Brief Report

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In vitro culture and characterization of enteric neural precursor cells from human gut biopsy specimens using polymer scaffold

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Summary ***In vitro*** **expansion and characterization of neural precursor cells from human gut biopsy specimens with or without Hirschsprung's disease using a novel thermoreversible gelation polymer (TGP) is reported aiming at a possible future treatment. Gut biopsy samples were obtained from five patients undergoing gut resection for Hirschsprung's disease (*n* = 1) or gastrointestinal disorders (*n* = 4). Cells isolated from the smooth muscle layer and the myenteric plexus were cultured in two groups for 18 to 28 days; Group I: conventional culture as earlier reported and Group II: using TGP scaffold. Neurosphere like bodies (NLBs) were observed in the cultures between 8th to 12th day and H & E staining was positive for neural cells in both groups including aganglionic gut portion from the Hirschsprung's disease patient. Immunohistochemistry using S-100 and neuron specific enolase (NSE) was positive in both groups but the TGP group (Group II) showed more number of cells with intense cytoplasmic granular positivity for both NSE and S-100 compared to Group I. TGP supports the *in vitro* expansion of human gut derived neuronal cells with seemingly better quality NLBs. Animal Studies can be tried to validate their functional outcome by transplanting the NLBs with TGP scaffolds to see whether this can enhance the outcome of cell based therapies for Hirschsprung's disease.**

Keywords: Enteric neural precursor cells, Hirschsprung's disease, thermoreversible gelation polymer (TGP)

1. Introduction

The part of the peripheral nervous system (PNS) that controls the peristaltic activity of the gut wall is the enteric nervous system (ENS). This is essential for propulsion of food in the digestive tract. The ENS is composed of a large number of neurons and glial cells, distributed throughout the length of the gut. These ganglion cells develop from the neural crest in the embryo. Hirschsprung's disease or congenital megacolon is the failure or delay of the complete colonization of the gut by these enteric neural crest cells during early development which results in the absence of ganglia or neurons in a portion of the gut, usually the colon, that results in aperistalsis and severe intestinal obstruction. Hirschsprung's disease affects

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1 in 5,000 newborns and affects boys more often than girls at a ratio of 4:1 (*1-3*). It appears either sporadically or has a familial basis and is often associated with other developmental defects. Surgical management continues to be the major treatment approach. The principle involves reconstruction of the intestinal tract by pulling the normal innervated portion of the colon down to the anus preserving the sphincter function. This pull-through surgery's recent variants include total transanal endorectal pull-through (TERPT) and the laparoscopic assisted pull-through (*3*). Inspite of advances in surgical approach, constipation, abdominal distension and enterocolitis are some of the long term obstructive symptoms observed in patients who undergo operative modalities for Hirschsprung's Disease (*3*). The extent of aganglionosis varies between patients and there are reports in the literature in which there are extreme forms of aganglionosis in which even a complete portion of the intestine is devoid of the ganglion cells (*4*). In such cases, surgery is impossible. At this juncture, cell-based therapies to replace the ganglion cells or enteric neuronal cells in the aganglionic portion of the gut aiming at restoring the function of the gut are being considered as a potential solution to Hirschsprung's disease (*5,6*). Earlier studies have reported the isolation of enteric neural precursor cells from normal gut tissues and Hirschsprung's disease affected gut tissues (*5,7*). In this study, we report *in vitro* culture and characterization of enteric neural precursor cells from full thickness gut biopsy samples of patients with or without Hirschsprung's disease in a novel polymer scaffold.

