Human Amniotic Epithelial (HAE) Cell Grafts Establish Connections with Host Hippocampal Cells and Suppress Aberrant Host Mossy Fiber Sprouting of the Dentate Supragranular Layer in a Rat Model After Trimethyltin Chloride (TMT) Lesion

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Research Article

Abstract: **Introduction:** 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. **Methods:** This study was conducted on the Wistar albino rats. Hippocampal disorder was induced by the intraperitoneal administration of trimethyltin chloride at 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 were 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. **Results:**The human amniotic epithelial cells become converted into neuron like cells and establish connections with host hippocampal cells and suppress aberrant host mossy fiber sprouting of the dentate supragranular layer. **Conclusion:** 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.

Introduction

Among the various systems present in the body, the nervous system provides a complex mechanism by which the living organisms can react to the ever changing external and internal environments. The drawback of these specialized cells 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 transplantation is to provide adequate number of cells to appropriate sites for useful cellular replacement to overcome the functional deficits caused by the lesion. Intracephalic implants of embryonic central nervous tissue were introduced in the early seventies as an experimental technique for studies on the formation of nerve connections in the adult rat CNS [1]. TMT is an organotin compound intermediate byproduct in the production of other tin compounds more commonly used in both industrial and agricultural settings, which is currently of interest more on account of its use as an experimental tool than in relation to environmental toxicology [2]. The rat hippocampus, after administration

of the neurotoxicant trimethyltin (TMT), offers a well-characterized model of neurodegeneration, with a distinct pattern of neuronal necrosis without appreciable demyelination, accompanied by a marked gliotic response [3]. The behavioral consequences of TMT- exposure include tremor, hyperreactivity and spontaneous convulsions [4]. Although these signs may reflect a generalized CNS dysfunction, TMT also produces several specific and persistent deficits in associative and cognitive functions. Sakuragawa [5], Sankar and Muthusamy [6] have demonstrated that the human amniotic epithelial (HAE) cells are non-immunogenic. The HAE cells have the potentiality to synthesize and secrete various neurotransmitters such as epinephrine, nor-epinephrine, dopamine [7] and acetyl-choline [8]. 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.

Materials 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

he 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 intraperitoneal administration of TMT (Sigma chemicals, U.S.A) at single dose of 7.5 mg/kg body weight or two divided doses of 3.75 mg/kg body weight for two days [9]. 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*., [10,11]. The connective tissue from the amniotic membrane (AM) 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 in a humidified atmosphere of 5% CO_2 in air at 37º C. The culture was maintained till the host animal was ready for transplantation **(Fig. 1).**

Transplantation

After midline incision, the skull was exposed and four burr holes were drilled using standard coordinates for hippocampal transplantation [12]. 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 (2×10^4 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.

Histological study

For histological confirmations of the lesion and the growth of the transplant in the hippocampus of rat brain, the animals were sacrificed after various post operative periods of 7, 15, 30, 60,120 and 150 days. For this, the animals were anesthetized with an over dose of intra peritoneal injection of pentothal sodium and transcardially perfused with 0.9% normal saline followed by 10% formal saline. Then the perfused animals were immersed and stored in 10% formal saline for one week. The brains were removed and stored in the same fixative for an additional four days and then processed for paraffin techniques. Paraffin serial sections were taken at 7 micron thickness for the following procedures. The sections were stained with 1. Cresyl fast violet 2. Haematoxylin and eosin 3. Silver and 4. Toluidine blue. Rapid Golgi impregnated brains were sectioned at 40 micron thickness. In silver impregnation method, normal nerve fibers appeared as dark brown fibers in a pale brown or yellow background.

Histological Observations

Normal Hippocampus

The hippocampal formation in albino rats is divided into two parts, the hippocampus proper and the dentate gyrus. The hippocampus proper is the larger part, which is folded in at the hippocampal fissure and forms a prominent ridge in the ventricle. The hippocampus is composed of three layers of cells, the molecular, pyramidal, and polymorphic, of which the pyramidal layer stands out clearly on account of the rather crowded arrangement of its perikarya. The dentate gyrus is likewise composed of three layers, in this case molecular, granular, and polymorphic. It runs along the edge of the hippocampus and is folded around it, the two points overlapping considerably. The cells of the granular layer are very closely packed, making it a conspicuous object in sections where they are stained **(Fig. 2).**

