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RESEARCH ARTICLE

DIFFERENTIATION OF HUMAN UMBILICAL CORD MESENCHYMAL STEM CELLS INTO INSULIN PRODUCING BETA CELLS FOR DIABETIC THERAPY.

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Abstract

Aim: The aim of the present study was to evaluate the potential of using human umbilical cord tissue as a source of mesenchymal stem cells to differentiate into functional pancreatic β-cells.

Method: Umbilical Cord Mesenchymal Stem Cells (MSCs), which have a low risk of immune rejection, were isolated, cultured *in-vitro* and characterized by Flowcytometry and immunocytochemistry. Further they were differentiated successfully *in-vitro* into functional pancreatic β-cells using H-DMEM (25 mM glucose/L) supplemented with β-Mercaptoethanol (0.1 mM/L), b-Fibroblast growth factor (10 µg/L), Taurine (10 mM/L) and Nicotinamide (10 mM/L).

Results: Umbilical cord sample of 8 cm length yielded $1.5*10^5 \pm 0.5*10^5$ cells after enzymatic digestion. These were passaged to confluence and were found by Flowcytometry to be CD-73$^+$, CD-90$^+$, CD-105$^+$, Vimentin$^+$, CD-34$^-$, CD-45$^-$ and HLA-DR$^-$ indicating that 95% of cells were MSCs. These were differentiated for 25 days and 20% stained positive for Dithizone, which is a specific marker for pancreatic beta cells. Insulin secreted in the medium was evaluated with and without glucose stimulation.

Conclusion: It is evident from this work that Umbilical cord tissue which is easily and ethically available is suitable to isolate MSCs, which have a potential to differentiate into insulin secreting β-cell *in-vitro*. These differentiated cells can be placed *in-vivo* either by Edmonton protocol into the pancreatic duct or embedded in omentum for managing both Type-1 and Type-2 Diabetes Mellitus. This is a promising treatment option, especially since recent literature proposes that pancreatic β-cell apoptosis is the underlying cause of both these pathologies.

Introduction:-

Diabetes is the major non-communicable disease of the decade with estimated 422 million individuals diagnosed globally, according to World Health Organization (WHO) 2016. Pancreatic β-cells are the predominant insulin

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producing cell types within the Islets of Langerhans and insulin is the primary hormone which regulates carbohydrate and fat metabolism, the absence or dysfunction of it results in DM [1, 2]. According to a recent paper β-cell apoptosis is the common underlying cause of both Type-1 Diabetes (T1D) and Type-2 Diabetes (T2D) [3].

The current therapy of Diabetes includes oral medication or exogenous insulin replacement and regular monitoring [4]. Although pancreatic allotransplantation is an alternative to conventional insulin therapy, it is not considered at present as an accepted standard of care due to the lifelong requirement of immunosuppressive therapy and scarcity of suitable healthy organs for the millions suffering with this disease [5, 6]. To overcome these obstacles cell based regenerative therapy would be a suitable alternative in which stem cells could be differentiated into insulin producing β-cells [7,8,9] and can be transplanted in a patient either by (i) invasive intra portal transplantation [6,10] or (ii) minimally invasive encapsulation [2] or by (iii) seeding in the omentum [11].

Mesenchymal stem cells (MSCs) were identified for the first time from bone marrow and were subsequently successfully isolated from several tissues and irrespective of its origin exhibited the same phenotypic properties [12]. MSCs have specific characteristics such as plastic adherence and exhibit certain specific cell surface markers like CD-105, CD-90 and CD-73[13, 14]. They have a capacity of self renewing and have the ability to differentiate into Adipogenic, Chondrogenic and Osteogenic cell lineages [15, 16].

In the present study umbilical cord (UC) tissue was selected as a source for MSCs, since sampling is non invasive, abundantly available and considered as a clinically waste material and discarded, hence very few ethical controversies are involved in it [17]. MSCs are present in the UC within the extracellular matrix of the Wharton's jelly, where a number of growth factors exist [18].

Aim:-
The aim of the present study was to evaluate the potential of using human umbilical cord tissue as a source of mesenchymal stem cells to differentiate into functional pancreatic β-cells and use them in the treatment of both Type-1 and Type-2 DM.

Materials and Methodology:-

Sample Collection:-
With prior informed consent as approved by the Hospital Institutional Ethical and Stem cell Committee, Human umbilical cord (HUC) tissues were collected after lower segment caesarean section (LSCS) or normal vaginal delivery and transported to the laboratory in sterile normal saline within 30 minutes.