2. Materials and Methods

Postnatal gut full thickness 2-4 mm biopsy samples were obtained from five patients undergoing gut resection surgery after proper informed consent. The work was conducted in accordance with the Declaration of Helsinki (1964). Of the five patients, one had Hirschsprung's disease (Patient I). The remaining four patients (Patient II-V) underwent gut resection surgery for conditions like biliary atresia and exomphalos major with rectal atresia. From patient I, biopsy samples from both the ganglionic portions and the aganglionic portions of the gut were obtained. All samples were washed in phosphate buffered saline (PBS) containing penicillin (100 U/mL), streptomycin (100 µg/mL) and amphotericin (100 U/mL). Using forceps, the outer smooth muscle layers along with the myenteric plexus were peeled off from the underlying tissue as strips. Fibronectin (2 µg/cm^2) (Sigma-Aldrich, USA) was coated on 6-well tissue culture (TC) Plates (Corning Inc., Corning, NY, USA) and the plates were undisturbed for at least two hours. The tissue strips were washed with phosphate buffered saline (PBS) Ca^{2+} and Mg^{2+} free and cut into small pieces for enzymatic digestion using collagenase (1 mg/mL) and dispase (1 mg/mL) and kept in a CO_2 incubator at 37°C for 30 min. Digested tissues were filtered using a 70 µm filter and centrifuged at 1,800 rpm for 8 min at 24°C. Cell count of the pellet was obtained using the Trypan blue dye exclusion method and the cells were divided into two equal portions. The cells thus divided were seeded as two groups, Group I (Gr.I): in DMEM/F12 (Gibco BRL, Gaithersburg, MD, USA) medium supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (2 mmol/L), and growth factors including basic fibroblast growth factor (bFGF) (20 ng/mL) (Sigma-Aldrich, USA) and epidermal growth factor (EGF) (20 ng/mL) (Sigma-Aldrich, USA) onto the fibronectin coated TC plates according to the protocol reported by Bondurand *et al.* (*2*); Group II (Gr.II): with thermoreversible gelation polymer (TGP). TGP was obtained in a lyophilized vial from Nichi-In Biosciences (P) Ltd, Chennai, India. The Thermo-reversible Gelation Polymer (TGP) used in this study is a copolymer composed of thermo-responsive polymer block [poly(*N*-isopropylacrylamide-co-n-butyl methacrylate) (poly(NIPAAm-co-BMA)] and the hydrophilic polymer block (polyethylene glycol [PEG]) as described by Yoshioka *et al.* (*8*). Because this polymer block is hydrophilic at temperatures below the sol-gel transition temperature (20°C for the TGP used in this study) and hydrophobic at temperatures above this sol-gel transition temperature forming a homogenous three-dimensional (3D) network of the gel in water, cells for culture can be embedded at temperatures lower than 20°C and cultured three dimensionally in the hydrogel state at 37°C. For the present study, the TGP was reconstituted with 10 mL of DMEM/F12 medium and incubated at 4°C overnight. A drop of TGP-DMEM tissue culture (TC) medium mixture was placed at the center of the 6-well Fibronectin coated TC Plates (Corning Inc., Corning, NY) and solidified at 37°C. The cells from the remaining pellet were suspended in the culture medium and placed over this solidified gel-TC mixture after which a drop of the gel-TC medium mixture was again placed to cover the cells. Thus, the cells were embedded within the TGP. Culture medium containing DMEM/F12 medium supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (2 mmol/L), bFGF (20 ng/mL) and EGF (20 ng/mL) was overlaid over the TGP. Cells were incubated at 37˚C with 5% CO_2 for 18-28 days. Cells were observed daily and a media change was done every 2-3 days.

3. Results and Discussion

The average cell number obtained from the ganglionic samples was 0.94 million cells. The cell number obtained from the aganglionic portion of Patient I was 0.19 million cells. In all samples (both Gr.I and Gr.II),

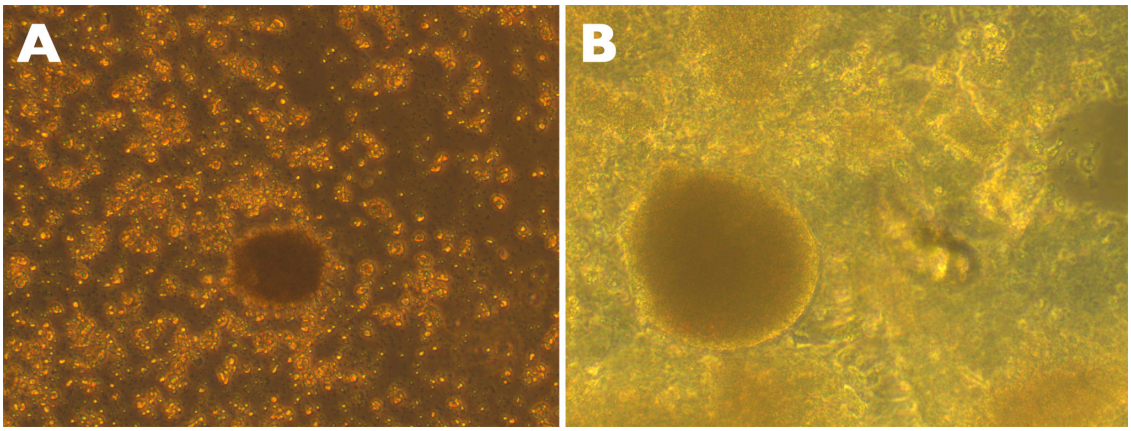

Figure 1. Neurosphere like body (NLB) observed. (A) Group I (Conventional). **(B)** Group II (TGP).

