OPTIMIZATION AND FUNCTIONAL CHARACTERIZATION OF A HEPATOCYTE ENDOTHELIAL CELL COCULTURE SYSTEM TOWARDS LIVER TISSUE ENGINEERING

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DECLARATION

I, **Anil Kumar PR**, hereby declare that I had personally carried out the work depicted in the thesis entitled **"Optimization and Functional Characterization of a Hepatocyte – Endothelial Cell Coculture System towards Liver Tissue Engineering"** under the direct supervision of **Dr. TV Kumary**, Scientist F, Division of Implant Biology, Biomedical Technology wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India, except where external help sought and acknowledged.

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CERTIFICATE

This is to certify that **Mr. Anil Kumar PR**, in the Division of Implant Biology of this institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the PhD degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The work relating to his thesis entitled **"OPTIMIZATION AND FUNCTIONAL CHARACTERIZATION OF A HEPATOCYTE – ENDOTHELIAL CELL COCULTURE SYSTEM TOWARDS LIVER TISSUE ENGINEERING"** was carried out under my direct supervision.

Dr. TV Kumary

To my parents

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Anil Kumar PR

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ABBREVIATIONS

AcOr	Acridine orange
ATR-FTIR	Attenuated Total Internal Reflection – Fourier Transform Infra Red
BAL	Bio Artificial Liver
BEC	Biliary epithelial cell
$CaCl_22H_2O$	Calcium chloride di hydrate
cpm	Counts per minute
Cyt P450	Cytochrome P450
Dil-Ac-LDL	1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethyl-indocarbocyanine perchlorate low density lipoprotein
DMSO	Di Methyl Sulphoxide
DNA	Deoxyribo nucleic acid
DPPIV	Di Peptidyl Peptidase IV (CD26)
EC	Endothelial cells
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
EtBr	Ethidium bromide
FBS	Foetal Bovine Serum
FHF	Fulminant Hepatic Failure
FVIII	Factor VIII (vWF)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte Growth Factor
KCl	Potassium Chloride
LAD	Liver Assisted Device
LCST	Lower Critical Solution Temperature
LSCM	Laser Scanning Confocal Microscope
mRNA	Messenger ribo nucleic acid
NaCl	Sodium chloride

NaOH	Sodium hydroxide
NO	Nitric oxide
NPC	Non parenchymal cells
PBS	Phosphate Buffered Saline
PC	Parenchymal cells
PFA	Paraformaldehyde
PIPAAm	Poly (N-Isopropyl acrylamide)
PVDF	Polyvinylidene fluoride
REC	Reticulo endothelial cell
SEC	Sinusoidal endothelial cell
SEM	Scanning Electron Microscope
TCPS	Tissue culture grade polystyrene
vWF	von Willibrand Factor (F-VIII)

SYNOPSIS

Liver diseases are increasing year by year all over the world including India. The currently available therapy for end stage liver failure is liver transplantation. Over the years survival of patients after transplantation has improved. Since there is an ever-increasing shortage of liver donors, investigations are going on to find an alternative to whole organ transplantation. Liver tissue engineering is one of the emerging approaches to address the problem.

Chapter 1 introduces the topic and chapter 2 reviews the literature on the topic. The four main currently available alternatives for end stage liver failure are – isolated cell transplantation, tissue engineering of implantable constructs, transgenic xenotransplantation and extracorporeal bio artificial liver devices. Cells in the liver can be broadly classified as parenchymal cells i.e. hepatocytes and non-parenchymal cells (NPC). The later includes endothelial cells (EC), Kupffer cells, ito cells, stellate cells and biliary epithelial cells. Primary hepatocytes are preferred for in vitro studies and clinical use. Although the majority of liver functions are through hepatocytes, significant independent roles are executed by the NPCs as well. The ability of hepatocytes to perform the multiple synthetic, metabolic and detoxifying functions of the liver is achieved by its interaction with the NPCs. However, under in vitro condition hepatocytes lose functional ability within 3–4 days mainly due to absence of other cells in liver which modulate function of in vitro cultured hepatocytes. Reports on hepatocyte coculture with pooled NPCs as well as individual NPC like stellate cells, Kupffer cells and biliary epithelial cells show the influence of cell-cell interactions in hepatocyte function. Since hepatocytes are closely positioned with SECs and utilize soluble factors of blood plasma through SEC layer the later is an attractive NPC type for coculture. To standardize hepatocytes and endothelial cell coculture system, rat hepatocytes were cocultured with HUVEC. Earlier reports on coculture have utilized either NPCs or uncharacterized SEC for coculture. In this study hepatocytes were cocultured with purified and characterized hepatic SEC for coculture. There are studies showing the feasibility of using hepatocyte spheroids for in vitro hepatotoxicity studies and as a cell component in bioartificial liver module. In such cases the culture environment is modified with non-adherent surface or growth stimulants to induce spheroid formation. In contrary to this, the present coculture system uses basic culture requirement for adherent cells which has the advantage of self organization of hepatocytes and tissue organization with EC. The disruption of cell-cell and cell-ECM interactions while retrieval by enzymatic or mechanical methods can be avoided by intact recovery of cell/cell sheets. Use of thermoresponsive surface facilitates intact cell sheet recovery and construction of tissue structures. In this work, a temperature responsive surface was synthesized by grafting poly(N-isopropyl acrylamide) on tissue culture plates.

Chapter 3 describes about the materials and methods adopted for carrying out the study. Whole experimental approaches are classified into various subheadings. The method of synthesis of thermoresponsive culture surface for cell retrieval and its characteristics are explained in detail. Cell retrieval by various methods is proposed that can be adopted for tissue engineering applications. Optimized cell isolation techniques for rat hepatocytes, rat liver sinusoidal endothelial cells and human umbilical vein endothelial cells are explained in detail under separate sections. The chapter also deals with the hepatocyte and endothelial cell interaction studies. Two methods adopted for the cell-cell interaction studies are coculture of hepatocytes with EC and use of EC conditioned medium. Hepatocytes were monitored for improvement both structurally and functionally. Morphological estimation was done by observing the spontaneous spheroid formation by hepatocytes in control and coculture. Physiological estimation of hepatocytes function was assessed by determining selected representatives of hepatocytes functions like protein synthesis, detoxification and metabolic ability. The cultures were maintained for 21 days to see the functional ability of hepatocytes in EC conditioned medium when compared to normal culture medium. The last portion of the chapter explains the experimental methods for hepatocyte retrieval for liver tissue engineering.

Chapter 4 gives all the results and the discussions of the various experiments and data. In-house synthesized thermoresponsive polymer was used for culturing various cell lines like L-929 (mouse subcutaneous connective tissue fibroblasts), NRK-49F (Normal rat kidney fibroblasts), SIRC (Rabbit corneal epithelial cells) and HOS (Human osteosarcoma) for its efficacy of cell adhesion and retrieval. The surface showed similar efficiency in generating tissue construct as well as non invasive method of cell sheet transfer compared to previously reported data. Cell sheet transfer with membrane support, without membrane support and synthesis of tissue construct with multi layer cell sheets are shown. The in vitro tissue construct were analyzed by various techniques like phase contrast, confocal and scanning electron microscopy (SEM). Analysis of tissue construct generated by using thermoresponsive polymer clearly evidenced the intact tissue like architecture with cell-cell contact proving it as a suitable substrate for cell sheet formation. The main three sections of chapter 4 present the standardization of hepatocytes and EC culture. Hepatocyte isolation methods were modified by using custom made perfusion apparatus to obtain hepatocytes yield with more than 85 % viability. An optimized micro environment for hepatocytes, HUVEC and SEC culture was standardized. The traditional problems like loss of function and viability of hepatocyte following enzyme treatment has been taken into account by optimized isolation technique and microenvironment. Hepatocytes were able to maintain in a basal culture condition without additional growth supplement. Similarly, HUVEC and SEC isolation was standardized and the microenvironment was optimized. Culture environments were provided for maintaining cells individually as well as in coculture. Isolated cells were characterized structurally and functionally before using for coculture. A common microenvironment was selected for maintaining cells independently as well as in coculture. Hepatocytes cultured on collagen surface formed bile canaliculi like structures and spheroids which is an indication towards improvement of functional characters. Two approaches have been employed to coculture hepatocytes with endothelial cells. The coculture study carried out in rat hepatocytes with SEC and HUVEC showed differentiated characteristics and function of hepatocytes at an early stage of 24 h. In contrast to the cocultures previously reported, this study used method of coculturing hepatocytes on monolayer of characterized SEC and HUVEC. Structural and functional characteristics were monitored in the coculture system, which showed improved hepatocyte differentiated characteristics. Morphological examination of coculture revealed spheroid formation which is considered as an indicator of improved hepatocyte function. Hepatocytes and EC reorganized into tissue like structures by forming heterospheroids. Spheroid formation of hepatocyte and endothelial cells has not been reported previously. Endothelial cells were found on the surface as well as inside the spheroids indicating the tissue like architecture. The involvement of both cell types in the heterospheroid formation shows the influence of these cell types on one another. The extra cellular matrix deposition in control and coculture with SEC and HUVEC compared by observing under SEM showed more ECM deposition on coculture with SEC than in control and HUVEC. This also shows the influence of one particular type of cell to hepatocyte on its in vitro functional ability. The coculture of hepatocytes has been previously reported with NPC as well as with many other extra hepatic cells. With a difference to previous reports this study standardized the coculture technique with hepatocyte and extra hepatic endothelial cells, HUVEC, and then used hepatic SEC for the coculture system. Hepatocytes were also cultured in SEC and HUVEC conditioned medium. Functional estimation of hepatocytes was assessed by albumin synthesis, urea synthesis and its ability in biotransformation of the substrate ethoxyresorufin by cytochrome P450 enzyme isoform. Functional assessment of hepatocytes revealed a steady level of hepatocyte function (urea and albumin) over a period of 21days. The results with EC conditioned medium for hepatocytes culture showed a steady functional ability of hepatocytes for 21 days in coculture where as the control showed variations. This showed the maintenance of hepatocyte function without loss after isolation. The hepatocytes expressing metabolic activity was found to be more in EC conditioned medium compared to control. Polarity assessment in EC conditioned medium revealed the protein expression pattern of hepatocytes in spheroid formation. A redistribution pattern of apical and basolateral proteins were observed in hepatocytes in conditioned medium, which has not been reported earlier. This information is substantial in studying the cell interaction study of hepatocytes with other cells. Cyt p450 activity staining showed more number of metabolically active cells in EC conditioned medium than in control. Hepatocytes on thermoresponsive polymer can form spheroid as normal and these spheroids are readily detached from the surface by lowering temperature. The retrieved hepatocytes were found to be functionally active in Cyt p450 staining after retrieval by temperature variation without using enzymes. Heterospheroid formed with HUVEC and SEC can be a good model for hepatotoxicity studies and as an alternate cell source for BAL.

Chapter 5 gives the summary and conclusions of whole work; Hepatocytes and EC formed tissue structures by forming heterospheroids which is supposed to be a spontaneous action of hepatocytes to enhance its differentiated functions. Functional ability like protein synthesis, detoxification and metabolic activity of hepatocytes was found to be maintained over 21 days when cultured in EC conditioned medium. Hepatocyte and endothelial coculture can be a general model to study spontaneous cellular arrangement towards multicellular structures with out supplementation of additional stimulants. Hepatocytes on thermoresponsive surface were retrieved without losing functional ability and cell architecture. The combined use of coculture and thermoresponsive surface will definitely maintain hepatocyte function under in vitro condition for a prolonged time. Spheroid formation involving the EC and presence of functionally active EC on surface as well as inside of heterospheroid, finds its utility as a cell culture model for epithelial–mesenchymal cell interaction studies and as a cell source in conventional bioartificial liver system.

Chapter 1

INTRODUCTION

1.1. LIVER DISEASES

Liver diseases are increasing year by year all over the world including India. With the onset of liver disease, the major cell type in the liver, the hepatocytes, gets injured or dead. Fulminent Hepatic Failure (FHF) has an abrupt onset and progresses over a relatively short time course. It has been defined as liver failure resulting in hepatic coma within 8 weeks after onset. This syndrome is marked by massive hepatocellular necrosis and rapid degradation of all liver-specific functions. FHF results in jaundice, coagulation disorders, hyperammonemia, disturbances in acid-base and fluid and electrolyte metabolism, deranged cardiovascular and respiratory function, and hepatic encephalopathy. Hepatic encephalopathy and increasing intracranial pressure are often the proximate cause of death. Chronic liver failure has a much more long time course of progression of disease and it has a different pathophysiology. If the injury is mild and reversible, the cells may regenerate and the patient may be left with an entirely normal liver. This remarkable capacity of the liver to regenerate is a unique feature of the organ. When the injury is more severe or sustained, regeneration may be incomplete or healing may occur with the development of fibrosis or scars leading to total liver failure. Once progressed to end stage disease, liver transplantation appears to be the only satisfactory procedure for saving the patient Tzanakakis *et al*, 2001).

1.1.1. Global status

World-wide, liver disease is a serious health problem. About 350 million people suffer from liver disease every year and at least 40% of them develop terminal illness.

Diseases of the liver are the third most common cause of death in Americans during their most productive years and the seventh most frequent disease-related cause of death in the United States overall. Liver disease accounts for up to $8 billion yearly in economic losses. (Underhill *et al*, 2006).

1.1.2. Indian status

In India the exact statistics for liver diseases is not currently available. However studies from different parts of the country shows that the main reason for liver disease is viral infection. Analysis done by Mall *et al* (2001) in 1612 subjects from different parts of India showed that almost 50% of children below 5 years of age are at risk for hepatitis A. A report by Bhowmick *et al* (2005) showed that the relative contribution of hepatitis A to acute viral hepatitis in children .has increased to over 80 % in 1994-1997 as compared to 51% in 1978-81. This retrospective analysis of data showed that 55 children below 15 years were diagnosed as having liver failure with mean age of 5.5 years. Of these, 22 (40%) hepatic encephalopathy, 12 chronic liver disease (22%), 11 acute liver failure (20%), 4 Fulminant hepatitis (7%) and 4 neonatal hepatitis (7%) were recorded. Hepatitis B viral infection is also proved to be involved in the progression of acute liver diseases in India (Kumar *et al*, 2005).

1.1.3. Treatment

The currently available therapy for liver failure at its end stage is transplantation. Over the last three decades liver transplantation has developed as a major advancement and therapy of choice for end stage liver disease in adults and children. As a result of technical advancement and better immunosuppression, liver transplantation has become a life saving procedure for patients with acute liver failure, end stage liver disease and some of the metabolic defects. Over the years survival of patients after transplantation has improved. Since then there is an ever increasing shortage of liver donors and investigations are going on to find an alternative to whole organ transplantation.

Liver tissue engineering is one of the emerging approaches to address this problem. Owing to the shortage of donor organs, end stage liver patients are good candidates for Bio-Artificial Liver (BAL) treatment, which may allow spontaneous recovery or at least survival until transplantation is possible. It is believed that a patient with an acute progression of chronic liver failure could be supported with a BAL device until liver transplantation or until the patient's own liver is able to recover enough function to allow survival.

1.2. TISSUE ENGINEERING

Tissue engineering was defined in 1988 by National Science Foundation as "The application of the principles and methods of engineering and life science toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissue and the development of biological substitutes to restore, maintain or improve functions".

Tissue engineering is the process of creating functional 3D tissues using cells combined with scaffolds or devices that facilitate cell growth, organization and differentiation. Tissue engineering approaches are poised to transform the way we study human tissue physiology and pathophysiology *in vitro*.

Tissue engineering research to restore, maintain and replace liver function is going on from past one decade. Many technical and biological reasons due to the peculiar characteristic of hepatocytes *in vitro* still keep this area under continuous research. For liver tissue engineering, important components required other than hepatocytes are selection of appropriate cell source, scaffolds, Extra Cellular Matrix (ECM), microenvironment, tissue specific functions and tissue organization.

Liver tissue engineering can be broadly sectioned under two headings – one is the *in vitro* stage of developing tissue like structures and the second is the use of these *in vivo*. The former has also got another use in using it as *in vitro* tissue equivalent for toxicity studies. The later is generally adopted by growing cells on appropriate substrate (ECM or Scaffold) in a defined medium to maintain hepatocyte differentiated functions. The *in vivo* application of tissue engineered liver construct is under active research with main implication coming to BAL or Liver Assisted Devices (LAD).

It is essential to find an appropriate environment for *in vitro* maintenance of hepatocytes. The methods under research focuses on 1) Use of defined culture medium, 2) Use of specific ECM, 3) Coculture – Heterotypic and conditioned medium, 4) *In vitro* tissue formation and most importantly 5) selection of appropriate cell source for the application.

Thus considering the *in vitro* and *in vivo* application of tissue engineering, liver tissue engineering procedures can be useful in developing *in vitro* tissue equivalents for cell transplantation, *in vitro* toxicity system or as a cell component in BAL.

1.2.1. Cell sourcing

The cell source is the vital component of any tissue engineering disciplines and has to be selected based on the end use application. Various cells have been studied for using in BAL (Allen *et al*, 2001).

For liver tissue engineering primary human cells would be ideal, but like whole organs, they are in limited supply. However studies with adult hepatocytes for BAL application and cell transplantation shows that it is very static in culture without proliferation. The growth limitations of primary cells have encouraged developing cell lines that can proliferate in culture while maintaining liver specific functions.

The regenerative capacity of liver is due to the presence of progenitor cells it harbors. Hence epithelial progenitor/stem cells from the fetal human liver has been isolated and studied for its clonogenic capacity and hepatocyte specific gene expression. Malhi *et al* (2002) demonstrated that under culture conditions progenitor cells proliferated for several months and differentiated into mature hepatocytes in immuno compromised mice.

It can be seen from the literature that primary hepatocytes are suitable cell source and foetal cells has a promising impact in liver tissue engineering.

1.2.2. Microenvironment

The microenvironment covers the ECM, medium and the appropriate physical environment like static or dynamic. Since hepatocyte receives stimuli from surrounding cells for the hepatospecific functions, studies have been done to supply the soluble growth stimulating substances in the medium. It has also been proved beyond doubt that hepatocyte receives autocrine and paracrine stimuli for expressing function. Hence coculture with other cell types (heterotypic) and conditioned medium from other cell types are used for maintaining hepatocyte functions.

1.2.3. *In vitro* tissue formation using thermoresponsive surface

Tissue formation *in vitro* is a distinguishing behavior of cultured hepatocytes on providing correct microenvironment. It is characterized by the formation of tissue specific structural features like bile canaliculi formation, tissue like organization and organoid formation. These organized structures are known as spheroids and can be achieved in simple as well as in coculture. While organoid formation, hepatocytes exhibit specific molecules on plasma membrane which are found on *in vivo* hepatocytes (apical and basolateral proteins). However tissue formation has to be stimulated *in vitro* by growth factor supply, non adhesive surface or using bioreactors.

The only non invasive method to create *in vitro* tissue structures is the use of stimuli responsive culture surface. The temperature-induced phase- separation experienced by some aqueous polymer solutions provides an excellent model to study the molecular interactions at play in thermoreversible polymers. Aqueous solutions of poly(N-isopropylacrylamide) (PIPAAm) exhibit this reverse temperature phase behavior. The solubility of the polymer in cold water can be attributed to its ability to form hydrogen bonds with water via the amide groups while inducing considerable ordering of the solvent through the apolar isopropyl substituents (Winnik *et al*, 1993). The polymer exhibits a characteristic reversible phase transition from hydrophobic to hydrophilic at a temperature of 32 °C which is known as Lower Critical Solution Temperature or LCST.

Using this property of the PIPAAm Yamada *et al* (1990) reported the development of novel culture surface by covalently grafting PIPAAm on culture surface by specific chemical immobilization reactions. The surface was responding to temperature to detach cells as monolayers. Later on the use of PIPAAm grafted surface was found to be useful for different type of cells for cell sheet engineering (von Recum *et al*, 1998).

Such Temperature responsive surfaces are also used for coculture as well as spheroid formation. Hirose *et al* (2000) reported the use of patterned cell surface using PIPAAm to create double layerd coculture system of hepatocytes and endothelial cells. Takezwa *et al* (1993) showed that fibroblast cells cultured on substratum composed of PIPAAm and collagen can be forceully detached as cell sheets. Tissue sheets when transferred to nonadherent surface formed spheroids with gap and tight junctions.

To engineer liver tissue it is important to understand the basic structure and biology of liver.

1.3. LIVER AS AN ORGAN

The liver is an extremely powerful organ that performs detoxification of toxic substances, synthesis of a range of plasma proteins such as albumin.

The liver is the most complex and metabolically active organ in the body which performs more than 500 vital functions. It is also the largest gland in the body situated in the upper and right parts of the abdominal cavity. The human liver has appearance of a wedge, the base of which is directed to the right and the thin edge toward the left. In the male it weighs from 1.4 - 1.6 kg, in the female from 1.2 - 1.4 kg (Williams).

1.4. LIVER STRUCTURE AND FUNCTION

The human liver possesses three surfaces, viz., superior, inferior and posterior. It is divided into four distinct lobes like left, right, quadrate and caudate by ligaments and fissures. The superior surface is attached to the diaphragm and anterior abdominal wall by a falciform fold of peritoneum, the falciform ligament. The line of attachment of the falciform ligament divides the liver into two parts, termed the right and left lobes. The inferior and posterior surfaces are divided into four lobes by five fossæ, which are arranged in the form of the letter H. The bar connecting the two limbs of the H is the porta (transverse fissure) in front of it is the quadrate lobe, behind it the caudate lobe.

The blood which circulates through the spleen, pancreas, stomach, small intestine, and the greater part of the large intestine is conveyed to the liver by the portal vein. The smallest radicals of these vessels inside parenchyma are distributed to channels called portal canal. Numerous nerves, derived from right and left vagus and the celiac plexus of the sympathetic nerves, accompanies the hepatic artery and portal vein. There will be three portal canals at the periphery of the lobule, about equally distant from each other giving a hexagonal shape. All these structures can be categorized into four systems – The Hepatic system, Biliary system, blood circulatory system and reticulo-endothelial system. The basic structure of liver in different species is almost same with minor differences. For example, there is no interlobular septum in human liver where as there is a distinct structure between pig liver lobules. Another slight difference between human and rat liver is that the later have five lobes.

The lobes of liver can be further subdivided into lobules that form chief mass of the hepatic substance measuring from 1 to 2.5 mm in diameter. In the humans lobular outline is very irregular whereas in some of the lower animals like pig they are well-defined with polygonal outlines. Each lobule is imperfectly isolated from the surrounding lobules by a thin stratum of areolar tissue which contains a plexus of vessels and ducts.

The lobules form the principal mass of the parenchyma and represent the functional unit of the organ comprising of different type of cells which can be broadly classified as parenchymal cells (PC) and non-parenchymal cells (NPC). While the hepatocytes form the PC, endothelial cells (EC), Kupffer cells, ito cells, stellate cells and biliary epithelial cells constitute the NPC. Although the majority of liver functions are done by hepatocytes, significant independent roles are executed by the NPCs as well (Talamini *et al*, 1998). Inside each lobule, hepatic cells are arranged in irregular radiating columns called hepatic plates (Figure 1).

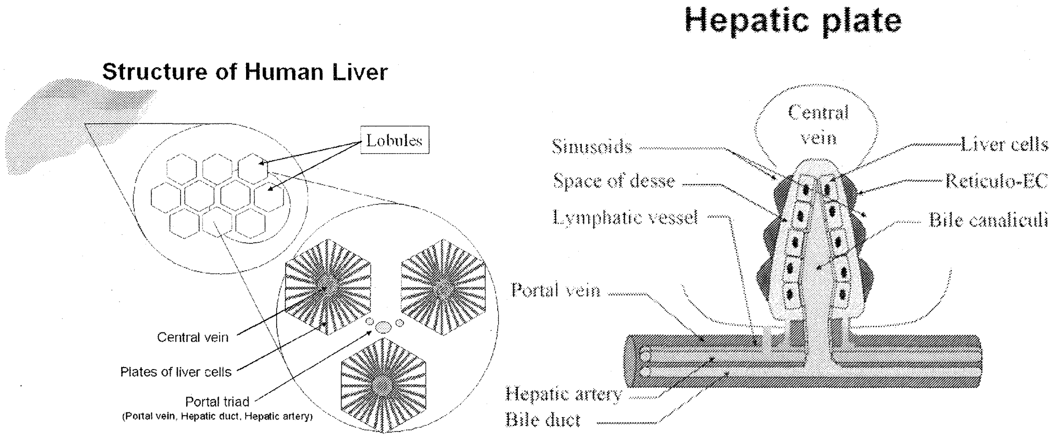

Figure 1 Structure of liver showing lobes, lobules and hepatic plates

There are channels in between the hepatic plates called sinusoids which convey blood from the circumference to the center of lobule. It ends in the intralobular vein, which runs through its center, to open at its base into one of the sublobular veins. Between the cells also there are minute capillaries known as bile canaliculi

The liver receives blood supply from two vessels – the hepatic artery and the portal vein. The hepatic artery ramifies to give nutrition to coats of vessels and ducts. It also gives off capsular branches which give lobular and interlobular branches to supply walls of interlobular veins and accompanying ducts. The lobular branch further enters the lobule and end in the net work of sinusoids between the cells

Thus sinusoids are low pressure irregular vascular channels that receive blood from terminal branches of the hepatic artery and portal vein at the periphery of lobules and deliver it into a vein at center on lobule called the central vein. Sinusoids are lined with endothelial cells and flanked by plates of hepatocytes. The space between sinusoidal endothelium and hepatocytes is called the space of Disse. Sinusoidal endothelial cells are highly fenestrated, which allows virtually unimpeded flow of plasma from sinusoidal blood into the space of Disse.

The blood from the sinusoids is collected to the rootlets of a series of veins called hepatic veins. The hepatic vein carry blood into the inferior venacava and then to heart. The blood in the portal vein carries certain products of digestion: the carbohydrates, which are mostly taken up by the liver cells and stored as glycogen, and the protein products which remain in solution and are carried into the general circulation to the various tissues and organs of the body. The hepatocytes are bathed in nutrient rich plasma derived from the portal vein. The plasma which collects in the space of Disse collects in lymphatic vessels through portal tracts and forms a large fraction of the body's lymph. Another important feature of hepatic sinusoids is that they house an important part of the phagocytic system. Sinusoids are populated by numerous Kupffer cells, a type of resident macrophage.

1.5. LIVER CELLS

1.5.1. Hepatocytes

Hepatocytes are the chief functional cells of the liver and perform an astonishing number of metabolic, endocrine and secretory functions. The hepatocytes are complex and highly differentiated cells of epithelial origin that forms the parenchyma of the organ and contribute to the 60% of cells and 80% of volume of liver. The cells are polyhedral in form and vary in size from 12 - 25 μm diameter with one or sometimes two distinct nuclei. The nucleus exhibits an intranuclear network and one or two refractile nucleoli. The cells usually contain granules; some of which are protoplasmic, while others consist of glycogen, fat, or an iron compound.

1.5.2. Sinusoidal Endothelial Cells

Liver sinusoidal endothelial cells (SEC) constitute the sinusoidal wall, also called endothelial lining. They are unique capillaries which differ from vascular capillaries because of the presence of open pores or fenestrae lacking a diaphragm and a basal lamina underneath the endothelium. The first description and electron microscopic observation of SEC fenestrae was given by Wisse in 1970 as groups of fenestrae arranged in sieve plates. In general, endothelial fenestrae measure 150–175 nm in diameter, occur at a frequency of 2.9–13 per μm , and occupy 6–8% of the endothelial surface (Braet *et al*, 2002). On the basis of morphological and physiological evidence, it was reported that the grouped fenestrae act as a dynamic filter for solutes and particles that are exchanged between the sinusoidal lumen and the space of Disse. Another functional characteristic of SEC is their high endocytotic capacity reflected by the presence of numerous endocytotic vesicles and by the effective uptake of a wide variety of substances from the blood by receptor-mediated endocytosis. In general, SEC can be regarded as a "selective sieve" and as a "scavenger system". This capability of SEC was studied and reported by Nedredal *et al* (2003) using experiments done on liver tissue and cultured SECs. Other functions of SECs are but not limited to Endothelin synthesis (Eakes *et al*, 1998), TGF β 1 (Ikejima *et al*, 2004) and Nitric Oxide (NO) modulation (Shah, 1997).

Chapter 2

REVIEW OF LITERATURE

2.1. HEPATOCYTE CULTURE

Hepatocytes have been studied *in vitro* for more than five decades especially to study the liver metabolism. During earlier stages, *in vitro* cultures were obtained from liver tissue by mechanical methods (Longmuir *et al*, 1956). Since then lot of research was going on with isolated hepatocytes with special emphasis on procedure of isolation.

The introduction of collagenase as a dissociating enzyme has presented a major progress in obtaining viable hepatocytes. In 1969 Berry *et al* (1969) proposed a well standardized technique for isolation of intact parenchymal cells by continuous in situ recirculating perfusion of the rat liver. The perfusion medium contained calcium free Hanks' solution containing 0 .05 % collagenase and 0.10% hyaluronidase and magnesium and calcium-free Hanks solution containing 2 M ethylenediaminetetracetate (EDTA). The procedure yielded conversion of about 50% of the liver into intact, isolated parenchymal cells. Biochemical and morphologic parameters measured in the isolated cells were comparable to normal hepatic parenchymal cells in situ in appearance and function.

Depending upon the requirement of isolated cells for biochemical study the isolation method is also modified accordingly. While many of the metabolic properties of liver *in vivo* can be reproduced with isolated parenchymal cell preparations, some concern about the membrane receptor integrity have been expressed in relation to the exposure of the cells to digestive enzymes for long periods. Berry *et al* (1983) also proposed a method for isolation of hepatocytes in good yield without enzymatic digestion.

Collagenase perfusion has two conflicting requirements (Neufeld) regarding presence of Ca^{2+}. One, the cells must be exposed to very low concentrations of Ca^{2+} to allow cleavage of hepatic desmosomes and the second, collagenase activity requires the presence of Ca^{2+}. To resolve this conflict two different procedures have been employed – one step and two step procedures. One step procedure takes advantage of the fact that the requirement of Ca^{2+} for collagenase activity is substantially less than that required for disruption of the desmososme. The two step procedure utilizes a preperfusion system with Ca^{2+} free medium during which the desmosomes are irreversibly cleaved followed by the addition of Ca^{2+} perfusate.

The advancement in the hepatocyte isolation by perfusion method was initiated by Seglen (Seglen, 1972). In his early reports Seglen proposed that best results are obtained when the liver was first perfused with Ca2+ chelating agent ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), followed by 5 min with enzymes in Ca2+ containing buffer. This sequential treatment converted the whole liver to a cellular suspension, in which 95 % of the cells were intact.

The main challenge of primary hepatocyte culture is its maintenance with desired level of viability and function. The initial attempt towards this was to maintain hepatocytes as monolayer for longer duration. One of the early works in this direction was reported by Bissell *et al* (1973) in 1973 showing the perseverance of differentiated functions of rat hepatocytes isolated from regenerating liver. They showed that non proliferating monolayer preparations of hepatocytes obtained by high density cell seeding, in a medium free of growth components, were able to express functions over a period of seven days.

The culture of primary rat hepatocytes in well defined serum-free media offers the opportunity to examine specific growth supplements in hepatocyte function and Deoxyribo Nucleic Acid (DNA) synthesis. The direct effects of glucose, insulin, dexamethasone, and triiodothyronine studied by Kurtz *et al* (1981) showed that effects of insulin and dexamethasone on the induction of Glucose-6-Phosphate Dehydrogenase enzyme were dose dependent and independent of DNA synthesis.

Current evidence suggests that extracellular signals play an important role in the preservation of the differentiated liver-specific functions of hepatocytes. Exogenous soluble factors comprising of hormones, growth factors, nutrients and trace elements were reported to have a beneficial effect on hepatocyte survival and function *in vitro*. In addition, other factors that favor hepatocyte survival and function *in vitro* are drug inducers such as sodium phenobarbital, isonicotinamide and a dipolar solvent dimethylsulphoxide (DMSO). Except for DMSO no soluble factor has been found to maintain well differentiated hepatocytes in pure culture beyond a few days. Guguen-Guillouzo *et al* (1990) reported that although not sufficient, several soluble factors are essential for improving cellular functions.

Primary culture of rat hepatocytes under conditions defined by the incubation medium of the culture affords the opportunity to study liver enzyme regulation. Studies with exogenous growth factor supplementation have made insight into the role of insulin, cyclic Guanidine Mono Phosphate (cGMP) and carbohydrate in the regulation of liver specific enzymes. The proliferative activity of rat hepatocytes growing on a collagen substratum cultured in presence of EGF, dexamethazone and insulin suggest that hepatocytes undergo binucleation in culture to a similar extent as during normal *in vivo* developmental growth. Both mononuclear and bi nuclear cells proliferate and like *in vivo*, the proliferative ability appears to be negatively correlated with ploidy (Mossin *et al*, 1994).

The low proliferation nature of hepatocytes in culture has brought lot of interest in improving microenvironment for culture. Block *et al* (1996) reported clonal expansion of adult rat hepatocytes in chemically defined medium under the influence of Hepatocyte Growth Factor (Scatter Factor) (HGF/SF), EGF, and Transforming Growth Factor α (TGF-α.) over a period of 28 days. The expanding populations of hepatocytes lose expression of hepatocyte specific genes [albumin, cytochrome P450IIB1 (cyt P450)] and acquired expression of markers expressed by bile duct epithelium. After population expansion and clonal growth, the proliferating hepatocytes could restore mature hepatocyte phenotype and function within 8 days on supplementation of appropriate ECM.

2.2. HEPATOCYTE FUNCTIONS

Although many investigators subsequently reported modifications, it is Seglen's method after series of meticulous studies has become the standard protocol till now (Strain, 1994). The technique involves perfusing the liver with a divalent cation free buffer, effectively loosening the desmosome and hemidesmosome junctions between the adjacent cells and ECM. This is followed by proteolytic digestion of the matrix with collagenase and careful maintenance of the temperature, pH and oxygenation. This procedure results in complete disaggregation of the parenchymal tissue without the mechanical disruption to get hepatocytes with consistently high viability and purity.

Primary hepatocytes in culture have been used to study liver physiology and pathophysiology for many decades. Isolated and cultured hepatocytes *in vitro* are important tool for the analysis of drug metabolism at the cellular level and hepatic disease study. The culture system has been extensively used in basic research of liver disease, pathophysiology, pharmacology and other related subjects. Since normal culture condition of hepatocytes differs greatly from the environment *in vivo*, it is difficult to maintain the physiological function of hepatocytes *in vitro* (Reid *et al*, 1984).

There are two serious disadvantages to the use of isolated, and particularly of cultured hepatocytes. First drawback refers directly to xenobiotic metabolism and second is general in nature (Skett, 1994). In terms of xenobiotic metabolism, one fact that limits the use of isolated and cultured hepatocytes for any extended period is the rapid loss of xenobiotic-metabolizing enzymes. This has been explained not as a general loss of enzyme activity but rather appears to be a "dedifferentiation" of the liver cells with a reversion to foetal-type metabolism. The usage of a wide variety of culture media for hepatocytes by different groups leaves a difficulty that results are not comparable between laboratories. Some groups tried a good medium for their particular assay whereas many others do not indicate the criteria for choosing their medium. Hence there is no common answer for the dedifferentiation of hepatoyctes in culture.

2.2.1. Role of culture medium and growth supplements

One of the first priorities for the European Center for the Validation of Alternative Methods (ECVAM) is to familiarize the current status of non-animal test development and validation. First meeting of ECVAM Angera, Italy in 1993, discussed about the practical applicability of hepatocyte cultures in routine testing and recommended use of isolated hepatocytes as *in vitro* system. A survey of the literature on metabolically competent long term culture models was performed with an ultimate goal to provide a proposal for industry and academia with a metabolically competent *in vitro* alternative for long term studies (Coecke *et al*, 1999). Coecke *et al* summarized the advantages and disadvantages of media components and soluble factors for *in vitro* maintenance of hepatocytes as given in the Table 1.

Table 1 Advantages and disadvantages in using media components and soluble factors for *in vitro* maintenance of hepatocytes

Media component	Advantage	Disadvantage
Serum	Cell attachment, survival and morphology	Dedifferentiation, inhibition of cyt P450 activity
Dexamethasone	Cell attachment, survival, tissue specific functions	Glutathione-S-Transferase (GST) isoenzyme expression
Insulin	Cell survival, attachment, aminoacid transport, stimulates Gprotein activity and DNA synthesis	
Glucagon	Acts on various hepatic enzyme activities, synthesis of urea and gluconeogenesis	Disrupts protein structure, and synthesis
Growth hormone	Regulates a class of Cyt P450 enzymes (CYP2C12, CYP2C11, CYP2A2)	Suppresses a group of cyt p450 enzymes (CYP3A, CYP4A)
EGF & HGF	Stimulate DNA synthesis, stimulate G1 to S phase progression	Suppresses Cyt P450 activity
Cytokines	IL-6 induces expression of fibronectin, stimulate G1 to S phase progression	A wide variety of interleukins and TNF inhibit Cyt p450 enzymes
Pyruvate	Prolongs survival and increases albumin secretion, urea synthesis and Adenosine Tri Phosphate (ATP) levels	Expresses fetal GST isoenzyme

Different basic media have been used with isolated hepatocytes but little comparative work has been published. Grant *et al* (1985) studied a range of media like William's E, Waymouth's, Dulbecco's, Eagle's and Ham F12 media supplemented with 5% foetal calf serum to find cyt P450 ability of hepatocytes in culture. Among the others William's E medium found to be good at maintaining some liver specific functions. But most groups have modified media for their own specific assay - for example Paine's P-450 medium (Padgham *et al*, 1992), modified Earle's medium, modified Leibovitz L-15

(Mitaka *et al*, 1991) and Chee's medium (Jauregui *et al*, 1986). Using hormonally defined medium with appropriate ECM is another option to partially overcome rapid dedifferentiation and maintenance of hepatocyte function (Waxman *et al*, 1990).

Culture medium has also got an important role while cryopreserving hepatocytes as reported by Calligaris *et al* (2002). It has been shown that the composition of the rewarming medium is an important factor in the maintenance of viability and ammonium detoxification ability of cryopreserved hepatocytes for cellular applications.

Since the hepatocytes removed from animal decreases transcription ability of liver specific genes in culture (Guguen-Guillouzo *et al*, 1983), attempts has been made to immortalize cells. At present different hepatocytes cell lines are available expressing specific functions. Main effort in genetic modification of hepatocytes is to maintain the differentiated function and long term *in vitro* passage. Kim *et al* (2000) developed a cell line that can be cultured with improved liver-specific differentiation and less oncogenicity. It is also advised that eventhough such improved function and proliferation of hepatocyte is expressed in earlier stages of culture, continuous passaging can make the dedifferentiation in cell lines.

Pain has reviewed (Pain, 1990) the treatments affecting the loss of Cytochrome P-450 in rat hepatocyte culture and explained the need for formulation of special culture medium to the study of drug metabolism and toxicity. William's E medium supplemented with serum and specific components were used to study the effect of culture systems on the expression of the multiple forms of Cyt P450 over a maximum of seven days.

2.2.2. Role of extra cellular matrix

Almost all the studies that are done to look at the functional aspect of hepatocytes *in vitro* were from hours to 2 weeks (Bissel *et al*, 1973). To maintain functional ability of isolated hepatocytes different approaches other than medium components are analyzed. It is now known that two factors that has role in improving hepatocyte function other than soluble factors are ECM components and cell-cell interactions (Guillouzo *et al*, 1990). Over the past two decades there has been lot of research going on to make use of these two factors for long term culture of functional hepatocytes.

Presence of ECM components in hepatic sinusoids as well as the ability of hepatocytes to secrete several collagen types and non-collagenous glycoproteins in culture has already been studied. This explains the influence of various matrix components in hepatocyte culture. To increase the longevity and function of hepatocytes in culture, various ECM components were supplied in different methods as coating (collagen, fibronectin or a mixture of components extracted from rat liver) on plastic dishes, floating collagen gels and collagen embedded in nylon meshes (Guillouzo *et al*, 1990).

2.2.2.1. Monolayer culture on ECM

The supply of ECM layer in general favors phenotypic changes in cells by promoting cell spreading and alterations in liver gene expression. All the data favor the conclusion that maintenance of hepatocellular functions requires the preservation of the globular cell shape (Guguen-Guillouzo *et al*, 1982).

2.2.2.2. Three dimensional (3D) culture in ECM

At present, research on 3D cell systems is more vital and productive than ever. One major advantage of 3-D cell cultures is their well-defined geometry, which makes it possible to directly relate structure to function and which enables theoretical analyses. Combining spatial resolution of 3D cultures with molecular analysis has clearly demonstrated that 3D cultures resemble more closely to *in vivo* situation with regard to cell shape and environment (Mueller-Klieser, 1997). Such monotypic 3D culture models described effectively approximate biochemical and spatial contributions required for tissue-specific function.

To preserve the phenotype of hepatocytes to improve its function, Dunn *et al.* (1989) cultured hepatocytes between two layers of type I collagen. In such a sandwich configuration, hepatocytes maintained a high albumin secretion rate for more than 40 days.

Excretory function of hepatocytes is also important and studies on that aspect is also undergoing. Liu *et al* (1999) demonstrated bile secretion by primary hepatocytes in collagen gel sandwich culture over a period of 5 days. This expression is due to the formation of extensive bile canalicular network by the hepatocyte in collagen sandwich.

LeCluyse *et al* (1994) demonstrated uniform formation of a contiguous anastomosing network of bile canaliculi throughout the 5 days culture with the development of the bile.

The method of formation of ECM is also given notice while preferring sandwich culture. Wang *et al* (2004) used a collagen gel mixture to immobilize hepatocytes after seeding. By such a method rat hepatocytes could uniformly mix in collagen gel, showing high density and a three dimensional structural characteristic. Over 9 days culture hepatocytes showed viability and urea synthesis ability. To preserve the morphology and to maintain metabolic function, hepatocytes have been cultured as aggregates inside gelatin microcarriers (Tao *et al*, 2003). Hepatocytes could maintain urea and albumin synthesis for 7 days in microcarrier culture.

2.2.3. Role of cell-cell interaction: Coculture

While analyzing the factors to improve hepatocyte functions it is necessary to consider the *in vivo* condition of hepatocyte from which it has been isolated for *in vitro* purpose. In liver, hepatocytes form cords of cells communicated by gap junctions that are closely surrounded by different sinusoidal cells and have direct contacts with biliary epithelial cells. Thus it should be noticed that both homotypic and heterotypic interactions can influence hepatocellular functions. There are evidences showing the role of NPC in the liver detoxicification function. Steinberg *et al* (1987) demonstrated that oxidative and postoxidative drug metabolizing enzymes are not restricted to parenchymal cells alone and similar but distinguishable complements of these enzymes are also found in Kupffer and endothelial cells. Hepatocytes can in turn influence other cell types like SEC (Krausal *et al*, 2000).

Bringing up the cell interaction characteristics of hepatocytes *in vitro* can be achieved by considering three cellular variables: homotypic hepatocyte interactions (the effects of hepatocytes on one another), the heterotypic interface (the amount of contact between cell populations) and the homotypic interactions of the non hepatocyte population (which may produce secondary effects on hepatocellular response). It is apparent that hepatocyte behaves differently in culture due to the change in cell interaction happening during cell isolation.

Homotypic cell interaction can be increased by a high seeding density supported by suitable medium and ECM to bring the functionality upto a limited level. However to achieve heterotypic interaction, coculture of hepatocyte has to be adopted for long term functional activity in culture.

Coculture systems has been tried and functional assessment was done for studying different biochemical roles. The cocultures systems summarized by Coecke *et al* (1999) reviewed different culture systems using rat hepatocytes with epithelial cell lines (3T3, C3H, MS monkey kidney epithelial cells, MDCK, MSL-132, Balb/3T3), NPC, liver mesenchymal cells, mouse embryo fibroblasts Ito cells, Oval cellline and human cells with 3T3.

To study the influence of one cell over the other, cell interactions can be controlled by two general categories: prevention of contact and modulation of the degree of contact (Bhatia *et al*, 1999). Prevention of contact has been achieved by cocultures with porous filter inserts, insertion of crude spacers, or conditioned media experimentation. Modulation cell contact can be achieved by using patterned culture surfaces.

