th day	15 th day	30 th day	60 th day	120 th day	150 th day
Group I	13.45 \pm 0.35	13.84 \pm 0.32	13.01 \pm 0.10	12.93 \pm 0.54	13.49 \pm 0.43	13.86 \pm 0.26
Group II	13.91 \pm 0.50	13.75 \pm 0.56	13.31 \pm 0.42	13.36 \pm 0.42	13.78 \pm 0.67	13.57 \pm 0.45
Group III	9.97 \pm 0.36 a ***	9.50 \pm 0.65 a ***	10.14 \pm 0.35 a ***	9.68 \pm 0.33 a ***	10.96 \pm 0.66 a *	10.78 \pm 0.64 a **
Group IV	13.32 \pm 0.79 a NS b***	12.83 \pm 0.68 a NS b**	13.76 \pm 0.24 a NS b***	13.48 \pm 0.58 a NS b***	13.28 \pm 0.61 a NS b	13.67 \pm 0.59 a NS b***

Values were measured stride length in cm; Each value represents mean \pm standard error of six rats; Group I: Control; Group II: Sham control; Group III: 6-OHDA lesioned; Group IV: HAE cells transplant. a- comparison between Group I Vs III & IV; b- comparison between Group III Vs IV; NS- not significant; $P \leq 0.001$ ***; $P \leq 0.01$ ** ; $P \leq 0.05$ *

Table 4: Stride length for right forelimb in wide runway after 6-OHDA lesion and HAE cells transplantation from 7th day to 150th days

Group	7 th day	15 th day	30 th day	60 th day	120 th day	150 th day
Group I	14.12 \pm 0.45	14.12 \pm 0.16	14.65 \pm 0.69	15.58 \pm 0.50	14.46 \pm 0.30	14.22 \pm 0.47
Group II	13.67 \pm 0.64	14.21 \pm 0.53	14.02 \pm 1.00.	15.44 \pm 0.72	15.09 \pm 0.95	14.74 \pm 0.42
Group III	10.99 \pm 0.32 a ***	10.92 \pm 0.44 a ***	10.67 \pm 0.26 a **	10.99 \pm 0.51 a ***	10.72 \pm 0.30 a **	10.90 \pm 0.38 a ***
Group IV	13.22 \pm 0.44 a NS b*	13.75 \pm 0.28 a NS b***	14.18 \pm 0.56 a NS b**	14.14 \pm 0.66 a NS b**	15.03 \pm 0.52 a NS b***	14.93 \pm 0.61 a NS b***

Values were measured stride length in cm; Each value represents mean \pm standard error of six rats; Group I: Control; Group II: Sham control; Group III: 6-OHDA lesioned; Group IV: HAE cells transplant. a- comparison between Group I Vs III & IV; b- comparison between Group III Vs IV; NS- not significant; $P \leq 0.001$ ***; $P \leq 0.01$ ** ; $P \leq 0.05$ *

Table 5: Stride length for right hind limb in wide runway after 6-OHDA lesion and HAE cells transplantation from 7th day to 150th days

Group	7 th day	15 th day	30 th day	60 th day	120 th day	150 th day
Group I	12.25 \pm 0.42	12.90 \pm 0.66	13.54 \pm 0.63	13.12 \pm 0.71	13.17 \pm 0.45	13.92 \pm 0.64
Group II	13.49 \pm 0.36	13.18 \pm 0.65	13.58 \pm 0.59	13.39 \pm 0.66	13.73 \pm 0.43	13.76 \pm 0.29
Group III	9.82 \pm 0.25 a **	9.96 \pm 0.53 a *	10.42 \pm 0.42 a **	9.88 \pm 0.38 a *	10.05 \pm 0.34 a **	10.49 \pm 0.45 a ***
Group IV	12.56 \pm 0.50 a NS b***	13.48 \pm 0.62 a NS b**	13.60 \pm 0.53 a NS b**	13.03 \pm 0.97 a NS b*	13.53 \pm 0.84 a NS b***	13.95 \pm 0.25 a NS b***

Values were measured stride in cm; Each value represents mean \pm standard error of six rats; Group I: Control; Group II: Sham control; Group III: 6-OHDA lesioned; Group IV: HAE cells transplant. a- comparison between Group I Vs III & IV; b- comparison between Group III Vs IV; NS- not significant; $P \leq 0.001$ ***; $P \leq 0.01$ ** ; $P \leq 0.05$ *