Features of hippocampus in Albino rats, 7 days after TMT lesion

In 7 days old TMT lesioned animals, the damage to the hippocampus was quite obvious, which was invariably bilateral and symmetrical. CA3 area of the hippocampus was severely affected where degenerating neurons were seen. Most of the neurons within the CA3 and CA1 regions had various degrees of degeneration. Moderate loss of cells in the CA2 and dentate gyrus was visible. And there was wide spread acute dendritic swellings in the stratum oriens and stratum radiatum of hippocampus **(Fig. 3).** Glial response after TMT lesion was observed. Maximal numbers of microglia were exhibited in the CA3, and some microglia assembled, forming clusters. Microglia with ameboid morphology were frequently observed in the CA3 and CA1 pyramidal cell layers. High magnification reveals a change in microglial morphology from finely ramified cells in the saline control to bushy reactive cells **(Fig. 4)**. Hypertrophied microglia oriented along the degenerated dendrites were observed in the stratum radiatum of the CA1. By day 7, many hypertrophied astrocytes were observed in the CA3 region. Silver staining demonstrated a dense band of sprouted terminals (aberrant host mossy fiber sprouting- AHMFS) from the dentate granule cells in to the dentate supra granular layer (DSGL) **(Fig. 5C2).**

Features of hippocampus in 15, 30, 60, 120 and150 days old lesioned animals

The coronal sections of these animals exhibited shrunken hippocampus and enlarged ventricles. Degenerating cells were observed throughout the CA fields, the hilar region and the dentate gyrus, with most pronounced damage in CA3. The loss of neurons in CA1 was obvious. Cell lose were characterised by condensation and clumping of nuclear chromatin, giving a fragmented appearance to the shrunken nucleus. Chromatolysis was observed in which the nucleus becomes eccentric and kidney shaped. The Nissl substance is lost in the central brightly eosinophilic cytoplasm. Dendritic swellings were seen in the regions (stratum oriens and radiatum) of the hippocampal pyramidal cells innervated by the associational and commissural fibers from CA3 cells in both hippocampi **(Figs. 6 &7).** In the sections of rapid Golgi stained slides, though reactive microglia were still observed in the CA1, their number decreased and their appearance reverted to a resting state (reversion of bushy morphology) in the CA3 by day 15. Hypertrophied astrocytes with longer, thicker processes in the CA3 were conspicuous by day 15 **(Fig. 8A)**. By day 30, astrocytes had decreased in size and number **(Fig. 8B)**. A reactive gliosis observed persisted up to 60 days post lesion **(Fig. 9C)**. In 120 and 150 days old lesioned animals microglia and astrocytes were seen in the resting state **(Figs. 9D&10E)**. In silver stained sections aberrant host mossy fiber sprouting (AHMFS) from dentate granule cells in to the dentate supragranular layer (DSGL) was seen prominently **(Figs. 11ABC).**

Features of hippocampus in 7 and 15 days old transplanted animals

The graft was seen well integrated with the host tissue. HAE cells were seen along the needle tract. The cell size varied from 4.68 to 6.23 microns. Nerve fibers from the host tissue, approaching the graft tissue were the dominant feature in 7 day's old grafted animals **(Fig. 12).**

Features of hippocampus in 30 and 60 days old transplanted animals

In 30 days old transplanted animals graft had a location close to the degenerated CA3 area. Graft was found well integrated with the host. Dead cells were seen among the transplanted cells. In 60 days old transplanted animals the intrinsic cytoarchitecture of the hippocampus was found destroyed by the growing transplant. The graft (Gr) placed into hippocampus has shown the extensive growth into the ventricle (V). The grafted cells were found arranged in a network fashion **(Fig. 13).** The cell size varied from 5.10 to 7.78 microns. Silver stained slides showed significant sprouting of the host mossy fibers (aberrant host mossy fibers- AHMF) towards the grafted cell mass **(Fig. 15A).** These bundles of fibers densely innervated the grafted cells

Features of hippocampus in 120 and 150 days old transplanted animals

Discrete transplants were seen in all the grafted animals after sectioning. The location of the graft within the hippocampus varied among the different cases: some transplants had a predominant fraction in the degenerated CA3 cell layer **(Fig. 14E1),** other transplants showed extension in to either the CA1 subfield or into the

ventricle **(Fig. 14F1).** In these animals transplanted HAE cells were seen dispersed along the needle tract. Glial scar is seen at the site of transplantation. There was a good integration between the host and the graft tissue. In silver stained slides processes were seen arising from the cell bodies of the transplanted cells. The grafted cells were seen moving maximum towards the degenerated CA3 cell layer of the host hippocampus **(Fig. 14E1).** The size of the transplanted cells varied from 5.64 to 12.46 microns. The shape of the developing cells varied from polygonal, fusiform to pyramidal type **(Fig. 14F2).** Angiogenesis was seen in the grafted area **(Fig. 14E2).** Fibers from the host tissue, entering the graft tissue at the host graft interface are a common feature in these sections. In the graft, cells are seen arranged in a network fashion **(Figs. 14E2&F2).** As in 60 days old transplanted animals, in 120 days old transplanted animals, Silver stained slides showed significant sprouting of the host mossy fibers (aberrant host mossy fibers- AHMF) towards the grafted cell mass. These bundles of fibers densely innervated the grafted cells **(Fig. 15B).** The grafted cells differed from that of the host cells in cytological architecture, size and staining intensity. The grafted cells stained darker than the host cells**.** They are spindle shaped, oval or polygonal and are smaller than the host cells. Their size varied from 4.68 to 6.12 microns on 7^{th} day to 6.30 to.12.46 microns on 150^{th} day**.**