2.2.3.1. Patterned coculture

The counter part for hepatocyte coculture has given a good number of choices of cell types that are hepatic and extra hepatic in origin. Hepatocyte and mesenchymal cells has been chosen to study the cell-cell interactions *in vitro*. Bhatia *et al* (1998) has developed a coculture of rat hepatocytes with NIH3T3-J2 fibroblasts in a patterned culture surface.

2.2.3.2. Monolayer coculture

To use the heterotypic cell–cell interactions in modulating long-term hepatocyte behavior Bhandari *et al* (2001) described a co-culture system of 3T3 fibroblast on primary isolated rat hepatocytes. Hepatocytes seeded on monolayer of fibroblasts and cultured over 18-days were found to be maintaining viability, well-formed canalicular systems and cyt P450 activity.

When the purpose of hepatoycte coculture to study liver specific function or as a toxicity evaluation system, NPC is always preferred as the counter part. Non transformed epithelial monolayers were used to coculture hepatocytes, showed the restoration of albumin secreting ability (Guguen-Guillouzo *et al*, 1983). Similarly rat adult primary hepatocytes in pure and mixed monolayer cultures compared by Niemann *et al* (1991) also showed that Cyt P450 activity was stable throughout 10 days while there is decrease in other liver specific parameters.

Hepatocyte and purified NPCs also favor maintenance of liver specific characters. An autologous human hepatocyte and biliary epithelial cell (BEC) coculture by Auth *et al* (1998) in collagen gel, in the presence of serum free Williams' medium and hepatotrophic growth factors, maintained albumin production for more than two weeks.

Since the hepatocytes are closely positioned with reticulo endothelial cell (REC) system in the liver, cells from the later is also used for coculture. Since Hepatic stellate cells (HSCs) are a type of NPCs and are present in the perisinusoidal space of Disse, Higashiyama *et al* (2004) cocultured hepatocytes with HSCs with the aim of maintaining differentiated liver functions *in vitro*. It was found that hepatocytes cultured over the HSCs for 6 days were exhibiting more functional ability than when HSC conditioned medium were used. Hepatocyte – Kupffer cell cocultre has been studied by Lelbach *et al* (Lelbach *et al*, 2001) to understand the regulation of insulin-like growth factor-I and of insulin-like growth factor binding protein 1, 3, 4 and by interleukin-6. Hepatocyte and Kupffer cell coculture on a micropatterned culture surface done by Zinchenko *et al* indicates that hepatocyte function can be modulated by controlling the heterotypic cell-cell contact (Zinchenko *et al*, 2006).

The main cell type in RES is the SEC which is supposed to be performing a significant role in hepatocyte function when compared to other NPCs. The role of NO in vascular tone of sinusoids is known and the role of SECs in NO production has been investigated. Shah *et al* (1997) with the help of an in situ perfusion system measured the endogenous NO release from hepatic vasculature in time dependent manner and demonstrated its role in modulation of portal pressure.

Morin *et al* (1986) established a coculture of freshly isolated hepatocytes with primary NPC from adult rat liver and pulmonary EC line. Freshly isolated EC were distinguished from Kupffer cells based on the presence of factor VIII related antigen and peroxidatic/phagocytosis activities. Using a co-culture system, the effect of sinusoidal liver cells on hèpatocyte functional activity was characterized. Hepatocytes from suckling rat in coculture showed stable state of differentiation and maintenance of albumin secretion. It has been particularly noted that dexamethasone was required for such beneficial effect as in the case of normal hepatocyte culture. The hepatocyte-stabilizing activity was also shown in the coculture with pulmonary endothelial cell line.

Role of extra hepatic endothelial cells, especially the vascular endothelial cells, on hepatocyte function has been described by various studies. It has been proved by many independent studies that hepatocyte released factors influence vascular cells and there by vascular tone and vise versa. Due to the complementary role of these cells in normal physiology, hepatocytes and vascular ECs were cocultured to explain the cell-cell interaction between PCs and NPCs. Talamni *et al* (1998) used a coculture system of primary rat hepatocytes and bovine aortic endothelial cells to explain cell-cell interaction. The study suggests that ECs modulate hepatocyte gene expression by direct cellular interaction. This proves that rat hepatocytes and ECs of vascular origin from another species could be used as a culture system for cell-cell interaction studies. Such reports used cell lines to understand the cell-cell interaction.

Song *et al* (2005) investigated the adhesive mechanical properties of different cell cycle human hepatoma cells to human umbilical vein endothelial cell line. There is no report on polarity with respect to spheroid formation in coculture of rat hepatocytes and human umbilical vein endothelial cells (HUVEC).

2.2.3.3. Three dimensional coculture: Organotypical models

A coculture with pooled NPC fraction is another option to provide PC and NPC interaction. When monotypic 3D culture models approximate the biochemical and spatial contributions required for tissue-specific function, it is possible to include different cell types to simulate organ function. In an organotypical model total or a part of organ environment is reproduced in culture system to see the significance of autocrine and paracrine interactions (Schmeichel *et al*, 2003).

A study conducted by Kaihara *et al* (2000) showed that NPCs or RECs have no measurable effects on hepatocyte function in their continuous flow culture system as shown by albumin and urea synthesis. Their study showed that hepatocytes survived and were functional on the 3-dimensional scaffolds under flow conditions for 7 days rather than in a coculture with NPCs.

Use of biological scaffolds or ECM may be very essential for the improved function of hepatocytes in coculture. Michalopoulos *et al* (1996) studied morphogenetic events in mixed cultures of rat hepatocytes and NPCs on biological matrices. Hepatocytes and NPCs were grown in chemically defined medium on collagen coated polystyrene beads in roller bottles, formed clusters of beads with proliferating hepatocytes and NPCs, including fenestrated endothelium forming vascular structures. When mixed cell clusters were implanted in Matrigel, hepatocytes grew in three dimensions, forming plates and ducts suggesting progressive linear assembly guided by hepatocyte specific structural parameters.

Bader *et al* (Badder *et al*, 1996) designed a 3D organotypical model by positioning NPC on top of PC enclosed as a monolayer within a collagen sandwich. In the coculture model cuboidal hepatocytes formed confluent layers below the NPC layer and expressed bilecanaliculi. Characteristic NPC including endothelial cells, Kupffer cells, and Ito cells completely covered this second matrix layer within a week with Kupffer cells on top of endothelial cells. Ito cells were intermingled and could be identified by their intracytoplasmic lipid droplets. Lipopolysaccharide (LPS) stimulation of cocultures resulted in a depression of albumin secretion. Interaction between NPC and PC in the coculture model could be demonstrated by a characteristic depression of the negative acute phase protein albumin secretion upon LPS stimulation. It is also shown that biotransformation of ethoxyresorufin was not influenced by the presence of NPC.

2.2.4. Spheroid formation

When hepatocytes are provided with appropriate micro-environment, it self assemble into 3D structures or spheroids that exhibit ultrastructural characteristics of native hepatic tissue with enhanced liver specific function. The spheroid formation process involves cell translocation and changes in cell shape, indicating organization of the cytoskeletal elements. The function of the cytoskeleton in, hepatocytes undergoing spheroid formation studied by Tzanakakis *et al* (2001) indicates that hepatocytes require an intact actin network for efficient self assemble into functional tissue like structures. Such spheroid formation was observed during 2 to 3 days in culture.

2.2.4.1. Spheroid formation in monolayer culture

Use of spheroids as *in vitro* models could be a good system to predict chemical induced hepatic cytotoxicity. Since spheroids can maintain function in culture conditions for a longer period than monolayer cultures, as indicated by P450 activity, urea, and albumin synthesis Xu *et al* (2003) characterized rat liver and HepG2 spheroids as *in vitro* models for application in hepatotoxicity studies.

From various data available on spheroid formation, it can be summarized that the microenvironment plays direct influence. Spheroid formation can be induced by culturing hepatocytes on modified non-adherent surface or as suspension in bioreactor.

Adult rat hepatocyte spheroid formation reported by Tong *et al* (1992) showed that hepatocytes were morphologically similar to those of newborn rat hepatocytes and could also form a monolayer of uniform liver PC when transferred on collagen-coated surfaces even after 2 months of culture. The culture required poly (2-hydroxyethyl methacrylate) (poly-HEMA) coated surface and hormonally defined media to form spheroids. Spheroid formation was described so far with rodent hepatocytes, has been reproduced using human hepatocytes (Tong *et al*, 1994).

Porcine hepatocytes culture system is used in various applications such as toxicity studies and in artificial devices. It has been reported that primary rat hepatocytes spontaneously formed spheroids in the pores of macroporous sponge like polyurethane foam (PUF). Similar observation was repeated by the same group (Nakazawa *et al*, 1999) using porcine hepatocytes. Primary porcine hepatocytes seeded into pores of flat PUF plate spontaneously formed spheroids within 24 – 36 h. The formed spheroids were attached to the bottom surface of the PUF pores, and their morphology and viability were maintained for more than 12 days. The cyt P450 activity in the spheroids of porcine hepatocytes demonstrated by detecting production of monoethylglycinexylidide from lidocaine were maintained for 12 days at a level twice as high as in the monolayer culture.

2.2.4.2. Spheroid formation in coculture

Hepatocyte heterospheroid formation with other cell types can also be used as organotypical model for cell based applications.

Hepatocyte and NPC coculture were also studied for spheroid formation. Juillert *et al* (1997) reported spheroidal aggregate formation by freshly isolated hepatocytes, endothelial cells, Kupffer and fat-storing cells from adult rat. Spheroid formed within 24 h when cultured under serum free and rotatory conditions. The ultrastructure analysis of intracellular organization and extracellular matrix deposition investigated expressed a histotypic organization. The aggregates preserved, over a period of at least 7 days showed high albumin mRNA expression. However Cyt P450 activity was found to be increasing only when stimulated with growth supplements.

Polymers supplied in culture medium are also found to be promoting hepatocyte spheroid formation. Yamada *et al* (2001) showed that addition of a synthetic polymer, Eudragit S100, a co-polymer of methacrylic acid and methyl methacrylate, to the culture medium as an artificial matrix has been found to promote cell aggregation and induce spheroid formation. Furthermore co-cultures of hepatocytes and liver non-parenchymal cells in such microenvironment enhanced the stable expression of liver functions. The values of albumin secretion, ammonia removal and urea synthesis were the highest when equal number of hepatocytes and non-parenchymal cells were mixed. The heterospheroids maintained a higher level of albumin secretion ammonia removal and cytochrome P-450 activity for over 4 weeks compared with cocultured cells without the polymer and spheroids consisting of hepatocytes solely.

A biodegradable substratum suitable for inducing spheroid formation is poly (DL-lactic acid) (PLA). Riccalton-Banks *et al* (2003) reported a system of rat hepatocytes cocultured with primary rat hepatic stellate cells on PLA biodegradable substratum. The coculture conditions encouraged rapid self-organization of three-dimensional spheroids that exhibited hepatocyte-specific functionality like Cyt-P450 activity and albumin secretion to almost two months in static culture.

Doo-Hoon Lee *et al* (2004) studied heterospheroid with primary rat hepatocytes and rat prostrate endothelial cell line (RPEn) for 20 h as spinner cultures. Heterospheroid formed were entrapped in a calcium alginate gel beads and were monitored for albumin synthesis and ammonia removal ability. Immunostained paraffin sections showed RPEn cells were located on surface of heterospheroids. This is an example of rat hepatocytes and extra hepatic endothelial cells forming spheroid in coculture.

2.2.4.3. Polarity of hepatocytes

One of the main functions of hepatocytes is bile production and excretion at the bile canaliculi. To perform this function hepatocyte requires polarity with specific domains on the plasma membrane. Development and maintenance of polarity in epithelial cells are a multistage process requiring cell-cell adhesion and specific organization of proteins and lipids on the cell surface. Isolated hepatocytes lose polarity and redistribute proteins of specific, plasma membrane domains over their whole surface.

Wanson *et al* (1977) studied the reappearance of polarity in isolated hepatocyte monolayer culture within 24 h in defined medium. Cells showed important morphological changes which correspond to differentiations taking place within the cytoplasm around the Golgi complex and concomitantly on the plasma membranes. Optimal cell attachment was obtained when Ham's F 12 medium was supplemented with 10% rat serum or 15% fetal calf serum. The study also suggested the need for collagen, optimal cell concentration (1×10^6 cells/ml) and constancy of the pH during the culture using HEPES buffer.

In liver transport of solutes such as bile acids and certain amino acids from blood occur in hepatocytes across its basolateral membrane, ie: sinusoidal and lateral sides. Sztul *et al* (1987) demonstrated that Na^+, K^+-ATPase is distributed asymmetrically and is restricted to the basolateral plasma membrane.

Bile canaliculi represent the apical side of hepatocytes, containing unique membrane proteins. Different canalicular enzymes such as 5' nucleotidase, leucyl aminopeptidase and alkaline phosphatase have been identified but these are also found in basolateral hepatocellular membranes or sinusoids. Other membrane bound enzymes like Mg^{2+} activated ATPase and Dipeptidyl peptidase IV (DPP IV) are reliable cytochemical markers for canalicular membranes and suitable for establishing functional integrity of bile canaliculi (Stecca *et al*, 1997).

Liu *et al* (1999) demonstrated the bile canalicular formation and bile secretion by rat hepatocytes cultured in collagen sandwich. Immunoblot analysis demonstrated that the canalicular multispecific organic anion transport protein (multidrug resistance-associated protein, Mrp2) was partially maintained and excreted fluorescent products in the canalicular networks.

Lecluyse *et al* (1994) examined the morphological and cytoskeleltal reorganization of collagen sandwiched rat hepatocytes during the de novo formation of complete canalicular network. The results illustrate the utility of the system as *in vitro* model to examine the regulation of cell membrane polarity and differential role of the cytoskeleton in the regeneration and maintenance of bile canalicular network. Bile canaliculi was found to be forming within 48h after collagen overlay.

One of the reasons that hepatocytes lose its typical physiological functions *in vitro* is mainly due to the absence of microenvironmental conditions. Falasca *et al* (1998) studied the role of retinoic acid in reestablishment of morphological characteristics including cell polarity, polyhedric shape and reformation of bile canaliculi and junctional complex.

Appropriate ECM and soluble growth factors are shown to be essential for the reestablishment of polarity by hepatocytes *in vitro*. Nishikwa *et al* (1996) showed that hepatocytes can produce bile duct like structures in 4-5 days in the presence of Type I collagen matrix and soluble morphogenetic factors like EGF and HGF in serum free Williams E medium containing insulin and nicotinamide.

Hepatocyte polarization by formation of bile canaliculi is also studied using poorly differentiated (HA22T/VGH, SK-HEP-1) and highly differentiated hepatoma (Hep3B, HepG2 and HuH-7) cell lines. A variety of human hepatoma cell lines were used by Lian *et al* (1999) to investigate hepatocyte secretory activities of developing bile canalicular domains. Cells were also cocultured with another cell line from human cervical carcinoma (HeLa cells). The results showed that an integral membrane protein, aminopeptidaseN (APN), was gradually delivered and accumulated at the developing canalicular domain. Targeting of APN seemed to correlate with activation of certain secretory functions. DPPIV bore no association with any secretory activity.

Specific cell lines are also developed for studying hepatocyte polarity. Hepatoma lines are often differentiated but not well polarized, as they exhibit few or no apical bile canalicular like structures. This has been partially overcome by fusing rat hepatoma cells (Fao) and human fibroblasts (WI38), thus generating hybrid cells, the WIF12-1 cells, that express liver specific functions. The WIF12-1 cells exhibit substantial polarity. However, they only grow to a low maximal density and many cells remain unpolarized.

A derivative of these cells designated as WIF-B, was selected specifically for its ability to grow to a high density. The use of these cells as a suitable model for *in vitro* studies was evaluated by Ihrke *et al* (1993).

Lecluyse *et al* (1994) showed the DPPIV expression in bile canalicular side of repolarized hepatocytes in collagen sandwich. This is one of the evidence showing DPPIV as a cell membrane marker for hepatocyte repolarization in culture.

When a biotransformable fluorescing substance is supplied to hepatocytes in culture the excreted byproduct shall be detected in the bile canalicular side. Barth *et al* (1982) has effectively demonstrated the use of fluorescein to visualize functional polarity of hepatocytes. It was proved that hepatocytes in culture show a functional polarity permitting the transcellular transport of substances bound for biliary secretion. This detailed work is supporting the use of fluorescein marker for functional polarity.

Since spheroids are structurally and functionally stable stage of hepatocytes *in vitro*, polarity studies in spheroid cultures also have importance. Primary hepatocytes self-assembled into spheroids possess tight junctions and microvilli lined channels.

Abus-Absi *et al* (2002) demonstrated the formation of polarity in hepatocyte spheroid, in suspension culture, by assessing DPPIV and localizing fluorogenic substrate. A fluorescent bile acid analogue, fluorescein isothiocyanate labeled glycocholate, taken up into the spheroids and excreted into bile canalicular channels was used to track the bile canalicular network.

2.3. LIVER TISSUE ENGINEERING

Recent advances in culture methods, stem cell research, and tissue engineering provide clues for making tissues *in vitro* that are functionally and structurally similar to hepatic tissues. Two approaches are employed to reconstruct hepatic organoids. One is by using cells in combination and the other using cells in scaffold (Mitaka *et al*, 2002). Various substrata have been used like biomatrices, proteoglycans, collagen gel, and Engelbreth-Holm-Swarm (EHS) gel as beds for the hepatocytes. The coculture of hepatic cells with mature hepatocytes, small hepatocytes, hepatoblasts and hepatic nonparenchymal cells has been reported to form hepatic organoids that possess differentiated hepatic functions. Hepatocytes 3D culture was shown to form specific structures consisting of biliary epithelial cells, connective tissue, mature hepatocytes, and endothelial cells.

Tanaka *et al* (2003) presented their study on a microchip-based system for a long-term hepatocyte culture which maintained hepatocyte functions. They designed a cell culture in microchip to mimic the *in vivo* hepatocytes microenvironment, in which cells are provided oxygen and nutrients through capillary systems with additional extra cellular matrix for cell adhesion function and viability. Toh *et al* (2005) have developed a technique for the in situ 3D immobilization of primary rat hepatocytes within a localized matrix in a microfluidic channel showed enhanced cytochrome P450 activity

The main impact of liver tissue engineering is expected in the development of a high performance LAD and BAL. The relative shortage of donor organs and lack of immediate availability is the main drawback of orthotopic liver. An effective temporary liver support system could improve the chance of survival with or without a transplant. Recent technological advances resulting in improved hepatocyte maintenance and bioreactor designs facilitate adequate development of BALs (Riordan *et al*, 1999).

In advanced tissue engineering approach towards the development of BAL use of primary hepatocytes have been significantly considered. However, primary hepatocytes encounter rapid dedifferentiation in culture that results in loss of function within 3–4 days (Croci *et al*, 1985) partially due to the absence of other cells in liver. Culture conditions with modification in ECM, medium containing growth factors and coculture of hepatocytes with other cells are alternatives for the crisis.

To study the feasibility of a coculture system in BAL, Washizu *et al* (2001) studied a hepatocyte-mesenchymal cell coculture using primary rat hepatocytes and mouse 3T3-J2 cell lines. The cocultures maintained upto 8 days in culture medium were then switched to heparinized human plasma containing 3-methylcholanthrene, Phenobarbital or no inducer for up to 7 days. Cytochrome P450 activities measured in situ based on the o-dealkylation of ethoxy- (EROD), methoxy- (MROD), pentoxy- (PROD), or benzyloxy- (BROD) resorufin. It was found that plasma alone increased PROD/BROD but not EROD/MROD. Furthermore, plasma CYP1A1 and CYP2B1/2 mRNA levels was found to be increased. It is also reported that decrease in Cyt

P450activities are enhanced by supplementation of amino acids, insulin, glucagon, and hydrocortisone in plasma.

Wu *et al* (1996) described a method of forming spheroids in spinner vessels which has significantly greater efficiency than formation in stationary petri dishes. More than 80% of inoculated cells formed spheroids within 24 h after inoculation, maintained a high viability, produced albumin and urea at constant rates throughout during 6 days culture. Transmission Electron Microscopy indicated extensive cell-cell contacts and tight junctions between cells within spheroids.

Recently, Nahmias *et al* (2006) has reported a coculture system of rat and human hepatocytes with 3T3 fibroblast, HUVEC and SEC to study the uptake of hepatitis C virus like particle by hepatocytes. Equal number of EC were seeded on hepatocytes cultured for 3 h and the cocultured for 24 h to show the influence of hepatocyte polarity and function in coculture.

2.4. AIM OF THE STUDY AND SCOPE OF THESIS

Considering the above facts and features of hepatocytes, the present thesis aims in optimization of culture condition, development of a coculture system of hepatocytes and endothelial cells, synthesis of temperature sensitive polymer to help in cell attachment and detachment without loss of cell function, functional assessment of coculture system and formation of tissue structures.

The following strategies were aimed for improving, maintaining and prolonging the differentiated function and viability of cultured hepatocytes (Figure 2)

a) To optimize culture conditions for hepatocytes, SEC and HUVEC
b) To optimize microenvironment for coculture
c) To synthesize, characterize and to study cell culture efficacy of thermoresponsive polymer
d) To utilize thermoresponsive polymer for hepatocyte culture
e) To assess structural and functional characteristics of hepatocyte spheroids

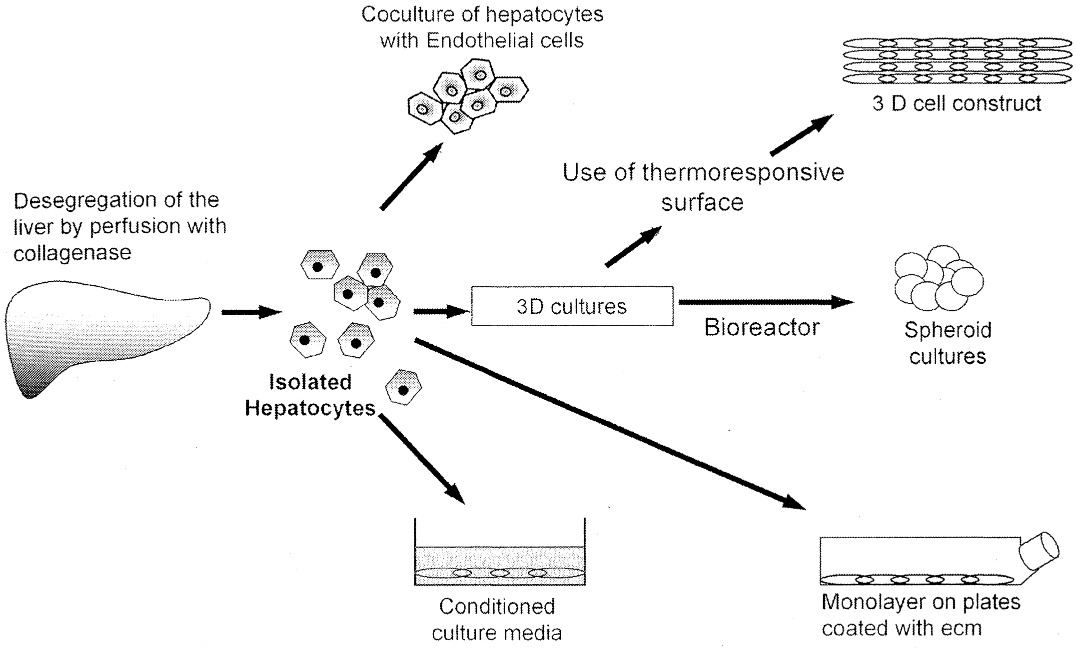

Figure 2 Strategies for improving, maintaining and prolonging the differentiated function and viability of cultured hepatocytes

Chapter 3

MATERIALS AND METHODS

3.1. TEMPERATURE RESPONSIVE SURFACE

The monomer in crystalline form was procured from Polysciences Inc. (USA).

3.1.1. Materials

i)	N-isopropyl acrylamide (NIPAAm)	-	Polysciences Inc. (USA)
ii)	2-Propanol (Isopropyl alcohol)	-	Merck Inc
iii)	Tissue Culture Grade Polystyrene (TCPS) Dishes	-	Nunclon USA
iv)	Pipettes	-	1 ml, 200 μl
v)	γ - irradiator	-	Panoramic Batch Irradiator (BARC, India) - PANBIT
vi)	Fourier Transform Infra Red spectrophotometer	-	Nicolet Inc (Madison, USA) - Impact 410
vii)	Contact Angle Goniometer	-	NRL Contact Angle Goniometer - 100.00
viii)	Scanning Electron Microscope	-	Hitachi - S 2500

3.1.2. Synthesis of thermoresponsive polymer

Thermoresponsive culture surface was synthesized by grafting poly(N-isopropyl acrylamide) (PIPAAm) on tissue culture dishes and plates.

3.1.2.1. Preparation of monomer

The monomer solution was prepared by dissolving NIPAAm at different concentrations (Table 2) in isopropyl alcohol.

Table 2. Composition of monomer for grafting

Polymer ID	Composition
i) B/35	35 % (w/w)
ii) Bx/45 (x = 1,2,3,4,5,6,7 & 8)	45 % (w/w)
iii) B/55	55 % (w/w)

3.1.2.2. Grafting PIPAAm on Tissue Culture Plates

A thin film of monomer solution was added uniformly and spread evenly on TCPS. The monomer was simultaneously polymerized and grafted on the dishes by γ-ray irradiation in PANBIT at a dose of 2.5 MGy. The grafted surface was washed thoroughly with cold sterile deionized water to remove un-reacted monomer and unbounded polymer and dried immediately in nitrogen atmosphere Sterilization and storage

The dishes were then sterilized by either γ-ray irradiation or Ethylene oxide and stored at room temperature until use.

3.1.3. Characterization of polymer

The PIPAAm grafted culture surface was characterized by Attenuated Total Reflection - Fourier Transform Infra Red spectrophotometry (ATR-FTIR), Scanning Electron Microscopy (SEM) and water contact angle measurement.

3.1.3.1. Spectrophotometric analysis of PIPAAm surface

A Fourier Transform Infra Red spectrophotometer and a horizontal Attenuated Total Reflection accessory with ZnSe crystal was used to obtain spectrum of the grafted and ungrafted surfaces of TCPS. Specific peak representing monosubstituted aromatic ring of TCPS and amide group of PIPAAm was analyzed at 1600 cm^{-1} and 1650 cm^{-1} respectively.

3.1.3.2. Surface analysis using Scanning Electron Microscope (SEM)

The grafted and ungrafted surfaces were sputtered with gold using an ion sputter device (Hitachi, E 101). The samples were observed at high magnification using SEM and surface morphology was compared.

3.1.3.3. Water contact angle measurements

Contact angle is a simple, fast and sensitive method to measure the wettability of a solid surface. Water contact angles of grafted and ungrafted surfaces were measured using deionized water (Millipore) at 37 °C and 27 °C. A minimum of five different fields were measured from each sample and the mean was calculated. Contact angles at different temperatures were represented as a mean ± standard deviation.

3.2. CELL ATTACHMENT ON POLYMER

3.2.1. Materials

i) Cell
ii) PIPAAm grafted dishes and normal TCPS
iii) Culture medium

3.2.2. Procedure

Cells were seeded on PIPAAm grafted culture dishes at a density of 2×10^5 cells/dish. The cultures were incubated at 37 °C until it reached subconfluency with media change at an interval of three days. Different cell types (Table 3) were cultured on PIPAAm surface. Cells were maintained in IMDM supplemented with 5 % FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin, at 37 °C in a 95 % humidified atmosphere with 5 % CO_2. Cell adhesion, growth and morphology on PIPAAm was monitored and compared with cells on ungrafted surfaces.

Table 3. Cell types cultured on PIPAAm surface

Cell line	Cell type	Source
L-929	Mouse subcutaneous connective tissue fibroblasts	American Type Cell Cultures (ATCC) - USA
NRK-49F	Normal rat kidney fibroblasts	National Center for Cell Sciences (India) - NCCS
HOS	Human osteosarcoma	NCCS
SIRC	Rabbit corneal cells	NCCS
Hepatocytes	Adult rat and human foetal	Primary culture
HUVEC	Human umbilical vein EC	Primary culture

3.3. CELL CULTURE STUDIES ON TEMPERATURE RESPONSIVE SURFACE

L-929, NRK-49F, SIRC and HOS culture on PIPAAm were transferred to new culture surface without enzyme treatment. Cells cultured on PIPAAm grafted culture dishes were retrieved and transferred by two methods. 1) Using a membrane support and 2) Without membrane support.

3.3.1. Cell sheet transfer with membrane support

3.3.1.1. Materials

i)	Cell	- L-929, NRK-49F, SIRC and HOS
ii)	Culture dishes	- PIPAAm grafted and normal TCPS
iii)	Culture medium	- IMDM with FCS
iv)	Forceps	- Flat tipped - Millipore
v)	Membrane	- Hydrophobic Poly Vinylidene Fluoride (PVDF) – Durapore

3.3.1.2. Procedure

Confluent cell layers (L-929, NRK-49F, SIRC and HOS) on PIPAAm grafted culture surface cultured as before were used for cell sheet transfer. The excess medium was removed and a hydrophilic PVDF membrane soaked with medium was placed over cell layer. An additional 200 µl culture medium was added over the membrane to avoid drying and the culture was incubated at temperature ≤ 20°C for 15-20 minutes. During low temperature incubation, the cells detached from grafted surface and adhered on to the membrane. The membrane together with the cells were peeled off using forceps and kept on a new culture dish with cell side facing down. About 200 µl fresh culture medium was added on membrane and incubated at 37 °C for 30 minutes. After incubation sufficient amount of fresh medium was added so that the membrane starts floating. The membrane was removed and culture was incubated at 37 °C. The transferred cells were monitored under phase contrast microscope before transfer, 0 h and 24 h after transfer. The steps involved in cell transfer using membrane support is illustrated in Figure 3

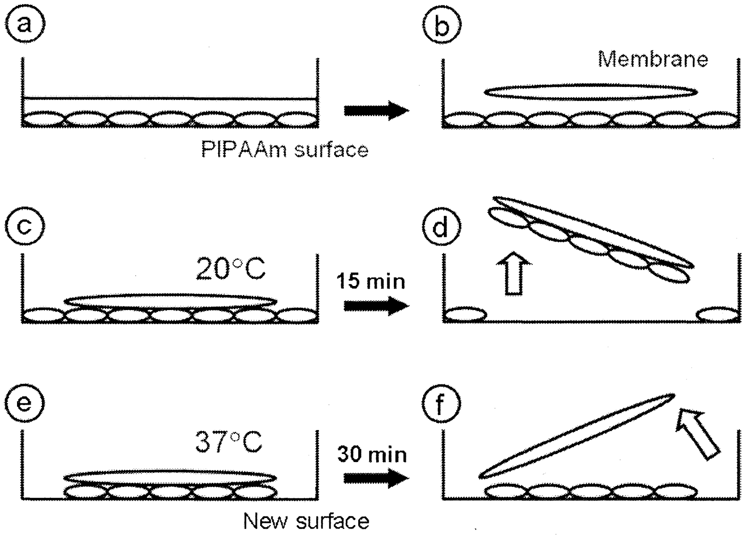

Figure 3 Method to transfer cell sheet using hydrophilic PVDF membrane.

3.3.2. Cell sheet transfer without membrane support

Cell monolayer from thermoresponsive surface can also be transferred without supporting membrane. Monolayer of SIRC detached by temperature variation was peeled off from the PIPAAm grafted culture surface.

3.3.2.1. Materials and Equipment

i)	Cell	SIRC, HOS
ii)	Culture dishes	PIPAAm grafted
iii)	Culture medium	IMDM with 5 % FCS
iv)	Forceps	Flat tipped – Millipore
v)	Scaffold	In house synthesized Hydroxyapatite (HA) – Porous (PHA) and Dense (DHA)
vi)	Glutaraldehyde	Sigma (25% in water) - G6257
vii)	Phosphate Buffered Saline (PBS)	0.1 M, pH 7
viii)	Phosphate Buffer (PB)	0.1 M, pH 7
ix)	Acridine orange (AcOr)	0.05 μg/ml
x)	Ethidium bromide (EtBr)	0.05 μg/ml
xi)	[3H] Thymidine	
xii)	Paraformaldehyde	Sigma
xiii)	Fluorescence microscope	Nikon Eclipse 600
xiv)	Phase contrast microscope	Leica inverted phase contrast

xv) Scanning Electron Microscope (SEM) - Hitachi S 2500 Japan
xvi) Critical point drying (CPD) - Hitachi, HCP-2
xvii) UV-VIS spectrophotometer - Hitachi
xviii) Liquid scintillation counter - Triathlar

3.3.2.2. Generation of peeled off cell sheets

A fresh 60 mm PIPAAm grafted culture dish was rinsed with culture medium and SIRC cells were seeded at density of 1×10^4 cells. Cells were allowed to form a monolayer inside CO_2 incubator with a medium change on every third day. For cell retrieval, culture medium was replaced with 0.5 ml fresh culture medium preincubated to ambient temperature. The culture was then kept below 10 °C for 10 min inside a refrigerator. Cell monolayer was then peeled off using a flat tipped forceps under a 3D-stereo microscope or phase contrast microscope as shown in the illustration (Figure 4).

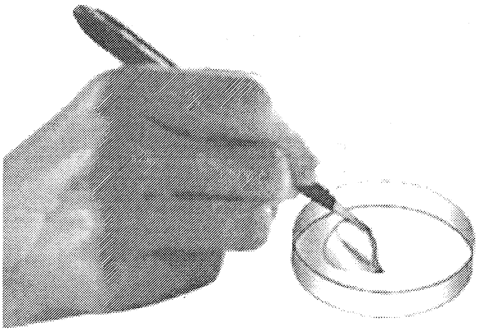

Figure 4 Peeling of cell sheet from PIPAAm surface using forceps

3.3.2.3. Generation of cell sheet patches

Osteoblast (HOS) monolayer was obtained on PIPAAm grafted surface as described above. In brief, culture medium was replaced with 0.5 ml fresh culture medium pre-incubated to ambient temperature and kept below 10 °C for 10 min inside a refrigerator. Cell sheets were retrieved as patches by gentle pipetting. The size of the patches was measured using image analysis software (QWin, Leica Germany).

3.3.3. Tissue engineering using cell sheets

To demonstrate the use of cell sheet structures in tissue engineering application, intact osteoblast cell sheet structures were uniformly distributed on to bone substitute material (Hydroxyapatite or HA) for rapid and complete cellularization without altering material characteristics.

3.3.3.1. Cell transfer to scaffold

The materials (PHA and DHA) were preconditioned by incubating in culture medium for 10 min at room temperature. Cell patches with varying sizes obtained by temperature variation from grafted dishes were taken in suspension and added on conditioned materials. Cell sheets were allowed to settle and adhere by incubating at 37 °C for 1 h. After adding sufficient medium, cultures were maintained in CO_2 incubator for 7 days with a medium change on every third day.

For functional assessment of osteobalst cells in cellularization of HA materials, cell patches were obtained from 35 mm culture PIPAAm dish in 1 ml suspension. To triplicate samples of conditioned PHA materials, 100 μl of cell patch suspension was added and allowed to adhere for 1 h and then sufficient media was added. Cell patches transferred to culture plates without materials were taken as control.

To compare functional ability of osteoblast cells during cell patch transfer and conventional trypsinization, HOS cultured on normal 35 mm TCPS was transferred to PHA after trypsinization. Cells obtained in 1 ml cell suspension by trypsinization were seeded on materials as described for cell patch seeding. Cells seeded on normal culture plates without materials were taken as control.

Experiment was carried out for a week with medium change on 1st, 3rd, 5th and 7th day. The medium replaced from culture was collected and stored below - 20 °C until used for enzyme assay. Alkaline Phosphatase (ALP) activity was assessed using Alkaline Phosphatase Kit (Glaxo, India) as per the product instruction sheet. Absorbance was read at 510 nm in a UV-VIS spectrophotometer .

3.3.3.2. Analysis of cellularized scaffold

Fluorescence and Confocal Microscopy. Cells on PHA and DHA samples were analyzed under fluorescence microscope. Samples were fixed using 4 % PFA in PBS at time intervals 1h, 2 days and 7 days. To visualize cells inside the pores, cells were stained with acridine orange for 5 min and observed under normal Fluorescence

Microscope (B-2A filter) and Laser Scanning Confocal Microscope (excitation at 488 nm).

Scanning Electron Microscopy: To observe the adhesion of cells and their morphology, samples were observed under SEM. Glutaraldehyde fixed samples were processed by dehydrating in graded alcohol, critical point drying, and gold sputtering followed by observation under Scanning Electron Microscope (SEM,).

Energy Dispersive X-Ray Spectroscopy (EDS): To determine the osteoblast cell function, PHA samples at 1h and 7 days were used for element analysis by EDS. The spectral peak from different fields of the sample representing carbon at × 600 magnifications was compared.

Live-Dead staining: Transferred HOS cell patch after culturing on PHA for 7 days was used for viability assay. Samples were incubated with a combination of acridine orange and ethidium bromide in phosphate buffered saline for 1 min. The scaffold was then analyzed at z plane under confocal microscope using 488 nm and 510 nm excitation wavelength in a sequential scanning mode.

[3H] thymidine uptake assay: Proliferation of cells on the materials was determined by [3H] thymidine (American Radio Labeled Chemicals) uptake assay as described elsewhere (Shivakumar *et al*, 1992). Cell patches were transferred as described above on to triplicate samples of PHA and DHA and monitored cell proliferation was monitored for 7 days. [3H] thymidine (2 μCi/ml) was added after 2h of seeding and continued culture for 7 days with additional supplementation of labeled medium on alternate days. At 2nd and 7th day, cells on materials were processed for determining acid-precipitable radioactivity using a liquid scintillation counter. Data obtained in count per minute (cpm) at two time points on both materials were compared.

3.3.4. Tissue construct with multi layer cell sheets

In vitro tissue construct was generated using a combination method of membrane support transfer and peel off technique. Here two or more PIPAAm grafted culture plates were used to create multilayer on another PIPAAm surface to detach tissue construct.

Osteoblast cells cultured on 2 or 3 PIPAAm grafted dishes was used for generating multilayerd tissue construct. A monolayer from one surface was transferred to a monolayer on another PIPAAm grafted culture dish by membrane support method. To this double layered cells third and fourth monolayer was overlaid to get 4 cell thick multilayered cell construct. Since the multilayer is cultured on a PIPAAm grafted surface, the temperature was lowered and the cell sheets were peeled off from the surface by peeling off technique describe above. Figure 5 shows the steps involved in generation of multilayer tissue construct. The peeled off multilayer was transferred to another culture dish.

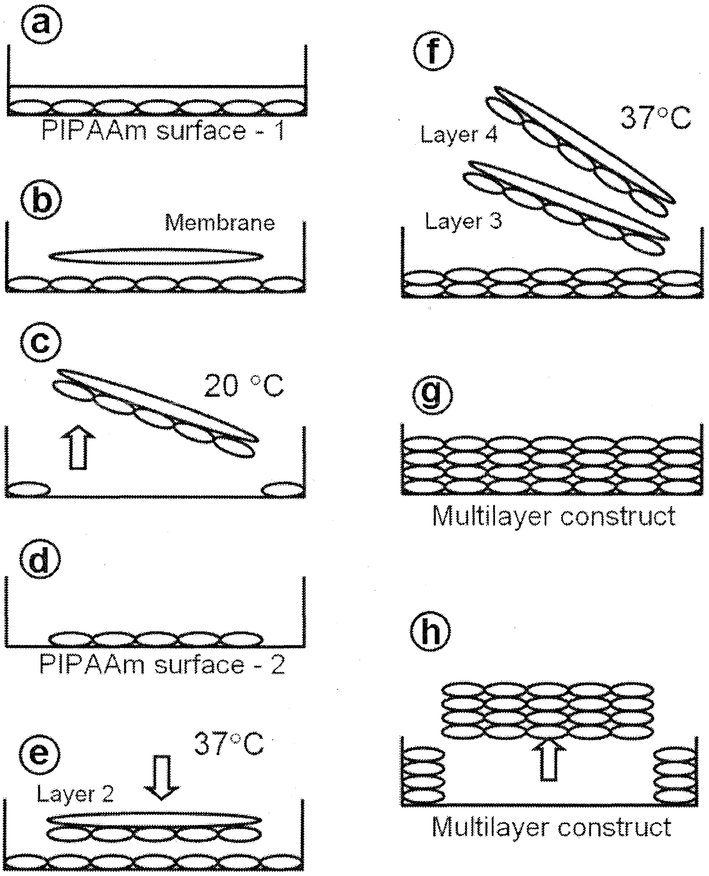

Figure 5 Illustration showing the steps involved in generating multilayered tissue construct..

To observe the tissue architecture, a small portion of multilayer cell sheet was fixed in 3 % glutaraldehyde, processed and observed under SEM. Samples were processed by dehydrating in graded ethanol as given below

25 % ethanol - 5 min (2 times)
50 % ethanol - 10 min (2 times)
75 % ethanol - 10 min (2 times)
95 % ethanol - 10 min (2 times)
100 % ethanol - 15 min (3 times)

The dehydrated samples were dried in Critical Point Dryer and then gold coated in sputter device. The samples were labeled and observed under SEM.

3.4. HEPATOCYTE CULTURE

3.4.1. Isolation of adult rat hepatocytes

Hepatocytes were isolated from adult male wistar rats weighing 230 – 250 g by two-step perfusion method as described by Seglen.

3.4.1.1. Materials

The materials given below were used for isolation of hepatocytes by two-step perfusion method.

a) Beakers
b) Cannula
c) Centrifuge tubes
d) Collagen coated multiwell and coverslips
e) Dissection board
f) Forceps
g) Haemocytometer
h) Heparin
i) Nylon mesh
j) Perfusion apparatus with oxygen supply and water bath
k) Petridish
l) Pins
m) Pipettes/Dispenser
n) Scissors

o) Sieve plate/dish
p) Sodium Pentobarbitone or xylazine and Ketamine
q) Surgical thread
r) Tray

3.4.1.2. Equipment

A perfusion apparatus was set up for isolating liver cells by perfusion (Figure 6).

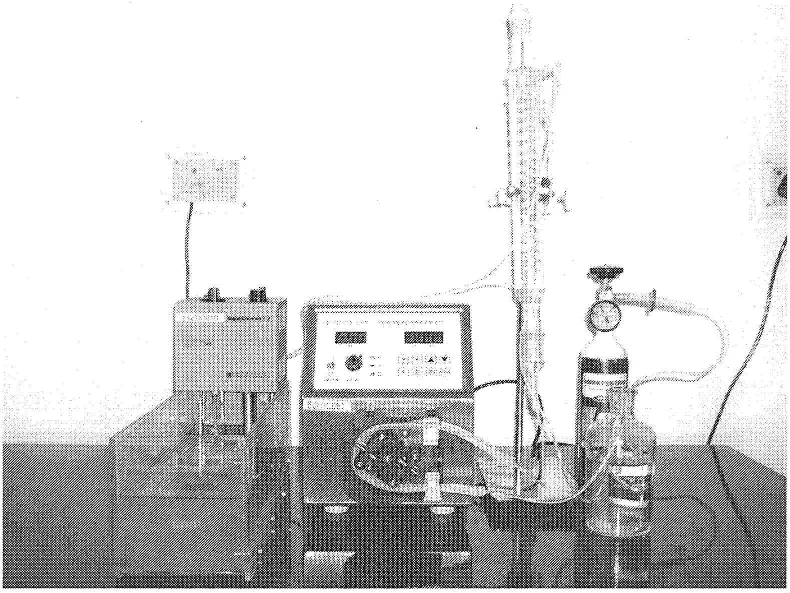

Figure 6 Custom made perfusion apparatus with perfusion pump, incubation and oxygen supply.

3.4.1.3. Reagents

3.4.1.3.1 ***Perfusion Buffer***

The perfusion buffer was prepared by adding NaCl - 4.15 g, KCl – 0.25 g, HEPES - 2.4 g, 1 M NaOH - 5.5 ml to 950 ml deionized distilled water. pH was adjusted to 7.2 and made up to 1000 ml. The buffer was sterilized by autoclaving and stored at room temperature (1 month).

