A DISSERTATION ON

Handling and maintenance of 2D and 3D cultures of human stem cells and application in toxicity assessment

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DECLARATION

I hereby certify that the work embodied in this dissertation entitled "Handling and maintenance of 2D and 3D cultures of human stem cells and applications in toxicity assessment" by Zaini Sultan Khan, Enrollment No. 1700102778, submitted to Integral University, Lucknow, is an authentic record of my own work carried out under supervision of Dr. Aditya Bhushan Pant. No scientific dishonesty or plagiarized material has been incorporated. Due recognition has been given to authors whose work has been summarized here by means of correct references and citations. Any part of this work will not be published in print media without prior permission from the supervisor. The matter presented in this dissertation has not been submitted in any other University/ Institute for the award of any other degree or diploma. The responsibility of any plagiarism related issue stands solely with me.

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Place: Lucknow (U.P)

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TO WHOM IT MAY CONCERN

This is to certify that the study conducted by **Ms. Zaini Sultan Khan** student of M.Sc. Biotechnology (IV semester), Integral University has completed her three months dissertation work entitled "**Handling and maintenance of 2D and 3D cultures of human stem cells and application in toxicity assessment"** successfully. She has completed this work from CSIR-Indian Institute of Toxicology Research, Lucknow, under the guidance of **Dr. Aditya Bhushan Pant.** This dissertation was a compulsory part of her M.Sc. Degree.

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INTRODUCTION

Stem cells are special type of undifferentiated cells which are able to divide into any type of cells. These cells are obtained from the tissues of endodermal, mesodermal and ectodermal lineage. They are capable of dividing and renewing themselves for long periods of time. When stem cells divide, each new cell has the potential to either remain a stem cell or differentiate into another type of cell with a more specialized function like muscle cells, red blood cell, etc.

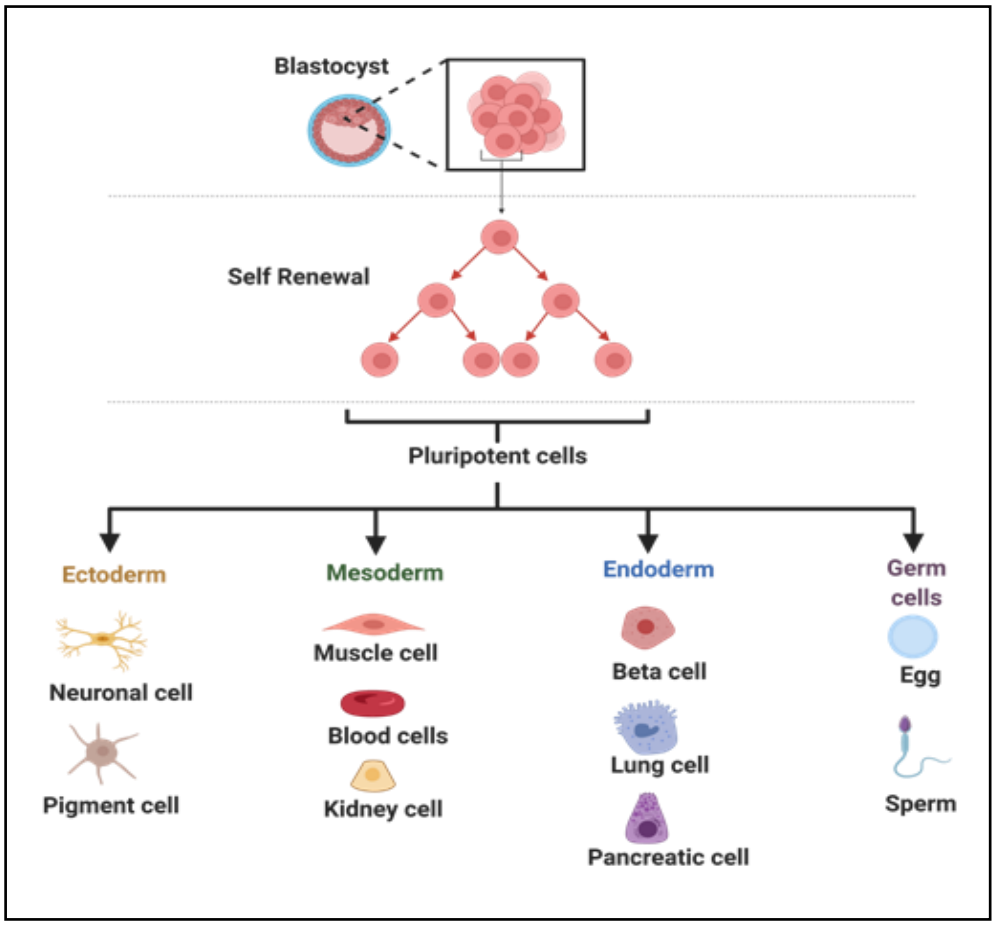


Figure 1: Schematic representation of origin of Stem Cells(*Image created using biorender.com*)

Classification of stem cell based on source of origin

(1) Embryonic stem cell(2) Adult stem cell

Embryonic type stem cells:Embryonic stem cells are pluripotent stem cells derived from inner cell mass of a blastocyst, an early-stage preimplantation embryo. Human ES cells measure approximately 14 micrometres.

Adult stem cells: These cells are undifferentiated cell, found among differentiated cells in a tissue or organ. These cells have the ability of self-renewal and can differentiate into special types of cells (tissues or organ).

Classification of stem cells based on difference in potency

1. Totipotent
2. Unipotent
3. Multipotent
4. Pluripotent

***Totipotent*:** These cells have the capability to produce all cell types of developing organism, it includes both embryonic and extra embryonic membrane.

Pluripotent: These cells make the cells of whole body except extra embryonic membrane. For instances iPSCs, ESCs

Multipotent: These cells can only make cells within a given germ layer but cells of same lineage like the cells of the blood can make all the cells of the blood only.

Unipotent: These cells make cells of single cell type. An example is a germ cell no other cell type.

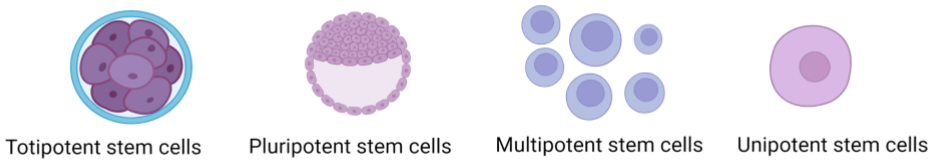


Figure 2: Classification of Stem Cells on the basis of potency*(Image created using biorender.com)*

We have further studied about following cells:

1. Neural stem cells (NSCs)
2. Induced pluripotent stem cells (iPSCs)

Neural stem cells

Neural stem cells (NSCs) are self-renewing, multipotent cells that firstly generate the radial glial progenitor cells that generate the neurons and glia of the nervous system of all animals during embryonic development. The characteristic marker genes: SOX2, Oct 4, Pax-6 etc.

Neural Progenitor Cells (NPCs)

NPCs are multipotent stem cells, which can give rise to the three cell types of the Central neural system (CNS) namely neurons, astrocytes and Oligodendrocytes. Physiologically, NPCs can be found during neural development as well as in adult mammalian brain. First evidence of postnatal neurogenesis in rats was shown in 1965(Altman and Das, 1965) [1]. Somatic NPCs are restricted to several neuronal subtype and glial cells with some NPC-type possessing a glial nature.

Induced Pluripotent Stem Cells (iPSCs)

Induced Pluripotent Stem Cells are derived from skin or blood cells that have been reprogrammed back into an embryonic-like pluripotent state that enables the development of an unlimited source of any type of human cell needed for therapeutic purpose. Most of the cells of multicellular organism become more and more restricted to specific cell lineage. For the treatment of many genetic diseases Human ESCs can be used. But due to some ethics we can’t use embryo for this purpose. To avoid this problem artificially iPSC came in picture create from normal somatic cells by ectopic expression of some genes which are responsible for the pluripotency. The discovery of induced pluripotent stem cells (iPSCs) by Shinya Yamanaka in 2006 was heralded as a major breakthrough of the decade in stem cell research.

Production of Induced Pluripotent Stem Cells (iPSCs)

Induced Pluripotent Stem Cells (iPSCs) are typically derived by introducing products of specific sets of pluripotency-associated genes, or "reprogramming factors", into a given cell type. The original set of reprogramming factors (also dubbed Yamanaka factors) is the transcription factors Oct4, Sox2, Klf4 and cMyc. While this combination is most conventional in producing iPSCs, each of the factors can be functionally replaced by related transcription factors, miRNAs, small molecules, or even non-related genes such as lineage specifiers. These cells are derived by the transfection of stem cells associated genes into non-pluripotent cell such as fibroblast, transfection by viral vector (retro virus). After 3-4 weeks small number of transfected cells become morphologically and biochemically similar to pluripotent stem cells. These cells are isolated through morphological selection, doubling time, or through a reporter gene and antibiotic selection. Then isolate and culture then donor cells, then transfect stem cell associated genes into the cells by viral vectors. Harvest and culture the cell according to embryonic stem cell culture. Then small subsets of the transfected cells become iPSCs cells and generate embryonic stem cell like colonies.

