DIFFERENTATION OF PLACENTAL MESENCHYMAL CELL TO PANCREATIC BETA CELL UNDER DIFFERENT SCAFFOLDS

MASTER OF SCIENCE IN MICROBIOLOGY

SUBMITTED BY

SUPRITA PANIGRAHI

SCHOOL OF BIOTECHNOLOGY, CAMPUS 11
KIIT UNIVERSITY
BHUBANESWAR, ODISHA, INDIA

UNDER THE SUPERVISION OF

DR. SHASHI SINGH

CHIEF SCIENTIST

CSIR CENTRE FOR CELLULAR AND MOLECULAR BIOLOGY
HYDERABAD, INDIA

CERTIFICATE

This is to certify the dissertation entitled "**DIFFERENTATION OF PLACENTAL MESENCHYMAL CELL TO PANCREATIC BETA CELL UNDER DIFFERENT SCAFFOLDS"** Submitted by '**Suprita Panigrahi**' in partial fulfillment of the requirement for the degree of Master of Science in Applied Microbiology, KIIT School of Biotechnology, KIIT University, Bhubaneswar bearing Roll No.1662019 & 16530850273 is a bona fide research work carried out by her under my guidance and supervision from '1st December' to '31st May'.

Further, it is also to certify that the above said work has not been previously submitted for the award of any degree, diploma, or fellowship in any Indian or foreign University. The data is generated is property of CCMB and cannot be submitted without the consent of PI.

Date:

Place:

Dr. Shashi Singh,
Chief Scientist

DECLARATION

I hereby declare that the dissertation entitled “”**DIFFERENTATION OF PLACENTAL MESENCHYMAL CELL TO PANCREATIC BETA CELL UNDER DIFFERENT SCAFFOLDS”** submitted by me, for the degree of Master of Science to KIIT University is a record of bona fide work carried by me under the supervision and guidance of ‘**Dr. Shashi Singh” Chief Scientist** ’, CCMB, Hyderabad, Telangana , India.

Date: **Suprita Panigrahi**

Place:

Abstract

Tissue engineering is the use of a combination of cells, materials, methods and suitable biochemical and physicochemical factors to improve or replace biological tissues. It involves the use of a tissue scaffold for the formation of new viable tissue for a medical or diagnostic purposes.

In this study, , we have used Mesenchymal cells in variety of scaffolds ranging f collagen based scaffolds and pancreatic ECM as such in order to optimize the conditions for better differentiation and functional potential. The collagen was prepared from the rat tails and crosslinked with glycans ; For pure pancreatic ECM we have decellularized the mouse pancreas.. These scaffolds are then used as 3D cell culture & differentiation of MSCs which could be then used to replace for the loss of pancreatic beta cell following degeneration. These scaffolds are also tested for their Injectability in order to use them as inks.

Diabetes mellitus involves the loss of function and/or absolute numbers of insulin-producing β cells in pancreatic islets. Islet transplantation is currently being investigated as a potential cure, and advances in tissue engineering methods can be used to improve pancreatic islets survival and functionality.

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Suprita panigrahi

Abbreviations

1. µl: micro litre
2. 2D :Two Dimensional
3. 3D: Three Dimensional
4. BME: Basal Medium Eagle
5. βME: Beta Mercaptoethanol.
6. DMEM: Dulbecco's Modifies Eagles Media
7. DNPH: Dinitrophenylhydrazine
8. ECM: Extra Cellular Matrix
9. EDTA: Ethylenediaminetetraacetic acid
10. ESCs: Embryonic Stem Cells
11. FBS: Fetal Bovine Serum
12. GAA: Gum Arabic Aldehyde.
13. IMDM: Iscove's Modified Dulbecco's Media
14. IPSCs: Induced Pluripotent Stem Cells
15. MSCs: Mesenchymal Stem Cell
16. PBS: Phosphate Buffer Saline
17. PEG: Poly Ethylene Glycol
18. RBCs: Red Blood Cells.
19. TE: Trypsin EDTA
20. WBCs: white blood cells.
21. ºC: Degree Celsius
22. ml: Milli litre.
23. µM: Micro Molar
24. mM: Milli Molar
25. TGF: Transforming Growth Factor.
26. IGF: Insulin Growth Factor.
27. SDS: Sodium Dodecyl Sulphate.
28. PAGE: Poly Acrylamide Gel Electrophoresis.
29. TEMED: Tetramethylenediamine.

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INTRODUCTION

CHAPTER 1

Background information

1.1 Cell :

The cell theory, or cell doctrine, states that all organisms are composed of similar units of organization, called cells. The concept was formally articulated in 1839 by Schleiden & Schwann and has remained as the foundation of modern biology.

The smallest unit of life with independent replicative potential was discovered by Robert Hooke in 1665, who named them 'Cell' because of their resemblance to the cells inhabited by monks in a monastery.

In 1839 Matthias Jacob Schneiden and Theodor Schwann, came up with the first cell theory. All organisms are composed of one more cells, that cells are the fundamental unit of life and they come from pre-existing cells, and that all cells contain the hereditary information for the regulation of all cellular functions and its transmission to the next generation.

There are multiple kinds of cells but all share the same structural features, The "Plasma Membrane", defines the boundary of the cell and holds all essential organelles. Inside the plasma Membrane is the liquid component "The Cytoplasm" has the cytosol and other components with specific functions and most importantly "The Nucleus" which in turn

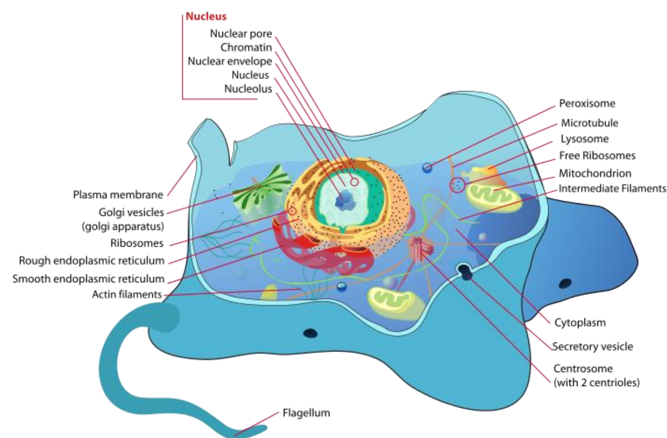


Figure 1.1 Structure of animal cell

1.2:Stem cell :

Stem cells are the body's natural reservoir – replenishing stocks of specialized cells that have been used up or damaged. Stem cells have the unique ability to produce both copies of themselves (self – renewal) and other more specialized type (differentiation) every time they differentiate.

Stem cells are unspecialized. Unlike a red blood cell, which carries oxygen through the blood stream, or a muscle cell that works with other cells to produce movement, a stem cell does not have any specialized physiological properties.

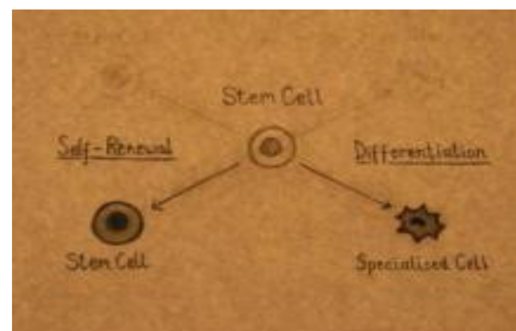


Figure 1.2: Structure of Stem cell types

Stem cells can divide and produce identical copies of themselves over and over again. This process is called self-renewal and continues throughout the life of the organism. Self-renewal is the defining property of stem cells. Specialized cells such as blood and muscle do not normally replicate themselves, which means that when they are seriously damaged by disease or injury, they cannot replace themselves.

Stem cells can also divide and produce more specialized cell types. This process is called differentiation. Stem cells from different tissues, and from different stages of development, vary in the number and types of cells that they can produce.

Types of stem cells:

1. Embryonic stem cells: grown in the laboratory from cells found in the early embryo
2. Induced pluripotent stem cells, or 'reprogrammed' stem cells: similar to embryonic stem cells but made from adult specialised cells using a laboratory technique discovered in 2006.
3. Tissue stem cells: found in our bodies all our lives.

1.3 **Properties of Stem cells:**

a) Self Renewal: Stem cell renewal can take place through two mechanisms

i) Obligatory asymmetric replication: In which a stem cell divides into two cells which is an exact copy of the mother while the other (daughter cell) is differentiated into different cells.

ii) Stochastic Differentiation: While one stem cell divides into two daughter cells another stem cell divides through mitosis to produce two cells identical to the mother cell that is how the balance gets maintained. But the molecular mechanisms governing this choice of cells are not yet known.

b) Potency: It is the ability of a stem cell to get differentiated into different cell types.

i) Totipotent (a.k.a. Omnipotent): These Stem Cells can differentiate into the embryonic and extra-embryonic cell types. They have the ability to form a complete individual and are produced from fusion between an egg and a sperm.

ii) Pluripotent: Since they are derived from the totipotent cells therefore, they have the ability to differentiate into the cells derived from the three germ layers along with the ESCs
.

iii) Multipotent: These cells can differentiate into more than one cell types but are limited than the Pluripotent stem cells. Adult stem cells and cord blood stem cells are considered Multipotent.

iv) Oligopotent: These can differentiate only into lymphoid or myeloid stem cells.

v) Unipotent: They can produce only their own cell type but can self renew and that is what distinguishes them from the Progenitor cells e.g. (adult) muscle stem cell.

1.4.Mesenchymal stem cells

Mesenchymal stem cells (MSC) are a group of cells present in bone-marrow stroma and the stroma of various organs with the capacity for mesoderm-like cell differentiation into, many types like osteoblasts, adipocytes, and chondrocytes. MSC are being use in the clinic for the treatment of a variety of clinical conditions. Cell-based therapies (CBTs) are quickly taking hold as a revolutionary new approach to treat many human diseases. Among the cells used for therapeutic functions, multipotent mesenchymal stromal cells, also often and imprecisely termed (MSC), are widely used because they are considered clinically safe, unique in their immune-capabilities, easily obtained from adult tissues, and easily developed and restore.

MSCs are non-haematopoeitic stromal cells that are capable of differentiating into and contribute to the regeneration of mesenchymal tissues like bone, cartilage, muscle, ligament, tendon, and adipose. . MSCs, are contact inhibited, can be grown in culture for about 20 to 25 passages, have an immunophenotype same to bone marrow MSCs positive (CD34, CD45, CD44, CD13, CD73, CD90, CD105) human leukocyte antigen [HLA]-ABC, and stage-specific embryonic antigen [SSEA]-4), can differentiate into osteocytes and adipocytes, and can be used as fibroblast is a type of cell to support the growth of undifferentiated hESCs (human embryonic stem cell). The able to developed MSCs from hESCs should prove useful to produce large amounts of genetically identical and genetically modifiable MSCs that can be used to study the biology of MSCs and for therapeutic applications (Emmaneul. *et al*., 2006).

MSCs are can be identified by the expression of many molecules including CD105 (SH2) CD73 and CD34, CD45. The properties of MSCs make these cells potentially ideal candidates for tissue technology. It has been prove that MSCs, when transplanted systemically, are ability to transport to sites of physical harm or damage in animals, suggesting that MSCs have migratory capacity. The mechanism of migration of MSCs remains unclear.

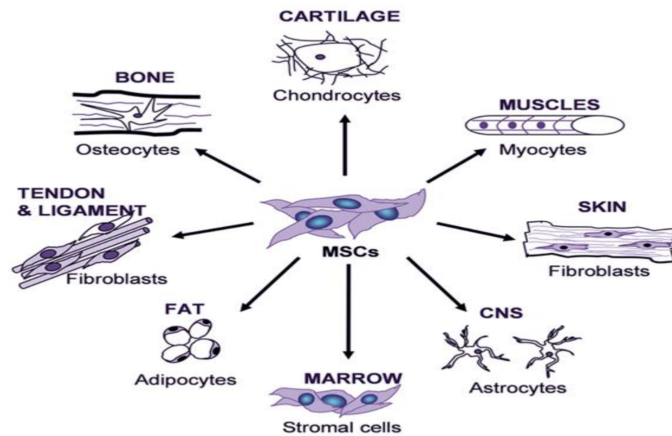


Figure 1.4: Differentiation of MSCs

1.5: Regenerative Therapy

It is a branch of Medicine that develops methods to grow, repair or replace any damaged or diseased cell, organ or tissue. It also includes the generation and use of therapeutic stem cells, tissue engineering and the production of artificial organs.

This promising field of therapeutics aims to restore structure and function of damaged tissues and organs that are permanently damaged or untreatable injuries and diseases.

In this following study we, have tried out the ways for generating mass of beta cells with properties of glucose induced insulin secretion, basically inducing the pancreatic beta cell differentiation from the human derived mesenchymal cell.

