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Cell-Sheet Technology: A Novel Method To Enhance Bone-Implant Integration

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Cell-Sheet Technology:

A Novel Method to Enhance Bone-Implant Integration

A thesis submitted in partial satisfaction

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in Oral Biology

by

Rajita Kodali Kanuru

2012

ABSTRACT OF THE THESIS

Cell-Sheet Technology:

A Novel Method to Enhance Bone-Implant Integration

By

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Master of Science in Oral Biology

University of California, Los Angeles, 2012

Professor Takahiro Ogawa, Chair

Abstract

Although dental implants have become a standard treatment procedure, there is still a growing demand to enhance their osseointegration capability to address complex cases, such as host sites with bone grafts and type IV bone. This study explored the viability and effectiveness of pre-osseointegrated implants to achieve faster establishment of bone-implant integration using cell sheet technology. Cells harvested from the rat bone marrow were cultured on a poly (N-isopropylacrylamide) culture dish. Taking advantage of the ability of temperature responsive conversion of the dish from hydrophobic to hydrophilic, the layer of the grown cells was detached as a sheet. For in-vitro experiments, the cell sheet (single or double layered) was transferred to acid-etched titanium disks and examined for its capability to show cell metabolic

activity, osteoblastic phenotypes and responsiveness to an osteogenic enhancer molecule, N-acetyl cysteine (NAC). For in vivo study, the experimental titanium implants (1 mm in diameter and 2 mm in length) were coated with the cell sheet and placed into rat femurs. After 2 weeks of healing, the implants were subjected to the biomechanical push-in test to evaluate the strength of bone-implant integration. Cell sheet grown on temperature sensitive dishes showed the expected cell metabolic activity levels and osteoblastic phenotypes of cells grown on regular culture dishes. Cell sheets, created from bone marrow cells, were successfully transferred as an intact sheet, to titanium disks and showed the alkaline phosphatase (ALP) activity. Double layers of the sheets showed 2-fold greater cell metabolic activity than single layered ones. The cell sheets treated with NAC showed significantly greater ALP activity in a NAC dose-dependent manner. The cell sheet-coated implants showed a 2.2-fold greater push-in value than uncoated implants. This is the first study that has established an osteogenic cell sheet applicable to titanium implants. Transferred cell sheets to titanium surfaces are capable to show reliable osteogenic phenotypes and to accelerate the bone-titanium integration, can be enhanced by foreign molecules and show increased cell metabolic activity levels depending on the number of layers of cell sheets transferred.

The thesis of Rajita Kodali Kanuru is approved.

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2012

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CHAPTER 1:

Background and Significance

1.1 Background and Significance:

In 1952 the Swedish orthopedic surgeon, P I Branemark, was interested in studying bone healing and regeneration, and adopted a specially designed rat chamber for studying the blood flow in rabbit femurs. After several months of study he tried to retrieve the chambers from the femurs and found out he was unable to remove them. He conducted further research and carried out several studies into this phenomenon where bone had grown into such close proximity with the titanium that it effectively adhered to the metal. He labeled the clinically observed adherence of bone to titanium as 'osseointegration'. He placed his first titanium implant into a human volunteer in 1965. Ever since then we have been trying to improve implant surfaces by plasma spraying, anodizing, acid-etching, or sand blasting to increase the surface area and roughness and osseointegration potential of the implant. After all these years since the first implant was placed, have we been able to achieve the ideal 100% bone-implant contact for the success of an implant?

Even with all the research and successful endeavors in improving the implant surfaces and related clinical protocols, several issues directly related to the biological capability of titanium remain unresolved. For dental implants, the protracted healing time required before restoring the implant has been a continuous challenge. Various risk factors, including host bone anatomy, diabetes, osteoporosis, smoking, and aging may limit the application of dental implants[1-3]. For orthopedic implants, the treatment outcome includes a high percentage of revision surgery ranging from 5 to 40% [4], not to mention the cost of removing and replacing a failed implant. Rapid and firm establishment of bone-implant integration has therefore been a persistent challenge in both fields. How do we address this issue of rapid and firm establishment of bone, is

it by improving the surface properties of the implant itself so we can achieve the highest level of bone-implant contact or by regenerating bone around the implants as soon as possible?

The biological capability of the implant, the reason why bone tissue does not form entirely around an implant is of extreme importance in trying to improve the success rates of implants although it seems to have been neglected or overlooked. The implant area eventually covered by bone(bone- implant contact percentage)remains at 45-60% [5], or 50-65% [6]. Most implants fail because of an incomplete establishment, or early/late destructive changes at the bone implant interface [7, 8]. As no implant related factors have been elucidated to answer this question, a limited supply of stem cells may be the hypothetical explanation for the incomplete bone regeneration.

Bone is a rigid organ of the body which is a type of dense connective tissue. There are different stages and factors involved in the regeneration of bone. Uninterrupted blood supply to the area, space creation and maintenance to facilitate for bone growth, i.e.: a scaffold, newly forming cells around the area and also signaling between the cells. The approach of seeding cells into biodegradable scaffolds has evolved as the hallmark of modern tissue engineering. One of the characteristics required by bone tissue engineering for scaffolds is that they must have a porous and interconnected structure, enabling the migration and distribution of cells. Several materials have been investigated as potential scaffolds for bone tissue engineering. The ideal scaffold should provide a suitable environment for tissue development. It should favor cell attachment, growth and differentiation, in vivo revascularization, integration with the host tissues, and the gradual replacement of the scaffold by newly formed tissues. At the same time, the materials and their degradation products must be non-toxic and non-immunogenic. Unfortunately, no such

scaffold currently exists. The biggest drawback to the use of scaffold-based designs is the strong inflammatory responses that are induced upon their biodegradation. It has been previously observed that the implantation of nearly all polymer materials, even if non-toxic, causes a non-specific inflammatory response. This host inflammatory response can damage the transplanted cells, thus resulting in failure of the engineered tissues. How do we overcome the disadvantages of tissue engineering bone?

Since Shimizu and colleagues proposed the novel tissue engineering methodology of layering cell sheets to construct 3D functional tissues without any artificial scaffolds, it has become an increasingly popular method to overcome the disadvantages and complications of the scaffolds. When cells are cultured confluently, they connect to each other via cell-to-cell junction proteins and ECM. With enzymatic digestions, these proteins are disrupted and each cell is released separately. In the case of cell-sheet engineering using PIPAAm-grafted surfaces [9], cell-to-cell connections are not disrupted and cells are harvested as a contiguous cell sheet by decreasing the temperature of the dish. Furthermore, adhesive proteins underneath cell sheets are also maintained and they play a desirable role as an adhesive agent in transferring cell sheets onto other culture materials or other cell sheets. These viable cell sheets are composed of cells and biological ECM without any artificial scaffolds. Various types of cell sheets have been successfully lifted up and transferred on other surfaces. Cell sheet technology has been applied to corneal epithelia, mucosal epithelia, periodontal ligament cells, bladder epithelia, and esophageal epithelia[10]. The cell sheets can also be layered one on top of the other to create three dimensional structures such as cardiac muscle. This method is being used to create functional organ-like structures.

Essentially, when we place an implant into the bone we are creating a defect in the bone which causes a loss of cells, signaling and bone structure. In order for the implant to be successful it has to be integrated into the bone which is dependent upon the magnitude of the bone directly deposited onto the titanium surface without any soft/connective tissue intervention. For any organ regeneration including bone, the three important factors needed are cells, signaling between the cells and the scaffolding. When placing the implant into the bone we disrupt all of these. From the perspective of tissue engineering, titanium surfaces appear to work as a scaffold, but as previously mentioned we are disrupting the cell signaling and removing bone structure from the implant surgery site. In the U.S, 10% of the adults and one-third of adults aged >65years are fully edentulous [11, 12]. Despite the need in an aging society, dental implant therapy has been employed in only 2% of the potential patient population.

Cell-sheet technology basically takes advantage of the ability of temperature responsive conversion of a specially made culture dish from hydrophobic to hydrophilic, which allows a layer of grown cells to detach as a single sheet without the use of proteolytic enzymes. We would like to use this technology and ability of cells to be detached as a single layer and apply it to enhance biomaterials and regenerate bone. We have made an experimental and scientific strategy that the surface of titanium implants could be coated with osteogenic cells in advance, before the placement of the implant into the bone, so that the process of osseointegration can be expedited and enhanced when the implant is placed into the host bone. A cell sheet made of a full layer of cells may provide an ideal and maximum capacity of cellular source that the implant can retain, solving the problem of insufficient and limited supply of necessary stem cells, it could solve the problem of an ideal scaffold for bone formation and it could also provide the signaling between the cells needed for the immediate regeneration of bone. A long term goal of this study is to

develop a cell sheet based therapy for osseous implants for rapid and firm establishment of enhanced osseointegration. This particular project proposes to examine the viability and effectiveness of osteogenic cell sheets in vitro and preliminary test cell-sheet layered implants in vivo for potential enhancement of osseointegration. In order to achieve this goal the following specific aims have been formulated:

Specific Aim 1: Determine the feasibility of fabricating osteogenic cell sheets and testing them for anticipated functional phenotypes and checking the responsiveness to biological signals before and after transferring them to titanium surfaces.

Specific Aim 2: Determining the effect of single and double-layered cell sheets on cell metabolic activity and the osteoblastic functional phenotype of the cells after transferring them onto the titanium.

Specific Aim 3: To evaluate the effect of an osteogenic enhancer molecule on the cell sheet potential.

Specific Aim 4: Examine the in-vivo effect of the osteogenic cell sheet-implant complex on the enhancement in the strength and speed of osseointegration.

CHAPTER 2:

Feasibility of fabricating an osteogenic cell sheet and performing pre and post transfer viability tests.

2.1 Introduction

The approach of seeding cells into biodegradable scaffolds has evolved as the hallmark of modern tissue engineering. Cell-based therapies make use of a protocol where individual cells are injected directly into the site where they are expected to remain. However, in case of bone generation, the injected cells cannot be retained around the target tissue and in case of implant placement; it is hard to deliver cells through an injection precisely around the implant surface soon after the surgery. One of the characteristics required by bone tissue engineering for scaffolds is that they must have a porous and interconnected structure, enabling the migration and distribution of cells. Several materials have been investigated as potential scaffolds for bone tissue engineering. Upon the degradation of the polymers, the cells seeded are believed to proliferate and migrate in order to replace the scaffold. However, the cells in the center of the scaffold might not receive enough oxygen and nutrients and strong inflammatory responses may also be present. The ideal scaffold should provide a suitable environment for tissue development. It should favor cell attachment, growth and differentiation, in vivo revascularization, integration with the host tissues, and the gradual replacement of the scaffold by newly formed tissues. At the same time, the materials and their degradation products must be non-toxic and non-immunogenic. Unfortunately, no such scaffold currently exists for bone regeneration. It has been previously observed that the implantation of nearly all polymer materials, even if non-toxic, causes a non-specific inflammatory response. This host inflammatory response can damage the transplanted cells, thus resulting in failure of the engineered tissues.

Since Shimizu and colleagues proposed the novel tissue engineering methodology layering of cell sheets to construct 3D functional tissues without any artificial scaffolds, it has become an increasingly popular method to overcome the disadvantages and complications of the scaffolds.

When cells are cultured confluently, they connect to each other via cell-to-cell junction proteins and ECM. With enzymatic digestions, these proteins are disrupted and each cell is released separately. In the case of cell-sheet engineering using PIPAAm-grafted surfaces, cell-to-cell connections are not disrupted and cells are harvested as a contiguous cell sheet by decreasing temperature. Furthermore, adhesive proteins (ECM) underneath cell sheets are also maintained and they play a desirable role as an adhesive agent in transferring cell sheets onto other culture materials or other cell sheets. These viable cell sheets are composed of cells and biological ECM without any artificial scaffolds. Various types of cell sheets have been successfully lifted up and transferred on other surfaces. Cell-sheet technology has been tested in regenerating cardiac muscle, corneal epithelia, liver tissue, bladder epithelia and periodontal ligament. This study is trying to use this technology in growing and retrieving the osteogenic cells as a single layer. The growing and layering of osteogenic cells as a sheet will help us transfer a maximum number of osteoprogenitor cells to the implant therapy site. We need new bone to generate around the bone-implant interface which will help in increasing the secondary stability of the implant, in turn increasing the osseointegration capacity and success rate of the implants. Cell-sheet technology consists of a temperature-responsive culture dish, which enables reversible cell adhesion to and detachment from the dish surface by controllable hydrophobicity of the surface. This allows for a non-invasive harvest of cultured cells as an intact monolayer cell sheet along with the deposited extra-cellular matrix. This monolayer cell sheet can be transplanted to host tissues without the use of biodegradable scaffolds.

2.2 Materials and Methods:

2.2.1 Thermo-responsive tissue culture dish:

PIPAAm(poly N-isopropylacrylamide) dishes which exhibit thermo-responsive hydrophobicity changes were used for the studies [13]. By irradiation of electron beam onto the monomer on commercial polystyrene dishes, the monomer is polymerized and covalently bonded with the dish surface. At temperatures below 32° C PIPAAm molecules are highly hydrated, so the PIPAAm grafted surfaces are hydrophilic. At temperatures above 32° C the surfaces suddenly change to hydrophobic nature. This change is completely reversible and dependant on the temperature of the dish.