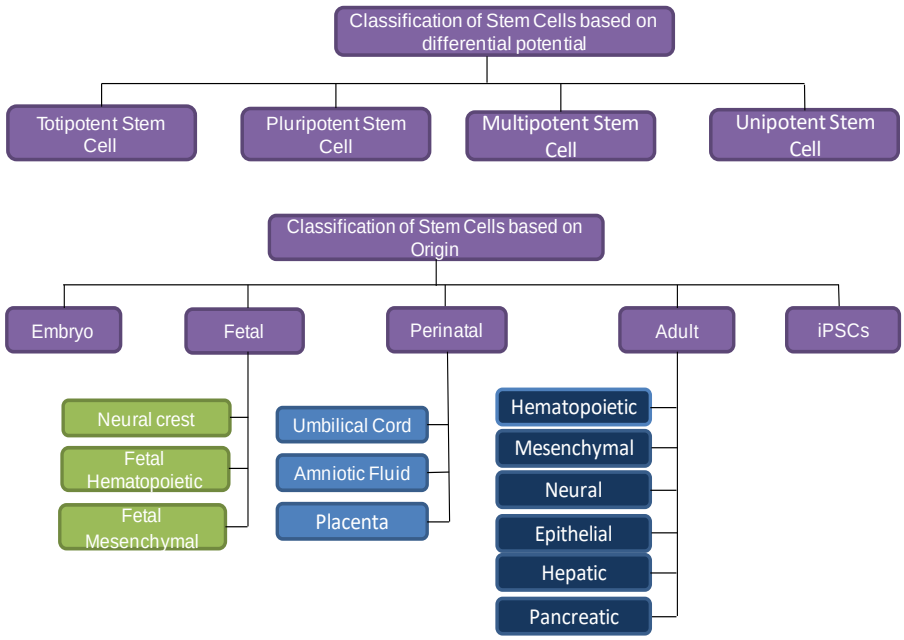


Figure 3: Diagram representing classification of Stem Cells (*Image created in Microsoft Office PowerPoint Presentation*)

Application of stem cells

The goal of any stem cell therapy is to repair a damaged tissue that can't heal itself. Ongoing research on stem cell therapies gives hope to patients who would normally not receive treatment to cure their disease but just to alleviate the symptoms of their chronic illness. Stem cell therapies involve more than simply transplanting cells into the body and directing them to grow new, healthy tissue. It may also be possible to coax stem cells already in the body to work overtime and produce new tissue.

Stem cells in neurotoxicity

As we all know the human nervous stem is the sole controller and coordinator of one's behaviour, and is responsible for perceiving and responding to the external stimuli, mediating communication with the external environment, coordinating the activities of all other organs systems thus playing important role in maintaining metabolic balance of body. Therefore, any kind of damage to the components or part of nervous system can be fervent. Severe neuronal injuries or damage can result in coma, convulsion, paralysis, dementia, incoordination, loss of memory, disturbed communication, improper motor function and even death. Some of the compounds can affect this ability of nervous systems. Such compounds or substances are called as neurotoxicants, and their study is called as neurotoxicity. With the increase in human activities to meet our demands, ultimately leading in alteration of environmental conditions, the exposure of neurotoxicants have increased in past few decades, thus raising the concern for the scientists to study neurotoxicity. Neurotoxicity refers to the damage caused to the brain or peripheral nervous system due to the exposure of toxic substances mainly neurotoxicants. Neurotoxicants can alter the natural activity of nervous systems in several ways which can disrupt or kill the components of nervous systems. Since neurons have high metabolic rate therefore, they are at greatest risk caused by neurotoxins followed by Oligodendrocytes, astrocytes, microglia, and capillary endothelium cells. Neurotoxicants may not necessarily damage the whole nervous system instead it can destroy a particular element of the system. Nonpolar substances are more

soluble in lipids and can therefore access the nervous tissue more easily than polar compounds, which are less soluble in lipids. The body's response to neurotoxins is influenced by numerous factors such as neurotransmitter affected, cellular membrane integrity and the presence of detoxifying mechanism. Some neurotoxins have polluted our environment to such a great extent that it has become extremely difficult to avoid its exposure. These include: - heavy metals like arsenic, mercury, lead etc., chemicals like solvents, chlorine, formaldehyde, phenol, insecticides, pesticides and drugs like stimulants such as amphetamine. Some of the effects of neurotoxicity may appear immediately while others can take months or years to manifest. The effects of neurotoxicity depends on the various factors such as characteristics of neurotoxins, the dose a person has been exposed to, ability to metabolise and excrete the toxin, the ability of affected mechanisms and structures to recover and how vulnerable a cellular target is common systems of neurotoxicity may include paralysis or weakness in the limbs, altered sensations, tingling and numbness in the limbs, headache, vision loss, loss of memory and cognitive function, uncontrollable OCD, behavioural problems, sexual dysfunction, depression, loss of circulation, imbalances etc. Other conditions that may develop as a result of neurotoxicity include chronic fatigue syndrome, attention deficit, hyperactivity disorder, chronic sinus asthma etc. Besides causing movement disorders, cognitive deterioration and dysfunction of the autoimmune nervous system, neurotoxicity has been reported to be a major cause of progressive neurological disorders such as Alzheimer's disease, Parkinson's and multiple sclerosis.

Stem cell in 3D culture

The 3D cultures often provide more complete cell-to-cell and cell-to-matrix interactions,mimicking the natural environment in which the stem cells reside better than the traditional 2D cultures.Furthermore, many desired cellular characteristics are maintained or even promoted in 3D cultures,further supporting their use in basic and translational research. In recent advancesin the 3D cultures of stem cells presents various stem cell types and their characteristics in 3D environments. This includes the effective use of induced pluripotent stem cells (iPSCs) to develop cardiac microtissue, neurospheres, and

cortical progenitors. In addition, this contains work on scaffolds with mesenchymal stem cells (MSCs) and embryonic stem cells (ESCs). Furthermore, research on generating brain organoids from stem cells and neurogenesis studies utilizing pluripotent stem cell. 3D culture system provides a similar physicochemical environment in vivo by facilitating cell-cell and cell-matrix interaction to overcome the limitations of traditional monolayer cell culture. In suspension culture, aggregates of adjacent cells form a spheroid shape having wide utility in tumor and cancer research, therapeutic transplantation, drug screening, and clinical study, as well as organic culture.

Stem cell in translational medicine and regenerative therapy

A number of stem cell therapeutics exist, but most are at experimental stages and/or costly, with the notable exception of bone marrow transplantation. Medical researchers anticipate that adult and embryonic stem cells will soon be able to treat cancer, Type 1 diabetes mellitus, Parkinson's disease, Huntington's disease, Celiac Disease, cardiac failure, muscle damage and neurological disorders, and many others. They have suggested that before stem cell therapeutics can be applied in the clinical setting, more research is necessary to understand stem cell behaviour upon transplantation as well as the mechanisms of stem cell interaction with the diseased/injured microenvironment. Bone marrow transplants (BMT) are a well-known clinical application of stem cell transplantation. BMT can repopulate the marrow and restore all the different cell types of the blood after high doses of chemotherapy and/or radiotherapy, our main defence used to eliminate endogenous cancer cells. The isolation of additional stem and progenitor’s cells is now being developed for many other clinical applications. Several are described below. The knowledge of stem cells has made it possible for scientists to grow skin from a patient’s plucked hair. Skin (keratinocyte) stem cells reside in the hair follicle and can be removed when a hair is plucked. These cells can be cultured to form an epidermal equivalent of the patients own skin and provides tissue for an autologous graft, bypassing the problem of rejection. It is also used in brain cell transplantation in which stem cells can provide dopamine - a chemical lacking in victims of Parkinson’s disease. It involves the loss of cells which produce the neurotransmitter dopamine. The first double-blind study of foetal cell

transplants for Parkinson's disease reported survival and release of dopamine from the transplanted cells and a functional improvement of clinical symptoms. However, some patients developed side effects, which suggested that there was an over sensitization to or too much dopamine. Although the unwanted side effects were not anticipated, the success of the experiment at the cellular level is significant. Diabetes affects millions of people in the world and is caused by the abnormal metabolism of insulin. Normally, insulin is produced and secreted by the cellular structures called the islets of Langerhans in the pancreas. Recently, insulin expressing cells from mouse stem cells have been generated. In addition, the cells self-assemble to form structures, which closely resemble normal pancreatic islets and produce insulin. Future research will need to investigate how to optimize conditions for insulin production with the aim of providing a stem cell-based therapy to treat diabetes to replace the constant need for insulin injections.

