

Human amniotic epithelial (HAE) cell grafts restore cell numbers in a rat model after trimethyltin chloride (TMT)-induced lesion

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Abstract

The main aim of present study is to provide adequate numbers of cells to appropriate sites for useful cellular replacement to overcome the functional deficits caused by the trimethyltin chloride lesion. Hippocampal disorder was induced by the intraperitoneal administration of trimethyltin chloride (Sigma chemicals, U.S.A) at a single dose of 7.5 mg/kg body weight or two divided doses of 3.75 mg/kg body weight for two days. The Human Amniotic Epithelial cells to be transplanted were isolated from the fetal surface of the human placenta obtained from uncomplicated elective caesarian. Using standard co-ordinates human amniotic epithelial cells injected at four sites of hippocampal formation. For histological confirmations of the lesion and the growth of the transplant in the hippocampus, rat brains were processed and stained after various post operative periods of 7, 15, 30, 60, 120 and 150 days. Trimethyltin chloride treatment caused a severe reduction in neuronal cell density in the CA1 and CA3 regions. The human amniotic epithelial cell grafts restored cell numbers in the above said regions to the near normal level. The present study concludes that the human amniotic epithelial cells may be used as a suitable donor tissue to alleviate various degenerative diseases in animal model before the clinical trial in humans, who are suffering from various degenerative diseases.

Keywords: Dentate gyrus, Hippocampus, Human amniotic epithelial cells, Transplantation, Trimethyltin chloride.

Accepted July 07 2011

Introduction

The draw back of the neurons is their inability to divide after their differentiation. Therefore, the loss of neurons in the central nervous system of higher animals leads to permanent damage of the structures involved. The main aim of present study is to provide adequate number of cells to appropriate sites for useful cellular replacement to overcome the functional deficits caused by the lesion. The rat hippocampus, after administration of the neurotoxin trimethyltin chloride (TMT), offers a well-characterized model of neurodegeneration, with a distinct pattern of neuronal necrosis without appreciable demyelination, accompanied by a marked gliotic response [1]. TMT is an organotin compound, intermediate by-product in the production of other tin compounds more commonly used in both industrial and agricultural settings, which is cur-

rently of interest more on account of its use as an experimental tool than in relation to environmental toxicology [2].

Moreover, the usage of HAE cells as donor tissue for cellular replacement in cases of neurodegenerative diseases does not invoke any religious, ethical or legal issues like human fetal cortical tissue. Keeping all these points in mind we selected HAE cells as donor tissue for the replacement of cells in the TMT-induced neurodegenerative disorder in the hippocampus of wistar albino rats.

Material and Methods

Study population

Wistar albino rats weighing 175 ± 25 g of either sex were used for the experiments. Animals were acclimatized to the animal house conditions (12:12 hr. light/dark cycle)

for a week. Standard pelleted feed (Hindustan Lever Limited, Bangalore) and water were provided *ad libitum*. This project was approved by Institutional Animal Ethical Committee (IAEC). The project approval number is IAEC No. 01/011/03.

Experimental Groups

The animals were divided into three groups. Each group consists of six animals for six post operative periods of experiment. The groups were as follows.

Groups	Experimental protocol
Group-I	Control
Group-II	Lesioned in which 7.5mg/kg body wt.of TMT was injected intraperitonially
Group-III	Lesioned and Human amniotic epithelial (HAE) cells transplanted

Experimental induction of hippocampal disorder

Hippocampal disorder was induced by the intra peritoneal administration of TMT at a single dose of 7.5 mg/kg body weight or two divided doses of 3.75 mg/kg body weight for two days [3]. Single dose was given to large animals whereas divided doses were given to small animals.