2.2.2 Bone marrow cell preparation:

Following the previously established protocol [14-18], bone marrow cells isolated from the femurs of 8-week-old male Sprague-Dawley rats were inoculated into alpha-modified Eagle's medium supplemented with 15% fetal bovine serum, 50 mg/ml ascorbic acid, 10 mm Na-β-glycerophosphate, and antibiotic–antimycotic solution. Cells were incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. At 80% confluency, cells were detached using 0.25% trypsin-1 mm EDTA-4Na and seeded onto temperature-responsive culture dishes and regular culture dishes with or without dexamethasone in the media at a density of 3×10^4 cells/ml. The culture medium was renewed every 3 days.

2.2.3 Titanium disk preparation:

Titanium samples were prepared from commercially pure grade 2 titanium by machining discs. To create microroughened morphology with peaks and valleys, titanium samples were acid-

etched with 67% H_2SO_4 at 120 °C for 75 s. The titanium disks were autoclave-sterilized and stored under dark ambient conditions for 4 weeks, which allowed sufficient aging of the surfaces and helped to standardize the surface energy. The surface energy was evaluated by the level of hydrophilicity defined as a contact angle of a 10 ml ddH2O drop placed on the titanium disk. The morphology of these surfaces was examined using a scanning electron microscope (SEM) (XL30, Philips, Eindhoven, Netherlands) and a laser profile microscope (VK 8500, Keyence, Osaka, Japan) to determine the average roughness (Ra), peak-to-valley roughness (Rz), and inter irregularities space (Sm). The elemental composition of the titanium surfaces was examined by an energy dispersive X-ray spectroscope (EDX). Disks which were 200 mm in diameter and 1.5 mm in thickness were prepared for the in-vitro experiments.

2.2.4 Osteogenic cell-sheet fabrication:

The culture medium in the temperature responsive dishes was renewed every three days until the cultures were confluent and ready to harvest between days 7-9. The culture medium was renewed with or without dexamethasone as needed. The sheets were observed every day and harvested with cell-sheet transfer disks which were used to specially transfer the cell-sheets onto titanium disks. After transfer the titanium disks were incubated for three hours before the removal of the transfer sheet.

2.2.5 Cell sheet transfer membrane:

Cell sheet transfer membranes made especially to help transfer cell sheets from the temperature responsive dishes to other substrates were ordered from cell seed company which is established in Japan. This transfer membrane adheres to the cell sheet in the temperature responsive dish

which allows us to use a forceps to detach the sheet as a single layer and transfer it to the titanium substrate.

2.2.6 Cell attachment, density, and proliferation assays:

The initial attachment of the cells was evaluated by measuring the number of cells attached to the culture plates and titanium disks after 3hrs of incubation. The density of propagated cells was measured at 24hrs or 48 hrs of culturing. These quantifications were performed using a tetrazolium salt (WST-1)- based colorimetric assay. (WST-1; Roche Applied Science, Mannheim, Germany). A culture well was incubated at 37° C for 4 h with 100 mlWST-1 reagent. The amount of formazan product was measured at 420 nm using an ELISA reader (Synergy HT, BioTek Instruments, Winooski, VT).

2.2.7 Cell morphology and morphometry

Confocal laser scanning microscopy was used to examine the intactness of osteogenic sheets transferred onto the titanium surfaces [19]. After 24 h of culture, cells were fixed in 10% formalin and stained using fluorescent dye rhodamine phalloidin (actin filament, red color; Molecular Probes, OR). Spreading and settlement behavior of cell-sheets on titanium surfaces were observed under confocal laser microscopy.

2.2.8 Alkaline phosphatase activity

The ALP activity of transferred osteogenic sheets was examined by a colorimetry-based assay. Cultured osteoblastic cells were washed twice with Hanks' solution, and incubated with 120 mm Tris buffer (pH 8.4) containing 0.9 mm naphthol AS-MX phosphate and 1.8 mm fast red TR for

30 min at 37 °C. For colorimetry, the culture was rinsed with ddH_2O and added with 250 µl p-nitrophenylphosphate (Lab Assay ATP, Wako Pure Chemicals, Richmond, VA), and then incubated at 37 °C for 15 min. The ALP activity was evaluated as the amount of nitro phenol released through the enzymatic reaction and measured at 405 nm wavelength using ELISA reader (Synergy HT, BioTek Instruments, Winooski, VT).

2.2.9 Statistical analysis

In vitro experiments were performed in triplicate (n=3). The difference among the experimental groups was examined by 1-way ANOVA. If needed, Bonferroni multiple comparison test was performed ad hoc; $p<0.05$ was considered statistical significant.

2.3 Results of pre-transfer osteogenic cell sheets:

2.3.2 Cell density and metabolic activity assay:

Cell density and cell metabolic activity was measured using WST-1 assay. The assay was performed at 48 hrs after seeding. The groups tested were cells seeded into regular culture dishes and temperature responsive dishes without dexamethasone in the media. The results showed that the activity and density of the cells grown in temperatures responsive dishes were similar to or slightly more than the cells grown in regular culture dishes. This proves that the PIPAAm coating on the dishes does not hinder the growth of osteogenic cells and osteogenic cells can be grown as a single layered sheet.

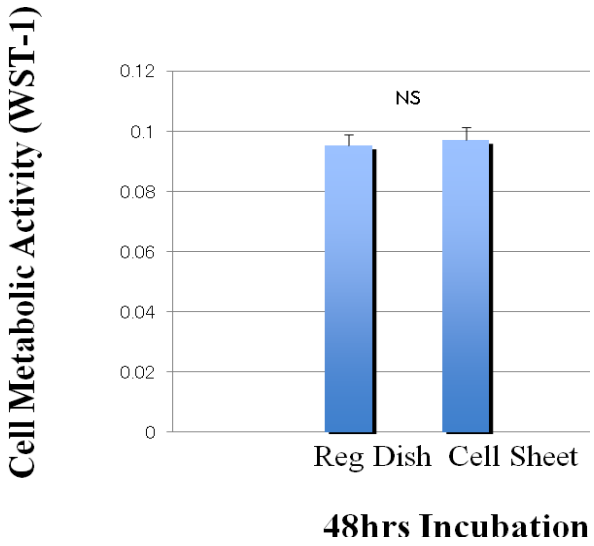


Fig. Cell Metabolic Assay at 48 hrs of incubation. The two groups being compared show similar results. This proves that the PIPAAm coating on the dishes does not hinder the growth of osteogenic cells and osteogenic cells can be grown as a single layered cell sheet with similar cell metabolic activity levels

2.3.3 Confirmation of osteoblastic phenotype in cell-sheets

To confirm the feasibility of the cell sheet technology and it's effect on the osteoblastic phenotype of the cells, Alkaline phosphotase activity to confirm the phenotypic activity was performed on the cell cultures at 7 days of culture. The results of the assay are shown in the figure below. At day 7 the assay shows significant difference in the differentiation levels of cells in the regular dishes and cell sheet dishes without dexamethasone as compared to the cells in the dishes with dexamethasone in the media. This increase in differentiation can be attributed to the presence of dexamethasone in the media which is an osteogenic inducer. This similar response in cell sheet dishes and regular culture dishes to known reagents added shows that the cell sheet is still viable at day 7 and we could successfully elicit similar responses which are not hindered by the PNIPAM coating of the dishes.

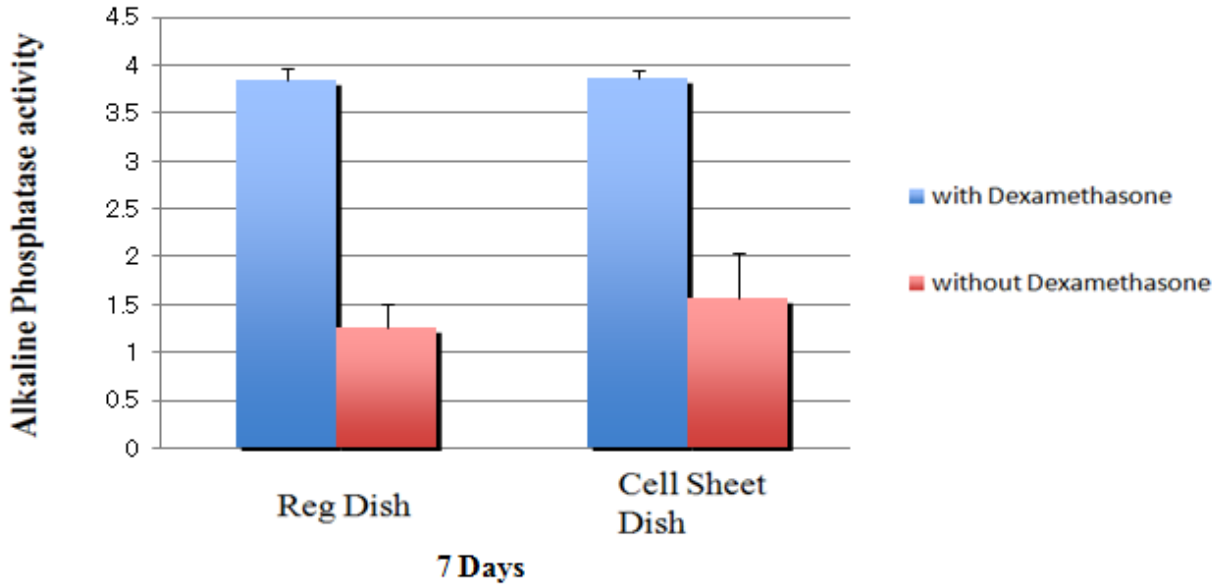
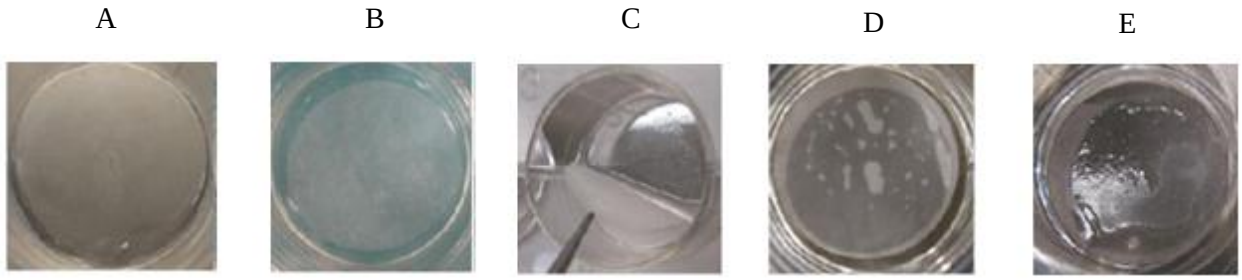


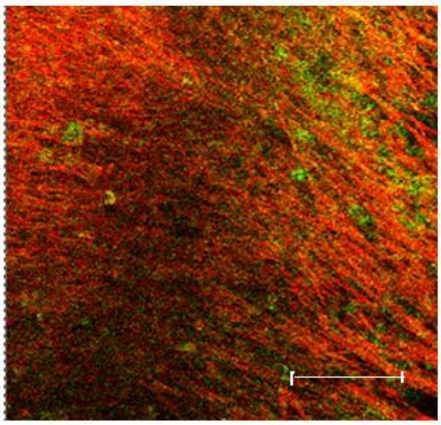
Fig 2. Confirmation of osteoblastic phenotype in the cell sheets. At day 7 we can see the differentiation is much more in the groups with dexamethasone added to the media. This proves that cell sheet is still viable at day 7 and is showing expected results to known reagents

2.4 Post Transfer Results:

2.4.1 Successful fabrication and transfer of cell sheet to titanium surface:

As explained in the materials and methods section rat BMC were harvested and grown in culture dishes to 80% confluency and then seeded onto the temperature-responsive dishes. After 7 days of culture the confluency of the sheet was checked under the microscope and the cell-sheet was harvested by a simple temperature change of the dishes. The osteogenic cell-sheet was then transferred to a titanium disc carefully, avoiding any air bubbles by the use of a cell-sheet transfer membrane. A confocal microscopic image confirmed that the cell-sheet was transferred as a single intact layer without any tears and hence the cell-to cell junctions were preserved.