1.6: Insulin ; Secreted by Pancreatic Beta cells

Beta cells are unique cells in the pancreas that produce, store and release the hormone insulin. Located in the area of the pancreas know as the islets of Langerhans (the organ's endocrine structures), they are one of at least five different

types of islet cells that produce and secrete hormones directly into the bloodstream. The main function of a beta cell is to produce and secrete insulin - the hormone responsible for regulating levels of glucose in the blood.

When blood glucose levels start to rise (e.g. during digestion), beta cells quickly respond by secreting some of their stored insulin while at the same time increasing production of the hormone. (The global Diabetes community)

Diabetic mellitus (DM), one of the leading causes of morbidity and mortality in many countries, is caused by an absolute insulin deficiency due to the destruction of insulin secreting pancreatic cells (type 1 DM) or by a relative insulin deficiency due to decreased insulin sensitivity, usually observed in overweight individuals (type 2 DM). In both types of the disease, an inadequate mass of functional islet cells is the major determinant for the onset of hyperglycemia and the development of overt diabetes. A limited supply of human islet tissues prevents this therapy from being used in patients with type 1 DM. Alternatively, much effort has been made to increase β cell mass by stimulating endogenous regeneration of islets or *in vitro* differentiated islet-like cells. Multipotent stem cells have been described within pancreatic islets and in nonendocrine compartments of the pancreas and these cells have the capacity of differentiating into pancreatic islet-like structures. Cells that do not reside within the pancreas, such as embryonic stem cells(ESC), hepatic oval cells, cells within spleen, have been differentiated into pancreatic endocrine hormone-producing cells in vitro and in vivo.
The current studies reports a potential means to generate insulin-producing cells, islet differentiation from bone marrow-derived stem cells. So from the above studies we can suggest that cells within the adult bone marrow (mesenchymal stem cells MSC) are capable of differentiating into functional pancreatic β cell phenotypes. (World J Gastroenterol. 2004)

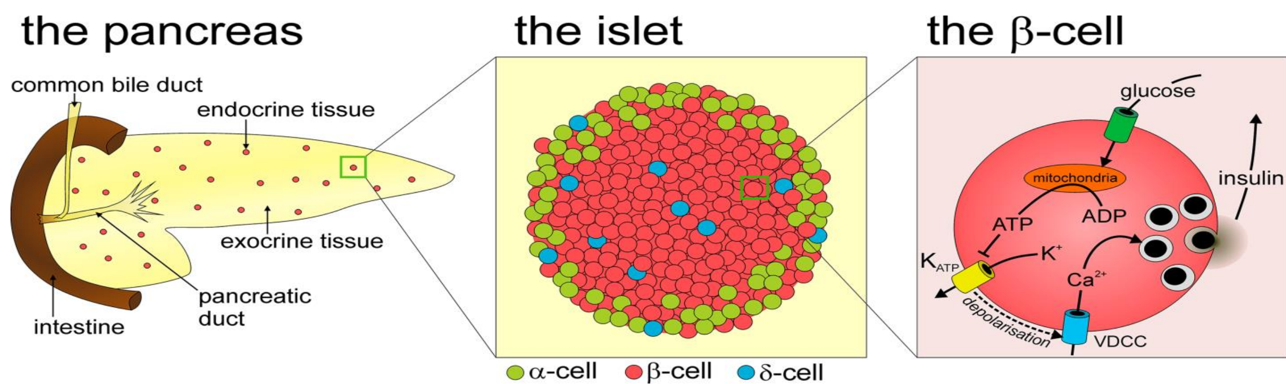


Figure 1.6: Pancreatic Beta cell

1.7. Bioengineered Scaffolds and its importance in 3D cultures

A **3D cell culture** is an artificially created environment in which biological cells are permitted to grow or interact with their surroundings in all three dimensions. Unlike 2D environments (e.g. a Petri dish), a 3D cell culture allows cells *in vitro* to grow in all directions, similar to how they would *in vivo*.

These three-dimensional cultures are usually grown in bioreactors, small capsules in which the cells can grow into scaffolds either made up of collagen or fibroblast or 3D cell colonies. Cells exist in 3D microenvironments with intricate cell-cell and cell-matrix interactions and complex transport dynamics for nutrients and cells.

3D scaffolds more closely used for the development of extracellular matrices. These matrices help the cells to be able to move within their scaffolds similar to the way cells would move in living tissue. The spheroids are thus improved models for cell migration, differentiation, survival, and growth. Furthermore, 3D cell cultures provide more accurate depiction of cell polarization,

The third dimension of cell growth provides more contact space for mechanical inputs and for cell adhesion, which is necessary for integrin ligation, cell contraction and even intracellular signaling. Normal solute diffusion and binding to effector proteins (like growth factors and enzymes) is also reliant on the 3D cellular matrix, so it is critical for the establishment of tissue scale solute concentration gradients.

3D cell cultures have greater stability and longer lifespan. This means that they are more suitable for long-term studies and for emonstrating long-term effects of the cells within the Scaffolds.3D environments also allow the cells to grow undisturbed.

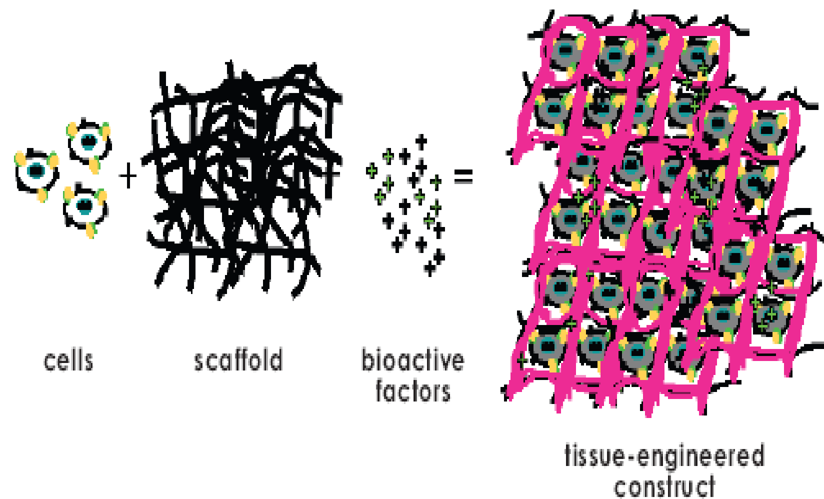


Figure 1.7.1 Scaffolds in 3D culture.

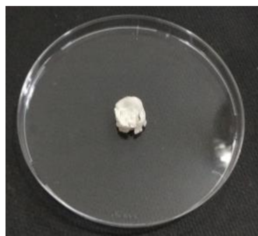
Type 1 collagen is most commonly used in 3D cell cultures and this can be because of a number of reasons including the ease of processing, low cost and the flexibility for live cell manipulation.

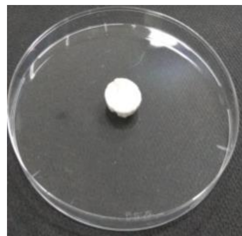
A good scaffold should posses the following properties and designing criteria like:

1. It should be biocompatible, so that the cells must adhere and function normally without any cytotoxicity.
2. It should elicit minimum immune response in order to prevent it from causing a severe inflammatory response when transplanted inside the body.
3. The pore sizes should be good enough to allow proper penetration of cells and adequate diffusion of nutrients to the cells and to the ECM.
4. It should easily get degraded in the body and should be replaceable with bodies own cells.
5. It should posses such mechanical properties which are consistent with the anatomical site where it has to be placed and should be strong enough to withstand surgical handling upon implantation.
6. It should be cost effective and should be possible to scale up from making one at a time in the laboratory to small batch production.

The criterion for scaffolds in tissue engineering, and the one which all of the criteria listed above are dependent upon, is the choice of biomaterial from which the scaffold should be made and the flexibility in the formation of its structure i.e. its pore size and flexibility can be varied by changing the concentration of collagen by introducing chemical cross-linking compounds, thus making it easy to change the structural properties of the gel .(Baker et al, 2009,2011, Harjanto and zaman, 2011)

In this study we have made scaffolds at increasing oxidation levels of Gum Arabica (10%, 20%,)upon cross linking with a fixed amount of collagen. Oxidation was carried out using Sodium periodate and the different oxidation levels attribute to the difference in levels of cross linking, porosity, morphology, stability and various other physical properties





10% Oxidation **20% Oxidation** **Collagen**

Figure 1.7.2 Scaffolds at different concentrations and collagen.

1.8 <u>Collagen:</u>

Collagen is the main structural protein in the extracellular space in the various connective tissues in animal bodies. As the main component of connective tissue, it is the most abundant protein in mammals, making up from 25% to 35% of the whole-body protein content. Collagen consists of amino acids wound together to form triple-helices to form of elongated fibrils. It is mostly found in fibrous tissues such as tendons, ligaments and skin.

Depending upon the degree of mineralization, collagen tissues may be rigid (bone), compliant (tendon), or have a gradient from rigid to compliant (cartilage). It is also abundant in corneas, cartilage, bones, blood vessels, the gut, intervertebral discs, and the dentin in teeth.

Collagen also has many medical uses in treating complications of the bones and skin.

Types of collagen :

Collagen occurs in many places throughout the body. So far, 28 types of collagen have been identified and described. They can be divided into several groups according to the structure they form:

- Fibrillar (Type I, II, III, V, XI)
- Non-fibrillar
- FACIT (Fibril Associated Collagens with Interrupted Triple Helices) (Type IX, XII, XIV, XIX, XXI)
- Short chain (Type VIII, X)
- Basement membrane (Type IV)
- Multiplex in (Multiple Triple Helix domains with Interruptions) (Type XV, XVIII)
- MACIT (Membrane Associated Collagens with Interrupted Triple Helices) (Type XIII, XVII)

The five most common types are:

- Type I: skin, tendon, vasculature, organs, bone (main component of the organic part of bone)
- Type II: cartilage (main collagenous component of cartilage)
- Type III: reticulate (main component of reticular fibers), commonly found alongside type I.
- Type IV: forms basal lamina, the epithelium-secreted layer of the basement membrane.
- Type V: cell surfaces, hair & placenta

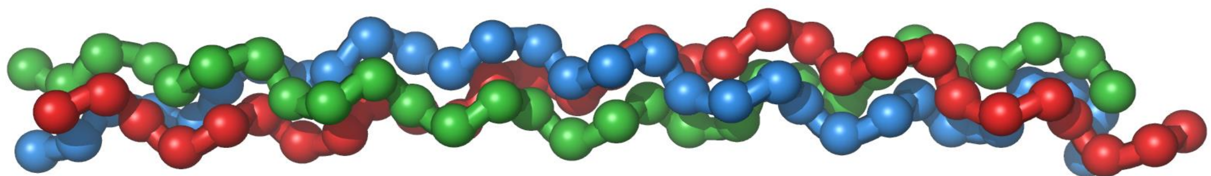


Figure 1.8: Structure of collagen triple helix

Uses in Tissue Engineering :

Collagen scaffolds are used in tissue regeneration, whether in sponges, thin sheets, or gels. Collagen has the correct properties for tissue regeneration such as pore structure, permeability, hydrophilicity and it is stable in vivo. Collagen scaffolds are also ideal for the deposition of cells.

LITERATURE REVIEW

CHAPTER 2

2.1 Role of Mesenchymal stem cells in differentiation :

A population of multipotent stromal cells exists within bone marrow and other adult tissues, which is able to differentiate into different skeletal tissues such as bone, cartilage and fat. These cells are frequently referred to as mesenchymal stem cells (MSCs) and offer significant therapeutic potential, particularly in orthopedic applications, but may also have broader roles in regenerative medicine, cancer treatment, as anti-inflammatories, immunosuppressive and vehicles for gene/protein therapy. Much attention has focused on understanding MSC biology and the regulation of differentiation to help realize these clinical aspirations. (pubmed)

Here we review some of the key molecular determinants of MSC function, with an emphasis on transcription factor control and the cell-cell signaling pathways that regulate MSC differentiation. The source information comes from a range of different models, including isolated human MSC cultures, animal-derived MSC-like cell lines, animal models and skeletal developmental processes to provide a wide-angled overview of the important players in MSC biology and tri-lineage specification.

When maintained under standard condition these cells must be plastic adherent and about 95% of MSC population must express CD 105, CD73 and CD 90 and must lack expressions of CD45, CD34 , CD14, CD11b, CD9a or CD19.

MSCs are quite rare in the body of adults; although they are abundant at the early stages of development and it is evident from the fact that tissue damage healing takes place much faster in children as compared to adults and this can be attributed to a number of factors, one of those is the presence of adult or progenitor cells including MSCs in abundance.

