Osteoblastic Differentiation of hMSCs exposed to Alternating Current Electric Fields

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Department of Biomedical Engineering

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I declare that this written submission represents my ideas in my own words, and where other's ideas or words have been included, I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that any violation of the above will be a cause for disciplinary action by the Institute and can also evoke penal action from the sources that have thus not been properly cited, or from whom proper permission has not been taken when needed.

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Approval Sheet

This thesis entitled – Osteoblastic Differentiation of hMSCs exposed to Alternating Current Electric Fields – by – Sundeep Bethapudi – is appróved for the degree of Master of Technology from IIT Hyderabad.

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Dedicated to

My Family and Friends

Abstract

Mesenchymal stem cells (MSCs) have become a major research area for the past decade. Their tremendous ability to regenerate and to differentiate into different cell types has given hope in tissue regeneration and wound healing. The ability to control their differentiation in vitro has made possible to develop various types of tissue engineered products. Osteoblastic differentiation of MSCs which is considered as the process involved in bone healing, can be achieved fast when exposed to alternating current (A.C) electric fields. Proving osteoblastic differentiation of MSCs when exposed to current electric fields in vitro can lead to the development of products that can help in bone healing process. In this thesis a bioreactor is designed to stimulate MSCs in culture.

Different designs were designed and implemented to discover the ideal conditions for Osteoblastic differentiation. Some of the designs were based on previous studies and some are designed by calculating the property of electric field intensity inside the bioreactor. The designs are simulated in Comsol Multiphysics software to observe the field distribution inside the bioreactor.

MSCs were successfully isolated from various sources and are grown in culture. These are exposed to different voltages of A.C fields for certain number of days and time to achieve osteoblastic differentiation. Day to day images has been taken in order to observe the changes in cell morphology and behavior. The differentiation results were analyzed by different staining methods and assays.

MSCs exposed to A.C field of 20mv/cm and 60 kHz frequency in Osteoinduction Media (OIM) has showed significant differentiation when compared to the MSCs which are grown in OIM without any electrical stimulation.

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Nomenclature

hMSCs: human Mesenchymal Stem Cells

hASCs: human Adipose-derived Stem Cells

hUVSCs: human Uumbilical Vein Stem Cells

hUVECs: human Umbilical Vein Endothelial Cells

bAECs: bovine Heart Aortic Endothelial Cells

PBS 1x: Phosphate Buffer Saline of 1x concentration

ALP: Alkaline Phosphatase

PG: Pico Green

AR: Alizarin Red

DMEM: Dulbecco's Modified Eagle Medium

EGCM: Endothelial Growth Conditioning Medium

OIM: Osteoinduction Medium

FBS: Fetal Bovine Serum

A.C: Alternating Current

D.C: Direct Current

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Chapter 1
Introduction

1.1 Background and Motivation

MSCs play a significant role in bone healing and tissue regeneration. Making the MSCs to differentiate into osteoblasts can significantly affect the bone healing process by time constraint. Electrical stimulation induces directional migration, reorientation, elongation and differentiation in different types of cells. Combining both the principles of differentiation and stimulation, the process of differentiation can be increased which can improve the bone healing process.

1.2 Contribution of the Thesis

The primary objective of this work is to achieve Osteoblastic differentiation of hMSCs in the presence of OIM and A.C fields. A bioreactor was successfully designed and simulated. Different types of cells are stimulated by A.C fields to set the optimum voltage for hMSCs to differentiate. Using the optimum voltage of 20mV hMSCs were stimulated inside the bioreactor and Osteoblastic differentiation was achieved.

1.3 Organization of the Thesis

The thesis is organized as follows:

Chapter 2: provides an overview of MSCs, Osteoblastic differentiation and various methods of stimulation.

Chapter 3: presents the isolation of different cells and various designs of bioreactor and their simulations.

Chapter 4: in this chapter, experimental setup and various procedures have been described and results were discussed.

Chapter 5: contains summary of Osteoblastic differentiation by A.C fields with conclusion and future work.