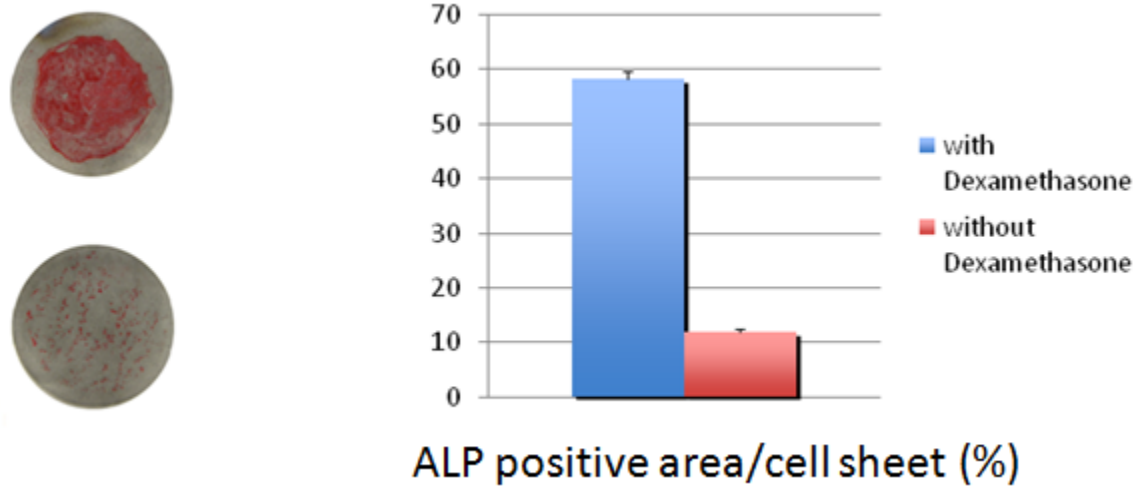




A step-by-step procedure shown in pictures. (A) Confluent cell-sheet ready to be harvested.(B) Cell-sheet with transfer membrane. (C) Harvesting of the cell-sheet as a single contiguous layer by a simple change in the temperature. (D) Cell-sheet transfer-membrane complex placed onto titanium disk. (E) Cell-sheet on the titanium disk without any bubbles or tears. (F) Confocal microscopic image of cell-sheet after cytoskeleton (red, rhodamine phalloidin) and vinculin(green) immunostain.

2.4.2 Confirmation of osteoblastic phenotype in cell-sheets:

To confirm the feasibility of the cell sheet technology for titanium implants, cell sheets transferred to the titanium disks were maintained on the disks without detaching by changing the media every 3 days. Cell sheets were cultured with and without dexamethasone after transferring to titanium. ALP staining showed a large area of the cell sheet cultured with dexamethasone as ALP positive, while a smaller area of the cell sheet cultured without the dexamethasone was stained positive, indicating a normal functional phenotype and an anticipated responsiveness of the cells to a known biological agent.



The figures shown here are representative of the ALP stained areas of the dex+ and dex- cell sheets. The sheet grown with the osteogenic inducer shows a high area of positive staining as compared to the sheet grown without it. The ALP positive area has been calculated and can be appreciated in the graph.

2.4.3 Cell Metabolic activity levels and osteoblastic phenotype after transfer of cell sheet to various titanium surfaces:

The osteogenic cell sheets were fabricated and transferred to various surfaces such as regular culture dish, machine surfaced titanium and acid-etched surface. We can appreciate the cell metabolic activity levels after the cell sheet has been transferred to the various surfaces from the figure. The acid etched surface showed the highest levels of activity when compared to the machined surfaces. The alkaline phosphatase activity levels of the cell sheet were also higher in the sheets transferred to the acid etched surfaces as compared to the cell sheets on machined surfaces and the regular culture dish. Hence, we performed all further assays for this study using

acid-etched surfaces. Acid-etched surface has been proven to have better affinity for osteoblasts as compared to the machined implant surfaces.

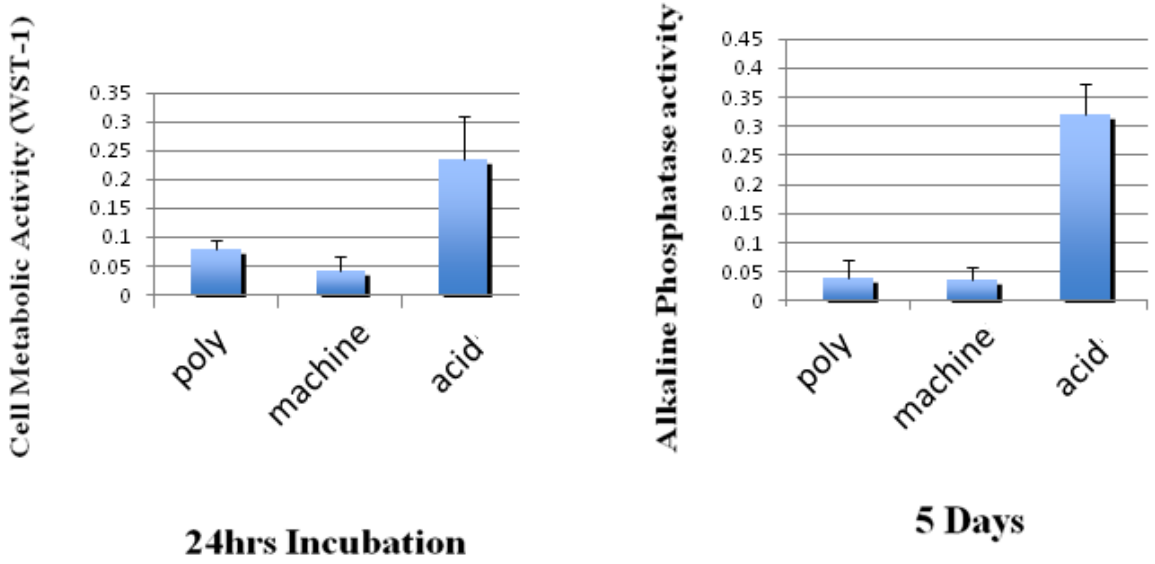


Fig Cell metabolic activity and alkaline phosphatase activity levels at 24hrs and 5 days of culture respectively. The cell metabolic activity was much more on the acid etched surface. The alkaline phosphatase activity also showed similar results at day 5 of culture where the differentiation levels were much more significant on the acid etched surfaces as compared to the machine surfaces and regular culture dish surface.

2.4.4 Cell attachment and metabolic activity levels:

Once we established that the acid etched surface has better affinity for the attachment of the cell sheets, we transferred the cell sheets grown with and without dexamethasone in the media to the acid etched surfaces. The fig below shows that the WST-1 assay performed in both dex+ and dex- groups at 48hrs did not show much difference. This indicates that dexamethasone which is an inducer of osteoblastic differentiation did not have effect on the transfer of the cell sheets. Hence, we can grow the cell sheets in a media devoid of dexamethasone which will help us in preserving the stem cell lineage of the cells. When these cells come into contact with osteoblasts they will differentiate towards that lineage.

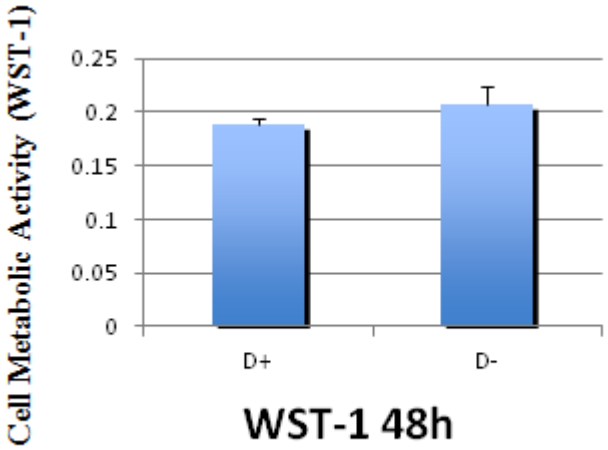


Fig: Cell attachment, proliferation and metabolic activity levels among cell sheets grown in dex+ and dex- media. The attachment, proliferation and cell metabolic activity levels did not differ significantly among the two groups of cell sheets compared.

2.5 Discussion:

This is one of the first studies to introduce the effect of a cell-sheet layered implant complex on the bone-titanium integration capacity of implants. Previous studies have established the fabrication of cell sheets in soft tissue regeneration. In this study we demonstrated the feasibility of fabricating an osteogenic cell-sheet and transferring or layering the cell-sheet onto the titanium surface. The cell-sheet transferred to the titanium disks showed increased phenotypic activity as compared to the control. The cell metabolic activity levels suggest that the fabrication of an osteogenic cell sheet is not only feasible but the cell sheet is viable and retains the cell metabolic activity levels and also the phenotypic activity. These results suggest the feasibility and effectiveness of cell sheet transferred to the titanium surface which can further be tested in enhancing the biological process of osseointegration.

By harvesting cell sheets we also preserve the adhesion molecules that cells secrete thereby allowing the cells to attach to the titanium surface. This allows for increase in signaling between the cells which have been transferred as a single sheet. Mesenchymal stem cells (MSCs) existing in bone marrow have the potential to differentiate into osteoblasts, adipocytes, chondrocytes, neurons and myogenic cells. Differentiation into osteoblastic lineage cells in in-vitro culture in osteoinductive medium is induced by the inclusion of dexamethasone (Dex), ascorbic acid phosphate (Vitamin C; VC) and β-glycerophosphate (β-GP). The increased ALP staining observed suggests the increased osteoblastic phenotype in cells cultured with the presence of dexamethasone in the culture media. With the fabrication of osteogenic cell-sheets we might be able to eliminate the use of scaffolds.

This study has shown the feasibility of fabricating an osteogenic cell sheet and transferring it to a titanium substrate. Although previous studies done with cell-sheet technology have proved that cell-sheets can be successfully harvested and transferred to human corneal epithelium and other organ regeneration was made possible, this is the first study that introduces this technique for implementation with titanium implants and hence it is still in the preliminary stages. Harvesting the cell sheet is very technique sensitive and has to be done very methodically. There is no set protocol established for harvesting the osteogenic cell sheets. It depends on the cell growth and differentiation capability of the seeded cells. We have established the protocol by observing the cell sheets consistently and we were able to transfer them to the titanium surfaces. Although further in vitro studies are required to accumulate the data, this study demonstrated the great potential of cell sheet technology for further use in improving the bone-implant contact and thus improving the osseointegration capabilities of the implants.

CHAPTER 3:

Determining the effect of single and double-layered cell sheets on cell metabolic activity and the osteoblastic functional phenotype of the cells after transferring them onto the titanium.

3.1 Introduction:

The approach of seeding cells into biodegradable scaffolds is the hallmark of tissue engineering. Tissue engineering has been used clinically to replace a variety of tissues and organs. Vascular smooth muscle and endothelial cells are being used in tissue engineering to reconstruct arteries. The clinical applications of tissue engineering have been widely used, but there are a few disadvantages to the use of it. In tissue engineering an organ we need to make use of a scaffold to create three dimensional structures. To survive the three dimensional cell dense tissues after transplantation, there are several factors that need to be looked into such as, improvements of hypoxia, nutrient sufficiency, blood supply to the centre of the cell dense tissue and so on. We need to overcome these disadvantages and obtain stable three dimensional structures in order to regenerate organs.

Instead of using biomaterials as scaffolding materials cell sheet technology uses thermo responsive polymer coated culture surfaces that facilitate non-invasive, non-disruptive harvest of cultured cells as an intact single layered sheet along with the deposited extra cellular matrix. This has been shown in the previous chapter by fabricating a single layered osteogenic cell sheet which has retained all the functional properties and could also be transferred to a titanium surface without any tears. The temperature responsive polymer, PIPAAm, makes it possible for us to harvest the cell sheets by simply controlling the incubation temperature. At temperatures above 32° C the thermo responsive culture surfaces are slightly hydrophobic, allowing various cell types to attach, spread and proliferate. By reducing the temperature the PIPAAm surface undergoes a spontaneous change and becomes hydrophilic. This controlled change allows us to harvest confluently cultured cells as an intact sheet. With non-invasive harvest, cell-to-cell junctions and ECM can be maintained.

Cell-sheets have been layered one on top of the other to construct three-dimensional structures such as cardiac muscle, bladder epithelia, and liver tissue as mentioned earlier. This will allow us to manage the thickness of the cell sheet. Why are we trying to increase the number of layers of the osteogenic cell sheets? We are trying to increase the density of the oeteoprogenitor cells delivered in order to improve the quantity of bone formed around the implant surface. Also, by increasing the number of layers of cell sheets we increase the number of cells delivered along with cell-to-cell signaling present between those cells. This will help in improving the cell-to-cell communication between newly forming bone and osteoprogenitor cells present on the implant surface. This study will test the layering effect of the cells with osteogenic cell sheets. Layering cells on titanium substrates will not allow the same blood supply as it is for layering cell sheets in soft tissue reconstruction. The ECM deposited by the cells retain the adhesive properties of the cells. Due to the preservation of the adhesive property, cell sheets can be easily transferred and attached to other culture surfaces and even host tissues. This property was helpful in reconstruction of corneal epithelia, bladder epithelia, liver tissue and cardiac tissue too. We wanted to use this unique property of cell sheets in this study and we tried to layer the osteogenic cell sheets onto the titanium surface to see if this improves the functional capability of the cells.