3.4.1.3.2 ***Collagenase Buffer***

Collagenase buffer was perfused to dissociate cells from the liver. Calcium containing buffer was prepared by mixing NaCl - 3.9 g, KCl - 0.5 g, HEPES - 2.4 g, 1 M NaOH - 66 ml, $CaCl_2.2H_2O$ - 0.7 g in 950 ml deionized distilled water. After adjusting the pH to 7.0-7.2 the volume was made up to 1000 ml. The buffer was autoclaved and stored at room temperature (1 month).

To 100 ml of buffer 50 mg Type IV Collagenase (Sigma, Clostridium histolyticum) was added and filtered through 0.22 μm Millipore filter.

3.4.1.3.3 ***Trypan blue solution***

Trypan blue (Sigma) in PBS at a final concentration of 3 μg/ml.

3.4.1.3.4 ***Culture medium***

IMDM (Gibco) supplemented with 2.5 % FCS (Sigma) along with 100 IU/ml Penicillin (Sigma) and 100 μg/ml Streptomycin (Sigma).

3.4.1.3.5 ***Collagen coating for hepatocyte culture***

Collagen solution at a concentration of 1.5 mg/ml in PBS was prepared as given below and used for coating coverslips.

Collagen acid soluble (3mg/ml, Vitrogen Inc. USA)	8 ml
NaOH (0.1 M)	1 ml
PBS (10 X)	1 ml
PBS (1 X)	6 ml

3.4.1.4. Procedure for isolation of rat hepatocytes

3.4.1.4.1 ***Pretreatment of rat for cell isolation***

The rats (male, Wistar) weighing 200-250 gm obtained from Animal Holding Units of Biomedical Technology Wing or National University of Singapore was anesthetized by one of the following method..

a) Using Pentobarbitone sodium
   Pentobarbitone sodium (Nembutal, Mrial Australia Pty limited, 60 mg/ml) was injected intraperitoneally so as to give a dose of 40–55 mg/Kg body weight of Pentobarbitone (van Zutphen *et al*, 1993). This was followed by 0.5 ml Heparin (LEO Pharmaceutical Products, Singapore) to avoid blood clotting and left the animal undisturbed until it was fallen deep sleep.

b) Using Xylazine and Ketamine

Xylazine injection (Indian Immunologicals Ltd, India) and Ketamine hydrochloride injection (Neon Laboratories, India) was injected intraperitoneally so as to give a dose of 10 mg/kg body weight and 90 mg/kg body weight respectively. Animal was left undisturbed until it was fallen deep sleep.

3.4.1.4.2 ***Perfusion of liver***

Perfusion was carried out using custom made perfusion apparatus with continuous oxygen supply during the procedure.

a) The anaesthetized animal was pinned on its back to the dissection board and performed laparotomy.

b) Alimentary canal was moved to the left side of the animal to expose portal vein.

c) After placing loose ligatures around the portal vein a small incision was made on it using pointed scissors.

d) A cannula was inserted into portal vein and secured in position using surgical thread.

e) Perfusion was started immediately with perfusion buffer at a low flow rate of 20 ml/min and the colour change of liver from deep red-brown to pale yellow was observed.

f) Immediately a deep cut was made in the inferior venacava

g) Liver was disconnected from other organs with care not to tear off tissues while the perfusion is continued through portal vein.

h) Portal vein was cut before cannulation point and liver was separated from the carcass.

i) Cannulated liver was transferred into a sieve kept on a beaker.

j) Perfusion was continued for 10 min at a rate of 30-35 ml/min.

k) Perfusion buffer was then changed with collagenase buffer and continued perfusion for 15 min in a re-circulating fashion.

l) Liver was disconnected from cannula and transferred to a pertridish.

m) After adding 25 ml of serum free medium, the Glisson's capsule was peeled off from the liver surface using forceps.

n) Cells were dislodged and obtained in serum free IMDM.

o) Enough medium was added and remaining tissue pieces were removed using forceps.

p) Cells were incubated at 37 °C for 30 min.

q) Cells were filtered through 60 - 100 μm nylon mesh and collected in a centrifuge tube.

r) Cell suspension was kept in ice for 10 min.

3.4.1.4.3 ***Purification of hepatocytes***

a) Cell suspension was centrifuged at 40 g for 2 min at 4°C.

b) Supernatant was removed (or collected for SEC isolation) and cells were resuspended in 30-50 ml serum free DMEM or IMDM.

c) Centrifuged again at 20 g for 2 min and discarded the supernatent.

d) Pellet was resuspended in 30 – 50 ml medium.

e) Performed step c and d twice.

f) Cells were resuspended in 5 - 10 ml medium and kept in ice..

3.4.1.4.4 ***Viability assessment***

Viability of hepatocytes was determined by trypan blue staining. Viable cells remained unstained while the dead ones retained the blue stain. Equal volume of cell suspension and trypan blue was mixed. Cells were counted using haemocytometer.

$$\text{Cell number} = \text{Average cell number} \times \text{Dilution factor} \times 10^4 \ \text{Cells / ml}$$

$$\text{Viability in \%} = \frac{\text{Number of unstained cells}}{\text{Total number of cells}} \times 100$$

Cell suspension having more than 85 % cell viability was used for experiments.

3.4.2. Optimization of microenvironment

Hepatocytes were cultured in different media like RPMI-1640, IMDM and MEM each one containing 5 % FCS and in serum free MEM. Cultures were maintained for 48 h to see the adhesion of cells and it survival.

3.4.3. Culture of hepatocytes

Hepatocytes were seeded at a concentration of 1×10^6 cells/well on a collagen coated coverslips in 12 well culture dishes (Falcon, Singapore or Greiner bio-one, Germany). Cultures were maintained in a CO_2 incubator at 37 °C, in 95 % humidified atmosphere containing 5 % CO_2.

Hepatocyte proliferation was determined by [3H] thymidine uptake assay for 9 days. Freshly isolated hepatocytes were seeded on collagen coated 96 well plates. After 2h of cell seeding, radiolabelled thymidine (2 μCi/ml) was added and continued culture. Half amount of medium was replaced with fresh thymidine medium on alternate days. On 2, 5 and 7 days the cells were used for determining the thymidine uptake in a liquid scintillation counter.

3.5. HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVEC)

3.5.1. Materials

a) Bovine brain
b) 0.1M NaCl
c) Homogenizer
d) Streptomycin sulphate powder
e) Dialysis tubing (50 KDa, Sigma)
f) Surgical thread
g) Cannula
h) Three way stop cock
i) Gelatin
j) Triton-X 100
k) Primary antibody - anti-human-FVIII mouse Ig Dako
l) Secondary antibody - FITC tagged rabbit anti-mouse IgG Sigma

m) Propidium iodide - 0.05 μg/ml in PBS

n) DiI-Ac-LDL

3.5.2. Preparation of Endothelial Cell Growth Factor (ECGF)

Crude ECGF was prepared from bovine brain according to the method of Maciag *et al* (1979). The procedure was started with whole bovine brain at ice-cold temperature and the sample temperature was maintained at 4°C during entire procedure. Brain was cleared from blood by rinsing with PBS and was cut into pieces of 1 cm^2. The pieces were homogenized for 5 min in 1L 0.1M NaCl in an ice-cold blender while keeping the pH at 7.0. The homogenate collected in a beaker was stirred for 2 h followed by centrifugation at 13,800 g for 40 min. The supernatant was collected and streptomycin sulphate powder was added to it in a concentration of 0.5 % w/v. The mixture was then allowed to stand for at least 1 h at and the pH was maintained at 7. The mixture was centrifuged again as above and the supernatant was collected into a dialysis tubing (50 KDa, Sigma) and dialyzed overnight against 0.1 N NaCl. The dialyzed solution was collected and centrifuged again as above to collect the supernatant rich in ECGF. The growth factor was then aliquoted, freeze dried and stored below -20°C.

3.5.3. Culture medium for HUVEC

Iscove's Modified Dulbecco's Medium (IMDM) containing 10 % FBS, 500 μg/ml ECGF, 100 IU/ml penicillin and 100 μg/ml streptomycin was used to culture endothelial cells.

3.5.4. Preparation of buffer for collecting cord (cord buffer)

The following chemicals were mixed in 1 L deionized water and filter sterilized

KCl - 400 mg, KH_2PO_4 - 60 mg, NaCl - 8000 mg, Na_2HPO_4(anhydrous) - 62.1 mg, $NaHCO_3$ – 300 mg and Glucose – 1000 mg

Antibiotic in high concentration (1000 IU/ml penicillin and 1000 μg/ml streptomycin) was added to the solution and used for collecting umbilical cord.

3.5.5. Endothelial Cell culture

Endothelial cells from human umbilical vein was isolated by Jaffe's method with modification (Jaffe *et al*, 1973). Umbilical cord was collected in ice cold cord buffer and used for cell isolation within 2 – 3 h. Clamped or otherwise damaged areas of the cord were discarded and it was cleaned from blood. Both ends of vein were cannulated and secured it in place using surgical thread. Vein was then flushed many times with cord buffer to remove blood. After connecting a three way stop cock to one cannula, the vein was filled with 0.1 % collagenase through the other side and incubated at 37 °C for 15 min. Cells were dislodged from the vein and collected into a centrifuge tube by flushing with culture medium. Cells were pelleted by centrifuging at 500 g for 10 min and resuspended in culture medium containing 20 % FBS, 500 µg/ml crude endothelial cell growth factor (ECGF) and antibiotics. Cells were seeded on culture dish pre-coated with 1 % gelatin and incubated at 37 °C. This was followed by a change in medium at the end of 2 h and on every alternate day thereafter. For subculture, cell monolayer was trypsinized using 0.25% Trypsin EDTA solution (Gibco). Cells from 3rd passage onwards were used for experiments.

3.5.6. Characterization of HUVEC

Endothelial cells were characterized by expression of von Willibrand Factor (vWF) or Factor VIII (FVIII) and the ability to uptake acetylated low density lipoprotein.

3.5.6.1. vWF expression

The characteristic vWF antigen expression by HUVEC was demonstrated by immunoflourescence staining. Cell monolayer formed on gelatin coated coverslips were rinsed with PBS and fixed in 4 % paraformaldehyde. Cells were permeabilised by incubating with 0.1 % Triton-X 100. The non-specific binding regions were blocked by incubating with 1 % BSA for 10 min. From every step hereafter the cells were rinsed with PBS for 3 times each in 5 min incubation. Cells were then sequentially incubated with primary antibody (1:100 rat anti-human-FVIII mouse Ig Dako, USA) and secondary antibody (1:100 FITC tagged rabbit anti-mouse IgG Sigma, India) for 1 h each in a humidified chamber. Cells were counter stained with 0.05 µg/ml propidium iodide to visualize nucleus and observed under confocal microscope (Carl Zeiss LSCM 510 Meta, Germany) using 488, 514 nm excitation filters and LP 505nm, LP 610nm emission filters.

3.5.6.2. Modified LDL uptake

HUVEC was further characterized by their ability to uptake DiI-Ac-LDL. Cells cultured on gelatin coated coverslips were incubated in medium containing 10 µg/ml

DiI-Ac-LDL at 37 °C for 4 h and then fixed in 4 % PFA. To visualize LDL uptake, cells were rinsed with PBS and observed under fluorescence microscope (Nikon G-2A filter, Japan).

3.6. RAT LIVER SINUSOIDAL ENDOTHELIAL CELLS (SEC)

The SEC isolation procedure was started from the step (3.4.1.4.3 b)). The supernatant during the first wash of hepatocyte was used for SEC isolation.

3.6.1. Materials

a) Uncoated TC plate

b) Collagen coated TC plate

3.6.2. Reagents

3.6.2.1. Percoll solutions

a) Stock Percoll Solution (SPS, 90% in PBS-10X)
Mix 10 ml PBS-10X and 90 ml Percoll.

b) Percoll gradient solutions
50 % percoll solution - Mix 5 ml SPS and 5 ml PBS (1X)
25 % percoll solution - Mix 2 ml SPS and 6 ml PBS (1X)

3.6.2.2. Culture medium

IMDM (Gibco) supplemented with 2.5 % FCS (Sigma) along with 100 IU/ml Penicillin (Sigma) and 100 μg/ml Streptomycin (Sigma).

3.6.3. Procedure

3.6.3.1. SEC separation

Liver SECs were isolated by two step percoll gradient technique with minor modifications from previously reported method (Braet *et al*, 1994). The supernatant collected after first centrifugation of hepatocyte isolation was centrifuged further at 100 g for 5 min. The supernatant was centrifuged at 350 g for 10 min and the cell pellet was washed in PBS. Cells were obtained in PBS and loaded on top of a two step percoll gradient as given below.

a) 50 % percoll (bottom) - 3.75 ml
b) 25 % percoll (middle) - 5.00 ml
c) Cell suspension in PBS (top) - 2.50 ml

This was immediately centrifuged at 900 g for 20 min. Cell layer formed between the two percoll gradient layers was carefully collected and transferred to another tube. With sufficient PBS it was again centrifuged at 900 g for 10 min to obtain cells as pellet.

3.6.3.2. SEC purification and culture

The pellet was resuspended in culture medium and seeded on uncoated culture dish and incubated at 37 °C for 20 min. By this time cells other than SEC adhered on the uncoated surface. The unattached SECs were transferred to collagen coated 12 well plates and allowed to form monolayer.

After 2 h, medium was changed to remove unattached cells and continued incubation with sufficient medium.

3.6.3.3. Characterization of SEC

3.6.3.3.1 ***Morphology***

SEC cultured on collagen coated coverslips was observed under phase contrast microscope to see fenestrations. For higher magnifications, SECs cultured on collagen coated coverslips for 24 h were processed for SEM as described before.

3.6.3.3.2 ***Modified LDL uptake***

Reagents required are Serum Free Medium, DiI-Ac-LDL (Molecular Probes, Cat No 3484, 1 mg/ml) - Mix 30 μl DiI-Ac-LDL to 270 μl Serum Free Medium, Paraformaldehyde (3.7 % in PBS), PBS, Confocal mounting medium.

Sinusoidal cells cultured on collagen coated chamber slides was characterized by DiI-Ac-LDL uptake as described earlier (3.5.6.2)

3.7. LIVER TISSUE SECTIONS: CONTROL FOR STRUCTURAL POLARITY AND METABOLIC ABILITY OF HEPATOCYTES

Liver tissue sections were used as control for analyzing the metabolic ability and structural polarity of hepatocytes in culture by confocal microscopy.

Liver tissue sections were obtained by vibratome sectioning of adult rat liver. Briefly, male wistar rat weighing 200-250 g was anaesthetized by administering Pentobarbitone Sodium intraperitoneally at a dose of 50 – 60 mg/kg bodyweight. This was followed by 0.5 ml Heparin to avoid blood clotting inside blood vessels. After laparotomy, a small incision was made on the portal vein and the liver was perfused with oxygenated Collin's solution ($MgSO_4.7H_2O$ – 7.394 g, $NaHCO_3$ – 0.840 g, K_2HPO_4 - 9.699 g, KCl – 1.118 g, KH_2PO_4 – 2.042 g, Glucose – 25.228 g, pH 7.00) at 3 - 4 °C. The intact liver was excised and tissue cylinders were prepared using 8 mm diameter coring tool on a motor-driven tissue-coring device (Alabama Research & Development, Munford). Tissue slicing was made in cold Collin's solution at a thickness of 250 to 300 μm using Vibratome microslicer (DTK-1000, Pelco International, Redding, USA).

Hepatocytes are metabolically active and highly polarized in liver. To visualize this tissue sections were stained for structural and functional behavior of hepatocytes. The metabolic ability of hepatocytes in liver tissue was visualized under confocal microscope by the formation of fluorescent compound by cytochrome P450 (cyt P4501A1, Ethoxy Resorufin O De-ethylase, EROD) enzyme from the substrate O^7-ethoxyresorufin. Liver tissue sections were incubated in medium containing 10μg/ml substrate for 5 h at 37 °C. Tissue sections were then rinsed and fixed in paraformaldehyde. Fixed sections were then used for immunofluorescence staining by combined use of primary antibodies and a common secondary antibody. Structural polarity of hepatocytes in coculture and HUVEC conditioned medium was determined by analyzing the localized distribution of apical and basolateral protein representatives. CD-26 or Dipeptidyl Peptidase – IV or DPPIV was selected as a marker for apical polarity and CD-147 or Basigin was taken as marker for basolateral polarity. Tissue slices were permeabilized with 0.2 % Triton X 100 for 2-3 min and then blocked with freshly prepared 1% BSA for 10 min. Slices were sequentially incubated for 1 h with combined primary antibodies anti-CD26 and anti-CD147 Ig followed by a common secondary antibody FITC- anti-mouse rabbit IgG with alternate rinse using PBS. Cells were observed under confocal microscope to visualize metabolically active red cells showing polarized green periphery.

3.8. HEPATOCYTE CULTURE IN ENDOTHELIAL CELL CONDITIONED MEDIUM

Hepatocytes in conditioned medium were monitored under phase contrast microscope (Leica DMIL, Germany) every day to see the morphological changes and cell characteristics. Cells cultured in normal IMDM medium were considered as the control.

3.8.1. Collection of EC conditioned medium

Medium was collected from HUVEC and SEC culture during medium change was used as conditioned medium for hepatocytes culture. The collected medium was stored at 4°C until the amount reached about 20 - 25 ml and it was centrifuged at 900g for 10 min to remove cells and cell debris. The supernatant collected was pooled and filtered using 0.22 millipore filter.

3.8.2. Hepatocytes in HUVEC conditioned medium

Freshly isolated hepatocytes were seeded on collagen coated culture plates and coverslips at a concentration of 1×10^6 cells/well. Cells were first incubated in IMDM containing 5 % FCS for 2 h to allow the cells to adhere. The first medium change was done with HUVEC conditioned medium at the end of 2 h and on every alternate day thereafter for 10 days. Cells cultured in normal IMDM with 5% FCS were considered as control.

3.8.2.1. Spheroid formation of hepatocytes

Hepatocytes were cultured in conditioned medium as described above and were monitored under phase contrast microscope (Leica DMIL) everyday to see the morphological changes and cell characteristics. Images were captured using a digital camera on 1, 3, 5 and 7 days to record the progression of spheroid formation.

3.8.2.2. Albumin synthesis

Hepatocytes were seeded on collagen coated 96 well plates. After 2h, multiple rows of cells were shifted to HUVEC conditioned medium. Medium was changed on every alternate day. A full row of cells were fixed in paraformaldehyde on 1, 3, 5 and 7 days. The cells were used for immunofluorescence staining using anti-BSA rat Ig and FITC-anti-rat IgG. Cell based assay was done with immunostained cells in a spectrofluorimeter (Spectra Max Gemini E). Relative Fluorescence reading was obtained with an excitation and emission of 495 and 520 nm respectively.

3.8.2.3. Ammonia detoxification (urea synthesis)

Hepatocytes were cultured in 12 well plates as described before. Triplicate samples were used to determine urea synthesis ability of hepatocyte cultured in normal medium and HUVEC conditioned medium. On 1st day cultures were incubated for 4 h in a serum free medium containing 0.1 M NH_4Cl. Hepatocytes detoxify ammonium chloride to urea. Urea containing medium was collected and stored below -20°C until tested. The same culture was maintained up to 7 days with estimation of urea synthesis on 3, 5 and 7 days. Amount of urea formed was estimated by diacetyl monoxime (DAM) method using commercially available kit (Glaxo, India).

3.8.2.4. Biotransformation (metabolic ability)

The metabolic ability of hepatocyte in conditioned medium was assessed by measuring the cytochrome P450 (CYP4501A1, Ethoxy Resorufin O De-ethylase, EROD) activity. A modified procedure based on earlier reports by Donato *et al* (1993) and Thum *et al* (2000) was followed to measure the Cyt P450 ability to convert the substrate O^7-ethoxyresorufin. Freshly isolated hepatocytes were cultured on collagen coated dishes in HUVEC conditioned and normal culture medium for 7 days in duplicates. Enzyme activity was measured on 1st, 3rd, 5th and 7th day. Cultures were incubated with medium containing substrate at a final concentration of 8 μM/well for 4 h. The medium was collected and the cultures were maintained for the remaining experiments. The 75 μl of collected medium was transferred to black 96-well plate. To each well 25 μl β-glucuronidase (15 Fishmann units) in sodium acetate buffer (pH 4.5) was added and incubated for 2h. The reaction was stopped by adding 100 μl Glycine buffer (pH 10). Fluorescence of supernatants was measured in spectrofluorometer at 530 nm excitation and 590 nm emission wavelengths and relative fluorescence were compared.

The metabolic ability was also observed using confocal microscope. Freshly isolated hepatocytes were cultured on collagen coated dishes in HUVEC conditioned and normal culture medium for 7 days in duplicates. First, 3rd, 5th and 7th day cultures were incubated with EROD substrate containing medium for 4 h. Cells were rinsed with PBS and fixed in paraformaldehyde until observed under confocal microscope (Carl Zeiss LSCM 510 Meta, Germany) using 543 nm excitation and BP 530 – 600 nm emission filters.

3.8.2.5. Structural polarity

Cells were fixed in paraformaldehyde on 1, 3, 5 and 7 days. Cells were subjected to double immunofluorescence staining using antibodies specific to apical (CD26) and basolateral (CD147) proteins. Fixed cells were rinsed 3 times with PBS and permeabilised using 0.1 % TritonX100. The non specific binding sites were blocked using freshly prepared 1% BSA in PBS. Cells were incubated in a humidified chamber sequentially with the antibodies anti-CD26 mouse Ig, FITC- anti-mouse rabbit IgG, anti-CD147 mouse Ig and TRITC-anti-mouse rabbit IgG at 1:100 dilution for 1 h each. Cells were observed under confocal microscope (Carl Zeiss LSCM 510 Meta, Germany) using 488, 543 nm excitation filters and long pass (LP) 505 nm, band pass (BP) 560 - 615 nm emission filters. Images were obtained in z-stack to see the polarized nature of hepatocytes.

3.8.3. Hepatocytes in SEC conditioned medium

Hepatocytes were cultured with SEC conditioned medium to know the influence of SEC secreted factors on hepatocyte functions. Hepatocytes were seeded on to collagen coated coverslips at a concentration of 1×10^6 cells/well in a 12 well plate. After 2 h incubation, medium was changed to remove unattached cells. Cells cultured on coverslips were fed with SEC conditioned medium and control cultures were maintained with normal culture medium. The cells in conditioned medium were compared with those in normal medium (control).

Hepatocytes were seeded on to collagen-coated culture plates and coverslips and cultured in SEC conditioned medium with medium change on alternate days.

3.8.3.1. Spheroid formation

Hepatocyte spheroid formation was monitored in SEC conditioned medium as described before (section 3.8.2.1).

3.8.3.2. Hepatocyte function assay

The metabolic ability of hepatocyte in SEC conditioned medium was studied as described before (3.8.2.4) using confocal microscope. Freshly isolated hepatocytes were cultured on collagen coated dishes in SEC conditioned and normal culture medium for 7 days in duplicates. On 1st, 3rd, 5th and 7th day cultures were incubated with EROD substrate containing medium for 4 h. Cells were rinsed with PBS and fixed in paraformaldehyde until observed under (Olympus 1X81) using 543 nm excitation.

3.8.3.2.1 ***Structural polarity***

Structural polarity of hepatocytes in coculture and SEC conditioned medium was determined by analyzing the localized distribution of apical and basolateral protein representatives as described before (section 3.8.2.5).

3.8.3.2.2 ***Functional polarity***

Fluorescein Di Acetate (FDA), a vital stain, is also used to detect the polarization of epithelial cells.

To determine the functional polarity, cells cultured in conditioned medium for 2 days and 7 days were used for FDA assay. Cells were incubated with serum free medium containing 0.5 μg/ml FDA and incubated at 37 °C for 20 min. Cells were then fixed in paraformaldehyde and observed under confocal microscope using 488 nm excitation and FITC filter

3.9. COCULTURE OF HEPATOCYTES AND EC

About 1 × 10^6 freshly isolated hepatocytes were seeded on subconfluent SEC and HUVEC. Cells were cultured for 7 to 10 days in IMDM containing 2% FBS. Cultures were maintained with medium change at the end of 1, 3, 5 and 7 days.

The morphology and structural characteristic like bile canaliculi formation.of hepatocytes and cocultures were observed under phase contrast microscope (Leica or Olympus)

3.9.1. Spheroid formation on collagen

Spheroid formation and other hepatocyte-specific characteristics when cultured on collagen, was monitored at the end of 1, 3, 5 and 7 days under phase contrast microscope.

The culture at the end of 10th day was examined under SEM to comprehend the surface structure and Extra Cellular Matrix (ECM) deposition. Hepatocytes cultured on collagen was taken as control for the coculture.

3.9.2. Heterospheroid formation on EC

Hepatocyte heterospheroid formation on HUVEC was monitored as above. Hepatocyte coculture with HUVEC on 10th day was observed under SEM to see the cellular content of heterospheroid in detail.

To differentiate HUVEC from hepatocytes and its presence in heterospheroids, cocultures were incubated with DiI-Ac-LDL as described earlier. Confocal images were obtained in z planes were used for depth analysis using "DepthCod" in Zeiss LSM Image browser (V 3.5) software The DepthCod feature provides the ability to recognize the focal plane of the cells throughout a z-stack by assigning a color code to the pixel intensity as a function of the z-depth. This was done to visualize whether DiI-Ac-LDL stained HUVECs were dispersed inside spheroid.

3.10. CULTURE OF PRIMARY HEPATOCYTES ON THERMORESPONSIVE SURFACE

To see whether hepatocyte needs supporting ECM for growth, cells were cultured on bare PIPAAm surface as well as PIPAAm dishes coated with collagen as described above. Attachment and spreading of cells on the thermoresponsive surface was compared after 24 h.

3.10.1. Retrieval of hepatocytes by temperature variation

Adult rat hepatocytes cultured on collagen coated PIPAAm grated surface were retrieved by temperature variation without enzyme treatment. Cells were cultured as described above on coated polymer for 27 days. For cell retrieval, cultures were incubated below 10°C for 10 min. The cells were retrieved as patches or clusters from the dish by gentle pipetting.

3.10.2. Metabolic ability of retrieved cells

To determine the functional ability of hepatocytes during cell sheet retrieval, retrieved cells were incubated with EROD substrate as described above and observed under fluorescence microscope

3.10.3. Culturing retrieved cells

Hepatocytes culture on collagen coated PIPAAm dishes were retrieved by temperature variation as described above. Cells retrieved on 1, 2, 3 and 4 days were transferred to new culture surface and allowed to grow.

3.11.HUMAN FOETAL HEPATOCYTE CULTURE

Non-viable human fetuses matured to 12 – 15 weeks discarded during Medical Termination of Pregnancy were collected from Sree Avittom Thiruanal Hospital (Trivandrum, India) under approval from the ethical committees of institute (Agenda Item No.9 dated 20-07-2002) and hospital (G1/3098/03/MCT dated 19-05-2003).

3.11.1. Cell isolation

The sample was used for cell isolation within 2 – 3 h of disposal and entire procedure was performed under aseptic conditions. Sample was cleaned with sterile gauze before dissection. A longitudinal incision was made at pubic symphysis and extended to xiphoid process along lateral sides. Ligaments and tissue connections between abdominal wall and viscera were disconnected. While holding umbilical cord, the flap of abdominal wall was removed by a circular cut around the umbilicus leaving a ring of tissue around the cord. Excess cord was cut off at a distance of 10 cm from the body. A cannula was connected to umbilical vein and secured using surgical thread. While holding the umbilical cord, whole liver was excised off and transferred to a sieve. Liver was then perfused by double perfusion method as described earlier for rat hepatocytes.

3.11.2. Characterization of cells

Liver cells were characterized by immunofluorescence staining of albumin as described before. Cells were counter stained with Hoechst to visualize nucleus. The samples were mounted in 1:1 PBS-Glycerol solution and observed under fluorescence microscope using FITC and UV-2A filter.

3.11.3. Actin cytoskeletal staining

To observe the actin cytoskeletal distribution, cells fixed in paraformaldehyde was incubated with FITC phalloidin as described before and observed under fluorescence microscope.

3.11.4. Differentiation capacity of liver cells to adipocytes

To assess differentiating capacity of foetal liver cells into different lineages, cell were cultured in adipogenic induction medium (IMDM containing 0.5 mM 1-methyl Isobutyl Xanthine, 10μg/ml insulin, 0.2μM indomethacin & 10 μM Dexamethazone) and supportive medium (IMDM with 10μg/ml insulin). Cells were cultured for 14 days with medium replacement on every 3 to 4 days for three cycles alternating between induction and supporting media.

The cells were monitored under phase contrast microscope for morphology changes. Cells fixed in 10% buffered formalin for at least 1 h was incubated at room temperature for 2 h with oil red O solution (0.5 g of oil red O in 100 ml of isopropyl alcohol). Cells were washed twice with distilled water and observed under microscope for lipid accumulation.

3.11.5. Culture of human foetal cells on PIPAAm surface

Human foetal liver cells were subcultured on PIPPAAm grafted culture dish for non invasive cell retrieval. After removing culture medium from the monolayer on PIPAAm, culture was incubated below 10 °C for 5 -10 min to detach cell sheet. Gentle pipetting with cold culture medium retrieved cells from the PIPAAm surface as patches without trypsin treatment.

Chapter 4

RESULTS AND DISCUSSION

Several approaches including extracorporeal devices, cell transplantation and tissue engineered constructs have been proposed as potential adjuncts or as replacement for transplantation. The strategies followed were - optimization of culture condition for hepatocytes, HUVEC and SEC, coculture of hepatocytes and endothelial cells, culture of hepatocytes in EC conditioned medium, spheroid cultures and 3D tissue construct using inhouse synthesized thermoresponsive polymer.

4.1. TEMPERATURE RESPONSIVE SURFACE

The main requirement of tissue engineering is the functional recovery of damaged tissue. Inter cellular communications among different cell types in tissue play critical roles in the maintenance of differentiated cellular functions. Obtaining cells in intact cell - cell and cell - ECM contact can facilitate maintenance of tissue functions. Utilization of thermoresponsive polymer may help in maintaining cell - cell and cell - ECM interaction. This was achieved in this study by using an in-house prepared thermoresponsive surface grafted on normal tissue culture dishes.

Poly (N-Isopropyl acrylamide) having LCST around 32 °C in aqueous medium is being studied as a thermoresponsive polymer for various biomedical research applications since last decade. This polymer finds application as attached to solid surface, cross linked hydrogels and biomolecules to create new grafted surfaces, comb type grafted hydrogels and modified thermoresponsive bioconjugate (Kikuchi *et al*, 1998). The polymer expresses temperature dependent wettability changes in the form of hydrated extended chain conformation below LCST and dehydrated compact form above LCST. The polymer cross linked with copolymers has been developed and used as thermal on – off switching surface for drug loading and releasing (von Recum *et al*, 1998). Zammaretti and Jaconi (2004) reviewed the different approaches for cardiac tissue engineering revealing significance of thermoresponsive surface for such purposes.

Okano *et al* (1993) showed that cells cultured on surface grafted with PIPAAm by electron beam irradiation detaches spontaneously by lowering the medium temperature . The use of thermoresponsive surface in cell culture enables to acquire cells from their culture environment with intact cellular arrangements and organizations as designed *in vitro*. During detachment as a confluent layer from the temperature sensitive surface, the cells maintain interactions with cells and ECM, whereas ECM interaction with grafted surface decreases.

For various applications of *in vitro* culture, retrieval of anchorage dependent cells is done by enzymatic or mechanical methods (Freshney, 2004) which hamper the cell-cell and cell-ECM binding. In this study an in-house prepared stimuli responsive culture surface was used to retrieve intact cells. The surface was synthesized by grafting PIPAAm with γ-ray irradiation, the most commonly used sterilization method, instead of previously reported methods of electron beam (Okano *et al*, 1993) or UV irradiation (Morikawa *et al*, 2002). This process of grafting eliminates the need for sophisticated equipment like electron beam accelerator. Previous reports of concentration of PIPAAm on grafted surfaces ranges from 35% to 55%. Here PIPAAm monomer at 35%, 45% and 55% were used to graft the culture surface. All components were found to be suitable for cell culture. In this work a total of 8 batches were prepared at 45% w/w concentration and all experiments have been carried out with this. For cell culture studies, different cell types like L -929, NRK-49F, HOS and SIRC have been used to culture on temperature responsive PIPAAm homopolymer.

The polymer grafted culture dishes were sterilized either by γ-ray irradiation or by ethylene oxide. There was no influence of sterilization method on growth of cells on grafted polymer. The PIPAAm grafted culture dishes stored upto two years at room temperature has been found to be suitable for cell culture.

4.1.1. Characterization of polymer

Poly (N- Isopropyl acrylamide) grafted tissue culture dishes were characterized by ATR-FTIR, water contact angle measurement and SEM analysis.

4.1.1.1. FTIR analysis

Fourier Transform Infra Red (FTIR) spectroscopy is a powerful analytical tool for characterizing and identifying organic and inorganic molecules by identifying chemical bonds and the molecular structure.

The FTIR spectrum of PIPAAm grafted surface depicted a shift in peak from 1600 cm^{-1} to 1650 cm^{-1} in comparison with ungrafted culture dish (Figure 7). This confirmed grafting of PIPAAm on culture dish by the presence of amide peak at 1650 cm^{-1} instead of the monosubstituted aromatic ring peak at 1600 cm^{-1}.

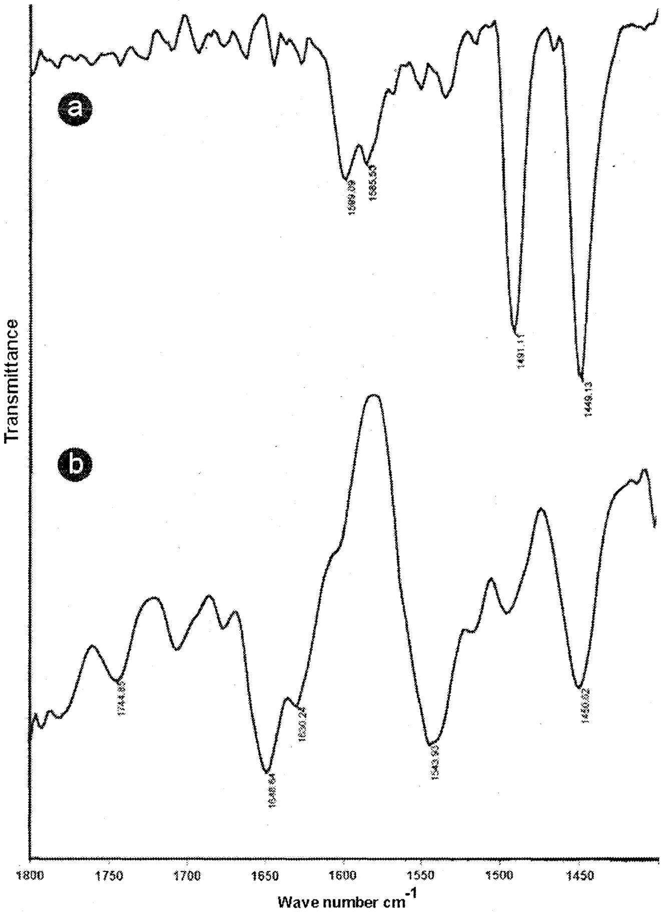

Figure 7 ATR FTIR spectrum of (a) commercial tissue culture dish depicting the peak of monosubstituted aromatic ring at 1600 cm^{-1} and (b) PIPAAm grafted tissue culture dish showing the amide peak around 1650 cm^{-1}.

The grafted TCPS by γ-ray method showed the presence of ATR-FTIR peak of the amide group at 1650 cm^{-1} in conformity with earlier reports (Yamato *et al*, 2000).

4.1.1.2. Surface analysis by SEM

SEM is used as an important tool for analyzing the surface characteristics of polymeric biomaterials (Merette *et al*, 2002) and for assessing cellular responses to biomaterials. SEM analysis of PIPAAm grafted surfaces did not show any surface alteration when compared to ungrafted TCPS surface (Figure 8).

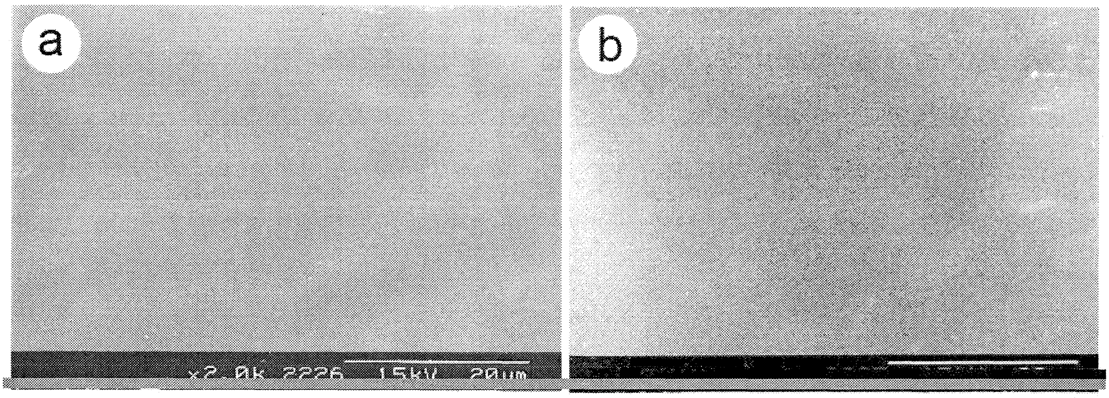

Figure 8 Scanning Electron Micrograph of (a) ungrafted and (b) grafted culture dish showing no alteration in surface morphology

4.1.1.3. Water contact angle

Water contact angle is a simple, fast and sensitive method to measure the wettability of a solid surface. Water contact angles of grafted and ungrafted surfaces were measured using deionized water (Millipore) at 37 °C and 27 °C. At 37 °C, water contact angle measurement of polymer grafted surface showed relatively hydrophobic contact angle when compared to contact angles at lower temperature of 27 °C. The contact angles of untreated culture dishes were almost same when compared to that of lower temperature at 27 °C (Table 4).

Table 4 Sessile drop contact angles for culture surfaces
(θ = Mean ± SD)

	θ at 27 °C	θ at 37 °C
Untreated TCPS	39.55 ± 2.99	40.6 ± 2.50
PIPAAm grafted TCPS	32.65 ± 1.02	35.8 ± 1.61

Contact angle measurements on the normal and grafted culture surfaces showed the hydrophilic-hydrophobic changes with variation in temperature. The difference in the contact angle is due to the temperature influenced hydration of surface grafted with PIPAAm. The grafted surface was more hydrophilic at room temperature and below, when compared to ungrafted surface. The results of water contact angle from different areas of grafted surface confirmed the even grafting of polymer using γ-ray irradiation method.

4.2. CELL ATTACHMENT STUDIES ON POLYMER

Thermorsponsive polymer surface was used to culture cell lines as well as primary isolated cells. In both cases the cells attached and grew as normal. Cell lines like L-929, NRK-49F, SIRC and HOS were able to grow without any further treatment (Figure 9 a-d). Primary cells normally require ECM coated surfaces for adherence. On grafted surface also ECM coatings can be done as required. Rat hepatocytes attached on collagen coated grafted surfaces and formed monolayer (Figure 9 e). However human foetal liver cells were able to adhere and proliferate on uncoated grafted surface (Figure 9 f).

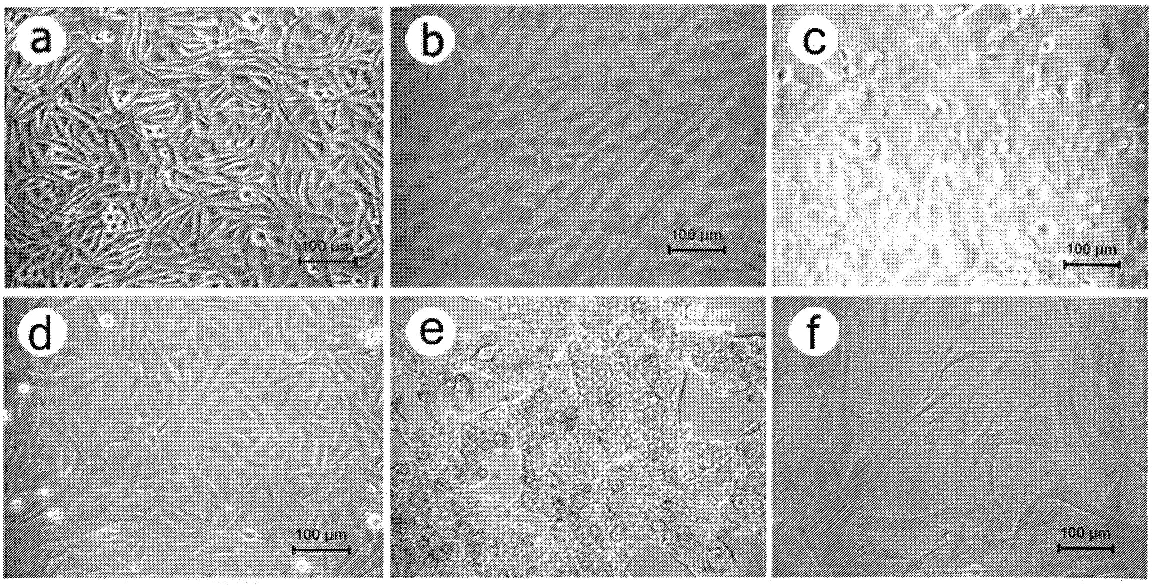

Figure 9 Different type of cells cultured on PIPAAm grafted culture dishes. (a) L-929, (b) NRK-49F, (c) SIRC, (d) HOS, (e) primary rat hepatocytes on collagen coated PIPAAm dish and (f) primary human foetal liver cells on PIPAAm

It has been previously reported that progenitor cells can be cultured on PIPAAm grafted thermoresponsive surfaces. This is the first attempt to study human foetal liver cells on PIPAAm grafted surface. Foetal liver cells were able to adhere and proliferate on grafted surface without modification using ECM coating.

To see the actin cytoskeletal organization of cells cultured on polymer, L-929 cells were stained with FITC – Phalloidin and cell viability was assessed by live-dead staining. Cells cultured on normal TCPS was used as control. There was no change in actin distribution of L-929 cells on polymer and control (Figure 10). The cells adhered on polymer was viable as evidenced by the uptake of live stain acridine orange by all cells (Figure 11).

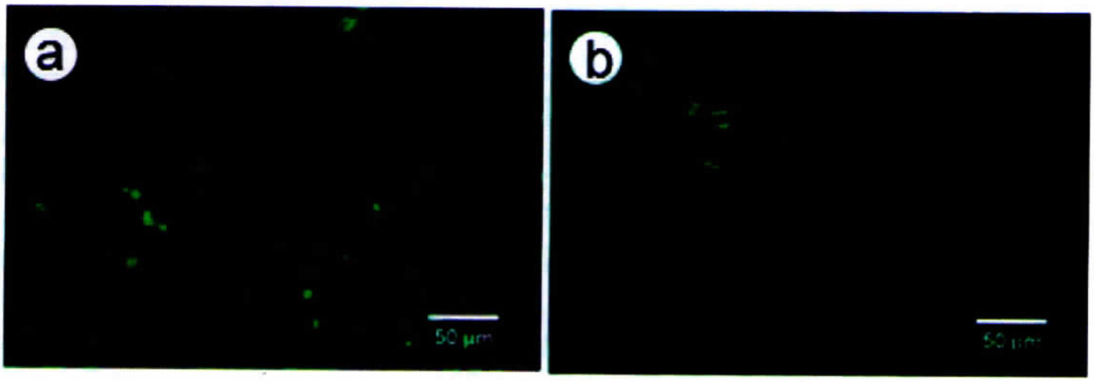

Figure 10 Actin cytoskeletal distribution of spread L-929 cells on (a) TCPS and (b) PIPAAm grafted culture surface.