REVIEW OF LITERATURE

In the upcoming years, stem cell therapy has become a very promising and advanced scientific research topic. The genesis of stem cells is followed by laboratory steps of controlled stem cell culturing and derivation. Quality control and teratoma formation assays are important procedures in assessing the properties of the stem cells tested. And it is quite difficult to set up proper environmental conditions in culturing media to control cell differentiation. Stem cell therapy provides hope for untreatable diseases. It is already available for treating several diseased conditions and their impact on future medicine appears to be significant. Currently, untreatable neurodegenerative diseases have the possibility of becoming treatable with stem cell therapy. Induced pluripotency enables the use of a patient's own cells. Tissue banks are becoming increasingly popular, as they gather cells that are the source of regenerative medicine in a struggle against present and future diseases [2]. Mueller, L.P., et al described about the toxicity related to the infusion of dimethyl sulfoxide-cryopreserved peripheral blood stem cells (DMSO-PBSC) mainly comprises cardiovascular events. Fatal neurotoxicity has been reported in the few studies DMSO represents the putative causative agent of such rare toxicities and elaborate strategies to replace DMSO would benefit from the identification of predisposing factors for DMSO-related toxicities. Scientists studied neurotoxicity which was observed in only one patient who suffered from a generalized tonic seizure upon infusion of DMSO-PBSC and for which the clinical course is reported herein. No neurotoxicity was observed in the group of patients with pre-existing neurological disease. Furthermore, no neurotoxicity was observed in patients who received particularly large volumes of DMSO. In all patients, mild non-neurological side effects occurred but besides the reported seizure no other severe adverse events were observed upon PBSC-infusion. [3] Various types of blood cell (lineages) are produced from rare haematopoietic stem cells that reside in the bone marrow. This process, known as haematopoiesis, provides an beneficial model for examining how genetic programs are established and executed in vertebrates, and also how homeostasis of blood formation is modify in leukaemia's [3]. Advances over the past decade suggest that generating functional β-cells from human iPSCs is

achievable. In particular the signalling pathways that instruct endocrine progenitor cells to differentiate into mature and functional β-cells are poorly understood. Other significant obstacles remain including the need for safe and cost-effective differentiation methods for large-scale generation of transplantation quality β–cells, methods to prevent immune rejection of grafted tissues, and amelioration of the risks of tumorigenesis. The discovery that human somatic cells can be reprogrammed into an embryonic stem cell-like state that appears to be phenotypically and functionally equivalent to hESCs represents a seminal moment for regenerative medicine. Induced pluripotent stem cells (iPSCs) may be an ideal source for cell replacement therapies because they can be derived from patients, including those with diabetes and eventually used to generate autologous therapeutic β-cells for transplantation [4]. Yamanaka et.al., (2007), were established the formation of Human induced pluripotent stem cells by induction of transcription factor i.e., c-Myc, Oct3/4, SOX2, Klf4 into human fibroblast. These human iPSC cells are indistinguishable to human ES cells in their morphology, their gene expression, and the epigenetic status of pluripotent cell-specific genes. Furthermore, these cells can differentiate into the cell types. This discovery of iPSC cells has opened up new avenues to generate patient- and disease-specific pluripotent stem cells. Human iPSC cells may be productive for understanding the mechanisms of diseases and for drug screening. However, iPSC cell technology has several issues that remain to be overcome, including the present low efficiency of iPSC cell generation without genetic alterations, the possibility of tumor formation in vivo, and unregulated growth of the remaining cells that are partially reprogrammed and refractory to differentiation. These issues must be solved before iPSC technology can be fruitfully used in clinical applications [5]. From the past 20 years, stem cell technologies have become an exceedingly attractive option to investigate and treat neurodegenerative diseases, Lunn, J.S., et al., have studied the process of extending basic stem cell research into translational therapies for patients suffering from neurodegenerative diseases. Then they explained the stem cell therapy in neurodegenerative diseases. Finally, they focus on current applications of stem cell therapies to specific neurodegenerative diseases, focusing on Parkinson’s disease, Huntington’s disease, Alzheimer’s disease, amyotrophic lateral sclerosis and spinal muscular

atrophy. Then they understands the capacity of stem cell technologies, there is greater public hope that stem cell therapies will continue to increased progress into realistic and efficacious treatments for neurodegenerative diseases [6]. Growing need for innovation in relation to recent evidence in the field of reconstructive therapy Understanding the molecular mechanisms underlying neurodegenerative disorders, and the advent of methods able to induce neuronal stem cell differentiation has allowed developing innovative therapeutic approaches offering the prospect of healthy and perfectly functional cell transplants, able to replace the sick ones. Hence the importance of deepening the state of the art regarding the clinical applications of advanced cell therapy products for the regeneration of nerve tissue. Besides representing a promising area of tissue transplant surgery and a great achievement in the field of neurodegenerative disease, stem cell research presents certain critical issues that need to be carefully examined from the ethical perspective. This paper shows an ethical assessment of tissue regeneration in neurodegenerative diseases with the aim to rule out the fundamental issues related to research and clinical translation [6].This study shows that neurons differentiated from these cells display important disease properties and, thus, have the potential to serve as cellular models to explore various aspects of Alzheimer's pathogenesis. Over the last decade, hPSCs have been cultured, propagated and differentiated predominantly under adherent cultured configuration, which renders them a two dimensional (2D) monolayer geometric configuration. Derivations of tissue-specific cells from iPSCs cultured under such 2D configurations have generally resulted in an immature phenotype resembling a fetal state. In the native tissue environment, the cells are exposed to a complex, heterotypic 3 dimensional (3D) environment supported by multiple other cells types and extracellular matrix (ECM). Such dynamic, reciprocal interactions are beginning to be realized as essential to reproduce the adult mature function of the hPSC-derived tissue. Thus recent efforts are focusing on generating tissue/organ-specific 'organoid' models (meaning 'resembling and organ') with the goal of reproducing the in vivo, tissue-specific niche environment The main goal of this familial Alzheimer's disease (FAD)-derived induced pluripotent stem cells (iPSCs) is to generate brain-like structures ("organoids") mimicking native brains. Three-dimensional (3D) systems, called cerebral organoids, can enumerate distinct

architectures of the human brain, such as fluid-filled cavities resembling brain ventricles and tissues organized in layers including progenitor ventricular and sub ventricular zones present in the native brain. Immortalized cancer cell lines typically served as the primary cell source, which is restrictive since its long term culture often results in accumulated genetic defects or chromosomal aberrations. In contrast, the iPSCs offer an unrestrictive cell source, they are genetically stable and are amenable to genetic manipulation either to reproduce a disease phenotype or correcting a genetic defect. Extracellular vesicles secreted from AD patient derived neurons contain a relatively low level of Aβ but have an increased Aβ42/ Aβ40 ratio; the majority of Aβ is situated on the surface of the EVs. Pluripotent Stem cell: (PSCs) can be amplifying indefinitely and differentiated into almost some organ-specific cell type. This has empowered the generation of disease relevant tissues from patients in scalable features. iPSC-derived organs and organoids are currently being evaluated both in regenerative therapy which are enhancing towards clinical trials, and for disease modelling, which are facilitating drug screening efforts for invention of novel therapeutics. This paper result shows the current efforts and advances in iPSCs technology and its applications and gives a brief commentary on future prospect of this promising technology [7]. The blood–brain barrier (BBB) is a critical component of the central nervous system that preserves neurons and other cells of the brain parenchyma from potentially injurious substances found in peripheral circulation. Gaining a thorough understanding of the development and function of the human BBB has been obstruct by a shortage of relevant models given remarkable species differences and limited access to in-vivo tissue. However, advances in induced pluripotent stem cell (iPSC) and organ-chip technologies now allow us to gain our knowledge of the human BBB in both health and disease. Their result focuses on the recent increment in modelling the BBB in vitro using Induction of human embryonic stem cell (hESCs) lines in 1998 was not only a major technological improvement in the field of regenerative medicine; this also triggered a passionate debate about the ethical issues associated with the utilization of human embryos for induction of hESCs lines. Successful induction of induced pluripotent stem cell (iPS) lines from human somatic cells with defined reprogramming factors by Shinya Yamanaka`s group in 2007 was another outcome that generated enormous

excitement and hope for the development of donor- specific personalized cell replacement therapies (CRT) without the ethical dilemma associated with it. It also explained the progress towards generating donor-specific somatic cell lineages from iPSC lines, especially the functional immune cell lineages, and progress towards advancing these findings to the clinic. Finally, they explain the detail of genome instability and inherent immunogenicity of hPSCs lines that need to be addressed to grow safe and effective iPSC-based CRT. Induced pluripotent stem cell-derived neurons from patients promise to fill an important niche between studies in humans and model organisms in interpret mechanisms and identifying therapeutic avenues for neurologic and psychiatric diseases. A main advantage of using hiPSC-derived neurons is that they offer the only practical way of studying the development and function of live human neurons.[8]

AIMS AND OBJECTIVES

The present study was carried out with following major objectives:

- Handling and maintenance of stem cells: iPSCs and NPCs
- Spheroid Formation
- Transcriptional studies

Techniques used in the study

Tools and techniques used during the study are as follows-

Microprocessor controlled vertical and horizontal laminar flow hoods, Bio-safety Cabinet, inverted and upright phase contrast cum fluorescent microscopes containing the software for live image analysis and live image transfer software, programmable cooling centrifuges of various rotating speeds, CO2 and O2 control incubators, single pan digital balance, programmable microprocessor controlled cell and particle counter, multiple well plate reader with absorbance, fluorescence, luminescence, milli Q two stage water purification unit, Real Time-PCR machine (RT-PCRq), Horizontal and vertical electrophoresis assembly, transblotting apparatus, Gel Documentation system, -80ºC,-20 ºC, liquid nitrogen cryocanes, etc. Isolation, quantification and separation for protein, DNA and RNA was conducted independently for at least more than 10 times, which mainly includes, isolation, Agarose gel electrophoresis, image analysis, and RT-PCR, PCR, etc.