3.2 Materials and Methods:

3.2.1 Titanium disk preparation

Titanium samples were prepared from commercially pure grade 2 titanium by machining discs. To create microroughened morphology with peaks and valleys, titanium samples were acid-etched with 67% H_2SO_4 at 120 °C for 75 s. The titanium disks were autoclave-sterilized and stored under dark ambient conditions for 4 weeks, which allowed sufficient aging of the surfaces

and helped to standardize the surface energy [3]. The surface energy was evaluated by the level of hydrophilicity defined as a contact angle of a 10 ml ddH2O drop placed on the titanium disk. The morphology of these surfaces was examined using a scanning electron microscope (SEM) (XL30, Philips, Eindhoven, Netherlands) and a laser profile microscope (VK 8500, Keyence, Osaka, Japan) to determine the average roughness (Ra), peak-to-valley roughness (Rz), and interirregularities space (Sm). The elemental composition of the titanium surfaces was examined by an energy dispersive X-ray spectroscope (EDX).

3.2.2 Bone marrow cell preparation

Following the previously established protocol [1,10], bone marrow cells isolated from the femurs of 8-week-old male Sprague-Dawley rats were inoculated into alpha-modified Eagle's medium supplemented with 15% fetal bovine serum, 50 mg/ml ascorbic acid, 10 mm Na-β-glycerophosphate, and antibiotic–antimycotic solution. Cells were incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. At 80% confluency, cells were detached using 0.25% trypsin-1 mm EDTA-4Na and seeded onto temperature-responsive culture dishes at a density of 3×10^4 cells/ml. The culture medium was renewed every 3 days.

3.2.3 Cell sheet transfer membrane:

Cell sheet transfer membranes made especially to help transfer cell sheets from the temperature responsive dishes to other substrates were ordered from Cell Seed Inc, a company which is established in Japan. This transfer membrane adheres to the cell sheet in the temperature responsive dish which allows us to use a forceps to detach the sheet as a single layer and transfer it to the titanium substrate.

3.2.4 Osteogenic cell-sheet fabrication

The culture medium in the temperature responsive dishes was renewed every three days until the cultures were confluent and ready to harvest between days 7-9. The culture medium was devoid of dexamethasone so as to have the stem cell lineage of the cells. The sheets were observed every day and harvested with cell-sheet transfer membranes which were used to specially transfer the cell-sheets onto titanium disks. After transfer the titanium disks were incubated for three hours before the removal of the transfer sheet.

3.2.5 Cell attachment, density, and proliferation assays:

The initial attachment of the cells was evaluated by measuring the number of cells attached to the culture plates and titanium disks after 3hrs of incubation. The density of propagated cells was measured at day 2 of culturing. These quantifications were performed using a tetrazolium salt (WST-1)- based colorimetric assay. (WST-1; Roche Applied Science, Mannheim, Germany). A culture well was incubated at 37 _C for 4 h with 100 mlWST-1 reagent. The amount of formazan product was measured at 420 nm using an ELISA reader (Synergy HT, BioTek Instruments, Winooski, VT).

3.2.6 Alkaline phosphatase activity

The ALP activity of transferred osteogenic sheets was examined by a colorimetry-based assay. Cultured osteoblastic cells were washed twice with Hanks' solution, and incubated with 120 mm Tris buffer (pH 8.4) containing 0.9 mm naphthol AS-MX phosphate and 1.8 mm fast red TR for 30 min at 37 °C. For colorimetry, the culture was rinsed with ddH_2O and added with 250 µl p-

nitrophenylphosphate (Lab Assay ATP, Wako Pure Chemicals, Richmond, VA), and then incubated at 37 °C for 15 min. The ALP activity was evaluated as the amount of nitro phenol released through the enzymatic reaction and measured at 405 nm wavelength using ELISA reader (Synergy HT, BioTek Instruments, Winooski, VT).

3.2.7 Statistical analysis

In vitro experiments were performed in triplicate (n=3). The difference among the experimental groups was examined by 1-way ANOVA. If needed, Bonferroni multiple comparison test was performed ad hoc; $p<0.05$ was considered statistical significant.

3.3 Results

3.3.1 Successful fabrication and transferring of cell sheets to titanium samples for in vitro testing

As explained in the materials and methods section rat BMC were harvested and grown in culture dishes to 80% confluency and then seeded onto the temperature-responsive dishes. After 7 days of culture the confluency of the sheet was checked under the microscope and the cell-sheet was harvested by a simple temperature change of the dishes. The temperature responsive dish with the osteogenic cell sheet was removed from the incubator to the hood and the media was carefully aspirated. The cell sheet transfer membrane was placed over it and we waited for 15 min before picking up the osteogenic cell sheet along with the transfer membrane. The osteogenic cell-sheet was then transferred to a titanium disc carefully avoiding any air bubbles. After transferring the first layer and waiting for it to be attached, we removed the cell sheet transfer membrane and layered the second sheet onto the first one.

3.3.2 Increased cellular activity by multiple layers of cell sheets:

The multi-layering of cell sheets was made possible by a carefully followed protocol. After the first layer was transferred, we had to wait for 30min to make sure it has attached to the titanium disc. We then removed the cell sheet transfer membrane and proceeded to harvest the second layer of cell sheet and transferred it onto the first layer. We had to wait for another 15 min before removing the transfer membrane. We tried to transfer a total of 3 layers. For the first assay performed at 24 hrs of incubation after transfer, the cell sheets showed a significant difference in cell metabolic activity among the three groups. However, for the assay performed at 48hrs, the third layer of osteogenic cell sheet started peeling off, hence we were not able to perform the WST-1 assay at the 48hr time point with three layers.

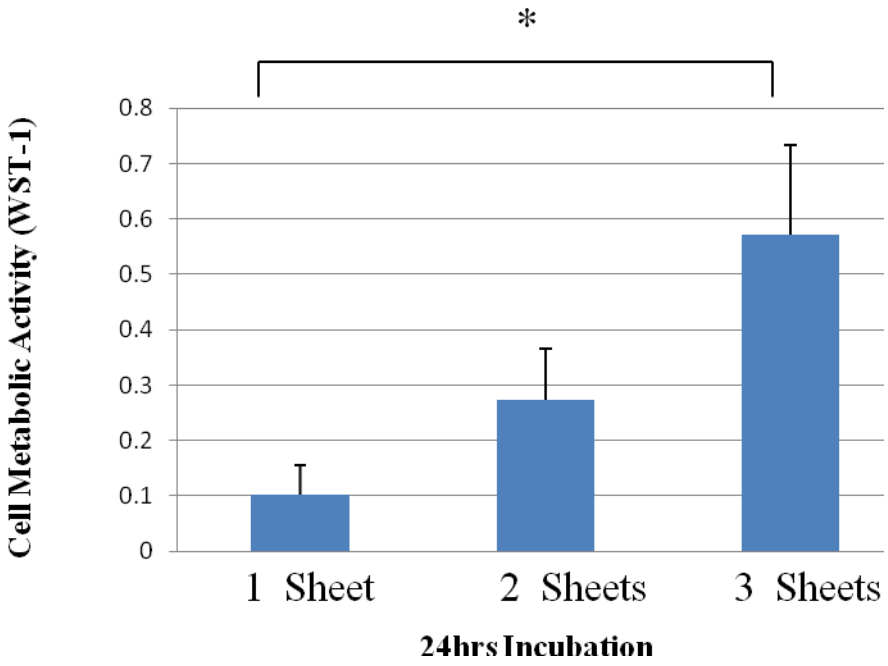


Fig: This figure shows the results of cell metabolic activity between single, double and triple layered osteogenic cell sheets after transfer to the titanium surface. The cell metabolic activity has increased correspondingly depending on the number of cell sheets layered which proves that the cell sheets attached to one another and they were viable.

The titanium disks with single and double layers of cell sheet were compared for cell metabolic activity at 24 hrs and 48 hrs of incubation after transfer to the titanium surface. WST-1 showed the increasing activity of the cells which corresponded to the number of layers stacked onto the titanium disc ($p<0.05$;). This proves that the cell sheets when transferred as two layers one on top of the other were still viable and retained the cell metabolic activity levels at 24hrs and 48hrs after transfer. In the case of soft tissue regeneration the cell sheets were being transferred to viable organs with inherent blood supply, but in this case we are transferring the cell sheets to titanium surfaces for in-vitro testing. It was hard to retain the sheets beyond the second layer for a longer period of time without the vasculature, nutrition and signaling to support the cells.

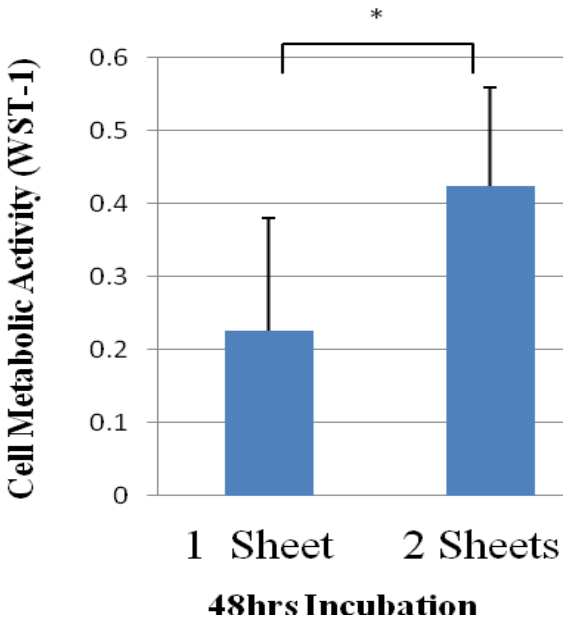


Fig: Fig shows the cell metabolic activity levels at 48hrs after transfer. The results show a significant difference between the single and double layer groups. The double layered groups show double the activity as compared to the single layered groups which is an expected outcome and shows that the cells were viable at 48hrs after transfer.

3.3.3 Confirmation of osteoblastic phenotype in one and two layered cell-sheets

To confirm the feasibility of the cell sheet technology for titanium implants, cell sheets transferred to the titanium disks were maintained on the disks without detaching by changing the media every 3 days. Cell sheets were cultured without dexamethasone after transferring to titanium. The Alkaline phosphatase activity when tested showed similar corresponding results which increased with the number of layers stacked ($p<0.01$;). This proves that the cell sheets were viable at day 5 after transfer to the titanium discs and they showed expected phenotypic activity when tested with the ALP assay.

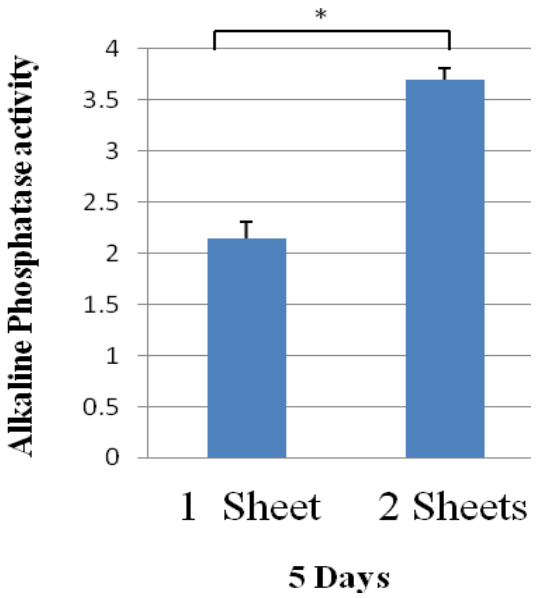


Fig: Fig shows similar results in the alkaline phosphotase activity where the double layered groups have double the activity as compared to the single layer group. This shows that the cells were still viable at day 5 and they showed normal functional response to a known reagent.

3.4 Discussion:

In this study we demonstrated the feasibility of fabricating an osteogenic cell-sheet and transferring or layering single or double cell-sheets onto the titanium surface. The cell-sheet transferred to the titanium disks showed increased phenotypic activity as compared to the control. The cell metabolic activity increased depending on the number of layers transferred to the titanium substrate as observed in the results. We have established an increased strength in osseointegration of the cell-sheet implant complex as compared to the control. These results suggest the feasibility and effectiveness of cell sheet coating on titanium implants in enhancing the biological process of osseointegration.

It should be noted that the cell metabolic activity was correspondingly increasing depending on the number of cell-sheet layers added to the titanium substrate which proves that providing an abundance of cells along with the implant complex has influenced the activity of cells around the implant and has set the stage for faster and improved bone-titanium integration. The delivery of an increased number of cells was made possible due to the osteogenic sheet as opposed to individual cells present around the implant. This is also confirmed by the corresponding increase in cellular metabolic activity depending on the number of cell-sheet layers stacked together. By harvesting cell sheets we also preserve the adhesion molecules that cells secrete thereby allowing the layers of cells attach to one another. This allows for increase in signaling between the cells which might be one of the reasons for improved cell metabolic activity levels. By treating the cell sheets with a biological enhancer we were able to induce an osteoblast differentiating environment which is much needed for the bone growth and differentiation. With the fabrication of osteogenic cell-sheets we were able to eliminate the use of scaffolds and we were able to

deliver a pre-osseointegrated complex in the form of a titanium implant coated with the osteogenic cell-sheet into the femurs of rats.

