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Research Article

NONIMMUNOGENECITY OF HUMAN AMNIOTIC EPITHELIAL CELLS IN CIRCULATION AND SUBCUTANEOUS TISSUE OF THE WISTAR ALBINO RATS

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ABSTRACT
The Human Amniotic Epithelial (HAE) cells have been used for allotransplantation in patient with the lysosomal storage disease due to lack of expression of HLA antigen. It also has been used for various neurodegenerative diseases in experimental animal model. However, the study on hematological changes and hypersensitivity after HAE transplantation in to rat have not been addressed. The present study explored that the immunogenic of HAE cells in circulation and subcutaneous implantation in the Wistar albino rats in group-II cultured HAE cells labeled with the 1, 1 diotadeyl 3, 3, 3'3' Tetramethylindecarbocyanin perchlorate (DiI) were infused into the internal jugular vein (IV-Group)**.** In group III (Subcut-group) animals, HAE cells were implanted into the subcutaneous region of the thigh. After five days, in both the groups blood was collected from retro-orbital venous puncture and the hematological parameters were studied. Histological section of subcutaneous tissues from group III were observed under the florescent microscope for the infiltration. There was no evidence of tumorigenicity in humans when isolated amniotic cells were transplanted into human volunteers to examine their immunogenicity or into patients in an attempt to correct lysosomal storage diseases (Toda *et al.,* 2007). Hao *et al.,* (2004) reported that both HAE and HAM cells express various antiangiogenic and anti inflammatory proteins such as interleukin (IL)-1 receptor antagonist; tissue inhibitors of metalloproteinase (TIMPs)-1, -2, -3, -4; and IL-10. In the present observation there was no major variation in the hematological parameters of both groups while comparing with the normal group. The HAE cells are anatomically and histologically specialized fetal tissue. Transplantation of HAE cells MHC molecules would be more clinically relevant for clinical trials by using neural xenografts to treat patients with Parkinson's disease rather than tissue from knockout mice. These studies would provide further insight into the mechanisms of HAE cells xenograft rejection and would be important in achieving the ultimate goal of overcoming xenograft rejection epithelial cells that normally exist less than 10 months in nature. Absence of scar tissue and cellular integrity with the host tissues support the absence of major histocampatability of HAE cells.

***Key Words*:** *Human Amniotic Epithelial Cell, Dii, Blood Suspension, Subcutaneous Tissue*

ABBREVIATIONS
HAE - Human Amniotic Epithelial; DLC - Differential Leucocytes Count; TLC - Total Leucocytes Count; RBC - Total Red Blood Cell; Hb – Hemoglobin; DiI - 1, 1 diotadeyl 3, 3, 3'3' Tetramethylindecarbocyanin perchlorate

INTRODUCTION
Human Amniotic Epithelial (HAE) Cells is one of the cell groups formed during early development stages of an embryo of mammals. In the past, amnion is considered to serve as a storage delimiter for amniotic fluid in which the developing baby baths. Initially the role of amniotic membrane / cavity / fluid was considered as protective only. However, recent researches indicate that HAE might play a significant

role in early development stages (Millar *et al.,* 2005). HAE cells have been used for disorders like the lysosomal enzyme storage disease (Sakuragawa *et al.,* 1992).

HAE cells synthesis and secretes various neurotransmitter (Elwan *et al.,* 1997 and 1998) and hormones (Matsuura *et al.,* 2001). These cells has less or absence of immunogenecity in rat as in other species (Kakishita, 2000 and 2003; Sankar *et al.,* 2003). HAE cells have implanted in the cornea of rabbit and studied the re-epithelial formation on transplanted region (He *et al.,* 1999). The implantation of either sheets of amniotic epithelial cells or cells culture *in-vitro* has many advantages over the use of bone marrow or fibroblast transplants. Recent studies revealed that the HAE cells have ability to secret albumin (Tatakashima *et al.,* 2004) for the liver transplantation in-in-vitro and for the Parkinsonism treatments in in-vivo (Kakishita *et al.,* 2003) HAE cells which exhibit characteristics of embryonic and pluripotent stem cells, could be utilized for cell therapy without legal or ethical problems. Intracerebroventricular HAE cells transplantation improved spatial memory in double-TG mice, and results suggested that increased acetylcholine levels in the hippocampus, released by surviving cholinergic neurites, were responsible for this improvement (Xue *et al.,* 2012).