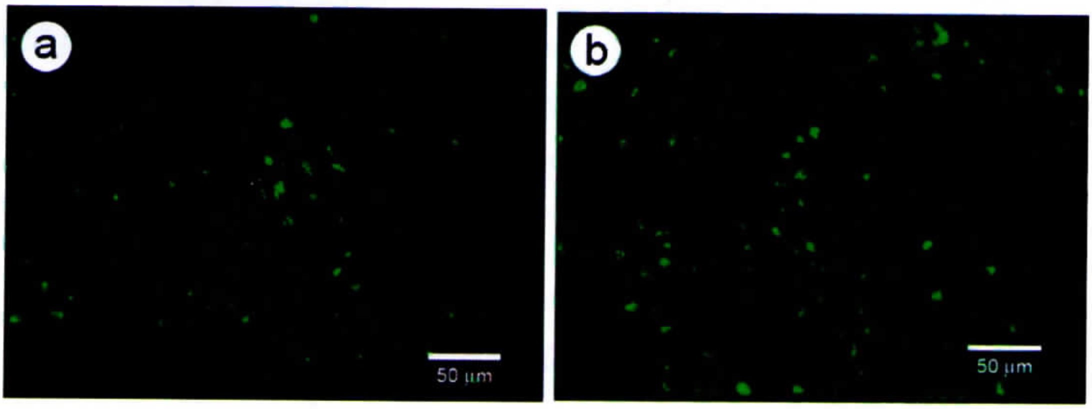

Figure 11 Live dead staining of L-929 cells on (a) TCPS and (b) PIPAAm grafted culture surface.

4.3. CELL RETRIEVAL STUDIES ON TEMPERATURE RESPONSIVE SURFACE

Thermoresponsive surface is a new tool for tissue engineering to achieve the preparation of artificial cell construct for transplantation as well as for the investigation of intact multilayered cell sheets or tissue like structures.

Cell attachment on hydrophobic surface involves tight binding of various proteins like albumin, myoglobin and fibronectin on which the cells attach (Kushida *et al*, 1999). Culture of cells on PIPAAm grafted surface above LCST is similar to that of cultured cells in normal condition. In this study different cell types like L-929, NRK-49F, SIRC and HOS cultured on PIPAAm were transferred without enzyme or mechanical treatment. During low temperature incubation at ≤ 20 °C, cells cultured on PIPAAm detached without any enzyme treatment.

4.3.1. Cell sheet transfer with membrane support

When cell monolayer on grafted surface was incubated at ≤ 20°C with a PVDF membrane placed over it, the cells detached as sheet and adhered on to the membrane. Low temperature initiated hydration of the surface and thereby cell detachment. At this stage, confluent cells adhered to the membrane retaining cell – cell contacts, other cellular arrangements and organization. The membrane together with the cells was peeled off and transferred to new surface and cultured as normal. Figure 12 and Figure 13 shows the transfer of L-929, NRK-49F, HOS and SIRC by this method. Observation of cells at 0h showed the transfer of intact cell patch to new surface. Upon reincubation at 37 °C the cells from the membrane adhered to new surface as intact cell sheet constructs. The transferred cells on new dishes grew with normal morphology (Figure 12 and Figure 13) within 24 h.

Low temperature treatment of cell culture on PIPAAm help in detachment of intact cell sheet structures. The detachment of cells might have occurred due to increase in hydration of surface as evidenced by the water contact angle measurement.

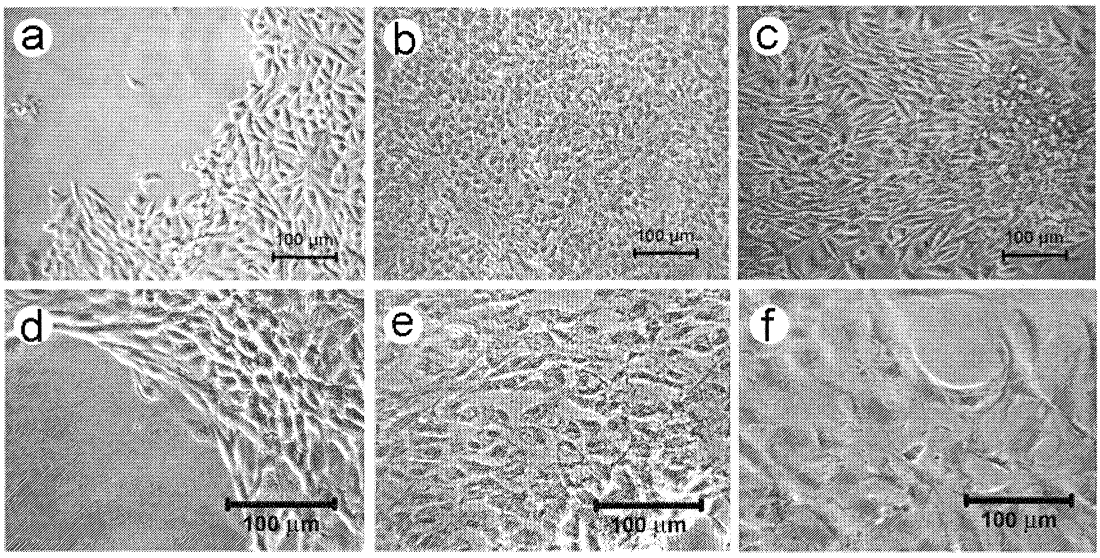

Figure 12 Cell sheet transfer with the help of membrane support. Images in each row show the removed cell monolayer, transferred cell sheet at 0h and transferred cell sheet after 24 h respectively. Cell lines used were L-929 (a, b and c) and NRK-49F (d, e and f).

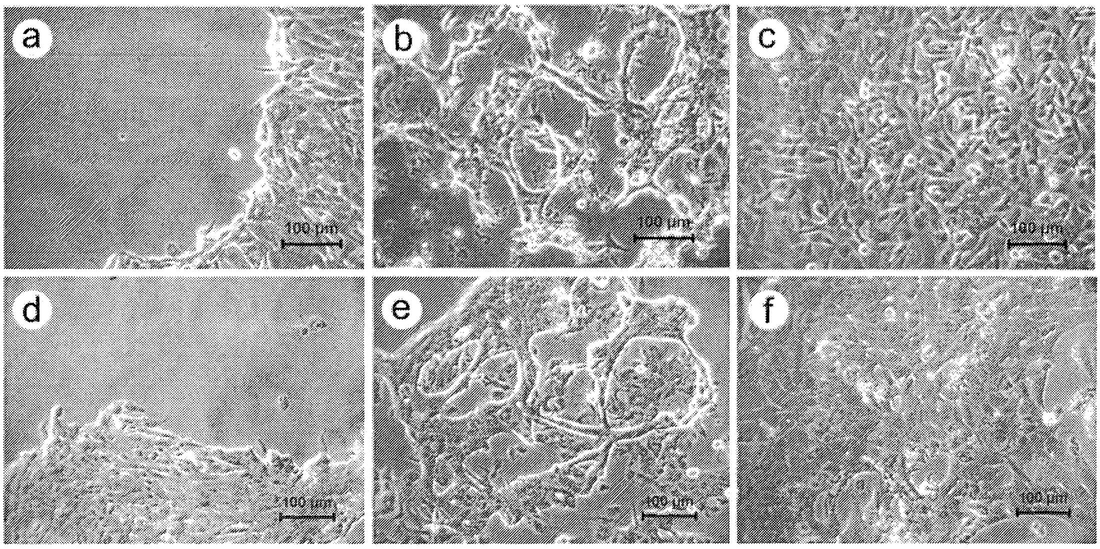

Figure 13 Cell sheet transfer with the help of membrane support. Images in each row show the removed cell monolayer, transferred cell sheet at 0h and transferred cell sheet after 24 h respectively. Cell lines used were HOS (a, b and c) and SIRC (d, e and f).

To assess the viability of cells after transfer, HOS cells were subjected to live-dead staining. All cells transferred to new surface were viable after 24 h culture (Figure 14).

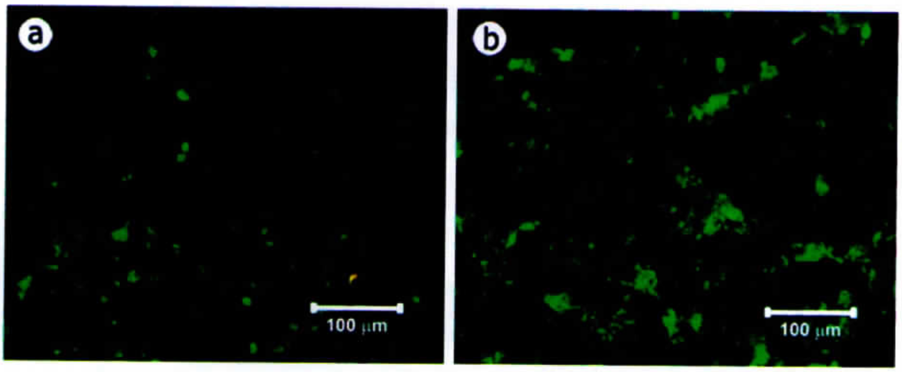

Figure 14 Live-dead staining of HOS cells cultured on PIPAAm surface. Cells showed viable staining (a) on polymer surface and (b) 24 h after transfer using membrane support to new culture dish.

4.3.2. Cell sheet transfer without membrane support

Monolayer on PIPAAm grafted polymer can also be retrieved without the use of support membrane (Zhang *et al*, 2006). Incubation at low temperature favors detachment of monolayer that can be physically separated from the culture dish. Low temperature treatment resulted in decrease in adhesion between the ECM and culture surface while maintaining the strong cell – ECM and cell-cell contact.

4.3.2.1. Generation of Peeled off cell sheets

L-929 and SIRC cells were able to be peeled of physically using a forceps as cell sheets from grafted surface by lowering the temperature. The method resulted in retrieval of cells as intact sheet like structure (Figure 15).

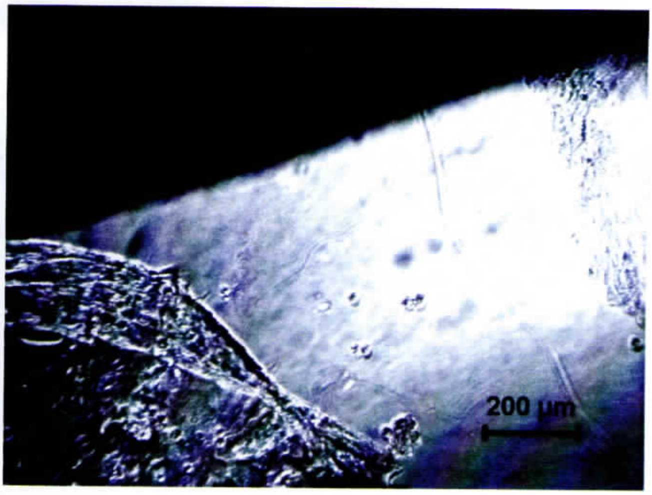

Figure 15 Photograph showing peeling off SIRC cell monolayer

Actin cytoskeletal staining is perfomed to analyze the adhesion characteristics of cells. To asses the cell cytoskeletal distribution of cells in retrieved cell sheet, L-929 cell sheets were stained with FITC-Phalloidin. The cells expressed cortical staining pattern inside the cell patch (Figure 16). Cortical staining of cells is noticed during low adhesion to substrate (Ball *et al*, 2004). Hence it can be deduced that cortical pattern observed is due to the non adhered stage of individual cells in sheet which evidences the maintenance of cell-cell and cell-ECM binding during retrieval of cell sheets.

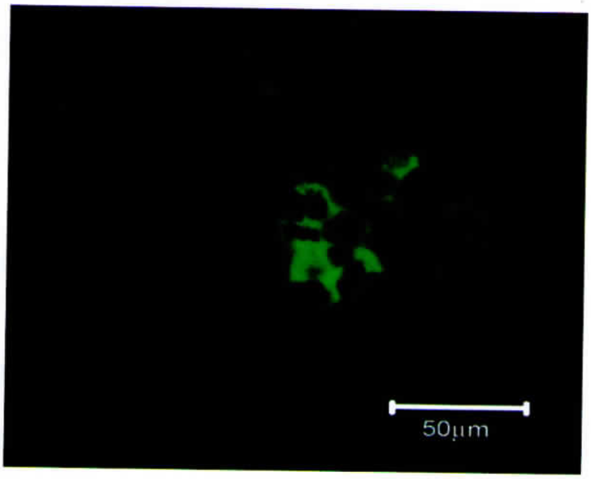

Figure 16 Cortical staining pattern of actin cytoskeletal distribution in L-929 cell patch obtained from PIPAAm surface by temperature variation.

4.3.2.2. Generation of cell sheet patches

Human osteoblast cell monolayer on PIPAAm grafted surface when incubated at ≤ 20 °C detached from the grafted surface as patches of cell sheet structures. Gentle pipetting yielded cell patches with varying sizes from 25 - 560 μm (Figure 17) which retained the intact cell - cell and cell – ECM contact.

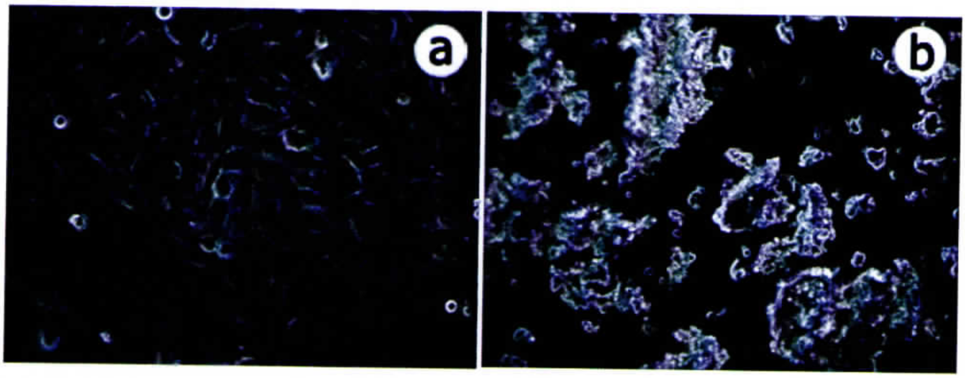

Figure 17 HOS monolayer on (a) PIPAAm grafted culture dish and (b) Cells retrieved patches by gentle pipetting.

Confocal images in different z plane of HOS cell sheet construct stained for actin cytoskeletal structures revealed cortical staining pattern (Figure 18a). Three dimensional reconstructed images created using images acquired at z planes gave illustrative expression of cell sheet structure (Figure 18b). The live-dead staining of retrieved cell sheet patches revealed the viable nature of cells (Figure 18c).

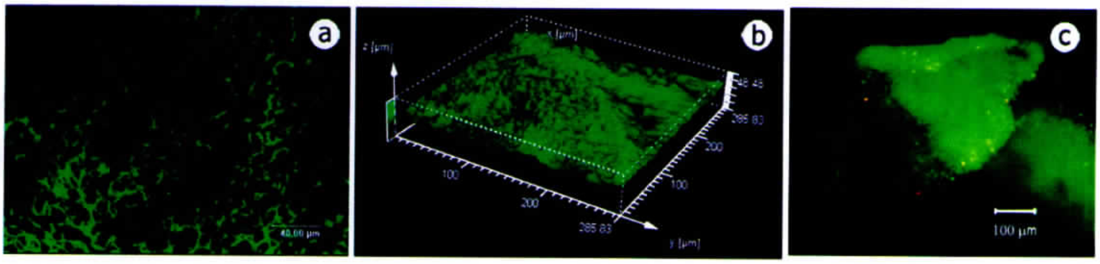

Figure 18. Actin cytoskeletal staining of HOS cell sheet observed under confocal microscope (a) showing cortical staining pattern. (b) 3D reconstructed image of cell sheet obtained in z plane showing the tissue architecture. (c) Live-dead staining of HOS cell sheet. Entire tissue construct represent viable staining (green).

4.3.3. Tissue engineering using cell sheets

4.3.3.1. Cell transfer to scaffold

Cell patches obtained as above were used to cellularize scaffold materials. One of the major challenges in creating Cell – Scaffold construct is to homogenously seed cells inside the scaffold. The available method of improving cell material interaction is by modifying material as well as adjusting the porosity (Cerroni *et al*, 2002). Furthermore, cell attachment and adhesion is a critical factor determining the proliferation and function of cells (Malik *et al*, 1992). When cells are loaded as suspensions to the surface of the scaffold, they will first adhere, grow and then migrate (Anselme *et al*, 2000). Moreover cells form a tissue layer on outer surface which will further inhibit the growth of cells inside the scaffold. This can hamper cell penetration and growth inside the scaffold which is of major concern in tissue engineering. In the current study cells are incorporated inside the scaffold as preformed patches to speed up the Cell-Scaffold formation.

Hydroxyapatite is a major component of the inorganic compartment of bone $Ca_{10}(PO_4)_6(OH)_2$. An inhouse synthesized calcium phosphate based bioceramic has similar composition as natural HA. Hydroxyapatite can be synthesized through wet chemical routes from calcium and phosphate containing reactants that can be made in the form of porous blocks, sintered rods, granules etc. Main clinical use is as bone substitute or marrow extender in various bone and dental defects. In this study osteoblast cells retrieved as cell sheet structures were used to cellularize HA materials for tissue engineering application.

4.3.3.2. Analysis of cellularized scaffold

Osteoblast cell sheets when top loaded on PHA and DHA adhered as patches within 1 h on both materials. Fluorescence staining showed cell patches with varying sizes that are homogenously adhered on DHA (Figure 19a). On PHA, cell sheet adhered on surface as well as inside the lining of pores (Figure 19b). Supporting the observations of fluorescence staining, SEM images of DHA (Figure 19c) and PHA (Figure 19d) confirmed even distribution of cell patches. In the case of PHA, cell sheets were able to penetrate into the pores and adhered inside the lining of pores as patches which is clearly demonstrated by fluorescence staining (Figure 19e) and SEM (Figure 19f).

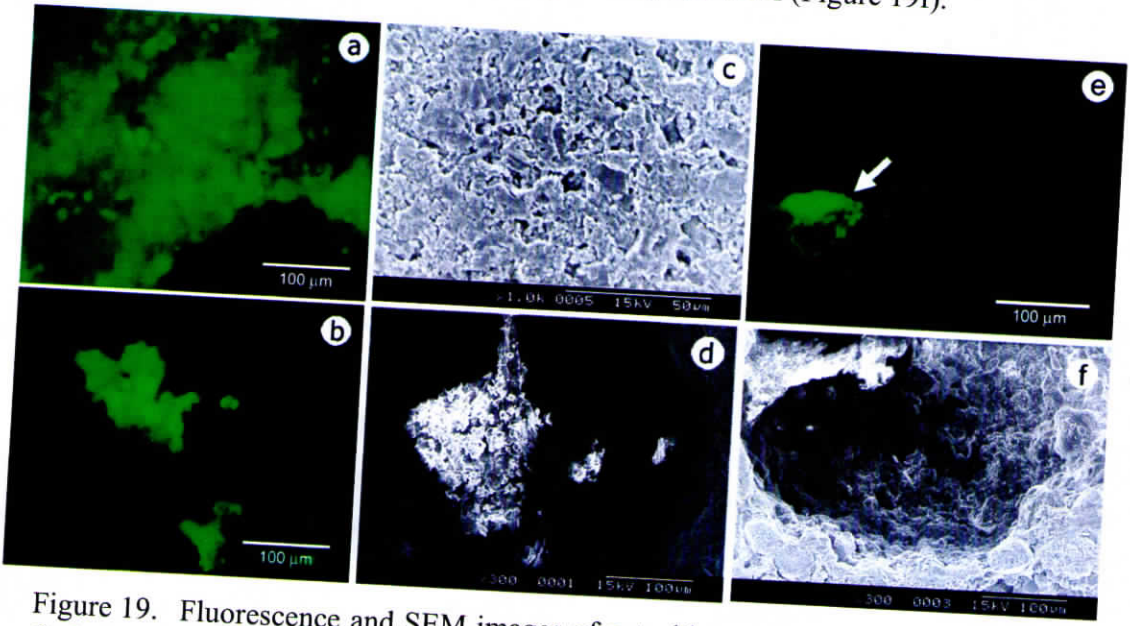

Figure 19. Fluorescence and SEM images of osteoblast cells (a & c) on DHA and (b & d) PHA demonstrating even distribution of cell patches. (e & f) Maintenance of porous nature of scaffold after 1 h.

After 2 days of incubation, osteoblast cell patches spread, forming monolayer on the surface showing characteristic morphology on both DHA and PHA (Figure 20 a & b). On DHA, cells adhered and grew as patches (Figure 20 c). On PHA scaffold, cells adhered on all surface as patches including lining the pores and spanned across the material (Figure 20 d). Cells lining the pores were visualized by confocal microscopy (Figure 20 e). Even though the cells covered and spanned the whole surfaces and channels, the microporous nature of the scaffold was maintained (Figure 20 f).

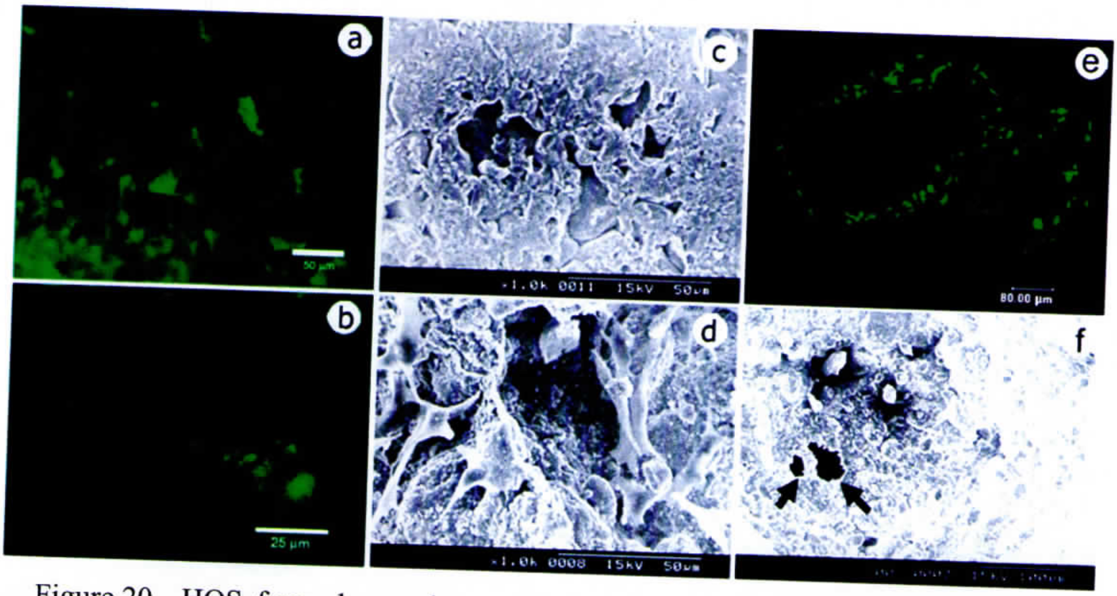

Figure 20. HOS formed monolayer and covered all surface after 2 days (a) on DHA and (b) PHA. SEM images showing surface morphology of cells on (c) DHA and (d) PHA. (e) Osteoblast cells lining the channels of PHA, when observed under confocal microscope. (f) Morphology of cells lining the pores showing monolayer formation under SEM. Cell growth did not block the porous nature (arrows) of PHA.

Culture of HOS cells on scaffolds for 7 days gave complete cellularization of the material. On DHA, cells formed thick monolayer with intact cell – cell contact. Fluorescence staining showed cells as thick patch (Figure 21 a). When observed under SEM, cells appeared as multilayered and clumped forming thick sheets over the surface (Figure 21 c). Cells on PHA covered as monolayer on material and formed complete lining on the surface (Figure 21 b & d). Only very little area of material was found to be cell free after 7 days (Arrow in Figure 21 d).

Complete cell coverage on materials was observed at 7 days culture due to favorable attachment and proliferation of cell patches (Figure 21 e). SEM images of HOS cell sheets showed characteristic cell morphology with ruffles. Even though complete cellularization was observed, there was exposed HA at discrete points. There was no topography change or recrystallization at cell free exposed surface of HA. Multilayered cells did not hamper the porous structure (Figure 21 f). The cells lining channels of PHA without affecting the porosity was better visualized under fluorescence microscope (Figure 21 g).

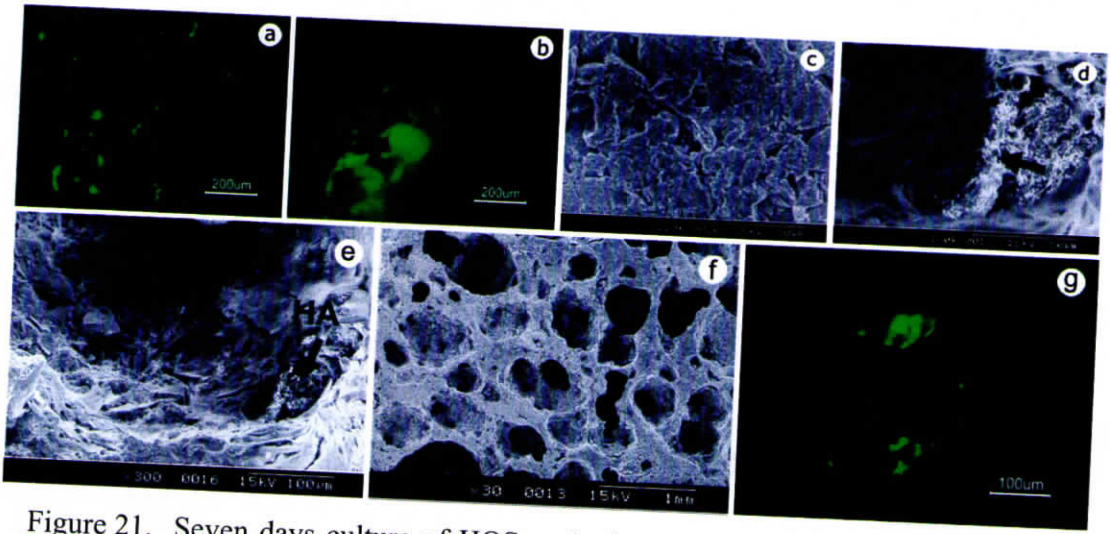

Figure 21. Seven days culture of HOS on hydroxyapatite. (a & c) cell growth on DHA forming thick sheets. (b & d) complete cell coverage on porous structure of PHA. Arrow in figure d indicates exposed HA part without recrystallization. (e) SEM image showing complete cellularization with exposed PHA at discrete points (arrow). (f) Porosity of the scaffold even after intense cell coverage. (g) Fluorescence image showing lining of cells on porous structure without hindering the porous nature.

The viability, proliferation and functionality of osteoblast in the scaffold are important in bone tissue engineering applications. EDS is one of the methods to analyze *in vitro* mineralization (Tsuru *et al*, 1988). Spectral peak representing carbon at 0.2 keV was compared. Cellularized PHA by cell patch transfer analyzed for EDS analysis showed an increase in carbon peak on 7th day (Figure 22 a & b). The appearance of carbon peak represents either carbonate substitution in the calcium apatite structure during remineralization due to osteoblast function or due to the increase in cell number. Proliferation of cells transferred as patches on PHA and DHA, determined by [3H] thymidine incorporation assay, showed different multiplication pattern. After 2 days, number of HOS cells on both materials was found to be similar. However after 7 days, cells in the PHA was found to be more when compared to cells on DHA (Figure 22 c).

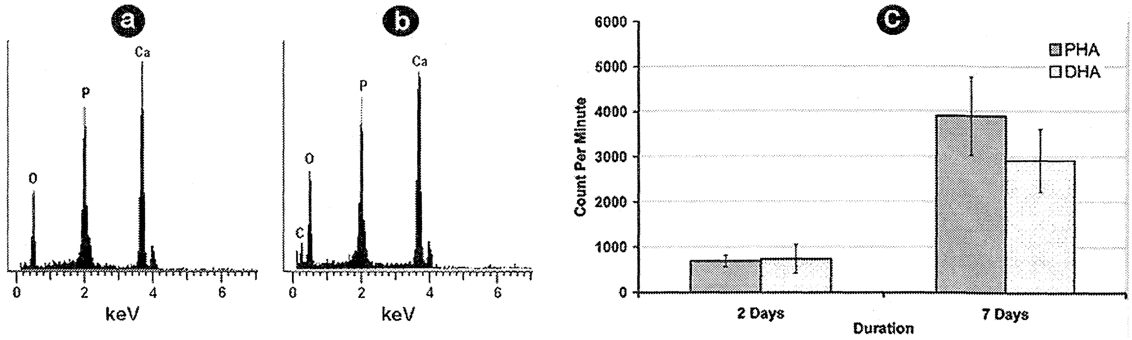

Figure 22. SEM/EDS spectrum of cellularized PHA showing HOS cell patches after (a) 1 hr and (b) 7 days showing appearance of carbon peak at 0.2 keV (labeled 'C'). (c) Proliferation of HOS cells transferred as patches on PHA and DHA, determined by [3H] thymidine incorporation assay. Graph shows average cpm ± standard deviation on 2nd and 7th day.

Alkaline phosphatase activity of HOS cells seeded on PHA by conventional trypsinization and cell sheet transfer were analysed. When cells were transferred to PHA and TCPS by conventional method of trypsinization there was not much difference in ALP activity during all 7 days (Figure 23 a & b). Enzyme activity of transferred cells as patches to PHA was found to be more at 1st day, showed a decrease on 3rd day followed by an increasing trend at the end of 7th day (Figure 23a) when compared to that of cells transferred by trypsin method. This decrease in ALP activity seen during cell sheet transfer on PHA could be due to the change in enzyme expression during mineralization process. This support the appearance of carbon peak in the EDS spectrum.

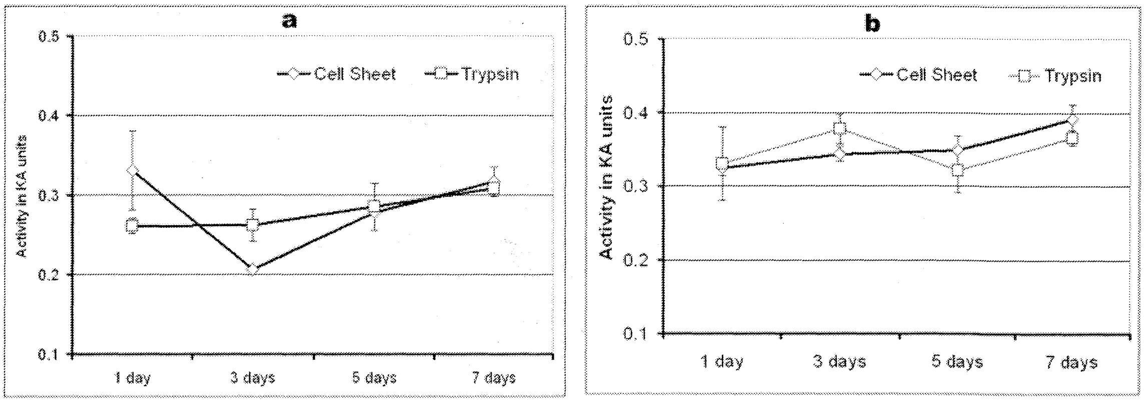

Figure 23. ALP activity of HOS cells transferred by cell sheet and trypsin method on (a) PHA (b) TCPS.

Osteoblast cells express characteristic morphology on hydroxyapatite (Vrouwennvelder *et al*, 1993). In this study osteoblast cells cultured on thermoresponsive polymer were retrieved as cell patches and seeded to porous and dense hydroxyapatite. Osteoblast cells retrieved as cell sheet structure when analyzed for actin distribution expressed cortical staining pattern. Cells during initial stages of adhesion with round morphology exhibit cortical staining pattern of actin filaments (Anselme *et al*, 2004). Cells maintained cell - cell contact and were held together tightly during manipulation. This compactness of cell sheet could be due to the strong cell – ECM interaction. Such cell sheets within the scaffold formed lining on the surface as well as inside the pores by 2 days and covered the entire surface of scaffold within 7 days. As the cell patch maintains cell - cell and cell – ECM interaction, the time delay for the cells to adjust with material is avoided favoring rapid cellularization.

This novel method of cell sheet transfer in bone tissue engineering maintains cell viability inside the scaffold.

4.3.4. Tissue construct with multilayer cell sheets

By membrane support method, cells were over layered to get multi layered tissue constructs. In this study 4 layers of HOS cells were cultured on PIPAAm grafted surface and detached as tissue construct by temperature variation and peel off method. Combination of membrane support and peel off method resulted in generating a tissue construct with 20 mm diameter (Figure 24).

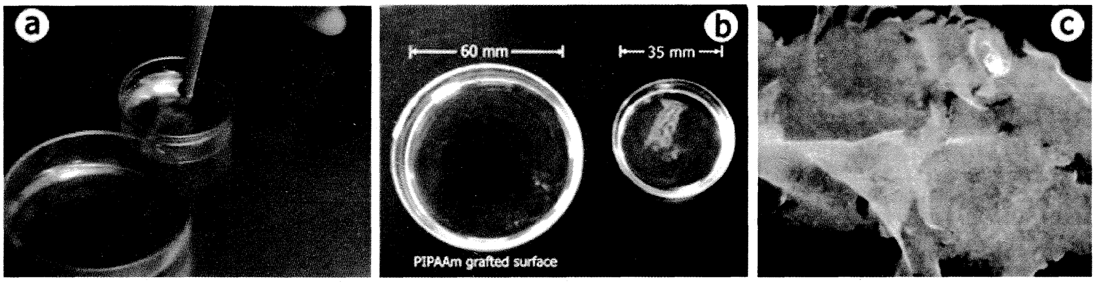

Figure 24. Multilayered tissue constructs generated using HOS cells (a) by combination of membrane support and peel off method. (b) A circular tissue construct with 20 mm diameter transferred to new dish and observed under (c) 3D stereo microscope

Scanning Electron Microscope analysis demonstrated intact tissue like architecture (Figure 25 a) and in high magnification multilayer were visible (Figure 25 b) hydroxyapatite. Osteoblast cells retrieved as cell sheet structure when analyzed for actin distribution expressed cortical staining pattern. Cells during initial stages of adhesion with round morphology exhibit cortical staining pattern of actin filaments (Anselme *et al*, 2004). Cells maintained cell - cell contact and were held together tightly during manipulation. This compactness of cell sheet could be due to the strong cell – ECM interaction. Such cell sheets within the scaffold formed lining on the surface as well as inside the pores by 2 days and covered the entire surface of scaffold within 7 days. As the cell patch maintains cell - cell and cell – ECM interaction, the time delay for the cells to adjust with material is avoided favoring rapid cellularization.

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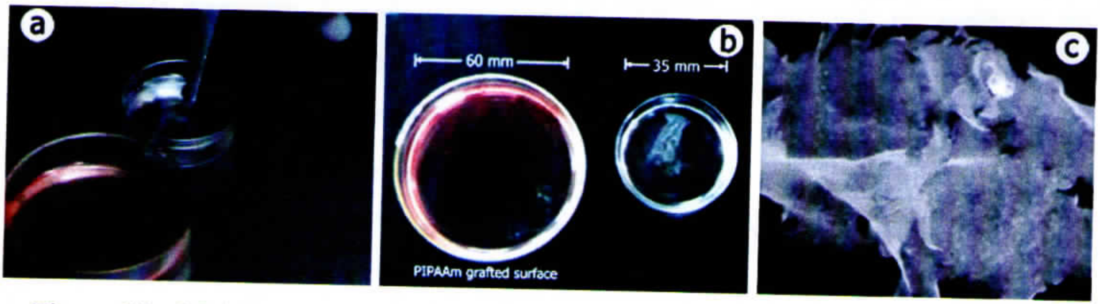

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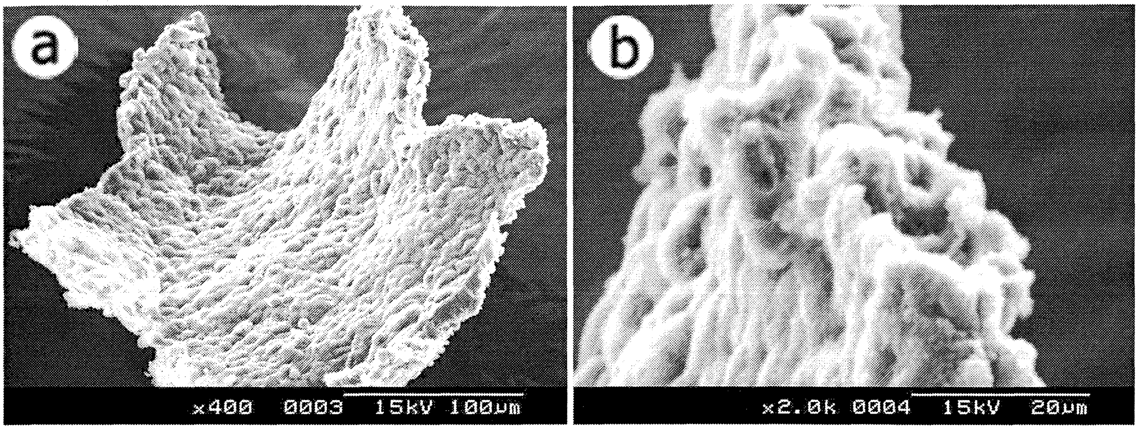


Figure 25. (a) Tissue construct observed under SEM. (b) Individual cell layers shown under high magnification

4.4. HEPATOCYTE CULTURE

4.4.1. Isolation of adult rat hepatocytes

Hepatocytes were isolated from rat liver by double perfusion method giving importance to the ECVAM guidelines using a custom made perfusion apparatus. More than 30 isolations were performed towards standardization of procedure and coculture experiments. Cells obtained by standardized method yielded 85 % - 95 % viability.

Isolated hepatocytes have got widespread use in a number of scientific disciplines, including fundamental and applied studies in biochemistry, pharmacology and toxicology. It is essential to obtain hepatocytes with high percent of viability and functional ability. The procedure followed in this study yielded more than 85 % viability of cells. Reasons for the popularity of this *in vitro* system include ethical and economic considerations, increased knowledge about the technical aspects of hepatocyte isolation and culture, the relative ease of obtaining a homogeneous preparation consisting of a single cell type, the high drug-metabolising capacities of hepatocytes and in particular, the scientific value of using a well-controlled biological model system for mechanistic studies.

The main goal of ECVAM is to promote the scientific and regulatory acceptance of alternative methods to reduce, refine or replace the use of laboratory animals. A workshop conducted by ECVAM in 19-23 October 1993 was on The Practical Applicability of Hepatocyte Cultures in Routine Testing. Based on the discussions which took place with scientists working in both academia and industry, a number of recommendations have been made concerning the use of hepatocyte cultures in testing chemicals (Blaauboor *et al*, 1994). The report highlighted the parameters to consider during hepatocyte isolation like animal strain, anaesthesia, use of heparin, buffers and enzyme, pH, osmolarity, temperature and oxygenation of perfusing buffer and viability of isolated cells.

In this study, hepatocytes were isolated considering the major recommendations made by the ECVAM. Hepatocyte isolation requires special attention like oxygen supply, stable pH and temperature in perfusion medium. For this, a perfusion apparatus was custom made to obtain cells with high viability and function. In the perfusion system, a water bath was included to preincubate perfusion buffer.

4.4.2. Optimization of microenvironment

Selection of suitable medium for hepatocytes, assessed by culturing in different media showed that IMDM with 5% FCS is suitable. Cells cultured in IMDM were able to adhere and spread at 24 h whereas it showed poor adhesion while cultured in RPMI, MEM and serum free IMDM (Figure 26).

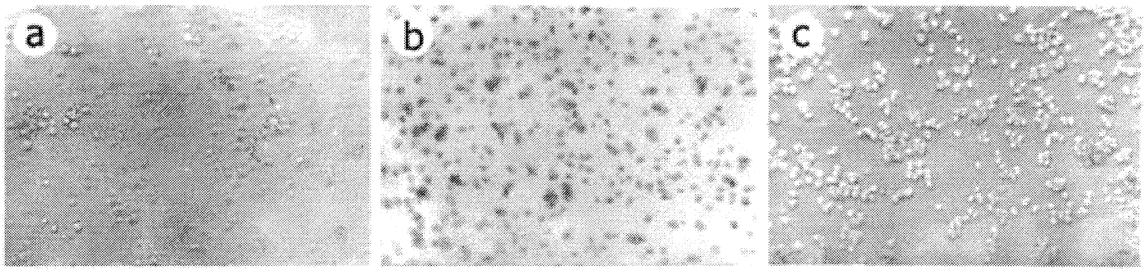


Figure 26. Adult rat hepatocyte cultured in (a) RPMI, (b) MEM and (c) IMDM after 24 h. Adhered and viable cells were more in IMDM with serum.

The presence of serum in media has been reported to improve cell attachment, survival, and morphology. However, serum generally promote growth and have a dedifferentiating effect on hepatocytes by inhibiting cyt P450 activity and favors attachment and proliferation of NPC (Dunn *et al*, 1989)

4.4.3. Culture of hepatocytes

Hepatocytes in selected medium (IMDM with 2 to 2.5 % FCS) on collagen-coated culture dishes adhered and spread with characteristic features and morphology (Figure 27a). Hepatocytes expressed spreading on collagen with binucleated polygonal morphology. A characteristic feature of liver growth *in vivo* is the progressive polyploidization resulting in increasing fractions of tetraploid and octaploid hepatocytes as a function of animal age. Polyploidization proceeds in two mitotic steps - binucleation and mononucleated with polyploid DNA content. It has been shown that hepatocytes growing on a collagen substratum undergo binucleation to a similar extent as during normal, developmental growth *in vivo* (Mossin *et al*, 1994). Binucleation of hepatocytes expressed in this study also indicate similar characteristics.

Bile canalicular like structures formed between adjacent hepatocytes is morphological evidence for hepatocyte differentiated characters. Observation of hepatocyte monolayer under high power magnification showed bile canaliculi like structures between adjacent cells (Figure 27b). Hepatocytes maintained on collagen spread continuously until forming a confluent monolayer within 24-48 h with the presence of punctate translucent sites between neighboring cells illustrating irregularity of cell borders indicating bile canaliculi generation (LeCluyse *et al*, 1994). This is due to the expression of apical domain characteristics of differentiated hepatocytes in between adjacent cells. Formation of bile canaliculi structures were also observed in hepatocyte culture with minimal culture requirement used for coculture studies. Extensive network of translucent spaces were observed in hepatocytes cultured in IMDM with 2 % FCS (Figure 27c).

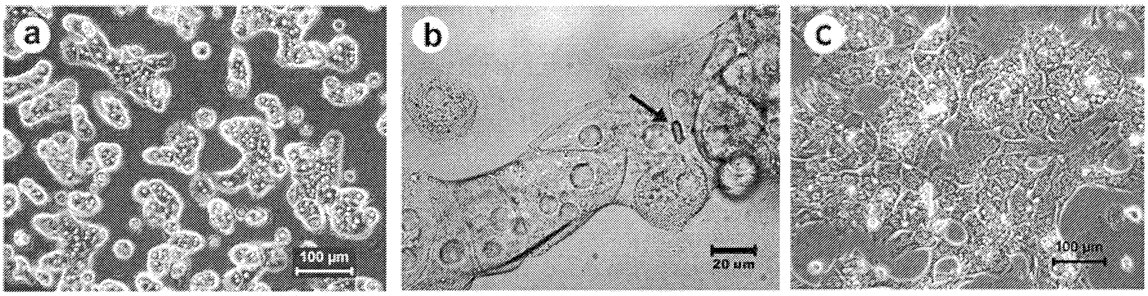


Figure 27 Hepatocytes on collagen after 24 h showing (a) binucleation (b) bile canaliculi (arrow) and (c) canalicular network.

Hepatocyte proliferation over 7 days determined by radio labelled assay indicated that the initial reading was increased after 5 days in culture (Figure 28) and then maintained upto 7 days. This shows that hepatocytes might have duplicated its nucleus or increased ploidy as described earlier (Mossin *et al*, 1994). This indicated characteristic behavior of hepatocytes in the restoration of differentiated conditions similar to *in vivo*.

