# ADIPOGENESIS ON BIOCERAMIC & COLLAGEN SCAFFOLDS FOR SOFT TISSUE RECONSTRUCTION

A DISSERTATION SUBMITTED

BY

**BALU V GOPAL**

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

**MASTER OF PHILOSOPHY**

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

THIRUVANANTHAPURAM – 695 011

# DECLARATION

**I, Balu V Gopal,** hereby declare that I had personally carried out the work depicted in the dissertation entitled **"Adipogenesis on Bioceramic & Collagen Scaffolds for soft tissue reconstruction"** under the direct supervision of **Dr. Annie John, Scientist E, Transmission Electron Microscopy Laboratory, Division of Implant Biology**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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**(An Institute of National Importance under Govt.of India)**

# CERTIFICATE

This is to certify that the dissertation entitled **"Adipogenesis on Bioceramic & Collagen Scaffolds for Soft tissue Reconstruction"** submitted by **Balu V Gopal** in partial fulfilment for the degree of Master of Philosophy in Biomedical Technology to be awarded by this Institute. The entire work was done by him under my supervision and guidance at the **Transmission Electron Microscopy Laboratory, Division of Implant Biology**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram-695012.

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**Date: August 10th, 2010**

**Dr.Annie John**

The Dissertation

Entitled

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Submitted

By

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For

**Master of Philosophy**

of

**SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY**

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**Balu V Gopal**

# ABBREVIATIONS

| | |
|---|---|
| ACBP | Acyl-CoA binding protein |
| ADAS | Adipose-derived adult stem cells |
| ASC | Adipose-derived Mesenchymal Stem Cells |
| BCP | Biphasic calcium phosphate |
| BSA | Bovine Serum Albumin |
| c-AMP | Cyclic-Adenosine monophosphate |
| CaP | Calcium phosphate |
| CD | Cluster of Differentiation |
| DLAS | Division of Laboratory Animal Sccience |
| DMEM | Dulbecco's Modified Eagles Medium |
| DMEM HG | Dulbecco's Modified Eagles Medium- High Glucose |
| DMSO | Dimethyl Sulphoxide |
| ECM | Extra cellular matrix |
| FACS | Fluorescent activated cell sorter |
| FBS | Foetal Bovine Serum |
| FT-IR | Fourier Transform Infrared Spectroscopy |
| GLUT 4 | Glucose Transporter 4 |
| H&E | Hematoxylin and Eosin |

| | |
|---|---|
| HA | Hydroxyapatite |
| hASC | Adipose-derived Mesenchymal Stem Cells |
| IAEC | Institutional Animal Ethics Committee |
| IBMX | 3-isobutyl-1-methyl Xanthine |
| IGF | Insulin-like growth factor |
| LDH | Lactate Dehydrogenase |
| LPL | Lipoprotein lipase |
| mA | milli ampere |
| mg | milli gram |
| MSC | Mesenchymal Stem Cells |
| $^{o}C$ | Degree Celsius |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PG | Picogreen |
| PLA | Processed lipo aspirate |
| PLA | Polylactic acid |
| PMMA | Polymethyl methacrylate |
| PPAR $\gamma$ | Peroxisome proliferator activated receptor gamma |
| SCTIMST | Sree Chitra Tirunal Institute for Medical Sciences & Technology |

| | |
|---|---|
| SEM | Scanning Electro Microscopy |
| SVC | Stromal-Vascular Cells |
| TE | Tissue Engineering |
| TG | Triacylglycerol |
| TGF-β1 | Transforming Growth Factor β1 |
| XRD | X-Ray Diffraction |

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# Synopsis

Soft tissue augmentation associated with traumatic injury, oncologic resection and congenital birth defects is a major challenge for plastic and reconstructive surgeons. Tissue engineering has emerged over the past 15 years as a multidisciplinary field focused on the development of bioengineered, living tissue constructs. To date, prototypes of numerous tissues and organs have emerged on the biomedical landscape and yet only recently has attention been focused on the engineering of adipose tissue to restore soft tissue defects.

The triad of Tissue engineering is a combination of biomaterials, growth factors and cells to repair failing organs. To successfully engineer a soft tissue substitute, it is critical to understand the basic physiology of adipose tissue and the process of adipogenesis. Adipose tissue is divided into two subtypes, white and brown fat. White fat is widely distributed and it represents the primary site of fat metabolism and storage, whereas brown fat is relatively scarce and its main role is to provide body heat, which is essential for newborn babies. Adipose-derived mesenchymal stem cells (ASCs) are multipotent and hold promise for a range of therapeutic applications. ASCs are primary, multipotent cells capable of differentiating to the osteogenic, chondrogenic and adipogenic lineages when stimulated under appropriate conditions and adipose tissue is easily accessible by lipoaspiration. Synthetic and natural scaffolds are used in adipose tissue engineering and materials selected should be capable of controlled non toxic degradation as they are replaced by healthy host tissues.

Aiming at soft tissue reconstruction with ceramic and collagen scaffolds, the major aspect is to establish the cell-material equation. Collagen scaffolds have been investigated aiming at adipose tissue regeneration. Ceramics are inorganic and non metallic materials. Most of the clinical applications of bioceramics relate to repair of skeletal systems, composed of bones, joints, teeth and to augment both soft and hard tissues. Here the challenge is to use ceramic as a scaffold for adipose tissue engineering.

Adipogenesis is the term used to explain the differentiation of stem cells or pre adipocytes to adipocytes. Adipocyte differentiation is characterized by sequential changes in the expression of specific genes that determine the specific adipocyte phenotype of the cells. Adipogenic differentiation can be induced in cells cultured in

DMEM supplemented with serum, dexamethasone, isobutyl-1-methyl Xanthine. Several other inducers are also been used along with this, like Indomethacin, Insulin, biotin and panthothenate. Pparg and C/EBP family of transcription factors, Pparg and C/EBP family of transcription factors must function cooperatively to activate adipocyte genes and thereby bring about adipocyte differentiation. *In vivo* demonstration of adipogenesis is also of prior importance because of its clinical relevance. Reviews state the use of rabbits, rats and mice as common models for adipose tissue engineering.

Collagen and Biphasic calcium phosphate (BCP- an in-house developed ceramic) were used as scaffolds for adipose tissue reconstruction. Scaffolds were characterized using scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FT-IR) and X-ray Diffraction (XRD). Primary cultures of ASCs from rat subcutaneous fat pad was isolated, expanded (confluent in 3-5 days), characterized for stemness using CD105 and CD44 by Flow Cytometry and differentiated into the respective osteogenic, chondrogenic and adipose multilineages.

Cytocompatibility of the collagen and ceramic scaffolds were tested using rat rASCs which supported cell viability (LDH, Acridine orange /Ethidium Bromide) and cell proliferation (Pico green) where cells favored the amorphous ceramic scaffolds compared to the collagen scaffolds. *In vitro* adipogenesis was demonstrated on glass cover slips using Nile red and Oil red O stains. Simultaneously Nile red stain was used in Flow cytometry analysis and the estimation of triglyceride accumulation in the cell population was done. Subsequently, cell-material interaction studies ensued for *in vitro* adipogenesis for evaluation by confocal microscopy exhibiting picturesque distribution of rich oil red globules within the cells which is a characteristic feature of adipocytes. However, the adipose specific gene expression was favored by the collagen scaffolds as demonstrated by real time -PCR from *in vitro* induced cells on the scaffolds.

*In vivo* experiments were performed on Sprague Dawley rats and the cell-construct (collagen and BCP) was implanted on the dorsal muscle on either side of the vertebral column. The samples were retrieved 21 days post -implantation and the histology was performed using different staining techniques (H & E, Picrosirius & Masson's Trichrome) to demonstrate adipogenesis with the bare and cell-seeded collagen and BCP implants - 21 days post implantation. The elliptical adipocyte-like cells within and near the collagen and ceramic scaffolds were confirmed as fat cells with comparison to subcutaneous fat pad in the normal rat skin and also to the normal muscle sections. Neo-angiogenesis in the vicinity of the scaffolds is a favorable sign for the support of fat cells to prevent tissue resorption and implant failure which would otherwise happen in the case of transplantation of fat grafts alone.

So in perspective of future applications, taking into account the merits of both the scaffolds, a combination of the two scaffold types – collagen in a 3D ceramic as a single unit, may be proposed to be the appropriate scaffold of choice for adipose tissue augmentation. The ceramic would remain to maintain the aesthetics and contour of the reconstructed tissue while the collagen degrades with time.

# Chapter-1

# Introduction

# 1. Introduction

Biomaterial Science and Tissue engineering (TE) in Regenerative Medicine is a multidisciplinary field that has evolved in parallel with recent biotechnological advances. Tissue Engineering has emerged from the role played by the surgeons in creating one body part for another to meet the needs of individual patients. Reconstructive Surgeries have the power to cure, repair and minimize deformities after injury or illness. Surprisingly, this concept has been with us since around 1600 BC. Reconstructive surgery was also practiced in India from around 500 BC. Susrata developed a technique to reform noses that had been cut off as a punishment or hacked off during battle. Again, a Sicilian family of surgeons practiced new suturing techniques and developed methods to repair wounds of ears and lips. Later in Bologna, a surgeon called Gaspare Tagliacozzi experimented with cutting flaps of skin, called pedicles, from one part of the body and sewing them to another. War was a catalyst for improvements of surgical techniques in this field.

The idea of improving the human system with synthetic and natural materials as substitutes for autologous tissues was gradually developed as technology advanced. The term reconstructive surgery came to be called tissue engineering when the focus of attention became the fabrication of living replacement parts for the body in the laboratory. The main challenge of a tissue-engineered product is the extent to which it could imitate Mother Nature or the living normal tissue.

Tissue engineering represents an innovative approach for the development of novel clinical modalities for the repair and reconstruction of human tissue defects. According to Langer and Vacanti (1993) tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain or improve tissue function or a whole organ. Tissue engineering has shown promising results for the development of tissue constructs to facilitate large volume of soft tissue augmentation in reconstructive and cosmetic plastic surgery. In the 1990's, tissue engineering emerged as a concept for regenerating biological tissues by seeding the patient's own tissue-forming cells in synthetic biocompatible polymers.

Over the past decades, scientific advances in biomaterials, stem cells, growth and differentiation factors and the biomimmetic environment have created unique opportunities to fabricate tissues in the laboratory from combinations of cells, bioengineered extra cellular matrices (scaffolds) and biologically active molecules (the triad of TE). According to the fabricated tissue of interest, tissue engineering may be classified as – Cartilage TE, Bone TE, Adipose TE, Cardiac TE, Pancreas TE, Skin TE, Liver TE, Lung TE, Vascular TE, corneal TE etc.

## 1.1 Adipose tissue engineering

Fat tissue engineering offers great potential in repealing limitations realized with classical approaches in reconstructive surgery. The clinical applications for tissue-engineered fat are vast and variable, including reconstructive, cosmetic and corrective indications. Because soft tissue is composed primarily of adipose tissue, adipose tissue engineering recently has been proposed as an alternative for current clinical treatments. One active area of investigation is the development of tissue engineering strategies for adipose tissue. Bioengineers, life scientists, and reconstructive surgeons are synergistically coupling expertise in areas such as cell culture technology, tissue transfer, cell differentiation, angiogenesis, computer modeling, and polymer chemistry to regenerate adipose tissue *de novo.*

A large proportion of plastic and reconstructive surgical procedures are performed for breast replacement and soft-tissue augmentation that result from tumor resection traumatic injury, extensive deep burns and congenital defects such as Romberg's disease and Poland syndrome. As an increasing number of patients seek aesthetic improvement through minimally invasive procedures, interest in soft tissue augmentation and filling agents is an all-time high demand.

## 1.2 Review of Literature

The success of using autologous fat tissue grafts to repair soft tissue defects is limited [Niemela *et al.,* 2008]. These autologous fat transplantation yields poor result with 40-60% reduction in graft volume and is due to insufficient vascularisation [Patrick *et al.,* 1998]. Currently clinical strategies for soft tissue augmentation involves autologous, allogenic and alloplastic materials [Katz *et al.,* 1999]. Free fat transfer yields unsatisfactory and unpredictable results because there is a varying degree of fat resorption reported due to the lack of supporting vasculature [Peer *et al.,* 1956]. Small defects can be corrected with injected autologous fat and even these applications require repeated treatments to maintain the desired volume [Huss *et al.,* 2002]. Alloplastic and allogenic materials is associated with immune rejection, allergic reactions, implant migration or resorption and failure to integrate into the host tissues [Katz *et al.,* 1999, Lin *et al.,* 2008]. The potential development of tissue engineered soft tissue represents a promising and innovative solution for many clinical challenges, especially in plastic and reconstructive surgery. Fat tissue engineering offers great potential in repealing limitations realized with classical approaches in reconstructive surgery. The clinical applications for tissue engineered fat are vast and variable, including reconstructive, cosmetic and corrective indications.

Adipose tissue is easily obtained in large amounts using the techniques of liposuction [Zuk *et al.,* 2000]. Fat tissue contains numerous different cell types that may be advantageous to tissue engineering applications and regenerative medicine. In recent years, adipose tissue-derived stem cells have been cultured and differentiated into several lineages with promising results indicating that these stem cells have therapeutic potential and utility for future tissue engineering applications and cell-based therapies [Niemela *et al.,* 2008].

### 1.2.1 Adipose tissue: structure and function

To successfully engineer a soft tissue substitute, it is critical to understand the basic physiology of adipose tissue and the process of adipogenesis. Adipose tissue is a dynamic and multi-functional tissue that is ubiquitous throughout the human body. It is commonly found in the subcutaneous region as a loose connective tissue, and also surrounds internal organs. Mature adipocytes constitute the majority of cells in an adipose tissue. Besides mature adipocytes, fat tissue contains several other cell types, including stromal-vascular cells (SVC) such as fibroblasts, smooth muscle cells, pericytes, endothelial cells, and adipogenic progenitor cells[Niemelä *et al.,* 2008].

Adipose tissue is divided into two subtypes, white and brown fat. White fat is widely distributed and it represents the primary site of fat metabolism and storage, whereas brown fat is relatively scarce and its main role is to provide body heat, which is essential for newborn babies.

White adipose tissue is the major energy reserve and its primary function is to store triacylglycerol (TG) in periods of energy excess and to release energy in the form of free fatty acids during energy deprivation [Julie *et al.,* 2007]. Adipocytes secrete various factors known to play a role in immunological responses, vascular diseases and appetite regulation. Leptin is a peptide hormone primarily made and secreted by mature adipocytes, and it has various biological activities, including effects on appetite, food intake and body weight regulation, fertility, reproduction and hematopoiesis [Niemela *et al.,* 2008]. Besides, adipose tissue is an important site for oestrogen biosynthesis and steroid hormone storage [Yu *et al.,* 2010].

In addition, adipose tissue secretes a variety of peptides, cytokines and complement factors, which act in an autocrine and paracrine manner to regulate adipocyte metabolism and growth, as well as endocrine signals to regulate energy homeostasis [Julie *et al.,* 2007]. Although adipose tissue is important to various normal processes of the human body, it has also many implications for human disease states such as obesity, cardiovascular diseases and hypertension. Obesity has also been associated to other pathological disorders, including some types of cancer, such as breast, ovarian, renal and colon cancer [Niemela *et al.,* 2008].

## 1.2.2 Adipose-derived Mesenchymal stem cells

The ideal source of ASCs for aesthetic use should be from a tissue that is easily available, accessible and readily expandable. ASCs are commonly found in tissue sources such as, bone marrow, adipose, blood, muscles, etc [Gimble *et al,* 2008]. The term "adipose-derived stem cell" (ASC) is used to refer the collective population of MSC and the more-committed adipogenic progenitors that are found within the stroma of adipose tissue [Lauren *et al.,* 2008]. Recent results have shown that stem cells within the stromal-vascular fraction of adipose tissue display a multilineage developmental potential. Zuk *et al.,* [2000] identified a putative stem cell population within the adipose stromal compartment, termed as processed lipo aspirate (PLA). ASCs isolated from human lipo aspirates and adipose tissue-derived stem cells differentiate to osteogenic, adipogenic, chondrogenic, myogenic and neurogenic lineages respectively [Niemela *et al.,* 2008; Patrick *et al.,* 2002; Kwang *et al.,* 2007]. ASCs have been shown to be very similar to marrow-derived stem cells in morphology and phenotype. In addition to their common multipotency, several CD marker antigens of marrow stem cells have also been found on the surface of ASCs [Cheryl *et al.,* 2006].

Researchers have developed several methods to use adipose tissue-derived mesenchymal stem cells (ASCs) to form new adipose tissue *in vivo* by means of tissue engineering [Sin-Daw Lin *et al.,* 2008]. As adipose tissue is readily accessible and abundant, ASC's isolated from this tissue may be a better candidate for cell therapy and tissue engineering [Kwang *et al.,* 2007]. Human adipose tissue is a good source for harvesting human adipose-derived mesenchymal stem cells (hASCs) and procedures for isolation of these cells either from liposuction or excised fat are not difficult [Lin *et al.,* 2008].

Cells that can be used for repair and regeneration include mature cells obtained from the patient or stem cells. If mature cells from the patient itself are used, the problem of immune rejection is out of question but these cells are not the best source for tissue regeneration because these cells are already differentiated and committed to a specific lineage [Pittenger *et al.,* 1998]. On the contrary, adipose tissue represents an abundant, practical and appealing source of donor tissue for autologous cell replacement where stem cells within the stromal-vascular fraction display a multilineage developmental potential [Niemela *et al.,* 2008].

### 1.2.3 Nomenclature of Mesenchymal stem cells

A variety of names have been used to describe the plastic adherent cell population isolated from adipose tissue. Mesenchymal stem cells derived from adipose tissue has been described with different terms like adipose-derived stem/stromal cells (ASCs), adipose-derived adult stem (ADAS) cells, adipose-derived adult stromal cells (ADASCs), adipose-derived stromal cells (ADSCs), adipose stromal cells (ASCs), adipose mesenchymal stem cells (ASCs), lipoblast, pericyte, preadipocyte, and processed lipoaspirate (PLA) cells. To address this issue, the International Fat Applied Technology Society reached a consensus to adopt the term "adipose-derived stem cells" (ASCs) to identify the isolated, plastic-adherent, multipotent cell population [Jeffrey *et al.,* 2007].

### 1.2.4 Identification of Mesenchymal stem cells

There are a number of surface markers available for mesenchymal stem cell which is also applicable for adipose-derived mesenchymal stem cells. [Patrick *et al.,* 2002; Lauren *et al.,* 2008; Cheryl *et al.,* 2006; Kwang *et al.,* 2007; Pittenger *et al.,* 1998]. Table 1 gives the list of surface positive and negative markers for adipose mesenchymal stem cells.

### 1.2.5 Scaffolds for adipose tissue engineering

Different varieties of scaffolds are used in experimental purpose for soft tissue augmentation in order to device an optimum one for use in adipose tissue engineering. The 3D scaffolds used should help in designing and maintaining the desired tissue volume [Lauren *et al.,* 2008]. Materials selected should be capable of controlled non toxic degradation as they are replaced by healthy host tissues [Lauren *et al.,* 2008].

There are both synthetic and naturally derived scaffolds used in research for adipose tissue engineering. Patrick *et al.,* [1999] have succeeded in the reconstruction of adipose tissues in the rat subcutis using a porous scaffold poly (lactic-co-glycolic acid) pre-seeded with autologous preadipocytes. Mauney *et al.,* [2007] has discussed the strategies of adipose tissue engineering *in vivo* and *in vitro* based on the scaffolds Poly Lactic acid (PLA), Collagen (COL) and Silk fibroins with adipose derived stem

**Table 1: Common stem cell markers**

| MSC Markers | Effect |
|---|---|
| CD9(tetraspan) | + |
| CD10 (CALLA) | + |
| CD11 (α-integrin) | - |
| CD13 (aminoepetidase) | + |
| CD (PECAM-1) | - |
| CD34 | +/- |
| CD44 (hyaluronate receptor) | + |
| CD45 (LCA) | - |
| CD54 (ICAM-1) | + |
| CD90 (Thy-1) | + |
| CD105 (Endoglin) | + |
| CD106 (VCAM) | +/- |
| CD117 (c-kit) | + |
| α-smooth muscle actin | + |
| Collagen type I | + |
| Collagen type II | + |
| Vimentin | + |
| Stro-1 | +/- |

**[Adapted from Gomillion *et al.*, 2006]**

cells for soft tissue reconstruction [Joshua *et al.,* 2007]. Lin *et al.,* [2007] worked on soft tissue reconstruction and used h-ASC on gelatin sponges, poly glycolic acid and poly propylene mesh. Adipose tissue of specific shape and three dimensional structures was generated *in vivo* using tissue engineering strategies. Fischbach *et al.,*

[2004] studied the response of induced cells *in vitro* on poly glycolic acid where the cells readily accumulated lipid within the scaffold and assumed a mature unilocular morphology. Karl & Crandall [1999] used Poly tetra fluro ethylene scaffolds that are non degradable and coated with collagen, albumin or fibronectin for use as an ideal scaffold for adipose tissue engineering. Fibronectin favored cell adhesion while lipogenesis was limited [Ugarte *et al.,* 2003]. Poly ethylene glycol - diacrylate hydrogels by Alhadlaq *et al.,* [2005] reported the encapsulation of human mesenchymal stem cells within the hydrogel both *in vivo* and *in vitro where* the scaffolds maintained the architecture and had excellent volume retention properties *in vivo.* Kang *et al.,* [2005] demonstrated oil red O stained lipid droplets depicting high levels of intra cellular lipid accumulation on non biodegradable Poly ethylene-terephthalate scaffolds (PET).