Therefore absence of the antigenic property it has been used for various experimental studies. Based on the previous investigation, the present study was focused on the implantation of HAE cells into the subcutaneous and intravenously infused into the adult Wistar albino rats and studied the post operative changes in Hematological parameters. The tissue infiltration by HAE cell were observed using skin biopsy, HAE cells blood labeling by a fluorescent marker (DiI)

MATERIALS AND METHODS

Animals

For this study 18 adult wistar albino rats weighing about 160g –240 grams of the body weight (King Institute, Chennai 32 India) were used for the antigenic reaction of the human amniotic epithelial cells. They were housed in pairs and allowed 7-10 days to acclimatize to the animal care facility before the collection of blood or surgery. They were maintained in a room at constant temperature and humidity (21^{o}C to 26^{o} C) less than 12 h light – dark cycles. Animals were allowed *ad libitum* access to food and water when not undergoing surgery. This experiment was conducted in accordance with the standard protocol and procedures of the Institutional Animal Ethical Committee (IAEC No: 01/001/01).

Before the infusion of HAE cells in to the albino rats the animals were divided into three groups as Control (6 animals) Group I, HAE cells injected into internal jugular vein (6 animals) (HAE -IV group or Group II) and HAE cells sub-cutaneously infused (6 animals) (HAE –subcutaneous group or Group III). One week prior to the infusion of the HAE cells into the both (HAE - IV and HAE - Sub cut.) groups including the control animals, the blood was collected from the retro-orbital venous puncture through capillary tube for the baseline value.

Isolation and Cultivation of Human Amniotic Epithelial **(HAE)** *Cells*

The human amniotic epithelial tissue was mechanically peeled from the chorion of a placenta obtained from an uncomplicated elective caesarean section after informed concerned Andra mahila saba hospital, Chennai India. The HAE cell layer was extensively scraped out to remove the underlying tissues such as the spongy and fibroblast layers to eventually obtain pure epithelial layers to eventually obtain a pure epithelial layer with basement membrane. The layer was then treated with 0.125 % trypsin (Himedia) three times each for 20 min. HAE cells, obtained usually after second and third treatment, were cultured in MEM (Himedia) and RPMI 1640 (Himedia)medium supplemented with 10% fetal calf serum and 1% antibioticantifungal solution (Gibco BRL) under a humidified atmosphere of 5% Co_2 in air at 37° C.

Smear Preparation and Blood Cells Count

Blood was collected from retro-orbital veins of all the experimental animals. The Leukocytes Differential Leukocyte Count (DC), Total Leukocytes Count (TLC) and total Red Blood Cell (RBC) counts were taken by using Automatic auto analyzer (YSVETO301). The hemoglobin (Hb) concentration also was measured with the Hb523 haemoglobinometer, HEM-010, DHT.

Labeling of HAEC with 1, 1 diotadeyl 3, 3, 3'3' Tetramethylindecarbocyanin perchlorate (DiI)

After the centrifugation with several wash with the Phosphate buffer saline (PBS) supernatant was discarded and the pellet was collected. One ml of cell pellet with equal amount of PBS and few crystals (1mg/10 ml of methanol) of DiI (sigma) were added and incubated for the 45 minutes at 37º C. The incubated HAE cells suspension was centrifuged at 800-1000 rpm for 10 minutes. Then supernatant was discarded leaving .5ml and the pellets was evenly diluted with PBS and.·1 ml of the cell suspension was injected into Internal Jugular Vein (IJV) of HAEC group under the general anesthetic condition (Sodium pentothal 40mg/kg body weight). The same amount of the HAE cells suspension was injected into the sub-cutaneously in the subcutaneous group.