neurosphere like bodies (NLBs) were observed in culture between the 8th day and 12th day (Figure 1) including the aganglionic sample from Patient I with Hirschsprung's disease. The neurosphere like bodies (NLBs) observed in the culture were then subjected to histological and immunohistochemical (IHC) studies for S-100 and neuron specific enolase (NSE), a neuronal specific marker. H&E staining showed that both ganglionic and aganglionic samples in both Gr.I and Gr.II had round to oval cells with a large nucleus and moderate amount of pale cytoplasm. The cell size varied greatly in all the smears. These cells did not form sheets or any other specific pattern. They were mostly loose clusters and vague, short filamentous extensions were seen from the cytoplasmic borders. H&E stains were positive for neural cells (Figure 2). In immunohistochemistry (IHC), morphologically there was no difference in cultured cells from ganglionic and aganglionic segments in Gr.I and Gr.II. However, the cellularity was relatively higher in Gr.II (TGP group). Though both groups showed a cytoplasmic granular appearance, the TGP group (Gr.II) showed more number of cells with intense cytoplasmic granular positivity for both NSE and S-100 (Figure 3B, 4B) compared to Gr.I (Figure 3A, 4A).

Earlier studies have reported the generation of NLBs containing functionally active neural progenitors from gut tissue (*1,9,10*). Almond *et al*. isolated and expanded progenitor/stem cells from the post-coitum embryonic mouse cecum and postnatal human myenteric plexus and successfully transplanted the differentiated neurons and glial cells into aganglionic murine hindgut. The implanted cells colonized postnatal aganglionic bowel and expressed neuronal markers including nitric oxide synthase and vasoactive intestinal polypeptide (*11*). Metzger *et al.* generated NLBs from postnatal human gut mucosal tissue and after transplantation; the cells from NLBs colonized aganglionic chick and human hindgut to generate ganglia-like structures, enteric neurons and glia (*1*). In this study, we have examined the feasibility of culturing NLBs obtained from routine

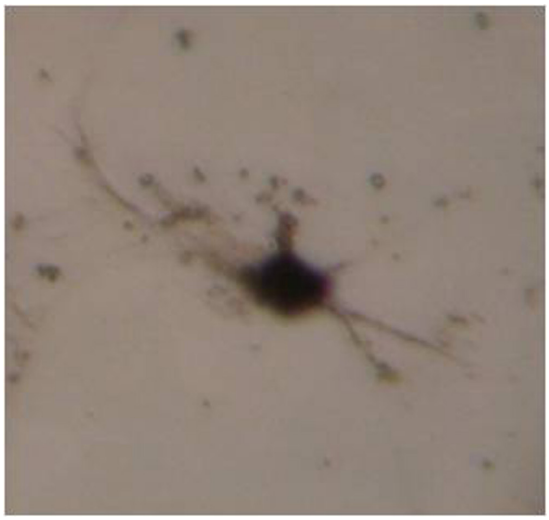

Figure 2. H&E staining of cultured Neurosphere like bodies (NLBs).

gut biopsy samples of patients undergoing surgeries for Hirschsprung's disease or other disorders of the gastrointestinal system in a Thermo-reversible Gelation Polymer (TGP) and compared it to conventional culture techniques. Though ENS progenitors from the ganglionic gut of children diagnosed with and without Hirschsprung's disease have been isolated, characterized and reported earlier (*12*), a study similar to ours using a TGP which has proven to yield an increased number of neural progenitors (*13*), makes this unique. Apart from this, among the various types of thermo-reversible hydrogels reported for cell culture in the literature, the TGP used in this study is novel because it is a purely synthetic hydrogel and does not contain any biological components like proteins such as Hen Egg White Lysozyme (*14*) or poly-saccharides such as Chitosan (*15*) which are used in hydrogels to improve properties like cell-adhesion (*14*). Further, the TGP has been proven to maintain the three-dimensional morphology (*16*) of different kinds of stem cells, pre-cursor cells and adult cells without alteration of their gene expression (*17*) for longer periods of time in contrast to other