This study has shown the feasibility of fabricating an osteogenic cell sheet and transferring it to a titanium substrate and layering cell sheets to improve the cell metabolic activity levels and also the phenotypic activity. Although previous studies done with cell-sheet technology have proved that cell-sheets can be successfully harvested and transferred to human corneal epithelium and other organ regeneration was made possible, this is the first study that introduces this technique for implementation with titanium implants and hence it is still in the preliminary stages. Harvesting the cell sheet is very technique sensitive and has to be done very methodically. With rat osteogenic sheets we were able to harvest and transfer sheets to the titanium for in-vitro testing. Although further in vitro and in vivo studies are required to accumulate more data, this study demonstrated the great potential of cell sheet technology for titanium as a novel cell therapy for dental implants.

CHAPTER 4

Measuring the enhancement of osteogenic cell sheets by examining the effect of adding a biological enhancer molecule to promote the functional differentiation of the cell sheet.

4.1 Introduction:

N-acetyl Cysteine (NAC) is an antioxidant amino acid derivative, which directly neutralizes ROS. NAC can be incorporated into a cell and deacetylated into L-cysteine, a precursor of glutathione, which plays a central role in intracellular redox balance. This antioxidant capacity can protect cells from oxidative stress by directly scavenging extracellular reactive oxygen species (ROS) and compensating for the depletion of intracellular glutathione levels. Oxidative stress in a biological system is caused by the harmful effects of ROS which causes potential biological damage [21]. It has a deleterious effect especially when there is an overproduction of ROS. ROS can also cause cell death via apoptosis when the cellular antioxidant capacity is insufficient [22, 23]. Intracellular levels of ROS can especially increase after surgical stress, like placing an implant into the bone. How do we overcome the stress caused by the implant surgery? Can a biological enhancer such as NAC be used to overcome the stress induced by surgery and can it also improve the biological activity of the cells?

It has been indicated that ROS has adverse effects on the osteoblastic phenotype and alkaline phosphatase activity of the cells. NAC has multiple therapeutic applications. It has been used as an additive in cancer chemotherapy, it has been used in respiratory disorders and it also works as a direct ROS scavenger. The effect of NAC on the proliferation and differentiation activity of osteoblastic cells has already been examined [24]. We wanted to apply the positive effects of adding NAC as a biological enhancer in fabricating an osteoblastic cell sheet. We wanted to see if it has similar effects on improving the cell metabolic activity and alkaline phosphatase activity of an osteogenic cell sheet. If we can establish the positive effects of NAC on the fabrication of a cell sheet, we will be able to use NAC as a biological enhancer which will improve the bone

regeneration capability of the cell sheet coated implant which can reduce the oxidative stress created by the implant surgery.

4.2 Materials and Methods:

4.2.1 Titanium disk preparation

Titanium samples were prepared from commercially pure grade 2 titanium by machining discs. To create microroughened morphology with peaks and valleys, titanium samples were acid-etched with 67% H_2SO_4 at 120 °C for 75 s. The titanium disks were autoclave-sterilized and stored under dark ambient conditions for 4 weeks, which allowed sufficient aging of the surfaces and helped to standardize the surface energy. The surface energy was evaluated by the level of hydrophilicity defined as a contact angle of a 10 ml ddH2O drop placed on the titanium disk. The morphology of these surfaces was examined using a scanning electron microscope (SEM) (XL30, Philips, Eindhoven, Netherlands) and a laser profile microscope (VK 8500, Keyence, Osaka, Japan) to determine the average roughness (Ra), peak-to-valley roughness (Rz), and interirregularities space (Sm). The elemental composition of the titanium surfaces was examined by an energy dispersive X-ray spectroscope (EDX).

4.2.2 Bone marrow cell preparation

Following the previously established protocol, bone marrow cells isolated from the femurs of 8-week-old male Sprague-Dawley rats were inoculated into alpha-modified Eagle's medium supplemented with 15% fetal bovine serum, 50 mg/ml ascorbic acid, 10 mm Na-β-glycerophosphate, and antibiotic–antimycotic solution. Cells were incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. At 80% confluency, cells were detached using 0.25%

trypsin-1 mm EDTA-4Na and seeded onto temperature-responsive culture dishes at a density of 3×10^4 cells/ml. The culture medium was renewed every 3 days.

4.2.3 Cell sheet transfer membrane:

Cell sheet transfer membranes made especially to help transfer cell sheets from the temperature responsive dishes to other substrates were ordered from Cell Seed Inc, a company which is established in Japan. This transfer membrane adheres to the cell sheet in the temperature responsive dish which allows us to use a forceps to detach the sheet as a single layer and transfer it to the titanium substrate.

4.2.4 Osteogenic cell-sheet fabrication:

The culture medium in the temperature responsive dishes was renewed every three days until the cultures were confluent and ready to harvest between days 7-9. The culture medium was devoid of dexamethasone so as to have the stem cell lineage of the cells. The sheets were observed every day and harvested with cell-sheet transfer disks which were used to specially transfer the cell-sheets onto titanium disks. After transfer the titanium disks were incubated for three hours before the removal of the transfer membrane.

4.2.5 NAC preparation:

We prepared a NAC stock solution [25] by dissolving NAC powder (Sigma-Aldrich, St.Louis, MO, USA) in HEPES buffer (1 mol/L stock, pH7.2) which had been shown not to have any influence on osteoblast viability. We prepared the NAC treatment solution by mixing the NAC

stock solution with alpha-modified Eagle's medium in a volume ratio of 1:49. We added this solution to the cell sheet after transfer to the titanium surface at different time-points.

4.2.6 Cell attachment, density, and proliferation assays:

The initial attachment of the cells was evaluated by measuring the number of cells attached to the culture plates and titanium disks after 24hrs of incubation. The density of propagated cells was measured at day 2 of culturing. These quantifications were performed using a tetrazolium salt (WST-1)- based colorimetric assay. (WST-1; Roche Applied Science, Mannheim, Germany). A culture well was incubated at 37 _C for 4 h with 100 mlWST-1 reagent. The amount of formazan product was measured at 420 nm using an ELISA reader (Synergy HT, BioTek Instruments, Winooski, VT).

4.2.7 Alkaline phosphatase activity

The ALP activity of transferred osteogenic sheets was examined by a colorimetry-based assay. Cultured osteoblastic cells were washed twice with Hanks' solution, and incubated with 120 mm Tris buffer (pH 8.4) containing 0.9 mm naphthol AS-MX phosphate and 1.8 mm fast red TR for 30 min at 37 °C. For colorimetry, the culture was rinsed with ddH_2O and added with 250 µl p-nitrophenylphosphate (Lab Assay ATP, Wako Pure Chemicals, Richmond, VA), and then incubated at 37 °C for 15 min. The ALP activity was evaluated as the amount of nitro phenol released through the enzymatic reaction and measured at 405 nm wavelength using ELISA reader (Synergy HT, BioTek Instruments, Winooski, VT).

4.2.8 Statistical analysis

In vitro experiments were performed in triplicate (n=3). The difference among the experimental groups was examined by 1-way ANOVA. If needed, Bonferroni multiple comparison test was performed ad hoc; $p<0.05$ was considered statistical significant.

4.3 Results

4.3.1 Successful fabrication and transferring of cell sheets to titanium samples for in vitro testing

As explained in the materials and methods section rat BMC were harvested and grown in culture dishes to 80% confluency and then seeded onto the temperature-responsive dishes. After 7 days of culture the confluency of the sheet was checked under the microscope and the cell-sheet was harvested by a simple temperature change of the dishes. The osteogenic cell-sheet was then transferred to a titanium disc carefully, avoiding any air bubbles by the use of a cell-sheet transfer sheet. Further in-vitro studies for the biological enhancement of the cell sheet were performed.

4.3.2 Increased cell metabolic activity in cell sheets treated with a biological agent:

The rat bone marrow cells were collected from the femurs of rats. They were grown to 80% confluency and then seeded onto the temperature responsive dishes for fabrication of osteogenic cell sheets. The cell sheets were checked for confluency and transferred to the titanium surfaces at day 7. We wanted to test the optimum time point at which NAC should be added to the transferred cell sheets. We prepared and added NAC at four different time points to the cell sheets, at 0hrs, 3hrs, 6hrs and 24hrs after transferring the cell sheets onto the titanium surfaces.

As seen from the figure below the cell metabolic activity levels were tested at 48hrs of incubation after transferring the cell sheets to the titanium surface. From the results below we can come to the conclusion that NAC added at 6hrs after transfer was the optimum time point for increase in cell metabolic activity levels.

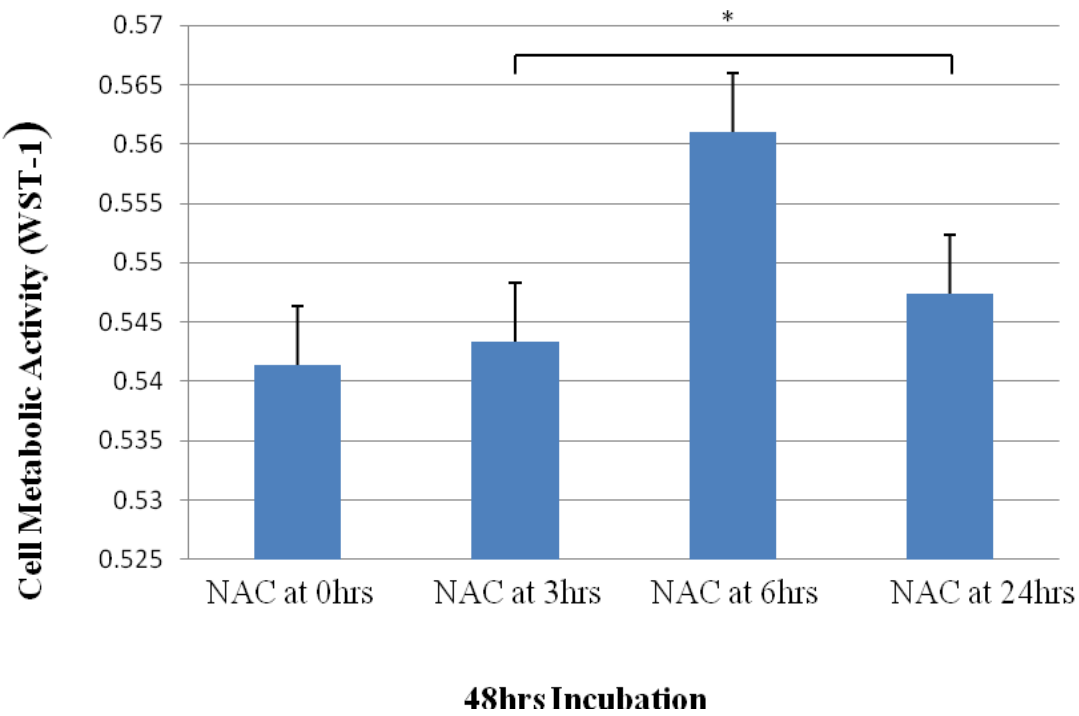


Fig: Comparison of the cell activity levels of NAC added at different time points. The graph below shows the cell metabolic activity levels of cell sheets with NAC added at 0hrs, 3hrs, 6hrs and 24hrs after transfer. The group with NAC added at the 6hr time point showed the maximum cell metabolic activity which can be appreciated in WST-1 assay performed at 48hrs after transfer of the cell sheet.

4.3.3 Alkaline Phosphatase activity of cell sheets:

Cell sheets transferred to the titanium disks were maintained on the disks without detaching by changing the media every 3 days. NAC was added to the culture dishes at the 0hr, 3hr, 6hr and 24hr time points after transfer. The media was changed after 3 days and the Alkaline phosphatase activity when tested showed similar results with a slight increase in the ALP activity of the NAC added at 6hr time point group. This proves that the cell sheets were viable at day 5 after transfer

to the titanium discs with the addition of NAC and they showed expected phenotypic activity when tested with the ALP assay. The NAC did not have any negative effect on the differentiation capability of the cell sheet.

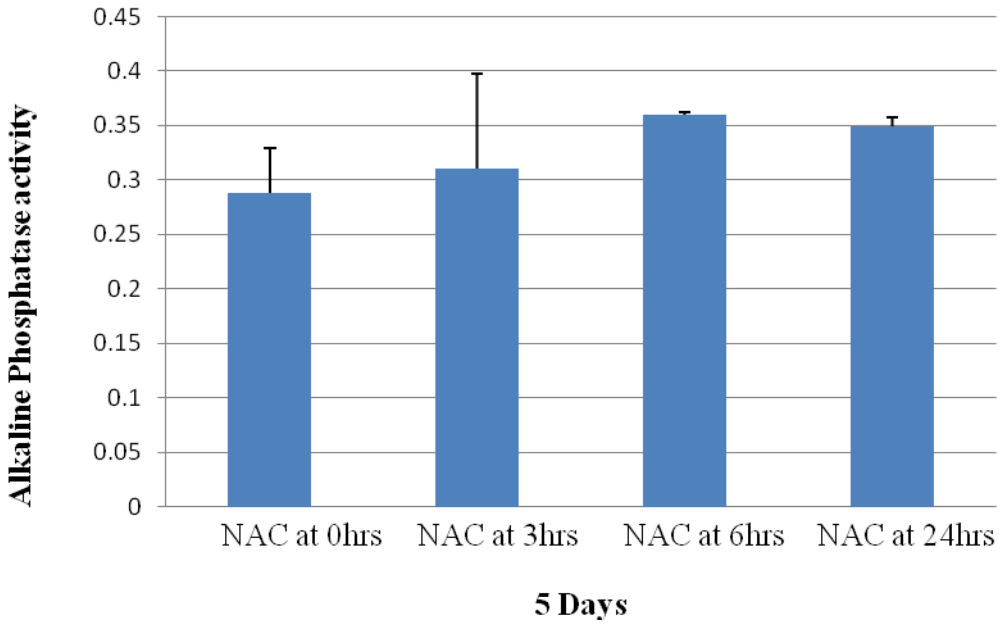


Fig: Alkaline phosphatase activity of cell sheets treated at 0hrs, 3hrs, 6hrs and 24hrs after seeding with NAC. This assay was performed at day 5 after transfer and shows a slight increase in the alkaline phosphatase activity of cell sheet treated with NAC at 6hr time point.