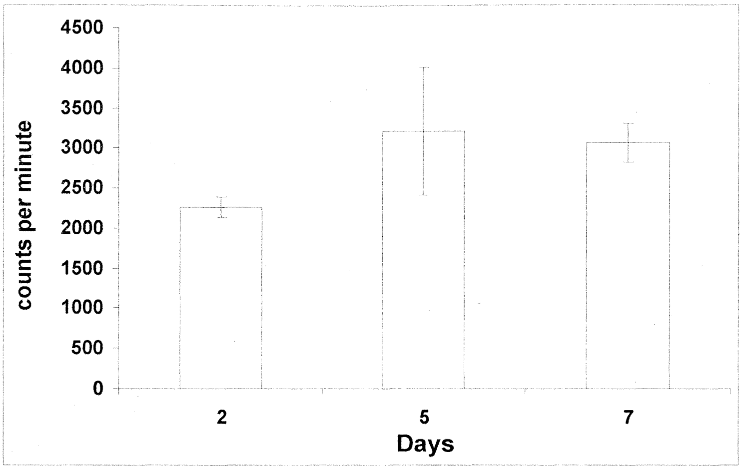


Figure 28 Tritiated thymidine uptake by primary hepatocytes cultured on collagen

4.5. HUMAN UMBILICAL VEIN ENDOTHELIAL CELL CULTURE

Endothelial cells (HUVEC) were used to standardize the coculture system with hepatocytes due to the easy cell isolation method and its proliferative nature *in vitro*. There are very few reports on hepatocyte - HUVEC coculture. To understand the paracrine role of hepatocyte and neuroblastoma cells in HUVEC cells, Beierle *et al* (2001) used conditioned media from cell line Chen hepatocyte (CCL 13) and neuroblastoma (IMR 32) to assess the HUVEC proliferation. It was reported that VEGF were found in both conditioned medium that favoured HUVEC cell growth. In this study rat hepatocytes were cultured on hepatic and vascular EC monolayer and conditioned medium. Hepatocyte self assembly and tissue organization was studied in the hepatocyte – EC coculture.

Endothelial cells cultured in growth factor isolated from bovine brain maintained EC characteristics and proliferation over passages. Endothelial cells from human umbilical vein were seeded on gelatin coated dishes in IMDM containing 10 % FCS with a medium change on every day or alternate days. IMDM has been used for endothelial cell culture for other studies also (Villars *et al*, 2002). Culture medium removed during change was collected as conditioned medium. Cells expressed characteristic cobble stone morphology (Figure 29) on subsequent passages by trypsinization. Cells at a minimum of third passage were used for experiments.

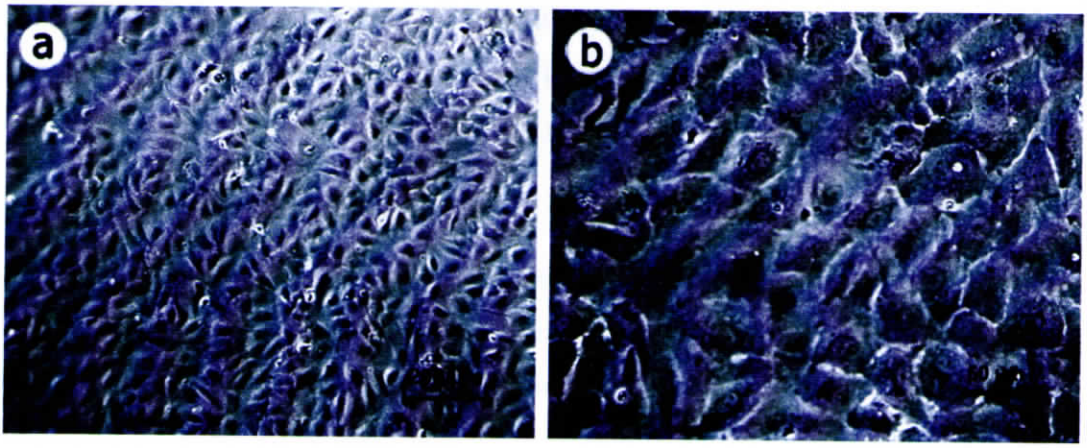


Figure 29 HUVEC monolayer (a) showing cobble stone morphology (b) monolayer under high magnification showing cell-cell contact.

4.5.1. Charaterization of HUVEC

4.5.1.1. vWF expression

The characteristic von Willibrand Factor (vWF) antigen expression by HUVEC was demonstrated by immunoflourescence staining. Cell monolayer on coverslips counter stained with 0.05 µg/ml propidium iodide and observed under confocal microscope (Carl Zeiss LSCM 510 Meta, Germany) using 488, 514 nm excitation filters and LP 505nm, LP 610nm emission filters showed vWF and nucleus (Figure 30a).

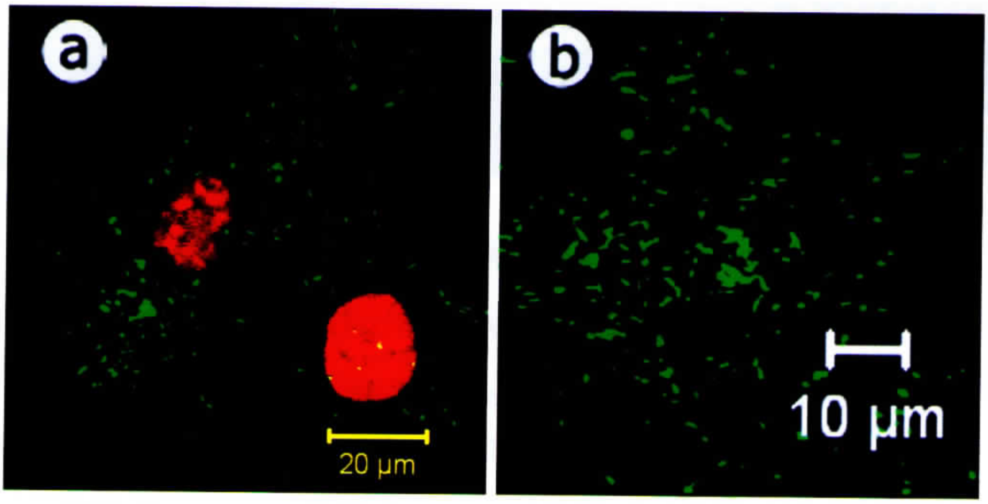


Figure 30 (a) vWF expression by HUVEC. (b) Rod shaped Weibel-Pallade bodies in zoomed image.

In physiology, Weibel-Palade bodies are organelles in the endothelial cells. The two major constituents of Weibel-Palade bodies are von Willebrand factor (vWF), a multimeric protein involved in blood coagulation and P-selectin, which binds to passing immune cells (leukocytes). vWF localized inside the rod shaped Weibel-Palade bodies was seen in immunostained endothelial cells under confocal microscope (Figure 30b).

4.5.1.2. DiI-Ac-LDL uptake

Modification of the lysine residues of the apoprotein B in low density lipoproteins (LDL) by acetylation (Ac) or acetoacetylation (AcAc) results in a dramatic acceleration of the plasma clearance of these lipoproteins when they are injected into animals. Cells with a scavenger pathway can take up modified LDL and it is generally done by cells in liver. It is known that LDL uptake is happening primarily in every endothelial cell with majority of clearance by liver sinusoidal endothelial cells (Pitas *et al*, 1985). Experiment with fluorescent tagged Ac-LDL (DiI-Ac-LDL) on isolated HUVEC cells demonstrated the uptake of modified lipids (Figure 31 a and b).

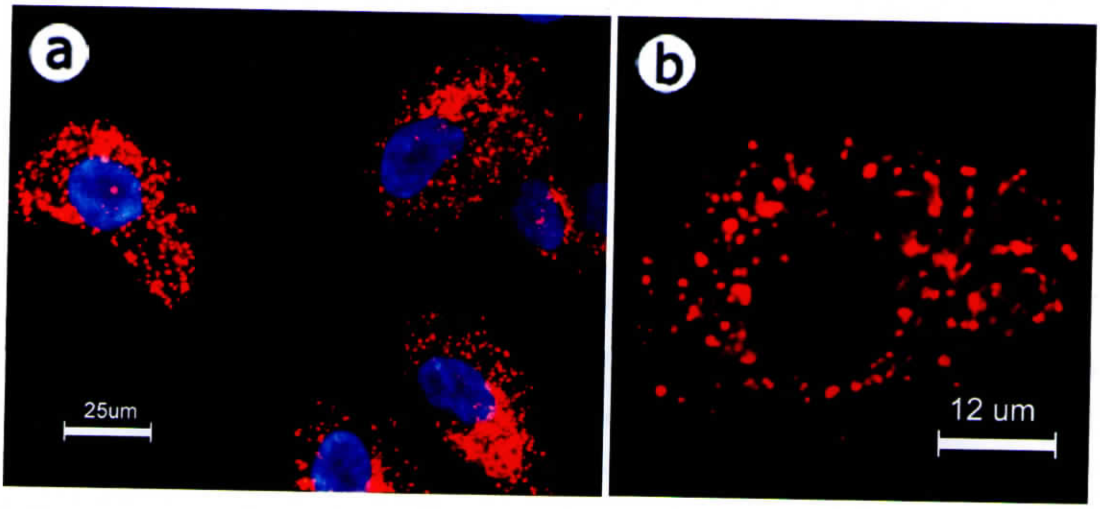


Figure 31 (a) LDL uptake by HUVEC. Nucleus counterstained with Hoechst. (b) Zoomed image showing localization of DiI-Ac-LDL inside cells.

4.6. RAT LIVER SINUSOIDAL ENDOTHELIAL CELL CULTURE

Sinusoidal Endothelial cells being the main non-parenchymal cells of the liver, participate in liver growth with respect to both their own proliferation, and effects on hepatocyte proliferation (Malik *et al*, 2002). In partial hepatectomized rat it has been shown that SEC generates TGF-β, HGF and IL-6 that exerts both positive and negative influences on hepatocyte proliferation and initiates reconstruction and reformation of matrix proteins.

SEC from rat liver was obtained during isolation of rat hepatocyte by two step perfusion method. SECs were separated from other cell types of liver by density gradient centrifugation using Percoll gradient. When cell suspension in PBS was loaded on to a two step percoll solution of 50% and 25% and centrifuged at high speed of 900 g for 20 min, SECs got separated at the intermediate layer between the two solutions (Figure 32).

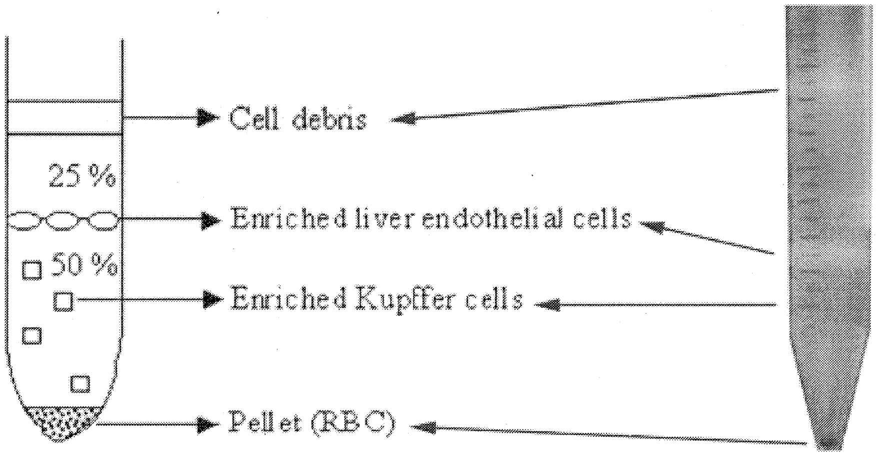


Figure 32. Two step percoll gradient for SEC isolation

The cells thus obtained were purified from Kupffer cells by selective adhesion. layer obtained at intermediate zone between percoll gradients was resuspended in IMDM containing 2.5 % FCS and 100 IU/ml penicillin and 100 μg/ml streptomycin. The cell suspension was seeded on uncoated Petri dish and incubated for 20 min at 37 °C in a CO_2 incubator. The Kupffer cells adhere more effectively than SEC on uncoated surfaces of normal tissue culture plates. The unattached SEC were transferred on to collagen coated culture dish and coverslips. The cells adhered on collagen coated surfaces and showed morphology as early as 2 h when the first medium change was done (Figure 33a). After 24 h, cells became large colonies good enough to be identified as SECs (Figure 33b).

Medium without serum is preferred for SEC culture, since large uptake of serum content by SEC favors its detachment from culture surface (Braet *et al*, 1994). However to culture SEC for long duration, it has also been cultured in serum containing medium by keeping the serum level at a low concentration of 2-2.5% but with additional growth factor supplementations (Tokairin *et al*, 2002). In this study IMDM with 2 % FCS was used for SEC culture to get monolayers on collagen coated surface. This culture system needed only minimum requirement of microenvironment to maintain SEC in culture for more than 10 days.

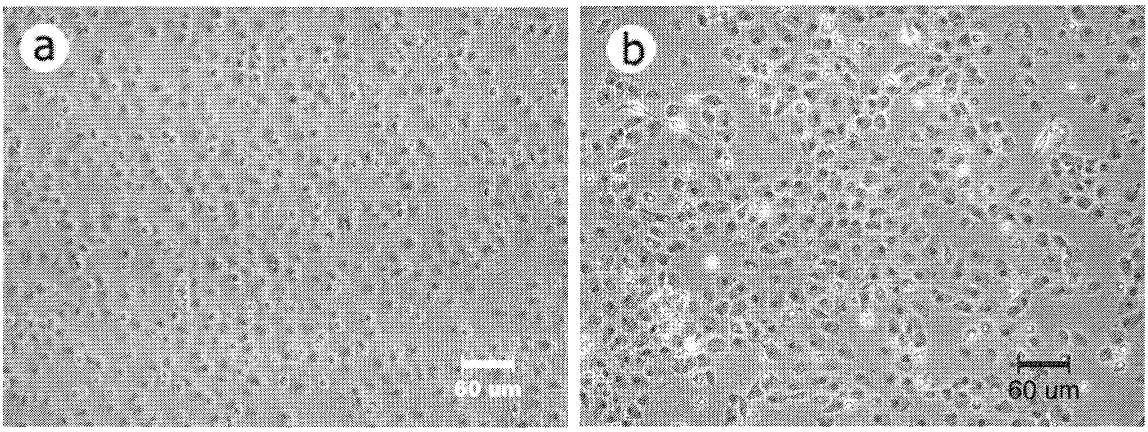


Figure 33. Primary rat liver Sinusoidal Endothelial Cells (a) 2 h after seeding (b) 24 h after seeding

4.6.1. Characterization of SEC

4.6.1.1. Fenestrated morphology

For the morphological characterization, cells were examined under low power magnification using phase contrast microscope and at higher magnification using SEM. The morphology identified under phase contrast microscopy (Figure 34) were confirmed by comparing with the SEM images (Figure 35). The cells revealed fenestrations on their surface which is considered to be the morphological characteristic feature of SEC.

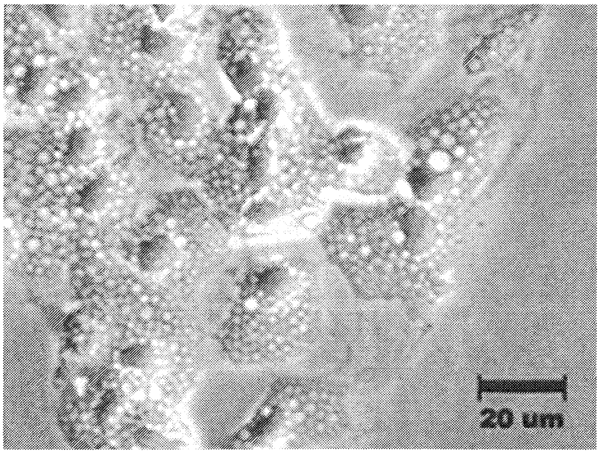


Figure 34. Characterization of SEC. The fenestrations on the cells can be clearly identified as white spots on cytoplasm

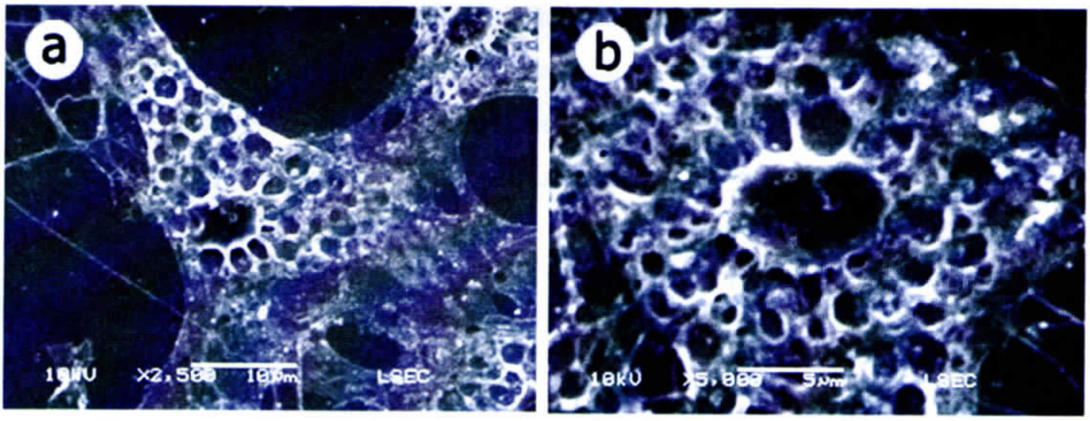


Figure 35. Demonstration of fenestrae of SEC by Scanning Electron Microscopy under (a) low magnification (scale = 10µm) and (b) high magnification (scale = 5µm)

4.6.1.2. Uptake of DiI-Ac-LDL

It has been previously mentioned that SEC are the primary cells for the clearance of modified LDL from plasma (Pitas *et al*, 1985). For the functional characterization, SEC cultured on collagen coated coverslips were used for DiI-Ac-LDL uptake assay. Upon incubation at optimal duration for 4 h, SEC internalized the fluorescent labeled modified LDL (DiI-Ac-LDL) molecules inside vacuoles which were visualized under confocal microscope (Figure 36).

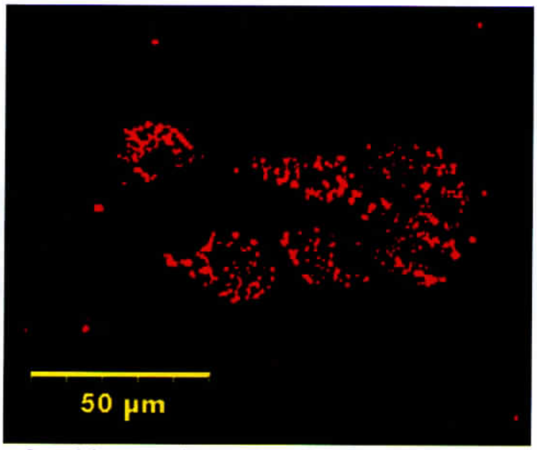


Figure 36 Confocal image demonstrating the DiI-Ac-LDL uptake by SEC

4.7. APPROACHES TOWARDS LIVER TISSUE ENGINEERING

In tissue engineering of the liver, for therapeutic or investigational purposes, different strategies have been looked into. The strategies envisaged towards liver tissue engineering were achieved by using direct and indirect hepatocyte and EC coculture.

Indirect approach was done by culturing rat hepatocytes in HUVEC and SEC conditioned medium whereas in direct method hepatocytes were cultured on HUVEC or SEC monolayer. Hepatocyte spheroid and heterospheroid formation, structural and functional polarity, metabolic ability and tissue organization were analyzed.

Several approaches including extracorporeal devices, cell transplantation and tissue engineered constructs have been proposed as potential adjuncts or replacement to transplantation

Whatever be the final design of a BAL the source of the cells and their availability for on-demand treatment is of crucial importance to their ultimate success. Human cell source would be ideal to serve in this task but are not available enough to be realistically considered as a possible donor source. However use of human cells is important in physiological studies of liver.

4.7.1. Control for structural polarity and metabolic ability of hepatocytes

Liver tissue sections were used as control for determining Cyt P450 activity and structural polarity. Liver sections subjected to biotransformation of EROD substrate was used for polarity staining to visualize both characteristics together. When observed under confocal microscope, the functionally active (EROD positive) hepatocytes in their highly polarized (CD26 and CD147 together) stages were visible. Since CD26 protein (DPPIV) is also expressed by biliary epithelial cells, bile duct cells were also shown up (Figure 37).

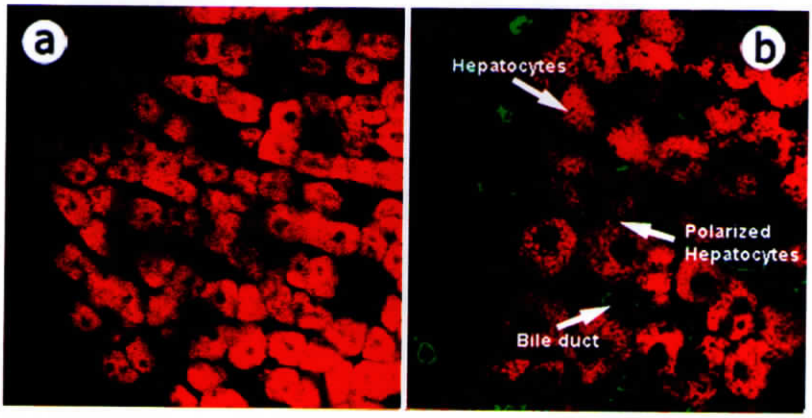


Figure 37 Liver tissue slice under confocal microscope. (a) Functionally active hepatocytes showing hepatic plate. (b) Polarized cells in liver tissue demonstrated by EROD assay and antibody staining

4.7.2. Hepatocytes and EC Coculture

Hepatocyte and endothelial cell interaction has been known to influence each other with respect to their function. Hepatocyte secreted growth factors like HGF has been found to be influencing a variety of cell types. HGF is reported to be a specific mitogen for endothelial cells. Similarly endothelial cell released factors also influence hepatocyte function (Ries *et al*, 2000). Since hepatocytes are in close apposition with sinusoidal endothelial cells, it brings lot of interest to study these two types of cells in coculture.

Hepatocytes with HUVEC and SEC interaction were studied broadly in two ways: Hepatocytes in EC conditioned medium and direct coculture. The metabolic ability and structural polarity of hepatocytes in culture was analyzed by confocal microscopy and spectrophotometric and spectrofluorimetric assays.

4.7.3. Hepatocytes in HUVEC conditioned medium

Hepatocytes in HUVEC conditioned medium was maintained for 21 days. The cultures monitored under phase contrast microscope showed normal cell growth (Figure 38).

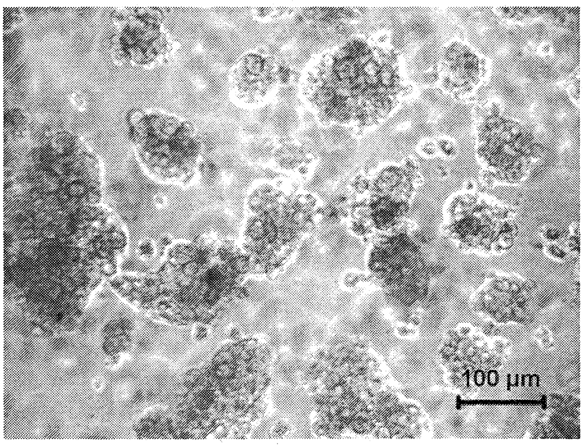


Figure 38 Hepatocytes cultured in HUVEC conditioned medium for 17 days. Spheroid formation was initiated on 3rd day and continued till 21 days.

4.7.3.1. Spheroid formation

Hepatocytes self organize into spheroid like structures in appropriate environment (Tzanakakis *et al*, 2001). Spheroids are the structurally stable form of hepatocytes in culture to express its differentiated functions. It has been previously reported that when rat hepatocytes were cultured on collagen coated surface in IMDM containing 2 % FBS, it formed spheroids (Figure 39a Figure 40a, Figure 41a and Figure 42a). Similarly hepatocytes cultured in HUVEC conditioned medium also formed spheroids (Figure 39b Figure 40b, Figure 41b and Figure 42b). This indicated that the optimized *in vitro* conditions have not affected the hepatocytes acclimatization. More adhesion and spreading of hepatocyte were observed on conditioned medium on initial day (Figure 39). Normally spheroid formation has to be induced by using non adherent surface, supply of specific growth factors or addition of polymeric growth factors in medium (Yamada *et al*, 2001). In this study spontaneous spheroid formation was observed after 24 h in culture without any external inducing factors.

Hepatocytes cultured on collagen-coated surface were used for coculture study using conditioned medium. During 7 days culture, cells expressed spheroid formation in static culture. After 24 h, hepatocytes spread well on the collagen both in normal and conditioned medium.

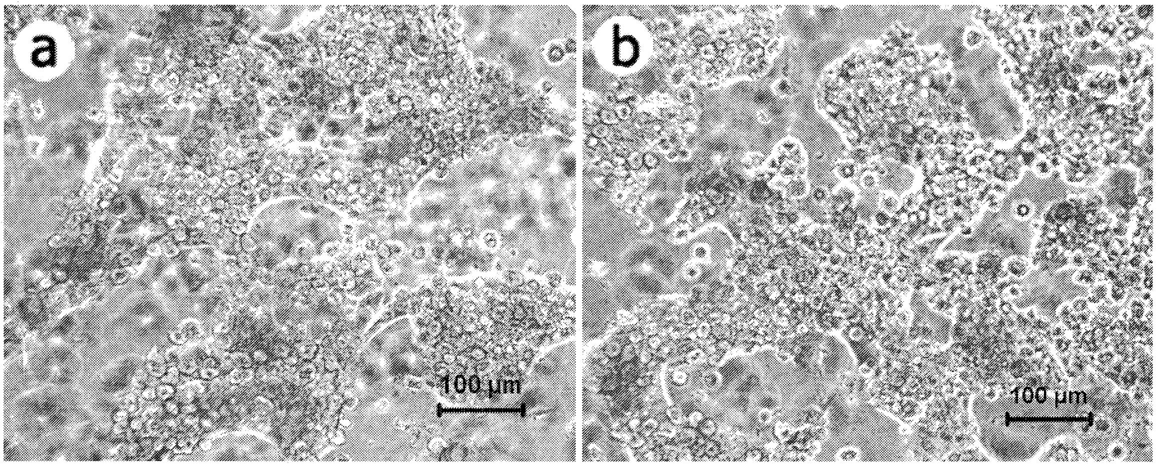


Figure 39 Hepatocyte coculture in HUVEC conditioned medium after 24 h. Cells formed patches in (a) normal medium and (b) Conditioned medium

On 3rd day, cells formed clumps and found to rise as an initiation towards spheroid formation (Figure 40). However it was noticed that cells tends to be in spread morphology in conditioned medium than in normal medium (Figure 40b).

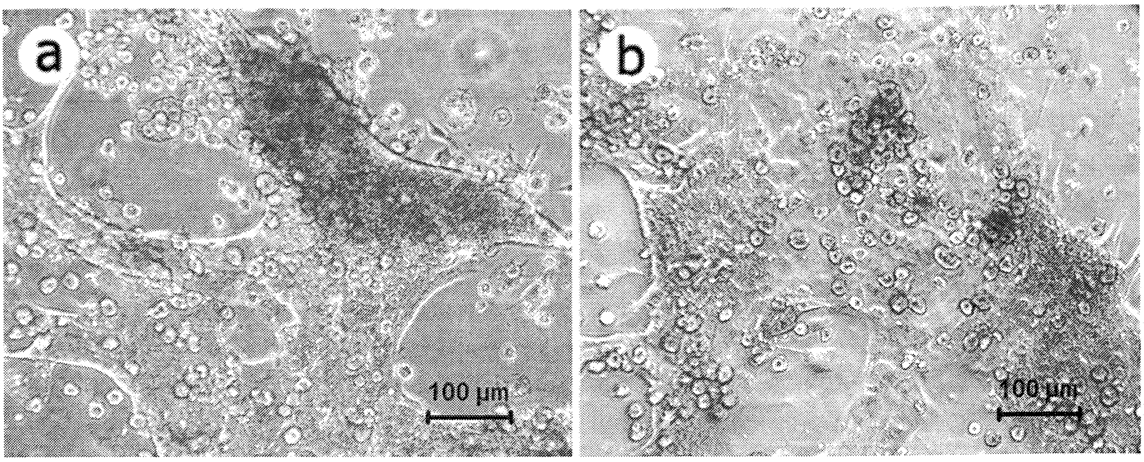


Figure 40 Hepatocyte started spheroid formation after 3 days in (a) Normal medium (b) Conditioned medium

The hepatocytes in spread morphology spontaneously started forming spheroids on 5th day (Figure 41). In conditioned medium, cells clumped to form very large aggregates and were lifted from the culture surface (Figure 41b)

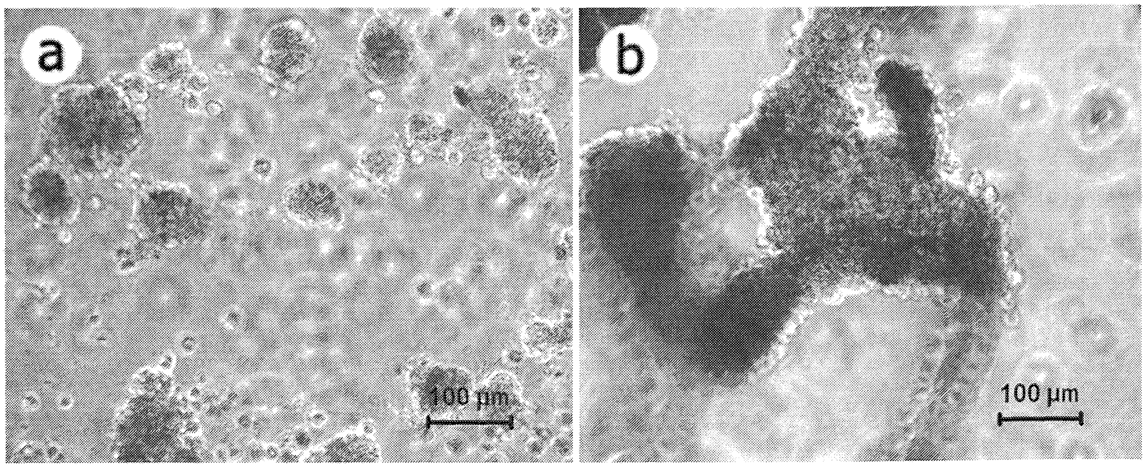


Figure 41 Hepatocyte spheroid formation after 5 days in (a) Normal medium (b) Conditioned medium

At the end of 7th day, more spheroid formation was observed (Figure 42) in both. The size of the spheroids in control cells were small (Figure 42a) when compared to spheroids in conditioned medium (Figure 42b).

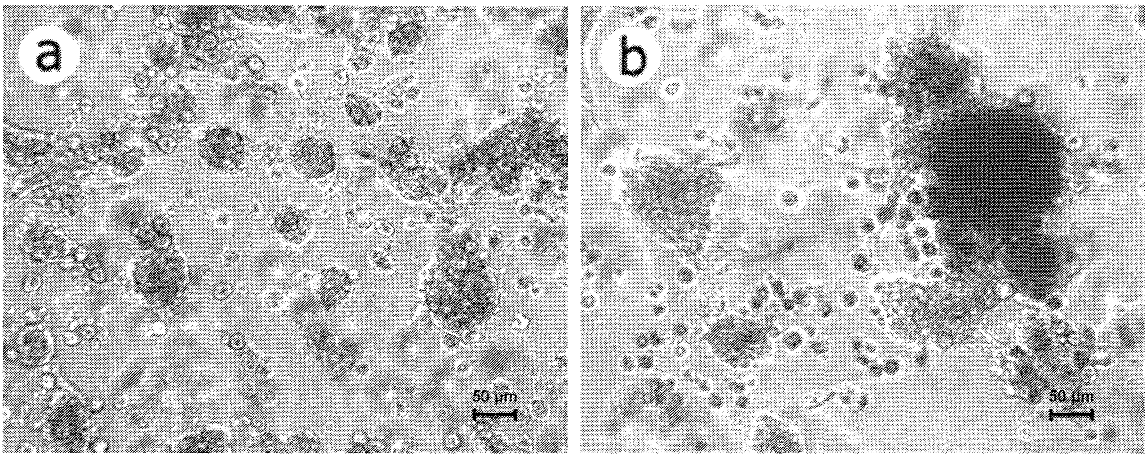


Figure 42 Hepatocyte spheroids after 7 days in (a) Normal medium (b) Conditioned medium

Hepatocyte function in HUVEC conditioned medium was assessed by estimating albumin synthesis, ammonia detoxification ability and biotransformation capacity.

4.7.3.2. Albumin synthesis

Albumin synthesis was studied over 7 days by cell based assay in spectrofluorimeter. Albumin synthesis expressed not less than 50% of initial response (Figure 43). Cells were maintained for 7 days with medium change on alternate days. During spheroid formation cells tend to detach from the surface and there is a chance for cell loss during medium change. This could be the reason for decrease in albumin concentration on later days when compared to initial day.

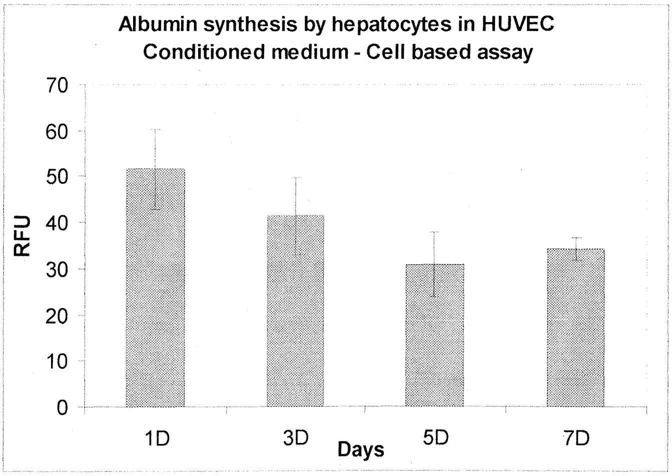


Figure 43 Cell based assay of albumin synthesis by hepatocytes cultured in HUVEC conditioned medium.

4.7.3.3. Ammonia detoxification (urea synthesis)

Ammonia is a toxic compound formed during amino acid metabolism, which is usually converted to urea in the liver. Inside hepatocyte, during urea cycle, ammonia reacts with carbomylphosphate and ornithine to form citruline which is then converted to arginosuccinate, L-Arginine and ornithine. Urea is released during the conversion of L-Arginine to ornithine (Watford *et al*, 2003). Thus detoxification ability can be assessed by quantifying the conversion of ammonia to urea.

Hepatocytes cultured in HUVEC conditioned medium were able to convert ammonia to urea in 7 days study (Figure 44) thereby showing the efficacy to maintain detoxification. It can be noted that decreased activity over 7 days could be due to the cell loss during medium change.

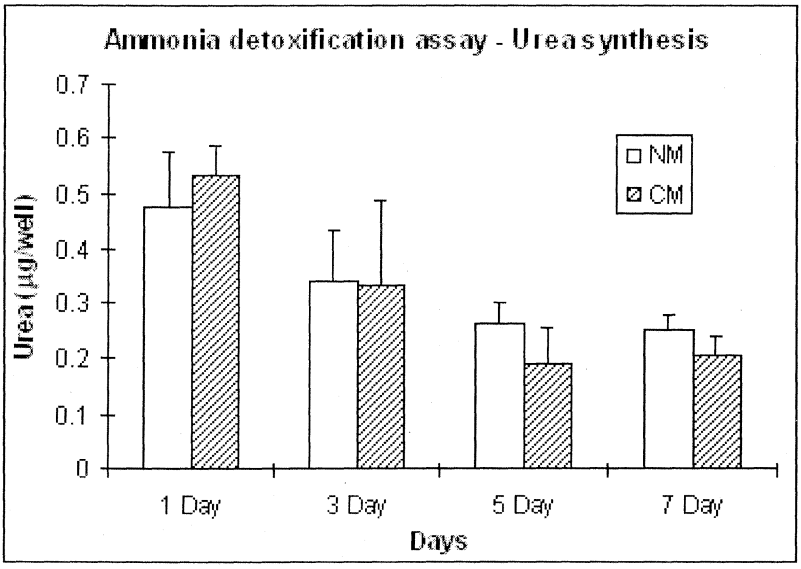


Figure 44 Urea synthesis by hepatocytes cultured in normal medium (NM) and HUVEC conditioned medium (CM)

4.7.3.4. Biotransformation (metabolic ability)

In biotransformation there are two major categories of metabolic reactions called Phase I and Phase II. "Phase I reactions" refers to a set of reactions that result in relatively small chemical changes that make compounds more hydrophilic. Phase I reaction also provide a functional group to the compound so that it can enter Phase II reactions. Cytochrome P450 enzymes are involved in the Phase I reaction of detoxification. The product of Phase I reaction enters into Phase II reaction where a set of transferase enzymes degrade the compound further.

One of the isoform of cytochrome P450 is the product of CYTP4501A1 gene known as Ethoxy Resorufin O-De ethylase (EROD) which biotransform the substrate O^7-Ethoxyresorufin to resorufin. Being highly fluorescent, the product can be visualized under fluorescence microscope or quantified using spectrofluorimeter.

Hepatocyte in HUVEC conditioned medium and normal medium were used for determining the functionality. Normally when hepatocytes are incubated with EROD substrate, cells convert it to an intermediate hydroxylated form of resorufin. The reaction is completed by addition of β-Glucoronidase to release resorufin. The released resorufin expressed in culture medium represents the metabolic activity.

It was observed that hepatocytes cultured in conditioned medium expressed more cyt P450 activity. Even though hepatocytes in normal medium were able to maintain cyt P450 activity, low level activity was noted on 7th day compared to 5th day. However in conditioned medium there was no change between 5 and 7 days (Figure 45).

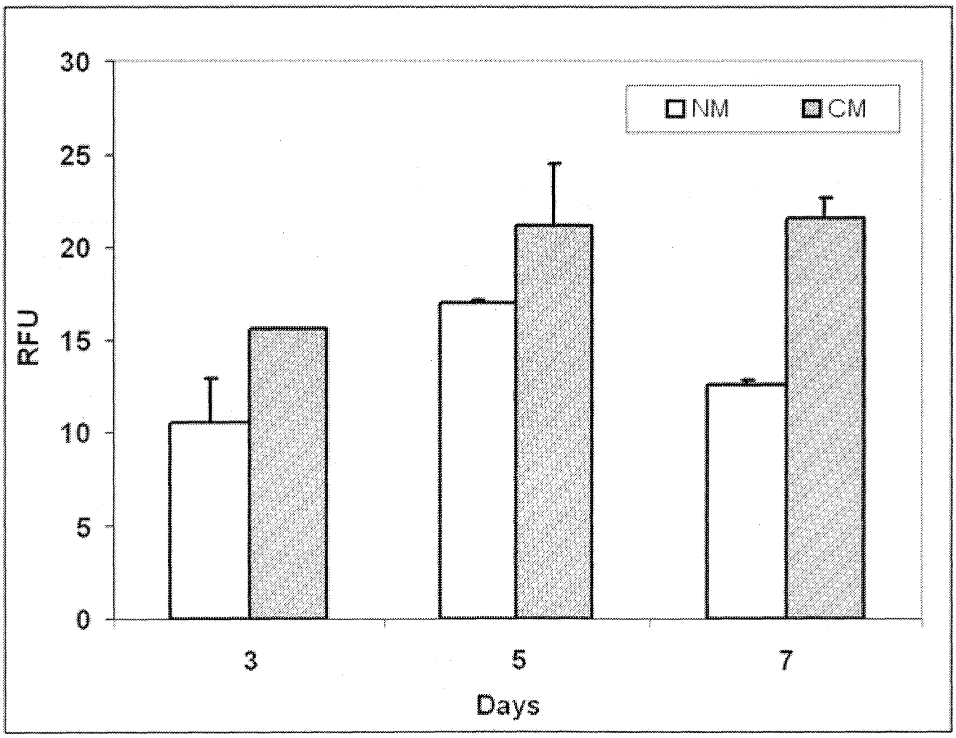


Figure 45 Spectrofluorimetric analysis of Cyt P450 activity expressed by hepatocytes (in Relative Fluorescence Unit, RFU) cultured in normal medium (NM) and HUVEC conditioned medium (CM)

Cytochrome P450 activity of hepatocytes cultured in conditioned medium was also studied using confocal microscopy. It has been shown that confocal microscopy is a technique to determine and quantify EROD activity (Kuleshova *et al*, 2004).

Under confocal microscope functionally active cells were appeared as red. Stained cells observed after 24 h in normal and conditioned medium (Figure 46) was compared. Usually hepatocyte in round morphology shows better metabolic activity than cells with flattened spread morphology. In conditioned medium, hepatocyte in spread morphology also showed positive staining (Figure 46b).

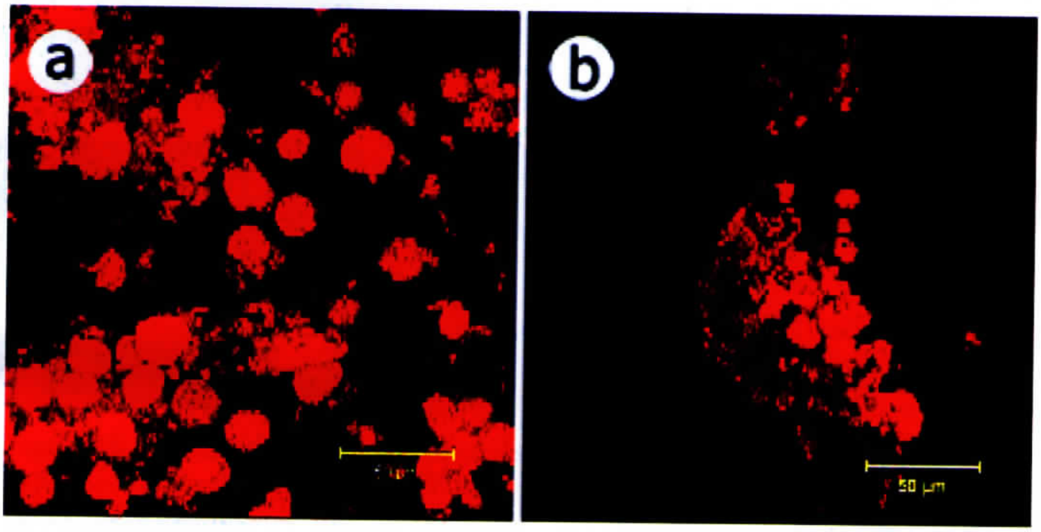


Figure 46 Functionality of hepatocytes after 24 h (a) in normal medium and (b) in HUVEC conditioned medium. Islands of spread cells showing functional activity in conditioned medium.

During 3rd and 5th day cells showed maximum activity, more specifically on the 5th day. On 3rd day hepatocytes started forming spheroids from the clumps. The cells in the spheroid remained functional (Figure 47 c & d), whereas in normal medium the cells tend to remain adhered as grouped together expressing functionality (Figure 47 a & b).

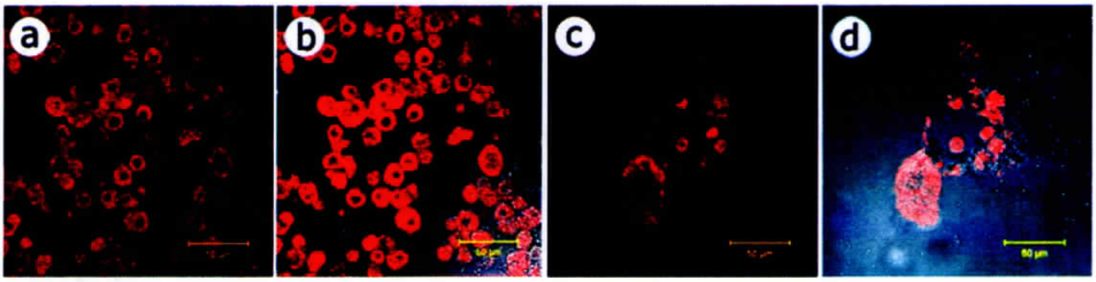


Figure 47 Functionality of hepatocytes on 3rd day. Cells started forming aggregates and spheroid in (a & b) normal medium and (c & d) HUVEC conditioned medium

On 5th day, hepatocytes formed thick clumps as the processes of spheroid formation continued. Even in the large thick clump of cells, functionality was found to be good (Figure 48).