In addition to their ability to differentiate to Mesenchymal cell types, MSCs secrete a lot of cytokines and factors that have autocrine and paracrine activities. In addition to producing Stem cell factor (SCF-1) it produces vascular endothelial growth factors (VEGF), granulocyte colony

stimulatory factor (G-CSF), Macrophage colony stimulating growth factors (M-CSF), interleukins (IL-1, 6, 7, 11, 15) and others.

Additional interesting and important aspects of MSCs that have come to light include their homing ability to the sites of tissue injury; particularly ischemic regions of the heart where they may prevent deleterious remodeling. MSCs have the ability to modify immune responses and engraft in allogenic recipients. These cells have surface molecules like HLA-1, VCAM-1, LFA-3 and ICAM-1 that allow close interactions with the cells of the immune system.

The considerable therapeutic potential of human multipotent mesenchymal stromal cells or mesenchymal stem cells (MSCs) has generated increasing interest in a wide variety of biomedical disciplines. Nevertheless, researchers report studies on MSCs using different methods of isolation and expansion, as well as different approaches to characterize them; therefore, it is increasingly difficult to compare and contrast study outcomes. To begin to address this issue, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed minimal criteria to define human MSCs. First, MSCs must be plastic-adherent when maintained in standard culture conditions (α minimal essential medium plus 20% fetal bovine serum). Second, MSCs must express CD105, CD73 and CD90, and MSCs must lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules. Third, MSCs must differentiate into osteoblasts, adipocytes and chondroblasts in vitro. MSCs are isolated from many adult tissues, in particular from bone marrow and adipose tissue. Along with their capacity to differentiate and transdifferentiate into cells of different lineages, these cells have also generated great interest for their ability to display immunomodulatory capacities. Indeed, a major breakthrough was the finding that MSCs are able to induce peripheral tolerance, suggesting that they may be used as therapeutic tools in immune-mediated disorders. Although no significant adverse events have been reported in clinical trials to date, all interventional therapies have some inherent risks. Potential risks for undesirable events, such as tumor development, that might occur while using these stem cells for therapy must be taken into account and contrasted against the potential benefits to patients.

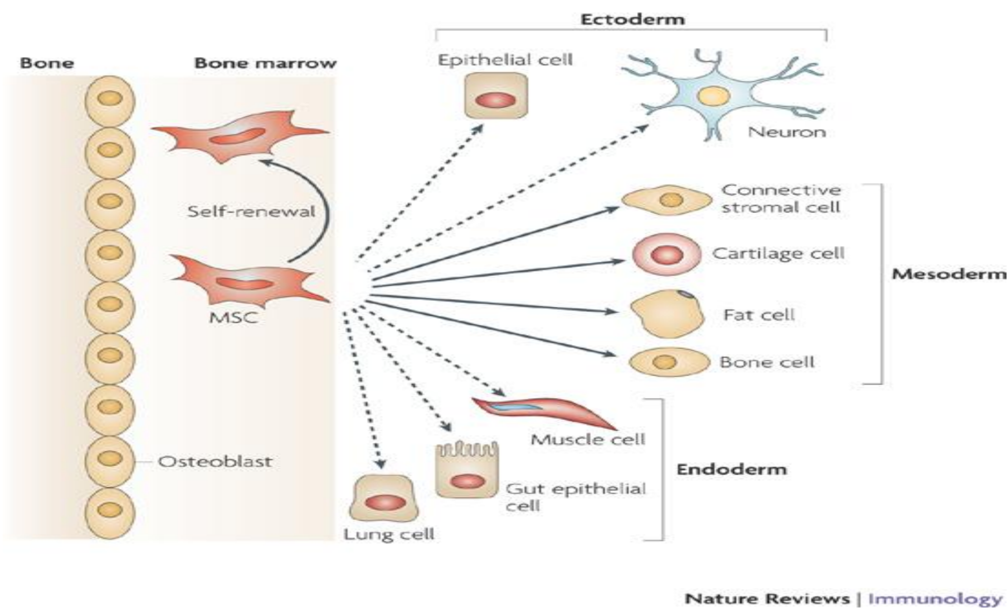


Figure 2.1: Multipotency of Mesenchymal stem cells

2.2 <u>Growth conditions, Cell morphology & Population in 2D & 3D culture</u>

Harrison carried out the first cell cultures in 1907 during research into the origin of nerve fibres . Since then, the method has been improved and used to observe the growth and differentiation of cells outside the body.

Nowadays, Primary cultures are isolated from living organisms and usually contain populations of different cell types present in the source tissue.

In ancient days, 2D cultures, cells grow as a monolayer in a culture flask or in a flat petri dish, attached to a plastic surface i.e. to adherent dish. The advantages of 2D cultures are associated with simple and low-cost maintenance of the cell culture and with the performance of functional tests. Unfortunately, adherent cultures also have numerous disadvantages.

First, 2D cultured cells do not mimic the natural structures of tissues . In this culture method, cell-cell and cell-extracellular environment interactions are not represented as they would be in the tumour mass. These interactions are responsible for cell differentiation, proliferation, vitality, expression of genes and proteins, responsiveness to stimuli, drug metabolism and other cellular functions. After isolation from the tissue and transfer to the 2D conditions, the morphology of

the cells is altered, as is the mode of cell division. The loss of diverse phenotype is also a result of 2D culturing. The changed morphology of the cells can affect their function, the organization of the structures inside the cell, secretion and cell signaling. Due to disturbances in interactions with the external environment, cells growing adherently lose their polarity which changes the response of those cells to various phenomena, such as to apoptosis . Another drawback of 2D culture is that the cells in the monolayer have unlimited access to the ingredients of the medium such as oxygen, nutrients, metabolites and signal molecules.
Owing to the many disadvantages of 2D systems, there was a need to find alternative models, better able to mimic a natural tumor mass, such as 3D culture systems.

Due to so many consequences, 3D culture media was taken for a solution to get rid of all these problems. 3D models can be divided into: i) suspension cultures on non-adherent plates ii) cultures in concentrated medium or in gel-like substances and iii) cultures on a scaffold.

Cells, when grown in 3D culture systems form aggregates or spheroids within a matrix, on a matrix, or in a suspension medium. In cell aggregates/spheroids, cell–cell interactions and cell–ECM interactions more closely mimic the environment found *in vivo.* So, cell morphology closely resembles its natural shape in the body. In addition, 3D spheroids are comprised of cells in various stages, usually including proliferating, quiescent, apoptotic, hypoxic, and necrotic cells. The outer layers of a spheroid, which is highly exposed to the medium, comprise mainly of viable, proliferating cells. The core cells receive less oxygen, growth factors, and nutrients from the medium, and tend to be in a quiescent or hypoxic state. Such cellular heterogeneity is very similar to the natural conditions, particularly in tumors. Since the morphology and the interactions of cells grown in 3D culture is more similar to what occurs *in vivo*, the cellular processes of these cells also closely emulate what is seen *in vivo*.
The 3D culture systems not only provide the spatial cell–cell interactions and cell–ECM interactions in monoculture for studying cell behaviors that mimic *in vivo* conditions, but also provide an opportunity for the co-culture of multiple types of cells to more closely mimic the *in vivo* conditions. Other types of cells interacting with the cells of interest play important roles in cell functions.

One important contribution for the ‘closer-to-*in vivo*’ behavior of cells when grown as 3D cultures is the matrices and scaffolds that are used for obtaining such cultures, The commonly used scaffolds are agarose, collagen, fibronectin, gelatin, laminin and vitronectin. From simple liquid overlay methods to complex co-cultures, several matrices are utilized; from low-melting agarose to complex synthetic compounds. Of significance is to note that a specific type of matrix or scaffold can be the most suited to elicit a particular type of morphological and physiological behavior in cultured cells. More than 100 types of matrices and scaffolds of both organic and inorganic nature are being currently used. The choice of such matrices and scaffolds is based on the cell types as also on the nature of the study.

Type I collagen matrix is used commonly in 3D culture system. This is due a number of reasons including the ease in processing, low-cost and the flexibility for live cell manipulation. Also, the pore size, ligand density and stiffness can be varied by changing the concentration of collagen or introducing chemical cross-linking compounds, thus making it easy to change the structural properties of the gel (Baker et al., 2009, 2011; Harjanto et al., 2010) 3D scaffolds are generated using various natural (collagen, gelatin, elastin, silk fibroin, chitosan, chitin, fibrin, fibrinogen, etc.) and synthetic polymers. The composite of natural and synthetic substances are also being used. These composites mimic the native extracellular matrix by porosity, fibrous, permeability and mechanical stability. The micro architecture enhances the biophysical and biochemical interaction of the adhered cells to be better expressed *in vitro*.

The 3D matrix provides a biologically active environment for the cells to proliferate, differentiate and secreted cell specific extracellular matrix which can be potentially used for a variety of applications. Agarose hydrogels are an example of simple materials to obtain 3D aggregates for a variety of cell types. However, each cell type requires markedly different optimal conditions as defined by the composition, concentration and volume of the agarose hydrogels. Also, different cell types behave differently when cultured using agarose hydrogels, thus proving to be simple, yet useful models.

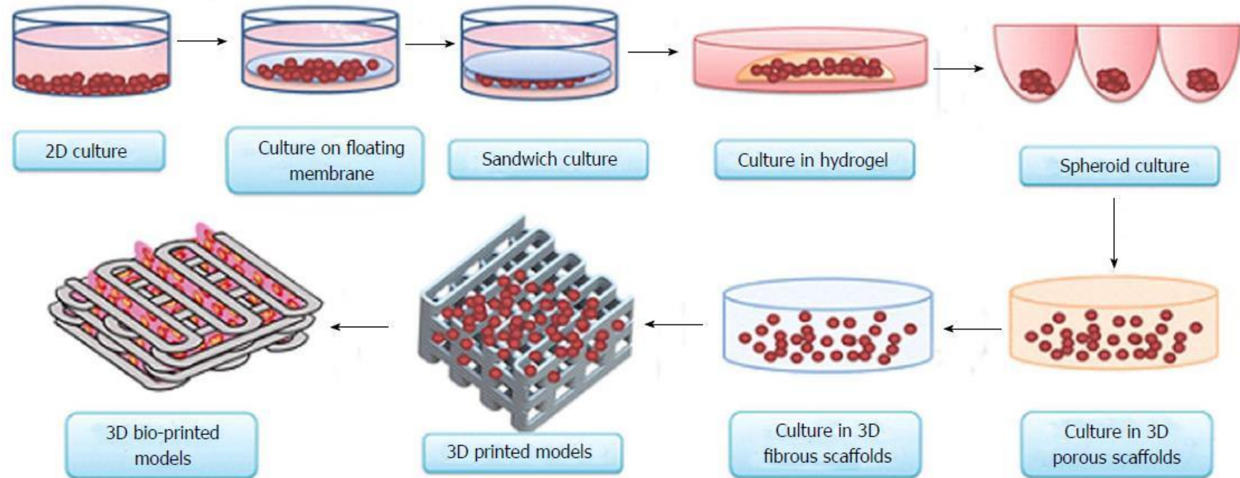


Figure 2.3 : 2D & 3D cell culture

2.3 : Stem cell differentiation is regulated by intrinsic extracellular matrices

Stem cell differentiation has always employed a cocktail of various growth factors, but differentiation is also linked to cell generated physical forces as recently explored. These forces usually originate from myosin bundles sliding along actin filaments and are transmitted to the extracellular matrix (ECM), a three-dimensional fibrillar protein scaffold that surrounds and anchors cells. Transduction of these signals employs a great array of adhesive proteins that assemble together to mechanically link the extracellular and intracellular worlds.

A good understanding of the interplay between development and ECM expression provides a starting point for tackling stem cell differentiation. ECM is composed mainly of three major structural proteins, i.e. collagen, fibronectin, and laminin, though it also includes elastin, fibrillin, tenascin, glycosaminoglycans, and proteoglycans. Each of these has a very specific distribution and assembly pattern in the body (Hay, 1991) that could contribute to, many developmental processes. For example, the matrix protein fibronectin regulates cell migration during the rearrangement process called gastrulation (Darribere and Schwarzbauer, 2000), which occurs just when a stem cell's fate is being decided and the body's plan and axis is being established. Moreover, interactions between specific germ layers and matrix proteins, e.g. mesoendoderm morphogenesis and the synergy site of fibronectin (Davidson et al., 2002), also suggest a link between intrinsic matrix properties and differentiation. On the other hand, adult stem cells, such

as bone-marrow-derived mesenchymal stem cells (MSCs), are multipotent meaning that they can mature into a specific subset of cells. Though they may not have temporally-changing matrix as with ESCs, they are known to migrate from the marrow to a variety of tissues with different matrix compositions (Pittenger et al., 1999), providing good cause to examine their susceptibility to matrix variation. Both cell types sit in contrast with lineage-specific progenitor cells, which can only become a single cell type and thus may behave more closely to that adult cell type. Many observations in modern cell biology have been performed on glass coverslips, often coated with a thin layer of ECM. However, such thin coatings do not mimic the normal mechanical environment of most cell types (Engler et al., 2004) and can lead to de-differentiation or loss of function in the cells (Engler et al., 2008). The correct mechanical properties of a cell niche are quite important and it varies as much as 300-fold from soft brain tissue to the rather rigid, calcifying bone and this illustrates a third component of ECM signaling: mechanics. Such natural variations in mechanics or elasticity also occur during development as both temporal and spatial changes in matrix (Krieg et al., 2008; Rozario et al., 2009; Zamir et al., 2003) may help guide cells as they mature and assemble into tissues,. While many variations in the concentration of growth factors may be more widely appreciated as compared to the variations in matrix elasticity, the importance of elasticity-directed differentiation as a novel and surprisingly sensitive cell regulator is now recognized (Engler et al., 2006). Undifferentiated MSCs grown on polymer gels mimicking the ECM elasticity of a given tissue were found to express precursor proteins for the cell type present in that tissue even in the absence of specific growth factors. Cells grown on gels of similar elasticity to brain express neuronal markers phenotype and are not tremendously contractile.