Isolation and Expansion of MSCs from HUC:-
HUC samples were processed within an hour after collection in a laminar air flow cabinet. Umbilical cord was opened lengthwise, blood vessels were dissected and the remaining tissue was scraped and minced mechanically in a sterile petri-dish. This was transferred to a 50 ml centrifuge tube along with normal saline digested enzymatically with Collagenase-II (50-200 U/ml, Gibco USA), at 37^{o}C in 5% CO_2 incubator for 35 minutes. The digested tissue was collected and centrifuged at 1500 RPM for 15 minutes. Cell pellet obtained was initiated in a T-25 flask and provided with Low Glucose-Dulbecco's Modified Eagles Medium (L-DMEM 4.5 mM glucose/L), streptomycin 0.1mg/ml and 10% FBS for cell growth and attachment. Every third day medium was replaced with fresh medium and the cells were passaged when they reached 70-80% confluence by treating with 0.25% Trypsin-EDTA and were split and reinitiated in new flasks with a cell count of 2000 cells/cm^2.

Cell count and Viability:-
Growth curve was plotted by culturing the cells in a 12 well plate. In which cells were harvested on a day to day basis and cell count was performed to establish the log phase of the cells. Cell count was established using the Neubar chamber: 10 µl of a cell suspension of unknown concentration was mixed with equal volume of 0.4% Tryphan Blue solution, incubated 2-5 minutes at room temperature. Evaluated under 10X magnification in a binocular inverted microscope (Nikon TS100, Japan).

The cell concentration was then calculated as follows: Cell concentration (in cells/ml) = average count x 2 x 10,000 x dilution factor of original cells.

Karyotyping:-
Karyotyping was performed on the cultured cells after each passage toanalyze numerical/structural changes in chromosomes if any. Metaphase images obtained in 100X and analyzed using DSS spectral imaging software system. International System for Human Chromosome Nomenclature (ISCN) 2013 used for analysis of karyotypes.

Flowcytometry:-
Fluorescent labeled direct antibodies CD-90^{+}, CD-105^{+}, CD-73^{+}, CD-34^{-}, CD-45^{-} and HLA-DR^{-} (Becton & Dickinson (BD) Biosciences, USA) were added to the cells from P1 passage, which were dislodged using 0.25 % Trypsin-EDTA and resuspended in Phosphate Buffer Saline (PBS) and incubated for 30 minutes at room temperature. The labeled cells were analyzed by BD-FACS Caliber (BD Biosciences, USA) by the method of Feisst et.al (2014).

Immunocytochemistry:-
Immunocytochemistry was performed using Vimentin antibody on isolated MSCs based on standard basic protocol of Wang et.al (2011).

Differentiation:-
MSCs were differentiated into insulin producing β-cells with High Glucose-Dulbecco's Modified Eagles Medium (H-DMEM 25mM Glucose/L) supplemented with β-Mercaptoethanol (0.1mM, Himedia), b-Fibroblast growth factor (10µg/L, Gibco USA), Taurine (10mM/L, Himedia) and Nicotinamide (10 mM/L, HiMedia) and incubated at 37^{o}C in a 5% CO_2 incubator.

Dithizone staining:-
Diphenylthiocarbazone, Dithizone (DTZ, Sigma) is a zinc-chelating substance which is used for identification of insulin granules in pancreatic β-cells. DTZ stock solution (10 mg/ml) was prepared in Dimethylsulfoxide (DMSO) and stored at –20ºC. Dithizone working solution was obtained by adding 10 µl of the stock solution to 1ml of the culture medium, followed by filtration through a 0.2 micron filter. Differentiated HUMSCs were incubated with the staining solution at 37ºC for 20 minutes and then examined [19].

ELISA:-
The Human Insulin Enzyme-Linked Immunosorbent Assay (ELISA) is an *in vitro* enzyme-linked Immunosorbent assay for the quantitative measurement of human Insulin in serum, plasma, cell culture supernatants or in urine. In our study ELISA was performed for quantitative estimation of insulin in cell culture supernatant with (50mM glucose/L) and without glucose stimulation [20].

Results:-
A total of 26 HUC tissues were collected out of which six were from normal vaginal delivery remaining 20 were after LSCS. Approximately 8 cm size cord was processed and MSCs were successfully isolated from 16 samples which were all obtained after LSCS. Cell attachment started on day four, small colonies observed on day six or seven, large colonies were observed by 12th day which were considered as P^0 population. Mean cell count observed was $1.5*10^5 \pm 0.5*10^5$, cell viability was found to be 98% (Figure 1.A, 1.B).