Discussion

Histological study

Histology was done to compare the structure of hippocampus of TMT lesioned and transplanted animals with that of normal 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.*, [13] increased extracellular glutamate after TMT treatment may have an excitotoxic effect on hippocampal neurons. Toggas *et al*., [14] 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. Xue *et al.*, [15] have demonstrated that intracerebrovetricular transplantation of human amniotic epithelial cells ameliorates spatial memory deficit in the doubly transgenic mice coexpressing APPswe and PSIΔE9-deleted genes. According to them the higher content of acetylcholine in hippocampus released by more survived cholinergic neurites is one of the causes of this improvement. The present study demonstrates that the HAE cells transplanted early into the TMT treated hippocampus cause a long-term suppression of TMT-induced aberrant sprouting of host mossy fibers (AHMF) into the DSGL by rendering an appropriate postsynaptic target. Host mossy fibers grew into all grafts regardless of their location within the lesioned hippocampus [12]. However, the grafts located near the degenerated CA3 cell layer demonstrated a higher density of mossy fibers compared to grafts located elsewhere in the hippocampus **(Figs. 15A&B).** It may be argued that a factor or a combination of factors released from HAE cells might have directed new growth of mossy fibers into the graft. As far as the immunological aspect of the present study is concerned, the immunosuppressant, cyclophosphamide was given initially at the dose of 5 mg/kg for 3 days for three animals, and three animals were observed without the immunosupppressant. As there were no differences between the immunosuppressed and non-immunosuppressed animals in histological study, immunosuppressant was not given to the rest of the transplanted animals. The reason for the absence of rejection in the present study could be due to [1] the brain is considered as a immunologically privileged site for transplants. [2] the non-immunogenic nature of HAE cells. In our present study neuronal cell density was found to be increased in the HAE cells transplanted group. However in long term study (150^{th}day) no noticeable difference could be observed. Sakuragawa *et al.*, in 1996 demonstrated the neuronal and glial characters exhibited by HAE cells [10]. In the work of Sankar and Muthusamy [6] human amniotic epithelial cells did not evoke 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.

The following conclusions were made from the present study:

Intraperitoneal administration of TMT produces severe and permanent damage in the hippocampus and can be used as a suitable model for hippocampal disorder. Due to the absence of surface antigens grafted HAE cells did not evoke any immune response. The transplanted HAE cells may be progenitor cells to generate neuron like and glia like cells. The transplanted HAE cells established synaptic connections with the host tissue. This study provides evidence that grafted HAE cells located near the degenerated CA3 cell layer can suppress the development of post- TMT aberrant mossy fiber circuitry in hippocampus on a long- term basis.

Acknowledgement

We thank Dr. S.R. Hegde, former Director of VIMS, Bellary, Karnataka, India ,Who was instrumental

in granting the required permission to take up this research work. We gratefully acknowledge Dr. M. Mallikarjun, Professor and H.O.D of Anatomy, Vijayanagar Institute of Medical Sciences, Bellary, Karnataka, India for the suggestions given by him to make this paper.

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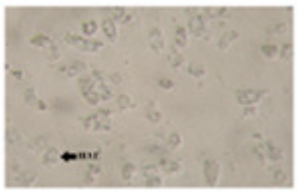
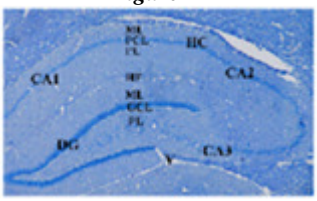


Figure 1



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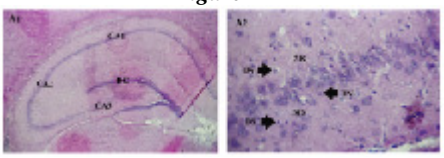

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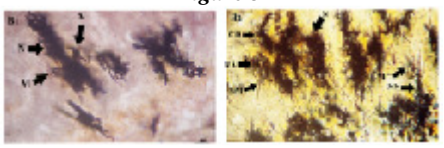

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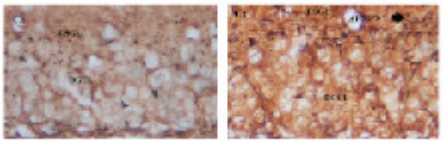

