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GRAFTS CONTAINING HAE CELLS AFTER TMT LESIONS IN THE HIPPOCAMPUS OF WISTAR ALBINO RATS, IMPROVE PERFORMANCE IN THEIR BEHAVIOR

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ABSTRACT

Introduction: The main aim of the present study is to transplant HAE cells as useful cellular replacement to overcome the functional deficits caused by the trimethyltin chloride lesion in the hippocampus. Methods: This study was conducted on the Wistar albino rats. The effect of transplanted human amniotic epithelial cells in hippocampus after trimethyltin chloride lesion was tested on eight-arm radial maze during different time duration. Hippocampal disorder was induced by the intraperitoneal administration of trimethyltin chloride 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. Human amniotic epithelial cells were isolated from placenta obtained from uncomplicated elective caesarian. Using standard co-ordinates human amniotic epithelial cells were transplanted at four sites of hippocampus. Results: It is observed that the transplanted human amniotic epithelial cells can aid in the partial recovery of performance, tested in eight-arm radial maze after the trimethyltin chloride lesion. Conclusion: The human amniotic epithelial cells may be used as a suitable donor tissue to alleviate various degenerative diseases in animal model before the clinical trials in humans, who are suffering from various degenerative diseases.

KEYWORDS: Hippocampus, Human amniotic epithelial cells, Radial arm maze, Trimethyltin chloride.

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INTRODUCTION

The striking disadvantage of a fully matured nervous system when compared to the other systems of the body is that the new nerve cells generally cannot be produced. The usage of human amniotic epithelial cells (HAE cells) as donor tissue for neuronal cellular replacement in cases of neurodegenerative diseases do not invoke any religious, ethical or legal issues like human fetal cerebral cortical tissue. Therefore in this study HAE cells were selected as donor tissue for the replacement of the trimethyltin chloride (TMT) induced neurodegenerative condition in the hippocampus of Wistar albino rats. The rat hippocampus after administration of the neurotoxin 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 currently of interest more on account of its use as an experimental tool than in relation to environmental toxicology[2]. Histological alterations both qualitatively and quantitatively, are shown to be associated with neurobehavioral changes, including severe learning and memory deficits, so TMT has also been regarded as a potential tool for the study of memory dysfunction in animal models, including Alzheimer's disease[3,4]. It produces the distinctive behavioral syndrome consisting of hyperactivity, seizures, learning disturbance and self-mutilation. The analysis of behavioral changes associated with brain damage in animals and humans is one of the oldest and most widely used methods in neurophysiology. The aim of the present study was to assess, whether the memory deficit was associated with the hippocampal lesions.

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* except for the animals which were trained in radial arm maze. The project was approved by Institutional Animal Ethical Committee (IAEC) [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 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[5]. 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* [6,7]. 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. Photomicrograph of human amniotic epithelial (HAE) cells in suspension stained with Trypan blue. 40X

Fig. 2. Photograph showing the transplantation of human amniotic epithelial (HAE) cells into the lesioned hippocampus using a syringe (S) with a 26 G needle, fitted to the electrode carrier of the stereotaxic instrument.

Fig. 3. Photograph showing the training of animal in Radial arm maze.

Transplantation procedure

The animals to be transplanted were anesthetized using intra peritoneal injection of thiopentone sodium (Pentothal, Abbott Laboratories, India) at a dose of 40 mg/kg body weight and fixed in to a stereotaxic apparatus. The plane of the incisor bar was set at 3.3 ± 0.3 mm below the interaural line. After midline incision, the skull was exposed and four burr holes were drilled using standard coordinates for hippocampal transplantation[8,9]
(Fig: 2). These coordinates were standardized earlier using Wistar rats. 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 10^4 cells/µl) was slowly injected into the lesioned 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 layeres. The animals were left undisturbed for two hours, then they were taken for post-operative management.

Antibiotic (Gentamycin 3 mg/kg/day) therapy was given till the wound heals. Human amniotic epithelial (HAE) cells are non-immunogenic[10], however initially the immunosuppressant, cyclophosphamide was given at the dose of 5 mg/kg for 3 days for three animals on trial basis. As there were no differences between the immunosuppressed

and non-immunosuppressed animals in histological study, the entire study was conducted without any immunosuppressant.