Two samples were used to establish the growth kinetics of MSCs, which showed that cells actively divided till 5th day when the initial cell count was 2000 cells/cm^2 and later reached a growth plateau (Figure 2A). Numerical and structural abnormalities were not found in the chromosomes till the 5th passage (Figure 2B).
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Flow Cytometry analysis was performed on two samples on P^2 population which showed that 95% cells expressed CD90, CD105, 80% cells expressed CD73, none of them expressed CD34, CD45 and 20% cells expressed HLA-DR (Figure 3). Immunocytochemistry was performed on one sample for Vimentin antibody in which all the cells showed positive staining (Figure 1.D, E).

4 samples were used for Differentiation in which one sample got contaminated and remaining samples were further processed for differentiation with the above mentioned growth factors. After 25 days of incubation cells stained positive for Dithizone (Figure 1). Amount of insulin released was measured by ELISA and was found to be four-fold higher when stimulated (8 IU/ml) compared with unstimulated (2 IU/ml).

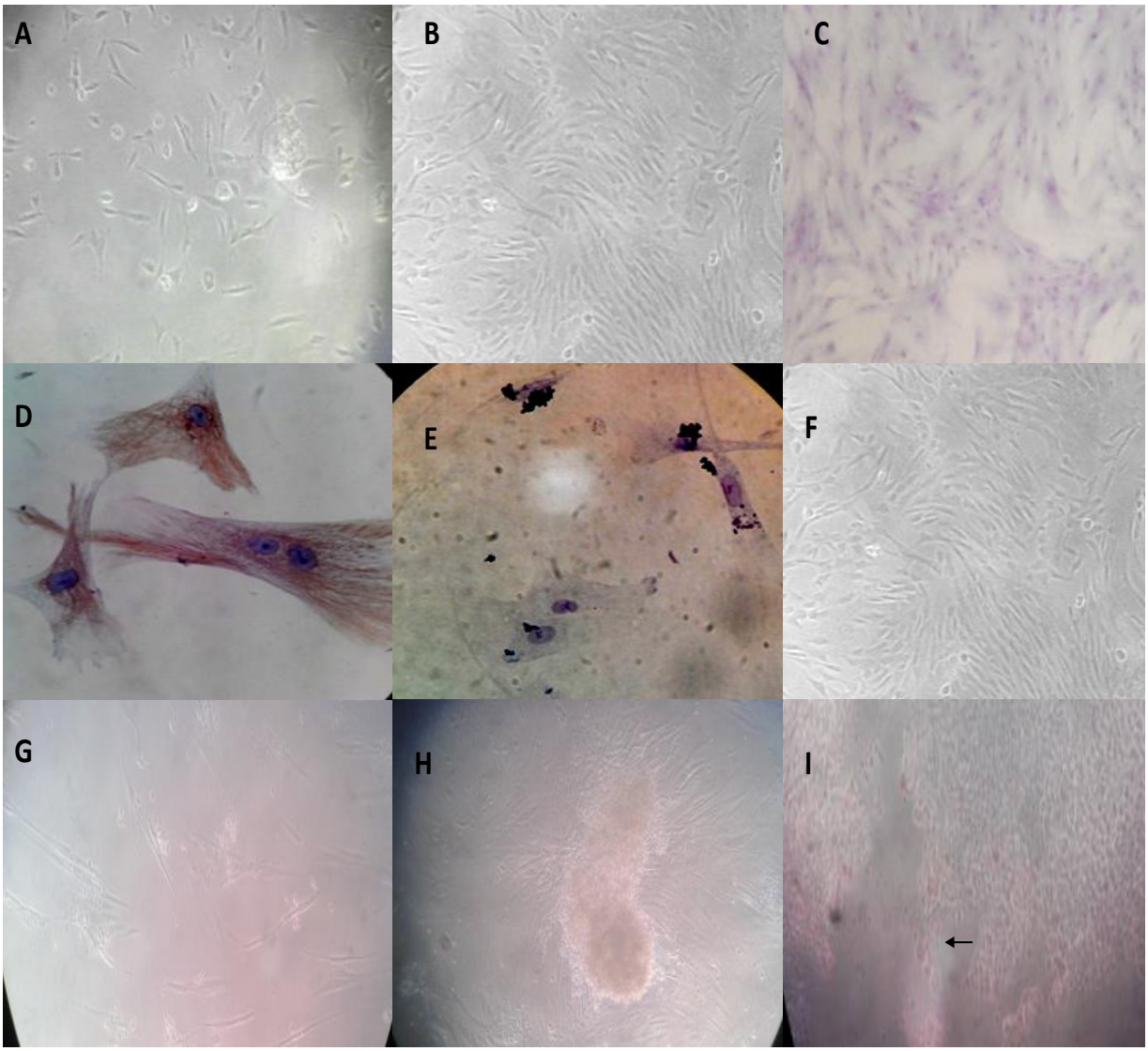

Figure 1:- (A) MSCs on day-6-small dividing colony (B) MSCs on day-12-colony attained confluency (C) Giemsa staining-Fibroblastic appearance of MSCs and its colony forming capacity. IHC: (D) Immunocytochemical staining- MSCs expressing Vimentin, which is one of the positive markers, (E) Negative control- Cells stained without Vimentin antibody. Differentiation: (F) MSCs showing 80% cell confluency on day 1 of differentiation (G) Day 2 of differentiation (H) Day 15 of differentiation-cells aggregated in clusters (I) At the end of differentiation (25 days) cells stained positive for DTZ stain.

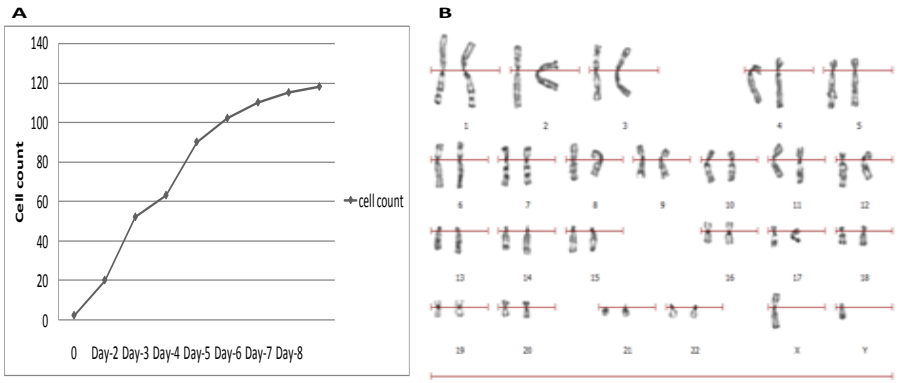

Figure 2:- (A) Growth curve- After day-5 cell senescence was observed. (B) Karyotyping- Genome integrity was observed till the last passage (passage-5).

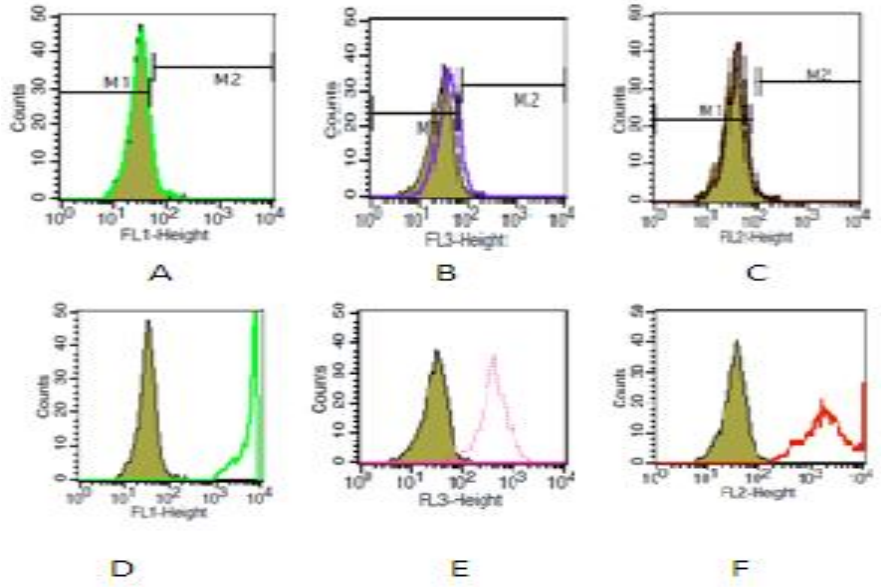

Figure 3:- Flowcytometry analysis- (A) CD34, 0%; (B) CD45, 0%; (C) HLA-DR,10%; (D) CD90, 95%; (E)CD105,95%;(F)CD73,80%; (CD34,CD45-Haematopoitic stem cell marker, CD90, CD73,CD105-Mesenchymal stem cell markers and HLA-DR is an MHC class II cell surface receptor).