Isolation and culture of HAE cells

HAE cell isolation was done as described by Sakuragawa *et al.*, [4, 5]. The connective tissue from the amniotic membrane was scrubbed and removed. The membrane was then cleaned with Dextrose normal saline (DNS) thoroughly and trypsinised in 0.125% trypsin (Hi-media) in DNS for 3 changes of 20 minutes each. The pellets so obtained after each treatment were re-suspended in DNS and pooled together and washed in fresh DNS for 3 times. The HAE cells so obtained were suspended in RPMI 1640 culture medium with HEPES (Hydroxy ethyl piperazine sulphonic acid) buffer (Himedia India), supplemented with 10% fetal bovine serum. The HAE cells were then maintained in a carbon dioxide incubator with a humidified atmosphere of 5% CO₂ in air at 37° C. The culture was maintained till the host animal was ready for transplantation.

Transplantation procedure

After midline incision, the skull was exposed and four burr holes were drilled using standard coordinates for hippocampal transplantation [6]. The coordinates include the following:

- (i) anterior-posterior (AP) = -3.3 mm, posterior to bregma, lateral (L) = 2.5 mm, and ventral (V) = 3.5 mm from the surface of brain;
- (ii) AP = -4.3 mm, L = 3.5 mm, and V = 3.5 mm. The syringe with a 26 G needle, fitted to the electrode carrier of the stereotaxic apparatus and 5 to 10µl of cell suspension (2 X 10⁴ cells/µl) was slowly injected into

the denervated hippocampus. After injecting the transplant, the needle was left in the place for 10 minutes and then withdrawn slowly. The surgical incision was closed in layers. The animals were left undisturbed for two hours, and then they were taken for post-operative management.

Morphometric study

Standard stereological principles, as outlined by Weibel and Elias, [7] were used to obtain morphometric data. The diameter is determined by using ocular micrometer. The ocular micrometer is a special circular piece of glass which bears a graduated scale which is 1 cm in length which is divided in to 10 divisions each measuring 1 mm. The ocular meter is inserted in to the eye piece in the plane of lens for taking the measurements.

The stage micrometer is a microslide bearing an engraved scale which is 1 mm in length and graduated at intervals of 0.01 mm or 10 microns. A calibration was made for each ocular lens, and objective used for observation. This was done by superimposing the image of ocular micrometer on the image of the stage micrometer and the divisions coinciding are noted and a calibration constant was obtained for each magnification. Using the ocular micrometer the diameter of the cells was measured and the axial ratio is obtained as follows:

$$\text{Axial ratio} = \frac{L+B}{2}$$

Where, L = greatest diameter and B = diameter at right angles to L.

The diameter is obtained by multiplying the axial ratio with the calibration constant. The size of the cells in the culture and in the histology sections was determined using the above method.

Estimation of numerical cell density of pyramidal cells

Numerical cell density was calculated using a multicoherent grid system, the reticule. The reticule consists of a square of 1 sq. cm. area, engraved on a circular glass piece. The square is internally divided into 100 small squares by 11 horizontal and 11 vertical lines. However, these lines are not taken into account for the purpose of calculation of numerical cell density. The reticule was placed in the focal plane of the objective. The number of pyramidal cells within the area of the reticule was counted in random fields. Numerical cell density was calculated using the following formula:

$$\frac{NA}{Nv} = \frac{1}{A(D+T)}$$

HAE cells restore cell numbers after TMT lesion.

Where, Nv = Numerical cell density expressed as number/cu.mm.

NA = Number of pyramidal cells within the reticule.

D = Average axial diameter of pyramidal cells.

A = Area of the reticule.

T = Section thickness in millimeter

Statistical Analysis

All the data expressed as Mean \pm SEM were analyzed by analysis of variance (ANOVA) followed by Tukey test

and P values < 0.05 were considered statistically significant.

Normal hippocampus

The vast majority of neurons in the hippocampus are of pyramidal type, seen in the pyramidal cell layer. The cell density of which varies from 3325.50 ± 37.52 to 3551.83 ± 21.68 cells/mm³ in CA1 area, 2724.23 ± 30.08 to 3041.34 ± 29.05 cells/mm³ in CA2 area and 2826.67 ± 24.76 to 2999.83 ± 37.65 cells/mm³ in CA3 area.