Biomaterials derived from silk fibrions were used *in vivo* and *in vitro* for their utility in adipose tissue engineering strategies. In the work done by Mauney *et al.,* [2007], the silk scaffolds supported the *in vivo* adipogenesis either alone or seeded with adipose-derived stem cells. The presence of exogenous cell sources substantially increased the extent and frequency of adipogenesis observed. This scaffold offers an important platform for cell-based adipose tissue engineering and its applications [Joshua *et al.,* 2007]. Naturally derived scaffolds include Matrigel™ which is derived from the reconstituted basement membrane of the mice sarcoma. Matrigel™ supported cell growth but its clinical applications are prohibited due to its tumerogenic origin [Vashi *et al.,* 2006]. HYAFF® scaffolds are a semi synthetic benzyl ester of hyaluronan (HA) developed by Aachen University of technology. The scaffolds were seeded with adipose stem cells and implanted subcutaneously in a mouse model. Only minimal adipose tissue formation was observed within HYAFF® [Lauren *et al.,* 2008]. Marler *et al.,* [2000] used injectable alginate scaffolds seeded with syngenic fibroblast. The adhesion of fibroblasts increased volume retention but the adipose tissue formation was not supported. Naturally derived placental decellularized matrix (PDM) and the PDM with cross linked hyaluronan (XLHA) scaffold are also been used in adipose tissue engineering for soft tissue augmentation [Flynn *et al.,* 2007]. Table 2 lists the scaffolds used for adipose tissue engineering.

**Table 2: Scaffolds used in adipose tissue engineering**

| Materials Used in place of Adipose Tissue in Reconstructive Surgery | Product | Vendor | Primary Component(s) |
|---|---|---|---|
| ECM/Tissue Matrix | Alloderm® | (Life Cell Corp) | Decellularized Human dermal Tissue |
| | Autologen® | (Collagenesis Inc) | Autologus human dermal collagen |
| | Cymentra™ | (Life Cell Corp) | Micronized Alloderm® |
| | Dermaloge™ | (Collagenesis Inc) | Allogenous human dermal tissue matrix |
| | Fibrel® | (Mentor Corp) | Fibrin Gel |
| | Hylaform® | (Biomatrix Corp) | Hyaluronic acid gel |
| | Restylane™ | (Q-Med) | Viscoelastic hylan gel |
| | Tissel® | (Baxter) | Human fibrin |
| | Zyderm® I | (Collagen Aesthetics) | Bovine dermal collagen(35mg/mL) |
| | Zyderm® II | (Collagen Aesthetics) | Bovine dermal collagen(65mg/mL) |
| | Zyplast® | (Collagen Aesthetics) | Zyderm® II with glutaraldehyde |
| Polymers | Artecoll® | (Fofil Medical Jut) | Polymethylmethacrvlate microspheres |
| | Bioplastique® | (Bioplasty Inc) | Cross-linked polydimethylsiloxane |
| | Gortex ® | (W.L. Gore & Associates) | Expanded polytetrafluoroethylene (ePTFE) |
| | Marlex® | (Davol Inc) | Porous/mesh-form polyethylene |
| | Softform™ | (Collagen Aesthetics) | Expanded polytetrafluoroethylene (ePTFE) |
| Silicone | Various fonnulas | (Dow Coming) | |

**[Adapted from Patrick. C. W. 2000]**

### 1.2.5.1 Collagen as a scaffold

In the 1970s, animal and human derived collagens were studied for soft-tissue augmentation. Collagen is an outstanding biocompatible natural ECM molecule in the connective tissue, which is a well used scaffold for adipose tissue engineering. As scaffolds, they are extensively studied as it has advantages of availability, biocompatibility and biodegradability [Nimni *et al.,* 1987; Kim *et al.,* 2000]. Collagens have been used in native form or in its denatured form, gelatin. Collagen type I is the most commonly used natural scaffold for tissue engineering techniques [Julie *et al.,* 2007]. The polypeptide collagen does not possess the desired degradation and mechanical properties desired for adipose tissue. However, they can be chemically crosslinked to yield polymer blends, possessing defined micro-architecture and desired degradation and mechanical properties. Glutaraldehyde (GA) is an effective crosslinking agent that is water soluble, highly efficient, and economical [Xuemei *et al.,* 2007].

Collagen scaffolds were investigated by a lot of Researchers aiming at adipose tissue regeneration. Freeze-dried bovine type 1 collagen scaffolds were investigated *in vitro* and *in vivo* seeded with human preadipocytes [Heimburg *et al.,* 2001]. *In situ* adipose tissue regeneration in fat tissue by collagen sponges and gelatin microspheres containing basic fibroblast growth factor (bFGF) was investigated by Kimura and co workers [2009]. They used type 1 collagen extracted from porcine tendon. Von Heminburg *et al.,* [2003] seeded collagen scaffolds with adipose stem cells but reduction of graft volume was reported while using collagen [Niemela *et al.,* 2008]. Gluteraldehyde crosslinked collagen-chitosan hydrogels for soft tissue augmentation was developed by Wu *et al.,* [2007] and the cells in the scaffolds were found visible for over a period of 10 days.

Commercially available collagen composites include bovine derived Zyderm I®, Zyderm II®, and Zyplast® (Inamed aesthetics, USA), Artecoll® (Canderm Pharma, Canada), a solution containing polymethyl methacrylate microspheres (PMMA) suspended in bovine collagen. CosmoDerm I®, CosmoDerm II®, and CosmoPlast® (Inamed Corporation, USA), contain human type I and III collagen. Autologen® (Collagenesis Corporation, USA) is an autologous collagen suspension, made of the patient's own tissue, containing 3.5% collagen. Cymetra® (Lifecell, USA), is the injectable form of Alloderm®, an acellular allogenic human dermal matrix derived from cadaveric skin [Ryssel *et al.,* 2008].

### 1.2.5.2 Ceramic as a scaffold

Ceramics are defined as the art of making and using solid articles that have as their essential components, inorganic and non metallic materials [Kingery *et al.,* 1976, Joon *et al.,* 1998]. Ceramic materials that are specially developed for use as medical and dental implants are termed bioceramics. Most of the clinical applications of bioceramics relate to repair of skeletal systems composed of bones, joints, teeth and to augment both soft and hard tissues. Ceramics used in fabricating implants can be classified as non-absorbable (relatively inert), bioactive or surface active (semi inert), and biodegradable or resorbable (non inert). Alumina, zirconia and carbons are inert bioceramics. Certain glass ceramics and dense hydroxyapatites are semi inert while calcium phosphates and calcium aluminates are resorbable ceramics [Joon *et al.,* 1998]. Several Ceramic biomaterials like bioactive glass ceramics and calcium phosphate ceramics such as hydroxyapatite (HAP), β tricalcium phosphate (β-TCP) or biphasic calcium phosphate (BCP) have been developed to fill and reconstruct bone defects [Guehennec *et al.,* 2004]. Their main characteristics rely on their ability to be biocompatible, resorbable and their osteoconductive property as a bone substitute.

Calcium phosphate (CaP) biomaterials are available in various forms and mixtures. Synthetic calcium phosphates are classified as hydroxyapatite (HAP), $Ca_{10}(PO_4)_6(OH)_2$ (Cerapatite®, Synatite®); tricalcium phosphate (ß-TCP), $Ca_3(PO_4)_2$ (Biosorb®, Calciresorb®, Chronos®), biphasic calcium phosphate (BCP) from mixtures of HA and β -TCP [Biosel®, Ceraform®, Eurocer®, MBCP®, Hatric®, Tribone 80®, Triosite®, TricOs®); and unsintered or calcium-deficient apatite. All these varients are used as Synthetic calcium phosphate bone substitutes.

**Table 3: Growth Factors influencing Adipose tissue engineering**

| Factors | Effect |
|---|---|
| α FGF | +/- |
| β FGF | +/- |
| EGF | - |
| Glucocorticoid | + |
| Growth Hormone | + |
| IGF-1 | + |
| IL-11 | - |
| Insulin | + |
| Interferon –γ | - |
| PDGF | +/- |
| Prostaglandins | + |
| TGF –α | - |
| TGF-β | - |
| Thyroid hormone | + |
| TNF-α | - |

**[Adapted from Niemela *et al.*, 2008]**

## 1.2.6 Current strategies of adipose tissue engineering

### 1.2.6.1 Scaffold guided tissue regeneration

Preadipocyte cells cultured on absorbable polymeric scaffolds and implanted *in vivo* such that simultaneous cellular proliferation and scaffold resorption results in mature adipose tissue [Burg *et al.*, 2003; Cavin *et al.*, 2005; Gomillion *et al.*, 2005]

#### 1.2.6.2 Injectable composite system

Injectable microcarrier beads combined with a hydrogel delivery medium to form a minimally invasive implant that will stimulate regeneration of host adipose cells and fill a soft-tissue void upon injection *in vivo* [Burg *et al.,* 2003; Cavin *et al.,* 2005; Gomillion *et al.,* 2005].

#### 1.2.6.3 Fragmented omentum based-tissue regeneration

The highly vascularized tissue of the omentum fragmented and combined with preadipocyte cells such that implantation *in vivo* results in a tissue mass consisting of high triacylglycerol content [Masuda *et al.,* 2004].

#### 1.2.6.4 *De novo* adipogenesis

A stimulus, such as appropriate growth factors, applied *in vivo* induces the migration of preadipocytes to the implant site. The cells subsequently proliferate and differentiate to form adipose tissue depots [Kimura *et al.*, 2002; Kawaguchi *et al.*, 1998].

Several strategies have been proposed and investigated in an attempt to develop successful adipose tissue engineering methods. These methods use cellular and polymeric-based scaffolds to support adipose cell growth, and the formation of adipose tissue to fill a void or defect site.

### 1.2.7 Adipogenesis

Adipogenesis is the term used to explain the differentiation of stem cells or pre adipocytes to adipocytes. Adipocyte differentiation is characterized by sequential changes in the expression of specific genes that determine the specific adipocyte phenotype of the cells. This is reflected by the appearance of various early, intermediate and late mRNA/protein markers and triglyceride accumulation [Niemela *et al.,* 2008]. Adipogenic differentiation of ASCs can be achieved by using adipogenic induction media. Adipogenic differentiation can be induced in cells cultured in DMEM supplemented with 10% FBS, dexamethasone [Niemela *et al.,* 2008; Patrick *et al.,* 2002; Joshua *et al.,* 2007; Cheryl *et al.,* 2006], IBMX (3-isobutyl-1-methyl

Xanthine) [Niemela *et al.,* 2008; Patrick *et al.,* 2002; Joshua *et al.,* 2007; Cheryl *et al.,* 2006]. Several other inducers are also been used along with this, include Indomethacin [Patrick *et al.,* 2002; Joshua *et al.,* 2007; Cheryl *et al.,* 2006; Pittenger *et al.,* 1998), Insulin [Niemela *et al.,* 2008; Patrick *et al.,* 2002; Joshua *et al.,* 2007; Cheryl *et al.,* 2006], biotin, panthothenate [Kwang *et al.,* 2007]. The medium containing some of these supplements in their optimum concentration is called adipogenic induction medium. The differentiated adipocytes from the induction medium are transferred to a final medium where the cells are maintained after adipogenic induction, the maintenance medium. The maintenance medium contains DMEM supplemented with 10% FBS and Insulin. The cells are maintained in this medium for around 2 weeks so that well developed adipocytes with a good storage of triglycerides are obtained [Pittenger *et al.,* 1998]. For most of the works in adipogenesis the primary culture is obtained from the adipose derived stromal vascular precursor cells [Niemela *et al.,* 2008].

During the growth phase, cells of preadipocyte lines as well as primary preadipocytes are morphologically similar to fibroblasts. At confluence, induction of differentiation by appropriate treatment leads to drastic change in cell morphology. The preadipocytes accumulate lipid droplets and attain a spherical shape, progressively acquiring the morphological and biochemical characteristics of the mature white adipocyte [James *et al.,* 2003]. When the cells are treated with adipogenic inducers Dexamethasome, IBMX (3-isobutyl-1-methyl Xanthine), the cells start accumulating lipids which results in the formation of intra cellular lipid containing vacuoles [Konieczny *et al.,* 1984]. A number of lipid soluble dyes are available to stain lipid vacuoles. It includes Nile red, Nile blue, Sudan black & Oil red O. These dyes are hydrophobic and accumulate in the lipid containing vacuoles. This helps in identifying adipocytes developed from differentiating MSC's using simple staining and by flow cytometry [Pittenger *et al.,* 1998].

The MSCs can be induced to adipogenic lineage by treating them with

1. Glucocorticoids
2. A compound which can up regulate the production of c-AMP
3. A compound which can inhibit the degradation of c- AMP.

These compounds play an important role in the gene expression favoring adipogenesis [Pittenger *et al., 1998*].

Dexamethasone is a synthetic glucocorticoid which can increase the intra cellular c-AMP level, which could favors adipocyte formation and can be used in a concentration from 0.5 to 2μM. It is employed for the differentiation of primary preadipocytes derived from fat depots of various species, including rodents, rabbits, pigs, and humans [James *et al.,* 2003]. Dexamethasone reduces the expression of preadipocyte factor-1 (pref-1), a negative regulator of adipogenesis and induces CCAAT enhancer binding protein (C/EBPα), which may account for some of its adipogenic activity [Niemela *et al.,* 2008]. Compounds like IBMX and Indomethacin can inhibit the activity of phosphodiesterase inhibiting c-AMP degradiation. This can be used in a concentration around 0.2-2mM [Pittenger *et al.,* 1998]. IBMX accelerates the differentiation of preadipose cell lines and primary preadipocytes [James *et al.,* 2003]. IBMX has been shown to increase expression of C/EBPβ, and this is required for subsequent PPAR-γ expression and adipocyte differentiation [James *et al.,* 2003]. IBMX is known to inhibit phosphodiesterases and stimulates adenylyl cyclase activity [Niemela *et al.,* 2008]. Dexamethasone stimulates the glucocorticoid pathway and IBMX and indomethacin stimulate the c-AMP dependent protein kinase pathway. High concentrations of insulin can also be used in combination with these inducing agents [Reusch *et al.,* 2000; Rosen *et al.,* 2000].

**Table 4: Agents that induce Adipocyte Differentiation**

| AGENT | EFFECT | COMMENTS |
|---|---|---|
| Insulin | + | Accelerates lipid accumulation |
| IGF-1 | + | Stimulates adipocyte differentiation |
| Glucocorticoids | + | Stimulates adipocyte differentiation |
| C AMP | + | Stimulates adipocyte differentiation |
| Thyroid hormone | + | Inducing effect on adipogenesis restricted to a pre adipose cell line |

**[Adapted from Niemela *et al.,* 2008]**

### 1.2.7.1 Transcriptional control of Adipocyte Differentiation

A number of molecules that are specific markers for adipocytes have been described and is used to characterize the adipocytes derived from ASCs. These mostly include proteins and enzymes. Differentiation to adipocytes is a complex process where a lot of genes and their products are involved [De Lany *et al.,* 2005; Katz *et al.,* 1999]. This includes growth arresting, induction of differentiation and expression of multiple adipogenic genes and triglyceride accumulation [De Lany *et al.,* 2005; Katz *et al.,* 1999]. These genes and its products can be used as markers for the biochemical characterization of adipose tissue.

Adipocyte differentiation is characterized by sequential changes in the expression of specific genes that determine the specific adipocyte phenotype of the cells. The regulation of the adipocyte gene occurs primarily at the transcription level. This includes CCAAT enhancer binding protein (C/EBPα) gene family and Peroxisome proliferator activated receptor γ (Pparg) that co-ordinate the expression of genes which create and maintain the adipocyte phenotype [Niemela *et al.,* 2008]. Pparg and C/EBP family of transcription factors must function cooperatively to activate adipocyte genes and thereby bring about adipocyte differentiation [James *et al.,* 2003]. Pparg is the most specific gene to adipogenic differentiation that induces the exit of cells from the cell cycle and triggers the expression of adipocyte-specific genes, resulting in increased delivery of energy to the cells [James *et al.,* 2003]. Members of the C/EBP family were the first transcription factors demonstrated to play a major role in adipocyte differentiation. C/EPB-α is expressed just before the transcription of most adipocyte-specific genes is initiated [James *et al.,* 2003].

The biochemical markers for adipocytes include Stearoyl-CoA-desaturase (SCDI), which is an enzyme used for conversion of fatty acid to triglycerides. Insulin responsive glucose transporter (GLUT4) [Patrick *et al.,* 2002; Joshua *et al.,* 2007; Pittenger *et al.,* 1998], The product of OB gene - leptin, a 16000 M.W polypeptide expressed only in adipose tissue, 422 Adipose P2 (422/ap2) a protein expressed during adipose differentiation [Cheneval *et al.,* 1991] also include the biochemical markers used to determine the induction of adipogenesis. aP2 is an adipocyte specific fatty acid binding protein which is an intermediate marker of adipocyte differentiation

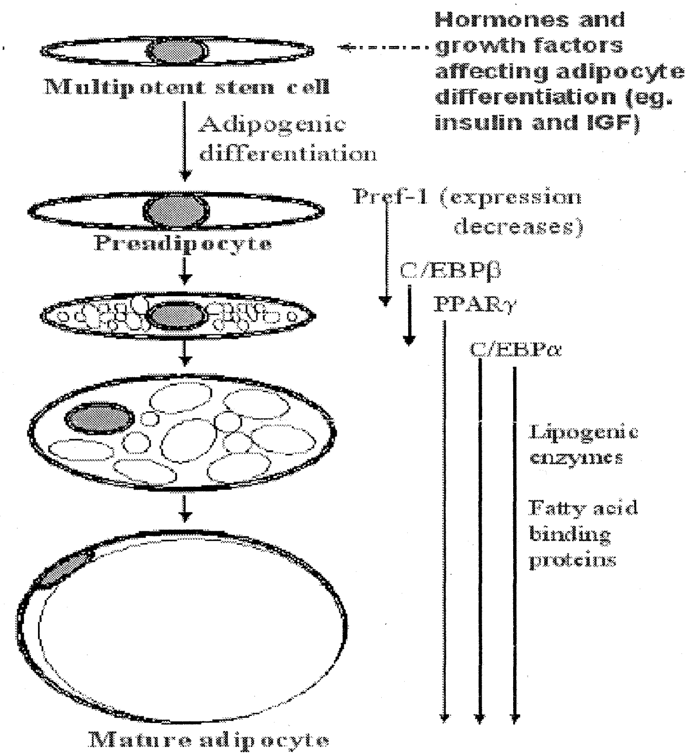
[Niemela *et al.,* 2008]. Lipoprotein lipase (LPL) is another enzyme that catalyses the hydrolysis of triglyceride molecules and it is abundant in adipose tissue. The expression LPL is a sign of adipocyte differentiation. The LPL is secreted by mature adipocytes. [Niemela *et al.,* 2008; Joshua *et al.,* 2007]. Pre adipocyte factor (pref-1) helps in maintaining pre adipose phenotype and a decrease in pref-1 is observed during adipocyte differentiation. Acyl-CoA binding protein (ACBP) is also a late marker for adipocyte differentiation. [Hansen *et al.,* 1991; Katz *et al.,* 1999]. During the terminal differentiation phase, adipocytes in culture show a marked increase in *de novo* lipogenesis and become sensitive to insulin [Niemela *et al.,* 2008].