2.4 Use of hydrogels in 3D cell culture

The development of biomaterials for tissue engineering applications has recently focused on the design of biomimetic materials that are capable of eliciting specific cellular responses and directing new tissue formation mediated by biomolecular recognition, which can be manipulated by altering design parameters of the material. Biomolecular recognition of materials by cells has been achieved by surface and bulk modification of biomaterials via chemical or physical methods with bioactive molecules such as a native long chain of extracellular matrix (ECM) proteins as well as short peptide sequences derived from intact ECM proteins that can incur

specific interactions with cell receptors. The biomimetic materials potentially mimic many roles of ECM in tissues. For example, biomimetic scaffolds can provide biological cues for cell–matrix interactions to promote tissue growth, and the incorporation of peptide sequences into materials can also make the material degradable by specific protease enzymes.

The works in scaffold engineering demonstrates that 3D synthetic microenvironments can be designed to promote cell viability and direct cell adhesion (Lee et al., 2008), differentiation (Salinas and Anseth, 2008), proliferation (Mann and West, 2002), and migration (West and Hubbell, 1999) through the controlled presentation of mechanical and biochemical cues. Such instructive materials are bridging the gap between promoting and permissive gels by incorporating biomimetic signals into synthetic materials that elicit desired cell–gel interactions. These scaffolds can be tailored to the specific cell culture requirements and design criteria and are providing novel and well-defined ECM mimics for controlled hypothesis testing in cell biology and regenerative medicine (Cushing and Anseth, 2007).

2.5 :Pancreatic islet cells differentiation of Mesenchymal Stem cell induced by collagen & matrigel based hydrogel.

The placenta is a temporary organ that accompanies pregnancy connected to the fetus via the umbilical cord. Besides playing a fundamental and essential role in fetal development, nutrition, and tolerance, placenta may also represent a reserve of progenitor/stem cells. Recently, the placenta was shown to be an important hematopoietic organ, containing cells in chorionic villi that showed hematopoietic cell lineage differentiation along with presence of the hematopoietic markers CD34 and CD45. In addition to hematopoietic stem cells, the placenta has been reported to contain a population of multipotent stem cells exhibiting some of the characteristics of pluripotent ESCs including expression of stem cell markers c-kit, Thy-1, oct-4, SOX2, hTERT, SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 . These cells resemble mesenchymal stem cells and can be induced to differentiate into hepatocyte, vascular endothelial, cartilage, and neural-like cells. Differentiation of these human placenta-derived mesenchymal stem cells (hPDMSCs) into insulin positive cells has raised hopes for the use of these cells as an alternative source for cell therapy in diabetes.

Cellular differentiation is a complex process; appropriate manipulation of ECM components in MSC cultures is crucial for guiding the differentiation and promoting insulin secretion. In vitro studies also indicated that culturing cells with various nano-sized scaffolds led to alterations in gene expression and differentiation. Collagen, an essential component of the ECM, has been widely used as a biomaterial in carrier systems to deliver drugs, proteins as well as genes and also functioned as a scaffold in tissue engineering. In addition to its excellent biocompatibility and biodegradable capacity, collagen can be easily fabricated into diverse forms and is useful in many biomedical and valuable applications. Nevertheless, the collagen matrices typically prepared have been usually limited to the sponge or thin-film structural forms and are less used in a nano-sized particulate form.

Matrigel, is a complex protein mixture obtained from the extracellular matrix (ECM) of a transplantable rat chondrosarcoma. It contains proteins commonly found in the basement membrane of epithelial structures, such as laminin, collagen, fibronectin, or entactin.For differentiation process matrigel is used. Matrigel is also known to contain growth factors required for cell homeostasis, differentiation, and tumor growth, such as basic fibroblast growth factor and epidermal growth factor. Due to these properties, Matrigel is widely used in cancer culture models to simulate the environment of a basement membrane. Using proper scaffold materials, a microenvironment should be designed that mimics the *in vivo* situation and create the environment to promote differentiation of MSCs. Scaffolds provide a three-dimensional (3D) microenvironment that allows cells to anchor, permits exchange of gas and nutrients, and promotes the synthesis of ECM (Risbud et al 2002) . A variety of materials based on both natural and synthetic polymers have been fabricated to serve as scaffolds for the study of differentiation in different forms, including fibrous structures, porous sponges, woven or nonwoven meshes, and hydrogels. Among them, embedding in hydrogels has been shown to be an effective culture system for differentiation of progenitor cells. (Risbud et al 2002, Coleman et al 2007, Bosnakovski et al 2006) Collagen hydrogels and collagen-based hydrogels were favorable scaffolds successfully applied in chondrogenic differentiation due to their biomimetic properties (No_th U et al 2007, De Chalain et al 1999). It has been reported of collagen substrates to modify the morphology, migration, and in some cases differentiation of cells (Kleinman HK et al 1981, Capito RM 2003). But these studies were always combined with growth factors. The effect of scaffolds on the induction of differentiation was not confirmed.

MATERIALS AND METHODS

CHAPTER 3

3.1 Isolation of collagen from rat tail tendons:

3.1.1 : Materials

- Rat tails
- Bone Cutter
- Glacial Acetic acid
- Dialyzing membrane
- Phosphate buffer
- NaCl
- Ethanol (70%)

3.1.2. Instrumentation

- Centrifuge
- Magnetic stirrer
- Lyophilizer

3.1.3 Solutions

Dialysis buffer**:** 12.5mM sodium dibasic (Na2 HPO4)

11.5mM sodium phosphate monobasic (NaH2 PO4)

0.15M Acetic acid: Dilute 8.6ml Glacial acetic acid (17.4M) to 1 litre.

0.5M Acetic acid: Dilute 14.4ml Glacial acetic acid (17.4M) to 500ml.

70% Ethanol

3.1.4: Procedure :

- One of the potential sources of collagen to be used in tissue engineering comes from rat tail tendons. Rat tail tendons if processed with acetic acid yield soluble Type I collagen.
- 23 rats were sacrificed by culling. The tails were dissected by making a full length incision in skin.

- Tendons were pulled out in bundles by grasping the bundle tip and pulling it with the help of a bone cutter.
- The tendons were then dissolved in 0.5M of Acetic acid and stirred at 4 °C for 4- 5 days still the solution became viscous. Solution was filtered to remove the undissolved components.
- The filtrate solution was then transferred to dialyzing membrane and dialyzed against phosphate buffer for 5-6 days until the solution in the membrane became turbid and collagen precipitated out.
- The semi solid collagen was centrifuged at 10000 rpm for 10 minutes; the pellet was the dissolved in 0.15M Acetic acid overnight at 4°C.Collagen was then salted out by adding NaCl to give a final concentration of 5%.
- Salted out collagen was centrifuged at 10000 rpm for 10 minutes.
- Acetic acid dissolution and salting out steps were done 2-3 times to ensure pure collagen extraction.
- Collagen was then sterilized using 70% Ethanol and centrifuged at the same conditions.
- It was transferred to pre weighed 50ml tubes and dried in a Lyophilizer.
- Dry weights of the collagen were noted.

3.2: <u>Separation of proteins based on SDS- PAGE.</u>

SDS- PAGE is a gel based separation technique for proteins which separates primarily by mass because the ionic detergent SDS denatures the proteins and binds to distributing uniform negative charge. Thus, under an electric field applied all the negatively charged protein will move towards the positive charged electrode (anode). Proteins of lower mass move more quickly through the gel as compared to the proteins of bigger mass because of the sieving properties of the gel matrix.

To obtain an optimal resolution of the proteins a stacking gel is cast over the resolving gel. The stacking gel has a lower concentration of acryl-amide for larger lower pH and a different ionic content. This allows the proteins to stack out during the first few minutes of electrophoresis before entering the resolved portion of a gel.

3.2.1 Materials:

1) 10 %Resolving Gel (10 ml):

- 4.6 ml distilled water.
- 2.7 ml Acrylamide mix (30%)
- 2.5 ml 1.5 M Tris (Ph 8.8)
- 100µl Ammonium per sulphate (10%)
- 100µl SDS
- 0.006µl TEMED

2) 5% Stacking Gel (5ml)l:

- 3.4 ml distilled water
- 0.83 ml Acrylamide mix (30%)
- 0.63 ml 1M Tris (Ph 6.8)
- 0.05 µl Ammonium per sulphate (10%)
- 0.05 µl SDS
- 0.005µl TEMED

3)TGS Running Buffer:

- Glycine: 14.4 gms
- Tris base : 3.02 gms
- SDS :1gm
- Make volume upto 1 litre

4) Loading Dye (4X):

- 4 ml of 100% Glycerol)
- 2.4 ml 1M Tris/HCl pH 6.8
- 0.8 gm SDS (8%)
- 4 mg Bromophenol blue (0.04%)
- 0.5 ml beta Mercaptoethnol (5%)
- 3.1 ml Milli Q water

5) SDS Coomassie Stain

- 1 gm of Coomassie R-250
- 100 ml of Glacial Acetic Acid
- 400 ml of methanol
- 500 ml of Milli Q water

6) SDS destaining solution

- 200 ml of Methanol
- 100 ml Glacial Acetic Acid
- 700 ml Milli Q water.

7. Gel Electrophoresis Unit

3.2.2 Procedure:

- 1 mg Dry collagen was dissolved in 500ml of loading Dye (1X).
- The mix was then denatured at 95℃ for 3 minutes and was then placed at 4℃.
- 8% resolving gel was prepared in the gel apparatus.
- 5% stacking gel was prepared once the resolving gel was polymerized.
- 1X TGS Buffer was made and was poured into the electrophoresis tank.
- The protein sample (5µl) was then added into the wells carefully.
- The electrophoresis was then carried out at 80-90 Volts.
- After the run for about 1-2 hours, the gel was carefully removed and then placed in SDS stain and was put on a shaker for 30 minutes.
- After 30 minutes the Gel was then placed in the SDS destaining solution for 1 hour on the shaker.
- The destainer solution was changed and was kept overnight.
- The gel was checked for bands.

- After the bands were confirmed another gel was run and was then processed for Western Blotting.