Observation of animals in radial arm maze[11,12]

The radial arm maze stood 70 cm from the floor and consisted of an octagonal platform (26cm) from which eight arms (42cm long; 12cm wide; 10cm height) radiated. Each animal was placed individually in the center of the maze and subjected to a reference and working memory task for 28 days, where the same four arms (numbers 2,3,5 and 8) were baited for each daily training trial. The other four arms (numbers 1,4,6 and 7) were never baited. The training trial continued until all four baits in the food cups had been consumed or until 5 min had elapsed. Measures were made of the number of reference memory errors (RME) (entering an arm that was not baited) and working memory errors (WME) (entering an arm containing food but previously entered). The total time/trial on the maze was recorded by stopwatch. Before creating lesion, animals in all the groups were trained. After lesioning, followed by treatment, the animals were once again subjected to maze learning (Fig: 3).

Statistical Analysis

All the data expressed as Mean ± SEM was analyzed by analysis of variance (ANOVA) followed by Tukey test and P values < 0.05 were considered statistically significant.

RESULTS

In the control Group (Group I) of animals reference memory errors were seen only up to the 60th day of the duration studied. The range of RME was from 3.17 ± 0.28 to 1.83 ± 0.28. The TMT lesioned (Group II) rats made more number of RME than the controls throughout the testing period. In this group, the RME was significantly increased in all the periods of study and was maximum on 60th day (128%). The lesioned (Group II) and HAE cells transplanted

(Group III) animals the RME was significantly decreased in all the study periods, which was maximum on 120th and 150th days (100%) and minimum on 30th day (33%), when compared to the Group II animals (Table 1). The group I animals showed the WME only during the initial stage (7th day). In hippocampal lesioned animals working memory (WM) was significantly impaired (P≤ 0.001). The errors in these animals ranged from 1.50 ± 0.20 to 3.00 ± 0.41 among the 4 baited and 4 non-baited arms. In Group III animals the WME was significantly decreased in all the study periods, which was maximum in the periods from 30th to 150th duration of study (100%) and minimum on 7th day (59%) over group II animals. Thus it appears that there were no significant differences between the control and the HAE cells transplanted groups in WME (Table 2).

The data for time per arm entry was calculated by dividing total time taken to complete a particular trial by dividing the number of arms entered in each trial. There was a gradual reduction in the time per arm entry in Group I from 7th to 150th days. The values ranged from 3.79 ± 0.42 to 9.67 ± 0.95 seconds. The Group II animals presented the range of 06.00 ± 0.41 to 15.15 ± 0.72 seconds and also showed significant increase in time per arm entry throughout the testing period which was maximum on 30th day (81%) and minimum on 120th day (47%) when compared to the Group I. In Group III animals the time per arm entry was significantly decreased when compared to the Group II animals (29% on 7th day; 36% on 15th day; 43% on 30th day; 30% on 60th and 120th days; and 33% on 150th day) and the values were almost close to the Group I values with the following difference: 06% on 7th day; 14% on 15th day; 03% on 30th day; 24% on 60th day; 03% on120th day and 06% on 150th day (Table 3).

Table 1

Reference memory error (RME) status in the control, TMT lesioned and HAE cells transplanted animals from 7th to 150th days in radial arm maze

Group	7th day	15th day	30th day	60th day	120th day	150th day
Group I	3.17 ± 0.28	3.00 ± 0.33	2.00 ± 0.33	1.83 ± 0.28	0.00 ± 0.00	0.00 ± 0.00
Group II	7.17 ± 0.28 a ***	6.00 ± 0.53 a ***	4.00 ± 0.41 a**	4.17 ± 0.28 a ***	3.17 ± 0.28 a ***	2.83 ± 0.44 a ***
Group III	4.00 ± 0.24 a NS b***	4.00 ± 0.33 a NS b*	2.67 ± 0.19 a NS b*	2.33 ± 0.30 a NS b**	0.00 ± 0.00 a NS b***	0.00 ± 0.00 a NS b***

*Mean ±SEM. (N=6). a- Group I Vs II & III; b- Group II Vs III; NS- not significant; P≤0.001***; P ≤0.01**; P ≤0.05*.*