### 1.2.7.2 Phases of Adipocyte differentiation

The primary prerequisite for adipocyte differentiation is growth arrest and not cell confluence or cell-cell contact [James *et al.,* 2003]. After growth arrest at confluence, preadipocytes receive an appropriate combination of mitogenic and adipogenic signals to continue through subsequent differentiation steps. Growth arrest and clonal expansion are accompanied by complex changes in the pattern of gene expression [James *et al.,* 2003]. Expression of lipoprotein lipase (LPL) mRNA has often been cited as an early sign of adipocyte differentiation. The two families of transcription factors, C/EBP and Pparg are induced early during adipocyte differentiation and are involved in terminal differentiation by activation of adipocyte-specific genes [James *et al.,* 2003]. Decrease in actin and tubulin expression is an early event in adipocyte differentiation that precedes changes in morphology and the expression of adipocyte specific genes. A dramatic decrease in pref-1 expression accompanies adipocyte differentiation; it is abundant in pre adipocytes and is not detectable in mature fat cells [James *et al.,* 2003]. It is the only known gene whose expression is completely down regulated during adipocyte differentiation. Fig.1 shows different stages of adipocyte induction and gene expression.

During the terminal phase of differentiation, adipocytes in culture markedly increase *de novo* lipogenesis and acquire sensitivity to insulin. The activity, protein, and m-RNA levels of enzymes involved in triacylglycerol metabolism including ATP citrate lyase, malic enzyme, acetyl-CoA carboxylase, stearoyl-CoA desaturase (SCD1), glycerol-3-phosphate acyltransferase, fatty acid synthase, and glyceraldehyde-3-phosphate dehydrogenase increase 10- to 100-fold [Niemela *et al.*, 2008]. Leptin, Pparg and C/EPBα are also increased during *in vitro* terminal differentiation [James *et al.*, 2003].

**Fig 1: Stem Cell to Adipocytes and Gene Expression**




**[Adapted from Niemela *et al.*, 2008]**

**Table 5: Differentiated Adipocyte Markers**

| | |
|---|---|
| Pre adipocyte marker | pref 1 |
| Early differentiation marker | C/EPB - β, α, LPL |
| Mid differentiation marker | PPAR γ, C/EPB - α |
| Late differentiation marker | Fatty acid translocase(FAT)- CD 36,<br>Fatty acid synthase (FAS),<br>aP2, Leptin, Adiponectin<br>GLUT4( Glucose transportase 4),<br>Glycerol-3-phosphate-dehydrogenase (GDPH),<br>HSL( hormone sensitive lipase), |

**[Adapted from Niemela *et al.,* 2008]**

### 1.2.7.3 Signal transduction pathways regulating adipogenesis

Adipocyte differentiation involves communication of extracellular signals and those of the ECM environment to the nucleus. Although the full complement of proteins involved in this process remains to be determined, IGF-1 has been shown to be an essential regulator of fat cell formation, and the requirement of IGF-1 and insulin in adipocyte differentiation has been clearly demonstrated [Niemela *et al.,* 2008]. The adipogenic effects of IGF-I indicate the involvement of a phosphorylation-dephosphorylation mechanism, subsequent to IGF-I receptor tyrosine phosphorylation intra cellular signaling system is activated during adipocyte differentiation. The role of Ras and Raf-1 as an integral component of the adipocyte differentiation promoting IGF-I signaling pathway has been already demonstrated [James *et al.,* 2003]. The effect of insulin on differentiation is shown to occur through cross-activation of the IGF-1 receptor [Niemela *et al.,* 2008]. Several studies indicate the role of the EGF receptor, such as EGF and TGF-α through which the growth factors act, in adipose tissue development. [James *et al.,* 2003]. Dexamethasone is believed to operate through activation of the glucocorticoid receptor, which is a nuclear hormone receptor in the same superfamily as Pparg [Niemela *et al.,* 2008] IBMX may function through increasing the accumulation of cAMP, which acts through cAMP response element-binding protein (CREB) and promotes the differentiation by inducing C/EBPβ.[ James *et al.,* 2003]. c-AMP, G proteins, and protein kinase C also plays a major role in accelerating the differentiation of preadipose cell lines and primary preadipocytes to mature adipocytes [James *et al.,* 2003].

#### 1.2.7.4 *In-vivo* models for Adipose tissue engineering

Reviews state the use rabbits, rats and mice as a common model for adipose tissue engineering [Patrick *et al.,* 2004]. *In vivo* study of adipogenesis on various scaffolds was carried out in nude mice and was explanted in a period of 2 weeks to demonstrate adipogenesis [Y.S. Choi *et al.,* 2005]. *In situ* adipose tissue regeneration in fat tissue by collagen sponges and gelatin microspheres containing basic fibroblast growth factor (bFGF) applied to larger-sized defects in side rabbits fat tissue was investigated for adipose tissue formation by Kimura *et al.,* [2010]. Adipogenesis on cell seeded gelatin sponge, polyglycolic acid mesh and polypropylene mesh was demonstrated *in-vivo* by implanting into the subcutaneous tissue of immune-deficient mice [Lin *et al.,* 2008]. Large animal models are also evaluated for adipose tissue regeneration [Patrick *et al.,* 2004]. Sheep and Yucatan MicroPigs are the large animal models that satisfy the criteria for adipose tissue engineering [Patrick *et al.,* 2004]

### 1.2.8 Future concepts in Fat tissue Engineering.

Recent advances in bioengineering and cell biology of fat tissue have led to innovative and new therapeutic potentials for regenerative medicine. Autologous human adipose tissue derived stem cells could have clinical applicability for cell-based therapies and tissue engineering purposes. Promising results suggest that adipose tissue will be a useful tool in biotechnology. There are several possible strategies for using adult stem cells for aesthetic applications. One approach is to harness the replicative capacity and plasticity of adult stem cells to engineer autologous grafts for soft tissue and facial skeletal augmentation. A second strategy is to simply deliver undifferentiated adult stem cells in high concentrations to a specific anatomic site. A third approach involves activating and manipulating endogenous adult stem cells *in situ* [Peter *et al.,* 2003].

One proposed strategy for breast tissue engineering is the liposuction or fat biopsy to obtain adipose tissue. Preadipocytes are isolated via enzymatic digestion and expanded *ex vivo*. The patient-specific scaffold shape, volume, and the number of cells required are obtained from a virtual breast simulator [Charles *et al.,* 2000]. The TRAM flap is also the most frequently used method for autogenous breast reconstruction, whether pedicled or microsurgical techniques are chosen. The harvested excess abdominal tissue is generally unwanted by the patient, the resulting contour is aesthetically pleasing, and the scars are well concealed [Aksu *et al.,* 2008].

Three-dimensional (3D) tissue models of mammary gland promise to overcome many of the shortcomings associated with both *in vivo* animal experimentation and two-dimensional (2D) tissue culture. Culturing cells with the correct 3D relationship maintains tissue function by establishing appropriate cell-signaling pathways and extracellular matrix interactions. Ultimately a meaningful 3D model system must be a compromise between supplying enough biochemical and mechanical properties to support diverse tissue processes, and also being simple enough to satisfy the constraints of diagnostic procedure, repeatability and quality control [Patrick C.W. 2008].

## 1.3 Scope of the study

The idea is to develop a tissue-engineered construct to mend soft tissue defects arising from traumatic injury, tumor resections and musculofacial reconstructions. A soft tissue defect is generally defined as a large tissue void within the subcutaneous fat layer of the skin that often results in a change in the 'normal' tissue contour [Patrick *et al.,* 2001]. Millions of plastic and reconstructive surgical procedures are performed each year to repair soft tissue defects that result from traumatic injury (i.e., significant burns), tumor resections (i.e., mastectomy and carcinoma removal), and congenital defects [Langstein *et al.,* 1999].

The American Society of Plastic Surgeons reported that over 5 million reconstructive procedures were performed in 2004, approximately 4 million of which were due to tumor removal In patients suffering from soft tissue defects, autologous transplantation of fat grafts has been applied clinically, and can help reduce defective regions or scars in the breast and other areas [Heimburg *et al.,* 2001]. Though various approaches including dermal fat graft, collagen injection, use of synthetic materials, and free adipose tissue grafts have been attempted, each method carries considerable disadvantages: for example, synthetic materials invariably result in foreign body reactions, and biologically derived materials shrink to an unpredictable extent [Dzubow *et al.,* 1998]. However, there is likely neither single nor ideal filler material that will satisfy all the clinical needs. Moreover, the clinical outcome of adipose tissue transplantation is unpredictable, with graft resorption due to a lack of vascularization resulting in poor cosmetics and impaired functionality. Synthetic implants are associated with immune rejection, implant migration and resorption, and a failure to integrate into the host tissues [Flynn *et al.,* 2007].

Fat tissue engineering offers great potential in answering the limitations realized with the classical approaches in reconstructive surgery [Niemela *et al.,* 2008]. In the reconstruction surgery of adipose tissues for the treatments of breast or subcutaneous adipose tissue defects, the grafting of autologous semi-liquid free fat tissue of a few millimeters in size has been clinically performed but problems like absorption and fibrosis of tissues are common to occur [Takashi *et al.,* 2010].

The adipose tissue-engineered triad concept is proposed here for soft tissue reconstruction. Adipose-derived mesenchymal stem cells (ASC) from male rats were used as the cell source and were induced to adipocyte lineage on both collagen and ceramic scaffolds. Collagen is a well proved scaffold for adipose tissue regeneration [Yu *et al.,* 2010]. As yet, there have been no reports of adipogenesis on ceramics. The concept of induction of adipogenesis on biphasic calcium phosphate may hopefully open up avenues in the field of Musculofacial reconstruction and soft tissue reconstruction

Therefore in this study, an attempt has been made to use the in-house synthesized and developed Biphasic calcium phosphate - BCP (Patented process - Bioceramics Laboratory, SCTIMST) as scaffolds for the induction of adipogenesis using adipose-derived stem cells. The fabricated cell-ceramic construct thus developed *in vitro* was implanted in female rats for a period of three weeks to demonstrate the effect of the efficacy of the TE construct in an *in vivo* environment.

# Hypothesis & Objectives

## 1.4 Hypothesis

To induce adipogenesis in a rodent model using a combination product of Adipose derived stem cells on Biphasic Calcium Phosphate and Collagen sponge and to prove the efficacy of adipose tissue constructs *in vivo*.

## 1.5 Objectives

1. To characterize Biphasic Calcium Phosphate (BCP) and Collagen sponge.
2. To isolate and characterize rat adipose-derived stem cells (ASCs) from Sprague Dawley rat subcutaneous fat tissue
3. To induce adipogenesis of ASCs on BCP and Collagen scaffolds *in vitro*
4. To fabricate cell-BCP and cell-collagen constructs *in vitro* for *in vivo* implantation in rat muscle to demonstrate adipogenesis.

Chapter-II

# Materials & Methods

# Materials and Methods

## 2.1 Materials

The bioactive ceramic (Biphasic calcium phosphate) and collagen sponges were the scaffold of choice used for the fabrication of a tissue engineering construct for fat tissue engineering.

### 2.1.1 Bioceramic (Biphasic calcium phosphate) Scaffolds

The bioactive ceramic used for this study was biphasic calcium phosphate (BCP), synthesized as part of the ongoing developmental programme at the Bioceramics Laboratory of the Biomedical Technology Wing, SCTIMST, Trivandrum, India.

### 2.1.2 Collagen Sponges

Bovine Collagen Type I (0.02 GA (wt%)) sponge was obtained from Nitta Gelatin; gifted by Prof. Yasuhiko Tabata, Institute for Frontier Medical Sciences, Kyoto University, Japan.

## 2.2 Sterilization

### 2.2.1 BCP

BCP materials were polished in the form of discs - 5mm diameter with 1-2mm thickness. The discs were washed with distilled water and subjected to ultrasonic cleaning (Cole Parmer) twice, 10 minutes each for the complete removal of fine powders that adhered over the surface. Subsequently, the materials were sterilized by steam (121°C, 15psi pressure for 15minutes) prior to the *in vitro* and *in vivo* studies.

### 2.2.2 Collagen Sponges

Collagen materials were punched out from the collagen sheet in the form of discs with 5mm diameter and 1-2mm thickness. Discs were gamma irradiated at Microtrol Sterilization Services Ltd, Bangalore with a minimum dose of 25 kilo gray prior to the *in vitro* and *in vivo* studies

## 2.3 Characterization of the scaffold materials.

### 2.3.1 Scanning Electron Microscopy (SEM)

Surface microstructure, topography, and porosity of the materials were observed by SEM (S2400-Hitachi).The scaffolds seeded with cells were treated with gradients (30%, 50%, 70%, 80%, 90%, and 100%) of ethanol (Merck) rinsed in distilled water and critically point dried (HCP2-Hitachi) to remove moisture. Specimens were then Gold coated (E101-Hitachi) in an ion sputter unit and viewed using SEM.

### 2.3.2 Fourier Transform Infrared Spectroscopy (FT-IR)

#### 2.3.2.1 FT-IR for BCP

Fourier Transform Infrared Spectroscopy analysis was conducted on Thermo Nicolet 5700 spectrometer, U.S.A. and the spectra were collected in diffuse reflectance (DRIFT) mode to determine the functional groups. BCP was powdered and mixed with optical grade KBr powder, while pure KBr was used as the background. The spectra were recorded at a resolution of 4 $cm^{-1}$ and scanned in the range 400 - 4000 $cm^{-1}$ with an average scan of 64.

#### 2.3.2.2 FT-IR of Collagen scaffold

Fourier Transform Infrared Spectroscopy analysis was conducted on Thermo Nicolet 5700 spectrometer, U.S.A. and the spectra were collected in attenuated total internal reflection (ATR) mode determine the functional groups. The spectra were recorded at a resolution of 4 $cm^{-1}$ and scanned in the range 640 - 4000 $cm^{-1}$ with an average scan of 64.

### 2.3.3 X-Ray Diffraction (XRD)

X- ray diffraction was performed to assess the phase purity and crystallinity of the sintered BCP. The samples were scanned between $2\theta=20^{\circ}$ to $35^{\circ}$ at a rate of $0.1^{\circ}$ $min^{-1}$ using Cu K$\alpha$ radiation at a voltage of 40 kV and a current of 30 mA (Siemens D-5005 X-ray Diffractometer, Germany). Materials were identified by comparing the data with the JCPDS files (Joint committee for powder diffraction standard).

## 2.4 Growth and Differentiation Media for cell culture procedures

### 2.4.1 Medium for isolation and expansion of Rat ASC culture

Medium used for isolation and expansion of Rat ASC culture was Dulbecco's Modified Eagles Medium (DMEM) High Glucose (HG) (Invitrogen) supplemented with 10% Fetal bovine serum (FBS) (Invitrogen) and 1% antibiotics (anti- anti mix, Invitrogen).

### 2.4.2 Media for Adipocyte Differentiation

Media used for adipocyte differentiation is DMEM HG (Invitrogen) supplemented with 10%FBS (Invitrogen) and 1% antibiotics (Invitrogen). For adipogenic differentiation two types of media are commonly employed

#### 2.4.2.1 Adipogenic induction Medium

Induction media (DMEM HG, 10% FBS and 1% antibiotics), contains adipogenic inducers with working concentration

- 0.5mM 3-iso-butyl methylxanthine (Sigma. USA)
- 1μM Dexamethasone (Sigma. USA)
- 50 μM Indomethacin (Lotus International),
- 5 μg/ml Insulin (Eli Lilly)

The medium with the inducers were filter sterilized (Millex GP, Millipore) and used.

#### 2.4.2.2 Adipogenic Maintenance Medium

The Adipogenic maintenance medium contains 10 μg/ml Insulin (Sun Pharmaceuticals) as an additional component. The medium with the inducers were filtered with 0.22μm filter (Millex, Millipore) and used.

### 2.4.3 Osteogenic Induction Medium

Medium used for osteogenic differentiation is DMEM HG (Invitrogen) supplemented with 15%FBS (Invitrogen) and 1% antibiotics (Invitrogen). The working concentration of the osteogenic inducers in the medium are

- 10mM β-glycerophosphate (Sigma. USA)
- $10^{-8}$ M dexamethasone (Sigma. USA)
- 0.05mg/ml L-ascorbic acid (Sigma. USA)

The medium with the inducers were filtered with 0.22μm filter. (Millex, Millipore) and used

### 2.4.4 Chondrogenic induction Medium

Chondrogenic medium contains DMEM-HG Invitrogen supplemented with 15% FBS Invitrogen and 1% antibiotics (Invitrogen). The chondrogenic inducers include

- 10ng/ml TGF-β1 (Imperial),
- 0.1% ITS Premix (BD Biosciences)
- 10-8 M Dexamethasone (Sigma. USA)

The medium with the inducers were filtered with 0.22μm filter. (Millex, Millipore) and used.

## 2.5 Culturing of Rat adipose derived mesenchymal stem cells (ASCs)

Mesenchymal stem cells were isolated from the subcutaneous fat pad of the dorsal subcutics of rat models. Male rats of 4-9 months were used for isolation of ASCs

### 2.5.1 Isolation of ASC from Rat

Tissue Isolation & Animal Experiments were carried out as per the guidelines of the Institutional Animal Ethics Committee (IAEC).

Fat tissue was isolated from rats, provided by the Division of Laboratory Animal Sciences (DLAS), BMT Wing, SCTIMST. Subcutaneous fat was collected under sterile conditions, into Phophate Buffer Saline (PBS). PBS containing 1% antibiotics (anti-anti mix Invitrogen), then the sample was washed with fresh PBS containing 1% antibiotics (Invitrogen). Collected adipose tissue was cleaned and well minced. Double the volume of 0.2% collagenase (Invitrogen) is added. Tissue was digested at 37°C for an hour. Digestion was stopped and the digest was filtered using a 180 micron nylon mesh membrane filter (Millipore). Cells were pelleted down at 14°C, 2500 rpm, 10 min (Hettich, Germany). Cells were plated in 5ml of DMEM HG, 10% FBS and 1% Antibiotics in a 25cm$^2$ flask (Nunc) and placed in the $CO_2$ incubator (Hereaus, Germany) at 37°C with 5% $CO_2$.

### 2.5.2 Expansion of Rat ASC in culture.

The isolated cultures at 90% confluency were trypsinized using trypsin (Invitrogen) and are passed on to the next Passage. One 25cm$^2$ flask was trypsinized with 1 ml of 0.25% trypsin (Invitrogen). Allow the cells to detach and then the action was stopped by adding medium (DMEM HG), centrifuged (Hettich, Germany) at 2500 rpm for 10 min and the pellet is resuspended and divided into two 25cm$^2$ flask (Nunc). The culture is further expanded until Passage 6 allowing the cells to form a monolayer at each Passage.

## 2.6 Characterization of Rat ASCs

### 2.6.1 Flow cytometry analysis of CD44 and CD105

Rat ASCs were cultured in 25cm$^2$ flasks (Nunc). Passage 2, passage 4 and passage 6 were taken for flow cytometric analysis. The cells at 80% confluency were taken, washed with PBS; trypsinized with 0.25% trypsin (Invitrogen) for 2-3 minutes and centrifuged (Hettich Germany) at 2500 rpm for 10 minutes. The pellet was equally divided into 3 Eppendorf and fixed with 500μl 3.7% paraformaldehyde in Sorensen phosphate buffer for half an hour; Centrifuged at 1500rpm for 5 minutes. The pellet was washed with PBS and incubated with respective CD markers. One of the eppendorffs were kept unstained and was taken as control

#### 2.6.1.1 Flow Cytometry for CD 105

The pellet was the incubated for one hour with the primary antibody CD105 (1:100 in 1%BSA) (rat monoclonal antibody Santa Cruz Biotechnology). After the primary incubation the cells were centrifuged and the pellet was incubated with FITC (1:100 in 1%BSA) (rabbit anti mouse antibody, Geni) for 1 h, centrifuged and resuspended in 300μl of PBS and the intensity of fluorescence was recorded under flow cytometry (BD Biosciences FACS Aria.

#### 2.6.1.2 Flow Cytometry for CD 44

The pellet was the incubated for one hour with the primary antibody CD44 (1:100 in 1%BSA) (Abcam). After the primary incubation the cells were centrifuged and the pellet was incubated with FITC (1:100 in 1%BSA) (rabbit anti mouse antibody, Geni) for 1 h, centrifuged and resuspended in 300µl of PBS and the intensity of fluorescence was recorded under flow cytometry (BD Biosciences FACS Aria.

### 2.6.2 Differentiation of Rat ASCs to Adipo, Osteo and Chondrogenic lineages

Rat ASC's were induced to multiple lineages using specific lineage inducing factors.