MATERIAL AND METHODS

Reviving induced pluripotent stem cells (iPSCs)

The wells of a 6-well plate (Nunc Catalog#140675) were coated with Matrigel: basement membrane matrix (Catalog#356234) and kept at room temperature for atleast 1hr. E-8 medium (Catalog#A1517001)supplementedwith Revita cell supplement (Catalog#A2644501)was pre-warmed to 37ºC. The iPSC vial stored at liquid nitrogen was immersed in 37ºC water bath (do not submerge the cap) while swirling the vial gently; and thawed until a single ice crystal remained. Transfer the cells from vial to a 15 ml tube then add 3ml medium (Revival medium) then rinse the vial with 1ml medium. Centrifuge at 1400 rpm for 5 mins. Discard supernatant and mix it with 1ml of E8 medium (Gentle pipetting requires), then seed it in matrigel coated dish. Place that matrigel coated dish into the incubator. On the next day replace the medium with fresh E8 complete medium. Until the cells gets 85% confluent.

Sub-culturing of Human iPSCs

Culturing of human induced pluripotent stem cells is done in Matrigel coated dishes with Essential 8 supplement (50X). These dishes were incubated for 1hr. at room temperature, then aspirated Matrigel from the culture dish was discarded. Cells were passaged and seeded directly onto the Matrigel coated dishes. The colonies were distributed equally in culture dish and kept at 37°C, with 5% CO2 in humidified incubator. The cells were routinely passaged everyday by using Accutase(SigmaAldrich, St. Louis, MO USA, Catalog#A6964), split into the 1:4 on every 4th to 5th days after reaching 80% confluence.

Cell Counting Using Automatic Cell Counter (Invitrogen Countess)

Automatic cell counter can provide a total count of mammalian cells and a live/dead ratio in a single step. Because there is no bias in counting, automated cell counters yield more accurate and reproducible results. Examples of processes that benefit from the speed and accuracy of automated cell counting

include flow cytometry, toxicology studies, viral production, high content screening, and high content analysis. 10µL of cell suspension and 10µL of Trypan Blue dye were mixed thoroughly and 10µL of this mixture was loaded in countess slide and number of live and dead cells per ml of the suspension was counted automatically by the cell counter.

Figure 4: Invitrogen Countess

Cryopreservation of iPSCs

After few passages, the cells were harvested and transferred to culture tubes & centrifuged at 1400 rpm for 4 min at room temperature. The supernatant was discarded and the cells were then suspended in pre-cooled freezing medium [recovery freezing medium] at an average density of 1X106 cells/ml. 1ml of cell suspension was added to each pre-cooled frozen vial and the vials were immediately transferred to 0ºC for 1hr, followed by -20ºC deep freezer for 2hr and subsequently to -80ºC for 24hr, and then to liquid nitrogen (-196.4°C).

Media preparation for neural progenitor cell (NPCs)

Prepare Dulbecco's Modified Eagle Medium (DMEM/F12 medium, Catalog#11320033) as per the manufacturer's instructions. Then add N2 supplement (100x)-5ml (Catalog#17502048), Normocin (Catalog#NC9273499) and B27 supplement (50x) (Catalog#17504044) in incomplete DMEM/F12 medium (Gibco). BFGF (20ng/ml) (Catalog#F3685)

growth factor is added on the day used in NBN medium (8µl) (Catalog#ab269506) in 40 mL DMEM medium.

Sub-culturing of NPCs

Healthy embryoid bodies obtained were seeded on Matrigel-coated dish with DMEM/F12 supplemented with N2, B27, Normocin, BFGF (20ng/ml) (NBNF medium). The medium was replaced every alternate day until neural rosette (Neural stem cell) were formed (day 15). Neural rosettes were manually picked under EVOSTM FL Auto imaging system and transferred into ultra-low attachment plate and allowed to grow for 2-3 days. After that, cells clumps grown in well, detached by using Accutase and plated onto the Matrigel-coated plate with NBNF medium. After 3-4 passages we obtained pure culture of Neural Progenitor Cells (NPCs). The NPCs were characterized by SOX2, Nestin and OLIG2 marker by immunocytochemistry. The NPCs obtained from neural rosette were maintained in NBN medium with BFGF (20ng/ml) medium and were used further for cryopreservation and differentiation into neuronal cells.

Cryopreservation of NPCs

After few passages, the cells were harvested and transferred to culture tubes & centrifuged at 1400 rpm for 4 min at room temperature. The supernatant was discarded and the cells were then suspended in pre-cooled freezing medium [cryostore medium] at an average density of 1X106 cells/ml. 1ml of cell suspension was added to 34 each pre cooled frozen vial and the vials were immediately transferred in a Frosty to 0ºC for 1hr, followed by -20ºC deep freezer for 2hr and subsequently to -80ºC for 24hr, and then to liquid nitrogen (-196.4°C).

Figure 5: Frosty for cryopreservation.

Characterization of Induced pluripotent stem cells

Immunocytochemistry

iPSCs were maintained in essential 8 medium in 60 mm dish. As 80-90 % confluence was observed in the culture dish the cells were seeded in the 4-chamber slide with E8 medium. On day2 the cells were fixed with 4% PFA for 20 minutes at room temperature. Then permeabilization was done by permeabilization solution including 0.5% Triton X 100 in Phosphate buffer saline (PBS) incubates for 10-15 minutes on the shaker at RT. Then blocking was done by adding blocking buffer (0.5% BSA, 0.1% TritonX100 in PBS) and incubated for 40 minutes at RT. Add primary antibody, incubate overnight at 4ºC then next day wash with PBS (two times) then add secondary antibody, incubate 42hrs at RT. Then again wash with PBS (two times). Prepare slide, remove PBS and add DAPI and incubate for 5 mins. Wash one time with PBS. Remove PBS and add mounting solution with (DAPI). Store slides at - 20ºC. Immunocytochemistry assay was done by antibodies for SOX2 and OCT4 markers.

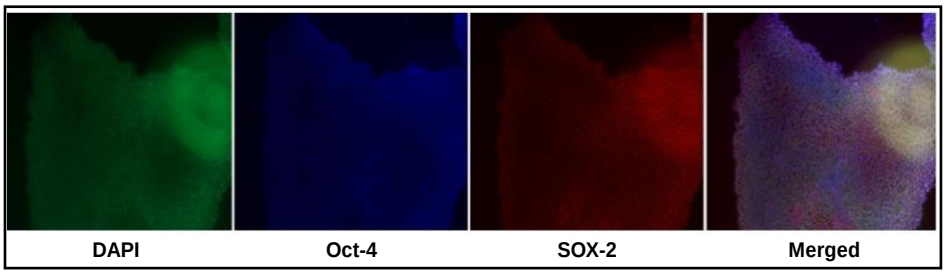


Figure 6: Immunocytochemistry images depicting the expression of OCT-4 and SOX-2 in cultured iPSCs colony images depicting the expression of OCT-4 and SOX-2 in cultured iPSCs colony.

Formation of NPCs from iPSCs

Once-healthy embryoid body was obtained, plated on Matrigel-coated dish with DMEM/F12 supplemented with N2, B27, Normocin, BFGF (20ng/ml) (NBNF medium). The medium was replaced every alternate day until neural rosette (Neural stem cell) was formed. Neural rosettes were manually picked under EVOSTM FL Auto imaging system (Thermo Cat#AMAFD1000) and transferred into ultra-low attachment plate and allowed to grow for 2-3 days. After that, cells clumps grown in well, detached by using Accutase and plated onto the Matrigel-coated plate with NBNF medium. After 3-4 passages we obtained pure culture of Neural Progenitor Cells (NPCs). The NPCs were characterized by Nestin and OLIG2 marker by immunocytochemistry. The NPCs obtained from neural rosettes were maintained in NBN medium with BFGF (20ng/ml) medium. For differentiation protocol, after achieving >70% confluency of NPCs, NBN medium was switched by alternate day followed by subsequent decreasing BFGF concentration i.e., 10ng/mL (Day4), 5ng/mL (Day2). At the day 0 cells seeding were done in medium with 2ng/ml BFGF followed by NBN without BFGF on day 2. From day 4 medium was replaced with differentiation factor (BDNF, GDNF, IGF) every alternate day with subsequent increase in concentration of differentiation factor i.e., 5ng/mL (Day4), 7.5ng/mL (Day6), 10ng/mL (Day8) along with Retinoic acid, Purmorphamine, Ascorbic acid. From day 8 onwards, the NPCs were allowed to differentiate in NBN medium supplemented with differentiation factor10ng/ml up to 34 days (replacing medium every alternate day). During this period, the cells were being examined under EVOSTM FL auto imaging

system. After 34 days, the differentiated cells were characterized for neuronal Tuj-1, MAP2.