Table 6: Neuronal cell density after 6-OHDA lesion and HAE cells transplanted animal's caudate-putamen in different periods

Group	30 th day	60 th day	150 th day
Group I	2183.00 \pm 44.64	2440.50 \pm 65.75	2409.50 \pm 28.60
Group II	2178.00 \pm 45.07	2455.83 \pm 37.54	2427.00 \pm 49.89
Group III	1543.66 \pm 57.12 a ***	1764.50 \pm 87.08 a ***	2177.00 \pm 26.80 a *
Group IV	2083.91 \pm 40.49 a*, b ***	2266.00 \pm 35.40 a*, b ***	2281.83 \pm 92.37 a*, b NS

Neuronal density was expressed as mean \pm SE. for six rats in each group. a Comparisons are made between Group I Vs . Group III and Group IV; b Comparisons are made between Group III and Group IV. NS: not significant; $P \leq 0.001$ ***; $P \leq 0.01$ ** ; $P \leq 0.05$ *

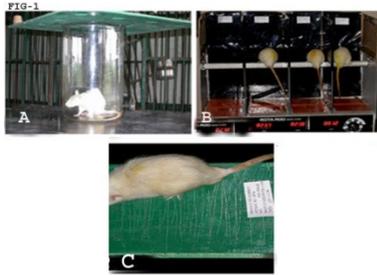


Figure 1: Behavioral instruments (A). Cylinder- Rotational behavior (B). Rotarod (C). Wide beam runway-Stride length

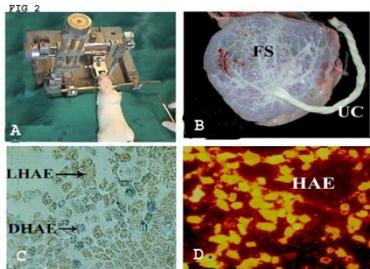


Figure 2: (A) Animal in the stereotaxic frame (B). Human Placenta

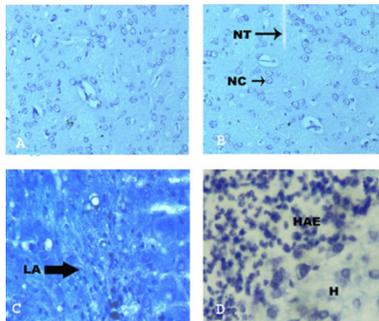


Figure 3: Micro-photograph showing the striatum stained with Toluidine blue and Cresyl Fast Violet magnified under 40X (A). Normal, (B) Sham Lesioned, (C). 6-OHDA lesioned, (D). 6-OHDA lesioned and Human amniotic epithelial cells transplanted. [NT – Needle Tract, NC – Neuronal Cell, LA- Lesioned Area, H- Host striatum, HAE- Human Amniotic Epithelial cells in striatum]

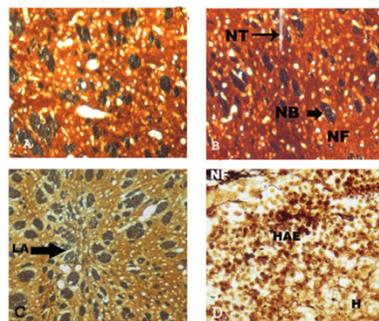


Figure 4: Micro-photograph showing the striatum stained with Silver nitrate magnified under 40X (A). Normal, (B) Sham Lesioned, (C). 6-OHDA lesioned, (D). 6-OHDA lesioned and Human amniotic epithelial cells transplanted. [NT – Needle Tract, NB- Nerve Bundle, NF- Nerve Fibre, LA- Lesioned Area, H- Host striatum, HAE- Human Amniotic Epithelial cells in striatum]

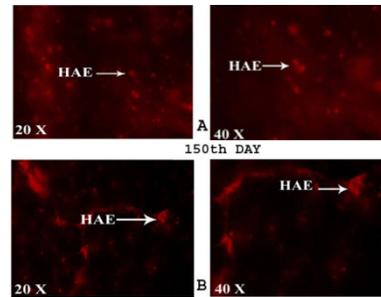


Figure 5: Micro- photograph showing the DiI labeled Human Amniotic Epithelial cells in the host striatum of rat. (A) 60 days after the transplantation, (B) 150 days after the transplantation. [HAE- Human Amniotic Epithelial cells in striatum].