Chapter 2

Mesenchymal Stem Cells and Osteoblastic Diffentiation

2.1 Introduction to Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are multipotent stromal cells. MSCs can differentiate into various cell types such as chondrocytes, myocytes, fibroblasts, astrocytes, stromal cells, adipocytes and osteocytes. MSCs are found in various tissues and parts in the human body such as bone marrow tissues, adipose tissues, umbilical cord, adult muscle, corneal stroma, and deciduous baby teeth. The youngest, most primitive MSCs can be obtained from the umbilical cord tissue – wharton's jelly and the umbilical cord blood. An extremely rich source for MSCs is the adipose tissue. There are more than 500 times more stem cells in 1gm of fat than in 1gm of aspirated bone marrow. MSCs eventually form a minimum of 29 different unique end organs. Because of the extreme ease in collection the umbilical cord is the most preferred source of MSCs. The umbilical cord is easily obtained after the birth of a newborn and is normally thrown away. MSCs from umbilical cord tissue have more primitive properties than the other adult MSCs obtained later in life.

MSCs have a great capacity for self renewal while maintaining their multipotency. MSCs are key to regenerative wound healing, they have a spatial memory and respond to local environment. MSCs orchestrate wound repair by cellular differentitaton, immune-modulation, secretion of growth factors that drive neovascularization and mobilization of resident stem cells [1].

In addition to multilineage differentiation, MSCs regulate immune response and inflammation and possess powerful tissue protective and reparative mechanisms, making these cells attractive for treatment of different diseases [2].

2.2 Differentiation

Cellular differentiation is the process by which a cell changes from one cell type to another. In other words it can be said that a cell of less specialized type becomes a more specialized type during its growth. Differentiation occurs numerous times during the development of a multicellular organism. Differentiation continues in adulthood as adult stem cells divide and create fully differentiated daughter cells during tissue repair or wound healing process. Differentiation dramatically changes a cell's size, shape, membrane potential, metabolic activity and responsiveness to signals. Each specialized cell type in an organism expresses a subset of genes and have a particular pattern of regulated gene expression. Cellular differentiation is thus a transition of a cell from one cell type to another and it involves a switch from one pattern of gene expression to another.

Molecular processes play a vital role in cellular differentiation via cell signaling. Signal molecules that convey information from cell to cell during the control of cellular

differentiation are called growth factors. Because of this reason, a special medium (induction medium) which contains necessary growth factors will be added which culturing cells in vitro conditions in order to achieve a particular differentiated cell type.

2.2.1 Osteoblastic Differentiation

Osteoblastic differentiation is the process whereby a relatively unspecialized cell acquires the specialized features of an osteoblast, a mesodermal or neural crest cell that gives rise to bone.

Osteoblasts are specialized, terminally differentiated products of MSCs. They synthesize very dense cross-linked collagen and several specialized proteins which comprise the organic matrix of a bone. Osteoblasts produce a calcium and phosphate based minerals called hydroxyapatite that is deposited into the organic matrix of the bone to form a very strong and dense mineralized tissue.

In the process of Osteoblastic differentiation, MSCs differentiate into osteoblasts under the influence of growth factors by forming calcium and alkaline phosphatase which is a key enzyme that provides high concentrations of phosphate levels.

2.3 Stimulation

Osteoblastic differentiation of hMSCs can be achieved by different types of Stimulation.

2.4 Types of Stimulation

2.4.1 Mechanical Stimulation

Mechanical Stimulation is one way by which osteoblastic differentiation can be achieved. MSCs can be differentiated into fibroblasts, chondrocytes and osteoblasts by the magnitude of mechanical stimuli. In some cases the magnitude and frequency of mechanical stimuli determines the proliferation rate of the cells. Numerous mathematical algorithms were designed and modeled to control the differentiation and proliferation patterns in the process of bone healing. Those models showed better results in bone fracture healing process.

2.4.2 Electromagnetic Stimulation

Electromagnetic Stimulation can also stimulate MSCs to differentiate into osteoblasts. This is purely a non invasive way of stimulating cells. No electric field is in direct contact with the media or the cells.