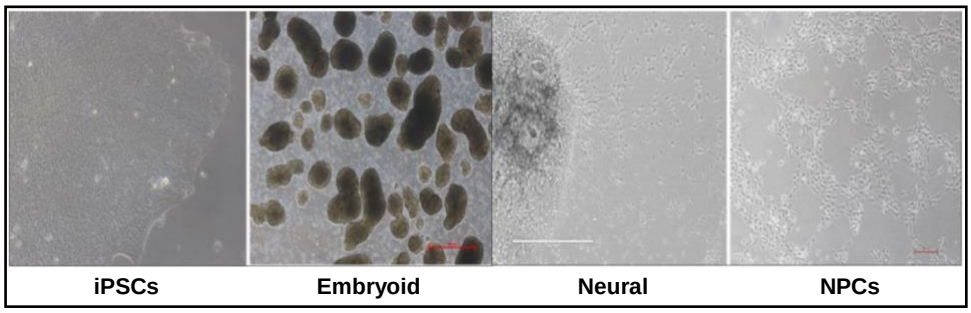
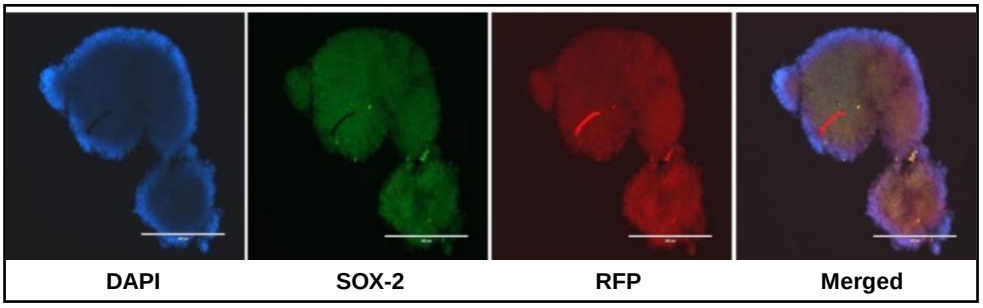


Figure 7: Neural Progenitor Cells Formation



Ectoderm Figure 8: Representative figure of the embryoid body showing Positive Marker for-SOX2 (green), RFP (red) marker for endoderm.

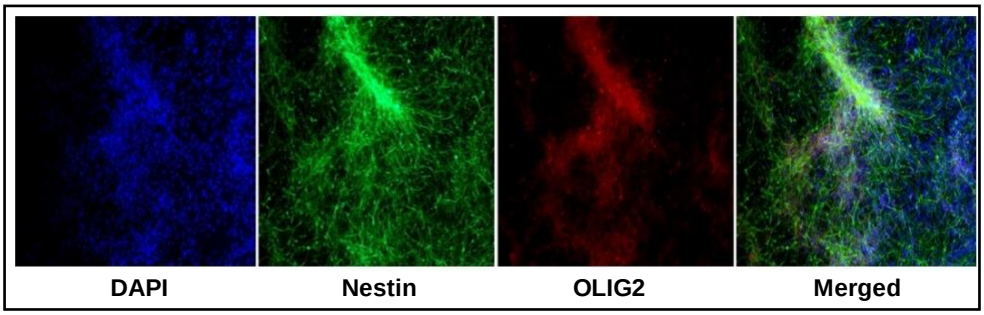


Figure 9: Representative Figure of Neural Progenitor Cells

Spheroid Formation

There are various spheroid culture methods such as hanging drop, gel embedding, magnetic levitation, and spinner culture. Lately, efforts werebeing

made to apply the spheroid culture system to the study of drug delivery platforms and co-cultures, and to regulate differentiation and pluripotency. However, spheroid cell culture system has limitations such as hypoxia and necrosis in the spheroid core.In a cell culture suspension, cells tend to aggregate and go through the process of self-assembly. Self-assembly means single cells constitute multi-cellular spheroids by themselves. Cell culture medium permeates inside the spheroids by diffusion. The gradient of diffusion was induced by increasing the spheroid size during spheroid culture. The process of spheroid formation is divided into several steps. Firstly, single cells present within the suspension agglomerate to form loosely adhesive cell spheroids. In this step, extracellular matrix fibers including complementary binding of peripheral cell surface to integrin encourages preliminary aggregation. Next, E-cadherin promotes strong adhesion of initial cell aggregate by creating homophilic binding between cadherins of peripheral cells. In addition, β-catenin complex facilitates cellular signal transduction. Actin can also affect agglomeration and stemness by promoting contacts between adjacent cells. As a result, strong adhesive multi-cellular spheroids were formed. The bigger the size attained by the spheroids, the harder it becomes for the medium to reach the core of the spheroids. In addition, the rate of production and consumption of factors can affect self-assembly. Adhesion and differentiation of cells affect the formation of multi-cellular spheroids. In particular, cadherin and integrin were directly related to the mechanism of spheroid formation.For the spheroid formation NPCs cell were seeded in 96 well ultra low attachment plate (ULA). The medium was replenished every alternate day till day 6.

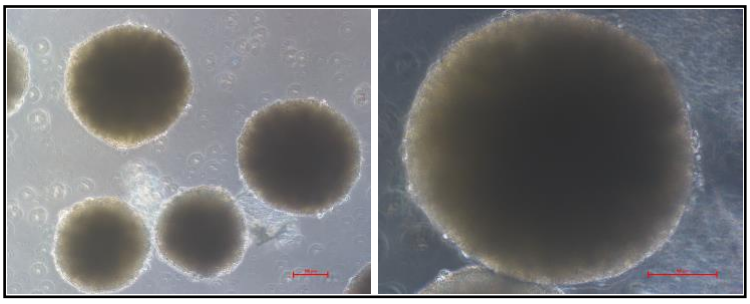


Figure 10: Cortical Organoids

Live-Dead Cell Viability Assay of 3D spheroids

The Live-Dead Cell Viability Assay is a quick and simple three-color assay to measure cell viability. The live-dead cell viability assay kit (Catalog#R37601) consists of Calcein-AM (stains live cells), Propidium Iodide (stains dead cells) and Hoechst 33342 (stains all cells). It has been optimized for 3D cell culture (spheroids, human organoids and 3D matrices) and 2D cell culture (multiple cell types). It can be used for flow cytometry, fluorescence microscopy and with fluorescence microplate readers. The kit determines viability of cells based on plasma membrane integrity and esterase activity.

2D Culture Reagent Preparation and Staining

Mix 1:1 ratio of cell culture medium and PBS. To this mixture add of Calcein AM, Propidium iodide, Hoechst to prepare dye solution. Mix the components. Aspirate the culture mediumfrom the 8-well plate and add appropriate volume of dye mixture to each well. Incubate the plate for 30 min at 37°C. Analyze the plate for cell count and viability using a suitable fluorescence microscope with the appropriate excitation and emission filters, or else use an appropriate system for automated image acquisition and analysis.

3D Culture Reagent Preparation and Staining

Mix cell culture medium PBS in a 1:1 ratio. To this mixture add Calcein AM, Propidium iodide and Hoechst 33342 to prepare dye solution. Mix the components. Aspirate the cell culture medium from the culture plate and add appropriate volume of dye mixture to each well.Incubate the plate for 60 min at 37°C.Analyze the plate for cell count and viability using a suitable fluorescence microscope with the appropriate excitation and emission filters, or else use an appropriate system for automated image acquisition and analysis.

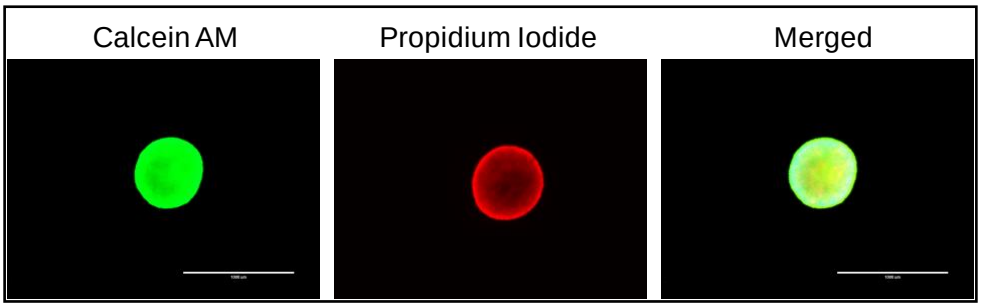


Figure 11: Live-Dead Cell Viability.

TRANSCRIPTIONAL STUDIES

RNA Isolation

The Invitrogen Life Technologies TRIzol Reagent (Total RNA Isolation Reagent) (Catalog#15596026) is a ready-to-use reagent for the isolation of total RNA from cells and tissues for use in PCR analysis. TRIzol solubilisation and extraction is a relatively recently developed general method for deproteinizing RNA. This method is particularly advantageous in situations where cells or tissues are enriched for endogenous RNases or when separation of cytoplasmic RNA from nuclear RNA is impractical.

Principle

TRIzol (or TRI Reagent) is a monophasic solution of phenol and guanidiniumisothiocyanate that simultaneously solubilizes biological material and denatures protein. After solubilization, the addition of chloroform causes phase separation (much like extraction with phenol:chloroform:isoamyl alcohol), where protein is extracted to the organic phase, DNA resolves at the interface, and RNA remains in the aqueous phase and is recovered by precipitation with isopropanol. The RNA is re-dissolved in Nuclease-free water. Patient RNA is stored for research requests. Therefore, RNA, DNA, and protein can be purified from a single sample (hence, the name TRIzol). TRIzol extraction is also an effective method for isolating small RNAs, such as microRNAs, piwi-associated RNAs, or endogeneous, small interfering RNAs.