#### 2.6.2.1 Induction of ASC to Adipogenic lineage

Rat ASC (cells in passage 4) were cultured in growth medium for 24 h in coverslips (Blue star) order to facilitate the cell adhesion and spreading. After 24 h the cells were induced to adipogenic lineage by maintaining in Adipogenic induction medium. The cells were maintained for 2 days in adipogenic induction medium and 21 days in adipogenic maintenance medium.

##### 2.6.2.1.1 Nile Red Staining

Rat ASC after 21 days induction to adipogenic lineage were fixed in 3.7% paraformaldehyde in sorrensen phosphate buffer for 24 hours. Washed with PBS stained with Nile red (Sigma. USA); stock solution contain 0.5 mg/ml in acetone; working solution is made by adding 10µl of the stock to 10 ml of a 75:25 glycerol-water mixture. Stir well and was diluted for optimum fluorescence if it turns out to be too strong and is observed under Confocal Laser Scanning Microscope-CLSM (Carl Zeiss LSM 510 META).

##### 2.6.2.1.2 Oil Red O Staining

Rat ASC after 21 days induction to adipogenic lineage were fixed in 3.7% paraformaldehyde in sorrensen phosphate buffer for 24 hours. Washed with PBS stained with Oil Red O (Sigma. USA). Stock solution is prepared by dissolving 300mg in 100ml Isopropanol (Nice Chemicals). Oil Red O Working Solution is obtained by diluting 6 parts Oil Red O stock with 4 parts distilled water and filtered. Prior to staining the cells are treated with 60% isopropanaol. The induced rat ASC are then stained with oil red O for 10 min and observed under Light Microscope (Leica DM 6000).

#### 2.6.2.2 Induction of ASCs to osteogenic lineage

Rat ASC (cells in passage 3) cultured in DMEM HG (10% FBS and 1% antibiotics) were trypsinized, pelletized and seeded on to the cover slips (Blue star). It was maintained for 24h in order to facilitate the cell adhesion and spreading. After 24h the cells were induced to osteogenic lineage by maintaining the cells in osteogenic medium. The osteogenic differentiation was confirmed through collagen, calcium and phosphorous staining.

##### 2.6.2.2.1 Collagen Staining

Rat ASC that are induced to osteogenic lineage for 28 days were fixed in 3.7% paraformaldehyde in sorrensen phosphate buffer for 24 hours. Later the cells were washed with PBS and incubated in Bouin's solution at 56°C for 30 minutes and stained with Masson's Trichrome (Sigma. USA) which stains collagen blue. The cells were again washed with distilled water and incubated with 0.5% glacial acetic acid for 1 minute followed b washing with PBS. Mounted with DPX and viewed under Light Microscope (Leica DM 6000)

##### 2.6.2.2.2 Calcium Staining

Rat ASC that are induced to osteogenic lineage for 28 days were fixed in 3.7% paraformaldehyde in sorrensen phosphate buffer for 24 hours. Washed with PBS and stained with 1% Alizarin red (Sigma. USA) to determine calcium deposition. Mounted with DPX and viewed under Light Microscope (Leica DM 6000)

#### 2.6.2.2.3 Phosphorous staining

Rat ASC that are induced to osteogenic lineage for 28 days were fixed in 3.7% paraformaldehyde in sorrensen phosphate buffer for 24 hours. Washed with PBS and stained with 5% silver nitrate (Merck India) in distilled water and exposed to UV light for 5 minutes. The cells are then washed with distilled water, air dried and viewed under Light Microscope (Leica DM 6000).

### 2.6.2.3 Induction of ASC to Chondrogenic lineage

ASCs in $25cm^2$ flask (Nunc) were incubated in growth medium (DMEM HG) until confluency was attained. Then the Cells were collected by trypsinization & pelleted. Culture was carried out in chondrogenic medium in micromass form in incubator (Heraeus) at 37° C and 95% RH with 5% $CO_2$.

#### 2.6.2.3.1 Alcian blue Staining

Induction of chondrogenesis was demonstrated by Alcian Blue staining. The stain is prepared by dissolving Alcian blue, 8GX (Sigma USA) in 3% Acetic acid solution (Rankum). Mixed well and pH adjusted to 2.5 using acetic acid.

### 2.6.2.4 Safranin O staining

Induction of chondrogenesis was demonstrated by Safranin O staining. The stain is prepared by dissolving 0.1% Safranin O Solution (Sigma. USA) in distilled water.

## 2.7 Material-Cell Interaction

To study the material cell interaction the adhesion of cells on to the BCP scaffolds and the viability of the cells on the same was studied.

### 2.7.1 Direct Contact

The rat ASC's were seeded onto the scaffold (BCP and Collagen) at a ratio of $4 \times 10^4$ cells/scaffold. The scaffolds were incubated in DMEM HG for 24h prior to cells seeding to improve the material characters. The cell material interaction was studied using phase contrast microscopy.

### 2.7.2 Adhesion of Rat ASC on BCP

The adhesion of rat ASC on BCP was evaluated using Scanning Electron Microscope (SEM) (S2400-Hitachi). The material was seeded with 4 x $10^4$ cells. Cultured for 3 days and fixed in 3% gluteraldehyde and proceeded for SEM analysis. The scaffolds were treated with gradients (30%, 50%, 70%, 80%, 90%, 100%) of ethanol (Merck) rinsed in distilled water and critically point dried (HCP2-Hitachi) to remove moisture, and coated with gold in an ion sputter unit (E101-Hitachi). Samples were viewed using SEM (S2400-Hitachi).

### 2.7.3 Biochemical evaluation for Cell Proliferation - Pico green

The proliferation of rat ASC on day 3 and day 6 days in culture was determined using Picogreen® dsDNA Quantitation reagent (Molecular probes). For this, the cell-seeded materials (n=3) were washed with PBS twice and kept in -80°C until analysis. Frozen cell samples were thawed for 20 min on ice and lysed with 1% Triton X-100 (300 µl) for 20 min with sonication for 10 min. The lysate (10µl) was then mixed with Picogreen in Tris-EDTA buffer (190µl) for 5 min and the intensity of fluorescence was measured with a multifunction microplate reader (HIDEX Chameleon) at an excitation and emission wavelength of 485 / 535 nm. Relative fluorescence units were correlated with cell number using a calibration line constructed from cell suspensions with increasing concentrations of cell numbers.

### 2.7.4 Biochemical evaluation for Cell Viability - LDH

The cell viability was determined quantitatively through the activity of total lactate dehydrogenase (LDH) in the cell lysates using LDH reaction buffer (Cytotox 96 kit, Promega, USA) It is measured in an enzymatic reaction that occurs in two steps (1) NAD+ is reduced to NADH/H+ by the LDH catalysed conversion of lactate to pyruvate. (2) The catalyst (diaphorase) transfers H/H+ of NADH/H+ to the tetrazolium salt INT which is reduced to formazan. This leads to colour change from pale yellow to red.

The viability of Rat ASC on day 3 and day 6 days in culture was measured. For this, the cell-seeded materials (n=3) were washed with PBS twice and kept in 80°C until analysis. Frozen cell samples were thawed for 20 min on ice and lysed with

1% Triton X-100 (300 μl) for 50 min with sonication for 10 min. An aliquot of each cell lysate (50μl) was mixed with LDH substrate (50μl) at room temperature and the enzymatic reaction was stopped after 30 min with 0.1 M acetic acid (50μl). The absorbance was read at 492 nm (HIDEX Chameleon). The absorbance (OD values) was correlated with cell viability using a calibration line constructed from cell suspensions with increasing concentrations of cell numbers.

### 2.7.5 Viability of Rat ASC on BCP and Collagen Scaffolds - Confocal Microscopy

The viability of Rat ASC after 3 days was qualitatively determined by confocal laser scanning microscopy using acridine orange and ethidium bromide. The cells after 3 days in culture were washed in PBS and stained with Acridine orange (1:100 dilution in PBS) and ethidium bromide (1:100 dilution in PBS) and incubated for 30 minutes in dark. It was then washed with PBS and imaged using CLSM (Carl Zeiss LSM 510 Meta) at an excitation and emission wavelength of 480/526 and 518/605 for Acridine orange and ethidium bromide respectively. The excitation was carried out with Argon 2 laser.

## 2.8 *In vitro* induction of adipogenesis in Tissue Culture Plates

### 2.8.1 Demonstration of Adipocytes with Confocal Microscopy-Nile Red Staining

Mesenchymal stem cells induced to adipocytes in culture for 21 days red a fluorescent dye which will specifically stain the lipids and was viewed using confocal laser scanning microscope. The cells seeded on cover slips were picked at confluency, fixed with 3.7% paraformaldehyde in Sorrensen phosphate buffer, stained with Nile red; stock solution contain 0.5 mg/ml in acetone; working solution is made by adding 10μl of the stock to 10 ml of a 75:25 glycerol-water mixture. Stir well; get rid of bubbles. Dilute to optimum fluorescence if it turns out to be too strong and is observed under Confocal Laser Scanning Microscope (LSM 510 META, Carl Zeiss). Nile red has an excitation at 485nm and an emission at 525nm.

The cells were double stained with DAPI (Sigma. USA) and Nile red to visualize both the nucleus and triglyceride accumulation in the cytoplasm respectively. The cells seeded on cover slips were retrieved at confluency, fixed with 3.7% paraformaldehyde in sorrensen phosphate buffer. Stained with DAPI (1:1000) in distilled water makes the working stock and 1:100 of the working stock in distilled water gives the working solution. Then double stained with Nile red; stock solution contain 0.5 mg/ml in acetone; working solution is made by adding 10µl of the stock to 10 ml of a 75:25 glycerol-water mixture. Stir well and is observed under Confocal Laser Scanning Microscope (Carl Zeiss).

### 2.8.2 Oil Red O Staining

Rat ASCs after 21 days induction to adipogenic lineage were fixed in 3.7% paraformaldehyde in Sorrensen phosphate buffer for 24 hours. Washed with PBS and stained with Oil Red O (Sigma. USA). Prior to staining the cells are treated with 60% isopropanaol. The induced rat ASC are then stained in oil red O for 10 min and observed under Light Microscope (Leica DM 6000).

### 2.8.3 Demonstration of Adipogenesis through Flow cytometry using Nile Red.

The cells in 25cm$^2$ flask after induction for 21 days were trypsinized with trypsin (Invitrogen), centrifuged at 2500 rpm for 10 minutes. The pellets were fixed with 3.7% paraformaldehyde in sorrensen phosphate buffer for half an hour and was washed with 1 ml PBS and then stained with Nile red (Sigma. USA): Stock solution (0.5 mg/ml in DMSO) was diluted 1:500 in PBS, 10 min before adding 500µl of this solution to each pellet. Cells were then incubated for 10 min at room temperature in the dark and then washed twice with 1 ml PBS. Pellets were finally resuspended in 500µl PBS. Samples were maintained on ice, avoiding direct light, and then acquired with the flow cytometer (BD FACS Aria) for acquisition. A total of 10,000 events were collected on the flow cytometry for each sample acquired. The cells without staining with primary and secondary antibodies but processed under the same conditions were taken as the control.

### 2.8.4 Biochemical Estimation of Triglycerides.

The triglyceride contents of the cells were estimated at different intervals to demonstrate triglyceride accumulation. The triglyceride content of a confluent $25cm^2$ flask was estimated at 7, 14 and 21 days with triglyceride estimation kit (Agappe Diagnostics). The cells were trypsinized with trypsin (Invitrogen) at confluency, centrifuged and the pellet was resuspended in PBS. The resuspended pellet was treated to ultra sonication (Cole Parmer) for 30 minutes. 100µl of the sample was taken and estimate with the triglyceride kit (Agappe Diagnostics) at 550 nm with Spectrophotometer (HIDEX Camilion). The blank and standard was provided.

The triglyceride concentration is calculated by the formula

$$\text{Triglyceride concentration} = \frac{\text{Absorbance of the sample} \times 200}{\text{Absorbance of standard}}$$

## 2.9 *In vitro* induction of adipogenesis on collagen & ceramic Scaffolds

### 2.9.1 Induction of adipogenesis on Biphasic Calcium Phosphate and collagen Scaffolds.

Rat ASC of passage 4 were cultured on BCP scaffolds. The Rat ASCs cultured on $25cm^2$ flasks were trypsinized with trypsin (Invitrogen) and centrifuged at 2500 rpm for 10 minutes. The pellet was resuspended in a required ml of medium and the cell number was counted manually using haemocytometer. $4 \times 10^4$ cells per material were seeded on each material

BCP and the collagen scaffolds were kept for conditioning for 24 hours prior to seeding. The cells were maintained in the culture for 2 days in adipogenic induction medium and for 21 days in maintenance medium. Later the samples were proceeded for *in vitro* evaluation.

### 2.9.2 Real time PCR.

*In vitro* evaluation of the cells on the scaffolds was done by Real time Polymerase chain reaction. Real time-PCR is a method that allows exponential amplification of short DNA sequences (usually 100 to 600 bases) within a longer double stranded DNA molecule. Real-time PCR is the most sensitive method that can quantitatively discriminate closely related mRNAs. Here real time-PCR was used as a tool to determine the expression of adipocyte specific genes on the scaffolds (BCP and Collagen). The Real time PCR was done in 3 steps (1) RNA isolation (2) cDNA synthesis and (3) cDNA amplification using specific primers.

#### 2.9.2.1 RNA Isolation

For the isolation of total RNA, rASCs were cultured on Collagen and BCP scaffolds for 2 days in adipocyte induction medium and were maintained in adipocyte maintenance medium for 21 days. RNA was isolated from 3 scaffolds each of BCP and collagen. At the end of 21$^{st}$ day the culture medium was removed from the scaffolds and approximately 250µl TRIsoln (M.J Reseaech) was added to each of the scaffold to lyse the cells and was transferred to -80°C. Later the lysate was collected and 150µl of chloroform (Ranbaxy) was added, mixed well and centrifuged (Heraeus) at 12,000 rpm for 10 min at 4°C. The aqueous phase containing RNA was collected, transferred to another sterile eppendorf tube and equal volume of isopropanol (Nice Chemicals) was added. Stored at room temperature for 5 min and then centrifuged at 12,000 rpm for 8 min. Pour dry the RNA pellet was then rinsed with 70% ethanol (Merck) and centrifuged again at 12000 rpm for 8 min. The RNA pellet was air dried, reconstituted in nuclease free water (Invitrogen) and quantified spectrophotometrically (Eppendorf BioPhotometer)

#### 2.9.2.2 cDNA Synthesis

The isolation of non degraded intact RNA is very essential for cDNA synthesis. cDNA was synthesized using RT-PCR reaction in a thermolcycler. (Eppendorf). For cDNA synthesis approximately 1000ng RNA was used. The reaction mix was prepared as given in the table (6). The reaction mixture was then incubated at 25° C for 10 minutes and then the reverse transcriptase step for 30 min at 48°C. The final incubation for 5 min at 95° C was given to inactivate the Reverse Transcriptase enzyme. The cDNA synthesized was stored at 4° C till use.

**Table 6: RT PCR Reaction components**

| Reagent | Volume |
|---|---|
| Rnase inhibitor (Stratagene) | 0.2μl |
| Random hexamer(IDT) | 0.5μl |
| 25mM MgCl2 (Geni) | 2 μl |
| Stratascript 10 X reaction Buffer (Stratagene) | 1 μl |
| 2.5mM dNTP (Fermentas) | 2 μl |
| Superscript III reverse transcriptase (Invitrogen) | 0.25 μl |
| Nuclease free water (Invitrogen) | 1μl |
| Template | 3.05 μl |

### 2.9.2.3 cDNA Amplification

The cDNA was amplified by real time PCR (MJ Research) using Full Velocity™ SYBER® Green QPCR Master mix. The reaction mixture was prepared as depicted in table (7) and PCR amplification was performed using specific primer pairs designed from reported gene sequences for different markers as given in table (9). Primer dilution is given in table (8). Cycling conditions were as follows.

Initial denaturation at 95 °C for 10 min, denaturation at 94 °C for 30s, specific annealing temperature as given in Table (9) for 45s and extension at 72 °C for 30 sec followed by plate reading and the analysis of melting curve. Later a final extension at 72°C for 10 min and is set at 4°C for ever. The PCR reaction was set for 35 cycles.

**Table 7: PCR reaction mixture for cDNA amplification**

| | |
|---|---|
| 2X SYBER Green I QPCR MM | 30μl |
| Passive reference dye (1:200) | 0.9 μl |
| C-DNA | 7.5 μl |
| Final volume without primers | 38.4μl |

**Table 8: Primer dilution**

| | |
|---|---|
| Forward primer | 2µl |
| Reverse Primer | 2µl |
| Rnase free water | 17.6µl |
| Final volume of primers | 21.6 µl |

The PCR reaction mix is mixed with the primer mix which will give a total volume of 60µl and can be used to run two reaction tubes of 30µl each

**Table: 9: Primer sequence and annealing temperatures of genes analyzed**

| Gene | Primer sequence | Annealing temperature (°C) | Product length (bp) |
|---|---|---|---|
| C/EBPa | FP-(5'-3') GGCGGGAACGCAACAA<br>RP- (5'-3') TCCACGTTGCGCTGTTTG | 54 | 1389bp |
| Pparg | FP-(5'-3') CTTGGCCATATTTATAGCTGTCATTATT<br>RP-(5'-3') TGTCCTCGATGGGCTTCAC | 54.7 | 1636bp |
| 28S rRNA | FP- (5'-3') GAATCCGCTAGGAGTGTGTAACAA<br>RP-(5'-3') GCTCCAGCGCCATCCAT | 54.2 | 4786bp |

### 2.9.3 Demonstration of adipogenesis on BCP scaffold by Confocal Microscopy

The BCP scaffolds seeded with rat ASCs were cultured in adipogenic media and was fixed with 3.7% paraformaldehyde in sorrensen phosphate buffer, stained with nile red; stock solution contain 0.5 mg/ml in acetone; working solution is made by adding 10µl of the stock to 10 ml of a 75:25 glycerol-water mixture. The scaffold was washed twice with distilled water at 10 min interval to remove the remaining stain on the scaffolds and is observed under Confocal Laser Scanning Microscope (LSM 510 META, Carl Zeiss).

## 2.10 *In Vivo* demonstration of Adipogenesis using bare & cell-seeded collagen & ceramic scaffolds

### 2.10.1 In Vivo Implantation Experiment

The animal experiments were carried out as per the guidelines of the Institute Animal Ethics Committee. The rats were obtained and the surgical procedures were carried out at the Division of Laboratory Animal Science of Sree Chitra Thirunal Institute of Medical Science and Technology (DLAS). The *in vivo* implantation was carried out on female Sprague Dawley Rats of 4 months age. Rats of weight between 190g and 210g were taken for implantation. 6 rats were used for implantation studies

**Table 10: *In Vivo* Experimental Plan**

| Study Period: 21 days | **Collagen*** | | **Ceramic*** | |
|---|---|---|---|---|
| | Collagen + Cell (Right Dorsal) | Collagen (Left Dorsal) | BCP+ Cell (Right Dorsal) | BCP (Left Dorsal) |
| Rats | 3 each | | 3 each | |
| Total | 6 Rats | | | |

(*2 subcutaneous implantation sites on either side of the vertebral column, giving a total of 4 implantation sites in each rat, of which 2 will be bare Collagen and the other two will be Cell-Collagen construct and likewise for Ceramic scaffolds).

*In vivo* experiments were planned on the dorsal muscle region in rats to demonstrate the efficacy of ceramic scaffolds in adipose tissue regeneration in comparison with collagen sponge as a control. Dorsal muscular region is a suitable ectopic region for implantation where normal fat tissue is absent. A short term study of 21 days were planned and thereafter these rats will be sacrificed and proceeded for histology to demonstrate adipose tissue regeneration

### 2.10.2 Surgical Procedure

The *in vivo* experimental groups comprise of albino rats. The animals are medicated with Tetracycline @ 10mg/kg body weight orally from one day prior to surgery and continued till the fifth post operative day. Rats will be anesthetized with Xylazine@5mg/kg body weight and ketamine@70mg/kg body weight as intramuscular injection. Under anesthesia, the dorsum of the animal is clipped and the site is sterilized with Povidone iodine solution. In each rat, under aseptic precautions, a skin incision is made at the mid-dorsum on the thoracic region and sub-cutis in the area is cleared off with fine dissection. The Test material is placed on the right side of the spinal cord over the musculature and secured *in situ* with the help of sterile 3-0 braided silk sutures (Ethicon). The control material is placed on the left side of the spinal cord over the musculature and secured with the help of sterile 3-0 braided silk sutures. Care is given not to apply pressure on the material during this fixing. Skin incision is closed using 3-0 braided silk sutures on Simple interrupted pattern. Neomycin antibiotic dust is applied over the incision site post operatively. Skin sutures are removed on the seventh post operative day. The implantation is done to evaluate the *in situ* adipogenesis due to the scaffold *in vivo*. Both cell seeded scaffolds and scaffolds alone were implanted to study the effects of cells in adipogenesis.