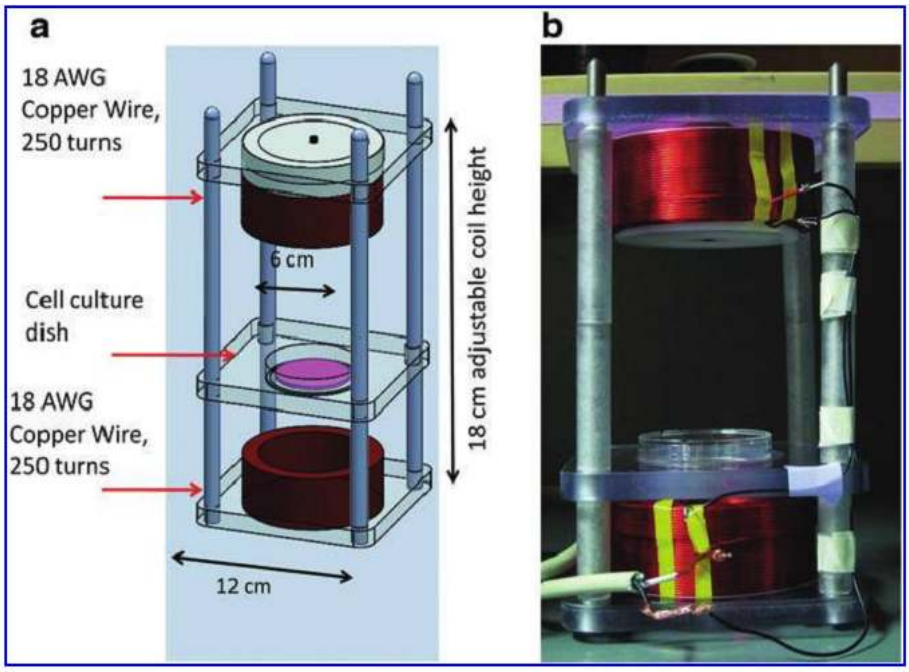

Figure 2.1: Electromagnetic stimulation chamber

2.4.3 Pharmacological or Chemical Stimulation

Adding different chemicals to the media in which the MSCs are grown, can effect significantly to make the cells to differentiate into osteoblasts. Normally chemicals like Dexamethasone, B-Glycerophosphate and Ascorbic acid induces differentiation in MSCs. Media containing these types of chemicals is normally termed as Osteoinduction Medium (OIM).

2.4.4 Electrical Stimulation

The biological electric field and current generated by human body is a well-known phenomenon. There have been case reports of bone healing by electrical stimulation as early as 18th century, but this method of treatment and trails did not progress until 1950. In 1953, an experiment conducted by applying continuous current on the femur of a rabbit for three weeks resulted in new-bone formation. It became known that electricity will have a significant effect on bone formation. To study the in depth mechanism of this process, researches around the world have been studying and exploring new methods starting from the cellular level. Osteoblastic differentiation in vitro conditions is a better way to understand and explore new ways of bone healing process. Direct current, capacitive coupling and inductive coupling are different modes of electrical stimuli used to enhance bone healing.

Both A.C and D.C electrical stimulation have been used in previous studies. Not all articles have reported differentiation when hMSCs exposed to D.C stimulation. A.C electrical stimulation has been used throughout this study.

2.5 Applications of Osteoblastic Differentiation

A variety of electronic devices are marketed as bone growth stimulators. These devices use electrical, electromagnetic, ultrasound and extracorporeal shock waves as stimuli to enhance the bone healing process [4].

Figure 2.2: Bone stimulators from ORTHOFLIX

2.6 Literature Survey

Different approaches have been introduced for Osteoblastic Differentiation especially when applying A.C electric fields.

A hypothetical voltage of 20mV/cm with a frequency of 60 kHz has been used to stimulate cardiomyocytes in culture. RPE [16]. [17] [18]. [19].

To calculate the electric field distribution inside the bioreactor gauss' law has been taken into consideration.