After establishing that the 6hr time point was the best for optimum cell activity, we wanted to see which concentration of NAC showed the optimum activity levels. NAC was prepared and added to the transferred cell sheets in different concentrations of 0, 2mM, 5mM and 10mM. This was done to see the most effective concentration of the NAC at which optimum cell metabolic activity is seen. WST-1 assay was performed at 48hrs after transferring the cell sheet to the titanium surface. As seen from the figure below, the cell metabolic activities of the cell sheets

increased in a dose dependant manner depending on the amount of NAC added. We chose 5mM concentration as the optimum for future studies with NAC and cell sheet as cells had increased activity at this concentration and there are no deleterious effects of NAC at this concentration.

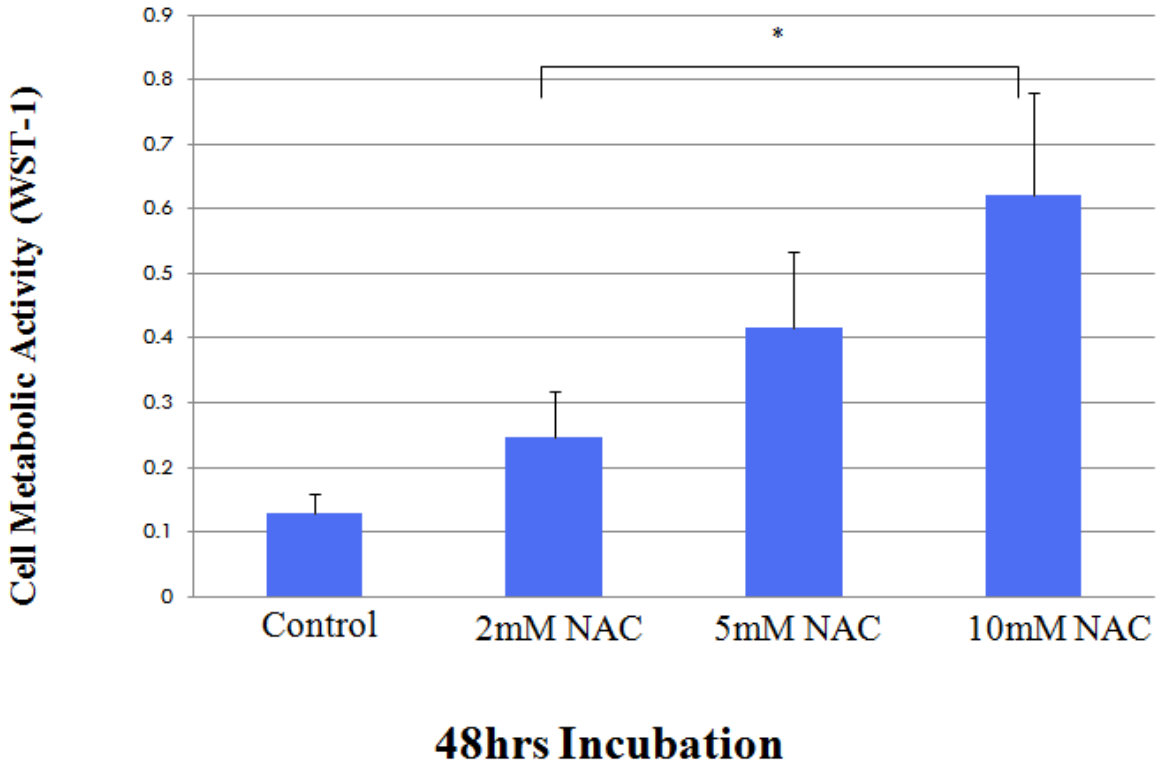


Fig: Comparison of the effect of different concentrations of NAC on the cell metabolic activity of the cell sheets. We can see from this graph that the cell metabolic activity increased in a dose dependant manner with the amount of NAC added to the culture dishes.

4.3.4 Increased cellular activity by multiple layers of cell sheets treated with biological enhancer:

Cells were seeded onto the temperature responsive dishes as explained. After fabricating the cell sheets at day7, the transfer of the first cell sheet was performed using a transfer membrane and

the second one was layered onto the first sheet carefully after making sure the first cell sheet had transferred and attached to the titanium disc. 5mM of NAC was added to the cell culture disc at 6 hrs time point after transfer. WST-1 assay was performed at 24hrs after transfer of the cell sheets. The results shown below show an increase in cell metabolic activity between single and double sheets with NAC added to them. We believe NAC acted as a biological enhancer in improving the cell metabolic activity levels and reducing the stress levels caused by the transfer when compared to the groups without the NAC.

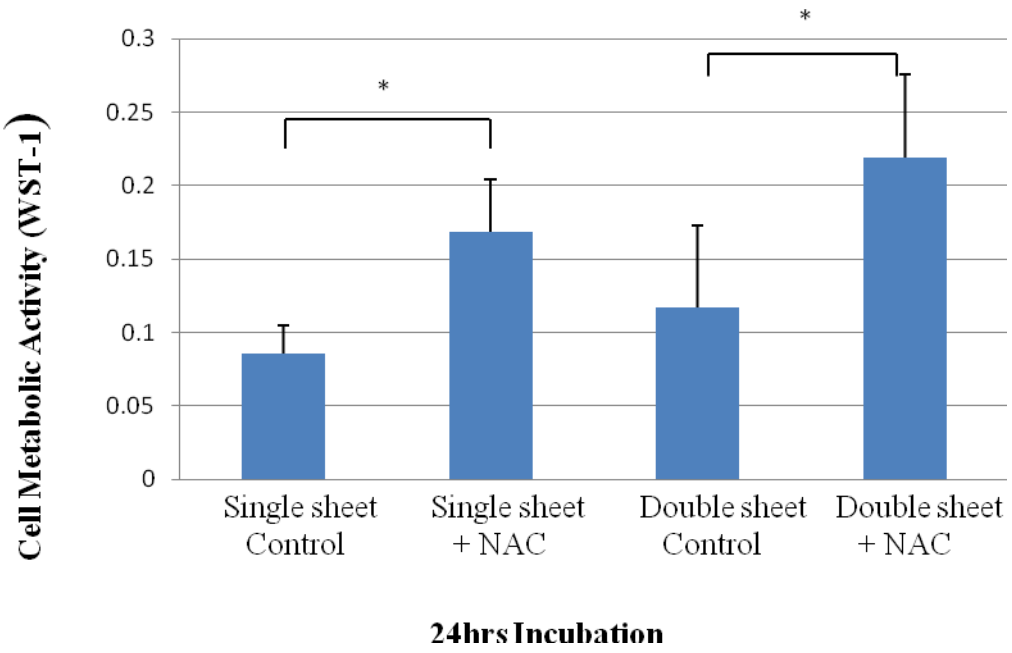


Fig:. Comparison of cell metabolic activity assays performed at 24hrs after transferring single and double layered cell sheets with and without NAC treatment. The results show an increase in cell metabolic activity of cells treated with NAC after transfer as compared to cells without NAC treatment. The double layering of sheets showed an increase in the cell metabolic activity as compared to the single layered sheets.

4.3.5 Alkaline phosphatase activity confirming the osteogenic phenotype in the cells:

When the cell sheets were 80% confluent at day 7, the cell sheets were transferred to the titanium surfaces and NAC was added to the cell sheets at the 6hr time point. ALP assay was performed at day 5 after transferring the cell sheet to the titanium surface. The results shown in the figure below confirm that the groups with NAC added to the media after transferring the cell sheets showed an increase in phenotypic activity when compared to similar groups without the NAC acting as a biological enhancer. Adding a second layer of NAC treated cell sheet increased the phenotypic activity of the cells as expected.

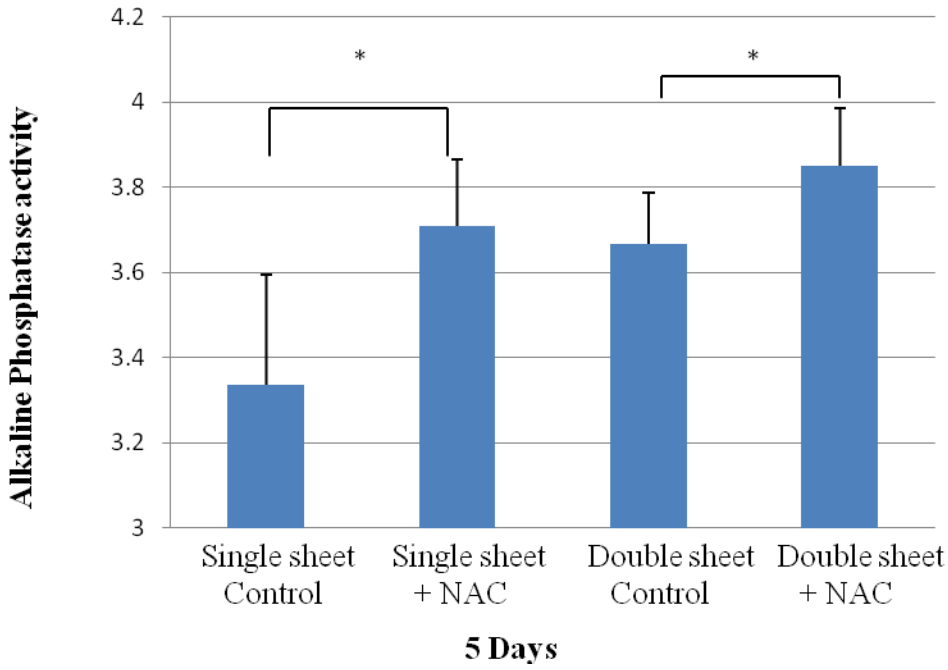


Fig: Confirmation of increase in osteoblastic phenotype of cell sheets treated with NAC and also increase in phenotypic activity of double layered cell sheets compared to single layer. The single sheet treated with NAC shows almost double the activity as compared to the single sheet which has not been treated with NAC. The double sheet treated with NAC shows 25% increase in phenotypic activity as compared to the double sheets without NAC.

4.4 Discussion:

In this study we established that the cell metabolic activity can be increased by adding a biological enhancer molecule, NAC to the transferred cell sheet. The cell metabolic activity also increased with layering the cell sheets one on top of the other due to an increase in the number of cells delivered to the titanium surface. The enhancement of cell metabolic activity by addition of NAC showed a dose dependant increase depending on the amount of NAC added and also depending on the number of layers of the cell sheets. These results suggest the feasibility and effectiveness of NAC treated cell sheet coating on enhancing the biological processes of the cells.

It should be noted that the cell metabolic activity was correspondingly increasing depending on the number of cell-sheet layers added to the titanium substrate which proves that providing an abundance of cells along with the implant complex has influenced the activity of cells around the implant surface and has set the stage for faster and improved cell metabolic activity levels. The delivery of an increased number of cells was made possible due to the osteogenic sheet as opposed to individual cells seeded onto a surface. This is also confirmed by the corresponding increase in cellular metabolic activity depending on the number of cell-sheet layers stacked together. By harvesting cell sheets we also preserve the adhesion molecules that cells secrete thereby allowing the layers of cells attach to one another. This allows for increase in signaling between the cells which might be one of the reasons for improved cell metabolic activity and also enhanced phenotypic activity.

By treating the cell sheets with a biological enhancer we were able to induce an osteoblast differentiating environment which is much needed for the bone growth and differentiation. The

mineralizing capacity of the implant interface is influenced by the degree of osteoblastic differentiation within the cell sheets when transferred to the titanium.

As mentioned previously, cell sheet technology can be very technique sensitive. The temperature responsive dishes are sensitive to the changes in temperature and hence when we remove the culture dishes from the incubator a decrease in temperature for an elongated period of time can lead to the detachment of cells from the culture surface. When performing the above assays and while changing the media in the cell culture dishes we had to take a lot of precautions to make sure the cells do not detach spontaneously. Addition of NAC as a biological enhancer has improved the biological capability of the osteogenic cell sheet and we will be able to use the effect of NAC treatment when transferring the cell sheet onto the titanium surfaces.

CHAPTER 5

Examine the in-vivo effect of the osteogenic cell sheet-implant complex on the enhancement by increasing the strength and speed of osseointegration and peri-implant bone morphogenesis.