There are two experimental groups each consists of three rats. One with cell seeded collagen and bare collagen in the right dorsal and left dorsal region on either side of the vertebral column respectively and the other group with cell seeded ceramic and bare ceramic in the right dorsal and left dorsal region on either side of the vertebral column respectively. Four scaffolds can be implanted on the dorsal muscular region of the rat, two cell seeded scaffold on the right dorsal and two bare scaffold materials in the left dorsal muscle. The rats in groups I & II will be sacrificed after 21 days by an overdose of anesthetic and the native tissue, including the scaffold was carefully taken out. The specimens proceeded for histology.

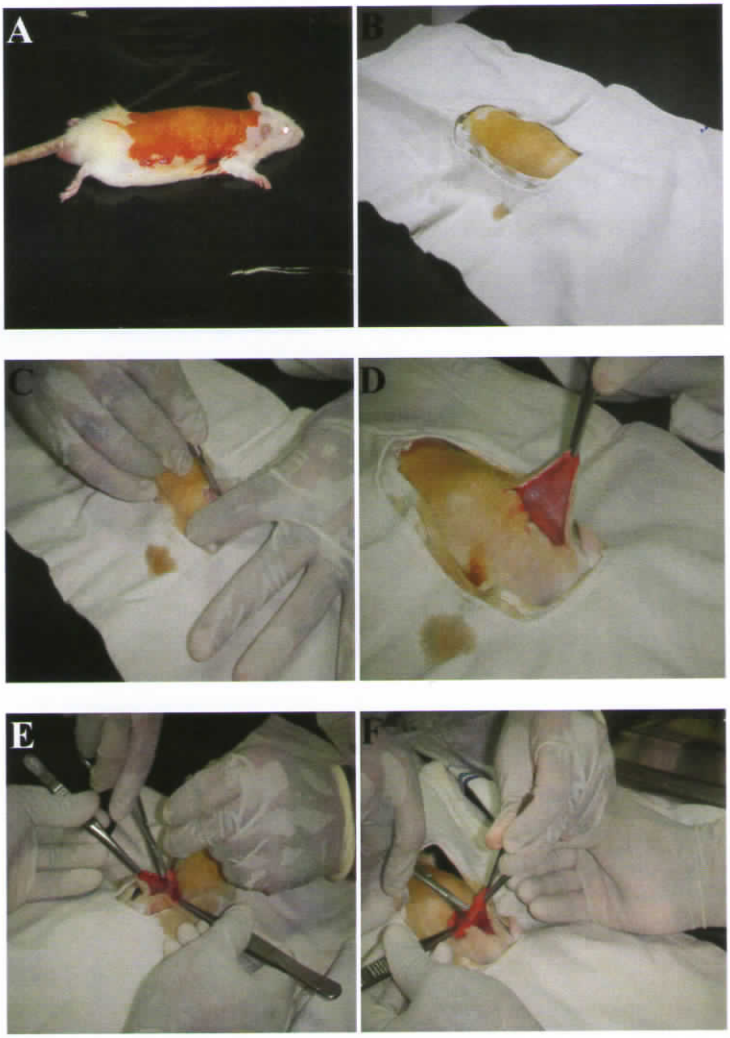



**Fig 2: Surgical Procedure for Implantation in rat model :- (A&B)** Preparation of Surgical site; **(C&D)** Incision made on the dorsal site to expose the dorsal muscle; **(E&F)** Implantation of tissue-construct in the dorsal muscle.

## 2.11 Histology of retrieved implants

### 2.11.1 Collagen implants

#### 2.11.1.1 Paraffin Processing

The retrieved tissues (Group I) with collagen 21 days after implantation were fixed in 10% neutral buffered formalin in Sorensen phosphate buffer for 2 days. It was then washed with tap water; dehydrated in graded ascending series of acetone (30%, 50%, 70%, 90% and 100% acetone) for 20 min each; infiltrated in xylene I (10 min) and xylene II (10 min) (Leica TP-1020 Histokinette); embedded in paraffin wax (SLEE MP3/P1 Paraffin wax embedder) for 1h (2 changes) and thin sections (5μm) were made using microtome (Leica RM2255). The sections were kept at 37°C overnight and proceeded for deparaffinization.

#### 2.11.1.2 Hematoxylin and Eosin staining

The sections were deparaffinised with xylene (3 changes, 10 min each); rehydrated in descending grade of ethanol (Merck) series (90%, 80% and 70% for 5 min); washed in tap water for 3 min; stained with Harris's Hematoxylin (Sigma. USA) for 15 min; washed in tap water for 3 min, differentiated in 1% acid alcohol for 30 sec and blued with 0.2% ammonia water for 2 sec. It was then rinsed with tap water for 5 min, counterstained with 1% eosin (Sigma. USA) for 4 min; dehydrated in ethanol (70% for 5 min, 100% ethanol for 5 min - 2 changes); cleared in xylene (3 changes, 10 min each); mounted in DPX and viewed under Light microscope (Leica DM 6000).

#### 2.11.1.3 Mason's Trichrome

The sections were deparaffinised and the deparaffinised sections was incubated over night in Bouin's solution, washed and stained with weigerts hematoxylin (Sigma Chemicals), differentiated and blued. Incubated in acid fuchsin (Sigma Chemicals), phosphomolybdic acid (Sigma. USA) and aniline blue (Sigma. USA). Rinsed in distilled water and then in 1% acetic acid. The slides were dehydrated and mounted with DPX.

#### 2.11.1.4 Picrosirius Staining

The sections were deparaffinised washed in tap water for 5 min and stained with weigerts hematoxylin, rinsed in tap water and stained with Picro sirius (Sigma. USA) for 60 minutes. Rinse in acidified water and then dehydrate and mount with DPX.

### 2.11.2 Ceramic implants

#### 2.11.2.1 Paraffin processing

One of the retrieved sample (Group II) of the ceramic cell seeded material was taken for paraffin embedding. The ceramic disc was carefully pulled out from the adjacent tissue and the adjacent tissue was proceeded for Paraffin processing.

#### 2.11.2.2 Hematoxylin and Eosin staining

The sections were deparaffinised with xylene (3 changes, 10 min each); rehydrated in descending grade of ethanol series (90%, 80% and 70% for 5 min); washed in tap water for 3 min; stained with Harris's Hematoxylin for 15 min; washed in tap water for 3 min, differentiated in 1% acid alcohol for 30 sec and blued with 0.2% ammonia water for 2 sec. It was then rinsed with tap water for 5 min, counterstained with 1% eosin for 4 min; dehydrated in ethanol (70% for 5 min, 100% ethanol for 5 min - 2 changes); cleared in xylene (3 changes, 10 min each); mounted in DPX and viewed under Light microscope (Leica DM 6000).

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#### 2.11.1.4 Picrosirius Staining

The sections were deparaffinised washed in tap water for 5 min and stained with weigerts hematoxylin, rinsed in tap water and stained with Picro sirius (Sigma. USA) for 60 minutes. Rinse in acidified water and then dehydrate and mount with DPX.

### 2.11.2 Ceramic implants

#### 2.11.2.1 Paraffin processing

One of the retrieved sample (Group II) of the ceramic cell seeded material was taken for paraffin embedding. The ceramic disc was carefully pulled out from the adjacent tissue and the adjacent tissue was proceeded for Paraffin processing.

#### 2.11.2.2 Hematoxylin and Eosin staining

The sections were deparaffinised with xylene (3 changes, 10 min each); rehydrated in descending grade of ethanol series (90%, 80% and 70% for 5 min); washed in tap water for 3 min; stained with Harris's Hematoxylin for 15 min; washed in tap water for 3 min, differentiated in 1% acid alcohol for 30 sec and blued with 0.2% ammonia water for 2 sec. It was then rinsed with tap water for 5 min, counterstained with 1% eosin for 4 min; dehydrated in ethanol (70% for 5 min, 100% ethanol for 5 min - 2 changes); cleared in xylene (3 changes, 10 min each); mounted in DPX and viewed under Light microscope (Leica DM 6000).

#### 2.11.2.3 Mason's Trichrome

The sections were deparaffinised with xylene (3 changes, 10 min each); rehydrated in descending grade of ethanol series (90%, 80% and 70% for 5 min); washed in tap water for 5 min; the deparaffinised sections was incubated over night in Bouin's solution, washed and stained with weigerts hematoxylin, differentiated and blued. Incubated in acid fuchsin, phosphomolybdic acid and aniline blue.Rinsed in distilled water and then in 1% acetic acid. The slides were dehydrated and mounted with DPX.

# Chapter-III

# Results & Discussion

# Phase I - Material Characterization

The ceramic scaffold (BCP) and collagen sponge were analyzed for their physico-chemical and morphological characteristics by Scanning Electron Microscopy (SEM), X-ray diffraction (XRD) and FT-IR spectroscopic methods

## 3.1.1 Scanning Electron Microscopy

### 3.1.1.1 Biphasic Calcium Phosphate

Scanning electron micrographs revealed the 3D rough surface topography of the biphasic calcium phospahte with micro- & macropores (pore sizes 120-150μm) and pore interconnections with a porosity of 30-40%. The porous nature of the ceramic provides favourable conditions for cell adhesion and proliferation since it offers cosy niches for cell habitat as well as interconnectivity facilitates easy nutrient and macromolecule transport. The ultrastructure of the surface topography revealed the roughness and porosity that encouraged proper adherence of the cells (Fig 3. A).

### 3.1.1.2 Collagen

The scanning electron micrograph of the bare collagen indicates a highly fibrous structure that is made up of interleaving fibres. The fibrous nature of the scaffold is evident at 500X, with fibres within 20 to 30 μm. The matted nature of (Fig 3. B) the collagen scaffold will provide ease of cell adhesion and an open architecture for nutrient flow, circulation.

## 3.1.2 FT-IR Analysis

### 3.1.2.1 FT-IR of Ceramic - BCP

The FT-IR spectrum obtained for BCP is presented in fig 4. This spectrum is a typical one similar to that of synthetic hydroxyapatite (HA). Peaks correspond to all the functional groups i.e.; hydroxyl and phosphate groups, present in the BCP ceramic and are clearly depicted by their vibrational frequency values. Hydroxyl stretch is observed at 3670 $cm^{-1}$ in the spectra BCP samples. Decrease in the hydroxyl band intensities of the sample powder may be attributed to an increase in the carbonate substitution. For the phosphate ions in BCP there are different sites present

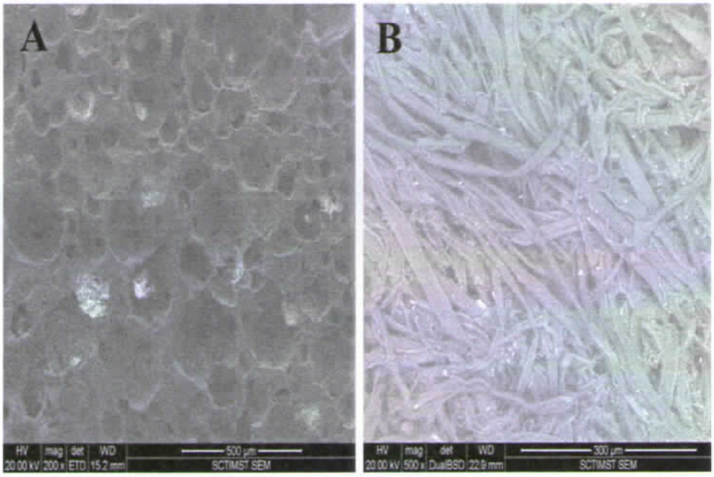


**Fig 3: Scanning Electron Micrographs:- (A)** Biphasic Calcium phosphate scaffold indicating open pores; **(B)** Fibrous collagen scaffold showing matted structure.

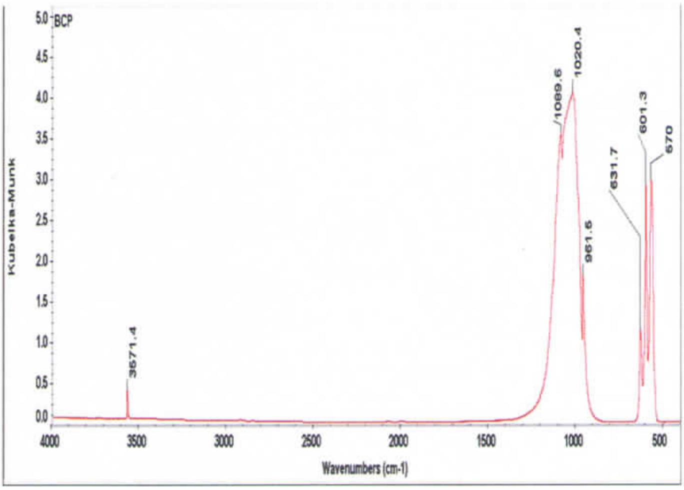


**Fig 4: FT-IR Spectrum:-** Biphasic Calcium Phosphate depicting Characteristic Hydroxyl (3671cm-1) and Phosphate group (1020cm-1, 670cm-1).

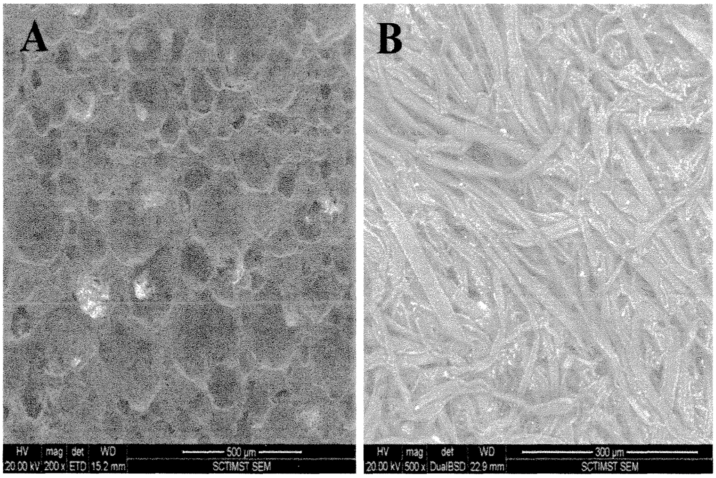


**Fig 3: Scanning Electron Micrographs:- (A)** Biphasic Calcium phosphate scaffold indicating open pores; **(B)** Fibrous collagen scaffold showing matted structure.

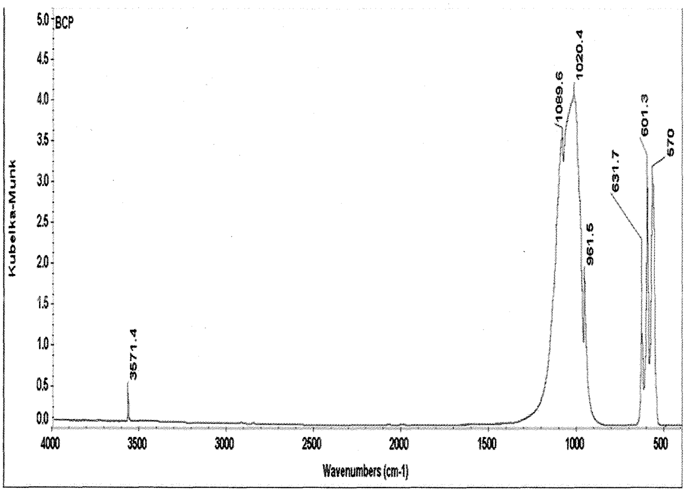


**Fig 4: FT-IR Spectrum:-** Biphasic Calcium Phosphate depicting Characteristic Hydroxyl (3671cm-1) and Phosphate group (1020cm-1, 670cm-1).

at 1085, 1056 cm and 961 $cm^{-1}$ as in HA. The different sites present in the region 1090-970 $cm^{-1}$ confirms presence of hydroxyapatite content in the sample rather than a carbonated hydroxyapatite which if present should have only a single peak for phosphate $\nu_3$ band centred around 1046 $cm^{-1}$. Band at 962.4 $cm^{-1}$ is the phosphate $\nu_1$ band and $\nu_4$ bands is observed in the region 660 – 520 $cm^{-1}$ and the presence of three sites at 629.9, 600.7, and 571.3 $cm^{-1}$ strongly indicate non-carbonated HA rather than carbonated HA which would have only two sites.

#### 3.1.2.2 FT-IR of Collagen

FT-IR spectra of type I collagen used is presented in Fig 5. Collagen exhibits most of the characteristic IR absorptions in the interval 1,800–900 $cm^{-1}$as revealed by the pick peaking. Collagen FTIR spectra exhibited absorptions at about 1,035 and 1,079 $cm^{-1}$, which arise from the ν(C–O) and ν(C–O–C) absorptions of the carbohydrate moieties. Although a precise absorption bands assignation is not available for several spectral intervals of collagen FTIR spectra, absorptions bands at 1,450, 1,404, 1,334, 1,282, 1,240, and 1,201 $cm^{-1}$ may be attributed to the $\delta(CH_2)$, $\delta(CH_3)$, ν(C–N), and δ(N–H) absorptions of collagens. Amides I and II absorptions could be found around 1,630 and 1,541 $cm^{-1}$, respectively.

### 3.1.3 X-ray diffraction (XRD)

X-ray diffraction pattern of the sintered BCP (Fig 5) was compared with the existing powder diffraction data files inorder to asses the phase and crystallinity of the prepared ceramic and are found to be well matched with the JCPDS files of hydroxyapatite (HA) and Tricalcium phosphate (TCP). The black colored scan pattern corresponds to the prepared BCP and by comparing with the standard patterns reveals the presence of TCP which matches with JCPDS # 29-0359; denoted by blue lines and that of HA JCPDS # 09-432 by red lines. The presence of both HA and TCP indicates the biphasic nature of the sample. The intense peaks observed in the pattern indicate well crystallized nature of BCP ceramic.

The characterization of these materials for microstructure, phase purity, crystallinity and functional groups are all necessary to meet the dire requirements for use as medical implants.

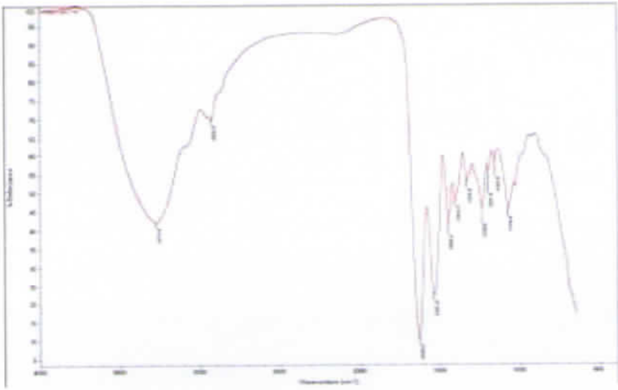

**Fig 5: FT-IR Spectrum:-** Collagen showing C=O (1640-1660cm$^{-1}$); C–N (1500-1600cm$^{-1}$); N–H (1200-1400cm$^{-1}$).

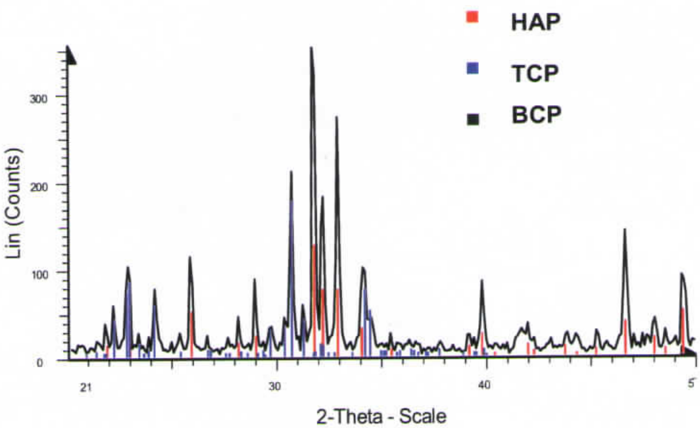


**Fig 6: X-ray diffraction Spectra:-** BCP (Black) in comparison with HAP (Red) and TCP (Blue) JCPDS standards.

# Phase II

## Rat adipose-Derived Mesenchymal Stem Cells (ASCs) - *in vitro*

## 3.2 Isolation, Expansion, and Characterization.

### 3.2.1 Harvesting Rat Adipose tissue

As per the guidelines of the Institutional Animal Ethics Committee (IAEC) rat ASCs were isolated from the subcutaneous fat pad of the rat which weighed around 180-200g. With the animal under anesthesia approximately 2-3g of the adipose tissue was collected.