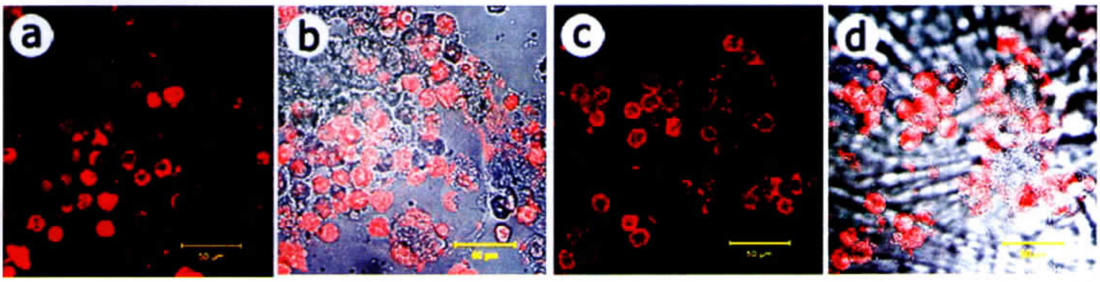


Figure 48 Functionality of hepatocytes on 5th day. Spheroid formation and expression of metabolic activity in (a & b) normal medium and (c & d) HUVEC conditioned medium

Number of functional cells on the 7th day was found to be similar to those in 5th day in both conditioned medium and control cultures. On comparing the conditioned medium with control culture, it was noted that the functionally active cells are more in conditioned medium (Figure 49 c & d) than in control (Figure 49 a & b)

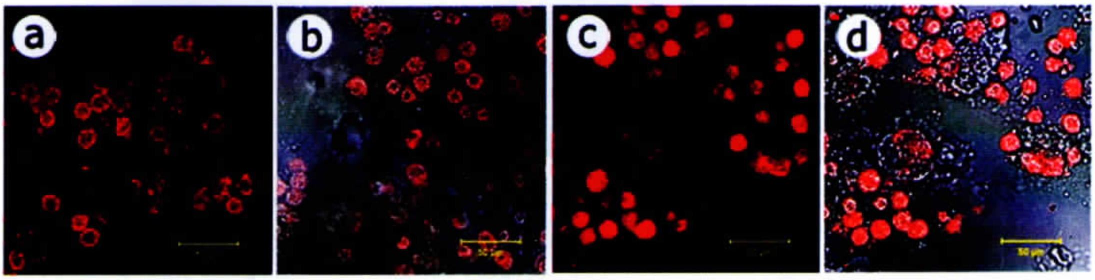


Figure 49 Functionality of hepatocytes on 7th day. Cells formed spheroid and more metabolically active cells were found in (c & d) conditioned medium than in (a & b) normal medium

4.7.3.5. Structural polarity

One of the morphological features that can ensure the polarized nature of hepatocytes is the development of bile canaliculi. Normally hepatocytes are cultured in basal medium containing specific additives like glucose, hormones and high concentration of serum. This enables the cells to become more polarized structurally (Barth *et al*, 1982). In this experiment IMDM with low concentration of 2.5 % FCS was used. It has been previously reported that IMDM is a suitable medium for primary adult rat hepatocytes (Anil Kumar *et al*, 2002). In this experiment it has been shown that IMDM with low concentration of serum can induce the structural polarity of hepatocytes as early as 24 h (Figure 50). The marker for apical domain, DPPIV or CD 26 was restricted to the gap between adjacent hepatocytes. Marker for the basolateral domain, CD147, was found to be present between lateral sides of hepatocyte, i.e. on lateral side of cells.

After 24 h culture, hepatocytes in control and conditioned medium showed apical and basolateral polarity. The polarity expression by hepatocytes in conditioned medium showed difference from normal medium. The apical protein was expressed by the cells in the gap (bile canaliculi) between hepatocytes. There was strong apical polarization in conditioned medium (Figure 50b) where as in normal medium it was scattered. However the basolateral polarity was observed only in normal medium (Figure 50a).

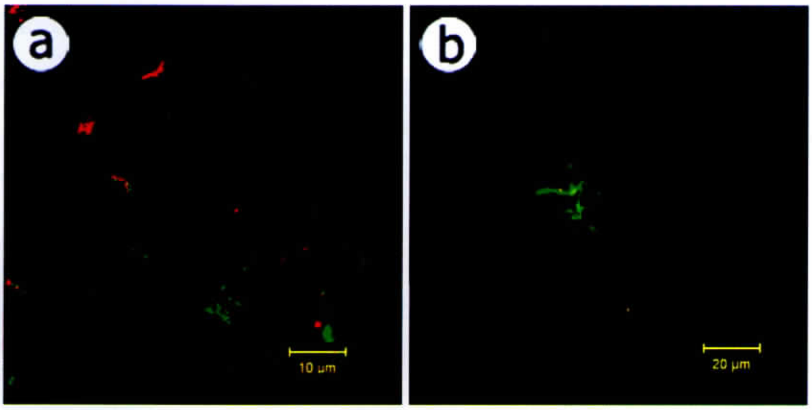


Figure 50 Polarity of hepatocytes in HUVEC conditioned medium after 1 day. Duel staining for Apical (green) and Basolateral (Red) domains showing polarized nature of cells in (a) normal medium and (b) conditioned medium.

After one day, basolateral polarity in conditioned medium disappeared and reappeared on 7th day.

On the third day basolateral protein expression was found to be decreased (Figure 51). In normal medium the apical polarity of hepatocytes were seen through the channels with decrease in basolateral polarization (Figure 51a). Hepatocytes in conditioned medium maintained the apical polarized nature as extensive networking with negligible basolateral protein expression. The networking could have been achieved by the initiation of spheroid formation and it was more in conditioned medium when compared to normal medium (Figure 51b).

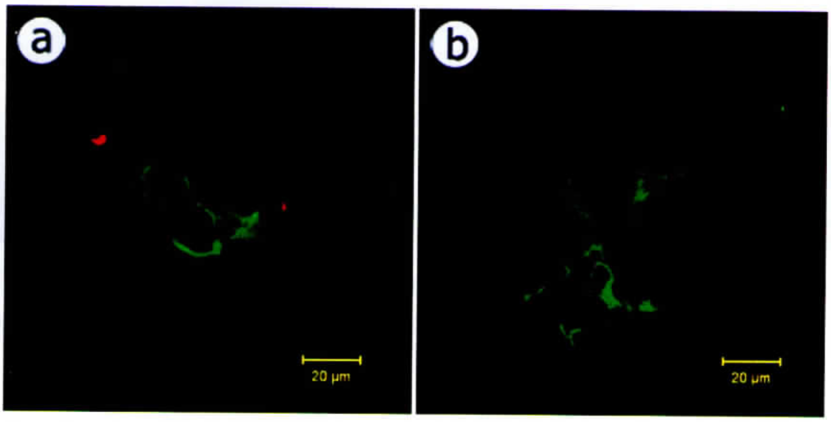


Figure 51 Polarity of hepatocytes on 3rd day in HUVEC conditioned medium for apical (green) and basolateral (Red) domains. (a) Polarity showing scattered green fluorescence and localized red fluorescence in normal medium and (b) extensive apical polarization with no basolateral expression in conditioned medium

The distribution of apical polarized characteristics on 5th day was different. There were no baoslateral proteins (CD26) in control (Figure 52a) as well as cells in conditioned medium (Figure 52b).

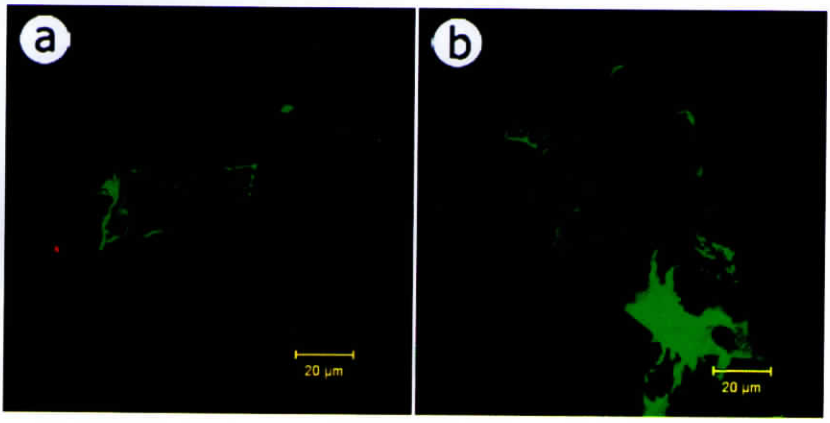


Figure 52. Polarity of hepatocytes on 5th day in HUVEC conditioned medium. Cells in normal medium hardly express apical proteins while basolateral polarity is diffused (a) in normal medium and (b) in conditioned medium

The bile canalicular network was found to increase in both cases. Apical polarity of hepatocytes in conditioned medium expressed extensive networking of bile canaliculi evidenced by images obtained in z-direction (Figure 53).

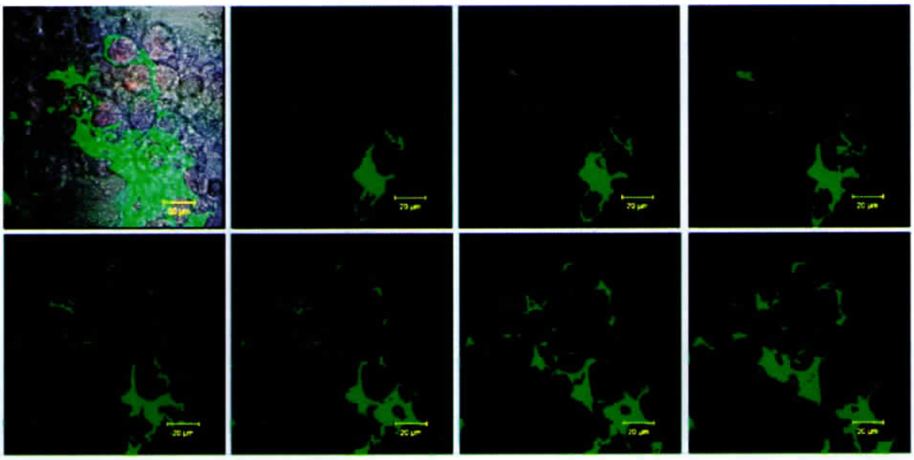


Figure 53. Polarity of hepatocytes in HUVEC conditioned medium on 5th day. Extensive networking of apical polarization in images obtained at z planes.

At the end of 7 days, both apical and basolateral polarity was restored in normal and conditioned medium. The basolateral polarity once disappeared on 3rd day was restored inside the spheroid while apical polarization was continuously maintained (Figure 54 and Figure 55).

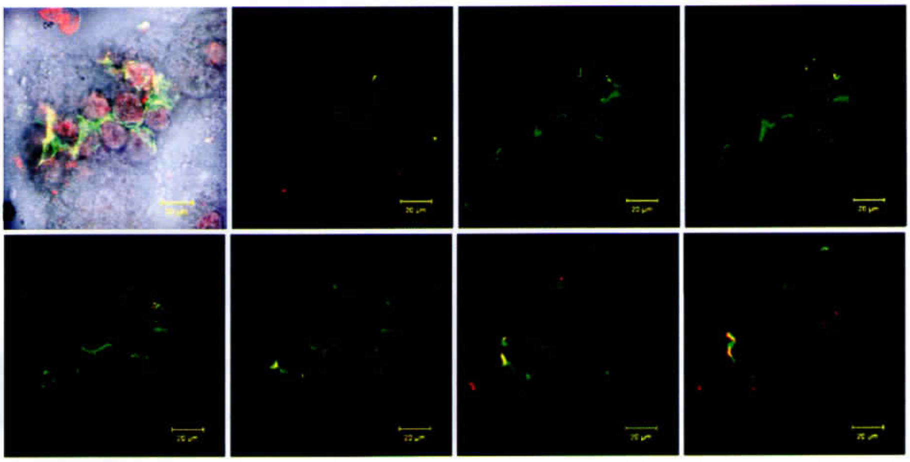


Figure 54. Restoration of apical polarity and redistribution of basolateral polarity by hepatocytes on 7th day in normal medium.

The repolarization was further observed by z-stacking the spheroid under confocal microscope. The outer cells in the spheroid expressed basolateral proteins whereas the cells inside expressed networking of apical polarity (Figure 55).

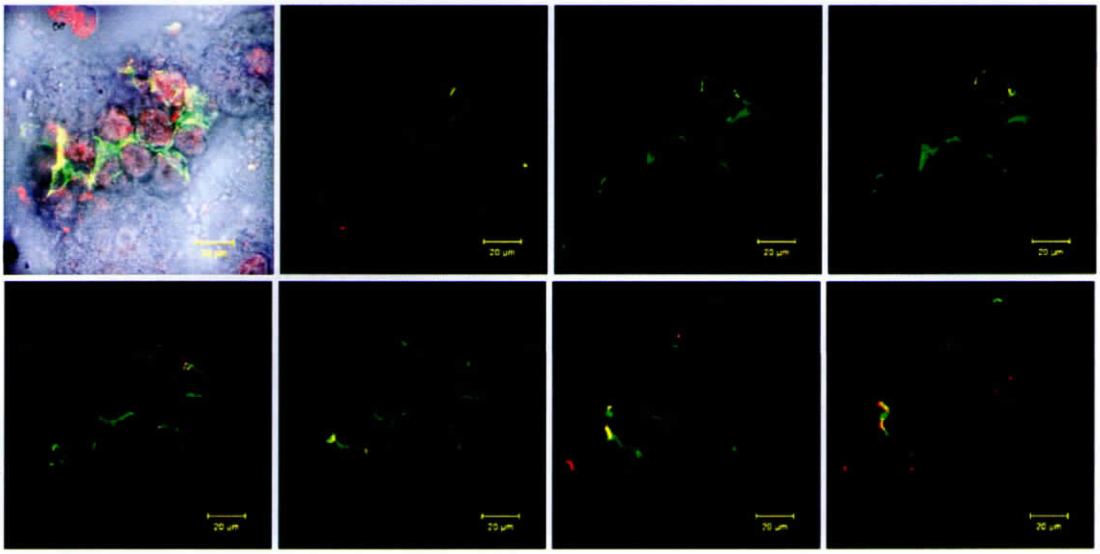


Figure 55. Restoration of apical polarity and redistribution of basolateral polarity by hepatocytes on 7th day in HUVEC conditioned medium.

4.7.4. Hepatocytes in SEC conditioned medium

Hepatocytes were cultured with SEC conditioned medium to know the influence of SEC secreted factors on hepatocyte functions. Structural and functional behaviors of hepatocytes were studied in the coculture with SEC by assessing the spheroid formation, bio-transformation of O^7-ethoxyresorufin, structural polarity, functional polarity and tissue organization.

4.7.4.1. Spheroid formation

Hepatocytes cultured on collagen-coated surface were used for coculture study using conditioned medium. Hepatocytes cultured as aggregates called spheroids have enhanced liver functions compared with cells spread on a culture substratum (Landry *et al*, 1985). During 7 days static culture, cells expressed spheroid formation. After 24 h, hepatocytes spread well on the collagen both in control and in conditioned medium (Figure 56). On 3rd day, cells formed clumps from the spread cells as an initiation towards spheroid formation (Figure 57).

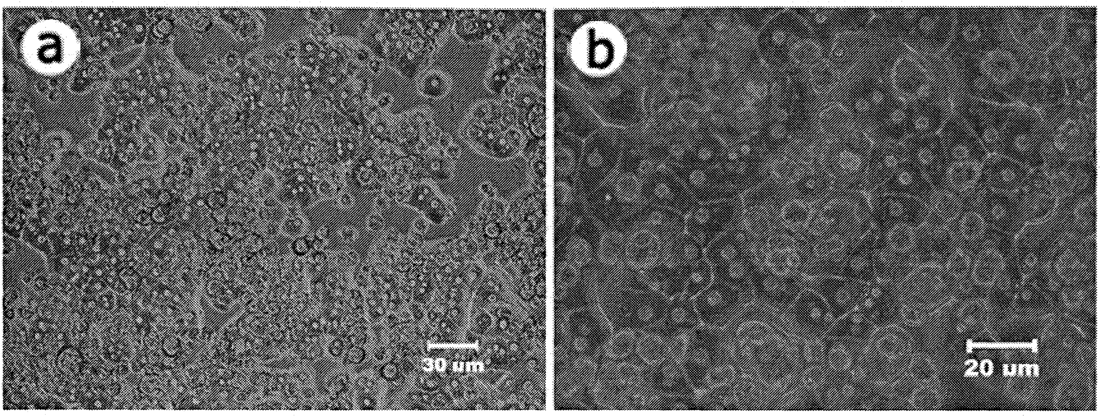


Figure 56 Hepatocytes coculture in SEC conditioned medium after 24 h in (a) normal medium and (b) SEC conditioned medium

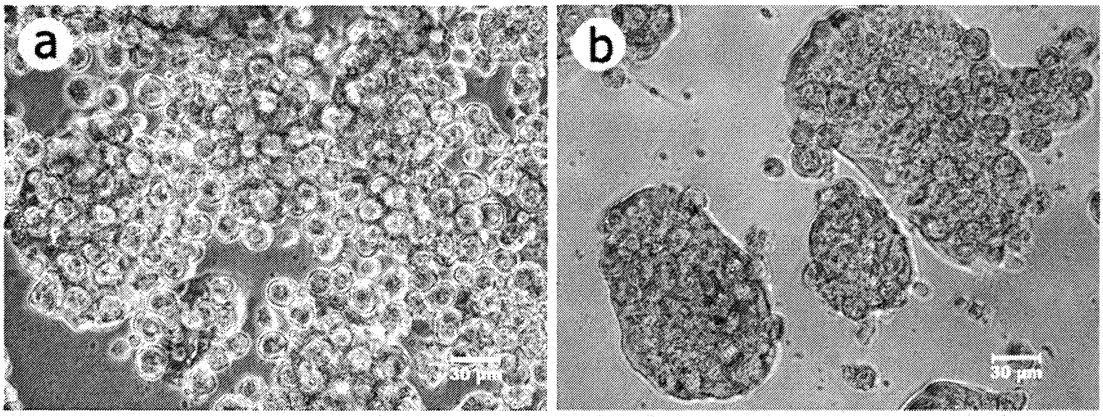


Figure 57 Hepatocyte spheroid formation after 3 days. (a) Cells started forming cell aggregates and clump together in normal medium. (b) spheroid formation in SEC conditioned medium

At the end of 5 days, hepatocytes in both normal and conditioned medium continued to form aggregates toward spheroid formation. Clumps with large size were observed in both cultures (Figure 58).

After 7 days, spheroids in SEC conditioned medium (Figure 59b) started floating leaving very few number of adhered cells. Even though spheroid formation was observed in the normal medium cell aggregates were found to be adhered on the surface (Figure 59a).

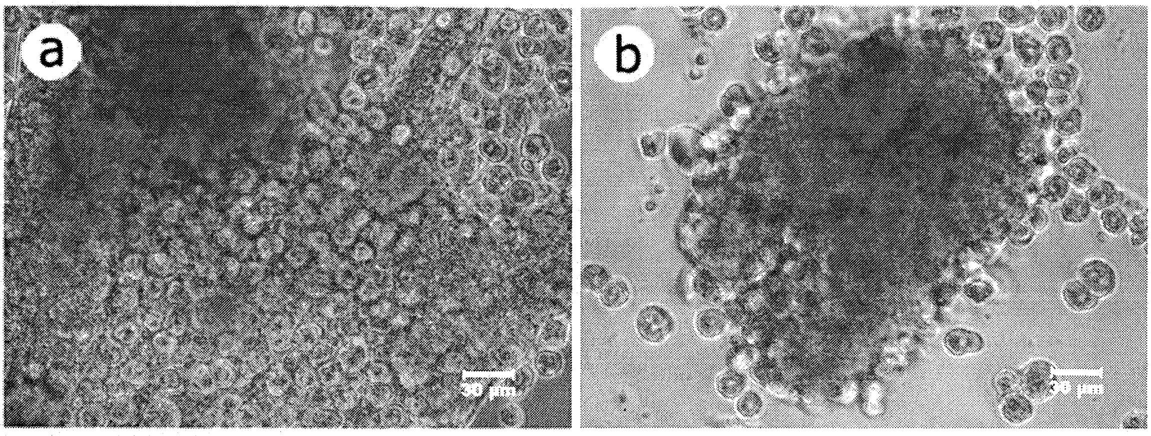


Figure 58. Hepatocyte spheroid formation after 5 days in SEC conditioned medium. Large clumps were formed in (a) normal medium and (b) SEC conditioned medium

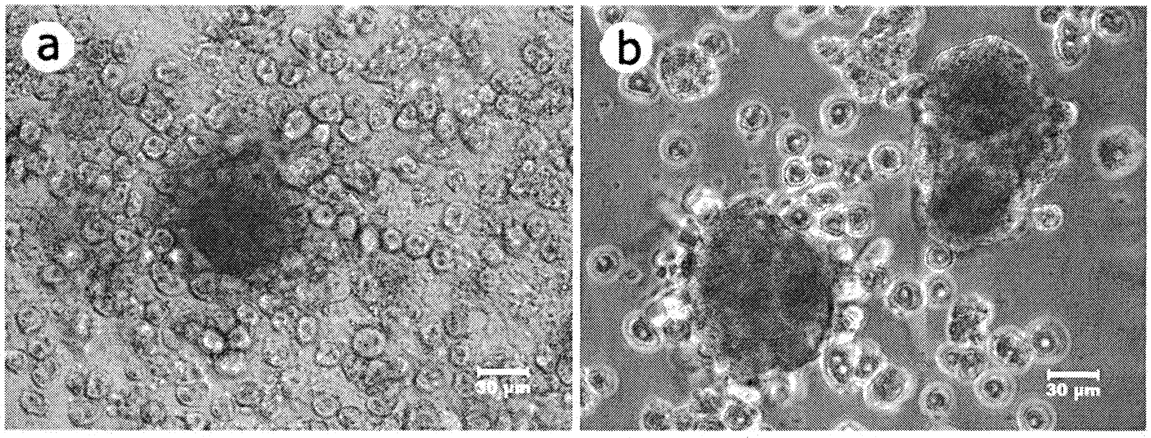


Figure 59 Hepatocyte spheroids after 7 days. (a) Cells formed spheroids and were adhered in normal medium. (b) Spheroids started floating in SEC conditioned medium

Spheroid formation within 4 days was also reported by other studies but with the supply of Eudragit S100 to culture medium as an artificial matrix (Yamada *et al*, 2001).

4.7.4.2. Functional activity

Hepatocyte in conditioned medium and normal medium were used for determining the functionality. Cyt P450 activity of cells cultured in conditioned medium was compared with control cells.

Functionally active red cells were observed under confocal microscope. After 24 h, the number of stained cells in conditioned medium (Figure 60 c & d) was more compared to control (Figure 60 a & b).

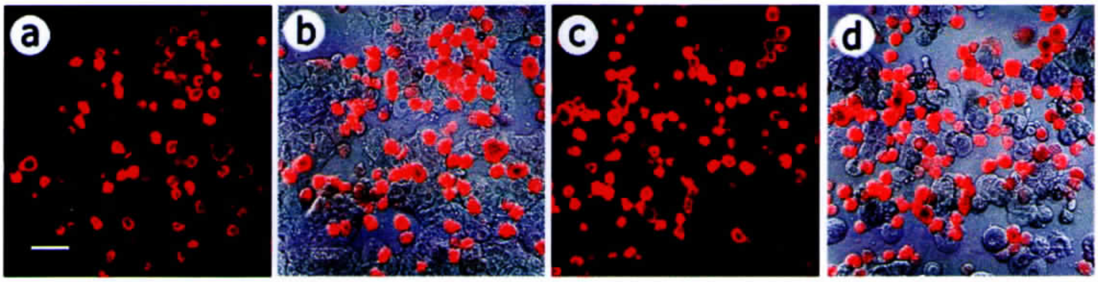


Figure 60 Functionality of hepatocytes after 24 h in (a & b) normal medium and (c & d) SEC conditioned medium.

Usually hepatocyte shows its dedifferentiated morphology as flattened spread cells that are not functional. But after 24 h culture, in normal as well in conditioned medium spread hepatocytes showed functionality similar to that expressed by the loosely bound cells. The functionally active spread cells in conditioned medium (Figure 61 c & d) was more in number than in control (Figure 61 a & b). This could be due to the optimized culture conditions selected for the coculture.

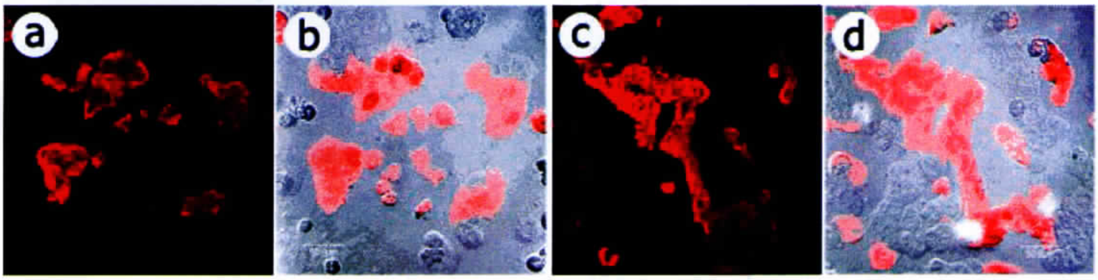


Figure 61 Functionality of spread hepatocytes on 1st day in (a & b) normal medium and (c & d) SEC conditioned medium. Islands of functionally active spread cells were more in number in conditioned medium

During the 3rd and 5th day the cells showed maximum activity, more specifically on the 5th day. On 3rd day hepatocytes started forming spheroids from the clumps. The cells in the spheroid remained functional and more spread in conditioned medium (Figure 62 c & d), whereas in normal medium the cells tend to remain grouped together expressing functionality (Figure 62 a & b).

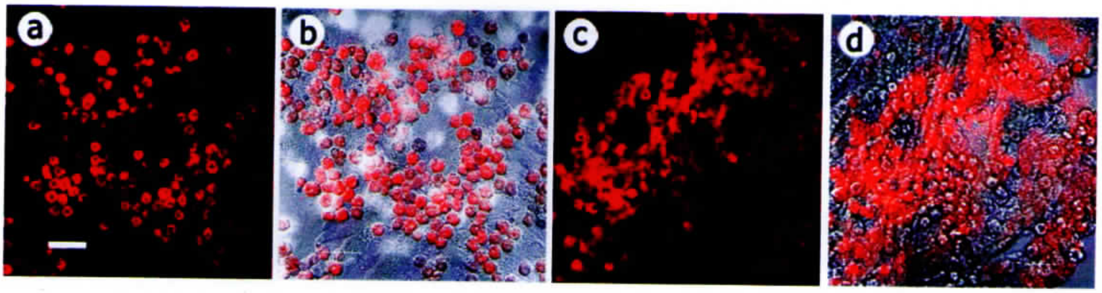


Figure 62. Functionality of hepatocytes on 3rd day in (a & b) normal medium and (c & d) SEC conditioned medium. Number of functionally active hepatocytes in spread morphology is more in conditioned medium

During the 5th day, hepatocytes formed thick clumps as the processes of spheroid formation continued. Even in the large thick clump of cells, functionality was found to be good. On comparing cultures in normal and conditioned medium on 5th day, it was obvious that functional hepatocytes in conditioned medium (Figure 63 c & d) were more in number than in control cultures (Figure 63 a & b).

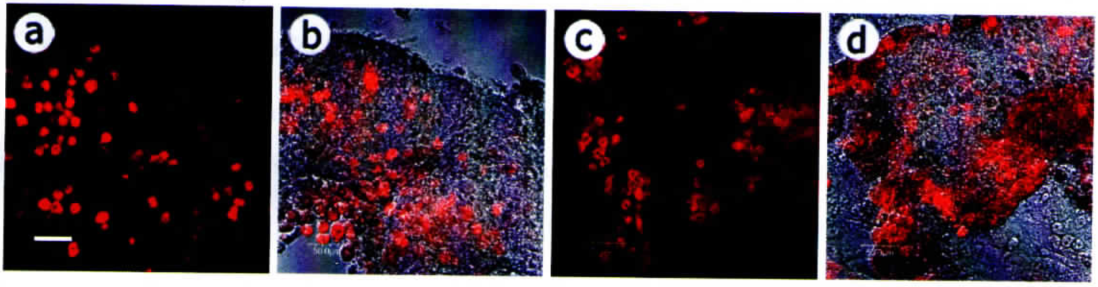


Figure 63 Functionality of hepatocytes on 5th day in (a & b) normal medium and (c & d) SEC conditioned medium. Cells inside the clump in conditioned medium is more than in normal medium

However, it was observed that number of functional cells on the 7th day was less than those in 5th day. This decrease was observed both in conditioned medium and control cultures. But when comparing the conditioned medium with control culture, functionally active cells were more in conditioned medium (Figure 64 c & d) than in control (Figure 64 a & b). Hepatocytes maintained functional activity in a stable pattern when cultured for 3 -5 days in IMDM.

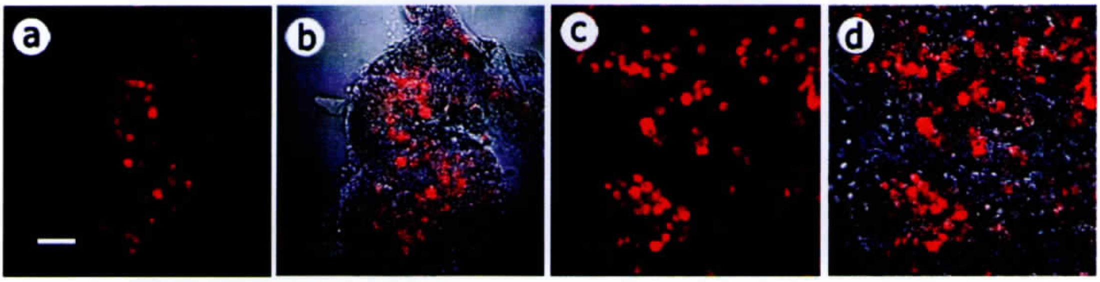


Figure 64 Functionality of hepatocytes on 7th day in (a & b) normal medium and (c & d) SEC conditioned medium. Cells showing functionality in conditioned medium was more in number than in control

4.7.4.3. Polarity of hepatocytes in SEC conditioned medium

Bile canalicular formation ensures the polarized nature of hepatocytes. Appropriate culture condition enables the cells to become structurally polarized. In this experiment it has been shown that IMDM with low concentration of serum can induce the structural polarity of hepatocytes as early as 24 h.

4.7.4.3.1 ***Structural polarity of hepatocytes***

After 24 h culture, hepatocytes in control (Figure 67 & Figure 68) and conditioned medium (Figure 65 & Figure 66) showed relatively less apical polarity and high basolateral polarity. In conditioned medium, hepatocytes showed localized distribution of apical and basolateral proteins.

The marker for apical domain, DPPIV or CD 26 in conditioned medium was restricted to the gap between adjacent hepatocytes (Figure 66). Marker for the basolateral domain, CD147, was found to be present between adjacent hepatocytes, i.e. on lateral side of cells. Observation in z plane under confocal microscope showed the tube like bile canalicular structure in between adjacent hepatocytes (Figure 66). The polarity expression by control cultures showed difference (Figure 67). The apical protein was expressed by the cells in the gap (bile canaliculi) between hepatocytes. This was visualized by z-stacking the hepatocyte clump (Figure 68). A weak fluorescence all over the clump showed incomplete polarization for apical domain. The basolateral polarity seemed to be intact and strong as visualized by the strong fluorescence between adjacent hepatocytes (Figure 67).

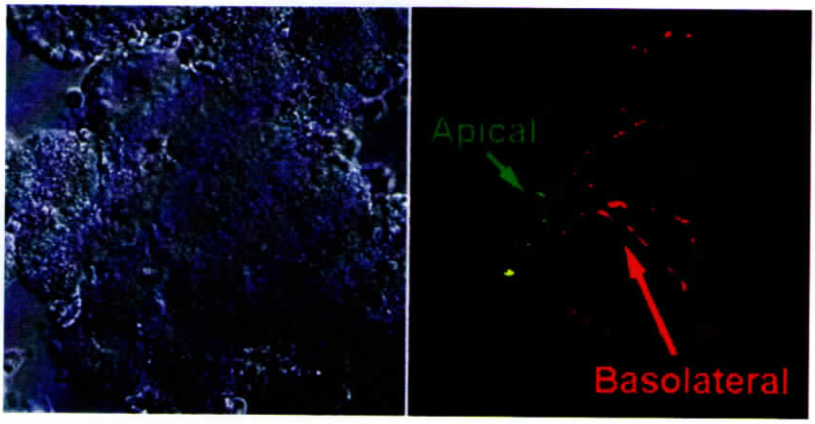


Figure 65. Polarity of hepatocytes in SEC conditioned medium after 1 day
Duel staining for Apical (green) and Basolateral (Red) domains shows localized fluorescence indicating the polarized nature of cells

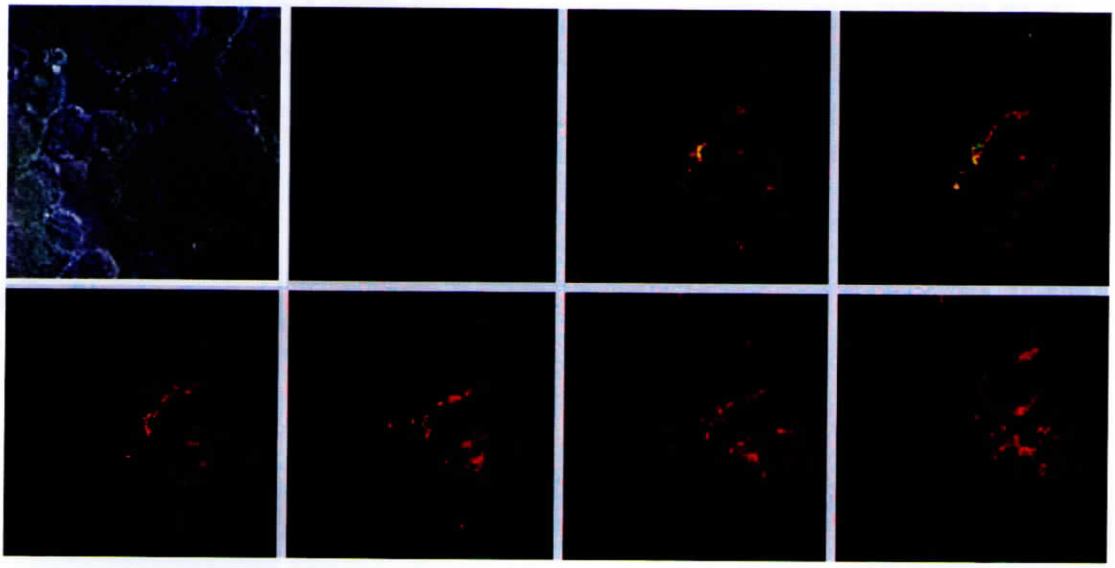


Figure 66 Serial sections showing polarity of hepatocytes in SEC conditioned medium after 1 day

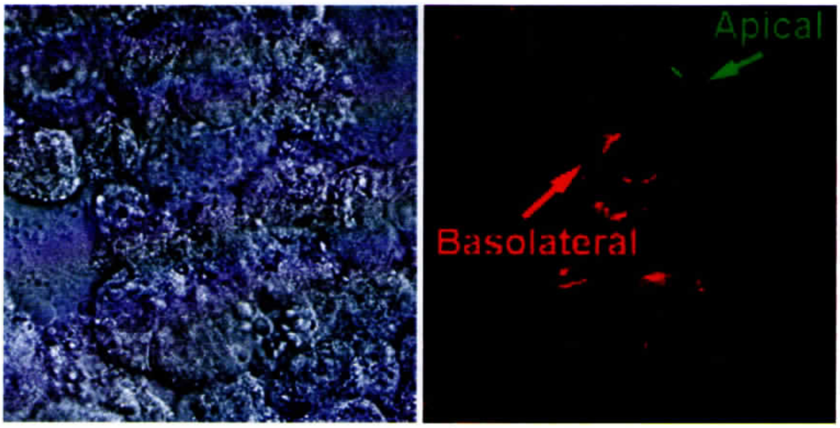


Figure 67. Polarity of hepatocytes in normal medium after 1 day. Duel staining for Apical (green) and Basolateral (Red) domains showed scattered green fluorescence and localized red fluorescence.

Observation in z plane under confocal microscope showed polarized nature of hepatocyte in normal medium with apical and basolateral markers (Figure 68).

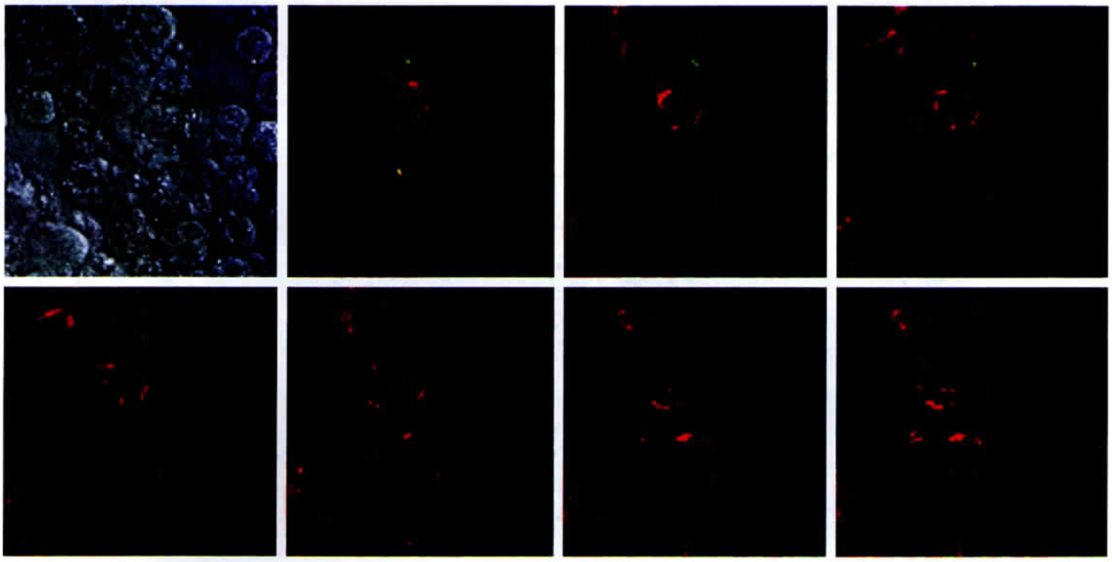


Figure 68. Serial sections in z stack showing polarity of hepatocytes in normal medium after 1 day

After one day, hepatocyte polarity in conditioned medium disappeared and reappeared along 3, 5 and 7 days.

On the third day apical protein expression was found to be decreased in control hepatocytes (Figure 69 a). Hepatocytes in conditioned medium maintained both apical and basolateral polarized characteristics (Figure 69 b).

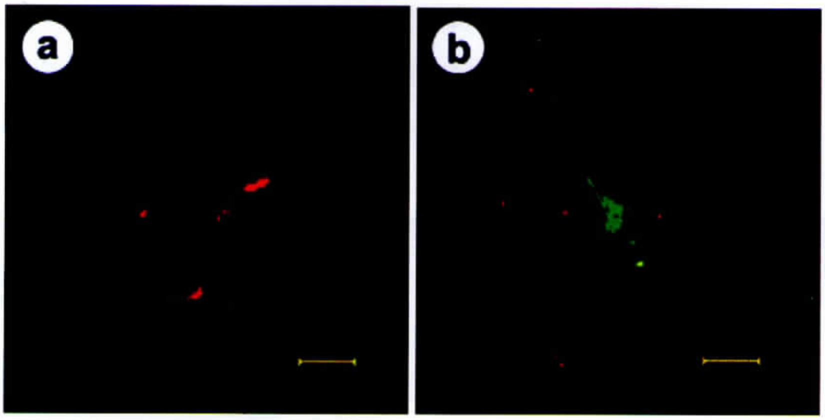


Figure 69. Polarity of hepatocytes on 3rd day. (a) Cells in normal medium hardly express apical proteins while basolateral polarity was retained. (b) In conditioned medium apical polarity was retained along with basolateral polarity.

The distribution of polarized characteristics of the cells on 5th day was entirely different. There were no apical proteins (CD26) in control (Figure 70 a) as well as cells in conditioned medium (Figure 70 b). However it was observed that cells maintained basolateral polarity by expressing CD147 in both cultures in a diffused behavior. Fluorescence in cellular outline representing the basolateral polarity was clear but dispersed.

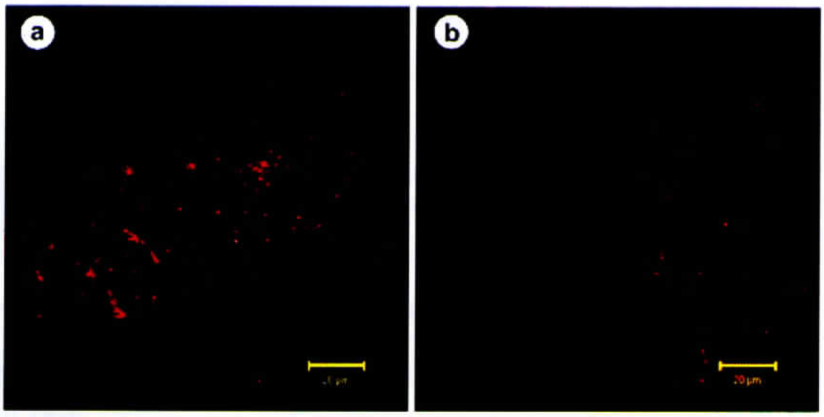


Figure 70. Polarity of hepatocytes on 5th day. Cells in (a) normal medium and (b) conditioned medium hardly expressed apical proteins while basolateral polarity was diffused

The cells formed spheroids after 7 days in culture. The apical polarity in conditioned medium was restored at the 7th day whereas the control cells still remained as in the 5th day (Figure 71). The apical polarity once disappeared on the 3rd day and the diffused basolateral nature on the 5th day in conditioned medium was restored inside the spheroid on 7th day. It was observed that the repolarization was different from those present during initial days. The outer cells in the spheroid expressed basolateral proteins whereas the cells inside expressed apical polarity (Figure 71b).

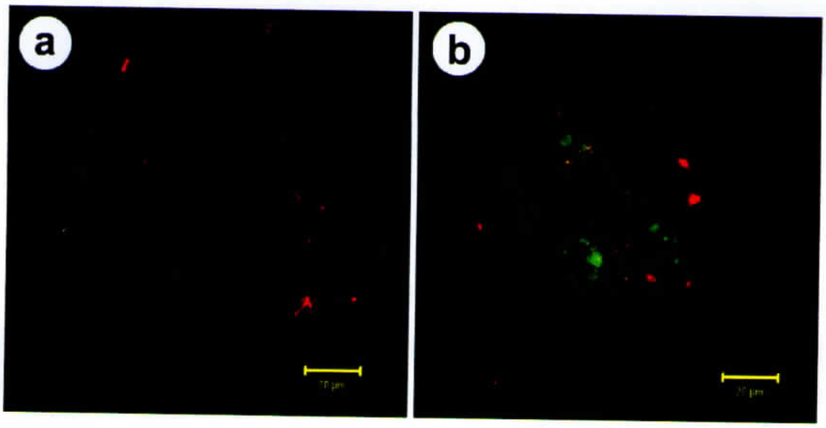


Figure 71. Apical polarity and redistribution of basolateral polarity by hepatocytes on 7th day in (a) normal medium and (b) conditioned medium. In conditioned medium apical polarity was restored in a redistributed fashion

4.7.4.3.2 ***Functional polarity of hepatocytes***

The functional polarity of hepatocytes in coculture with conditioned medium was determined by FDA assay. The polarity expressed by hepatocytes at 2nd day and 7th day, in normal and conditioned medium was compared. Functionally polarized cells convert FDA into fluorescing compound and transport outside the cells through the apical region. Hence highly polarized cells show intense fluorescence at their apical side.