Gaussian law is given by:

$$E = \frac{1}{2\pi\varepsilon}\frac{\rho}{r} \qquad \text{...Equation 2.1}$$

The above law is modified for cylindrical coordinates by the following equation:

$$E_x = \frac{V_{ab}}{2ln\left(\frac{a}{d-a}\right)}\left[\frac{x-\frac{d}{2}}{\left(x-\frac{d}{2}\right)^2+\gamma^2} - \frac{x+\frac{d}{2}}{\left(x-\frac{d}{2}\right)^2+\gamma^2}\right] \qquad \text{...Equation 2.2}$$

[21]. [22]. [23]. [24]. [25]. E is the electric field strength intensity at a particular point inside the bioreactor say (x, y). V_{ab} is the voltage drop across the graphite rods which is a known quantity which can be applied by a voltage source like function generator. The variable a is the radius of the graphite rod. d is the distance between the two graphite

rods. x is the distance of the point (x, y) in x-direction from the center of the graphite rod which is considered as anode. y is the distance of the point (x, y) in y-direction from the center of the graphite rod which is considered as anode. The same Equation 2.3 can be considered to calculate the electric field strength intensity due to the cathode by considering the distance of the point (x, y) with respect to the second rod which is considered as the cathode. The above parameters related to the experimental setup can be seen in the figure .

Chapter 3

Methods, Materials, Designs & Simulation

3.1 Isolation of hUVECs :

Step by step procedure (protocol) of hUVECs isolation:

1. Institutional ethics committee approved informed consent form was obtained from patient before parturition.
2. Umbilical cord was collected aseptically after child birth and was processed in IITH-RMS-tissue culture laboratory.
3. The cord was rinsed, washed with sterile PBS 1x for 2 to 3 times.
4. Umbilical vein was cannulated with vein cannula. The lumen cavity of umbilical vein was flushed with sterile PBS 1x to remove blood clots. Cord vein was blocked using an-artery forceps.
5. 0.15% collagenase type IV was incubated for 20 min at 37^0 C. the clamp was opened at the end of incubation period and flushed using DMEM.
6. The cell soup or digest was centrifuged at 1500 rpm for 10 min.
7. Cell pellet was washed twice by centrifugation and final pellet was re-suspended in endothelial growth medium (EGCM) containing 20% fetal bovine serum (FBS) and seeded on non-coated tissue culture plates.
8. After 12hrs of incubation hUVECs were fed with complete EGCM containing 20% FBS, 2mM L-Glutamine and incubated at 37C at 5% $C0_2$.

The seeded hUVECs of day0 and the confluent hUVECs on day 12 are shown in Figure 3.1

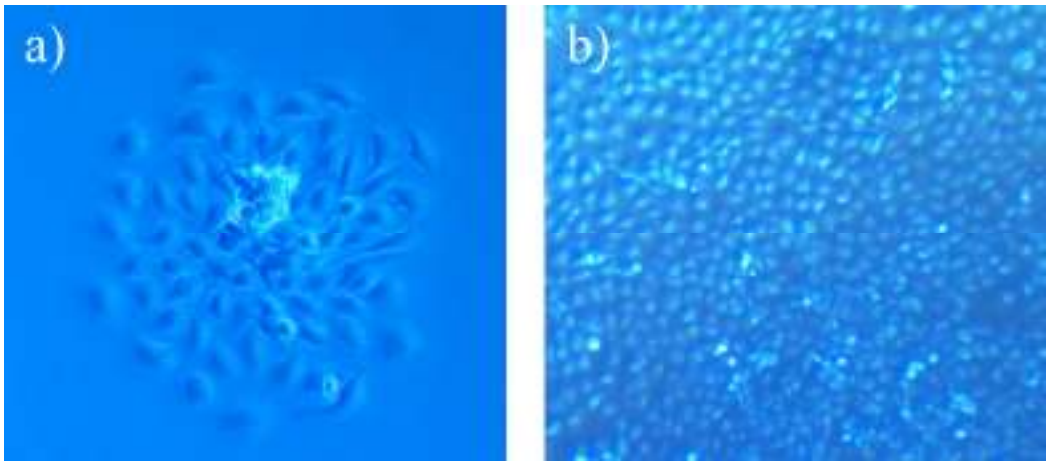

Figure 3.1: a)hUVECs on Day 1 b) hUVECs on Day 12

3.2 Isolation of hUVSCs:

Step by step procedure (protocol) of hUVSCs isolation from umbilical cord by explant method:

1. Institutional ethics committee approved informed consent form was obtained from patient before parturition.
2. Umbilical cord was collected in a falcon tube filled with DMEM and transported to the IITH-RMS-Tissue culture lab for cell harvesting.
3. The umbilical cord was cleaned to make it free from blood and blood clots.
4. A 5mm small cut was made in the Wharton jelly region and the pieces were rinsed in PBS 1x.
5. Care was taken before cutting in order to avoid a cut on venous and arterial parts of Wharton jelly which contains smooth muscle cells.
6. About 8 pieces of Wharton jelly explants were immersed and incubated in 0.1% collagenase solution for 5 min and further washed with PBS 1x.
7. The explants were directly placed in 100mm petri-dish as shown in
8. The explants were fed with DMEM which contains 20% FBS for 24hrs.
9. The explants were fed routinely for 5 days with a time interval of 24hrs.
10. The dissolved explants were removed and hUVSCs were refreshed and fed with DMEM which contains 20% FBS and 2mM L- Glutamine and incubated at 37^0C at 5% CO_2.

hUVSCs of passage 3 are used in Experiment 2.

3.3 Isolation of hASCs:

Step by step procedure (protocol) of hASCs isolation from lipoaspirate sample obtained from cosmetic surgery:

1. Institutional ethics committee approved informed consent form was obtained from patient before parturition.
2. A minimum volume of 250ml lipoaspirate is collected. The lipoaspirate sample is diluted with an equal volume of PBS and divided evenly in an screw cap conical bottom centrifuge tube.
3. Centrifuged for 10min. After centrifugation, the target cell containing lipid phase is removed from the top and poured in to a fresh screw cap conical bottom centrifuge tube. Again diluted with equal amount of PBS. This step is repeat one more time.
4. The aspirated lipid is diluted with an equal volume of the collagenase digestion solution. The mixture is transferred to a 1L storage bottle.
5. The mixture is incubated at 37^0C for 30min on a pre-warmed orbital shaker rotated at 250 rpm.
6. Equal volume of enzyme stop medium is added after 30 min.

7. Cell mixture is poured into fresh screw cap conical bottom centrifuge tube and centrifuged for 10min.
8. The supernatant is aspirated and discarded.
9. The cell pallet is resuspended in 10ml of NH expansion medium.
10. The cell suspension is passed through a 100 μm cell strainer, filtrate is collected in 50 ml conical tube and centrifuged for 10min.
11. Supernatant is discarded and remaining is resuspended in 5ml of NH expansion medium.
12. The cell suspension is passed through a 40 μm cell strainer, filtrate is collected in 50 ml conical tube.
13. Cells are seeded in 75cm^2 cell culture flask and cultivated at 37^{0}C 5%CO_2 in incubator.

hASCs were refreshed and fed with DMEM which contains 20% FBS and 2mM L-Glutamine and incubated.

3.4 Materials & Design of Stimulation Chamber

To study the behavior and osteoblastic differentiation of different cells under the influence of electrical stimulation, a particular design (Design1: Figure 3.2.) which was mentioned again and again across different articles was implemented in the Experiment1. Apart from that, different designs were modeled and implemented across different Experiments.

Material property is a major concern when it comes to cell behavior. Sometimes materials can harm, contaminate and can affect a cell in different ways. Care is taken while choosing materials for electrical stimulation process. The following are the different materials which were used across different designs.

3.4.1 Petri Dish

Tissue culture plate or simply known as petriplate was used for culturing cells. Circular Petri plate (NEST BIOTECH) of 60mm diameter was used in all the designs and experiments.

3.4.2 Graphite Rods

Graphite Rods which were used in most electroporation applications were used as cathode and anode to stimulate the cells. Graphite rods of research grade were purchased from LADD Research industries, USA.

3.4.3 Platinum Wires

Platinum is the best conductor of electricity apart from Gold. Researchers across globe used gold as the ideal material for cell based studies. Due to financial constraints, Platinum was used as the conducting material in the experiments. Platinum wires of research grade were purchased from LADD Research industries, USA.

3.4.4 Design 1:

All the designs are sketched using the software AUTOCAD 2010. Platinum wires are placed exactly at center of the graphite rods.