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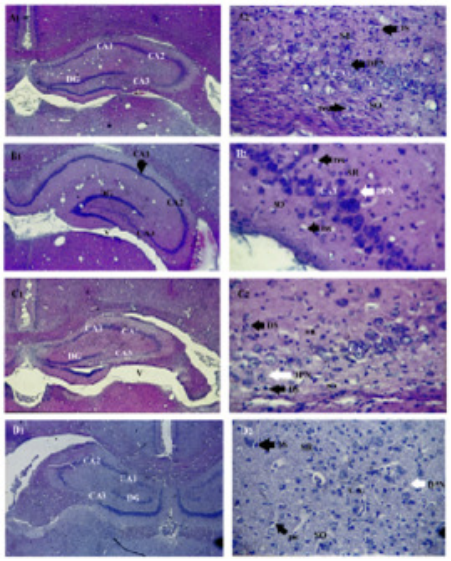

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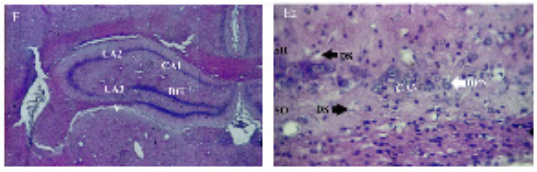

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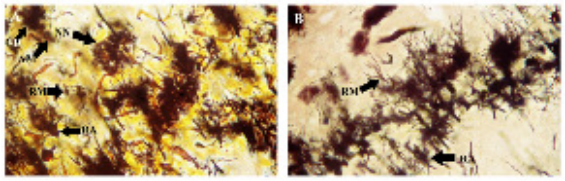

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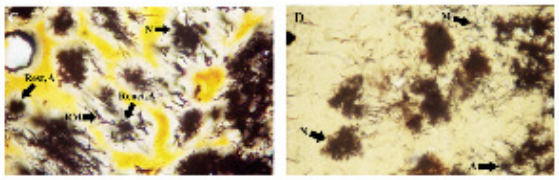

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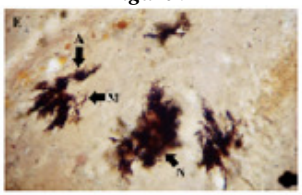

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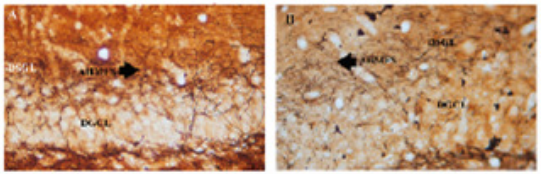

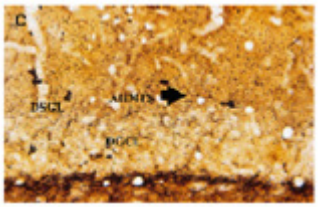

Figure 11

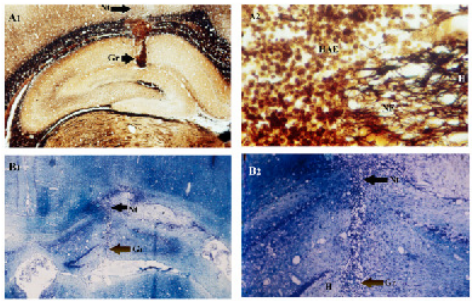

Figure 12

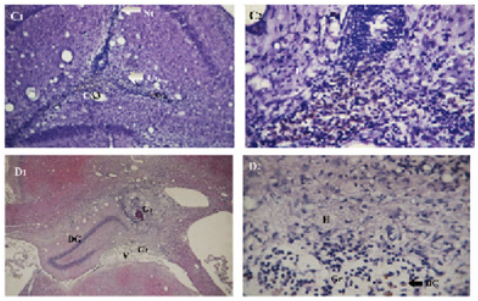

Figure 13

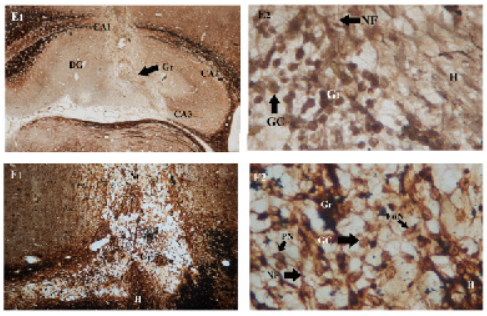

Figure 14

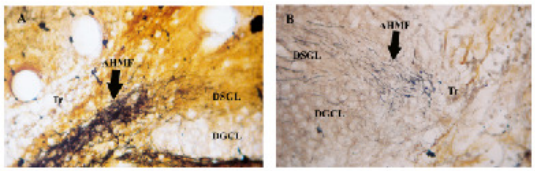

Figure 15