Reagent used: TRIzol reagent, Chloroform, isopropanol, Nuclease frees water, 75% ethanol.

Homogenization with Tissue Lyser

In a chemical fume hood, add 0.5 mL of TRIzol reagent to an appropriately labelled 2 mL SafeLock tube and place on wet ice.Frozen tissue should remain buried in dry ice until added to TRIzol. The optimum sample size was between 50-70 mg. (combine these after homogenization).Place the cell into the Safe-Lock tube containing TRIzol.Once all of the samples were in their labelled tubes, place the tubes into the TissueLyser adapter sets.Fix the adapter sets into the clamps (arms) of the TissueLyser. Homogenize the samples for 3 minutes. If the sample is not fully homogenized, repeat with decreased time and decreased frequency for no more than 2 additional minutes. For optimal operation, the TissueLyser must always be balanced. Working in a chemical fume hood, transfer the homogenized sample by pipet to a new 2 mL screw cap tube containing 0.5 mL of TRIzol. Invert tube to mix. Place the tube on wet ice temporarily until ready to proceed to Phase Separation, or on dry ice and then move into a -80° freezer for storage of homogenized sample for future extraction.

Phase Separation

Incubate the homogenized sample for 5 minutes at room temperature. In a chemical fume hood, add 0.2 mL of chloroform per 1 mL of TRIzol reagent. Cap sample tubes securely and shake vigorously by hand for 15 seconds. Incubate the samples at room temperature for 3 minutes. Following the incubation, centrifuge the samples at 11,600 x g for 15 minutes at 4°C. Following centrifugation, the mixture separates into a lower red phenolchloroform phase, an interphase and a colourless, upper aqueous phase. RNA remains exclusively in the aqueous phase (which is about 60% of the volume of TRIzol reagent used for initial homogenization). However, the organic phase can be saved for subsequent DNA and protein extraction.

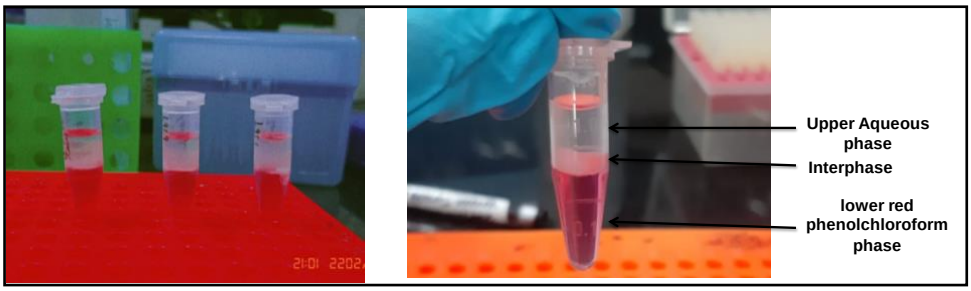


Figure 12: Phase Separation.

RNA Precipitation

Add 0.5 mL of isopropanol per 1 mL of TRIzol reagent originally used to a new tube.Transfer the aqueous phase to the labelled isopropanol tube. Precipitate the RNA from the aqueous phase by pipetting up and down gently. (Do not vortex.)**.** Incubate the samples for 10 minutes at room temperature, then centrifuge at 11,600 x g for 10 minutes at 4°C**.** The RNA precipitate forms a translucent gel-like pellet on the side and bottom of the tube.

Re-suspending the RNA

Remove supernatant with a pipet. Quickly spin in a capsule centrifuge to collect any remaining 75% ethanol to the bottom. Remove as much of the remaining ethanol with a pipet and air dry the RNA pellet by leaving the tubes open on the counter for approximately 15-30 minutes. When the pellet is dry, there must be no visible ethanol in the tube. Do not over dry the pellet as it may be difficult to re-dissolve.Resuspend the RNA pellet appropriate volume of nuclease-free water by pipetting up and down gently and incubating in a 60°C water bath for 10 minutes (can incubate longer if necessary, up to one hour). Flick(do not vortex) gently to mix and quickly spin to collect the solution. After resuspend NFW take reading by spectrometer machine.

Determination of Purity and Yield

The RNA was evaluated by measuring the sample absorbance spectrum between 240 and 300 nm. The A270 served as a measure of phenol

contamination, while the concentration of RNA was calculated from the A260 and A280 values.

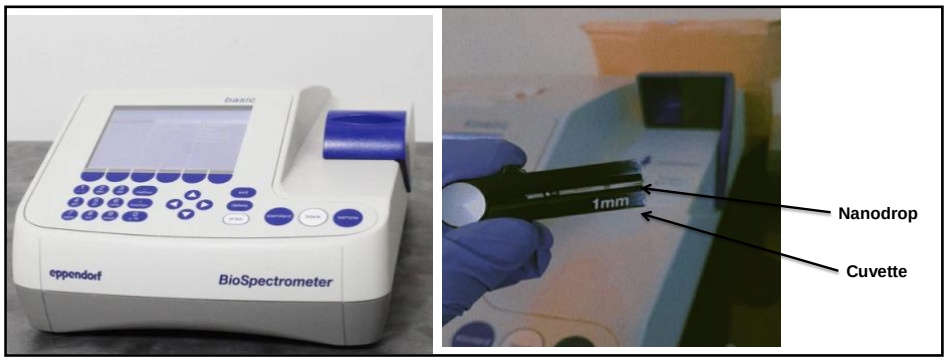


Figure 13: BioSpectrometer

Agarose-Gel Electrophoresis

Agarose gel electrophoresis is a method of gel electrophoresis used in biochemistry, molecular biology, genetics, and clinical chemistry to separate a mixed population of macromolecules such as DNA, RNA or proteins in a matrix of agarose, one of the two main components of agar. The proteins may be separated by charge and/or size (isoelectric focusing agarose electrophoresis is essentially size independent), and the DNA and RNA fragments by length. Biomolecules are separated by applying an electric field to move the charged molecules through an agarose matrix, and the biomolecules are separated by size in the agarose gel matrix. Agarose gel is easy to cast, has relatively fewer charged groups, and is particularly suitable for separating DNA of size range most often encountered in laboratories, which accounts for the popularity of its use. The separated DNA, RNA may be viewed with stain, most commonly under UV light, and the DNA, RNA fragments can be extracted from the gel.

Agarose gel electrophoresis of total RNA isolated using method 1 allowed for the resolution of the high molecular weight rRNA. The resolution of the low molecular weight RNA was poor with only a diffuse band observed around the 100 bp DNA standards. Smearing throughout the lane indicated degradation of the RNA by RNase despite attempts made to limit RNase contamination. The high molecular weight rRNA molecules from the total RNA isolated using method 2 could not be visualized by agarose gel electrophoresis. There was an absence of bands around 2900 and 1500 bp where the 23S rRNA and the 16S

RNA would be expected to run. Although the diffuse low molecular weight bands are still visible around the 100 bp DNA standard, smearing was observed throughout the lanes indicating that a portion of the RNA had been subject to degradation by RNase. The absence of the larger rRNA bands suggests possible preferential degradation of the large rRNA molecules. The increased degradation noted in the RNA sample isolated by method 2 may be partially attributed to extended storage at -20°C, repeated freezing and thawing and more opportunities for RNase contamination due to increased handling of these samples.

Reagents and Materials: Tank, tray, comb, Diethylpyrocarbonate, 0.1% DEPC (Diethylpyrocarbonate) H2O: mix 1 ml DEPC in 1000 ml H2O and autoclave, normal melting agarose powder, 10 x TBE buffer solution, Etbr gel stain, microwave, Erlenmeyer flask, measuring cylinder, scales.

Gel preparation:Prepare sufficient 1 x TBE electrophoresis buffer (1:10 dilution of TBE:DEPC H2O), Prepare agarose gel for a 1% agarose gel: 1 g agarose / 100 ml 1 x TBE buffer in erlenmeyer flask, stain the agarose solution: 3 µl Etbr / 100 ml gel, load the mixtures slowly into the slots, examine the gels, Carefully put it on an ultraviolet transillumunator.

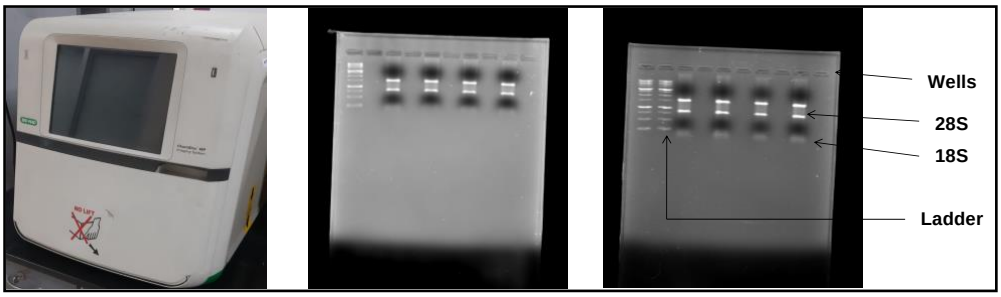


Figure 14: Agarose Gel Electrophoresis.