### 3.2.2 Isolation of rat mesenchymal stem cells from adipose tissue

Mesenchymal stem cells (ASCs) represent a class of multipotent progenitor cells that have been isolated from multiple tissue sites. Of these, adipose tissue and bone marrow offer advantages in terms of access and abundance. Adipose tissue, like bone marrow, is derived from the mesenchyme and contains a supportive stroma that is easily isolated. Adipose tissue is reported to have mesenchymal stem cells that have multi lineage potential [Zuk *et al.,* 2000].

In the present study ASCs were isolated from the subcutaneous fat pad of male rats. The tissue was digested with collagenase, filtered, centrifuged and the pellet was plated with medium where the cells were isolated based on its property of plastic adherence [Gomillion *et al.,* 2006]. Most of the non-adherent cells were removed during the first media change after 11-16 hours. Thereafter these fibroblastic cells exhibited extensive proliferation and reached confluency within 4 to 6 days (Figure 7. A & B).

### 3.2.3 Expansion of rat ASC in culture

The rat ASC's at 90% confluency were trypsinized using trypsin (Invitrogen), centrifuged and seeded in 25cm$^2$ flasks (Nunc) to expand the cell number The primary isolation flasks (25cm$^2$) attained 90% confluency in 4-6 days, while the successive passages were confluent in 3-4 days (Fig 7. C&D). A passage of healthy cells were

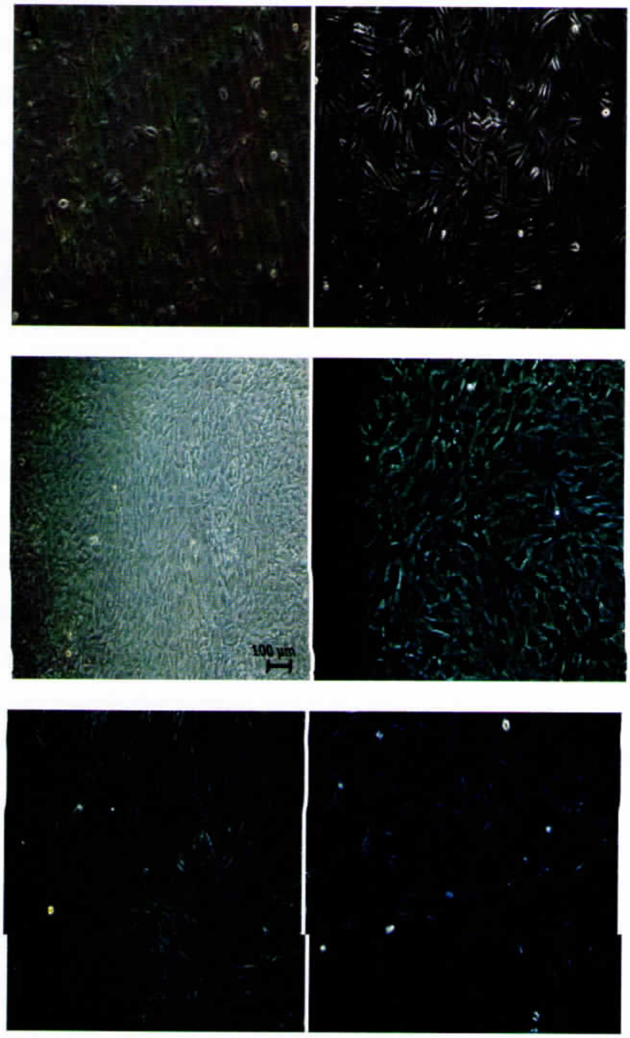


**Fig 7: Phase contrast Micrographs of Adipose-derived mesenchymal stem cells:-**
**(A)** Primary culture - Day 2; **(B)** Primary culture - Day 4; **(C & D)** Cells in Passage 4; **(E&F)** loss of fibroblastic morphology in Passage 9.

observed until $6^{th}$ or $8^{th}$ passage and thereafter the $10^{th}$ passage, cells showed trends of morphological changes (Fig 7. E& F)

### 3.2.4 Characterization of rat mesenchymal stem cells

#### 3.2.4.1 Flow Cytometry

To confirm whether the cells isolated represented mesenchymal stem cell population, characterization of the cells were done using CD markers. While there is no consensus regarding a single surface antigen that identifies an ASC, a panel of several positive and negative markers has been identified [Gimble *et al.*, 2008]. So to characterize the cell population in this study, CD profile was examined with CD105 (Endoglin) and CD44 (Hyaluronate) markers (Fig 8).

Cell populations at P-4 and P-6 were evaluated for FACS and both CD105 and CD44 were found positive for rat ASC cell population. The dot plot (Fig 8. A) shows the gating pattern. Unlabelled cell population was used as control and the histogram of the control is given (Fig 8. B). CD105 showed 86.5% positivity (Fig 8. C) and there was a strong expression of CD44 (Fig 8. D) observed during the FACS analysis and similar results have been reported by Minguell *et al.,* (2001). CD44 is a receptor for various ligands like hyaluronan and osteopontin, which plays a central role in the organization of the extracellular matrix in Mesenchymal stem cells. CD105 and CD 44 were reported to be one among the surface positive antigens for mesenchymal stem cells [Krampera *et al.,* 2006]. So for further confirmation of the cell population as adipose-derived mesenchymal stem cells (ASCs), their differential potential to multiple lineages were determined.

#### 3.2.4.2 Differentiation to Multiple lineages.

ASCs have been initially identified in bone marrow [Minguell *et al.,* 2001] and later in adipose tissue as non haematopoietic stem cells and are reported to differentiate into tissues of mesodermal origin, such as adipocytes, osteoblasts, chondrocytes and skeletal myocytes. ASC's can also differentiate into tissues of ectodermal orgin (neurons) and endodermal orgin (hepatocytes) [Krampera et al., 2006].

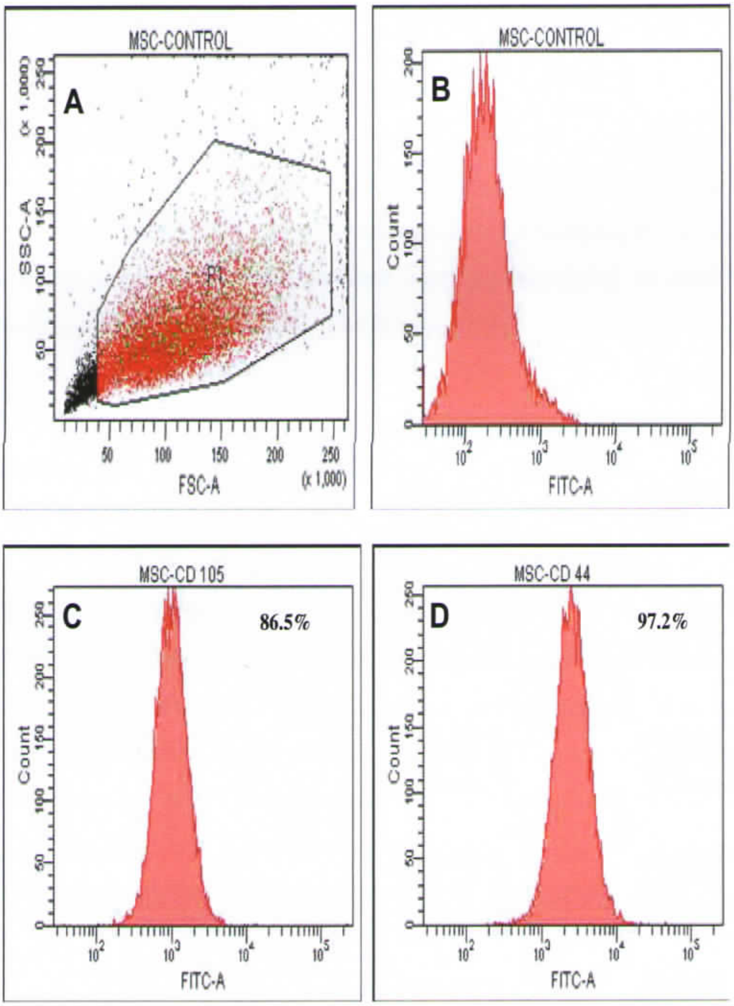


**Fig 8: Flow Cytometry analysis of Adipose-derived mesenchymal stem cells :-** (**A**) Dot plot showing gating pattern; (**B**) Histogram of Unlabelled cell population (control); (**C**) Histogram of CD105 surface positive marker; (**D**) Histogram of CD44 surface positive marker.

Apart from the plastic adherence property and presence of surface positive antigens for mesenchymal stem cells, the cell population isolated was proved to have the capacity to differentiate into multiple lineages like to adipocytes, osteocytes and chondrocytes.

#### 3.2.4.2.1 Differentiation of Rat ASCs to Adipocytes

For adipocyte induction the cells were treated with Adipocyte induction medium containing 0.5mM 3-iso-butyl methyl xanthine, 1µM Dexamethasone, 50 µM Indomethacin, 5 µg/ml Insulin for 2 days for the activation of adipocyte specific genes and transcription factors and in a maintenance medium containing 10µg/ml insulin. The cells were maintained for 21 days in maintenance medium for lipid accumulation. Presence of insulin acts as a substrate to activate the IRS (insulin receptor substrate) pathway which plays a major role in Adipocyte differentiation (Miki *et al., 2000)* The adipocyte induction was confirmed through triglyceride specific staining dyes like Nile Red and Oil Red O.

##### 3.2.4.2.1.1 Nile Red Staining

Nile Red is a lipophilic stain that stains intracellular lipid droplets red. Nile red is also intensely fluorescent, with a strong yellow-gold emission in a lipid-rich environment with its excitation at 485 nm and emission at 525 nm. The lipid globules in adipogenic induced rat ASCs will take up the dye, where they are visualized as intense red fluorescence under confocal microscopy (Fig 9. A). Evident lipid accumulation was observed in these cells (Fig 9. B) as most of the cells took up the dye.

##### 3.2.4.2.1.2 Oil Red O Staining

Oil red O is a commonly used dye for staining tissue cholestrol, esters and triacylglycerols. It is a fat soluble dye which provides a deeper red color for lipid globules. The lipid soluble dye is taken up by the adipogenic induced rat ASCs which will give red color in light microscope (Fig 9. C).

#### 3.2.4.2.2 Differentiation of Rat ASCs to Osteogenic Lineage

For osteogenic induction 10mM β-glycerophosphate, $10^{-8}$ M dexamethasone, 0.05 mg/ml L-ascorbic acid was used and the cells maintained in osteogenic induction medium for 28 days were stained with von kossa, Alizarin red and Masson's Trichrome. Formation of a collagen rich matrix and the deposition of abundant mineralized matrix composed of calcium and phosphorous was the indication towards osteogenesis.

##### 3.2.4.2.2.1 von Kossa staining for Phosphorous

Another relevant detection method for osteogenesis is the use of the von Kossa stain. Induction of osteogenesis with the osteogenic induction medium and staining with von Kossa after 28 days showed the presence of brownish black precipitates which indicated the presence of Calcium phosphate deposition. The staining principle is a precipitation reaction in which silver ions react with phosphate (not calcium) in the presence of an acidic material resulting in the photochemical degradation of silver phosphate to silver in presence of a strong light source (Fig 10. A). Csaki *et al.,* [2007] demonstrated osteogenesis by using von Kossa staining technique in- vitro in mesenchymal stem cells.

##### 3.2.4.2.2.2 Alizarin Red Staining for Calcium

Alizarin Red is used to determine the presence of calcium deposition by cells of an osteogenic lineage. As such it is an early stage marker (days 10–16 of in vitro culture) of matrix mineralization, a crucial step towards the formation of calcified extracellular matrix associated with true bone. The reaction is not strictly specific for calcium, since magnesium, manganese, barium, strontium, and iron may interfere since it will bind to any divalent cation, but these elements usually do not occur in sufficient concentration to interfere with the staining. Calcium forms an alizarin red – calcium complex in a chelation process where it appears dark red in color (Fig10. B). Aksu *et al.,* [2007] demonstrated osteogenesis in mesenchymal stem cells by alizarin red staining.

#### 3.2.4.2.2.3 Masson's Trichrome for Collagen staining

The rat ASCs cultured in osteogenic induction medium for 28 days showed collagen deposition which was stained with Masson's Trichome imparting a blue color in places of collagen deposition (Fig10. C). As the name implies, it makes use of three dyes for selectively staining muscle, collagen fibers, fibrin, and erythrocytes. The formation of collagen rich matrix is an indication towards osteogenesis. Aksu *et al.,* [2006] demonstrated osteogenesis in mesenchymal stem cells by masons trichrome.

#### 3.2.4.2.3 Differentiation of Rat ASCs to Chondrogenic lineage

For Chondrogenic induction, chondrogenic inducers like TGF-β1, ITS Premix and Dexamethasone were added to the growth media which will enhance chondrocytes induction of ASCs. Chondrogenesis was demonstrated on mesenchymal stem cells by alcian blue staining. Csaki *et al.,* [2007] used the same technique to demonstrate chondrogenesis on mesenchymal stem cells.

#### 3.2.4.2.3.1 Alcian blueStaining

Alcian blue is a water-based dye that stains acid mucosubstances and acetic mucins located on the cartilage, permitting the examination of cartilage formation. The stained parts are blue to bluish-green (Fig 11. A). Cells were observed to have secreted acid muco substances that indicate chondrogenesis.

#### 3.2.4.2.3.2 Safranin O Staining

Safranin O has been widely used to demonstrate the staining of proteoglycans in chondrogenesis studies. The staining process highlights the accumulation of proteoglycans (aggrecan, related proteoglycans) demonstrating a shift to the chondrogenic lineage and accumulation of the relevant extra cellular matrix components which is stained red (Fig 11. B).

The cell population isolated from Sprague Dawley rat subcutaneous adipose tissue were confirmed as ASCs based on the surface positive antigens (CD 105, CD 44) detected by FACS and their potential capacity to differentiate towards multiple lineages – Osteogenic, Adipogenic and Chondrogenic lineage.

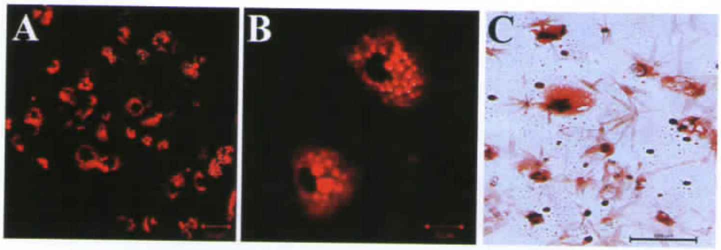

**Fig 9: Adipogenic Differentiation of Rat Adipose-derived mesenchymal stem cells– 21 Day in induction medium:-** **(A)** Confocal Micrograph of Nile Red stained cell population with red colored lipid globules; **(B)** Confocal Micrograph of Cells indicating discrete spherical morphology of accumulated lipids- Nile Red stain; **(C)** Light Micrograph of Oil Red O stain - depicting red oil droplets.

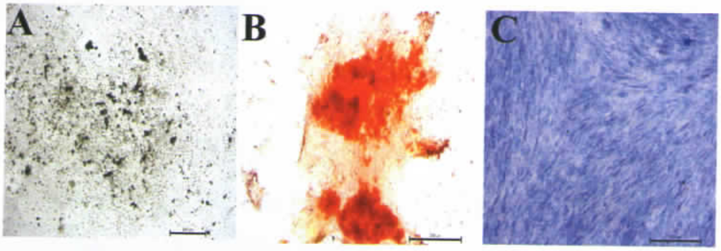

**Fig 10: Light micrographs of Osteogenic Differentiation of Rat Adipose-derived mesenchymal stem cells – 28 days in induction medium:-** **(A)** von Kossa Staining – Brownish black indicating phosphorus deposition; **(B)** Alizarin Red – Stains calcium ions red; **(C)** Masson's Trichrome – Stains collagen deposition blue.

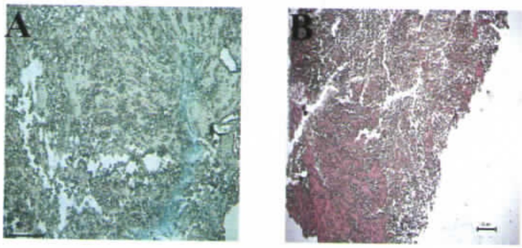

**Fig 11: Light Migrographs of Chondrogenic Differentiation of Rat Adipose-derived mesenchymal stem cells - 28 days in induction medium:-** **(A)** Alcian Blue – Stains acid mucosubstances bluish-green; **(B)** Safranin O – Stains proteoglycans red.

# Phase III

# *In-vitro* Studies – ASCs on collagen & ceramic scaffolds

## 3.3.1 Material-Cell Interaction: *in-vitro*

Cell material interaction is an important concept that has to be elucidated initially before fabricating a tissue-engineered construct. Four methods were adopted to determine the cell-material interaction to demonstrate whether the material has any inhibitory action on the cell population.

Tissue-engineered constructs were fabricated using BCP & Collagen in conjunction with rat ASCs. Prior to cell seeding the materials were conditioned, which was performed to improve the characteristics of the material surface.

### 3.3.1.1 Ceramic and collagen scaffolds in Direct Contact with ASCs

Simple microscopic evaluation of the cell seeded material showed excellent cell proliferation around the scaffold. The cells exhibited normal morphology even when in contact with the scaffolds (BCP and Collagen). The images of direct contact with cells in the immediate neighborhood of the scaffolds, BCP (Fig 12. A) and collagen (Fig 12. B) were taken 3 days after seeding. Both the collagen and ceramic scaffolds proved to be non-cytotoxic and cytocompatible supporting cell growth.

### 3.3.1.2 Adhesion of rat ASCs on bioactive Ceramic-BCP

The initial adhesion of rat ASC on the BCP was evaluated by SEM which showed fibroblastic morphology for most of the cells and the cells were found well spread within 48 hours after seeding. Adhesion of cells on materials is very important which influences their ability to proliferate and differentiate.

The adhesion of cells on material depends on the initial adsorption of serum proteins like vitronectin and fibronectin (Anselme *et al.,* 2000) whose strong affinity with ceramic substrates has been reported (Zreiqat H. 1996). From literature it is understood that from the third day of cell culture, the cells completely spread along the available surface of the ceramic scaffold. On day 1, the cells are seen to spread along the margin of the pores and from day 3 they initiate to form cluster colonies. On BCP also we had similar observations of cell cluster formation (Fig 12. C & D).

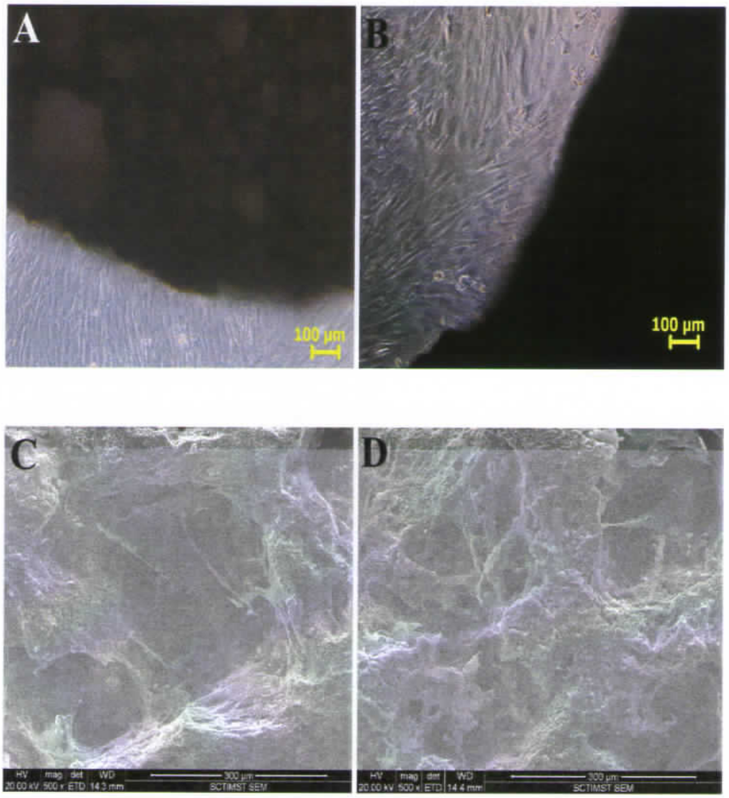


**Fig 12: Material - Cell Interaction:- Phase Contrast Micrographs of (A) Adipose-derived mesenchymal stem cells in direct contact with Ceramic disc (BCP) showing normal morphology; (B) ASCs in direct contact with Collagen showing normal morphology; (C&D) Scanning electron micrographs of cell adhesion on BCP**

Adhesion of cells to the surface is a very important factor in evaluating the cytocompatibility of materials (Anselme *et al.*, 2000) which is observed in various natural phenomenon such as embryogenesis, maintenance of tissue structure, wound healing, immune response and metastasis as well as tissue integration of biomaterial. Thus attachment, adhesion and spreading of cells belong to the first phase of cell/material interaction and the quality of this first phase will influence the cells capacity to proliferate and to differentiate on the surface of the implant. The behaviour of different cell types on materials has indicated that they react differently according to surface roughness (Chesmel *et al.,* 1995), which appears to be a relevant criterion for cell adhesion and proliferation.