Diameter of Culture Plate = 55mm
Length of the Graphite Rods = 50mm
Diameter of the Graphite Rods = 3mm
Cover slip size = 22mm X 22mm
Distance between the Graphite Rods = 10mm
Applied Electric Potential = 40mV
Applied Frequency = 60 kHz

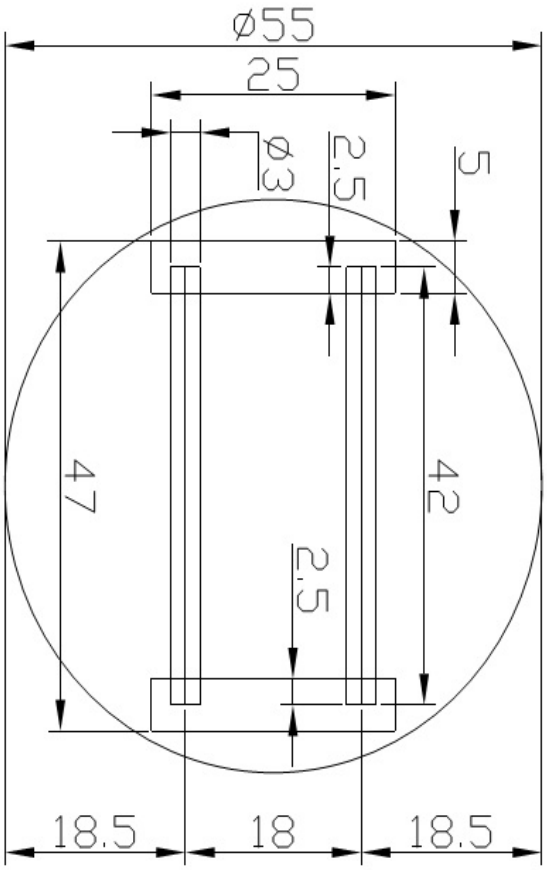

Figure 3.2: Stimulation Chamber Design 1 sketch

3.4.5 Design 2:

All the designs are sketched using the software AUTOCAD 2010. Platinum wires are placed exactly at center of the graphite rods.

Diameter of Culture Plate = 55mm
Diameter of the Graphite Rods = 3mm
Distance between the Graphite Rods = 20mm
Applied Frequency = 60 kHz
Length of the Graphite Rods = 50mm
Cover slip size = 22mm X 22mm
Applied Electric Potential = 20mV

3.4.6 Design 3:

All the designs are sketched using the software AUTOCAD 2010. Platinum wires are placed exactly at center of the graphite rods.

Diameter of Culture Plate = 55mm
Diameter of the Graphite Rods = 3mm
Length of the Graphite Rods = 30mm
Distance between the Graphite Rods = 10mm
Applied Electric Potential = 20mV
Applied Frequency = 60 kHz

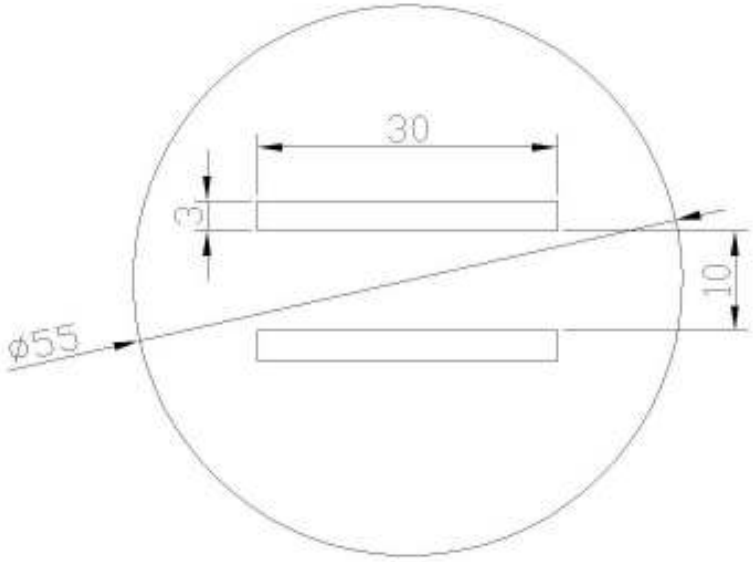

Figure 3.3: Stimulation Chamber Design 3 sketch

3.5 Comsol Multiphysics Simulation

Comsol multiphysics is a powerful tool that can modulate certain designs and parameters which are sometimes difficult to implement in practical cases. This tool runs on the mathematical models of Finite Element Methods and Mesh Analysis.