Table 1. Neuronal cell density in CA3 region of hippocampal formation in the control, TMT- lesioned and HAE cells transplanted animals from 7th to 150th days

Group	7 th day	15 th day	30 th day	60 th day	120 th day	150 th day
Group I	2998.83 41.90	\pm 2953.67 \pm 48.61	2999.83 \pm 37.65	2993.33 \pm 42.18	2827.67 \pm 62.67	2826.67 \pm 24.76
Group II	1935.67 07.80 a ***	\pm 1965.17 04.98 a ***	\pm 2403.33 \pm 36.65 a ***	1614.00 \pm 21.66 a ***	1964.83 \pm 45.85 a ***	1799.33 \pm 43.50 a ***
Group III	2974.33 14.50 a ^{NS} b***	\pm 2878.50 31.62 a ^{NS} b***	\pm 2961.00 \pm 07.98 a ^{NS} b***	2953.83 \pm 17.93 a ^{NS} b***	2216.67 \pm 52.70 a *** b*	1902.67 \pm 26.09 a *** b ^{NS}

Neuronal cell density was expressed as mean \pm SEM of six rats; Group I - Control; Group II – TMT-lesioned; Group III – HAE cells transplanted. a- comparison between Group I Vs II & III; b- comparison between Group II Vs III; NS- not significant; P \leq 0.001***; P \leq 0.01**; P \leq 0.0.

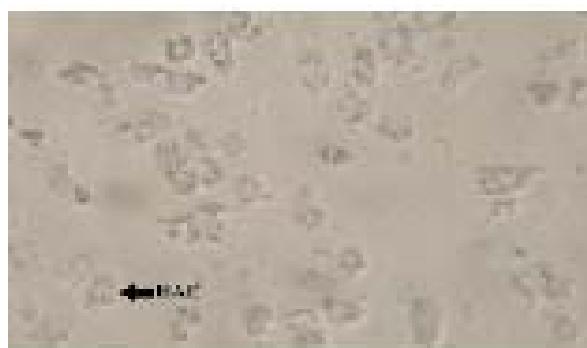
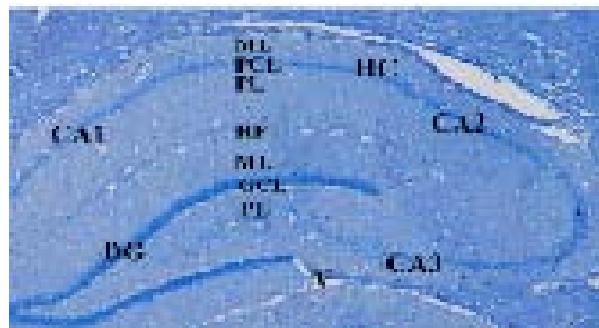
Table 2. Neuronal cell density in CA1 region of hippocampal formation in the control, TMT lesioned and HAE cells transplanted animals from 7th to 150th days

Group	7 th day	15 th day	30 th day	60 th day	120 th day	150 th day
Group I	3551.83 \pm 21.68	3448.50 \pm 21.48	3520.33 \pm 34.53	3452.33 \pm 44.91	3379.83 \pm 30.50	3325.50 \pm 37.52
Group II	2616.67 \pm 63.28 a***	2758.00 \pm 48.84 a***	2725.17 \pm 25.69 a***	2629.50 \pm 91.14 a***	2873.33 \pm 30.66 a***	2996.67 \pm 55.64 a***
Group III	3443.33 \pm 37.61 a ^{NS} b***	3394.33 \pm 28.70 a ^{NS} b***	3420.17 \pm 27.69 a ^{NS} b***	3250.33 \pm 11.80 a ^{NS} b***	3089.83 \pm 87.72 a** b*	3149.00 \pm 28.45 a * b ^{NS}

Neuronal cell density was expressed as mean \pm SEM of six rats; Group I - Control; Group II - TMT lesioned; Group III – HAE cells transplanted. a- comparison between Group I Vs II & III; b- comparison between Group II Vs III; NS- not significant; P \leq 0.001***; P \leq 0.01**; P \leq 0.