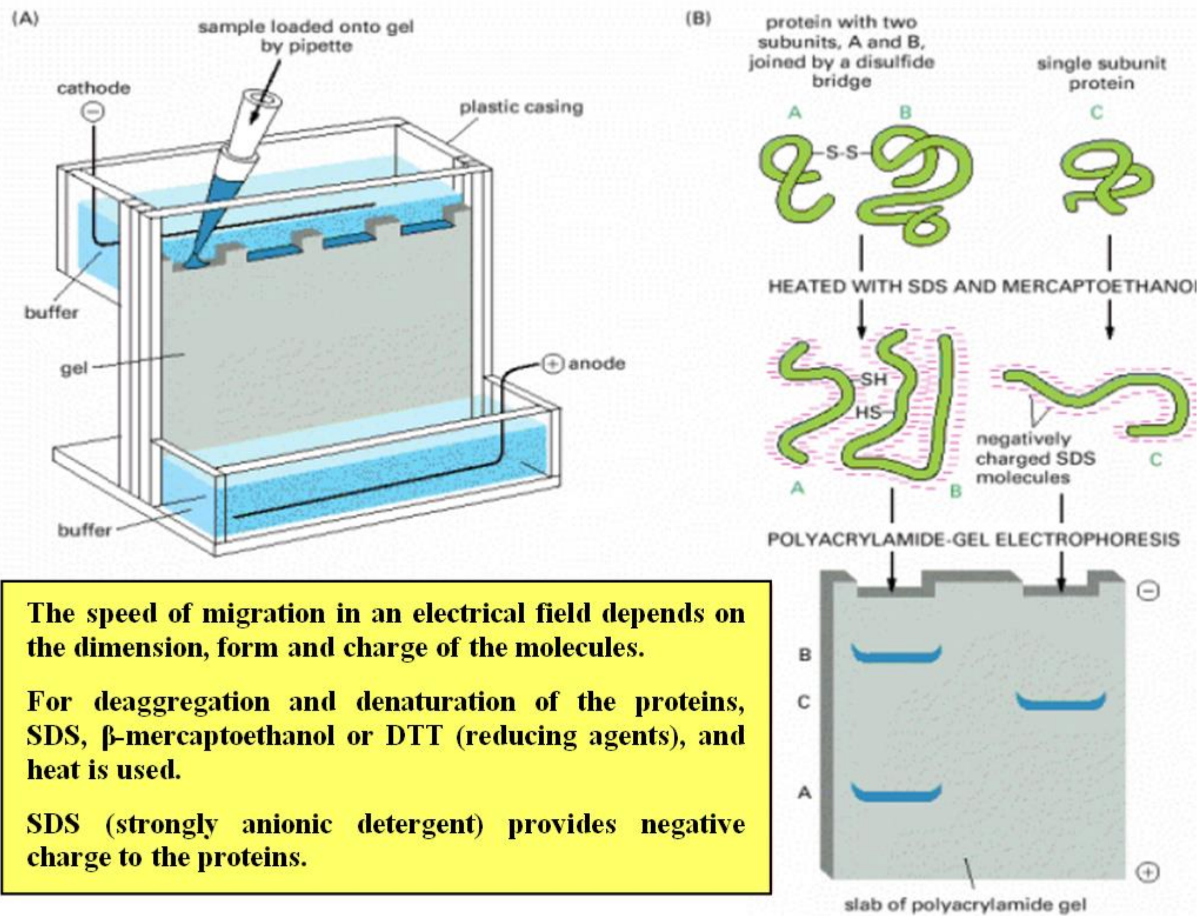


Figure 3.2.2.1. SDS- PAGE

3.3: Western Blotting

Western Blotting refers to the transfer of proteins samples from gel to a membrane and their subsequent detection on the surface of the membrane generally a nitro cellulose or PVDF membrane. The transfer is usually electrophoresis transfer of proteins involving placing a protein-containing polyacrylamide gel in direct contact with a piece of suitable membrane and sandwiching this between two electrodes submerged in a conducting solution. When an electric field is applied,

the proteins start migrating. Finally, a copy of the protein that was originally in the polyacrylamide gel is obtained on the membrane. Transfer efficiency can vary dramatically among proteins. Based upon the ability of a protein to migrate out of the gel and its ability to bind to the membrane under a particular set of conditions.

The efficiency of the transfer depends on the factors such as composition of the buffer, complete contact of the gel with the membrane, the right orientation of the electrodes, transfer time, size and composition of proteins, field strength.

After transfer and before proceeding with immunoblotting, total protein on the membrane is often stained with a dye such as Ponceau to check the transfer sufficiency. The gel may also be stained to confirm that the protein has been moved out of the gel. Dyes may interfere with antibody binding and detection, a protein stain that is easily removable is ideal. Ponceau stain is the most widely used reagent for reversibly staining proteins on a membrane.

3.3.1 Materials:

- Polyvinylidene fluoride (PVDF) membrane.
- Whattman filter papers.
- Milli Q water
- Coomassie stain
- Ponceau stain
- Transfer unit.

3.3.2 Procedure:

- After the polyacrylamide gel runs almost 80% the gel was stopped.
- The gel plates were detached carefully.
- The remaining empty gel was cut along with the stacking gel
- Whattman filter papers were cut equal to the size of the gel and were soaked in the buffer.
- PVDF membrane was cut of the same size and soaked in the buffer.
- 2-3 pieces of the filter paper were placed on a semi dry transfer apparatus, followed by the PVDF membrane.

- The gel was then carefully placed over the membrane and to cover the gel 2-3 more filter papers were placed.
- The entire set was carefully pressed with a glass rod to remove any air bubbles in between which hinder the current flow.
- The apparatus was then connected to a power source and was allowed to run for 50 minutes at 100mA.
- After the run was finished, the membrane was taken out on a small tray and was then added Ponceau's stain for making sure the transfer of bands.
- The gel was stained with Coomassie to ensure complete transfer of proteins.

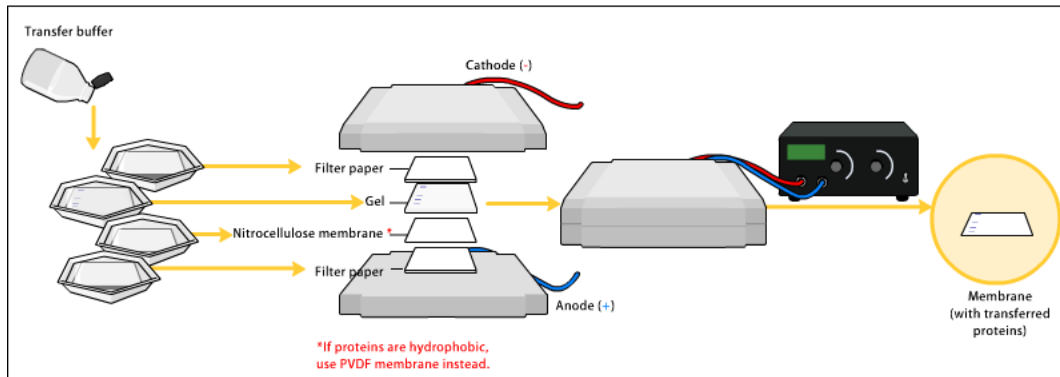


Figure 3.3.2.1. Western Blot Diagram

3.4 : <u>Preparation of Gum Arabic Aldehyde</u>

3.4.1 : Materials

- Gum Arabic from Sigma Aldrich
- Sodium Periodate from Sigma Aldrich.

3.4.2 : Instrumentation

Centrifugation

- Rotor
- Lyophilizer

Gum Arabic is a branched polysaccharide and can be oxidized by Sodium Periodate. After the oxidation with periodate the free aldehyde groups are released which can then further form cross linkages with other compounds.

3.4.3 Procedure:

- To prepare GAA 0.4 grams of Gum Arabica was dissolved in 5 ml distilled water.
- 5 test tubes each with 3ml of GAA solution is mixed with the increasing concentration of sodium periodate dispensed into 5 test tubes each mixed with the increasing concentration of sod. periodate as shown in the table 1.1.
- The mixture is allowed to rotate on a rotor for 6 hours in dark.
- The mixture is then stirred with Ethylene glycol for 1 hour and centrifuged.
- The supernatant is precipitated with acetone (in the ratio of 4:1) and further washed with acetone thrice**.**
- A purified pellet is obtained and lyophilized and recovery was calculated using the below formula:
- **Recovery = (Weight of dried Gum Arabic)/ (Initial weight of Gum Arabic Taken)*100**

Percentage of oxidation	**Amount of Sodium periodate**
10% oxidation	0.062gms
20% oxidation	0.124gms

Table 3.1: Table showing the weights of sodium periodate taken for various levels of oxidation.

3.5 **Preparation of 2, 4-Dinitrophenylhydrazine reagent**:

3.5.1 Materials:

- DNPH
- Conc. H_2SO_4
- Ethanol

3.5.2 Procedure:

2, 4-Dinitrophenylhydrazine, also called as Brady's or Borche's reagent is a substituted hydrazine which is commonly used to qualitative test carbonyl groups including ketones and aldehydes. Ketones or aldehydes give yellow colored precipitate (known as dinitrophenylhydrazone) upon reaction with DNPH signaling a positive test .The hydrazines react with carbonyl compounds to form hydrazones by undergoing Schiff's Base reaction. In order to prepare 2, 4-Dinitrophenylhydrazine, 60mg of DNPH was added to 4.50ml conc. H2SO4. Once the dye completely dissolved, 4.5 ml of ethanol was added and the final volume was made to 15 ml using distilled water.

3.6 Estimation of Aldehyde groups in GAA

DNPH test was employed to determine the aldehyde content in oxidised Gum Arabic prepared at increasing oxidation levels.

Methodology involved the following steps:

- Standard was prepared again in sets of two by taking 1µl formaldehyde at varied concentrations (0.5%, 1%, 2%, 5% and 10%) , water was used as blank.
- Samples were prepared by taking 1µl of 1% solution of Oxidized Gum Arabic (sets of two)
- To all the tubes, 500 µl of freshly prepared solution of DNPH was added and incubated at room temperature for 1 hour.
- After 1 hour incubation, all the tubes were centrifuged for 20 minutes at 12000 rpm.

- The supernatant was carefully collected without disturbing the pellet and was put in a 96 well plate in duplets.
- The absorbance of unreacted DNPH in the supernatant fluid was measured at 357 nm wavelength using UV visible multimode plate reader.
- The moles of aldehyde generated were calculated using the following equation:

Aldehyde Conc. (No. Of moles) = Reacted DNPH/ 198.14

Where 198.14 is the molecular weight of DNPH.

3.7 : Preparation of Cell Scaffolds using GA at different oxidized levels and Collagen.

3.7.1 Materials:

- Collagen
- GAA
- 0.1M Borax Buffer
- 0.15M Acetic acid
- Mesenchymal stem cells
- Dulbecco's Modified Iscove's Media (DMEM)

3.7.2 Procedure:

- 2 % & 1% solution of Collagen in 0.15M acetic acid was made and were kept at 4°C until it dissolved.
- It was then mixed with 10% solution of GAA. The 10% solution was prepared by dissolving in 0.1M Borax.
- GAA and collagen were mixed in equal proportions and vortexed till it started gelling.

- The foamy solution was then mixed with MSCs after trypsinization and was put into a 96- well or 24- well plate, under a laminar hood, according to requirements.
- The cell Scaffold was allowed to polymerize properly.

The Scaffolds were made in 2 sets.

Set 1 (2% Collagen)	1. Scaffolds were subjected to cross linking after lyophilization. 2. Cells were seeded in it. 3. Pancreatic Differential media was added
Set 2 (1% Collagen)	1. Scaffolds were left for cross linking after lyophilization. 2. Cells were seeded in it. 3. Pancreatic Differential media was added.
Fibrinogen	Cells were embedded in fibrin clots and incubated for differentiation.
Matrigel	Cells were embedded in matrigel and differentiated.
Decellularized Scaffolds	Cells were trapped in solubilized decellularized matrix and differentiated.

Table 3.2: Modes of Scaffold Preparation.

3.8. Media preparations

3.8.1 DMEM (Dulbecco's Modified Eagles) Media.

DMEM is a modification of Basal Medium Eagle (BME) that contains a four-fold higher concentration of amino acids and vitamins, as well as additional supplementary component DMEM is considered one of the more common and less complex in comparison to enriched media which are utilized for more specialized cell types and are the basis for more unique serum-free media formulations. The original DMEM formula contains 1000 mg/L of glucose and was first reported for culturing embryonic mouse cells. However, further alteration with 4500 mg/L glucose has proved to be optimal for cultivation of certain cell types.

3.8.1.1 Additional components of DMEM media.

Fetal bovine serum (**FBS**) or **fetal calf serum**

It is the fraction of blood that remains after the natural coagulation of blood, which is then centrifuged to get rid of any blood cells. Fetal bovine serum comes from the blood drawn from a bovine fetus. Fetal bovine serum is the most widely used serum-supplement for the in vitro cell culture of eukaryotic cells, because of the presence of low antibodies and more growth factors allowing for versatility in many different cell culture applications

Antibiotics (penicillin and streptomycin)

The Antibiotics used in Cell culture prevent Bacterial contamination due to their combat against both gram +ive and gram –ve bacteria.

3.8.2 IMDM (Iscove's Modified Dulbecco's) media

A totally reduced serum free medium supplemented with albumin, transferrin, lecithin, and selenium as determined by Guilbert and Iscove can be used to culture the precursor cells of erythrocytes and macrophages. This medium is an alteration of Dulbecco's Modified Eagle's Medium (DMEM) containing selenium, additional amino acids and vitamins, sodium pyruvate, HEPES buffer, and potassium nitrate (KNO_3) instead of ferric nitrate ($Fe(NO_3)_3$). Further studies demonstrated that Iscove's Medium would support murine B lymphocytes, hematopoietic tissue

from bone marrow, B cells stimulated with lipopolysaccharide, T lymphocytes, and a variety of hybrid cells. IMDM media are a highly enriched synthetic media beneficial for rapidly proliferating, high density cell cultures.