Polymerase Chain Reaction (PCR)

PCR is a revolutionary method developed by Kary Mullis in the 1980s. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a pre-existing 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to

delineate a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (amplicons).

Principle of PCR

The basic PCR principle is simple. It is a chain reaction and one DNA molecule is used to produce two copies, then four, then eight and so forth. This continuous doubling is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands. To do their job polymerases require a supply of DNA building blocks, i.e. the nucleotides consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). They also need a small fragment of DNA, known as the primer, to which they attach the building blocks as well as a longer DNA molecule to serve as a template for constructing the new strand. If these three ingredients are supplied, the enzymes will construct exact copies of the templates. PCR is a method used to acquire many copies of any particular strand of nucleic acids. It's a means of selectively amplifying a particular segment of DNA. The segment may represent a small part of a large and complex mixture of DNAs e.g. a specific exon of a human gene. It can be thought of as a molecular photocopier. PCR can amplify a usable amount of DNA/RNA (visible by gel electrophoresis) in ~2 hours. The template DNA need not be highly purified a boiled bacterial colony. The PCR product can be digested with restriction enzymes, sequenced or cloned. PCR can amplify a single DNA molecule, e.g. from a single sperm. The polymerase chain reaction relies on the ability of DNA copying enzymes to remain stable at high temperatures. PCR has transformed the way that almost all studies requiring the manipulation of DNA fragments may be performed as a result of its simplicity and usefulness. In Mullis's original PCR process, the enzyme was used in vitro. The double-stranded DNA was separated into two single strands of DNA by heating it to 96°C. At this temperature, however, the E.Coli DNA polymerase was destroyed, so that the enzyme had to be replenished with new fresh enzyme after the heating stage of each cycle. Mullis's original PCR process was very inefficient since it required a great deal of time, vast amounts of DNA-Polymerase, and continual attention throughout the PCR process. The most

substantial milestone in PCR utilization was the introduction of the concept of monitoring DNA amplification in real time through monitoring of fluorescence (Holland et al., 1991; Higuchi et al., 1992). In real time PCR (also denoted as quantitative PCR—qPCR; usage of RT-PCR is inappropriate as this abbreviation is dedicated to reverse transcription PCR), fluorescence is measured after each cycle and the intensity of the fluorescent signal reflects the momentary amount of DNA amplicons in the sample at that specific time.

The ability to synthesize DNA from an RNA template, via reverse transcription, enables researchers to study RNA with the same molecular approaches used for DNA investigations. cDNA generated by reverse transcription can be amplified using polymerase chain reaction (PCR). it is an important method for detecting specific nucleic acid molecules in a particular cell or small populations of cells. cDNA was reverse transcribed from the extracted RNA as per the following methodology (values per reaction).

Components	**Amount**
10X RT Buffer	2µl
25X dNTPs mix	0.8µl
10X RT Random Primer	2µl
Multi Scribe	1µl
Nuclease free water	4.2µl

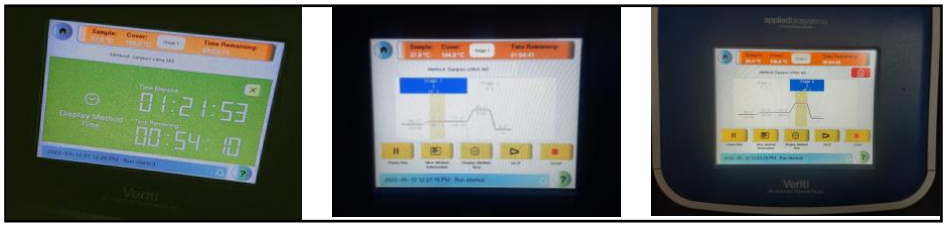


Figure 15: PCR Thermal Cycler (cDNA synthesis).

Real-Time PCR

Real-time PCR is a variation of the standard PCR technique that is commonly used to quantify DNA or RNA in a sample. Using sequence-specific primers, the number of copies of a particular DNA or RNA sequence can be determined. By measuring the amount of amplified product at each stage during the PCR cycle, quantification is possible. If a particular sequence (DNA or RNA) is abundant in the sample, amplification is observed in earlier cycles; if the sequence is scarce, amplification is observed in later cycles. Quantification of amplified product is obtained using fluorescent probes or fluorescent DNA-binding dyes and real-time PCR instruments that measure fluorescence while performing the thermal cycling needed for the PCR reaction.

Figure 16: QuantStudio 6 Flex and 12 Flex RT-PCR System

Real-time PCR steps

There are three major steps that make up each cycle in a real-time PCR reaction. Reactions are generally run for 40 cycles.

Denaturation: High temperature incubation is used to "melt" double-stranded DNA into single strands and loosen secondary structure in single-stranded DNA. The highest temperature that the DNA polymerase can withstand is typically used (usually 95°C). The denaturation time can be increased if template GC content is high.

Annealing: During annealing, complementary sequences have an opportunity to hybridize, so an appropriate temperature is used that is based on the calculated melting temperature (Tm) of the primers (5°C below the Tm of the primer).

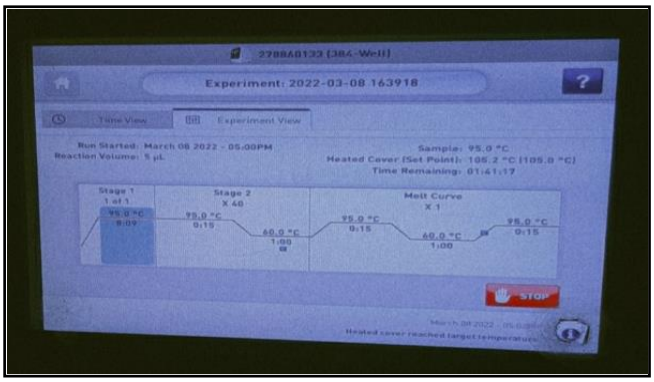
Extension: At 70-72°C, the activity of the DNA polymerase is optimal, and primer extension occurs at rates of up to 100 bases per second. When an amplicon in real-time PCR is small, this step is often combined with the annealing step using 60°C as the temperature.

Reaction Mixture for RT-PCR for 384 well plate was prepared as follows-

S.No.	**Component**	**Amount**
1	SYBR Green	2.5 µl
2	Forward Primer	0.25 µl
3	Reverse Primer	0.25 µl
4	c-DNA	As per the calculation
5	NFW	As per the calculation

Two-step qRT-PCR

Two-step quantitative reverse transcriptase PCR (qRT-PCR) starts with the reverse transcription of either total RNA or poly(A)+ RNA into cDNA using a reverse transcriptase (RT). This first-strand cDNA synthesis reaction can be primed using random primers, oligo(dT), or gene-specific primers (GSPs). To give an equal representation of all targets in real-time PCR applications and to avoid the 3’ bias of oligo(dT) primers, many researchers use random primers or a mixture of oligo(dT) and random primers. The temperature used for cDNA synthesis depends on the RT enzyme chosen. Next, approximately 10% of the cDNA is transferred to a separate tube for the real-time PCR reaction.



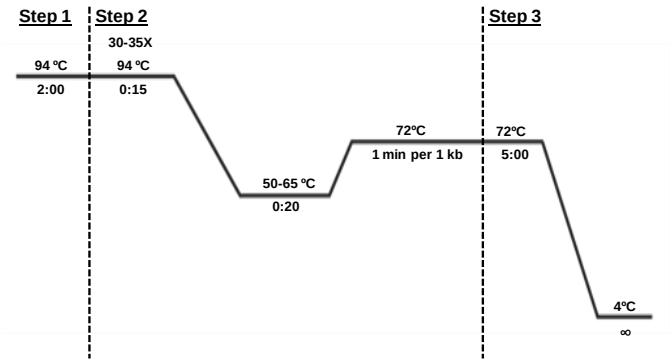


Figure 17: Real-Time PCR Cycle.