5.1 Introduction:

Despite successful endeavor to date in developing, establishing, and improving implant therapy, several issues directly related to the biological capability of titanium remain unresolved. For dental implants, the protracted healing time, various risk factors that still exist, including but not limited to host bone anatomy, diabetes, osteoporosis, smoking and aging may limit the application and reduce the success rate. For orthopedic implants, the treatment outcome includes a high percentage of revision surgery ranging from 5-40% (average, 25%). Rapid and firm establishment of bone–implant integration has therefore been a persistent challenge in both fields.

To address these challenges, there still is an unknown, critical question why bone tissue does not form entirely around implant surfaces. The question should be of extreme importance but seems to have been unanswered or overlooked in the history. The implant area eventually covered by bone (bone–implant contact percentage) remains at 45-60%, or 50–65% in the literature. Most implants fail because of an incomplete establishment or early/late destructive changes at the bone–implant interface. We hypothesized that (1) a finite supply of stem/osteoprogenitor cells around implant surfaces may explain the bone-implant contact that is far below an ideal level of 100% in the literature, and (2) implant surfaces can be coated with a sufficient number of osteogenic cells in advance to overcome the problem and the process of osseointegration can be enhanced and expedited.

We utilized the cell sheet technology, literally a technology to fabricate a sheet of cells using chemically modified culture dishes to fabricate and harvest an osteogenic cell sheet which can be layered onto the implants. Because the cell sheet is harvested as a full density (over-confluency) of cells, it enables a local delivery of a maximum number of cells. When an autologous source of

cells is used, this technology provides opportunities to directly apply cell sheets to patch and repair damaged and diseased tissues and organs.

As mentioned earlier, one of the characteristics required by bone tissue engineering for scaffolds is that they must have a porous and interconnected structure, enabling the migration and distribution of cells. Several materials have been investigated as potential scaffolds for bone tissue engineering. The ideal scaffold should provide a suitable environment for tissue development. It should favor cell attachment, growth and differentiation, in vivo revascularization, integration with the host tissues, and the gradual replacement of the scaffold by newly formed tissues. At the same time, the materials and their degradation products must be non-toxic and non-immunogenic.

Essentially, when we place an implant into the bone we are creating a defect in the bone which causes a loss of cells, signaling and bone structure. In order for the implant to be successful it has to be integrated into the bone which is dependent upon the magnitude of the bone directly deposited onto the titanium surface without any soft/connective tissue intervention. For any organ regeneration including bone, the three important factors needed are cells, signaling between the cells and the scaffolding. When placing the implant into the bone we disrupt all of these. From the perspective of tissue engineering, titanium surfaces appear to work as a scaffold, but as previously mentioned we are disrupting the cell signaling and removing bone structure from the implant surgery site. Along with this, the various risk factors for dental implants such as, host bone anatomy, diabetes, osteoporosis, various systemic conditions, smoking and aging lead to the protracted healing time (4-6 months) and failure of implant integration into the bone and invariably reduce the success rate. In the U.S, 10% of the adults and one-third of adults aged

>65years are fully edentulous. Despite the need in an aging society, dental implant therapy has been employed in only 2% of the potential patient population.

We hypothesized that by providing the essential factors needed for bone regeneration through the layering of an osteogenic cell-sheet onto the implant in advance, this will provide an implant with the scaffolding and cells in abundance along with the signaling between the cells intact which can improve the bone-titanium integration by regenerating bone in between the implant and the host bone and thereby improving the osseointegration and increasing the success rate of implants. After performing the in-vitro studies as stated earlier, in this particular study we wanted to implement the cell sheet technology to fabricate an osteogenic cell sheet, layer it onto the implant surface and place it into the femurs of rats to study the enhanced osseointegration capabilities of the cell sheet layered implant complex.

5.2 Materials and Methods:

5.2.1 Titanium mini-implant preparation:

Titanium cylinders (1mm diameter, 2mm length) were prepared by machining Ti–15Mo–5Zr–3Al alloy. To create microroughened morphology with peaks and valleys, titanium samples were acid-etched with 67% H_2SO_4 at 120 °C for 75 s. The titanium mini-implants were autoclave-sterilized and stored under dark ambient conditions for 4 weeks, which allowed sufficient aging of the surfaces and helped to standardize the surface energy. They were then used for layering the cell sheets for in-vivo surgeries.

5.2.2 Bone marrow cell preparation:

In order to perform a surgical placement of a cell sheet layered implant it was essential that we collect bone marrow cells from rats, fabricate sheets and place the cell sheet layered implant into the same rats to avoid any host-cell reaction. The first surgery involved collecting bone marrow cells from the left femurs of 8-week-old male Sprague-Dawley rats which were inoculated into alpha-modified Eagle's medium supplemented with 15% fetal bovine serum, 50 mg/ml ascorbic acid, 10 mm Na-β-glycerophosphate, and antibiotic–antimycotic solution. Cells were incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. At 80% confluency, cells were detached using 0.25% trypsin-1 mm EDTA-4Na and seeded onto temperature-responsive culture dishes at a density of 3×10^4 cells/ml. The culture medium was renewed every 3 days. At day 7 of culture the cell sheet grown in the incubator was carefully layered onto the mini-implant by rolling the mini-implant in the cell sheet dish. This cell-sheet layered implant was then placed into the right femurs of the rats.

5.2.3 Osteogenic cell-sheet fabrication:

The culture medium in the temperature responsive dishes was renewed every three days until the cultures were confluent and ready to harvest between days 7-9. The culture medium was devoid of dexamethasone so as to have the stem cell lineage of the cells. The sheets were observed every day for confluency and used for surgical placement once they were ready at day 7. The mini-implant was coated with the cell sheet.

5.2.4 Animal surgery:

Twelve eight-week old male Sprague-Dawley rats were used in this study. The rats were anesthetized by inhalation with 1-2% isoflurane. After their legs were shaved and scrubbed with 10% providine-iodine solution, the distal aspects of the left femurs were carefully exposed via skin incision and muscle dissection. The knee was exposed and by making a small hole by drilling with a 0.8mm bur, the bone marrow cells (BMCs) from the femur was collected by aspiration. Surgical sites were then closed in layers. The collected BMCs were then cultured on temperature responsive dishes and the cell-sheets were harvested after a week. The second surgery was performed after one week, the rats and the cultured dishes were labeled, the distal aspects of the right femurs were carefully exposed. The flat surfaces of the distal femurs were selected for implant placement. The implant site was prepared 11mm from the distal edge of the femur by drilling with a 0.8mm round bur and enlarged using reamers. Profuse irrigation with sterile isotonic saline solution was used for cooling and cleaning. One cylindrical titanium implant layered with the autologous cell-sheet was placed into one femur. Surgical sites were then closed in layers. Muscle and skin were sutured separately with reasonable suture thread. The rats were sacrificed after two weeks. The protocol was approved by the Chancellor's Animal Research Committee at the University of California at Los Angeles (UCLA) and all experimentation was performed in accordance with the United States Department of Agriculture(USDA) guidelines of animal research.

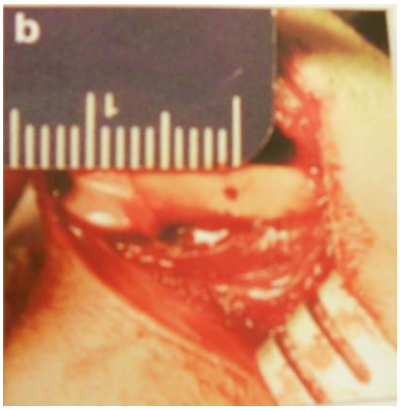


Fig: Mini-implant layered with a cell sheet placed into the right femur of the rat.

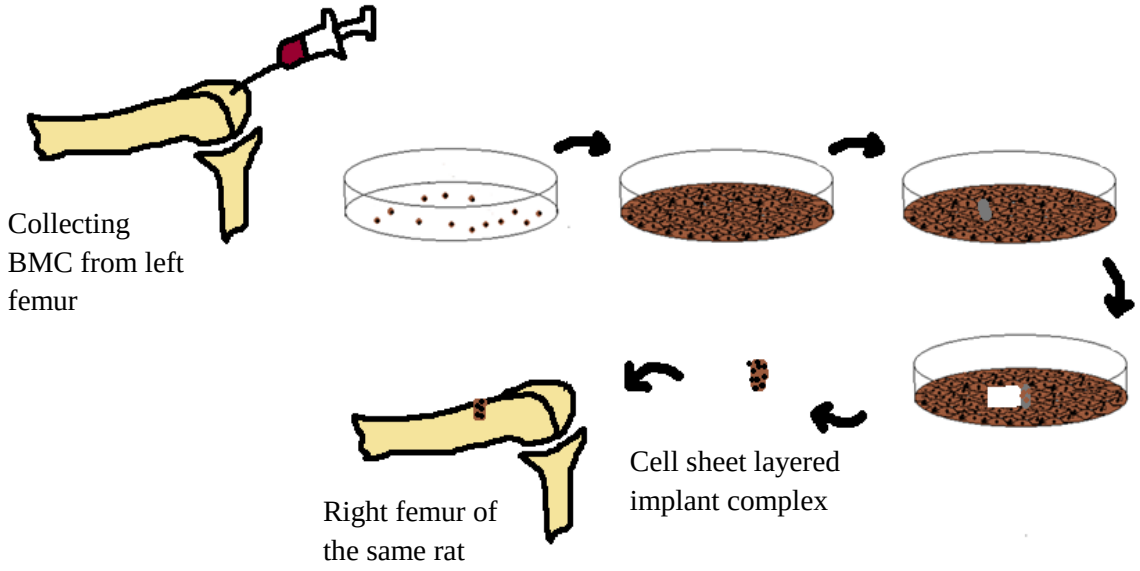


Fig: Step-by-step representation of collecting, seeding, transferring and placing an implant layered cell sheet complex into a rat femur. By following a carefully designed protocol the bone marrow cells were collected from the left femurs of the rats and the implant was placed into the right femur.

5.2.5 Implant biomechanical push-in test:

The established implant biochemical push-in test was used to assess the biomechanical strength of bone-implant integration[26, 27]. At week 2 of healing, the femurs containing a cylindrical implant were harvested and embedded into autopolymerizing resin with the top surface of the

implant being horizontal. The testing machine (Instron 5544 electro-mechanical testing system; Instron, Canton, MA) equipped with a 2000N load cell and a pushing rod (diameter=0.8mm) was used to load the implant vertically downward at a crosshead speed of 1mm/min. The push-in value was determined by measuring the peak of the load-displacement curve.

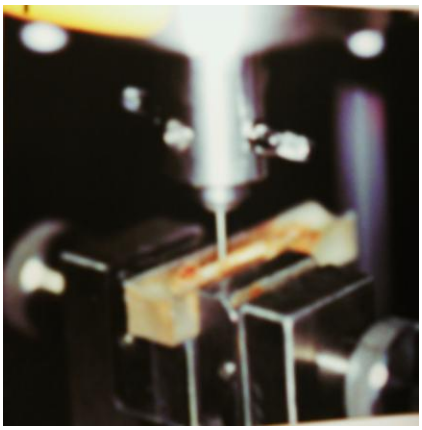


Fig: Picture of an instron machine used to perform the implant biomechanical push-in test.

5.2.6 Morphological and elemental analyses of implant/tissue interface after push-in test:

Morphological and elemental analyses of implant surfaces after the push-in test were found to be useful for examining the quality of bone- implant integration, in particular, adhesion property at the interface, and morphological and structural properties of the bone [14]. After push-in test, the bone implant complexes from selected implant specimens were carefully exposed and soaked in agitated water for 1 hr and dried under heat and vacuum. After coating with carbon sputter, the specimens were examined by SEM. The elemental compositions of the tissues and the implant tissue interface were analyzed by EDX spectroscopy(JSM-5900LV, Joel Ltd, Tokyo, Japan). Both control and cell-sheet layered implants were retrieved and observed under SEM. One implant from each group, depicting representative morphology of tissue, was presented.

5.2.7 Statistical analysis

In vivo experiments were performed in six rats (n=6). Three samples were used for biomechanical and histomorphometric studies and nine samples (n=9) were used for roughness and elemental analyses of titanium samples. The difference among the experimental groups was examined by 1-way ANOVA. If needed, Bonferroni multiple comparison test was performed ad hoc; $p<0.05$ was considered statistical significant.

5.3 Results:

5.3.1 Increased strength of osseointegration for cell-sheet layered implants:

After establishing the advantages of the cell sheet technology in improving the cell metabolic activity and osteoblastic phenotypic activity through in-vitro studies performed, we wanted to determine whether the level of bone-implant integration by a cell-sheet layered implant was higher than the control. Bone marrow cells were collected from the left femurs of the rats as mentioned earlier, seeded onto temperature responsive surfaces and grown to day 7 by changing media every three days. The cell sheet-layered implant was placed into the right femur of the same rat surgically. Controls were placed into the left femurs of the rats. The femurs were harvested from the rats two weeks post surgically. The established Instron biomechanical push-in test was performed. The biomechanical push-in test showed that the osteogenic cell sheet layering of the implant resulted in a significant increase in the push-in value after the two week healing period. The push-in values showed that the cell sheet-coated implants showed a 2.2-fold stronger osseointegration than non-coated implants ($p<0.01$). From these results we can interpret that bone formation around the cell sheet layered implant was much stronger than the control.