3.8.2.1 Additional components of IMDM media.

Mercaptoethanol

Beta Mercaptoethanol (BME-$HOCH_2CH_2SH$) it is a potent reducing agent used in cell culture medium to reduce reactive oxygen species. It can act as a biological antioxidant by scavenging hydroxyl radicals and is suitable for reducing protein disulfide bonds. It is a hybrid of ethylene glycol. The hydroxyl group confers solubility in water and lowers the volatility. Mercaptoethanol is not stable in solution so most protocols needs daily supplementation.

Hydrocortisone

Hydrocortisone or glucocorticoid is a steroid hormone. In cell culture hydrocortisone is used to induce cell growth and prevent the growth of macrophages. It also increases the proliferation of MSCs and aids in protein and carbohydrate metabolism.

Antibiotics (penicillin and streptomycin)

Antibiotics in cell culture are used to stop endogenous microbial infection and also to protect against intra- and inter-laboratory environmental factors. Penicillin is a group of antibiotics derived from penicillium fungi, it is known to act by inhibiting the last step in cell wall synthesis, the cross linking of different peptidoglycan strands. Streptomycin derived from the actinobacterium, streptomycin acts by binding to the 30s subunit of the bacterial 70s ribosome and blocking the initiation complex.

3.8.2.2 Materials for the preparation of IMDM media.

- Cryopreserved Cell line
- IMDM powder
- Sodium bicarbonate

- Beta Mercaptoethanol
- Hydrocortisone
- Antibiotics (penicillin and streptomycin)
- MSC FBS (Fetal Bovine Serum)
- PBS (Phosphate Buffer Saline)
- Autoclaved MilliQ
- Phosphate buffer solution

3.8.2.3 Instrumentation

- Sterilized Vacuum and pressurized filters
- Vertical laminar air flow
- Light microscope.
- Water jacketed CO_2 Incubator (37°C-5% CO_2)

3.8.2.4 Preparations

- A stock solution and a working solution were required
- **For the preparation of stock solution** 3.024 gms of Sodium bicarbonate ($NaHCO_3$) and 17.7 gms of 1x IMDM powder were dissolved in 900 ml of distilled water and a total volume of 1000 ml was made up.
- The solution was filtered using a vacuum filter.
- **For the preparation of working solution (100 ml**):-
- 15 ml of MSC FBS was taken in a milk bottle.
- 1 ml of antibiotic (0.6 gms penicillin and 0.5 gms streptomycin in 100 ml of PBS was taken and filtered ,the total volume was made upto 20ml with alcohol, and was stored at 4°C)
- 100µl of Beta Mercaptoethanol was added and a total volume of 100 ml using the stock solution was made up.
- The solution was then filtered using a pressurized filter

3.8.2.5 : Preparation of Pancreatic differentiation media

Differentiation media 1

- DMEM F12
- 1% BSA
- 1* ITS
- 4nm activin A
- 1Mm sodium Butyrate
- 50um 2- Mercaptoethanol
- Penicillin & streptomycin

Differentiation media 2

DMEM F12

1%BSA

1* ITS

0.3Mm Taurine

Differentiation media 3

- DMEM F12
- 1.5% BSA
- 1* ITS
- 1% PSA
- 3Mm Taurine
- 100 (Naugine)
- 1Mm Nicotinamide
- 1* NEAA
- After adding these components the volume was made upto 50ml and filtered for the addition of media to the cells

3.9. Cell Revival and Maintenance.

Human placental derived MSCs were obtained from previously Cryopreserved cells. These cells were thawed properly and were revived in culture flasks. They were then subjected to incubation for 24 hours at 37 °C -5% CO_2. The previous media was removed after 24 hours and a PBS washing was given to get rid of all the DMSO used during cryopreservation of the cell. The Cell culture was then maintained by changing media after every 2 days till 80-90% of confluence was attained it the flask.

3.10. Passaging of Cells.

After the attainment of 80%-90% of confluence in an adherent culture flask, the cells were subcultured for reducing their density. IMDM of the culture flasks were aspirated out and the cells were washed with PBS .0.1% TE (trypsin EDTA) 1 ml was added to the flask and the flask was kept in the CO2 incubator for 3-5 minutes. The flask was visualized for complete upliftment of the cells .2ml media was added to stop the enzymatic action and this media was aspirated to break down the clumps. The cell solution was then divided into two or three fresh flasks and IMDM was added again.

3.11. MTT Assay.

The **MTT** Assay is a colorimetric assay for assessing cell metabolic activity. This assay reflects the number of viable cells present.NAD (P) H dependent cellular oxidoreductase enzyme under suitable conditions reduce the tetrazolium dye MTT 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolim bromide to its insoluble formazon which has a purple color.

3.11.1 Materials:

- PBS (Phosphate Buffer Saline)
- MTT dye
- Isopropyl Alcohol
- HCl
- TE (Tris EDTA)
- GA-Collagen gel.

3.11.2 Instrumentation:

- 96 Well plate.
- Incubator
- Hemocytometer
- Centrifuge.
- UV visible multi mode plate reader.

3.11.3 MTT dye preparation:

5mg/ml of the dye was made, i.e. 75mg in 15 ml of PBS.

3.11.4 Procedure for MTT assay

Scaffolds were made In a 96 well plate using GA-collagen gel.

- Scaffolds were lyophilized, unlyophilized or made a mixture with cells.
- A confluent Cell dish was taken (up to 90% confluence)
- This dish was trypsinized.
- Cells were pelleted down using a centrifuge.
- DMEM media (1ml) was added to the cells.
- The cells were further diluted using additional DMEM as required for the experiment.
- Cells were seeded in the scaffolds in a 96 well plate using the pattern shown in table 3.3
- DMEM media was added after the cells were seeded.
- It was kept for 24 hours in the incubator at 37℃ and 5% CO_2.
- Media was removed from the plate the next day.
- One PBS wash was given to the Scaffolds.
- 100μl of MTT dye was added to all the scaffolds.
- The 96 well plate was then kept in the incubator (37℃) for 4 hours.
- Equal volumes of Isopropyl Alcohol and HCl (0.1M) were now put.
- This plate was centrifuged at 3000rpm for 5 minutes.
- The supernatant was carefully collected & Reading were noted using UV visible multi mode plate reader at 557nm.

3.12 .<u>Scaffold Fixation and Its processing for Histology.</u>

The Scaffolds with cells were incubated for 1 week for differentiation and fixed in 10% formaldehyde and were then processed for Histology.

3.12.1 Processing.

3.12.1.1 Materials:

- 10% Formalin.
- PBS.
- Milli Q water.
- Different Grades of Alcohol (50%, 70%, 80%, 95% and 100%).
- Coupling Jars.
- 100% Chloroform.
- Paraffin Wax.
- Wax Blocks.
- Rotor.

3.12.1.2 Procedure:

Day -1:

- 10% Formalin was made in PBS.
- After Proper Fixation in Formalin for at least 1 week, the samples were placed in Milli Q water and were rotated in a rotor for 1 hour.
- It was then replaced with 50 % Isopropyl Alcohol and rotated on a rotator for 1 hour.
- 70 % Isopropyl Alcohol was added to replace the previous solution and rotated on a rotator for 1 hour.
- 80% Isopropyl Alcohol was added to replace the previous solution and was rotated on a rotator for 1 hour.
- One wash with 95% Isopropyl Alcohol was given to the sample for 1 hour on the rotator.
- It was then kept overnight in 95 % Isopropyl Alcohol on the rotator with minimum speed.

Day -2:

- 100% Isopropyl Alcohol was added to the sample and was rotated for an hour on the rotator.
- 100% Isopropyl Alcohol was again added to the sample and rotated for an hour on the rotator.
- It was then replaced with 100% Chloroform twice and was rotated for 1 hour each.
- Chloroform was carefully drained and the samples were places in cassettes along with the labels.
- All the samples were placed in liquefied Paraffin wax 1 kept in a water bath at 62ºC for 1 hour.
- The samples were then shifted to Wax 2 for 1 hour
- It was then shifted to Wax 3 for another 1 hour.
- The samples were now ready for embedding.

3.12.2 Embedding.

- After the processing steps were over the samples needed to be embedded in wax blocks.
- For that, wax moulds were filled with liquefied wax on an embedding stage.
- The samples along with the labels were taken out from the wax cassettes and were placed in the moulds.
- The blocks were allowed to solidify by placing them on a cool surface.

3.12.3 Section Cutting.

- The wax blocks were allowed to solidify overnight after which they were trimmed in a microtome.
- 4 microns section was cut of each block and was placed on Probe On plus slides.
- The slides were neatly labeled and were kept overnight for drying.

3.12.4. H and E Staining.

Hematoxylin and Eosin (H & E) staining is the most common staining technique in histopathology. This uses a combination of two dyes, Hematoxylin and Eosin used for demonstration of nucleus and cytoplasmic inclusions in clinical specimens.

Procedure:

- The sections were deparaffinized by keeping them on a hot plate for 45-60 minutes.
- And then placing them in Xylene for 2 minutes (two changes with Xylene were given).
- The sections were hydrated by passing through decreasing concentration of alcohol baths for 1-2 minutes each and then into tap water for 5-10 minutes.
- Staining with Hematoxylin was done for 2 -10 minutes.
- It was then washed in tap water for 5-10 minutes.
- Staining with Eosin was done for 15-45 seconds.
- 3 changes of 95 % Alcohol washes were given and the slides were cleaned after the first wash.
- 100% alcohol wash was given for 1-2 minutes
- 4 changes with xylene were also given
- It was then mounted with DPX & was now ready for microscopic imaging

3.13. <u>Extraction of Fibrinogen & Thrombin from Blood</u>

3.13.1. Materials

- Sodium citrate
- Glass beads
- Syringe (10ml)

3.13.2. Procedure

For Fibrinogen, 1ml sodium citrate was taken in a test tube.

- 10ml blood was collected in that test tube.
- Then it was centrifuged at 3500rpm for 15min.

- After centrifugation, Plasma was collected and alcohol was in the ratio of 4 (plasma) : 1 (Alcohol).
- Again Centrifuged at 930g for 5 min at 4˚C.
- Then the supernatant was discarded and pellet was dissolved in $CaCl_2$.

  For Thrombin, 15 to 18 glass beads were taken in a test tube.
- 10ml blood was collected in a test tube without any anticoagulant.
- It was then centrifuged at 3500 rpm for 15min at 4˚C.
- Then supernatant was collected and used as Thrombin.

After the extraction of Fibrinogen and Thrombin, clots were made and cells were seeded with Differentiation media in it for differentiation to pancreatic Beta cell cluster formation.

3.14. Immunohistochemistry

Imunohistochemistry (IHC) involves the process of selectively imaging antigens (e.g. proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues.

3.14.1. Material

- Glass slides
- BSA
- Primary antibody
- Secondary antibody
- PBS (1X)
- Coverslips

3.14.2. Procedure

- After removal of differentiation media from the 96 well plates, PBS washing step was done.
- Then each well was shifted to a cover slip & 70% methanol was added for fixing the cells.
- Then after PBS washing, BSA was added into it.
- After 10 minutes, Primary antibody (Insulin, Neurogenin, PDX, PPY) was added in 1: 200 dilution with PBS & kept it at 4 °C for overnight.
- On the next day, after PBS washing for 10mins 3 times, Secondary antibody was added in 1:500 dilution with PBS in dark & kept covered for 1hr incubation.
- After 1hr, washed with PBS for 10 mins 3 times.
- Then the cover slips were mounted with DAPI and examined under confocal microscope.

3.15. <u>Decellularization of mice Pancreas</u>

Decellularization is the process used in biomedical engineering to isolate the extracellular matrix (ECM) of a tissue from its inhabiting cells, leaving an ECM scaffold of the original tissue, which can be used in artificial organ and tissue regeneration.

3.15.1. Materials

- Pancreas of mice
- Needles
- Petri dishes
- Thread
- Forceps
- PBS
- Saline bottle
- Roller bottle

3.15.2. Procedure

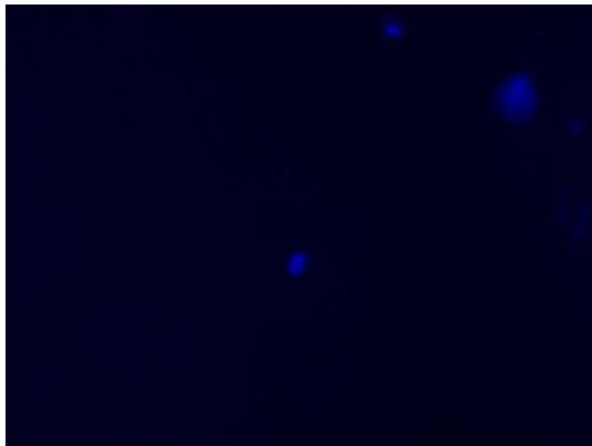
- Mice pancreas was carefully taken out after scarifying the mice.
- Then it was hanged inside a small container in such a way with the help of knot and needles were injected into it which is connected with the saline bottles pipe.
- 300ml of PBS was poured into the saline bottles and PBS was allowed to pass through the pancreas by drop wise.(In 1 minute only 4 -6 drops will go)
- After 4- 5 hr, Detergent was added (Triton + Ammonia) into the same saline bottles.
- On the next day, It was appearing like a white scaffold. After removal of the detergent, PBS washing was done for 2-3 hr.
- Then DNase was added into the left over PBS and it was allowed to flow through the pancreas whole day.
- Next day, PBS and antibiotic was poured into that saline bottle and completely passed through it.
- Finally, after these long procedure we got pancreas scaffold in which cells were seeded for checking its behavior inside an ECM.