Five days after the infusion of the HAE cells, blood from the both groups were collected and studied up to 21 days. The HAE cells sub-cut group animals were observed for the Hypersensitivity in the infused site then sacrificed with overdose of sodium pentothal and per fused by normal saline and followed by 10 % formal saline for 25 minutes through the left ventricle and incised through the right atrium. Then the animals were fixed in formal calcium fixatives. The skin around the HAE cells infused site was removed and processed with graded sucrose for frozen section.

The tissue fixed in tissue holder using cryo-embed (Bright Instrument company, England) and section were taken at 10 micron thickness then immediately were observed under Nikon microscope with the red filter and microphotograph was taken using Kodak 200 ASP.

Blood Suspension Preparation for Dii Labeled Hae Cells

6-8 ml of blood collected in EDTA coated centrifuge tube. It divided into two equal volumes in a pair of 10 ml of plastic centrifuge tubes, centrifuged both tubes at 2500 rpm for 5 minutes at 10º C. All plasma removed from both tubes and 0.9% of saline was added to give final volume of approximately 6 ml. Then 2 ml of saline was added mixed well, but gently by repeated inversion. The tubes was allowed to stand at ambient room temperature for approximately 30-45 minute or until cells settled down sufficiently (red blood cells occupied about the lower level). Leucocytes containing supernatant carefully removed from both tubes and transferred to a pair of fresh, clean 10 ml centrifuge tubes. The red cells were discarded. Centrifuged all tubes at 3,000 rpm (1500 x g) for 2 min at 10 degrees Celsius. Poured off supernatants and retained the leucocytes pellets with 1 ml of saline. The pellets saline was collected in the cavited slide and observed in fluorescent microscope using red filter.

Statistical Analysis

The data were analysed using one-way ANOVA followed by Turkey multiple comparison test to determine the statistical significance. Significance was calculated by comparing the control with HAE -IV (group II) and HAE- Subcutaneously (group III) infused groups. SPSS 10.0 p valve >0.05

RESULTS

Human Amniotic Epithelial (HAE) Cells

The viability of cultured HAEC were more than 85 % at the time of infusion into the skin and internal jugular vein, and the cell suspension were photographed with the low and high power magnification before the DiI incorporation (Fig. 1A).

Human Amniotic Epithelial Cells with DiI label (HAE - DiL)

The DiI incorporated human amniotic epithelial cells were fixed in formalin vapor and these cells are shown in (Fig. 1B).

Differential Count

In normal adult the predominant of white blood cells were lymphocytes which may constitute nearly 86% of the leucocytes, the neutrophils average is 14-20%, monocytes constitute a modest percentage (up to 6%) of the usual differential count, eosinophils range from 1-4%, and basophiles were rare or absent(Table-2).

Lymphocytes: Like the human, rat lymphocytes may range in size from approximately 6 to 15µm diameter. The lymphocytes count were significantly increased in Group II and Groups III in 9th day F (3,

15) =14.626 and 5th day F (3, 15) 13.215 in group III at P< 0.05 level. There is no significant increase or decrease in Group II and Group III in 15th day and 21st day when compared with the normal group (Table-2).

Neutrophils: These cells are approximately 11µm in diameter and the nucleus is coiled or twisted and nuclear lobulation was not pronounced. The count of the cells was not significantly altered in all the duration studied in both groups. However in 5th F (3, 15) =12.079 and 9th days F (3, 15) =114 in group III were significantly decreased (19 %, 15 %) with the control animals (Table-2).

Monocytes: This is largest of all leucocytes, has a convoluted nucleus, abundant cytoplasm, and some azurophilic or reddish-purple granulation. It has the general characteristics of monocytes of other species. Monocytes count in group II and group III were not significantly changed in all the duration studied when compared with control animals (Table-2).

Eosinophils: The cells, approximately the size of Eosinophils, contain a nucleus that has completely annular and large round granules usually fill the cytoplasm. Eosinophil count in group II and group III the 5th day, 9th day. 15rth day and 21st day were not significant from the control animals (Table-2).