Discussion:-

Diabetes is a group of disorders in which defective insulin production or function results in the deregulation of blood glucose levels. T1D and T2D are the most common forms of Diabetes. Irrespective of the etiology of diabetes the disorder is being diagnosed at an alarming rate and has been declared as a global epidemic by WHO, 2016. The disease burden and the complications associated with this progressive pathology is responsible for major morbidity and mortality causing both social and economic burden to developed and more so to developing countries. Given that diabetes is identified in both rural and urban populations in India, there is an urgent need for intervention at regional and national levels to mitigate the potentially catastrophic health complications [21]. Apart from life style modifications, several categories of drugs are available for managing diabetic patients and to delay the associated complications [22]. Recombinant insulin production revolutionized diabetic therapy especially for T1D [23, 24]. There are conflicting reports regarding the benefits of early insulin treatment in T2D [25].

Since Insulin replacement partially carries out, but does not match the precision of functioning β-cells, total pancreatic transplant surgery was considered as an option in 2000 [26]. However, there are several drawbacks to this approach which include: (i) mortality rate associated with the surgery, (ii) lifelong immunosuppression associated with adverse health effects and (iii) limited availability of donor pancreases [27]. Hence, human islet fetal transplant was considered as another alternative as they have a better self renewable capacity and can differentiate into pancreatic endocrine cells. However, inconsistencies in the islet yield, short term success rates and ethical issues restricted this treatment modality [2].

More recently stem cell therapy is an attractive alternative for the treatment of several diseases including Diabetes. Adult autologous Stem cells can be extracted from different parts of the body [28]. In the present study umbilical cord tissue which is usually considered as a clinical waste and discarded after the delivery of the baby was used. UC is easily accessible, available source of MSCs without ethical conflicts [19, 29].

For more than a decade several researchers are working on developing pancreatic islet insulin expressing cells from cord blood/tissue for use in the treatment of diabetes [8,30]. In a country like India, with an estimated birth rate of 21.8 per 1000 people would be one of the largest sources of umbilical cord tissue to isolate MSCs in the world [31].

Present study indicated that collecting UC tissue after LSCS from the operation theatre gives better culturable MSCs when compared to vaginal deliveries, when the same number of samples were collected from each mode of delivery (n=12). This is the first time to the best of our knowledge that this aspect has been evaluated. Since there were contradictions about the method of isolating MSCs, some studies have described disadvantages with enzymatic method such as yield, cost, contamination and process time [32,33], while others supported enzyme digestion over mechanical method [34]. Hence, we used a combination of both mechanical and enzymatic methods. It gave us an yield of $1.5*10^5 \pm 0.5*10^5$cells/8cm of UC after 12 days of processing (Figure 1). These numbers were relatively low when compared to reports from other groups [17, 35] and may be due to the variation in the initial quantity of the cord tissue used.

In the present investigation, growth curves indicated that MSCs, reached a 70-80% confluency, on day five when 2000 cells/cm^2 were seeded for P^1 population and karyotyping did not show any structural and/or numerical abnormalities in the cells cultured in-vitro till the 5^{th} passage Based on these results we performed differentiation after the 2^{nd} passage (Figure 2(A&B)).

Based on published studies CD-73, CD-90, CD-105, Vimentin, CD-34, CD-45 and HLA-DR markers were used to characterize the MSCs. In the present study 95% of undifferentiated cells expressed CD-73, CD-90, CD-105,Vimentin, none of the cells expressed CD-34, CD-45 and low expression was observed for HLA-DR after the second passage, confirming that these are MSCs. (Figure 3), which is similar to other published reports [14,36].

Different protocols have been published to differentiate HUMSCs into insulin producing β-cells in-vitro [8]. Wang et.al (2011), had earlier differentiated HUCMSCs into pancreatic β-cells using H-DMEM (25mM glucose/L), β-Mercaptoethanol (0.1mM), b-Fibroblast growth factor (10µg/L) and Nicotinamide (10mM/L). We achieved similar results after 25 days of incubation by additionally adding Taurine as it was shown to be an efficient endodermal differentiation factor [19]. Under the above conditions only some of the HUMSCs differentiated into insulin-producing cells which released insulin on glucose stimulation, which might represent β-cells and stained positive with Dithizone which is an islet specific stain (Figure 1).

Conclusion:-

It is evident from this study that HUMSCs have a potential to differentiate into insulin secreting β-cell in-vitro. Efficient differentiation protocol and suitable homing methods would help in translating the technology from laboratory bench to the clinic in near future to treat DM.

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Conflict of interest:-
Authors declare no conflict of interest.

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