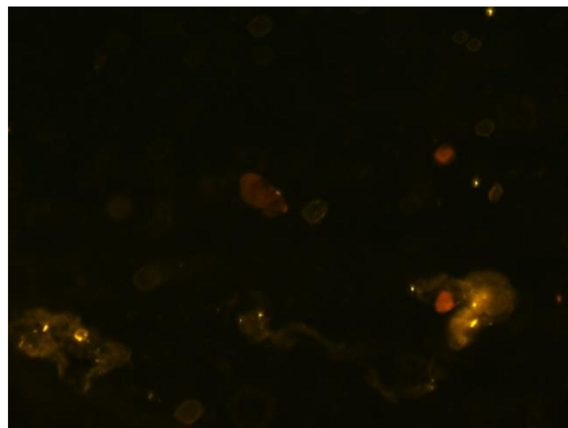
RESULTS & DISSCUSSION

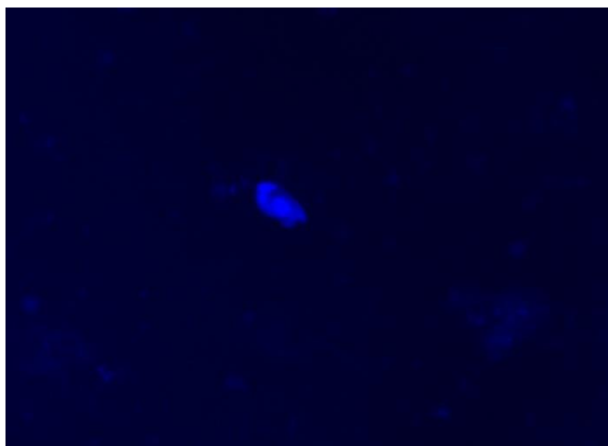
CHAPTER 4

Differentiation of MSC into beta cells

Placental derived mesenchymal stem cells form clusters in non- adherent plates after differentiation. This procedure took around 7 days in which cells after trypsinization were suspended in differentiation media MSC were incubated in differentiation medium in a sequence of steps that resulted in formation of clusters and these clusters had cells which expressed markers of beta cell type. The cells formed islet like clusters only when incubated in nonadherent dishes. Immunostaining of these cells with beta cell markers showed positive staining.







Same protocol was tried in presence of various scaffolds to check if any of the scaffolds will be suitable to provide in situ like conditions to allow proper differentiation and formation of islets. The first step was to make scaffolds and observe differentiation of cells.

4.1: Gum Arabica collagen scaffolds

Gum Arabica was oxidized using sodium periodate which helps it to crosslink proteins based on Schiff's reaction. The aldehyde group generated due to oxidation of glycan moieties was estimated.

When DNPH assay was performed with oxidized gum, the hydrazine in DNPH react with aldehydes and provide the peculiar yellow precipitate. In this study, it was seen that with the increasing oxidation, 20% oxidation level gum Arabica had more number of moles of aldehyde. More oxidation led to the inclusion of more aldehyde groups into the construct. Aldehyde groups show high reactivity and can potentially form linkages with other biopolymers like proteins. Oxidation of hydroxyl group on the backbone of polysaccharides leads to the inclusion of aldehydes.

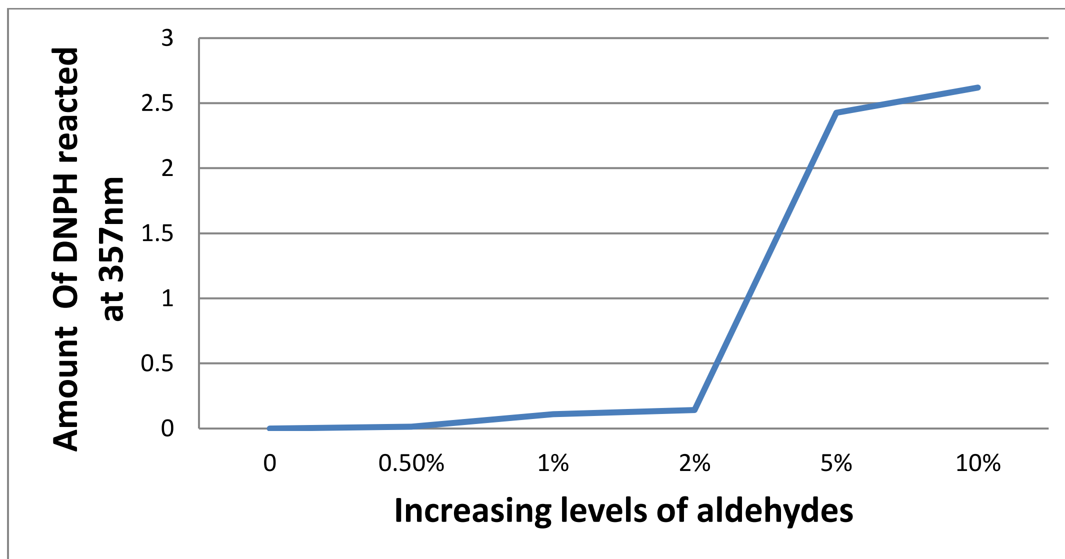


Figure 4.1.1 Curve showing the amount of DNPH reacted at increasing level of formaldehyde.

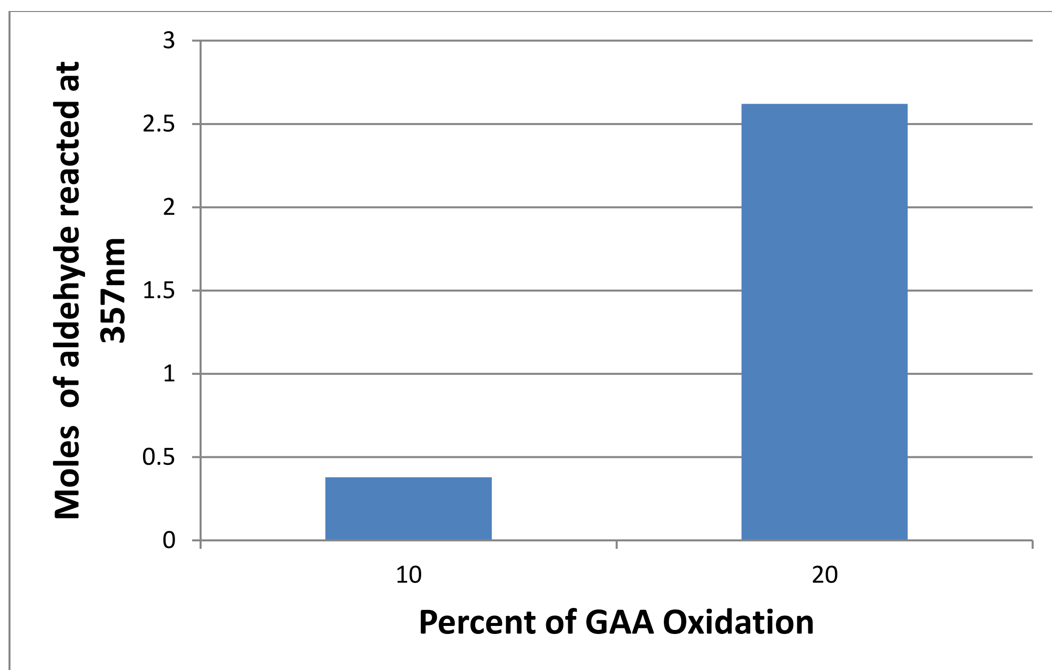


Figure 4.1.2 Curve showing increase in no of moles of aldehyde per gm of gum Arabica.

4.2 Extraction and purification of collagen from rat tail tendons.

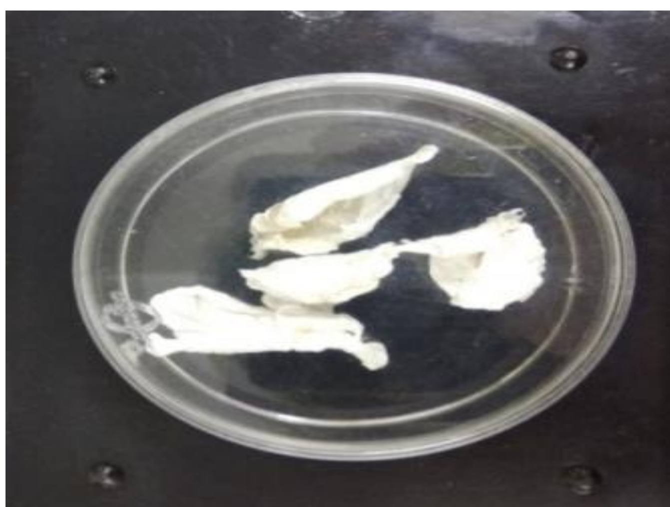


Figure 4.2 .Purified collagen after extraction (Source from Dr. Shashi singh's Lab , CCMB)

To check the purity of collagen SDS gel run was done. Monomeric collagen type 1 consists of alpha 1 and alpha 2 chains. Alpha 1 is around 250 KDa and Alpha 2 is 125KDa.

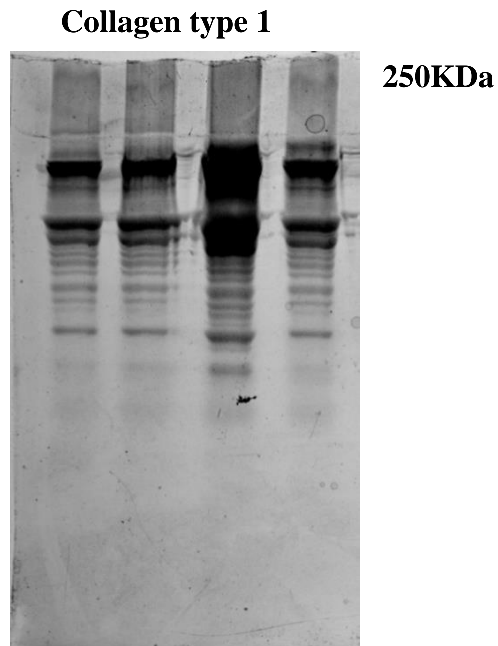


Figure 4.2 SDS PAGE for collagen type 1 extracted from rat tail tendons

Scaffold formation

Freshly oxidized gum Arabica was dissolved to make a solution of 10% in borate buffer. At the same time collagen was dissolved to a concentration of 2% in 0.15 M acetic acid. Both the solutions were mixed at a volumetric ration of 1:1 and vortexed for a minute to allow proper mixing. The gel like solution was poured into culture plates along with the cells. These gels were left for 10 mins to settle down and after 10mins differentiation media were added over it & kept it in incubator.

4.3 MTT Assay

Scaffolds were laid with cells and incubated overnight in culture conditions. The viability of the cell and their penetration in the scaffolds was examined by MTT assay.

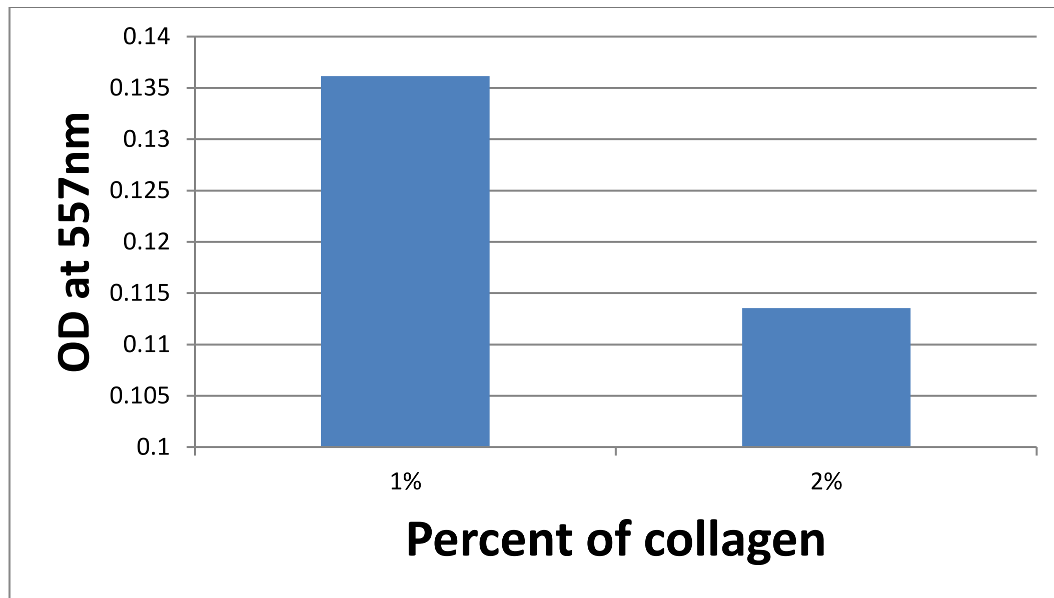


Figure 4.3 Curve representing the number of viable cells in scaffolds

MTT assay for the scaffolds indicated that the number of viable cells present after the differentiation process.