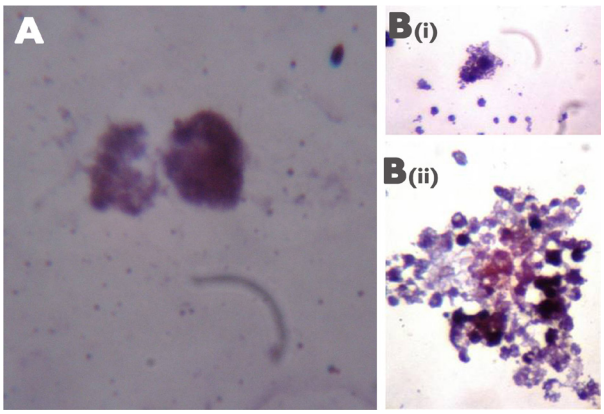

Figure 3. Neuron specific enolase (NSE) positive Neurosphere like bodies (NLBs). (A) Group I (Conventional). **(B(i)** and **B(ii))** Group II (TGP).

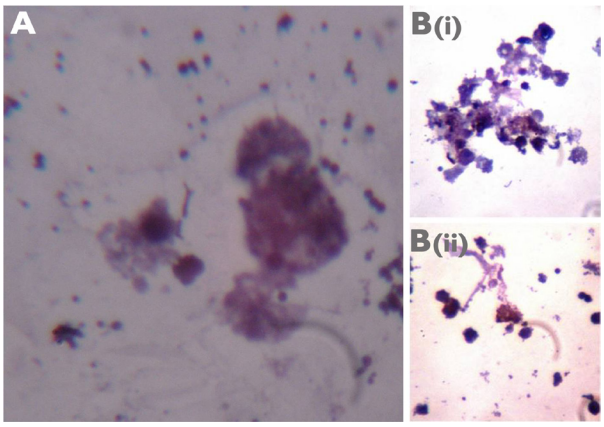

Figure 4. S-100 positive Neurosphere like bodies (NLBs). (A) Group I (Conventional). **(B(i)** and **B(ii))** Group II (TGP).

thermo-reversible hydrogels reported (*14*). Previous studies have suggested that from the 10th day onwards, NLBs can be observed in culture and NLBs have been grown in culture up to 28 days (*2,10*). In our study, the NLBs were observed in culture between the 8th day and 12th day in all samples in both Gr.I and II including that of the aganglionic tissue. Ability of the cells to generate NLBs in the aganglionic sample demonstrates the presence of neural progenitors in that portion of the gut also. However, further characterization and studies are required to identify whether the NLBs from the ganglionic portion and the aganglionic portion have similar characteristics and capability to colonize the aganglionic portion in animal models of Hirschsprung's disease. Furthermore, this would shed light upon the fact that perhaps neural crest progenitors do migrate to the distal colon but fail to proliferate or differentiate due to micro-environmental abnormalities in the distal colon (*3*) and due to *in vitro* culture conditions, they might form NLBs. The relatively higher cellular staining in Gr.II (TGP group) demonstrates that TGP supports growth of NLBs. Because transplantation of neurospheres from fetal and post natal intestine derived neural crest cells has already proven to produce functional neurons in the post natal colon of mice (*18*), further studies asking whether TGP can be used as a carrier for NLB transplantation in aganglionic gut models to help retain the neural progenitors in the region where they should re-colonize and form neural; glial cells are warranted. Suggesting TGP as a carrier for transplantation of NLBs is based upon earlier studies in which TGP was employed for transplantation of stem cells, progenitor cells and adult cells in animal models (*19-21*). The present study is only a preliminary study and extensive studies are warranted before this approach could be standardized and considered for treatment of Hirschsprung's disease.

4. Conclusion

We successfully isolated and expanded human enteric neural precursor cells in the form of NLBs from postnatal gut biopsy samples of patients with Hirschsprung's disease as well as other gastrointestinal disorders. Because the NLBs in the TGP group showed a higher positive in IHC staining compared to the group without TGP, there could be potential to utilize the NLBs cultured in TGP in cell-based therapies for Hirschsprung's disease, after confirming their efficacy in appropriate animal models. The transplantation of NLBs encapsulated in TGP or along with TGP is also another area for further experimentation because it might enhance the outcome, comparing it to earlier published studies.

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