In general, after 24 h incubation, hepatocytes cultured on collagen-coated surface did not show exact functional polarity both in normal and conditioned medium. Instead, almost all spread cells indicated that the cells were polarized (Figure 72). However some of the spread cells showed functional polarity by localized fluorescence on apical side (bile canaliculi - BC). More interestingly, the cells that are not spread or settled on the surface of spread cells were found to be non-fluorescing (unstained cells – UC).

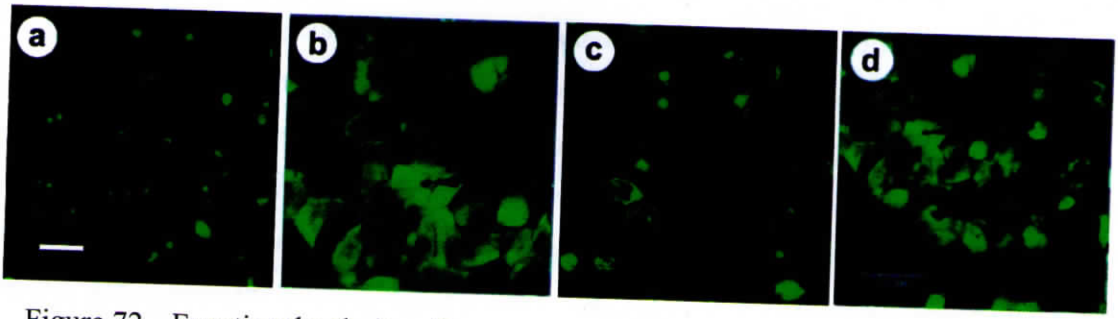


Figure 72. Functional polarity of hepatocytes by FDA staining after 2 days in (a & b) normal medium and (c & d) conditioned medium. Functionally polarized spread cells showing bile canaliculi (BC) and unstained round cells (UC) not showing functional polarity

After 7 days, hepatocytes formed spheroid and cells inside the spheroid with round morphology were also found to be positive for FDA staining. This was expressed by hepatocytes in both normal (Figure 73 a) and conditioned medium (Figure 73 b)

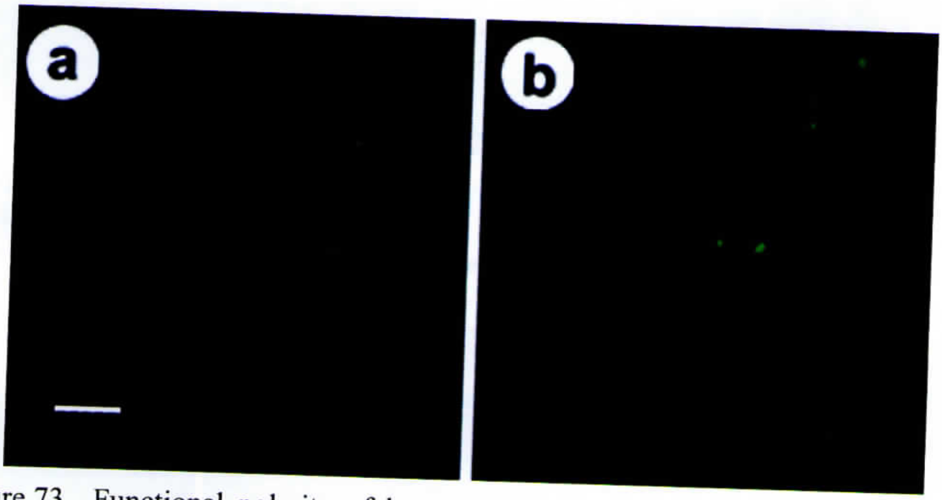


Figure 73 Functional polarity of hepatocytes cultured for 7 days in (a) normal medium and (b) conditioned medium. All cells inside the spheroid were positive for FDA staining.

4.7.4.3.3 ***Functional polarity and functional activity of Spheroids***

On comparing functionality (Cyt P450 activity by EROD staining) and functional polarity (FDA), it was visible that the cells that were functionally polarized at the initial stages were lacking Cyt P450 activity. The cells that were non-polarized and unstained in FDA staining were found to be more functional in EROD staining. The observation obtained from hepatocytes cultured in normal (Figure 74) and conditioned medium (Figure 75) was same.

The structural polarity is an indication for the functional activity of the cells. Hence to determine whether the polarized nature found in phase contrast microscope is contributing to the functional ability, a vital dye staining using FDA was done on hepatocyte culture. The cellular uptake and canalicular secretion of the dye was visualized under confocal microscope. It is previously reported that, FDA is used as a marker for hepatocyte polarity (Barth *et al*, 1982). In this study there were two cell morphology noticed in the primary hepatocyte culture – well spread polygonal cells and round cells adhering to the underlying spread cells. The spread cells were expressing the polarized nature even though not all cells were alike. The cells that are loosely bound were not fluorescing due to lack of functional polarity.

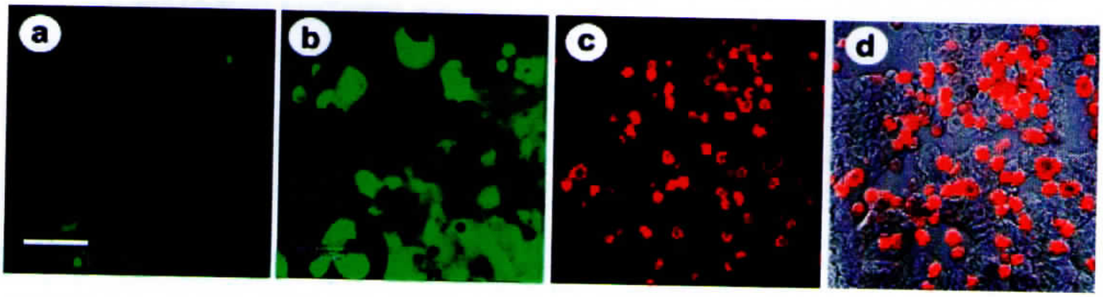


Figure 74 FDA staining and Cyt P450 assay of hepatocytes in normal medium after 1 day. Polarized spread cells (green) showing (a & b) functional polarity and (c & d) round (red) cells showing Cyt P450 function.

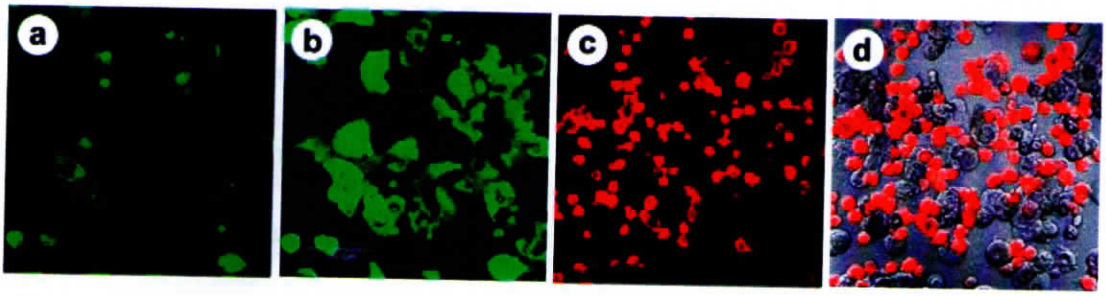


Figure 75 FDA staining and Cyt P450 assay of hepatocytes in conditioned medium after 1 day. (a & b) Green cells show functional polarity while (c & d) red cells indicate functional activity

After 7 days in culture both the control cells and cells in conditioned medium formed spheroid. Hepatocytes formed large clumps and spheroids retained functional polarity expressing green fluorescence.

The functional polarity and functional activity relation seemed to be different at 7th day. The cells inside the spheroids were found to be both functionally polarized as well as functionally active. There were no significant difference between the functional polarization and functionality between hepatocytes in normal (Figure 76) and conditioned medium (Figure 77).

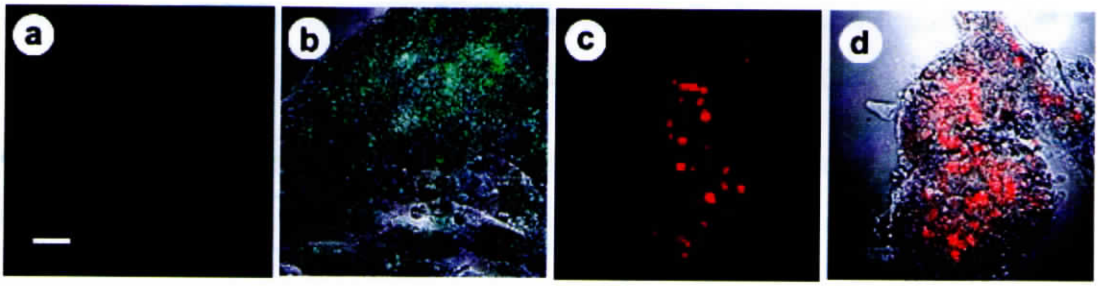


Figure 76 FDA staining and Cyt P450 assay of hepatocytes in normal medium after 7 days. Green cells show functional polarity while red cells indicate functional activity. Hepatocytes formed spheroid and all cells show (a & b) functional polarity and (c & d) functional activity.

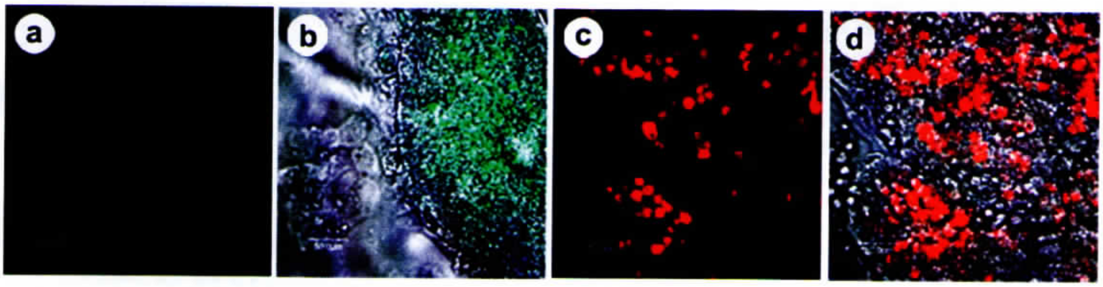


Figure 77 FDA staining and Cyt P450 assay of hepatocytes in conditioned medium after 7 days. Green cells show functional polarity while red cells indicate functional activity. Hepatocytes formed spheroid and all cells show (a & b) functional polarity and (c & d) functional activity.

From the polarity analysis of hepatocytes in culture, it was evident that there was depolarization of hepatocytes. The highly localized distribution of apical and basolateral proteins of hepatocytes in liver tissue (Figure 37 b) was found to be diffused all along its surface upon isolation. In other words, structural polarity of hepatocytes was found to be lost after cell isolation.

Hepatocytes express both structural as well as functional polarity for expressing its differentiated function. To compare the structural and functional polarity of hepatocytes, cells were subjected to EROD (metabolic functional) staining as well as FDA (functional polarity) staining. After 24 h in culture, hepatocytes showed functional polarity even in spread cells. The cells that were round and loosely bound on the spread cells remained unstained and hence were negative for functional polarity. But in the initial days, the metabolic activity by Cyt P450 of spread cells were found as low and loosely bound cells showed high functional activity (Figure 60 a). This showed that during initial days in culture, hepatocytes in its spread morphology expressed secretory function rather than metabolic ability. Likewise, hepatocytes that were loosely bound in round morphology showed more metabolic ability than secretory function.

When the above observations were compared with experiments done after 7 days in culture it was apparent that the functional polarity expressed by the round cells increased while the metabolic activity continued to be maintained. The functional polarity, which indicated the secretory function, found to be lacking in initial days were restored during the 7 days in culture (Figure 76). From this, it could be concluded that while forming spheroid, loosely bound cells with round morphology retained metabolic activity over 7 days. The restoration of functional polarity did not affect the metabolic activity of the cells. Cells continuously expressed Cyt P450 activity irrespective of restoration of functional polarity. However it was indicative that the number of functional cells found in conditioned medium was more than in control (Figure 76 and Figure 77).

4.7.5. Coculture of hepatocytes and EC

Coculture of rat hepatocytes on HUVEC and SEC monolayer was studied by analyzing spheroid and heterospheroid formation and ECM deposition using phase contrast microscope, SEM and LSCM.

The hepatocytes attached to collagen as well on EC formed good patches. Both the cell types in the coculture were in good morphology. Bile canalicular like structures were seen in between adjacent hepatocytes in the cellular patches both in control as well as in coculture as early as 24 – 48 h.

Hepatocytes are highly proliferative and metabolically active in liver. Both these characteristics are lost when it is isolated and cultured *in vitro*. Soon after isolation hepatocytes lose its polarity and when cultured on ECM-coated surface adopt flat morphology without much of its differentiated characteristics like bile canaliculi formation (Abu Absi *et al*, 2002). Hence bile canaliculi formation in culture is considered as an evidence for restoration of differentiated characteristics of hepatocytes. It has been previously shown that primary rat hepatocytes when cultured on non-adherent surfaces re-aggregate into small spheroids wherein they organize into tissue like structures (Landry *et al*, 1985). Results from this study showed the organization of hepatocytes into tissue-like structures when grown on EC monolayer in a two dimensional culture (Figure 78).

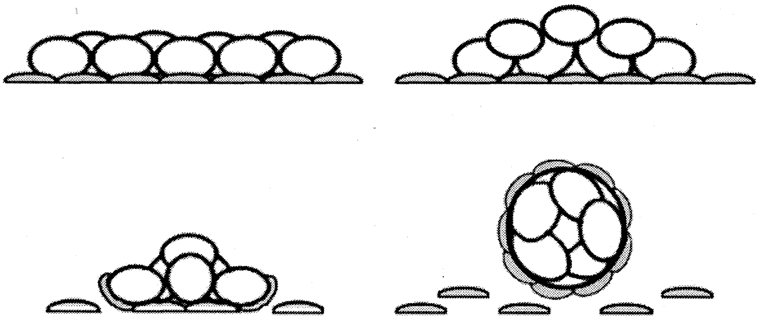


Figure 78. Schmatic diagram showing heterospheroid formation by hepatocytes on EC in a two dimensional coculture.

In tissue engineering of the liver, for therapeutic or investigational purposes, cocultivation has been attempted with primary hepatocytes and many different cell types. Hepatocytes cultured as aggregates or spheroids have enhanced liver functions compared with cells spread on a culture substratum (Landry *et al* 1985, Braet *et al* 1994). Thus, the use of hepatocyte spheroids in liver tissue engineering application should help to improve the performance of tissue functions.

4.7.5.1. Heterospheroid formation on HUVEC

Hepatocyte – HUVEC coculture is not much studied *in vitro*. The first information on this coculture system reported by Nahmias *et al* (Nahmias *et al*, 2006) was on seeding hepatocytes in tube structures of HUVEC using freshly isolated rat hepatocytes. Hepatocytes were seeded on patterned vascular structures to mimic sinusoidal structures. In this study freshly isolated rat hepatocytes were seeded on HUVEC monolayer to study the tissue organization under minimal *in vitro* condition.

4.7.5.1.1 ***Phase contrast microscopy***

Heterospheroid formation of hepatocyte with HUVEC was monitored under phase contrast microscope with comparing hepatocytes on collagen. Hepatocytes seeded on HUVEC monolayer attached and spread showing characteristic morphology on 1st day (Figure 79). On 3rd day, control cells started forming spheroid by clump formation.

However in coculture the cells were showing large polygonal morphology covering the underlying HUVEC (Figure 80).

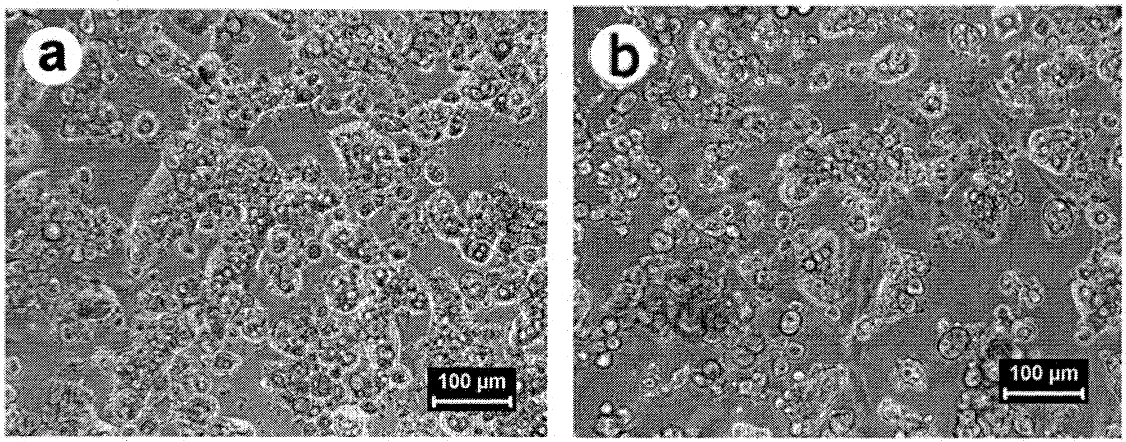


Figure 79. Hepatocyte spheroid formation on (a) collagen and (b) HUVEC on 1st day. Hepatocytes adhered on HUVEC and formed cell patches

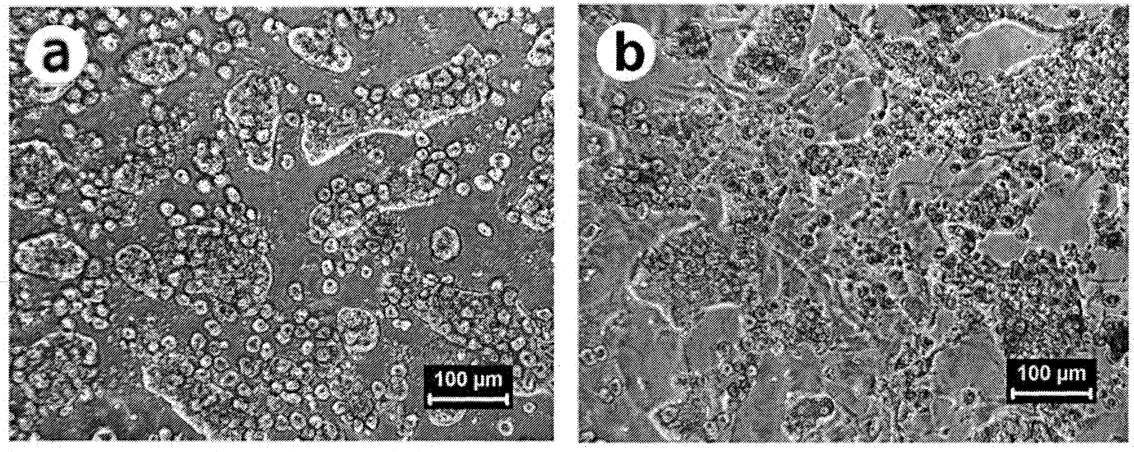


Figure 80. Hepatocyte spheroid formation on (a) collagen and (b) HUVEC on 3rd day. Cells spread well on HUVEC monolayer.

On 5th day, spheroid formation was started by hepatocytes on collagen as large patches of cells clumping together (Figure 81a). Hepatocytes on HUVEC monolayer continued to express spread morphology when compared to hepatocytes on collagen (Figure 81b). Heterospheroid formation was complete on 7th day, without leaving any cell patches on the culture surface (Figure 82) on both control and coculture. This short delay in hepatocyte spheroid formation on HUVEC coculture could be due to the role of ECM secreted by the EC or underlying HUVEC monolayer.

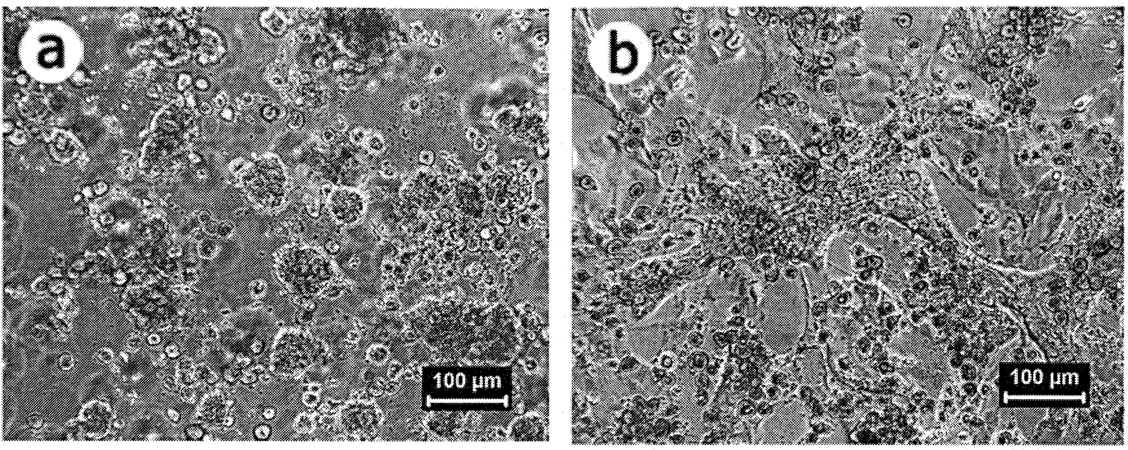


Figure 81. Hepatocyte spheroid formation on (a) collagen and (b) HUVEC on 5th day. Cells started forming spheroid on collagen but continued to be in spread morphology on HUVEC

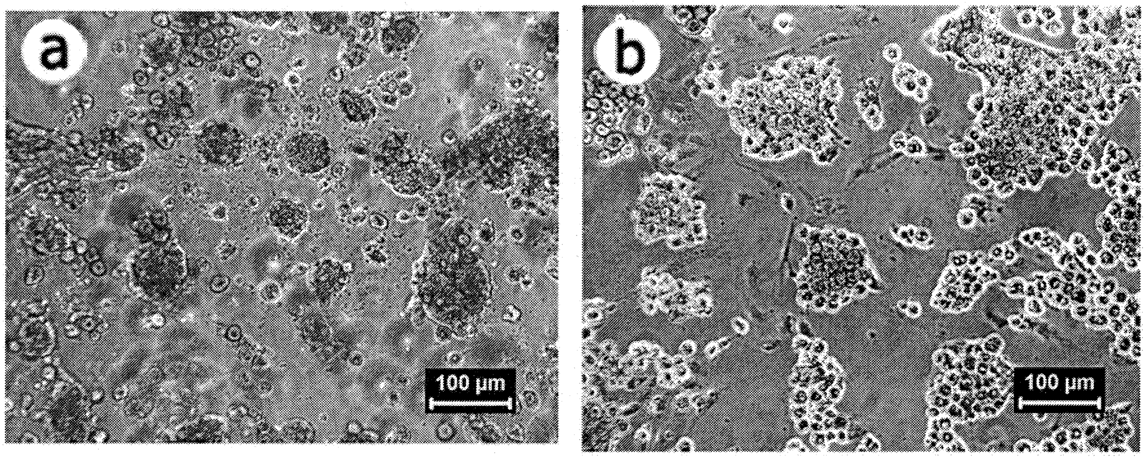


Figure 82. Hepatocyte spheroid formation on (a) collagen and (b) HUVEC after 7 days. Cells on both collagen and HUVEC monolayer formed spheroids. Very little cell patches were left on the surface while majority of patches formed spheroid.

4.7.5.1.2 ***Scanning Electron Microscopy***

The hepatocyte-HUVEC coculture was also observed under SEM. Over 10 days in IMDM, hepatocytes formed spheroid in both normal and coculture. The spheroid formed in control hepatocytes which were seeded on collagen showed an outer covering (Figure 83a). The outer covering of hepatocyte spheroid formed on HUVEC monolayer was smooth (Figure 83b).

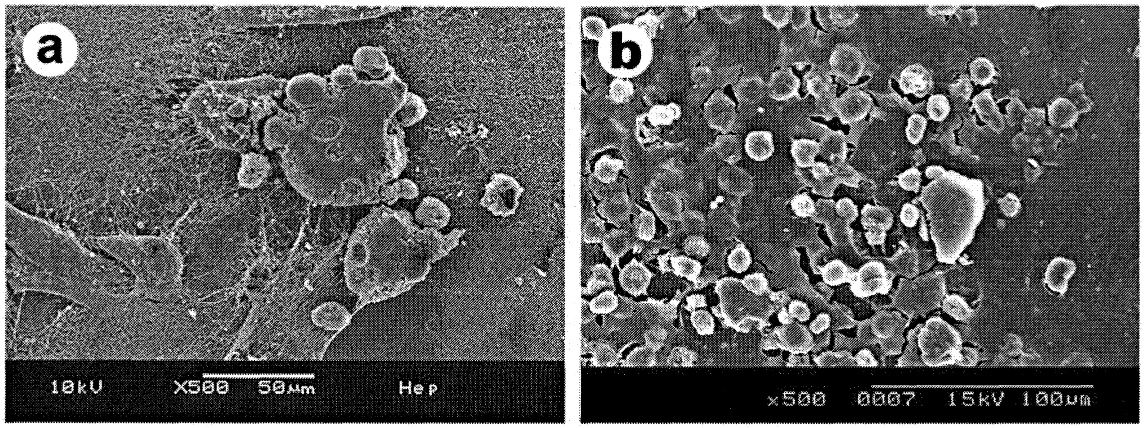


Figure 83. Spheroid formed on (a) collagen and (b) HUVEC monolayer.

In control cells, there were ECM like structure found below the spheroid (Figure 84a). Fiber like structures was left behind the surface when hepatocytes formed spheroids on collagen. However these structures were not seen in HUVEC coculture. This could be due to the increased proliferation of HUVEC masking the ECM (Figure 84b).

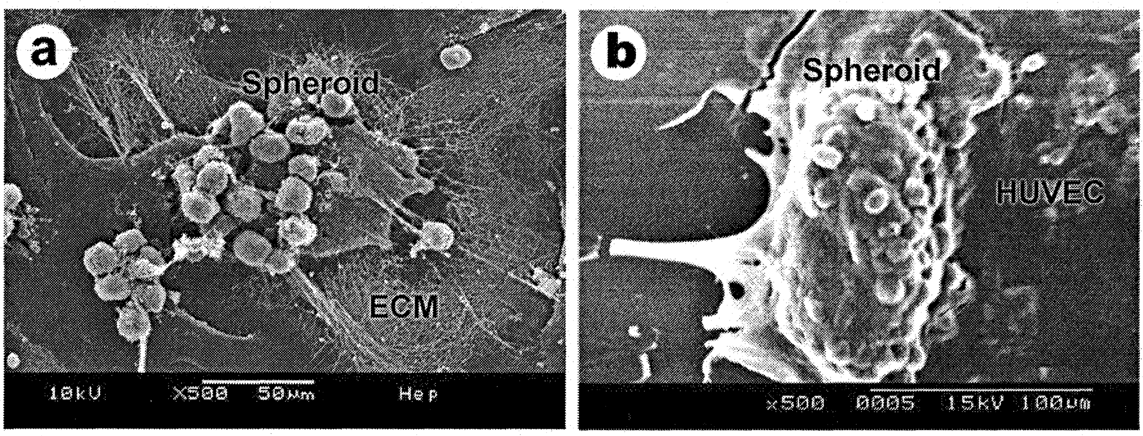


Figure 84. ECM deposition during hepatocyte spheroid formation in (a) collagen and (b) HUVEC monolayer.

4.7.5.1.3 ***Laser Scanning Confocal Microscopy***

Heterospheroid when analyzed for DiI-Ac-LDL uptake after 10 days in culture, showed functionally active interpenetrated HUVEC inside the spheroid. DepthCod analysis of confocal images obtained in different z planes to a height of 30 μm confirmed HUVEC cells inside the spheroid (Figure 85). In DepthCod analysis, images obtained in z-plane are assigned a color code to the pixel intensity as a function of the z-depth

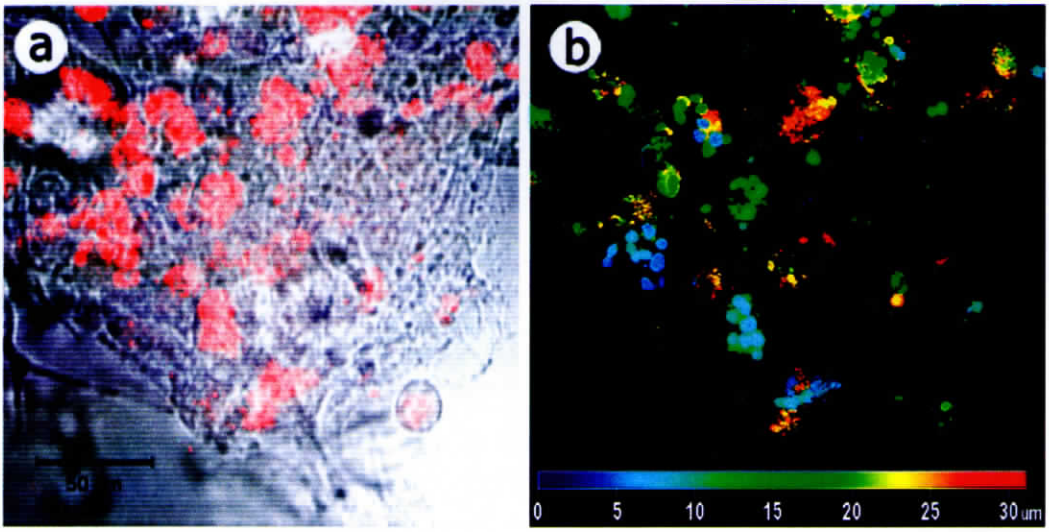


Figure 85. Tissue organization by hepatocytes with endothelial cells. (a) Projected representation of images obtained in transmittance and confocal mode at z plane. Red fluorescence represents EC and unstained cells represent hepatocytes. (b) Same image in 'a' representing the DepthCod. Blue colour denotes cells at bottom of spheroid and red colour denotes the cells on top. The gradient in between blue and red shows the distribution of EC inside the spheroid

Nahmias *et al* (2006) reported the absence of LDL uptake by rat hepatocytes when cocultured with HUVEC in 24 h. In the results presented (Figure 85), similar observation was seen with a rat hepatocyte- HUVEC coculture upto 10 days

4.7.5.2. Heterospheroid formation on SEC

Due to the physiological role and anatomical position of SEC in liver, it is evident that SEC is the appropriate cell type for coculture to simulate *in vivo* condition. In this study hepatocyte were cultured for 7 days on SEC monolayer to observe the spheroid formation.

*4.7.5.2.1 **Phase contrast microscopy***

The coculture of hepatocyte with SEC was observed under Phase Contrast Microscope. The hepatocytes attached to collagen-coated coverslips and formed good patches of cells (Figure 86a). Similarly hepatocytes seeded on SEC patches showed comparable morphology as in control (Figure 86b). Both the cell types in the coculture were in good morphology. Bile canalicular like structures were seen in between adjacent hepatocytes in the cellular patches both in control (Figure 87a) as well as in coculture (Figure 87b) as early as 24 – 48 h.

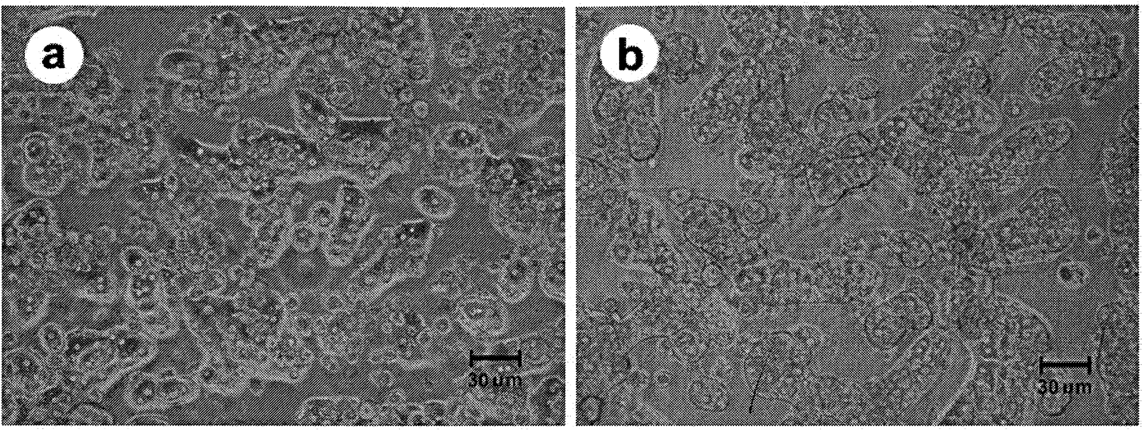


Figure 86. Hepatocyte culture on (a) collagen coated coverslips and (b) SEC monolayer

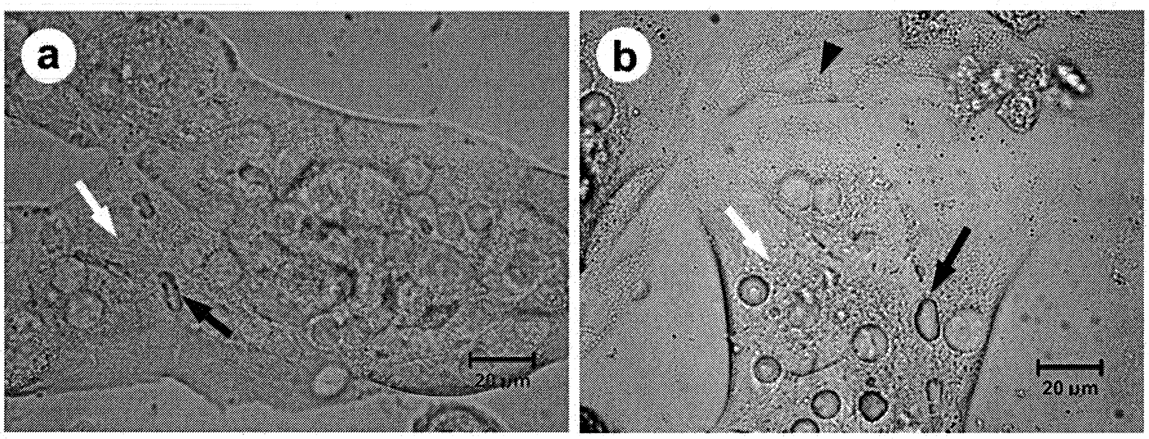


Figure 87. Bile canaliculi in hepatocytes (a) on collagen and (b) SEC cell patches. (White arrow – hepatocytes, Black arrow – Bile canaliculi, Arrow head – SEC)

The cells cultured on collagen-coated coverslips and SEC patches spread with intact cell – cell contact (Figure 87). Both the control hepatocytes and coculture revealed initiation of spheroid formation from the cellular patches at 3rd day in culture (Figure 88). Upon incubation for 5 days, the cell spheroid formation increased and started detaching from remnant cell patch (Figure 89). On 7th day, almost all cell patches formed spheroid and floated in the culture medium (Figure 90).

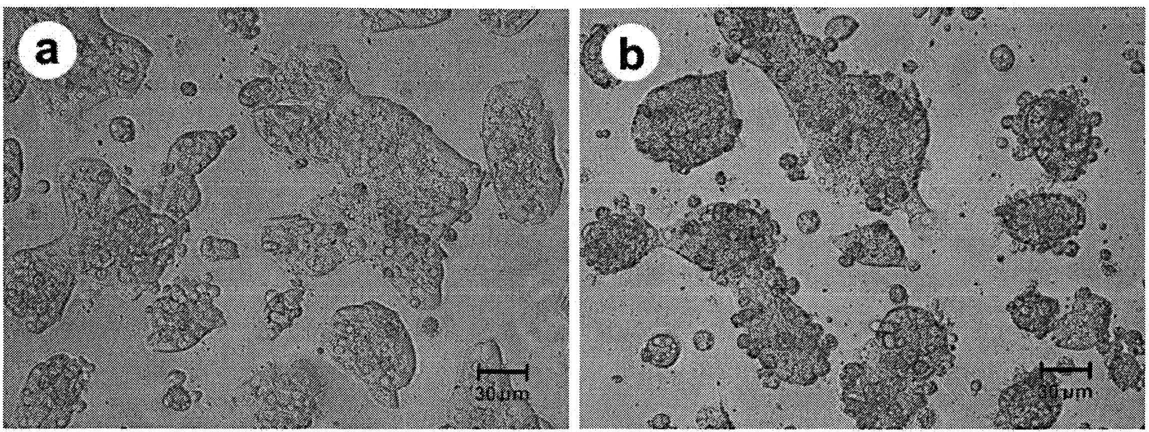


Figure 88. Hepatocyte spheroid formation on 3rd day (a) on collagen and (b) on SEC cell patch

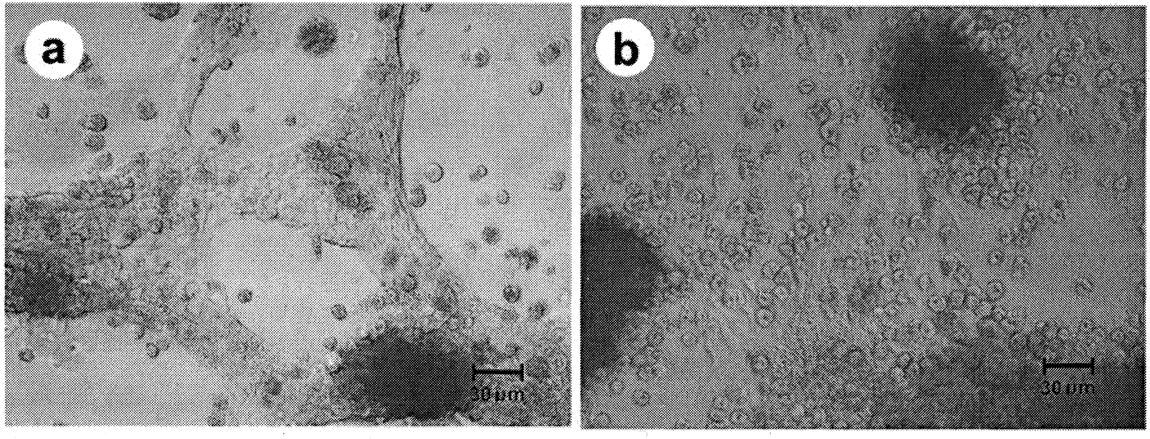


Figure 89. Hepatocyte spheroid formation on 5th day. Cells started forming spheroid from the cell patch (a) on collagen and (b) on SEC cell patch

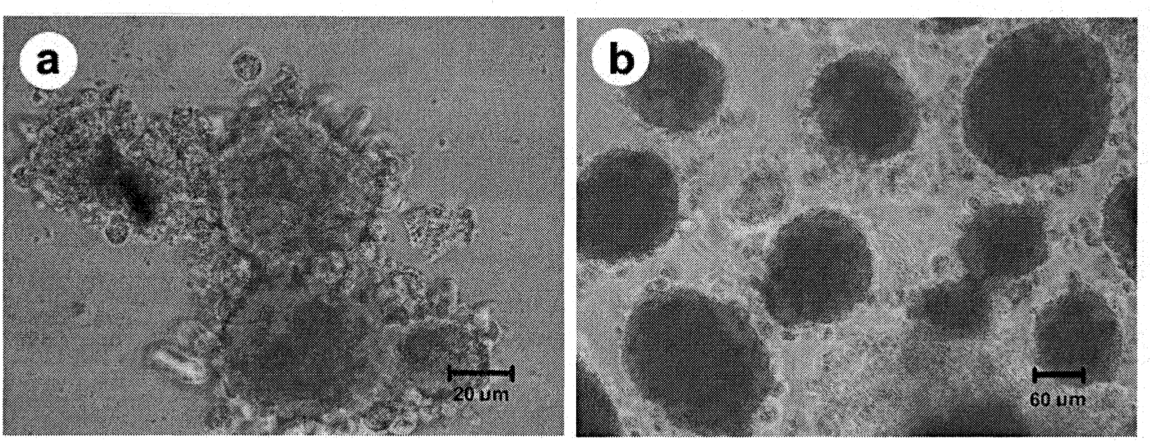


Figure 90. Hepatocyte spheroid formation on 7th day. Cells formed spheroid and started floating (a) on collagen and (b) on SEC cell patch

The hepatocyte spheroid formation in coculture with SEC is comparable with the control hepatocytes. During spheroid formation, hepatocyte patches glided over the underlying SEC, leaving the later undamaged (Figure 91a). Moreover, it was noted that the SEC expressed its characteristic feature, the fenestrations, during spheroid formation by hepatocytes (Figure 91b).

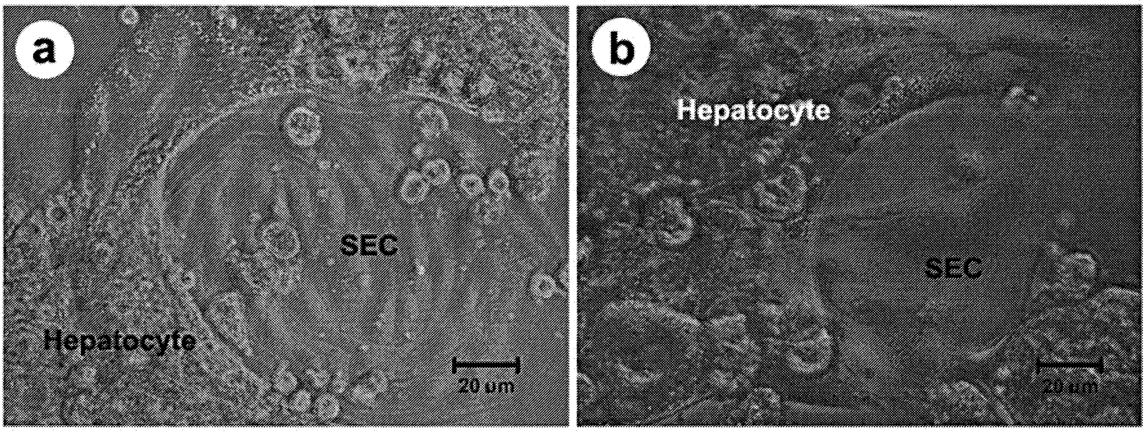


Figure 91. Hepatocytes and SEC during spheroid formation (a) Hepatocytes spheroid formation without disturbing underlying SEC and (b) Expression of fenestration by SEC while hepatocytes formed spheroid.

4.7.5.2.2 *Scanning Electron Microscopy*

The hepatocyte-SEC coculture was also observed under SEM for analyzing ECM deposition. Over 10 days in IMDM, hepatocytes formed spheroid in both normal culture (Figure 92a) and coculture (Figure 92b).

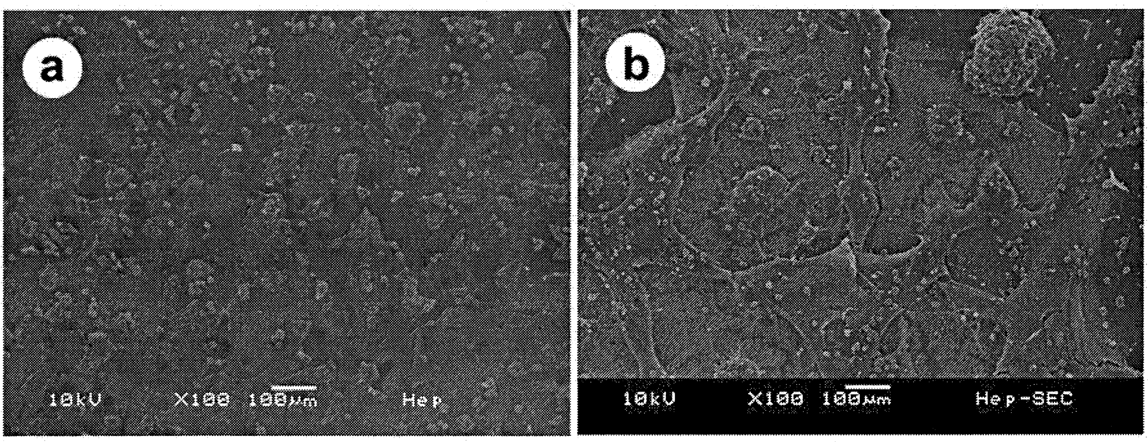


Figure 92. Formation of hepatocyte spheroid after 10 days (a) on collagen and (b) SEC patch.