3.1. Behavioral observations

3.1.1. 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. The 6-OHDA lesioned animals showed significantly increased contralateral rotation in all the period. It was for first week 26.40 folds, second week 27.04 folds, third week 25.75 folds and fourth week 25.72 folds when compared with the control non lesioned animals. The group IV animals showed decreased contralateral rotation for 1st-4th week 96%, 97%, 98% 97% respectively over 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 cells graft within the denervated striatum.

3.1.2. Stride length

There was no alteration in the sham control animals of both left forelimb and hindlimb SL compared with control rats. The average of SL in control animals for left forelimb was minimum on 7th day (12.12cm) and maximum on 120th day (14.48cm), whereas in right forelimb it ranged from 14.12cm to 15.58cm. For the left hindlimb it ranged from 12.93cm to 13.83cm and for the right hindlimb 12.25cm to 13.92cm. The overall stride length for both contralateral limbs was decreased in group III animals. The SL for left forelimb on 7th day, 15th day, 30th day, 60th day, 120th day and 150th day were 27%, 35% 22% 23 % 19% and 27% respectively (Table-2). The SL for left hindlimb on 7th day-150th day respectively were 26%, 31%, 25%, 25%, 19% and 22% in 6-OHDA lesioned animals, when compared with the group I animals (Table-3). However in HAE cells transplanted rats, the SL were increased in left forelimb on 7th day - 150th day respectively were 30%, 45%, 22%, 15%, 30% and 46% (Table-3). The SL left hindlimb 7th day-150th day were 33%, 35%, 36%, 39%, 21% and 27%, when compared with lesioned rats (Table-4). The right side SL in group I animals ranged from 14.12 to 15.58cm for

forelimb and 12.25 to 13.92cm for hindlimb. In the group III animals, the SL was slightly reduced. The highest reduction for forelimb was on 60th day (29%) and lowest reduction was on 7th day (22%) over the group I animals (Table-4), whereas in hindlimb the SL was from 20% to 25% over the group I animals (Table-5). The HAE cells transplanted animals showed significant increase in SL both limbs in all periods of study. For forelimb the maximum improvement was at 120th day (40%) and minimum was at 7th day (20%) and for the hindlimb also recovery occurred in same period (120th day 35% and 7th day 22 %) compared to the group III animals (Table-5, 6).

3.2. Histology

The injection of 6-OHDA in to the striatum, which induces retrograde loss of dopaminergic neurons may occur slowly and progressively. Considering these observations our results might reflect that there may be two mechanism in 6-OHDA induced neurotoxicity; the slow progressive neuronal death as shown in the 6-OHDA uptake mechanism in the dopaminergic terminals and the TH phenotype loss by the direct effect of 6-OHDA on cell bodies of the dopaminergic neurons. In the present work the reduced number of neuronal density was present up to 60 days, after the 6-OHDA injection and supported the work of Oiwa *et al.*, [23] (Table.6). In transplantation the question of an immunological response to the transplanted HAE cells need to be addressed in detail. This may support the work of Li *et al.*, in in-vitro and that of Kakishita *et al.*, in vivo [24,25]. These in-vitro results, support that the HAE cells itself would secrete the certain immunosuppressive factors such as migration-inhibitory factors (MIF) for the macrophage. Kakishita *et al.*, in their in vivo observation showed the PKH 26 labeled HAE cells were survived in the midbrain and there was a significant increase in the number of TH positive cells up to 88 % of the intact side [25]. DiI labeled cells could identified in 60th and 150th day. These results might suggest that the survival and secretion of certain neurotrophic factors from the graft might facilitate the recovery of the behavioral and reduction of the muscle rigidity; produced by the 6-OHDA induced degeneration.

4. Discussion

Information has been accumulating about the use of intracerebral grafting to treat disorders of the nervous system. Although the fetal grafts have been successfully used in human Parkinsonian patients, there are still several issues to be resolved such as biological and ethical concerns. Since the amnion is an early product during fetal development, it was presumed that the HAE cells are multipotential or there are multipotent mesenchymal stem cells in the amnion. Hao *et al.*, reported that several anti-angiogenic and