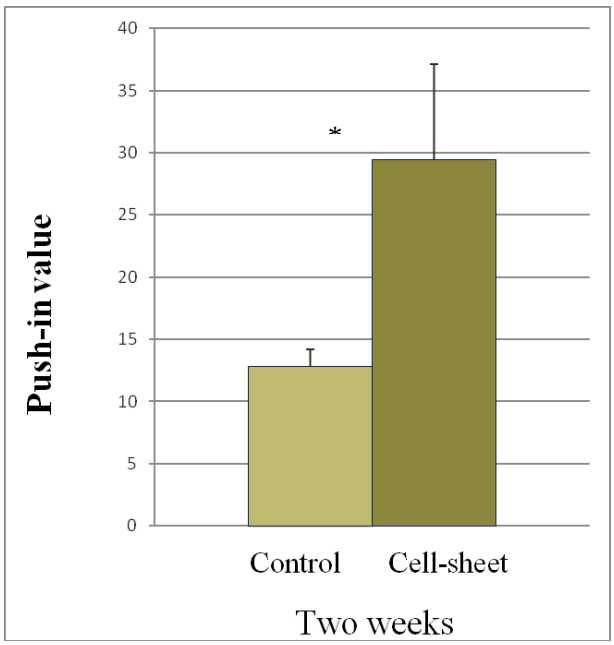


Fig: Push-in values of control and cell sheet layered implants. The cell-sheet layered implant showed a 2.2 fold increase in strength of osseointegration as compared to the control.

5.3.2 Morphological and elemental analyses of implant/tissue complex after push-in test:

The implant-tissue interface after the push-in test was examined by SEM and EDX for morphology and elemental composition, respectively. The SEM images of implant-tissue complexes of control and cell sheet layered implants retrieved from 2 week specimens are presented here. As shown in the images we can see that both control and cell-sheet layered implants showed biological structures remaining at the interface. The biological structures from both implants contained elemental peaks of Ca and P which demonstrate bone tissue. These results show that the coverage of bone tissue was present in both the implants. However, there were high peaks of Ca in all three zones of the cell-sheet layered bone when compared to the Ti peaks, especially in the bone-marrow zone which is why we had to split the graph. The Ca/Ti ratio in this zone for the cell-sheet layered implant is much higher than the control. This can be interpreted as an increase in bone formation around the cell-sheet layered implant surface as

compared to the control. This proves that the cell-sheet layered implant induced bone formation much faster than the control.

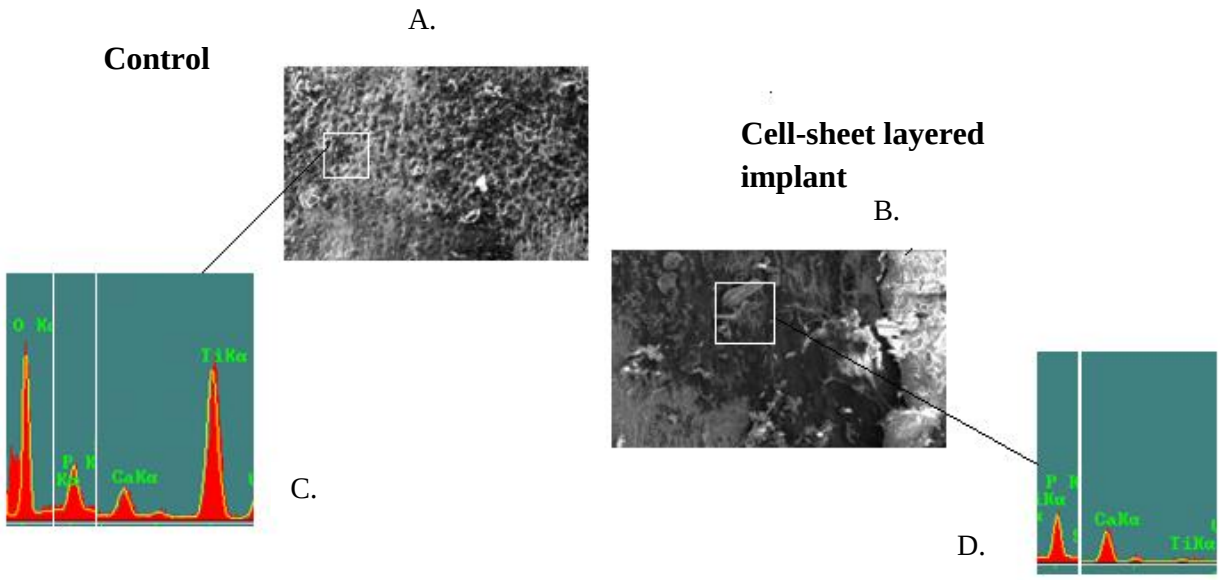
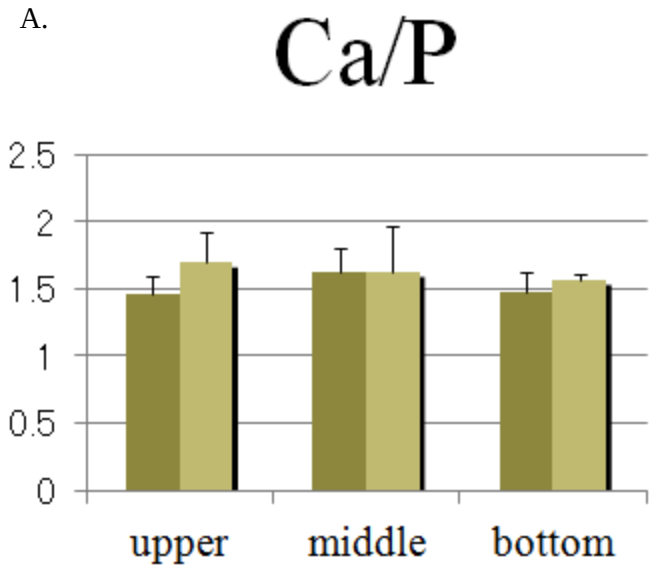


Fig: Morphological and elemental analyses of the control and cell-sheet layered implants at an early healing stage of week 2. The implants were retrieved after push-in test and tissue surfaces were exposed and analyzed by SEM and EDX.(A,C) are high magnification image and EDX spectra showing the Ca and P levels with a high Ti peak. (B,D) are high magnification image and EDX spectra of a cell-sheet layered implant showing the Ca and P levels but very little Ti peak. These results show that the bone formation was more in the cell-sheet layered implant.

A.



B.

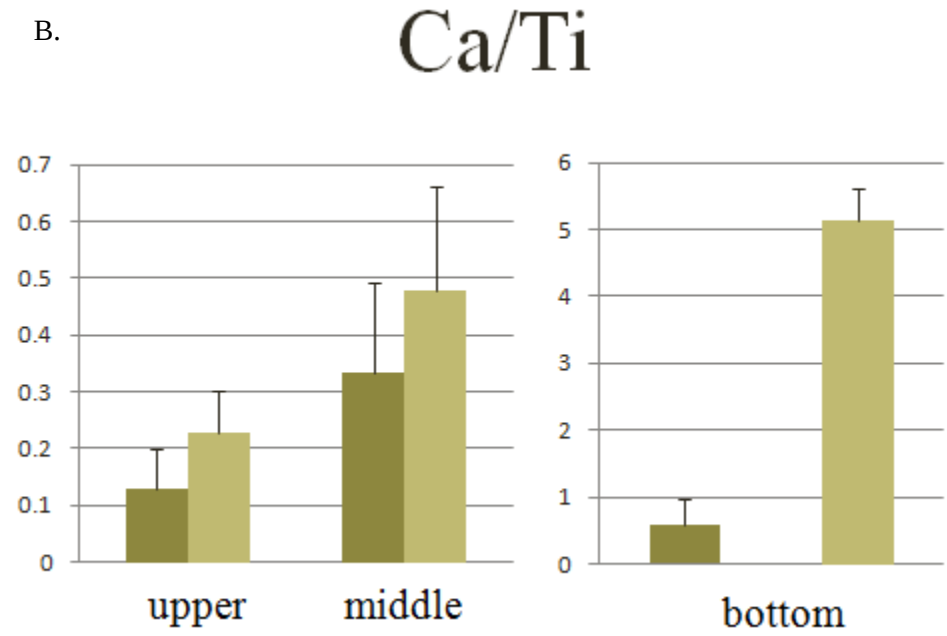


Fig: Fig A represents the Ca/P ratio of the elemental analyses in all three zones of control and cell-sheet layered implants. These results show that there was bone tissue around both implants.

Fig B represents the Ca/Ti ratios which show that the cell sheet layered implant showed more Ca peaks as compared to the Ti peaks in all three zones of the cell sheet layered implant when compared to the control. The bone marrow zone (bottom) showed an even higher Ca/Ti ratio and hence had to be split from the graph. This shows increased bone formation around the cell sheet layered implant.

5.4 Discussion:

This is the first study to introduce the effect of a cell-sheet layered implant complex on the bone-titanium integration capacity of implants. In this study we demonstrated the feasibility of fabricating an osteogenic cell-sheet and transferring or layering the cell-sheet onto the implant. We have established an increased strength in osseointegration of the cell-sheet implant complex as compared to the control. These results suggest the feasibility and effectiveness of cell sheet coating on titanium implants in enhancing the biological process of osseointegration.

It should be noted that the cell metabolic activity was correspondingly increasing depending on the number of cell-sheet layers added to the titanium substrate which proves that providing an abundance of cells along with the implant complex has influenced the activity of cells around the implant and has set the stage for faster and improved bone-titanium integration. The delivery of an increased number of cells was made possible due to the osteogenic sheet as opposed to individual cells present around the implant. By harvesting cell sheets we also preserve the adhesion molecules that cells secrete thereby allowing the layers of cells attach to one another. This allows for increase in signaling between the cells which might be one of the reasons for improved osseointegration. Mesenchymal stem cells (MSCs) existing in bone marrow have the potential to differentiate into osteoblasts, adipocytes, chondrocytes, neurons and myogenic cells. With the fabrication of osteogenic cell-sheets we were able to eliminate the use of scaffolds and we were able to deliver a pre-osseointegrated complex in the form of a titanium implant coated with the osteogenic cell-sheet into the femurs of rats. By placing this complex into the femurs we have delivered an abundant number of cells, which still had the signaling present between them and hence provided an environment for faster and better bone formation around the implant. Thus the osteogenic cell-sheet layered bone-implant complex provides increased initial

anchorage and stability for the implant and hence increases the initial bone-implant contact levels and also enhances the signaling between the cells which establishes the initial factors needed for the bone regeneration.

This study has shown the feasibility of fabricating an osteogenic cell sheet and transferring it to a titanium substrate and a mini-implant to create an osteogenic-cell-sheet complex for experimentation in rats. Although previous studies done with cell-sheet technology have proved that cell-sheets can be successfully harvested and transferred to human corneal epithelium and other organ regeneration was made possible, this is the first study that introduces this technique for implementation with titanium implants and hence it is still in the preliminary stages and clinical research with human subjects has to be done to confirm the results with humans. Harvesting the cell sheet is very technique sensitive and has to be done very methodically. With rat osteogenic sheets we were able to harvest and transfer sheets to the mini-implants as there was an abundance of sheet area. Although further in vitro and in vivo studies are required to accumulate the data, this study demonstrated the great potential of cell sheet technology for titanium as a novel cell therapy for dental implants.

CHAPTER 6

Conclusion

6.1 Conclusion:

This study introduced cell-sheet technology in improving bone-titanium integration. The first part of the study implemented cell sheet technology to fabricate an osteogenic cell sheet which was viable and showed expected cell metabolic activity levels and osteoblastic phenotypic activity which were comparable to cells grown on the regular culture dishes. We then successfully transferred the osteogenic cell sheet, created from rat bone marrow cells, as a single intact layer to a titanium surface without any bubbles or tears and we were able to maintain the cell metabolic activity levels and phenotypic activity levels post transfer. Because of the deposited ECM and cell-to-cell attachment we were able to transfer the cell sheet and also layer it one on top of the other. These double layered sheets were able to retain the functional capability and phenotypic activity of the cells and showed a 2-fold greater cell metabolic activity than the single layered sheets. The addition of a biological enhancer, NAC showed increase in the cell metabolic activity levels and greater ALP activity in a dose dependant manner. Finally, we were able to implement the cell sheet technology for in-vivo testing by layering a cell sheet around the implant and creating a unique osteogenic cell sheet layered implant complex. These implant complexes showed a 2.2 fold increase in push-in value when compared to the uncoated implants.

This is one of the first studies that has implemented cell-sheet technology in improving the osseointegration capability of implants. Transferred cell sheets to titanium surfaces are capable to show reliable osteogenic phenotypes and to accelerate the bone-titanium integration, can be enhanced by foreign molecules and show increased cell metabolic activity levels depending on the number of layers of cell sheets transferred.

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