The outer covering formed by the hepatocyte spheroid on SEC monolayer was smooth and large when compared to spheroids on collagen (Figure 93a). Spheroid formation and heterospheroid formation left ECM like structures on culture surface. The morphology of the ECM structures observed in both cases was different. The fiber like structures left behind the surface when hepatocyte spheroids formed on collagen were less and scattered. In contrast to this the ECM structures formed in hepatocyte-SEC coculture was more and dense (Figure 93b)

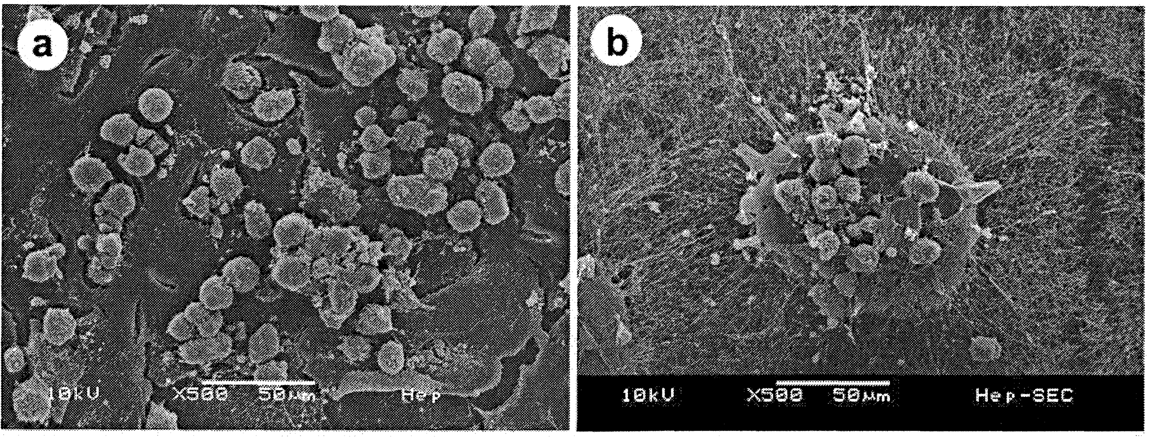


Figure 93. SEM images of hepatocyte spheroid on (a) collagen (b) SEC showing ECM deposition

In the coculture system, SEC under the hepatocytes was strongly bound to surface even after 10 days. While spheroid formation, the SEC patch expressed fenestrations (Figure 94).

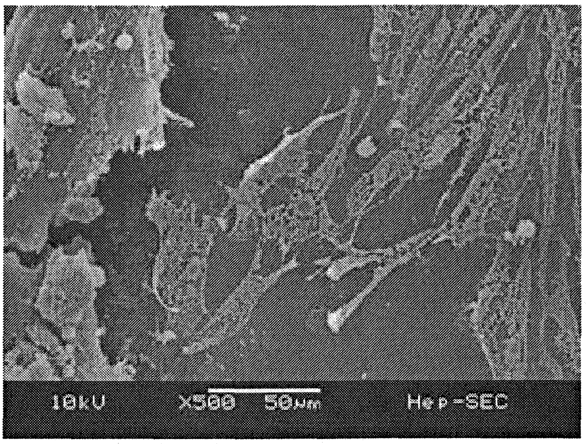


Figure 94. SEC in hepatocyte-SEC coculture showing fenestrations (10 days)

Under higher magnification, fenestrae like structures were observed over the surface of spheroids (Figure 95). Such structures found in the spheroids of hepatocyte-SEC coculture (Figure 95 e-h) were different from those in control hepatocyte spheroids (Figure 95 a-d) when compared in different magnifications

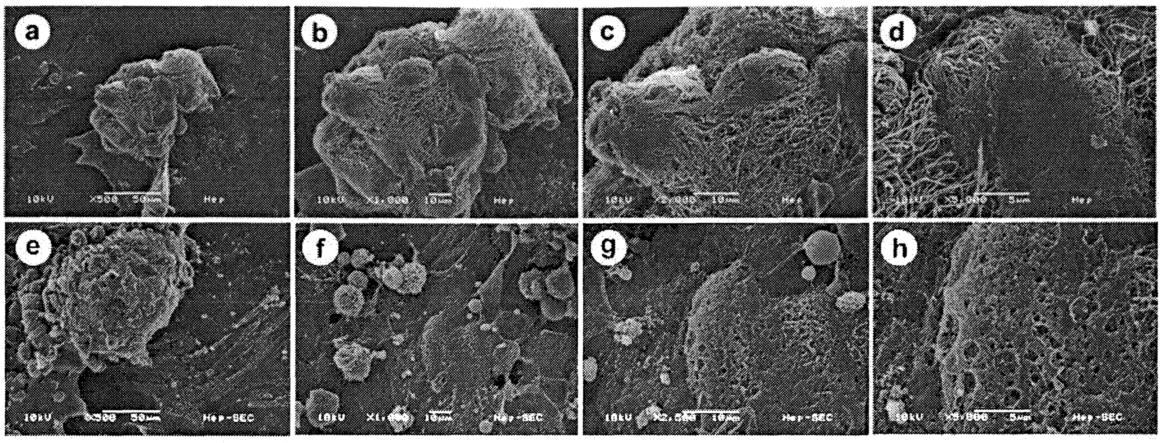


Figure 95. SEC fenestrae like structures seen on (a,b,c,d) spheroid and (e,f,g,h) hepatocyte SEC heterospheroid observed under diffenent magnifications.

4.7.6. Hepatocyte culture on thermoresponsive surface

To assess the culture requirement of hepatocyte on thermoresponsive surface, freshly isolated cells were cultured on uncoated and collagen coated PIPAAm dishes. It was found that adult rat hepatocyte requires collagen coating on PIPAAm surface for adhesion and growth. When observed after 24 h of cell seeding, hepatocytes on collagen coated PIPAAm dishes were more when compared to that on uncoated PIPAAm dish (Figure 96). This clearly showed that the adult hepatocytes required collagen coating on PIPAAm surface.

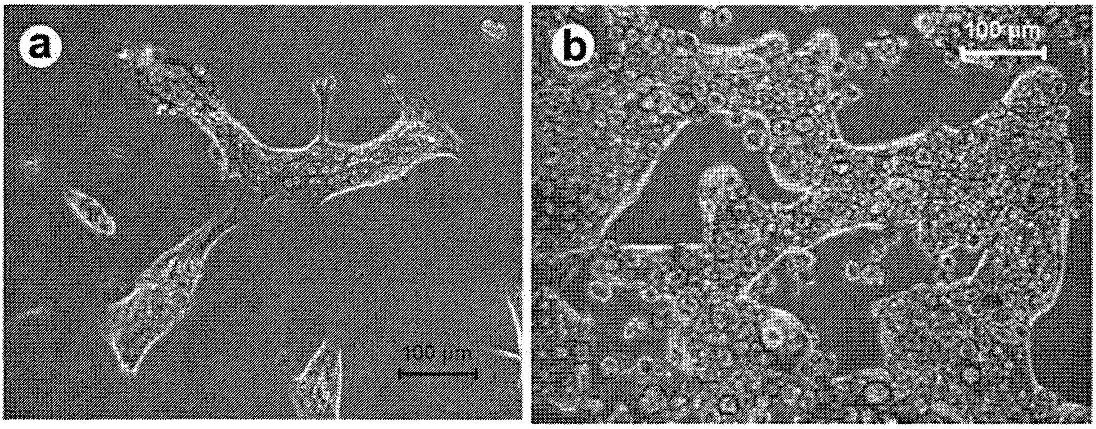


Figure 96. Adult rat hepatocytes cultured on (a) uncoated and (b) collagen coated PIPAAm dishes observed after 24 h. More number of cells were adhered on collagen coated surface.

Adult rat hepatocytes cultured on collagen coated thermoresponsive culture surface favoured normal cell adhesion and growth. Cells formed spheroid as in normal culture dishes within 4 days (Figure 97). Cells formed monolayer on 1 day formed clumps and spheroids on 3rd day. Spheroid formation continued up to 4 days showing normal acclimatization to culture on PIPAAm surface.

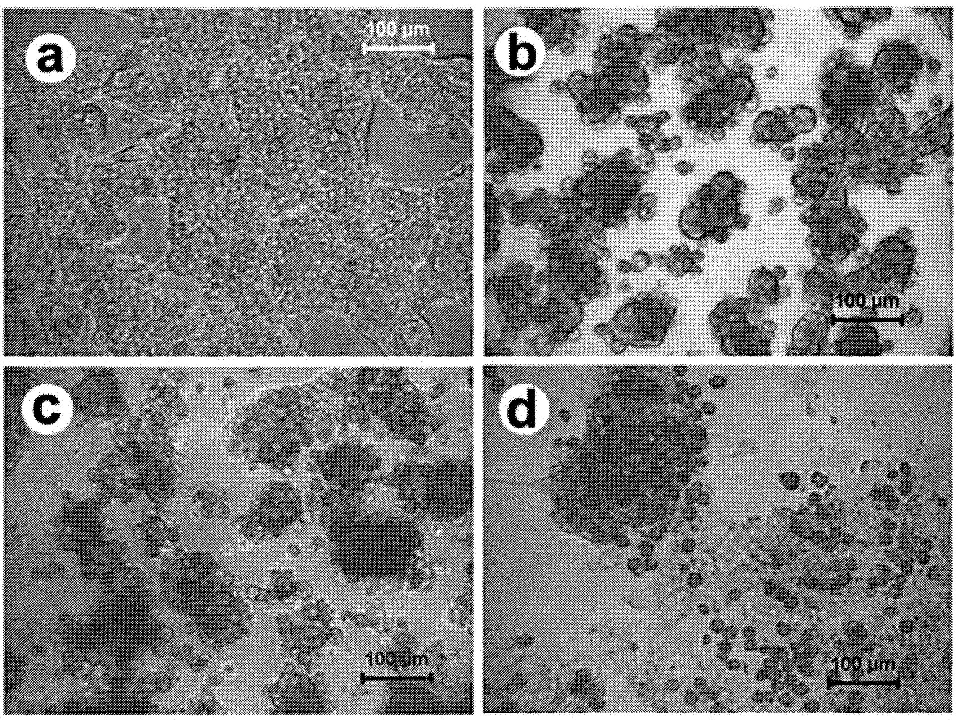


Figure 97. Adult rat hepatocytes cultured on collagen coated PIPAAm dishes observed at (a) 1 day (b) 2 days, (c) 3 days and (d) 4 days.

4.7.6.1. Retrieval of hepatocytes by temperature variation

The advantage of using thermoresponsive surface for hepatocyte culture is to retrieve the cells without enzyme treatment, thereby preserving the native structure and function. Primary hepatocyte monolayer on collagen coated PIPAAm surface detached from the surface when temperature is lowered below 10°C. Cells could be retrieved during different stages of spheroid formation (Figure 98).

4.7.6.2. Metabolic ability of retrieved cells

Cells retrieved by temperature variation were subjected to metabolic assay by EROD staining method. Cells retrieved by temperature variation were able to maintain the biotransformation ability of converting EROD substrate (Figure 99). This showed that hepatocyte retrieval from temperature responsive surface did not affect the metabolic functional ability of cells.

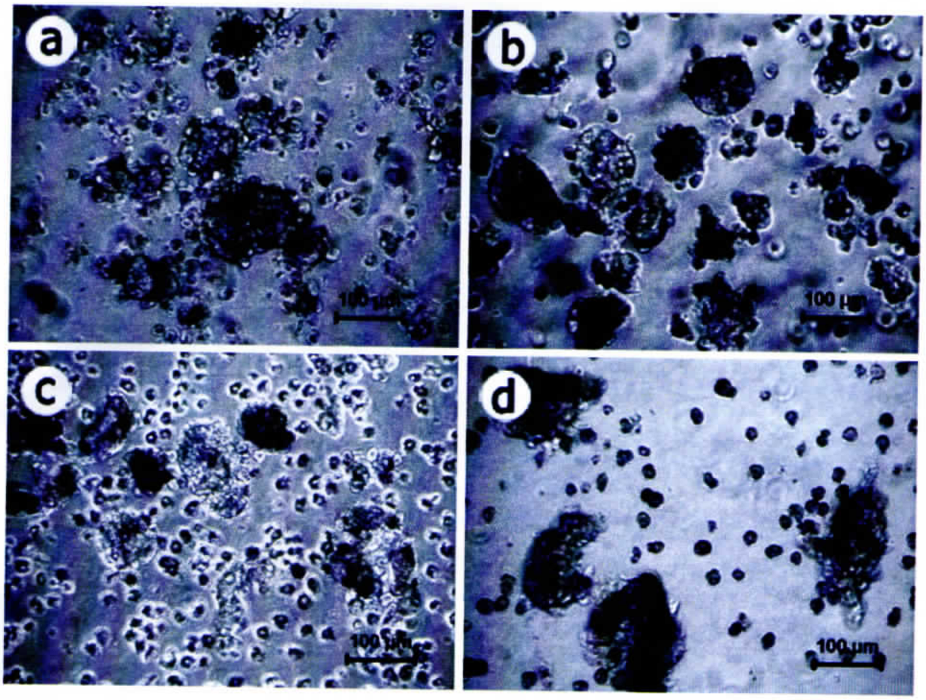


Figure 98. Hepatocyte cultured on PIPAAm surface were retrieved during different stages of spheroid formation (a) 1 day (b) 2 days, (c) 3 days and (d) 4 days.

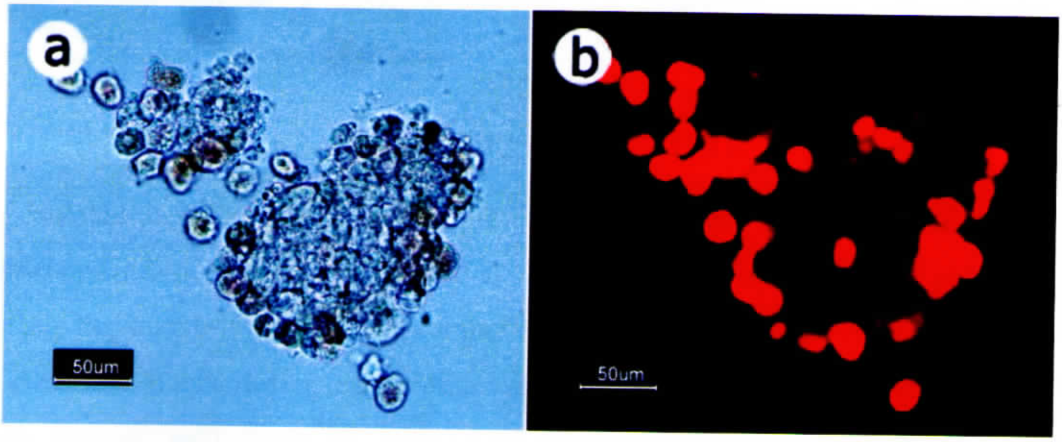


Figure 99. Expression cyt P450 activity by hepatocytes retrieved from PIPAAm grafted surface by temperature variation under (a) bright field and (b) fluorescence

4.7.6.3. Culturing retrieved cells

One of the requirements in using PIPAAm surface is to transfer intact cells to new surfaces avoiding enzyme treatment. Hepatocytes retrieved and transferred by temperature variation were able to grow on new dishes (Figure 100).

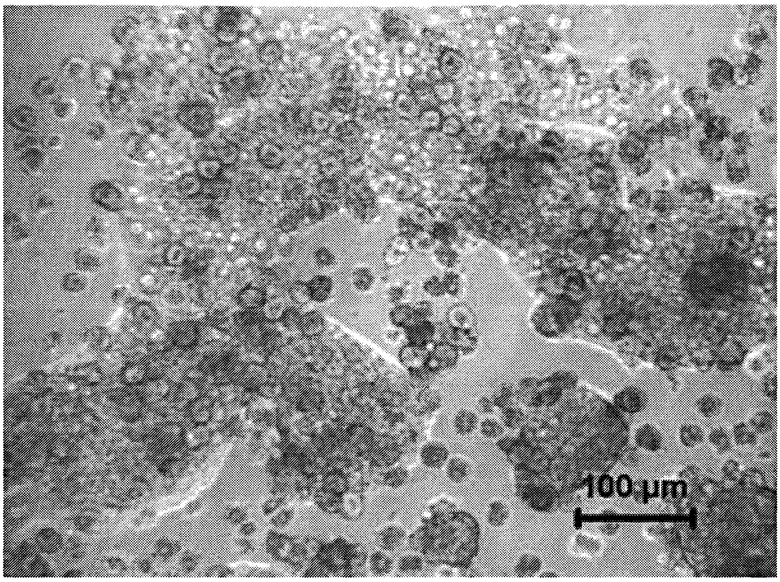


Figure 100. Hepatocytes retrieved from PIPAAm surface by temperature variation transferred to new culture dish grew as normal cells

One of the main hindrance in hepatocyte cryopreservation is the damage caused by membrane disorders manifested as a result of enzymatic digestion. The cell survival after thawing can be improved by cryopreserving hepatocytes inside collagen matrix by preserving native structure (Koebe *et al*, 1996). Cell retrieval by temperature variation explained above can be a good alternative for obtaining intact hepatocytes for cryopreservation and cell transplantation.

4.8. HUMAN FOETAL LIVER CELL CULTURE

Isolated hepatocytes have been found immense use in number of scientific disciplines from fundamental to advanced applied aspects. There are a lot of publications on human hepatocyte isolated from human samples. Human hepatocytes are isolated from discarded livers during transplantation, surgical waste material remaining after reduced size or split liver transplantations and material from partial hepatectomy specimens. Hence there is drastic difference among various tissue sources of human hepatocytes. Scarcity of donor human livers, absence of proliferation in culture and poor viability after cryopreservation impose restrictions in using adult human hepatocytes. To overcome these difficulties, hepatocytes of foetal origin has been used. Freshly isolated human foetal hepatocytes express similar function as that of adult hepatocytes (Cai *et al*, 2002). There are also reports showing fetal liver with large number of Progenitor/stem cells (hepatoblasts) which proliferates for several months retaining normal karyotypes. The progenitor cells differentiated into mature hepatocytes in mice with severe combined immune deficiency (Hamreet *et al*, 2002). However isolation of hepatocytes from human fetus is not so popular when compared to that from adult liver tissues. In this study an easy and simple technique was adopted to isolate and culture cells from human foetus by two step perfusion.

Hepatocytes isolated from discarded non-viable human foetal liver adhered and grew on uncoated and collagen coated tissue culture bottles showing good proliferation (Figure 101 a and b). Cells attained flat broad polygonal morphology and formed monolayer within 3 weeks. Cells were able to subculture by normal trypsinization and were maintained upto 3 passages (Figure 101 c and d).

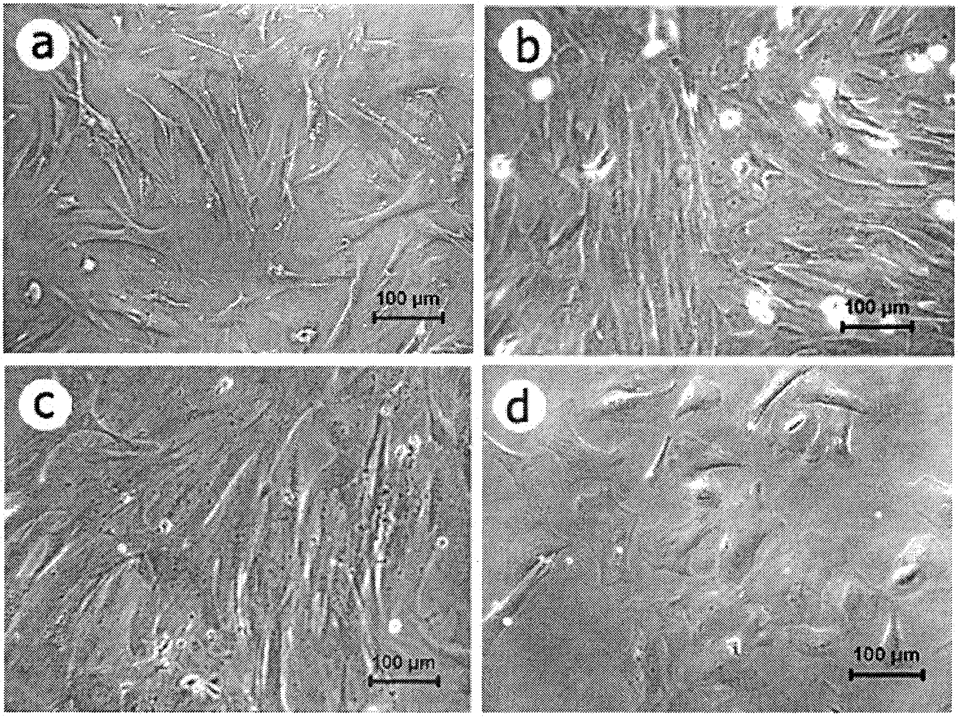


Figure 101. Human foetal liver cells in culture. Cells grew on (a) uncoated and (b) collagen coated culture dishes. Subcultred cells at (c) passage 1 and (d) passage 2 stage

4.8.1. Characterization of liver cells

Cells from foetal liver expressed albumin synthesis (Figure 102 a and b) as evidenced by immunofluorescence staining. It has been reported that freshly isolated human foetal hepatocytes express similar function as that of adult hepatocytes (Cai *et al*, 2002). Thus it is evident that foetal liver cells show hepatocyte specific characteristics of albumin synthesis.

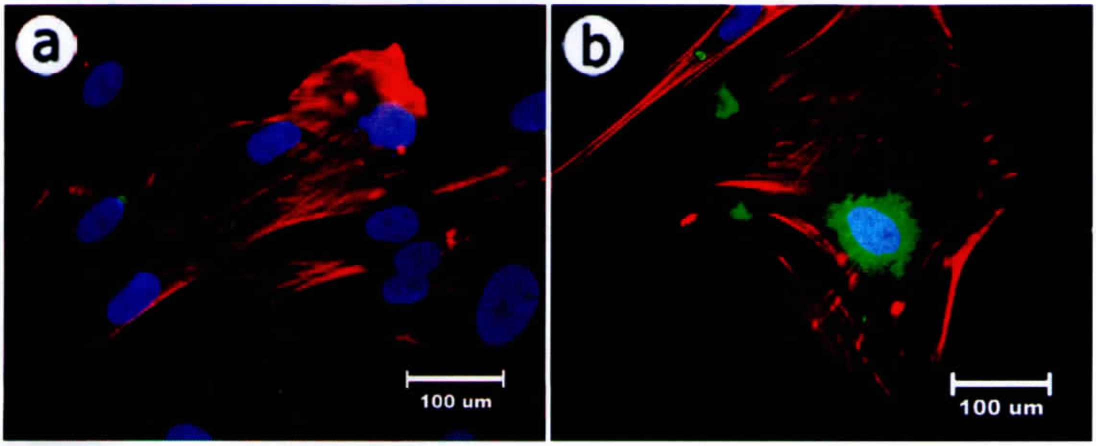


Figure 102. Liver cells (a) expressing albumin and actin cytoskeletal structures. Green colour represents albumin, red color represents actin and blue region represent nucleus. (b) Single cell showing albumin synthesis and extensive actin network denoting the adhesion and spreading of the cells.

4.8.2. Differentiation of liver cells into adipocytes

Fluorescence staining of liver cells showed different distribution pattern of actin filaments (Figure 103). This could be due to the presence of different types cells obtained from foetal liver. It is also understood that foetal liver harbours progenitor cells.

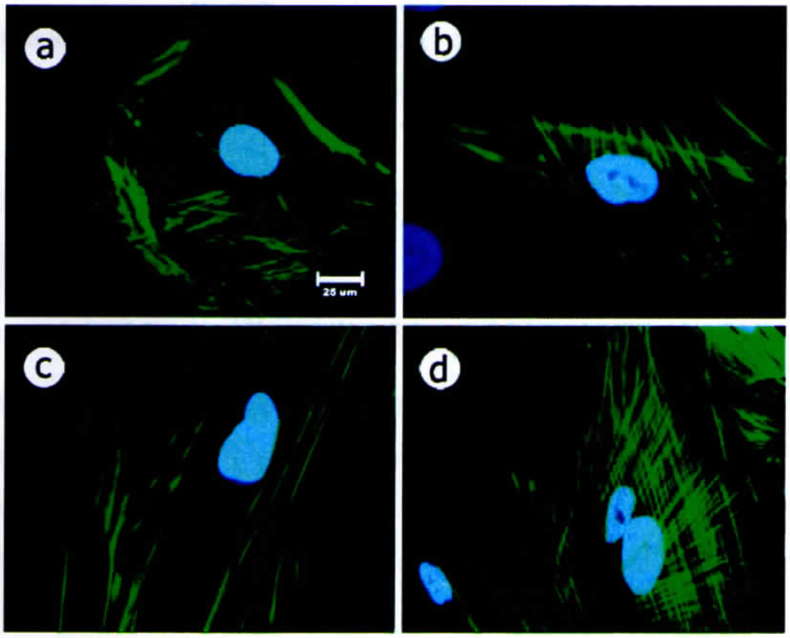


Figure 103. Cytoskeletal staining of foetal liver cells showed different patterns

Since liver represents the hematopoietic site in foetus, cells in foetal liver also have capacity similar to stem cells. As liver serves as the hematopoietic site in foetus, it also harbors cells with functional similarity to Mesenchymal Stem Cells (MSC)

(Gotherstorm *et al*, 2003). To assess capability of foetal liver cells to differentiate into different lineages, cells were differentiated using adipogenic medium. After one week, the cells morphologically expressed adipocyte characteristics (Figure 104a). Differentiated cells showed a positive reaction of Oil Red-O staining specifically demonstrating accumulation of lipid droplets. The intracellular lipid accumulation of the adipogenic differentiated cells seemingly increased with time of incubation (Figure 104b).

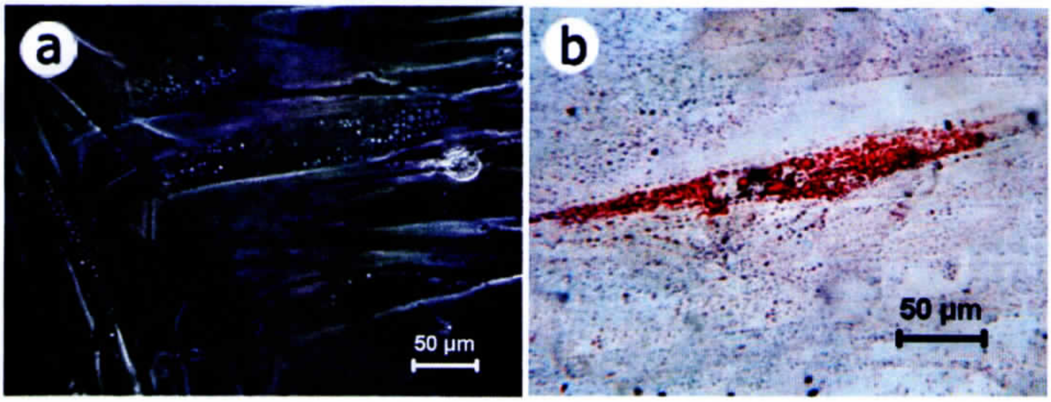
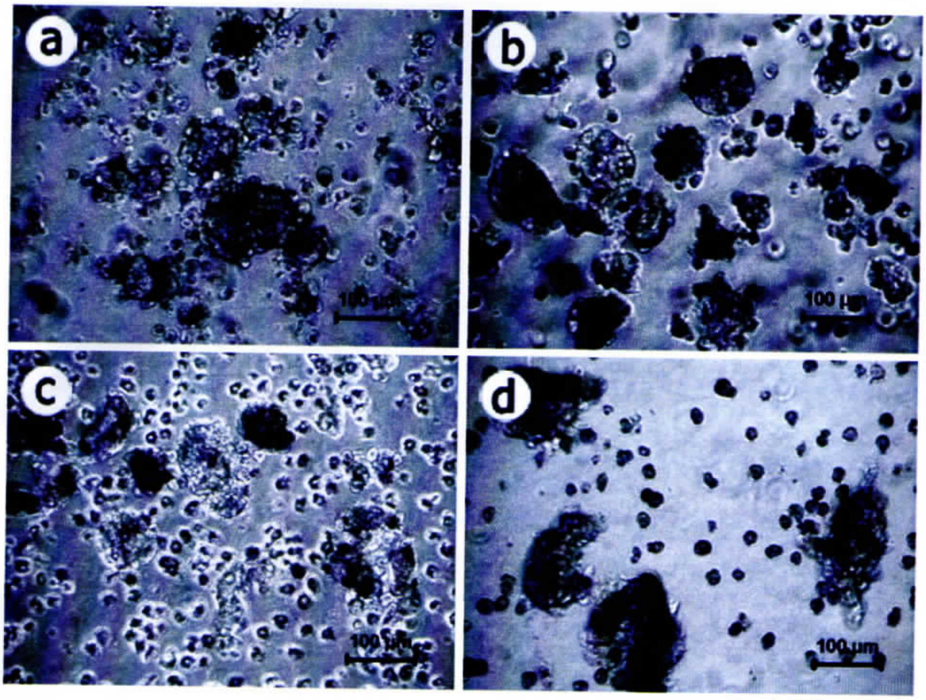


Figure 104. Differentiation of foetal liver cells to adipocytes. (a) Cells expressed lipid accumulation in adipogenic medium (b) Oil Red O staining

It is evident from the above results that human foetal liver cells cultured in IMDM with low serum supplement can be a good culture system to maintain liver cells of hepatic lineage with differentiating potential. Moreover double perfusion method of foetal liver is easy and gives large number of liver progenitor cells. A high differentiation ability of foetal progenitor cells to adipocytes manifest its importance as another cell source for tissue engineering.

4.8.3. Human foetal liver cells on PIPAAm

Foetal liver cells adhered and proliferated to form monolayer on PIPAAm grafted surface (Figure 105a). When the culture was incubated at low temperature cell adhesion on the polymer was reduced and cell sheets were retrieved as patches by pipetting with cold medium (Figure 105b). Human foetal liver cell culture and retrieval from the PIPAAm surface has not been reported earlier.



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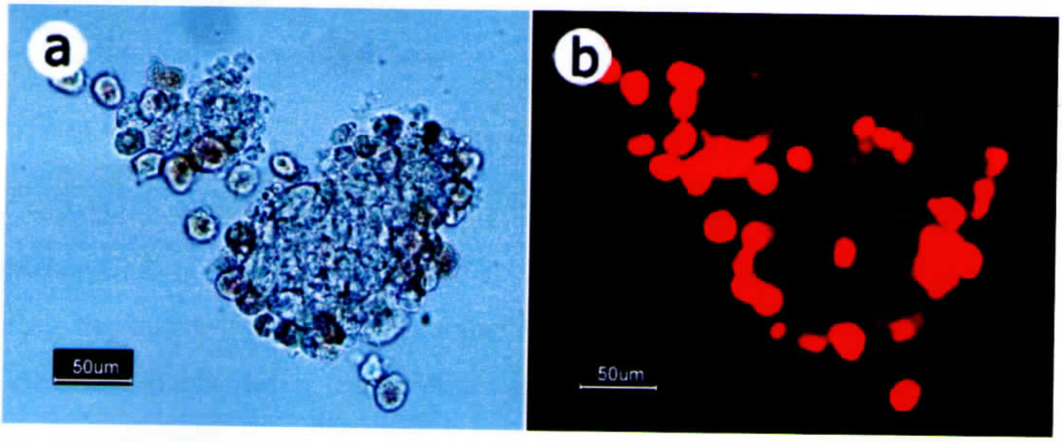

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4.7.6.3. Culturing retrieved cells

One of the requirements in using PIPAAm surface is to transfer intact cells to new surfaces avoiding enzyme treatment. Hepatocytes retrieved and transferred by temperature variation were able to grow on new dishes (Figure 100).

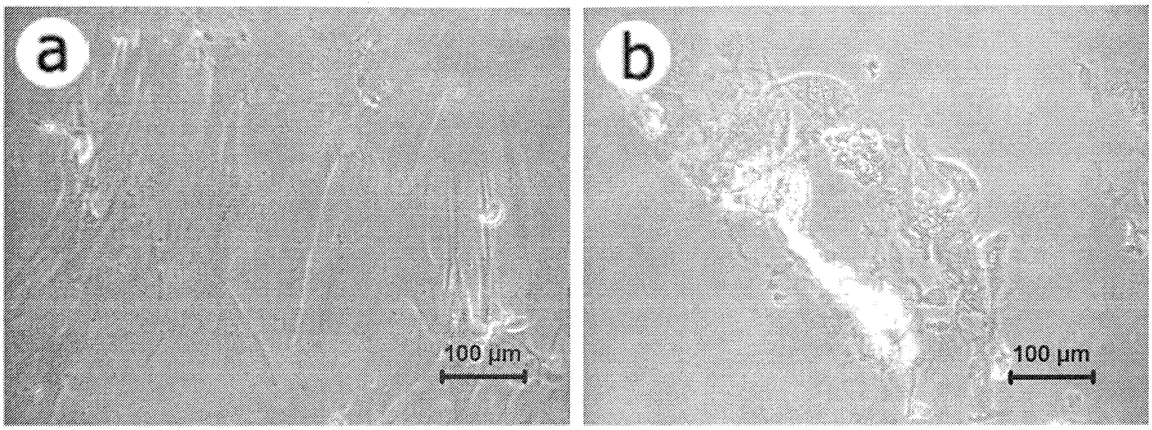

Figure 105. Human foetal liver cells on PIPAAm surface. (a) Cells formed monolayer on grafted surface. (b) Cell patch retrieved by gentle pipetting after incubating at a low temperature.

Chapter 5

SUMMARY, CONCLUSIONS AND FUTURE PROSPECTS

5.1. SUMMARY AND CONCLUSIONS

Liver diseases are increasing year by year all over the world mainly due to the injury to hepatocytes. In many cases if the injury is mild and reversible, the patient may be left with an entirely normal liver due to the remarkable capacity of the organ to regenerate. During end stage disease, liver transplantation is the only satisfactory treatment for saving the patient. There is an increasing demand of donor organs far beyond the availability. Liver tissue engineering is one of the emerging approaches to address this problem. Bioartificial Liver is a tissue engineered product which allows spontaneous recovery of damaged liver or survival of the patient until transplantation. Important components required for liver tissue engineering are appropriate cell source, scaffolds, ECM, microenvironment, tissue specific functions and tissue organization. Liver tissue engineering procedures can be useful in developing *in vitro* tissue equivalents for cell transplantation, *in vitro* toxicity testing module or as a BAL.

The main challenge of primary hepatocyte culture is its maintenance with desired level of viability and function. Attempts with high density cell seeding (Bissel *et al*, 1973), use of different growth supplements (Kurtz *et al*, 1981) and several soluble factors (Geoffrey *et al*, 1996) on hepatocyte functions have been studied. Long term culture models showed advantages and disadvantages of media, role of ECM and soluble media components. Three dimensional cultures resemble more closely to *in vivo* situation with regard to cell shape and environment. The conventional method of hepatocyte 3D culture is to sandwich hepatocytes in ECM. Both homotypic and heterotypic interactions influence hepatocellular functions. It is apparent that hepatocyte behaves differently in culture due to the change in cell interaction happening during cell isolation. Homotypic and heterotypic cell interaction can be accomplished *in vitro*. All the previous studies utilized either NPCs or extrahepatic primary cells or celllines. When hepatocytes are provided with appropriate environment, it self assemble into 3D structures or spheroids that exhibit ultrastructural characteristics of native hepatic tissue with enhanced liver specific function.

The present study aimed at optimization and functional characterization of a coculture system for enhanced maintenance of *in vitro* hepatocyte function as these cells are well known for losing its differentiated function immediately after isolation. To achieve this, culture conditions were optimized for coculture of hepatocytes and endothelial cells thereby providing both homotypic and heterotypic cell-cell interaction. A thermosensitive culture surface was inhouse synthesized to help cell attachment and cell retrieval retaining naïve cell-cell and cell-ECM contact and therby avoiding loss of cell funtion.

A temperature sensitive culture surface was prepared by grafting polymerized form of NIPAAm on tissue culture dishes. The PIPAAm grafted culture surface was characterized by Attenuated Total Reflection - Fourier Transform Infra Red spectrophotometry (ATR-FTIR), Scanning Electron Microscopy (SEM) and water contact angle measurement. Different cell types like L-929, NRK-49F, HOS, SIRC, primary hepatocytes, and HUVEC were cultured and retrieved by temperature variation from grafted surface to prove its efficiency to support any type of cells. Cells on PIPAAm grafted culture dishes were transferred using a membrane support and without membrane support. For tissue engineering application, the grafted surface was used as a cell retrieval tool for intact osteoblast cell sheet transfer to bone substitute material for rapid and complete cellularization. Analysis of the cellularized scaffold by fluorescence microscope, confocal microscope, SEM, EDS, ALP activity and [3H] thymidine uptake assay confirmed that this novel method of cell sheet transfer for bone tissue engineering favour rapid cellularization with maintenance of cell viability, proliferation and cell function. A combination method of membrane support transfer and peel off technique enabled the construction of multilayered, viable and intact *in vitro* tissue construct.

Hepatocyte isolation and microenvironment with minimal culture requirements were optimized using a custom made perfusion apparatus. Optimization of culture conditions proved that IMDM with 2 - 5 % serum can replace specific and expensive media like Hepatozyme. Hepatocytes in culture exhibited characteristic features like binucleation and bile canalicular formation as early as 24 h without external induction. Increase in [3H] thymidine uptake by hepatocytes showed restoration of similar *in vivo* characteristics.

Optimization of endothelial cells from human umbilical vein and maintenance using IMDM containing 10 % FBS, 500 μg/ml crude ECGF showed characteristic vWF expression and DiI-Ac-LDL uptake. Cells could be maintained by subculture without losing characteristic functions.

Rat liver sinusoidal ECs isolated, characterized and maintained upto 10 days in IMDM with 2.5% FCS, expressed characteristic fenestrated morphology and ability to uptake DiI-Ac-LDL.

The strategies followed for enhancing hepatocyte function were culture of hepatocytes in minimum culture requirements, using HUVEC and SEC conditioned medium, coculture with ECs and detachement of hepatocytes from thermoresponsive polymer.

This study used purified and characterized SEC and SEC conditioned medium for hepatocyte coculture. Hepatocytes formed spheroids in both SEC conditioned medium and HUVEC conditioned medium without any external growth factors or use of nonadhering surface or bioreactor. Hepatocytes maintained metabolic ability in EC conditioned medium as evidenced by EROD activity. Coculture system using conditioned medium retained viability and functionality for more than 21 days. Hepatocytes were metabolically active during spheroid formation in EC conditioned medium. Hepatocytes in spread morphology (a dedifferentiated stage) also showed functionality similar to that expressed by the loosely bound cells. Restoration of polarity being important in determining hepatocyte specific function and *in vitro* characteristics, polarity assessment using apical and basolateral markers revealed the polarization characteristics of hepatocytes during spheroid formation. Polarization of hepatocytes during spheroid formation in EC conditioned medium has not been reported earlier.

Eventhough spheroid formation was similar in both HUVEC and SEC conditioned medium difference in polarity was noticed. In SEC conditioned medium apical and basolateral polarity expressed during initial days were restored and reappeared in a redistributed fashion. The cells lining spheroid expressed more basolateral proteins whereas the cells inside spheroid expressed only apical proteins. Hepatocyte in HUVEC conditioned medium maintained apical polarity through out the spheroid progression. Spheroids formed in HUVEC conditioned medium showed extensive bile canaliculi networks inside the spheroid compared to spheroids in SEC.

Assessment of secretory ability by functional polarization showed spread hepatocytes as more functionally polarized than round cells during 1st day. After 7 days all cells inside the spheroid expressed functional polarity. Comparing metabolic activity with functional polarization during 1st day, spread cells were functionally polarized while round cells expressed metabolic activity. After spheroid formation at the end of 7 days, cells revealed functional polarization as well as metabolic activity. This is the first attempt to study hepatocyte polarity and metabolic ability together during spheroid formation in coculture with SEC. Specific expression pattern of polarized characteristics and metabolic ability of hepatocytes was confirmed using liver slices.

Hepatocytes maintain structural and functional attributes by formation of heterospheroids. Hepatocytes formed heterospheroids when cultured on SEC and HUVEC monolayer in 2D culture within 7 days under minimum culture requirements. A delay in heterospheoid formation was noticed in hepatocyte coculture with HUVEC compared to coculture with SEC. This result shows the significance of other cell-cell interaction and cell - ECM interaction in coculture. This study describes minimal culture requirement for heterospheroid formation by hepaptocytes on endothelial cell monolayer by spontaneous reorganization into functional tissue structures. It was found that the optimized culture condition in homotypic and heterotypic cocultures with hepatic and extra hepatic ECs has undergone tissue like organization by forming spheroids.

As human hepatocytes are more ideal for any application, an attempt was made to isolate, characterize and maintain human foetal liver cells. A simple and efficient perfusion technique was standardized to isolate foetal liver cells. The culture conditions were optimized to grow liver cells in minimal culture conditions without supply of additional ECM. Eventhough albumin expression revealed the isolated liver cells as hepatocytes, different pattern of actin cytoskeletal structures confirmed the presence of different cell types. Foetal liver cells behaved as mesenchymal stem cells as evidenced by its differentiation to adipocytes in adipogenic medium.

Non enzymatic retrieval of hepatocyte using PIPAAm grafted surface avoided functional loss and retained structural integrity. Optimization of microenvironment and systematic approach from the very beginning giving importance for intricacies in isolation etc might have played an important role in success of coculture system.

5.2. FUTURE PROSPECTS

An optimized *in vitro* system with minimal culture requirements described here can find application in liver tissue engineering. Since the major function of cell component in BAL is detoxification, this coculture system finds application in BAL as an efficient cell component. This system also finds utility in *in vitro* toxicity systems for drug-drug interaction (for both inhibition and induction) as liver (hepatocytes) is involved in the metabolic fate of most of the drugs. Use of human cell source is important as it is allogenic and it can be extrapolated to human physiology. The standardized isolation procedure for culturing human foetal liver cells finds application in cell transplantation due to its low antigenicity and in tissue engineering due to its preponderance as MSCs compared to bone marrow. Since hepatocyte spheroid formation occurs in this optimized culture condition, spheroids and heterospheroids can be retrieved at any stage by using temperature sensitive polymer without enzyme treatment. Combined use of thermoresponsive polymer and the coculture system will be advantageous in generating *in vitro* tissue constructs for toxicity studies or as cell component in BAL.

Chapter 6

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ANNEXURE - I

LIST OF EQUIPMENTS

1. γ-ray irradiation - Panoramic Batch Irradiator, Trombay
2. Autoclave - Sanyo, Japan
3. Centrifuge - Hettich Universal 32R and Biorad
4. CO_2 Incubator - Nuaire, USA
5. Contact Angle Goniometer - NRL Contact Angle Goniometer - 100.00
6. Critical point drying (CPD) - Hitachi HCP-2, Japan
7. Deep freezer -85°C -
8. Energy Dispersive X-Ray Spectroscopy (EDS) - Hitachi, Japan
9. Fluorescence microscope - Nikon Eclipse 600
10. Fourier Transform Infra Red spectrophotometer with Attenuated Total Internal Reflection (ATR-FTIR) - Nicolet Inc (Madison, USA) - Impact 410
11. Image analysis software - (QWin, Leica Germany)
12. Laminar Flow bench - Klenzaids
13. Laser scanning confocal microscope - Olympus IX51, Leica LCM SP2, Ziess LSM 510 Meta
14. Liquid scintillation counter - Triathlar, Germany
15. Perfusion apparatus - Custom made
16. Phase contrast microscope - Leica DMIL, Germany and Olympus IX51, USA
17. Refrigerator - Godrej, India
18. Scanning Electron Microscope - Hitachi - S 2500
19. Spectrofluorimeter - Spectra Max Gemini E, USA
20. Triathler - Hidex, Finland
21. UV-VIS spectrophotometer - Hitachi, Japan
22. Water bath - Julabo, USA

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