Basophiles: The classic or circular basophiles are rare in the rat. Tissue basophils or mast cells can be found on examination of the buffy coats in cardiac and tail blood (Hardy 1967). In our observation there was no basophiles have been identified (Table-2).

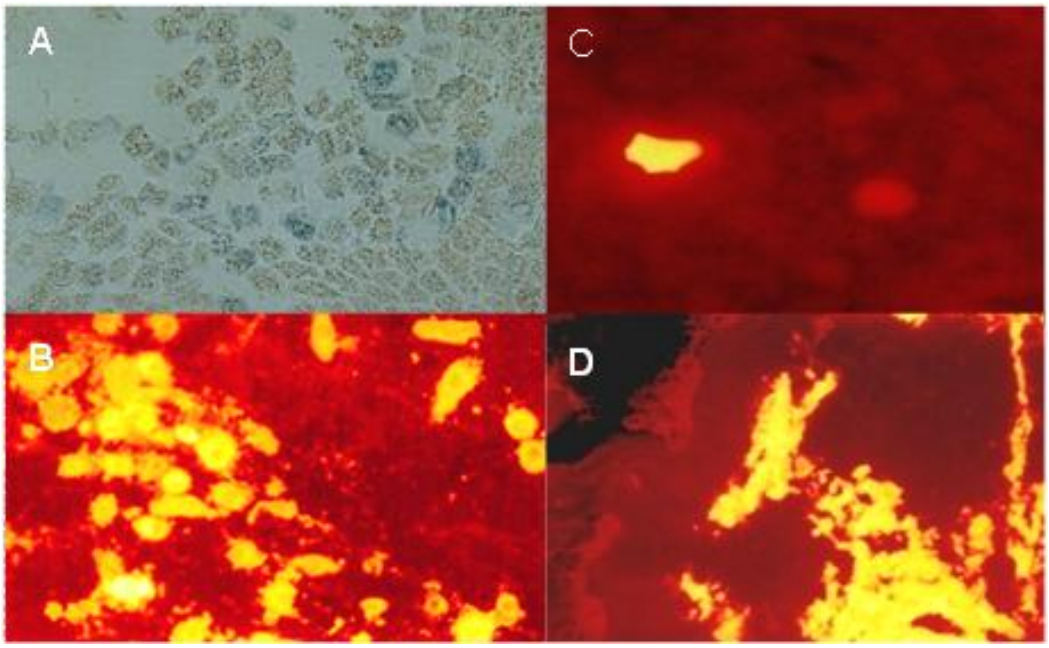

Figure 1: A-Normal Human Amniotic Epithelial (HAE) Cells (Cultured). **B-**Dil-Labeled Human Amniotic Epithelial (HAE) Cells. **C-** Human Amniotic Epithelial Cell in blood Suspension. **D-** Subcutaneous tissue with Dil-labeled Human Amniotic Epithelial Cell

Total Leukocyte Counts (TLC)

The leucocytes average is 9000 in rats with rang of 6000-18000. In survey of the literature, Schermer (1967) found tremendous differences in means leucocytes counts. Blood collected from the animals, which had been used for the intravenous infusion of HAE cells and sub-cutaneously injected animal. The total leucocytes count of group II in 9th day F (3, 15) = 6.262 and in both groups (Group II and Group III) in 21st day F (3, 15) = 10.850; F (3.15) = 16.004 were significantly increased (17 %, 11 % and 11 %) when compared with the ontrol animals (Table1). However there were no significant changes observed in 5th and 15th days in both groups and 9th and 21st days' in-group III (Fig 4; Table-1).

Total RBC Count

In this study RBC count were significantly reduced in both groups (Group II and Group II) in all the duration of study when compared with the control animals at P< 0.05 level (Fig 3;Table-1).