inflammatory proteins were expressed in amniotic epithelial and mesenchymal cells, including IL-1 receptor antagonist and IL-10[26]. Kakishita *et al.*, reported that human grafts of AEC into the striatum of a rat model of Parkinson's disease resulted in partial amelioration of apomorphine-induced rotational asymmetry [27]. In the present study, we found that the striatal neurons were atrophied markedly after the 6-OHDA lesion. After the transplantation of HAE cells, the atrophy was ameliorated and the motor and sensory-motor behaviors partially restored. Hypokinesia of gait with reduced stride length is characteristic for many basal-ganglia-related disorders. Although clinically relevant, gait performance has not widely been investigated in laboratory animals; Fernagut *et al.*, described stride-length performance in mice after pharmacological- and/or sub acute neurotoxin-induced Parkinsonism[22]. Based on these results, we hypothesized that Parkinsonian gait would express a side asymmetry after a unilateral 6-OHDA-lesion. From our observation, the overall stride length was reduced in the lesioned animals than the normal control animals. The stepwise regression model gives information on the unique prediction of each test on motor impairments based on the amount of cell loss in striatum. Iancu *et al.*, and Clarke and Still also observed the decrease in SL after 6 OHDA lesion in mice [28, 29]. The HAE cells transplanted animals showed significant increase in stride length of the paw prints. Klein *et al.*, reported that ectopic intrastriatal transplantation of E14 ventral mesencephalon-derived cells promotes recovery of gait balance and stability, but does not ameliorate the shuffling gait pattern associated with 6-OHDA lesions [30]. The reasons for selective neuronal degeneration in majority of neurological disorder are completely unknown. Krishnamurthi *et al.* have reported that 6 OHDA injected in to the corpus striatum. The long-lasting effect on motor co-ordination suggests the treatment is not simply symptomatic. Thus, Cyclo-L-glycyl-L-2-allylproline (NNZ-2591) is likely to provide a reparative intervention rather than a preventive and symptomatic treatment. Given its good central uptake, NNZ-2591 may have potential as a desirable therapeutic agent for neurodegenerative disorders[31]. It is reported that the dopaminergic cell loss in striatum could be apoptotic in nature after the 6-OHDA lesion [32]. Considering these observations our results might reflect that there may be two mechanism in 6-OHDA induced neurotoxicity; the slow progressive neuronal death as shown in the 6-OHDA uptake mechanism in the dopaminergic terminals and the TH phenotype loss by the direct effect of 6-OHDA on cell bodies of the dopaminergic neurons. In this work the reduced number of neuronal density was present up to 60 days, after the 6-OHDA injection and

supported the work of Oiwa *et al.*, [33]. They said that the decrease of TH immunoreactive cells were dose and duration dependent, the less number of TH immunopositive cells in SN were observed on 56th day after 20µg of 6-OHDA injection. The HAE cells survived for a long time (8 weeks) and integrated into the host spinal cord without immune rejection [34]. In transplantation the question of an immunological response to the transplanted HAE cells need to be addressed in detail. This may support the work of Li *et al.*, in in-vitro and Kakishita *et al.*, in in-vivo [24, 25]. These in-vitro results, support that the HAE cells itself would secrete the certain immunosuppressive factors such as migration-inhibitory factors (MIF) for the macrophage. Anastasia *et al.*, have studied the effect of enriched environment on 6-OHDA induced neuronal death [35]. They have assessed the TH Immunostaining and the fluorogold retrograde labeling. Yang *et al.*, observed that the transplantation of HAE cells into the ventricle of PD model rats may ameliorate rotational asymmetry are complicated and concluded that the neurotrophic factors secreted by human amniotic epithelial cells may slow down the apoptosis of dopamine neurons induced by 6-OHDA. The surviving dopamine neurons can secrete more dopamine for PD rats [36].

5. Conclusion

The 6-OHDA induced lesion in the striatum produced behavioral impairments; apomorphine induced asymmetrical rotations. The overall behavioral improvement in all the periods of the study was recovered in HAE cells transplanted animals from the 6-OHDA induced impairments. This would suggest that the HAE cells may be a suitable donor tissue to alleviate various degenerative diseases in animal model before the clinical trial in human who are suffering from the various degenerative diseases. Survival of HAE cells in grafted striatum and anatomical integration with host brain have been confirmed in the present study. DiI labeled HAE cells could identify in the grafted striatum and showed transformation in its morphology during the long periods such as 60th and 150th day's survival. The present study indicates that the transplantation of HAE cells could be a viable therapeutic strategy to slow down the progressive degeneration of striatal DA neurons in the animal model of PD. Thus the neurological degenerative disease can be treated by the grafting the HAE cells and can be attempted for faster and sustained recovery of functional loss.

Conflict of interest

The authors declare that they have no financial or other conflict of interests.

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