Table 3. Diameter of transplanted HAE – cells in Hippocampus from 7th to 150th days

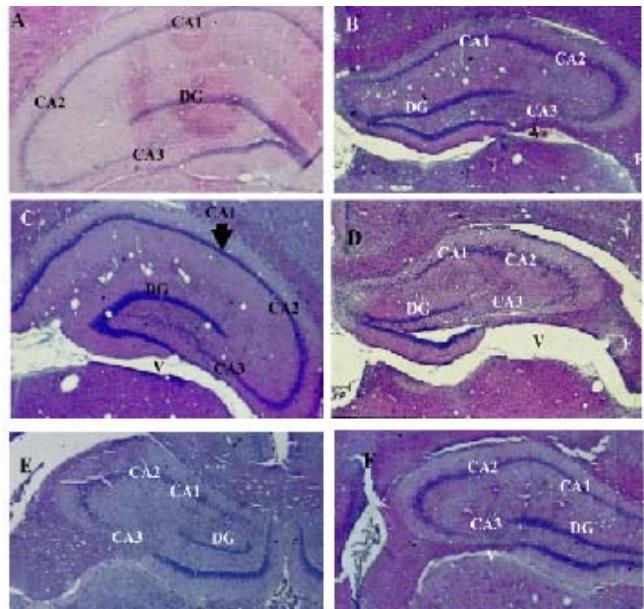
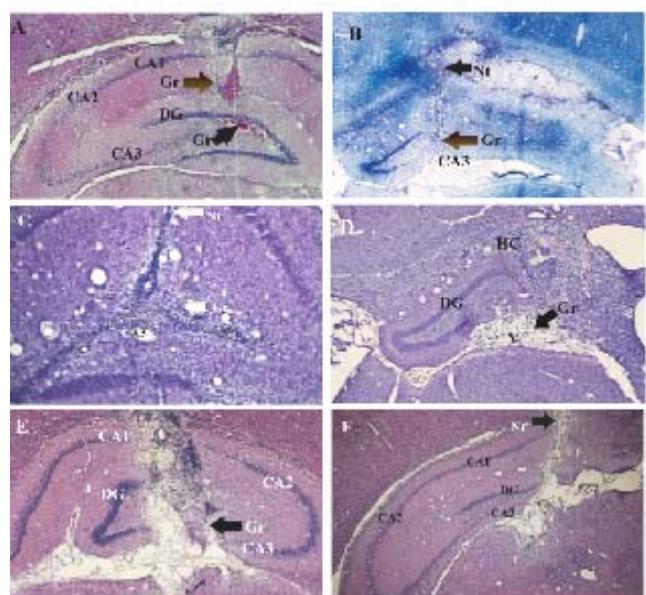
SL No.	Days	Fields counted	Cell size in microns	
			Range	Average
1	7	50	4.68 - 6.12	5.4
2	15	50	4.91 - 6.23	5.57
3	30	50	5.10 - 7.4	6.25
4	60	50	5.22 - 7.78	6.5
5	120	50	5.64 - 11.74	8.69
6	150	50	6.30 - 12.46	9.38

**Figure 1.** Photomicrograph of human amniotic**Figure 2.** Photomicrograph showing the layers of hippocampus

Histomorphometrical observations

Cell types

The vast majority of neurons in the hippocampus is of pyramidal type, in the pyramidal cell layer. Pyramidal cells have a basal dendritic tree that extends into the stratum oriens and an apical dendritic tree that extends to the hippocampal fissure (**Plate 8, Fig. 1**). The pyramidal cell layer also contains a population of basket cells of various sizes and shapes. A variety of non-pyramidal cell types are found in the stratum oriens, stratum radiatum,

**Figure 3****Figure 4.**

TMT-treated hippocampus

Among the regions of the CNS in rats, the hippocampus subsequently became the focus of research in trimethyltin neurotoxicity. TMT treatment caused a severe reduction in neuronal density in the CA1 and CA3 regions (**Table 1&2**); the CA2 region and DG showed a reduction in cell packing density that was not statistically significant.