A major parameter that was supposed to be measured inside the petri plate is the Electrical Potential or the potential difference (E). This parameter cannot be measured with the experimental step that was used in this project. This parameter can be simulated in a computer by drawing the models of the designs.

Comsol Multiphysics ver 4.4 is used to simulate the electric field potential inside the petri plate. Different designs were implemented on COMSOL Multiphysics (The MathWork, Inc). Program runs on a laptop computer (64-bit OS, Intel(R) Core(TM) i5-2400 CPU@3.10GHz, 4GB RAM).

Assuming water as the medium (conductor) of electricity inside the petri plate, all the parameters of water are given as input in the medium properties.figure1 shows the medium parameters field in the comsol multiphysics software. To generate mesh, 0.00263mm is taken as element size as it is almost equal to the size of a human cell.

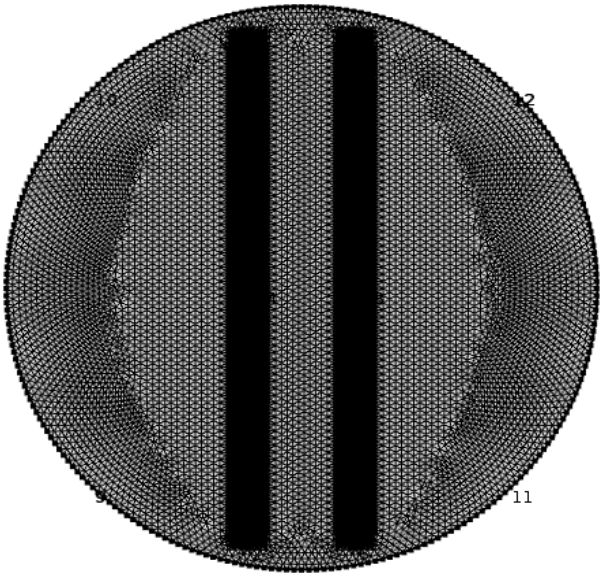