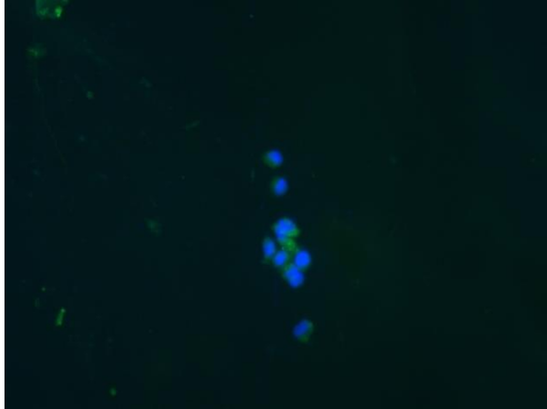
4.4 Differentiation of msc into beta cell in different scaffolds :

From the MTT assay result we got to know that cells were remain viable after the differentiation process.So, while making collagen & gum araabica crosslinking scafflods cells were added into it and incubated in differentiation media.

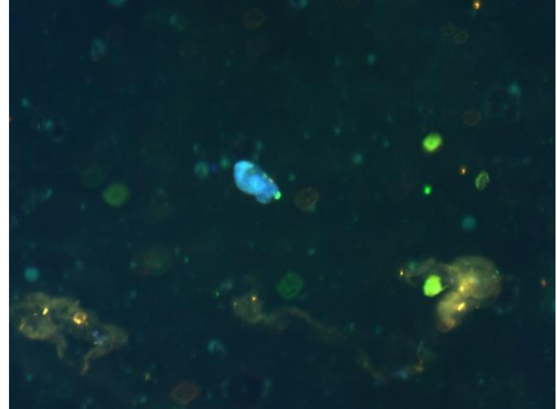
The material impaired the visibility of cells in blocks so we performed immunostatining of the cells in the blocks to examine the differentiation sttus and morphology of islets like structures.

4.5 Immunostaining:

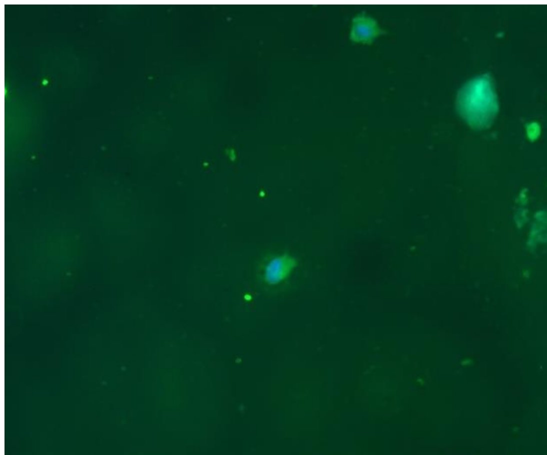
<u>Cells in Scaffolds</u> :



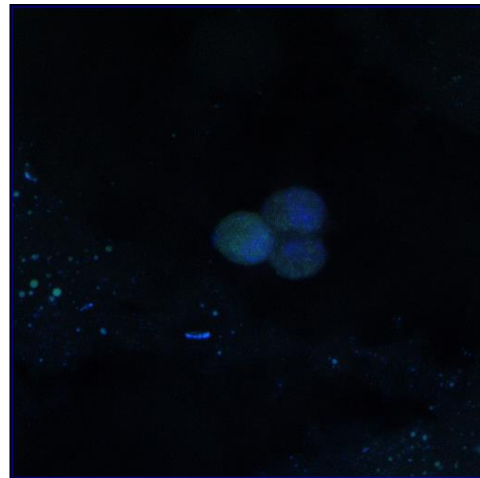
10% GAA oxidation in 1% collagen Concentration (NGN3)



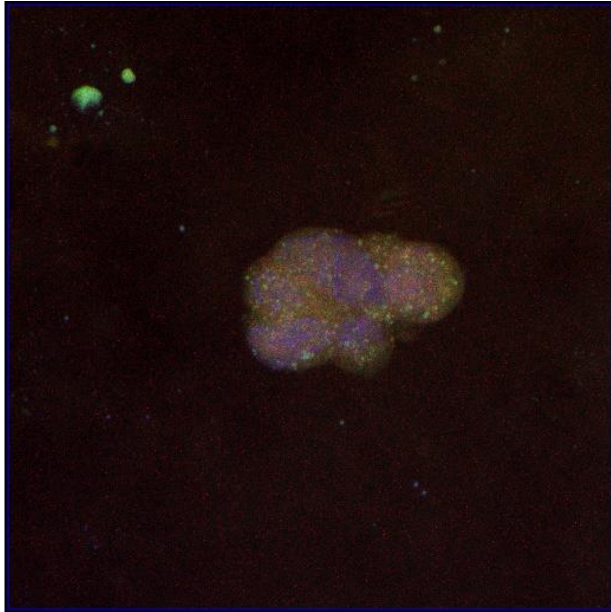
10% GAA oxidation in 2% collagen concentraion (PPy- PDX)



20% GAA oxidation in 2% collagen Concentration (INS)

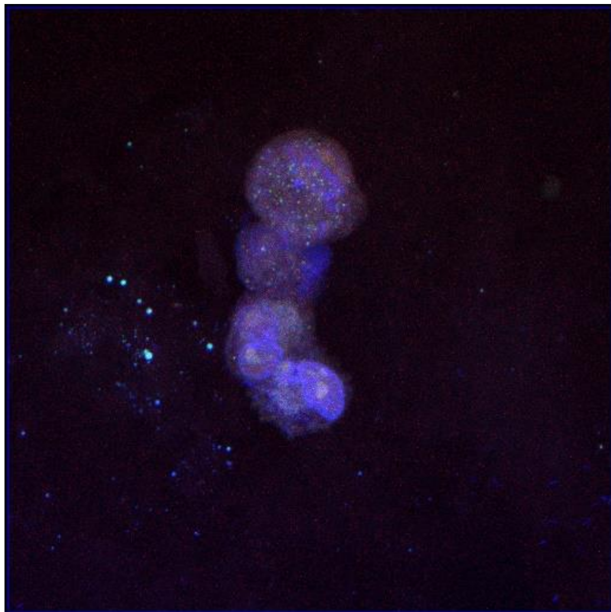


20% GAA oxidation in 1% collagen concentration (PPy – PDX)

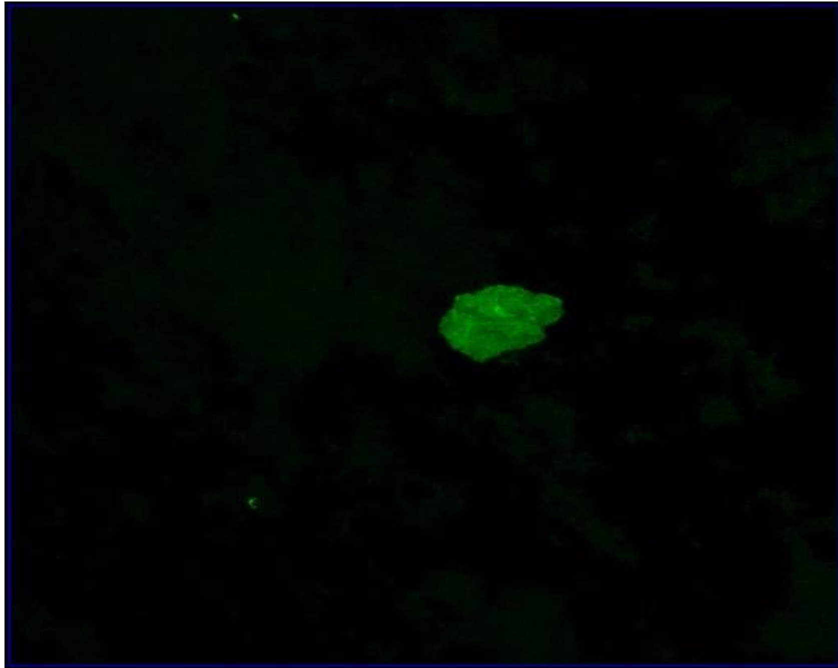


20% GAA oxidation in 1% collagen concentration PPy – PDX1

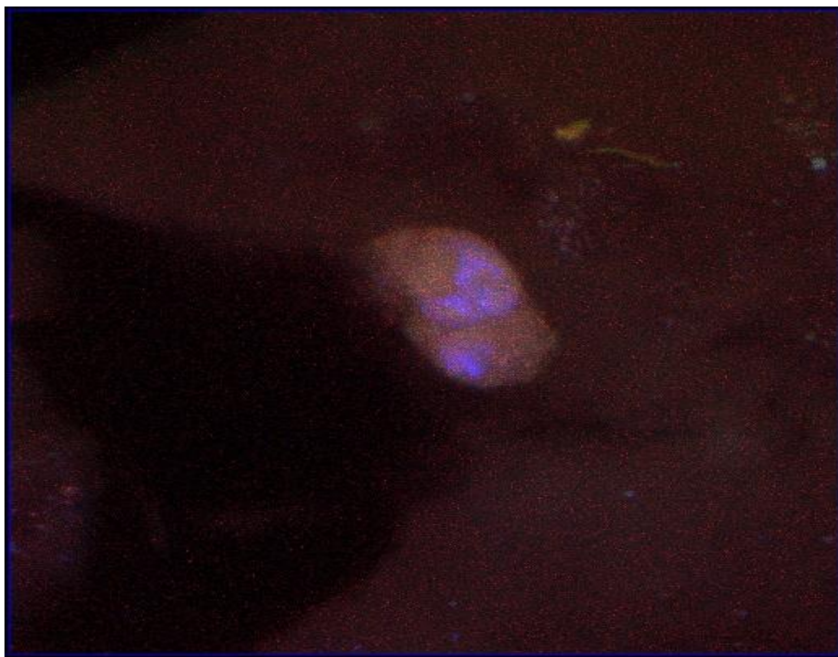
Cells in Matrigel :



Cells treated with PPy – PDX1



Cells treated with PPy – PDX1



Cells trated with PPy – PDX1

Immunostaining of the embedded cells gave the signals for PPy- PDX, INS & NGN3 which confirmed that differentiation of MSCs to Pancreatic beta cells.The green fluorophore was used against NGN & red fluorophore for PPy- PDx.DAPI was used to stain the nucleus.

Disscussion

From the above histochemistry, we get to know that the cells are more viable in scaffolds and surviving in the scaffolds.

Table 1 :

Oxidation	1% collagen	2% collagen
10	+ +	+
20	+ + +	+ +

Table 2 :

Matrigel	+ +
Fibrinogen	-ve, i.e.the fibrin clot in which cells were present further processed for histology.
Extracellular matrix	Processed for Histology for the presence of cells in it.

So, we got an idea from the table that in 20% oxidation and 1% collagen concentration is more realiable for cells to get differentiated & formation of clusters ocuured.

In case of matrigel, cells were also performed differentiation but ralatively less than collagen and gum arabica scafflold.

In case of Fibrinogen, after the formation of fibrin clot the cells into it get solidified also.So, we didn’t get any differentiation of cells in it, so further we do processing it for histology.

In case of ECM, also further processed for histology work.

Chapter 5

Conclusion & Future work

The combination of cells with an engineered 3D matrix is more reliable for monitoring Mesenchymal cell growth and differentiation as compared to 2D cultures as 3D cultures provide good cell to cell and cell to ECM communication. The uses of these scaffolds in 3D cell cultures are pillars for regenerative therapy to outrun Diabetes, as if insulin secreting beta cells get damaged it cannot regain normal functioning of the body. A collagen based scaffold cross linked with Gum Arabic Aldehyde, Matigel, Fibrinogen & a decellularized ECM can be conductive for such cultures and can also be used for the differentiation of Placenta derived Mesenchymal Stem Cells into Pancreatic beta cells.

Future insights of this work can be to optimize to do 3D bioprinting of these crosslinked scaffolds to replace the damaged beta cells in case of a diseased person.

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