Table 1

Parameters	**5th day**	**9th day**	**15th day**	**21st day**
RBC				
Control	7.0667 ± 0.25	7.3183 ± 0.42	7.3650 ± 0.46	7.4117 ± 0.23
IV-Group	6.2667 ± 0.39	6.3050 ± 0.35	6.1367 ± 0.21	6.4450 ± 0.44
Subcut- group	6.5167 ± 0.42	6.5183 ± 0.48	6.3883 ± 0.36	6.5867 ± 0.36
Hb				
Control	13.7167 ± 0.40	13.1000 ± 0.45	13.0667 ± 0.73	13.4667 ± 0.60
IV-Group	12.2500 ± 0.47	12.1333 ± 0.29	11.8333 ± 0.86	11.7833 ± 0.44
Subcut-Group	12.0167 ± 1.01	12.6333 ± 0.72	12.8500 ± 0.20	12.5333 ± 0.58
TLC				
Control	10.3523 ± 0.27	10.2937 ± 0.30	10.7117 ± 0.17	10.8742 ± 0.17
IV-Group	11.6505 ± 0.35	12.0387 ± 0.85	11.8542 ± 0.58	12.0842 ± 0.59
Subcut-Group	11.3432 ± 1.80	10.9493 ± 1.74	11.7350 ± 1.25	12.0643 ± 0.65

a) p> 0.05 compared with IV
b) p>0.05 compared with SC

Table 2

Parameters	**5th day**	**9th day**	**15th day**	**21st day**
Lymphocyte				
Control	59.8333 ± 2.14	60.6677 ± 1.86	63.5000 ± 2.74	63.3333 ± 1.86
IV-Group	63.1667 ± 4 .21	65.1667 ± 3.12	66.3333 ± 5.28	65.3333 ± 5.95
Subcut-Group	66.6667 ± 3.08	66.8333 ± 3.19	64.8333 ± 1.72	62.5000 ± 3.33
Neutrophil				
Control	38.5000 ± 1.38	35.1667 ± 1.55	34.6667 ± 2.34	35.8333 ± 1.72
IV-Group	35.1667 ± 4.12	33.1667 ± 3.06	32.0000 ± 5.18	33.0000 ± 5.73
Subcut-Group	32.3333 ± 3.08	31.3333 ± 3.33	34.3333 ± 1.86	35.3333 ± 3.01
Eosinophils				
Control	1.5000 ± 1.05	1.1667 ± 0.41	1.5000 ± 0.55	1.0000 ± 0.63
IV-Group	1.1667 ± 0.41	1.5000 ± 0.55	1.3333 ± 0.52	1.3333 ± 0.52
Subcut-Group	0.8333 ± 0.98	1.3333 ± 0.52	0.8333 ± 0.75	1.3333 ± 0.52
Monocytes				
Control	0.0000 ± 0.00	0.1667 ± 0.41	0.0000 ± 0.00	0.3333 ± 0.52
IV-Group	0.1667 ± 0.41	0.0000 ± 0.00	0.3333 ± 0.52	0.1667 ± 0.41
Subcut-Group	0.1667 ± 0.41	0.1667 ± 0.41	0.0000 ± 0.00	0.6667 ± 0.52

a) P> 0.05 compared with IV
b) P>0.05 compared with SC

Hemoglobin

In this finding after 5 to 21 days of HAE cells injection in both groups, the animals did not showed much physiological changes Hb concentration levels and anatomical disruption, such as tissue infiltration. There are few studies had been observed in xeno-transplantation model, which also reported the less or lack of immuno-genecity. There was no alteration in of Hb level in the entire group studied during different period. This will support for the use of HAE cells in rats for the experimental studies on various systems (Fig. 2, Table-1).