**3.3.1.3 Biochemical evaluation for Proliferation - Pico green**

Cell lysate was evaluated at 3 days and 6 days for proliferation by quantifying DNA content and comparing to a known number of cells. On both ceramic and collagen there is an increase in number of cells seen over time from 3 to 6 days indicating a proliferating cell population. The numbers of cells were higher on the ceramic scaffold when compared to the collagen at 3 and 6 days indicative of the larger surface area (porous nature) of the ceramic scaffold which favors cell adhesion (fig 13. A).

**3.3.1.4 Biochemical evaluation for Viability - LDH**

Viability of the cells on the BCP and collagen scaffolds was assessed by total LDH estimation from the cell lysate. The cell populations were assessed at two time intervals of 3 and 6 days. Increase in cell viability over time was observed on both scaffolds. On comparison (Fig 13. B) the ceramic scaffold supported higher cell viability over a period of 6 days. Enhanced cell viability is indicated by the increased assessment of LDH in ceramic samples at the $6^{th}$ day when compared to the collagen (Fig 13. B).

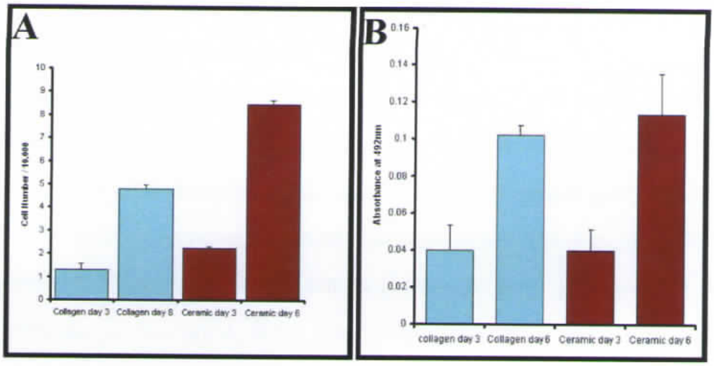


**Fig 13: Biochemical Estimation (A)** Pico green Assay for Cell Proliferation on Collagen and ceramic scaffolds; **(B)** LDH Estimation for Cell Viability on Collagen and ceramic scaffolds.

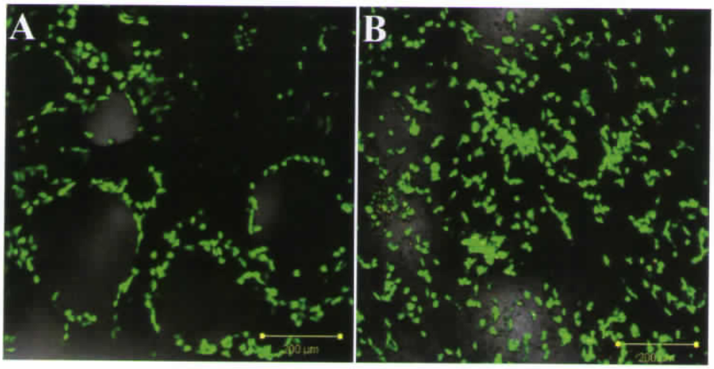


**Fig 14: Confocal micrographs of Live – Dead Staining (Acridine Orange and Ethidium Bromide) of Adipose-derived mesenchymal stem cells on (A)** Ceramic Disc (BCP) and **(B)** Collagen. Green color indicates the viable cells.

#### 3.3.1.5 Viability of rat ASCs on BCP and Collagen Scaffolds – Confocal Microscopy

Rat ASCs seeded on BCP and collagen was stained and observed for viability after 48 hours in culture. Acridine orange and Ethidum bromide are the stains used and observed under confocal microscope.

Acridine orange is a nucleic acid selective fluorescent cationic dye. It is cell-permeable, and interacts with DNA and RNA by intercalation or electrostatic attractions respectively. DNA intercalated Acridine orange fluoresces green (525nm); RNA electrostatically bound Acridine orange fluoresces red (>630nm). Ethidium bromide is a nucleic acid intercalating agent which fluoresces with a red-orange color. Since ethidium bromide is excluded from intact cell, live cells will not take up ethidium bromide and so the dead cells fluoresce red.

Appreciable number of cells was found viable on BCP (Fig 14. A) and Collagen (Fig 14. B) after 48 hours in culture. Confocal micrograph revealed that rat ASCs were homogenously adhered, distributed on Collagen. On BCP rat ASCs were homogenously distributed and rimmed around the macro pores. This indicated that the scaffolds used provide a cell friendly environment encouraging cells to proliferate on BCP and collagen scaffolds.

### 3.3.2 *In vitro* evaluation of Adipogenesis

The rat ASCs were seeded on cover slips and maintained in culture until confluency. Later at confluency these cells were induced to the adipogenic lineage using adipogenic inducers like dexamethasone, 3-isobutyl-1-methyl-xanthine (IBMX), Indomethacin and Insulin. The cells at confluency were maintained for 2 days in adipocyte induction medium and later transferred to maintenance medium which contains only insulin to enhance triglyceride accumulation. Dexamethasone is a synthetic glucocorticoid which can increase the intra cellular C-AMP level and thereby favor adipocyte formation. Dexamethasone also reduces the expression of pref-1, a negative regulator of adipogenesis and induces C/EBP-α, which may account for some of its adipogenic activity [Nilema *et al.*, 2008]. IBMX, Indomethacin can inhibit the activity of phosphodiesterase inhibiting C-AMP degradation. IBMX has been shown to increase expression of C/EBP-β, and this increase is required for subsequent Pparg expression and adipocyte differentiation [Gregoire *et al.,* 1998]. High concentrations of insulin can also be used in combination with these inducing agents [Reusch *et al.,* 2000]

### 3.3.2.1 Lipid soluble dyes can be used as markers for adipocyte differentiation

Lipid soluble dyes are able to satin lipid vacuoles in adipocytes. These include Nile red, Oil red O, Sudan Black and Nile blue [Pittenger 1998]. Each of these hydrophobic dyes has the propensity to accumulate in lipid containing vacuoles in the developing adipocytes and can readily identify the adipogenic cell population of differentiating adipocytes. Nile red and oil red O was used in this experiment for evaluating adipocytes.

#### 3.3.2.1.1 Nile Red and Oil Red O Staining

The rat ASCs were induced for 2 days and maintained in maintenance medium for a period of 7, 14 and 21 days and was stained with Nile Red and Oil Red O. Evident lipid accumulation was observed from day 7 itself. Both are lipophilic dyes which stain the triglyceride in the cell cytoplasm. Nile red stained cell population is demonstrated in Fig (15. A) and the Oil Red O staining is demonstrated in Fig (15. C & D) Nile red staining was further modified to determine the location of the nucleus in the cell. In normal adipocytes the lipid accumulation will push the nucleus aside to a corner with the whole cytoplasm containing the lipid globules. Similar observation was observed when double stained with DAPI and Nile red. Nucleus took up a blue color and the cytoplasm fluoresce red (Fig 15. E). Nile red stained ASC showing evident lipid globules is shown in Fig (15. F)

Theoretically from day four itself cells start accumulating triglyceride [Nielema *et al.,* 2008] if there is enough substrate available in the cellular environment. The cells when maintained for a longer duration in maintenance medium, are said to accumulate more and more lipid molecules in their cytoplasm [Nielema *et al.,* 2008]. Oil red O is the most common lipophilic dye used as a marker for adipocyte differentiation [Pittenger 1998].

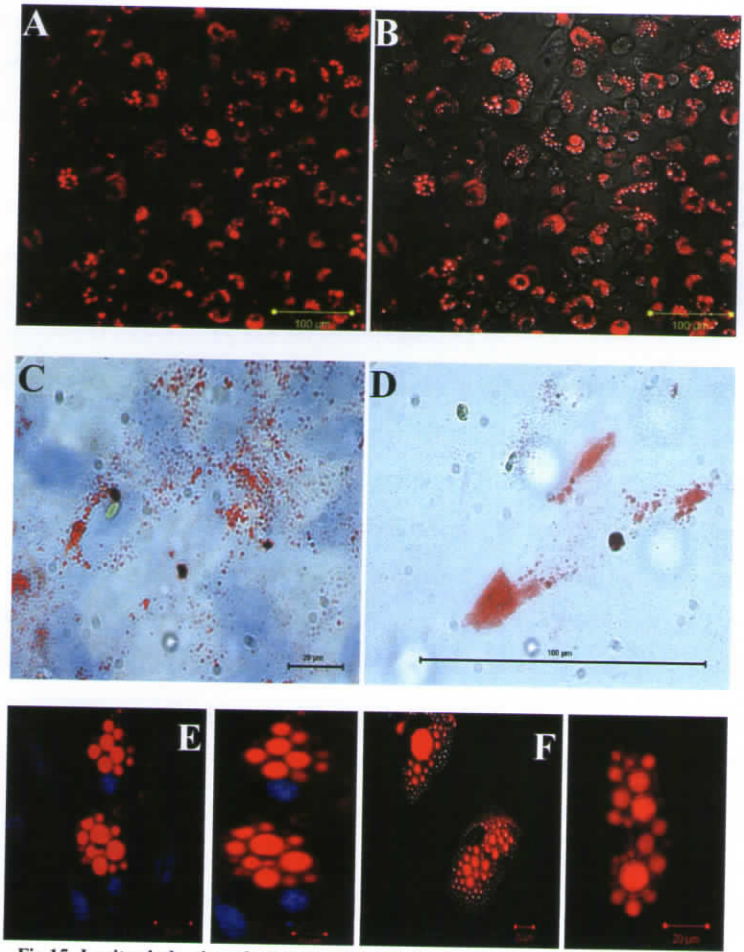

**Fig 15: In vitro induction of adipogenesis:– (A)** Confocal Micrograph of adipocytes stained with Nile red showing red colored oil droplets **(B)** Confocal Micrograph (DIC) of Nile red stained adipocytes; **(C&D)** Light micrographs of Oil red O Stained Adipocyte-like cells **(E)** Confocal micrograph of adipocytes double stained with DAPI & Nile red, DAPI stains the nucleus blue and the lipid accumulation in cytoplasm is stained with Nile red; **(F)** Confocal Micrograph of a single adipocyte stained with Nile red depicting red colored oil droplets.

#### 3.3.2.1.2 Demonstration of Adipogenesis with Flow cytometry using Nile Red.

To analyse adipogenesis using Flow cytometry or to analyse the number of cells induced to adipogenic lineage from the total cell population, a lipid specific dye called Nile red was used. Nile red will be taken up by the triglyceride storage within the cells, if the cells are induced to the adipocyte lineage. Nile red stained adipocytes has an exitation at 485nm and an emission at 525nm which was read by FACS (BD FACS Aria). Above 90% of the cells were recorded positive for Nile red staining which indicated that out of every 100 cells, above 90 cells are induced towards the adipogenic lineage (Fig 16).

### 3.3.2.2 Biochemical estimation for triglycerides (TG)

The triglyceride accumulation in a given cell population was estimated by a triglyceride detection kit (Agappae Diagnostics) which quantitatively estimates the amount to triglyceride present in a given population of induced rat ASCs. A total of four flasks were maintained in $25cm^2$ flasks, three flasks were induced to adipogenic lineage and the other one was maintained as a control which was not induced. The induced culture flasks were estimated at different intervals of 7, 14 and 21 for triglyceride accumulation. There was a notable increase in the triglyceride content in the rat ASC population at 7, 14 and 21 days (Fig 17). This states that the cell population shows an increase in their TG content with time. Initially triglycerides are formed as small globules at the initial stage of adipogenesis and later these globules accumulate to form big lipid vacuoles [Nielema *et al.,* 2008] which are denoted by the increase in triglyceride accumulation on day 21.

### 3.3.2.3 Induction of adipogenesis on BCP and Collagen Scaffolds.

The materials – collagen and BCP were conditioned initially by placing them in DMEM with 10% FBS for 24 h prior to cell seeding. The conditioning was done to improve the characteristics of the material surface. $4 \times 10^4$ rat ASCs were seeded on to BCP and collagen scaffolds and were induced towards the adipocyte lineage by maintaining them for 2 days in induction medium and for 21 days in the maintenance medium. The tissue-engineered constructs was thus fabricated using BCP and Collagen in conjunction with rat ASCs.

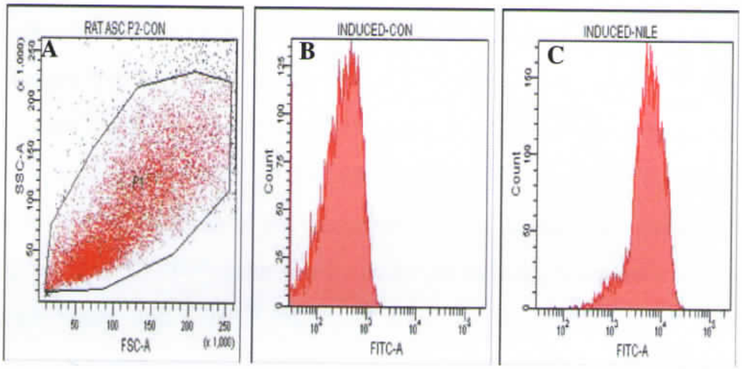


**Fig 16: Flow Cytometry analysis for adipocytes:– (A)** Dot plot showing gating pattern of induced Adipose-derived mesenchymal stem cells; **(B)** Histogram of unstained induced cell population – Control; **(C)** Histogram of Nile red stained Adipose-derived mesenchymal stem cells induced to the adipocyte lineage.

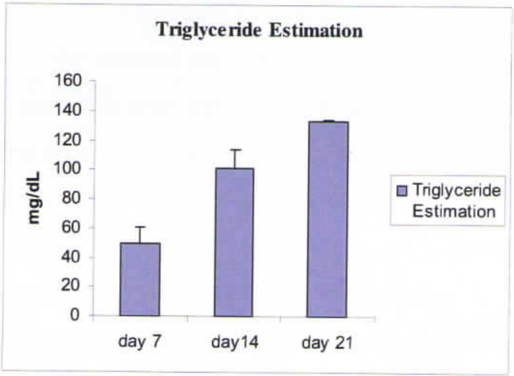


**Fig 17: Biochemical estimation of Triglycerides of adipogenic cells on 7, 14 and 21 Days in culture**

### 3.3.2.4 Demonstration of Adipogenesis on Ceramic and collagen scaffolds

#### 3.3.2.4.1 Real time PCR

Real-time PCR analysis of adipocyte specific genes was done to confirm the differentiation of rat ASC's into adipogenic lineage. Adipogenic specific genes were expressed on both ceramic and collagen scaffolds. C/EPBα and Pparg are the two transcriptional factors that are very important for adipocyte differentiation and was found to be expressed on the induced rat ASC population eluted from the scaffolds. The expression of these genes indicates the diffrentiation process. The regulation of the adipocyte gene occurs primarily at the transcription level which includes CCAAT/ enhancer binding protein (C/EBP) gene family and Peroxisome proliferator activated receptor γ (Pparg). Pparg and C/EBP family of transcription factors must function cooperatively to activate adipogenic specific genes and thereby bring about adipocyte differentiation and maintain the adipocyte phenotype [Nilema *et al.,* 2008].

#### 3.3.2.4.2 Analysis of Real-Time PCR

Molecular phenotype determination and the effect of scaffolds on the differentiation process of the induced rat ASCs were analyzed using real-time PCR. Collagen and ceramic scaffolds were analyzed for gene expression on $10^{th}$ and $20^{th}$ day. Pparg and C/EPBα were the genes used to evaluate the molecular phenotype. Cells eluted from both Ceramic (BCP) and Collagen on day 10 and 20 was analyzed and the gene expression was found to have increased more than 2 fold. This demonstrates the effect of the scaffolds in supporting and enhancing adipogenesis. Fig (18. A) Shows the fold expression of adipogenic specific genes cultured on ceramic scaffold and Fig (18. B) shows the fold expression of adipogenic specific genes cultured on Collagen scaffold. 28s rna was the house keeping gene used.

Comparing Ceramic and Collagen scaffolds for gene expression, Collagen was found to support the gene expression more than the ceramic scaffolds. So from the relative gene expression data (Fig 18. C) of adipogenic specific genes obtained from both the scaffolds Collagen seem to favor the differentiation process than that of Ceramic. This may be due to the fact that Collagen is a component of the natural ECM which may provide the cells a more appropriate environment and enhance the differentiation process towards the adipogenic lineage.

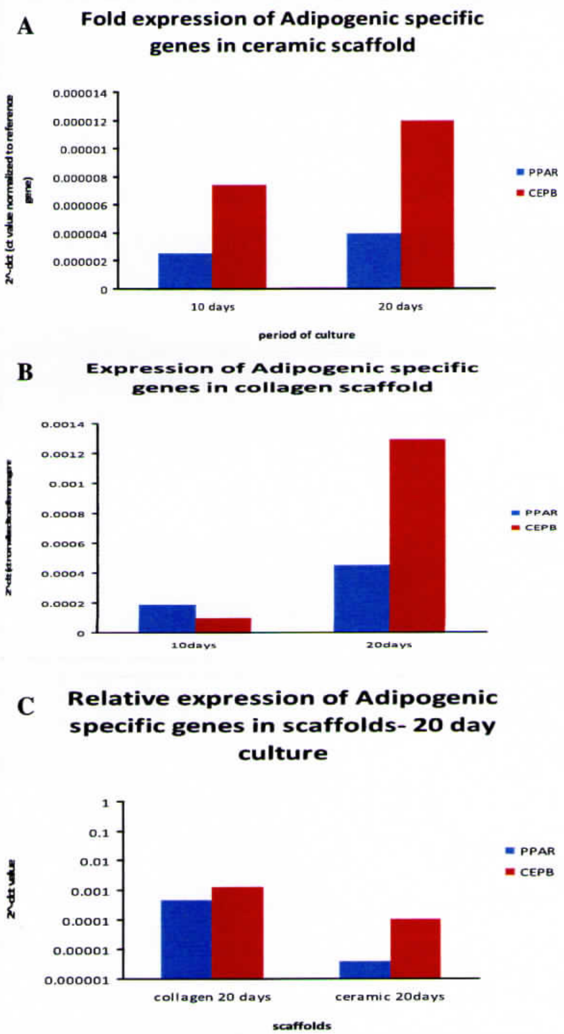


**Fig 19: Real time PCR analysis of Pparg & C/EPBα:- (A)** Molecular phenotype expression on ceramic (BCP); **(B)** Molecular phenotype expression on collagen; **(C)** Relative expression comparing Collagen and Ceramic.

#### 3.3.2.5 Demonstration of Adipogenesis on BCP by Confocal Microscopy

The cell seeded BCP scaffold was stained with Nile red and was observed using confocal microscope which showed evident lipid accumulated cells on the ceramic scaffold and was uniformly distributed throughout. The differentiated cells took up the Nile red stain which is a lipophilic fluorescent dye and fluoresces red. Differentiated cells were found both on the surface (Fig 19. A & B) and on the rim of the pores (Fig 19. C). The depth code in Fig (19. D) shows the differentiated cells within the pores upto a depth of 60μm. From the microscopic evaluation it is evident that BCP scaffold can also support adipogenesis even though it is meant generally as a bone substitute for osteogenesis.

It was not possible to carry out this evaluation on collagen scaffolds as they auto fluoresce.