Table 2

Working memory error (WME) status in the control, TMT lesioned and HAE cells transplanted animals from 7th to 150th days in radial arm maze

Group	7th day	15th day	30th day	60th day	120th day	150th day
Group I	1.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Group II	2.83 ± 0.37 a ***	1.50 ± 0.20 a ***	1.83 ± 0.28 a ***	3.00 ± 0.41 a ***	2.67 ± 0.30 a ***	1.83 ± 0.28 a ***
Group III	1.17 ± 0.15 a NS b***	0.50 ± 0.20 a NS b**	0.00 ± 0.00 a NS b***	0.00 ± 0.00 a NS b***	0.00 ± 0.00 a NS b***	0.00 ± 0.00 a NS b***

*Mean ±SEM. (N=6). a- Group I Vs II & III; b- Group II Vs III; NS- not significant; P≤0.001***; P ≤0.01**; P ≤0.05**

Table 3

Time per arm entry status in the control, TMT lesioned and HAE cells transplanted animals from 7th to 150th days in radial arm maze

Group	7th day	15th day	30th day	60th day	120th day	150th day
Group I	9.67 ± 0.95	8.44 ± 0.80	7.04 ± 0.31	7.56 ± 0.42	6.68 ± 0.99	3.79 ± 0.42
Group II	14.54 ± 0.77 a **	15.15 ± 0.72 a ***	12.73 ± 0.79 a ***	13.33 ± 0.81 a ***	9.84 ± 0.44 a *	6.00 ± 0.41 a **
Group III	10.26 ± 0.68 a NS b **	9.66 ± 0.59 a NS b***	7.26 ± 0.42 a NS b***	9.37 ± 0.67 a NS b**	6.88 ± 0.32 a NS b*	4.02 ± 0.11 a NS b**

*Mean ±SEM. (N=6). a- Group I Vs II & III; b- Group II Vs III; NS- not significant; P≤0.001***; P ≤0.01**; P ≤0.05*.*

DISCUSSIONS

The aim of the present study was to assess, whether the memory deficit was associated with the hippocampal lesions. TMT produces selective hippocampal *i.e.,*mainly CA3 and CA1 lesions similar to that caused by convulsants which interact with the brain excitatory amino acid transmission. These results offered additional evidence that CA3(Cornu Ammonis3) pyramidal neurons and their connections play an important role in radial-arm maze performance. Similar to the previous study by Walsh *et al*[13]. in this study TMT treated animals made more reference and working memory errors in radial arm maze compared to that of control animals. The time taken to complete the trial is also significantly greater in lesioned animals compared to that of control animals. Therefore TMT influences memory functions[14].

Of the known neurotransmitters, acetylcholine is most frequently associated with memory functions, decreasing the density of muscarinic receptors. In TMT treated rats, Earley *et al*[15] have also reported a decrease in the density of muscarinic receptors. The results of the study by Ishida *et al*[16] have shown a significant increase of Choline Acetyl Transferace [ChAT] activity in CA3. These results may be coincident with the selective degeneration of CA3 cells in the present TMT model, and further indicate that the altered cholinergic functions impair the memory. TMT treated rats with maximum post operative interval, revealed marked impairments on spatial discrimination tasks as assessed by the Morris water maze, and that fetal septal grafts, rich in cholinergic neurons, ameliorated place-navigation deficit in the experiment conducted by Kato *et al*[17]. In HAE cells transplanted group there was a decrease in the number of working and reference memory errors and the time taken to complete the trial compared to the lesioned rats. The study results indicate that the decrease in number of reference and working memory errors and time taken to complete the trial after the HAE cell transplantation may be attributed to the restored neural circuit in the hippocampal area and the secretion of neurotrophic factors by the graft.

CONCLUSIONS

The present study can be concluded with the following observations.

Intraperitoneal administration of TMT produces severe and permanent damage in the hippocampus and can be used as a suitable model for hippocampal disorder.

Secondly, the overall improved performance of HAE cells treated group from the TMT lesioned group, studying the various behavioral parameters using the radial arm maze indicate that the HAE cells may be used as a suitable donor tissue to alleviate various neurodegenerative diseases in animal model before the clinical trial in humans who are suffering from various neurodegenerative diseases.

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