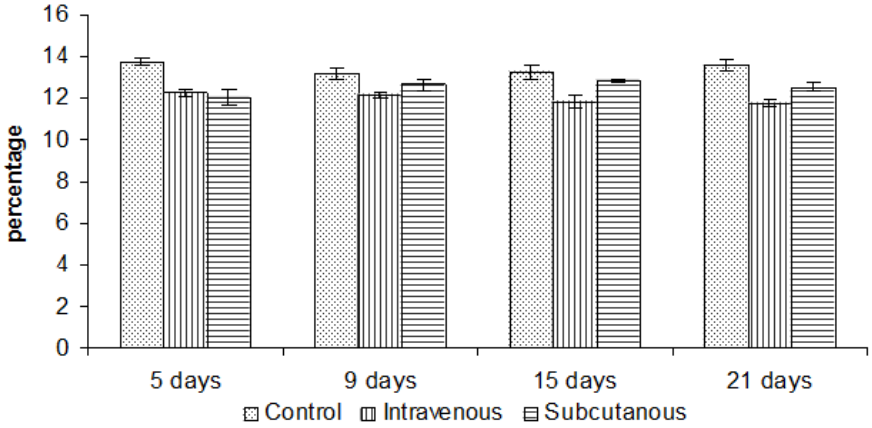

Figure 2: Hemoglobin concentration in various groups after the human Amniotic Epithelial cells transplantations

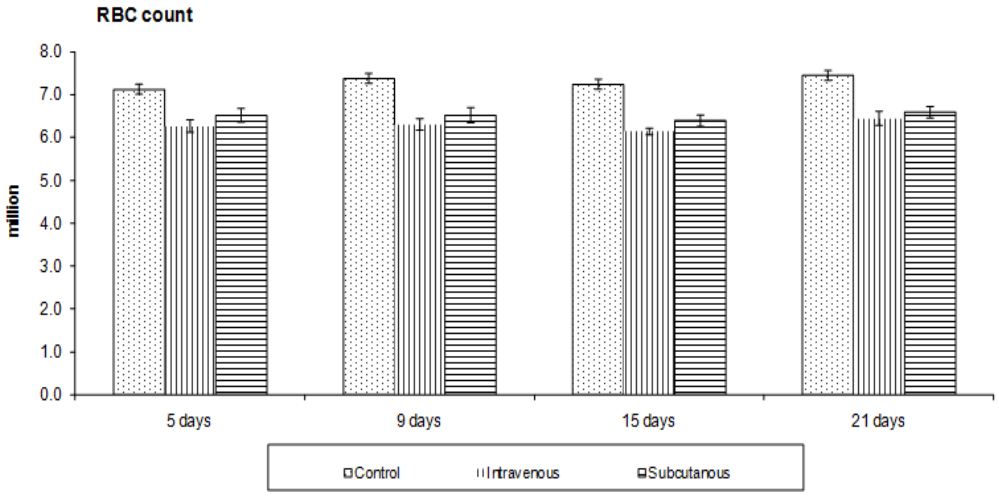

Figure 3: Red blood cells count in various groups after the human Amniotic Epithelial cells transplantations

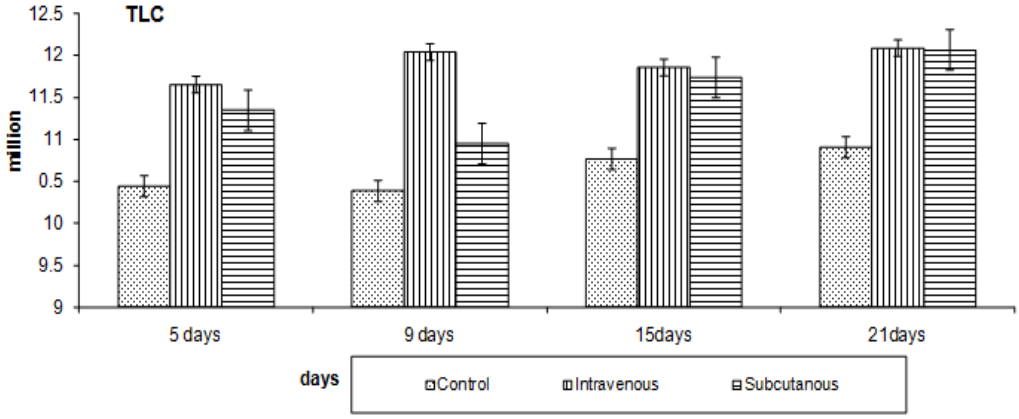

Figure 4: Total Leucocyte cells count in various groups after the human Amniotic Epithelial cells transplantation p>0.05

Blood Suspension

Blood suspension of the intravenously infused animals was collected and DiI labeled HAEC were identified in fluorescent microscope (Fig. 1C).