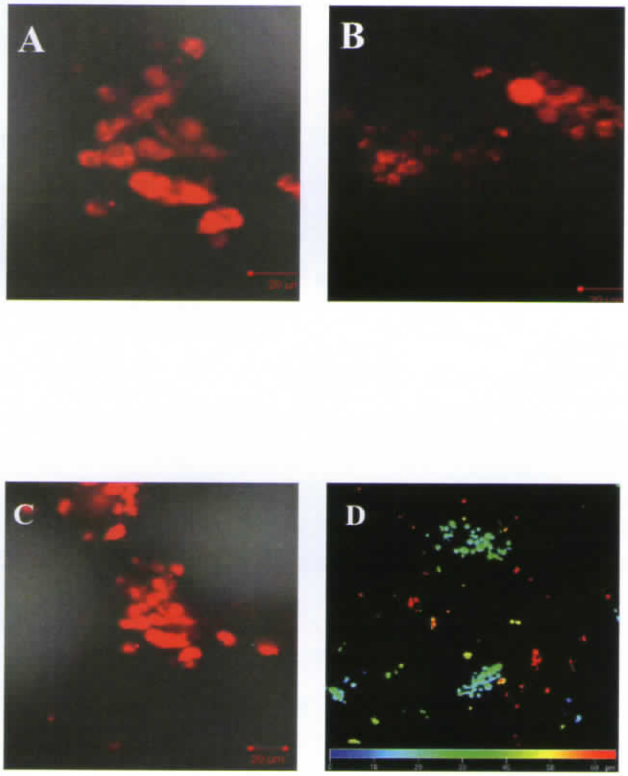


**Fig 18: Confocal micrographs of Adipogenesis induced Rat Adipose-derived mesenchymal stem cells on Ceramic Scaffolds – 21 days in culture:-**
**(A, B&C)** red color indicates Nile Red stained adipocyte-like cells; **(D)** Depth coded micrograph shows distribution of cells up to a depth of 60µm within the ceramic.

# Phase IV

## *In vivo* Studies on adipogenesis in rat muscle model

### 3.4 *In vivo* demonstration of Adipogenesis

The fabricated tissue engineered construct with Rat ASCs seeded on the BCP and collagen scaffolds were implanted in the rat dorsal muscle on either side of the vertebral column.

The rats from which the cells were isolated for implantation and the experimental group of rats belong to a syngenic population. The word "syngenic means genetically identical and immunologically compatible as to allow for transplantation. Even though the cells from an allogenic source are used, all rats belong to a syngenic population of closely in-bred strains with minimum genetic variations between them. Since ASC's are immunologically privileged cells, syngenic population can be successfully used for transplantation therapies.

#### 3.4.1 Histology

The retrieved tissue sections with the scaffolds were demonstrated using three different stains

a) **Hematoxylin and Eosin** to demonstrate the general cell morphology. Hematoxylin stains collagen in pale pink, muscle in deep pink, nuclei blue and erythrocytes cherry red.

b) **Picrosirius** stains the collagen scaffold, where collagen takes up a deep red color and

c) **Masson's Trichrome** stains the muscle tissue red, the collagen scaffolds blue.

Four groups of sections were evaluated using histological staining methods to demonstrate adipogenesis on Ceramic and Collagen scaffolds. It was difficult to paraffin process the Ceramic construct as it is hard when comparing to the collagen scaffolds. The ceramic pullout method was adopted. The BCP disc was carefully removed from the post-implanted retrieved tissue section. The rest of the tissue was proceeded for paraffin embedding.

#### 3.4.1.1 Bare Collagen Scaffold

The bare scaffold was just the collagen alone implanted and stained with H & E (Fig 20. A) depicted the implanted collagen amidst the muscle tissue. Picro sirius stained the bare collagen red (Fig 20. B). Masson's Trichrome stained the muscle tissue dark pink color and the collagen fibers were stained blue (Fig 20. C). Bare scaffold shows just the muscle architecture and the collagen scaffold. No fat cell regeneration was observed in the bare material.

#### 3.4.1.2 Induced cell-seeded-collagen construct

The Induced cell-seeded sections were stained with Hematoxylin and Eosin (H&E), which demonstrated morphology of fat cells along with the muscular architecture and the collagen fibers of the scaffold. The nucleus was stained blue, the muscles were stained deep pink and the collagen was stained pale pink (Fig 21. A).

The adjacent sections were also stained with Picrosirius to demonstrate the morphology of the collagen fibers. The collagen scaffolds in the retrieved implanted tissues stained red (Fig 21. B). The differential stain with Masson's Trichrome stained the muscles as dark pink, collagen as blue and erythrocytes as red (Fig 21. C).

The cell-seeded constructs showed few adipocyte-like cells within the collagen scaffolds. There was a network of elliptical cells with a prominent cytoplasm and the nucleus pushed aside to a corner. Networks of such cells were found in all the three staining techniques. Adipose tissue is said to be highly vascular and substantiating this fact, neo angiogenesis was found within the implant area which shows the role of the collagen scaffold in promoting neo-angiogenesis (Fig 21). No inflammatory reactions were observed in the implant region

#### 3.4.1.3 Histology of Ceramic Scaffolds.

To facilitate the processing of tissues via paraffin method for histological assessment the scaffold was removed from the soft tissue. The surrounding tissue was paraffin processed and stained with H&E (Fig 22. A) demonstrating a faint network of

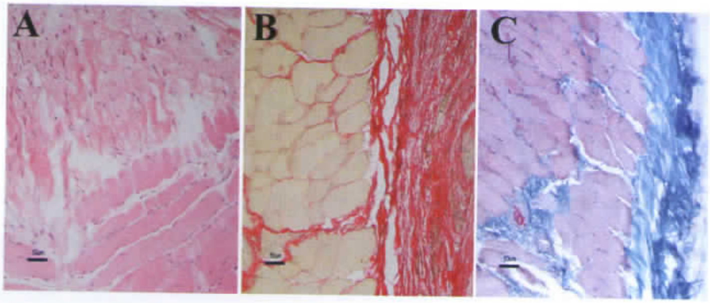

**Fig 20: Light Micrographs of bare collagen scaffold, 21 days post-implanted stained with- (A)** H&E; **(B)** Picrosirius; **(C)** Masson's Trichrome.

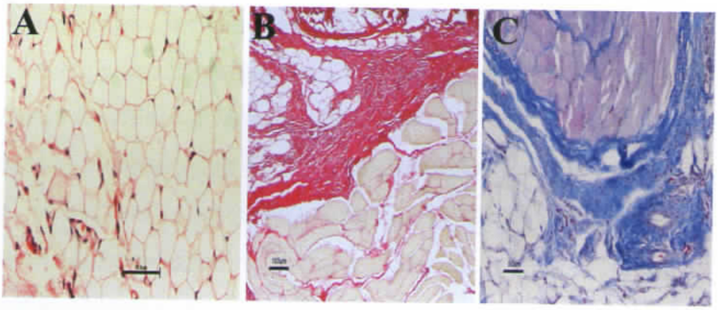

**Fig 21: Light Micrographs of post-implanted tissue-engineered construct (21 days) depicting adipocyte-like cells (asterisks) stained with (A)** H&E; **(B)** Picrosirius ; **(C)** Masson's Trichrome.

"empty" spaces indicating lipid removal during paraffin processing. Masson's Trichrome staining in consecutive sections identify the network of extra cellular matrix network containing the adipocyte – like cells (Fig 22. B&C).

#### 3.4.1.4 Neo-vascularisation

Neo-vascularisation is indicated by incipient capillary formation and is evident from the scaffold histology (Fig 23. A&B). This results in the formation of functional microvascular networks with red blood cell perfusion. It has been reported that adipose tissue contain progenitor cells with angiogenic potential and that therapy based on adipose tissue derived progenitor cells may constitute a promising cell therapy in patients with ischemic disease. Development of the capillary network is required to ensure adipose tissue remodeling and lack of vascularization may promote adipose tissue loss underlining a crucial link between adipose cells and the capillary network. Adipose lineage cells have been shown to release potent angiogenic factors such as monobutyril, vascular endothelial growth factor (VEGF), and leptin and to differentiate into endothelial cells in vitro [Moon *et al.,* 2006]

Compared to the bare scaffold, cell-seeded constructs depicted an interesting observation of adipocyte-like phenotype cells within the vicinity of scaffolds. To further confirm the cellular morphology and fat architecture, the histology of normal muscle and rat skin subcutaneous fat pad was performed.

#### 3.4.1.5 Normal Muscle

Paraffin sections of normal muscles were obtained from cadaver rats and were stained with H&E (Fig 24. A), Picrosirius (Fig 24. B) and Masson's Trichrome (Fig 24. C). The normal muscle morphology was evident from the staining techniques employed. Normal muscle was taken as a second control. It just showed that the muscle architecture had very limited amount of collagen within the muscle tissue. Comparing this normal muscle to the bare and the cell-seeded collagen scaffold, it was easy to distinguish the synthetic collagen scaffold and the normal collagen in the extra cellular matrix of the muscle tissue.

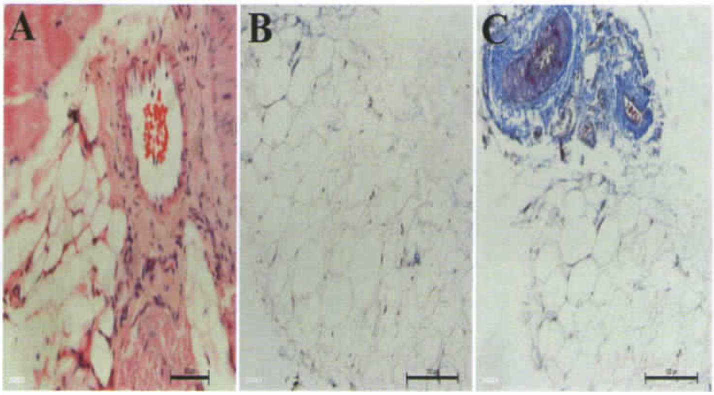


**Fig 22: Light Micrographs of muscle sections after ceramic pullout showing adipocyte-like morphology- (A)** H&E stain; **(B&C)** Masson's Trichrome.

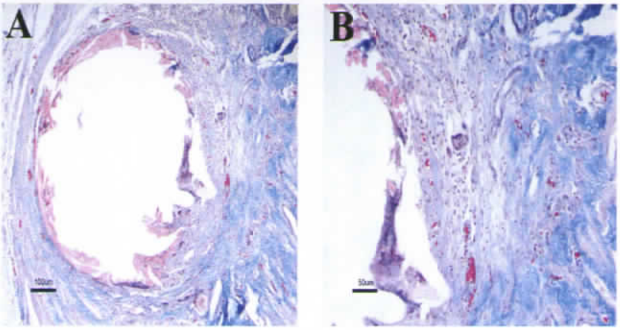


**Fig 23: Light Micrographs of 21 days post-implanted tissue-engineered construct stained with Masson's Trichrome showing sprouting blood capillaries (asterisks) - Neo-angiogenesis (A&B).**

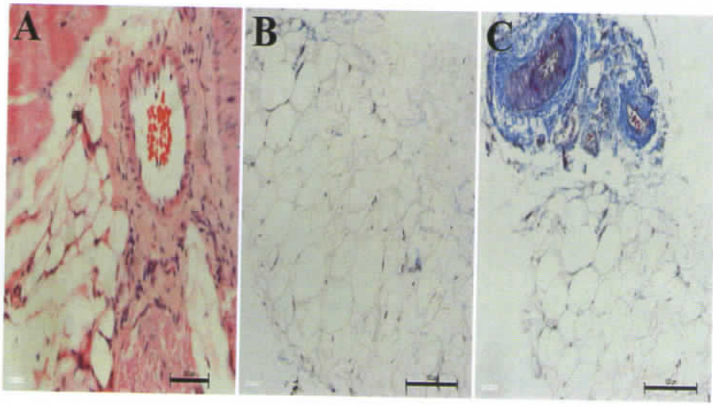


**Fig 22: Light Micrographs of muscle sections after ceramic pullout showing adipocyte-like morphology- (A)** H&E stain; **(B&C)** Masson's Trichrome.

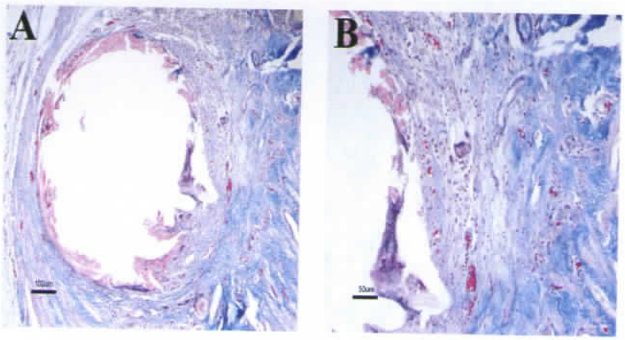


**Fig 23: Light Micrographs of 21 days post-implanted tissue-engineered construct stained with Masson's Trichrome showing sprouting blood capillaries (asterisks) - Neo-angiogenesis (A&B).**

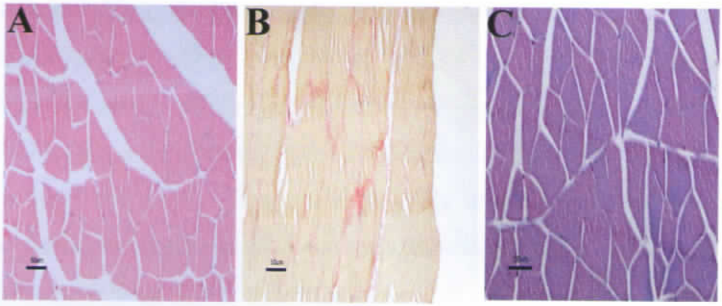


**Fig 24: Light Micrographs of stained normal rat muscle - (A)** H&E; **(B)** Picrosirius; **(C)** Massons Trichrome

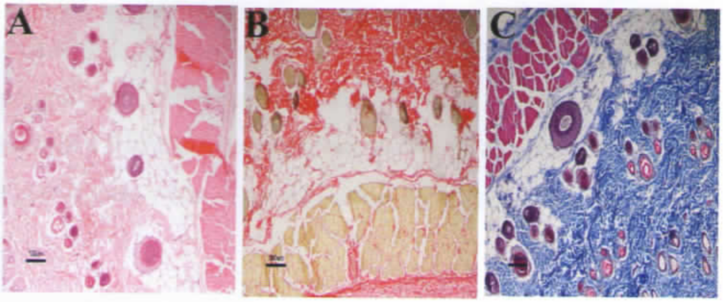


**Fig 25: Light Micrographs of stained normal rat skin- (A)** H&E; **(B)** Picrosirius; **(C)** Masson's Trichrome

### 3.4.1.6 Rat Skin

Rat skin was taken as a positive control and was stained with H&E (Fig 25. A), Picrosirius (Fig 25. B) and Masson's Trichrome (Fig 25. C). Rat skin was taken as a positive control to look up on the fat cell morphology and architecture. Normal vasculature around the fat cells is also seen in the sections of rat skin.

Comparing the data collected from all the sets of tissues and the staining methods employed, the elliptical cells observed with a prominent cytoplasam and an adipocyte-like morphology can be identified as a fat cell mass. Picrosirius stained the collagen scaffold around the muscle tissue and also the fat-like cells within the scaffold. Trichrome also showed similar results. Since the muscle tissue is an ectopic region of normal fat regeneration and when comparing with the bare scaffold, where such fatty-like cells were absent, it could be concluded that, there is no other factor that played a role other than the rat ASCs that was seeded on the scaffold in the formation of adipocyte-like cells. The normal adipocyte morphology of the rats was obtained from the sections of the rat and was compared to the *in vivo* differentiated adipocyte-like cells. Similar morphology as in the rat skin was observed in the muscle tissue which is an ectopic area for fat regeneration. From all the above experimental evidences it was concluded that the cells observed were regenerated fat cells formed in the muscle tissue post-implantation of rat ASC-seeded collagen tissue construct.

## 3.4.2 Conclusion

*De novo* formation of adipocytes at sites of implantation was assessed in terms of histological examination. Fat cells or adipocytes are very large (up to 200 μm in size) and contain major lipid inclusions. In mature adipocytes of white or unilocular fat, the nucleus and cytoplasm are pushed to the periphery and appear as a thin rim surrounding the lipid droplet(s), resulting in a "signet ring" appearance. The fat is usually dissolved in routine sections (processing removes fat), leaving a large, empty vacuole. The loss of lipid leads to a "chicken wire" appearance. However in larger masses or pre-formative stages they can be distorted by cell to cell contact. Close correlation between capillaries and adipose tissue was also observed in control sections of rat skin that supported the constant exchange between blood and adipose tissue, which correlated to the existence of fat-like cells in this study.

# Summary and Conclusion

Tissue engineering (TE) represents an innovative approach in Regenerative Medicine for the development of novel clinical modalities for the repair and reconstruction of human tissue defects. A large proportion of plastic and reconstructive surgical procedures performed each year are to repair soft tissue defects that result from traumic injury, tumor resection and congenital defects. These defects are typically due to the loss of large volumes of adipose tissues.

Adipose tissue is a good source for harvesting adipose-derived mesenchymal stem cells (ASCs), and procedures for isolation of these cells from liposuction or excised fat are not difficult.

In this study, an attempt has been made to use adipose-derived stem cells for the application of soft tissue augmentation. Rat ASCs were isolated and characterized for their stemness (CD 105, 44) and differentiation potential towards the osteogenic, adipogenic and chondrogenic lineages respectively. For adipogenesis, specific inducing agents were used to activate the genes that help in lipid accumulation by converting the stem cells to adipocytes. This was evaluated *in-vitro* by Oil Red O staining which is specific for lipids and also by biochemical estimation of triglycerides. The genes which play a role in adipocyte induction were analyzed by Real time-PCR.

Scaffolds selected for defect repair are expected to be biocompatible as they are replaced by healthy host tissues. As yet there are no reports of bioactive ceramics as scaffolds for Adipose Tissue engineering. Though it is an accepted fact that ceramics are popular as bone substitutes, the objective of the study was to demonstrate and evaluate an in-house developed biphasic calcium phosphate (BCP), an amorphous bioactive ceramic scaffold aiming at adipose tissue regeneration. Simultaneously, a comparative study was performed with collagen sponge which is a known scaffold in adipose tissue regeneration.

Cytocompatibility of these scaffolds were tested using rat rASCs which supported cell viability (LDH, Acridine orange /Ethylene Bromide) and cell proliferation (Pico green). *In vivo* experiments were done in dorsal muscle sites of Sprague Dawley rats to demonstrate the efficacy of these scaffolds in adipose tissue regeneration. Histology using different staining techniques (H & E, Picro Sirius & Masson's Trichrome) was employed to demonstrate adipogenesis with the collagen and BCP implants - 21 days post implantation. The elliptical adipocyte-like cells within and near the collagen and ceramic scaffolds were confirmed as fat cells when compared to the fat cells in the normal skin fat pad.

To conclude, adipocyte-like cells were observed with both the collagen and ceramic scaffolds *in vivo.* Real-time PCR data and *in vitro* observations supported the molecular phenotype expression. However, results have shown that the differentiated cells favored the collagen scaffolds to the amorphous ceramic scaffolds. Neo-angiogenesis in the vicinity of the scaffolds supported fat cells which in turn may help to prevent tissue resorption and implant failure.

So in perspective of future applications, a combination of the two scaffold types – collagen in a 3D ceramic as a single unit may be proposed to be an appropriate scaffold of choice for adipose tissue augmentation. The ceramic would be retained to maintain the aesthetics and contour of the reconstructed tissue while the collagen degrades with time. So here is another option towards the strategies adopted in Adipose tissue engineering

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# Annexure

# ANNEXURE

**Dulbecco's Modified Essential Medium-High Glucose (DMEM-HG)**

| | |
|---|---|
| DMEM- HG | 1litre |
| Antibiotics | 1ml |
| NaHCO3 | 3.7g |
| FBS | 100ml |

Dissolved in 1000ml deionised water and then filter sterilized.

**Phosphate Buffer saline (PBS) - 1000 ml**

| | |
|---|---|
| NaCl | 8.0 g |
| KCl | 2.0 g |
| $Na_2HPO_4$ | 1.15g |
| $KH_2PO_4$ | 2.0 g |

Dissolved in 1000ml deionised water and then autoclaved at $121^0$C for 20 minutes.

**Osteogenic Medium (100ml)**

| | |
|---|---|
| B Glycerophosphate | 100µl |
| Dexamethasone | 0.2µl |
| Ascorbic acid | 0.05 mg/ml |

DMEM-HG containing10% FBS

**Sorensen's Buffer (100ml):-**

| | |
|---|---|
| NaH2PO4 | 19ml |
| Na2HPO4 | 81ml |

**Paraformaldehyde (100ml):-**

| | |
|---|---|
| Paraformaldehyde | 3.7g |
| Sorensen's buffer | 50ml |
| Distilled water | 50ml |

**3% Gluteraldehyde (50ml):-**

| | |
|---|---|
| Gluteraldehyde (25%) | 6ml |
| Sorensen's buffer | 44ml |