In TMT-treated hippocampus, the pyramidal cell densities in CA3 area were significantly reduced when compared with the control values (35% on 7th day, 33% on 15th day, 20% on 30th day, 46% on 60th day, 31% on 120th day and 36% on 150th day) (**Table 1**).

The reduction in pyramidal cell densities in CA1 area were 26% on 7th day, 21% on 15th day, 23% on 30th day, 24% on 60th day, 15% on 120th day and 10% on 150th day respectively, when compared with the value of control animals (**Table 2**).

HAE cells transplanted hippocampus

In HAE cells-transplanted group the cell densities in CA3 area ranges from 1902.67 ± 26.09 to 2974.33 ± 14.50 cells/mm³. The increase in the neuronal cell density in the CA3 area is statistically significant except on 150th day when compared to the TMT- treated group values (54% on 7th day; 46% on 15th day; 23% on 30th day; 83% on 60th day; 13% on 120th day and 6% on 150th day). The increased neuronal cell densities in CA1 area of HAE cells-treated group compared to the TMT-treated group were 32% on 7th day; 23% on 15th day; 26% on 30th day; 24% on 60th day; 08% on 120th day and 05% on 150th day.

There was a gradual increase in the average diameter of transplanted HAE cells from 7th to 150th days and change in shape from oval to polygonal, indicating the conversion HAE cells into neurons of that particular region (**Table 3**).

Discussion

Histomorphometrical study: Histomorphometry [7] was done to quantify the extent of lesions and recovery after the transplantation. Number of neurons in hippocampus was estimated from the representative sections in different groups of animals. The neurons can be readily identified by the presence of Nissl bodies in the cytoplasm of large and small cells. The size of the neurons appeared to be larger in normal hippocampus and the average diameter was reduced in the lesioned hippocampus. The mechanism of TMT neurotoxicity and underlying selective vulnerability of neuronal sub-population have not been fully elucidated. According to Patel *et al.*, [8] increased extracellular glutamate after TMT treatment may have an excitotoxic effect on hippocampal neurons. Toggas *et al.*, [9] have hypothesized that the cDNA encoding the 88-

amino acid peptide (stannin) was cloned as a TMT-related molecule using subtractive hybridization strategies, by comparing control and TMT-treated brains.

Sankar and Muthusamy [10] reported that human amniotic epithelial cells did not produce immunological rejection in the spinal cord of Bonnet monkey, since there were no infiltration of leucocytes at the site of implant in short term as well as in long term experiments. The in-vitro work of Li *et al.*, [11] has shown that the HAE cells themselves would secrete certain immunosuppressive factors such as migration-inhibitory factors (MIF) for the macrophage. The observations by Mason *et al.*, [12] and Wood *et al.*, [13] on intra-cerebral neural xenograft did not appear to elicit hyper-acute rejection. This is due, at least in part, to the fact that cellular neuronal xenografts were vascularised by the host [14, 15].

The increased cell density in the lesioned hippocampus after HAE cell transplantation, the reconstruction of damaged circuitry and the secretion of certain neurotrophic factors from the graft might have facilitated the recovery of the behavioral features and reduction of the cognitive impairment produced by the TMT degeneration.

Acknowledgements

We thank Dr. S.R. Hegde, former Director of VIMS, Belalur, Karnataka, India who was instrumental in granting the required permission to take up this research work. We gratefully acknowledge Dr. Gopalakrishna, Tutor, Department of Anatomy, Hassan Institute of Medical Sciences, Hassan, Karnataka, India for the suggestions given by him to make this paper

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