Tissue Biopsy

There was small scar tissue were found at the site of injection of HAE cells in the skin of the experimental rats. After 21 days injection the subcutaneous tissue along with the striated muscle were removed from the sub-cutaneously infused animals. One centimeter of the tissues were cut and processed for frozen section and sections were taken in 15 microns thickness. In histological observation the dermis of skin with diffused human amniotic epithelial cells were present in the subcutaneous (Fig. 1D) with less or absence of infiltration in infused skin.

DISCUSSION

It has been shown that the human amniotic membrane and HAE cells transplantation is an effective surgical procedure that promotes the reduction of chronic inflammation, increases re-epithelialization, decreases recurrent erosion and cause regression of neo-vascularisation in affected area (Kim *et al.,* 1995; Kim *et al.,* 1995 and Mellte *et al.,* 2000).

HAE cells derived factors inhibit migration of neutrophils and macrophages in vitro. mRNA analysis revealed that HAE cells produced migration inhibitory factors (MIF), a potent inhibitor of macrophage migration and coincidentally, a potent inhibitor or Nature killer (NK) cell mediated lytic activity (Apte *et al.,* 1998). HAE cells also secrete factors that inhibit cells of adaptive immune system, as demonstrated by the steep reduction in T and B cell proliferates to mitogens. HAE cells reconstituted the corneal epithelium and did not undergo immune rejection during 10 days after transplantation on to denuded corneal surface of rabbits (He *et al.,* 1999). Under normal condition HAE cells do not express classic major histocompatibility complex (MHC) class I antigen (Adinolf *et al.,* 1981) and thus enjoy a degree of immune privilege. Addition to the absence of MHC class I antigen, the HAE cells secreted immune suppressive factors that inhibit both innate and adaptive immune response, therefore it reinforcing the immune privilege of HAE cells (Haochuan *et al.,* 2005).

The present study showed very limited rejection of human amniotic epithelial (HAE) cells in-vivo infusion in the Wister albino rats. In early few studies were done in-vitro (Sakuragawa *et al.,* 1992 and 1996; Elwan *et al.,* 1997 and 1998; Terada *et al.,* 2000 and Matsuura *et al.,* 2001). But the present study demonstrated the in-vivo evaluation of human amniotic epithelial cell in infusion and implantation. Similar to in-vitro observation, in-vivo infusion and implantation of human epithelial cells also showed good positive response against the host.

All surgical procedure were done under sterile conditions and the experiments were repeated with a different batch of HAE cells, suggesting that the inflammatory reaction reflected an Immunological response to hetero-specific tissue rather that an exogenous infection. No such inflammation response had been observed when the human amnion membrane preparation was implanted into a lesion cavity in rat brain (Davis *et al.,* 1987). A minor foreign body reaction has been demonstrated in human amnion membrane preparations used as micro-vascular graft in rats (Gray *et al.,* 1987).

For successful transplantation of tissue-cultured epithelial cells, it is very imported to determine the appropriate cell type for the function that is to be accomplished by the transplanted cells (i. e adhesion, spreading and proliferations). Using amniotic epithelial cells as donor cells for tissue cultured epithelial transplantation is initiated by successful use of amniotic membrane in full-thickness skin wound dressing (Stern, 1913) and repair of some damaged mucous membranes (Treford *et al.,* 1979) including the ocular surfaces (Roth, 1940 and Adinolf *et al.,* 1982). In such conditions, the major component of amniotic membrane basement materials was thought to be responsible for these successes (Lee *et al.,* 1997). It was shown that these basement membrane components are produced by amniotic epithelial cells (Aplin *et al.,* 1985). From the present observation, we conclude that though HAE cells as xenogenic origin it survived for short time duration (hypersensitivity exist for 21 days after the implantation) without much changes in

the circulation as well in skin. So it might be used for an experimental in animals and human clinical trail to treat various degenerative diseases including neurodegenerative disease such as Parkinson's Diseases (PD) and Alzheimer's Diseases (AD).

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