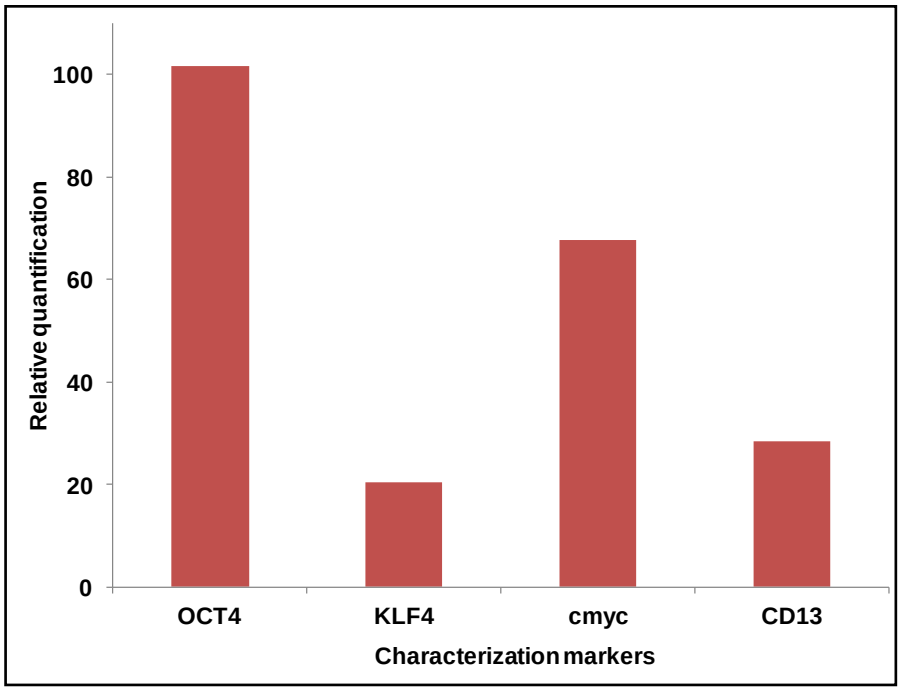


Figure 18: iPSCs characterisation

TRANSLATIONAL STUDIES

Western Blotting (ProteinBlotting or Immunoblotting)

A western blot is a laboratory method used to detect specific protein molecules from among a mixture of proteins. This mixture can include all the proteins associated with particular tissue or cell type. Western blotting refers to the electrophoretic transfer of proteins from sodium dodecyl sulphate gels, polyacrylamide gels to sheets of PVDF membrane followed by immunodetection of proteins using antibody with fluorescent or chemiluminescent detection. It is based on the principle of immunochromatography where proteins are separated into polyacrylamide gel according to their molecular weight.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS page, earlier known as Leammli method after its inventor Ulrich K. Leammli, is the most common method for separating proteins on the basis of their mass. SDS used in the gel preparation is basically an anionic detergent which denatures the proteins before they are electrophoresed. It denatures the proteins by binding to their hydrophobic core and gives a protein an overall negative charge proportionate to its mass. A polypeptide chain binds amounts of SDS in proportion to its relative molecular mass. The negative charges on SDS destroy most of the complex structure of proteins, and are strongly attracted toward an anode (positively-charged electrode) in an electric field. This results in a denatured and linear form of protein complex such that it becomes easy to separate out proteins on the basis of their mass. Proteins having smaller mass move farther than larger proteins on SDS page. Since the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in relative molecular mass of polypeptides. Since this method involves protein separation on the basis of mass therefore it can be used to estimate the molecular weight of a protein.

Purpose of resolving gel

Once the protein-SDS complex enters the resolving gel, owing to molecular sieving property of the gel separation of the proteins in the sample is observed. Smaller proteins move more easily and hence move further when compared to larger proteins. Bromophenol blue, being a small molecule moves further and forms the electrophoresis front.

Purpose of stacking gel

Stacking gel is used to concentrate the protein sample into a sharp band before it enters the resolving gel. This is achieved by utilizing the differences in ionic strength and pH between the electrophoretic buffer and the stacking gel, which involves the phenomenon known as isotachophoresis. Stacking gel has larger pore size, which allows the protein samples to concentrate and move freely under the effect of electric field. Band sharpening is attained by the difference in the electrophoretic mobility of glycinate ions, protein-SDS complex and chloride ions in the loading buffer. For having a steady electric circuit all the ionic species have to migrate at the same speed under the influence of the applied field. Field strength is inversely proportional to conductivity and is proportional to concentration.

$$[Cl^-] > [\text{protein-SDS}] > [\text{Glycinate}]$$

Due to lower concentration of protein-SDS complex they concentrate in a very tight band between glycinate and chloride ion boundaries. Once glycinate ions reach the resolving gel due to the higher pH environment they get easily ionized and their mobility increases.

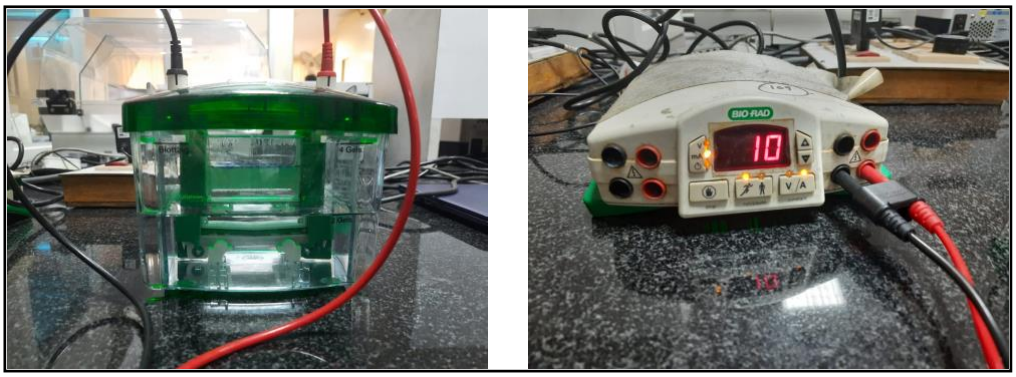


Figure 19: SDS-PAGE: running the gel

Western Immuno-Blotting

Western immuno-blotting is a technique, which is used to detect and determine the presence of a particular protein with accuracy. For doing a western blot, first we need to run the protein samples in a denaturing SDS-PAGE. The protein samples, separated by SDS-PAGE are then transferred onto a nitrocellulose/PolyvinylideneDifluoride membrane using capillary blotting or electroblotting where the proteins are irreversibly bound by hydrophobic interactions. The resolved protein bands were transferred onto PVDF (Polyvinylidene fluoride) membrane using the concept of Electroblotting. The gel was placed in the 'transfer sandwich' i.e., between filter paper-gel-membrane-filter papers, cushioned by pads and pressed together by a support grid. This supported gel sandwich was then placed vertically in the tank and filled with transfer buffer. This process was carried out at 250mA for 125mins.

Blocking protein on PVDF membrane

The membrane supports used in Western blotting have a high affinity for proteins. Since the membrane has been chosen for its ability to bind protein and as both antibodies and the target are proteins, therefore, it is important to block the remaining surface of the membrane to prevent nonspecific binding of the detection antibodies during subsequent steps. A variety of blocking buffers ranging from milk or normal serum to highly purified proteins have been used to block free sites on a membrane. Therefore, after the transfer of the proteins from the gel, it is important to block the remaining surface of the membrane to prevent nonspecific binding of the detection antibodies during subsequent steps. The blocking buffer should improve the sensitivity of the assay by reducing background interference and improving the signal to noise ratio. The ideal blocking buffer will bind to all potential sites of nonspecific interaction, eliminating background altogether without altering or obscuring the epitope for antibody binding.

Incubation with Primary Antibody

During the detection process the membrane is "probed" for the protein of interest with a modified antibody which is linked to a reporter enzyme; when

exposed to an appropriate substrate, this enzyme drives a colorimetric reaction and produces a colour. After blocking, a dilute solution of primary antibody (generally between 0.5 and 5μg/mL) is incubated with the membrane under gentle agitation. The antibody solution and the membrane are incubated together for anywhere from 30 minutes at room temperature to overnight at 4°C.

Incubation with Secondary Antibody

After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody, called as secondary antibody. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhance the signal. Most commonly, a horseradish peroxidase-linked secondary is used to cleave a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. After incubation with primary antibody, the blot was again washed with washing buffer five times. Then the membrane is incubated with 5mL of respective secondary antibody for 2hrs at room temperature with gentle agitation.

Blot Development

After the unbound probes are washed away, the western blot is ready for detection of the probes that are labelled and bound to the protein of interest. The colorimetric detection method depends on incubation of blot with fluorescent probe tagged secondary antibody. The blot was scanned using Image Studio or LI-COR Acquisition Software. The image is analysed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density.

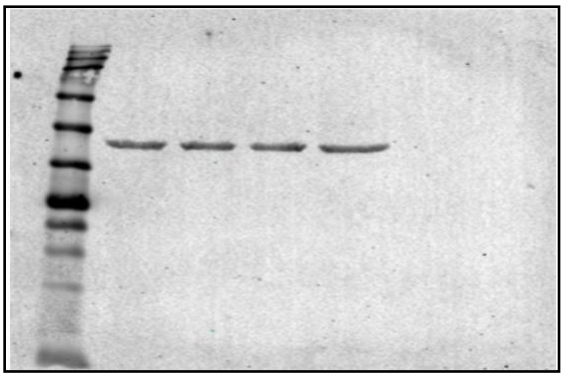


Figure 20: Representative blot for Beta-Actin.

Conclusion

The present study was designed to impart a hand on training experience on working with stem cells and their derived cells. We successfully cultured induced pluripotent stem cells (iPSCs) and iPSC derived neural progenitor cells (NPCs) under 2D and 3D in vitro conditions under the influence of specific growth supplements. The study also encompasses formation of 3D spheroids derived from NPCs. These spheroids were toxically challenged with sodium arsenite, a well known neurotoxicant and the cellular viability was estimated using Live/dead cell assay kit. The successful spheroid formation using iPSCs and NPCs indicate that these cells can be used as experimental model systems to study the neurodegenerative diseases and using such 3D models can restrict the need of animal use in research to certain extent.

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