Figure 3.4: Stimulation Chamber Design 1 mesh size = 0.00263mm

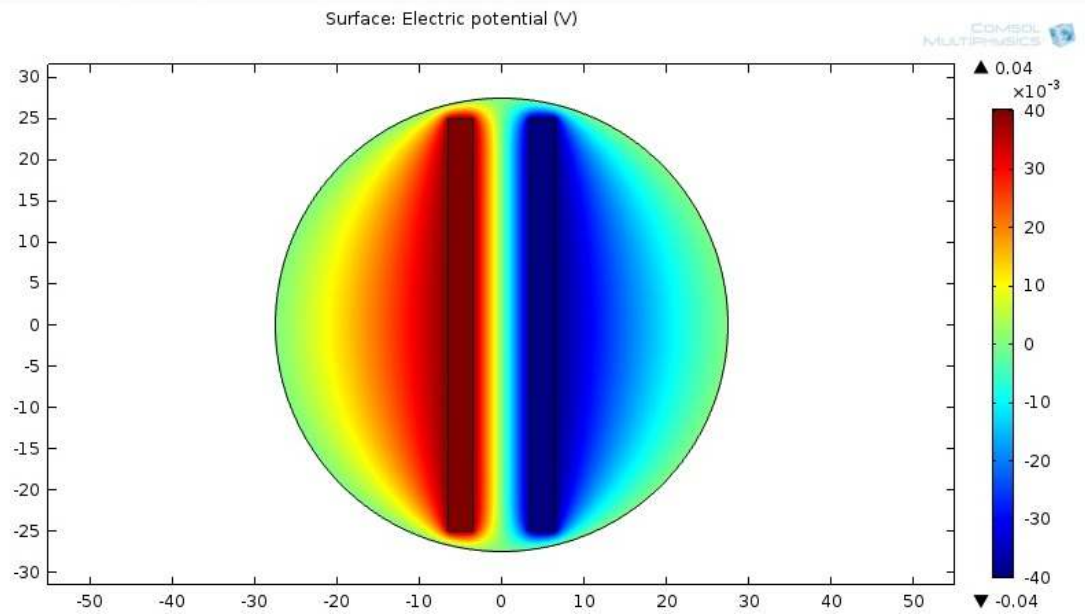

Figure 3.5: Electric Potential distribution of Stimulation Chamber of Design 1

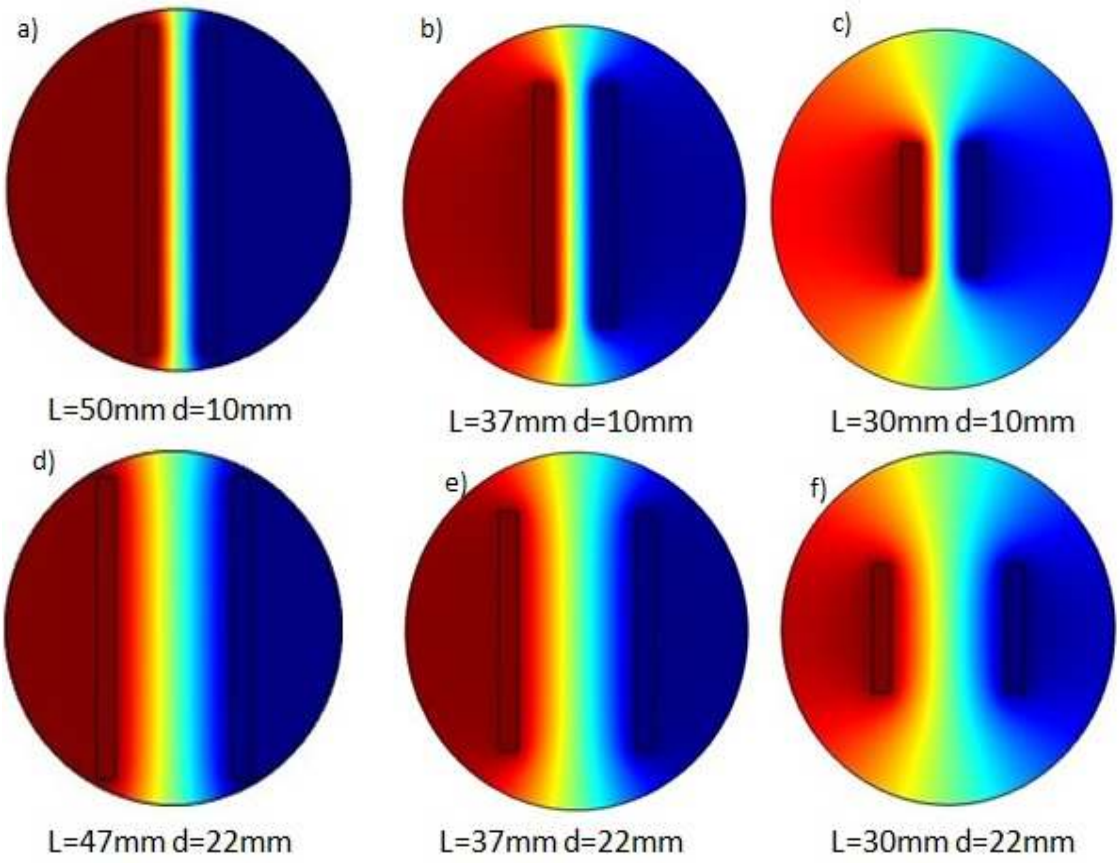

Figure 3.6: Electric Potential distribution of Stimulation Chamber with different parameters.

Figure 3.7: f) Considered as Design 2 c) Considered as Design 3

Based on the simulation results and considering different parameters figure 3.6 a, f and c are considered for experimentation. According to the parallel plate capacitor principle, the length of the graphite rods should be twice the distance between the rods to achieve the charge coupling effect. The design3 is expected to give optimum results for the experiments.

Chapter 4
Experiments and Results

4.1 Experimental Setup

A voltage source namely Function Generator (TEKTRONIX) was connected to the platinum wires of the stimulation chamber through normal copper wires which have alligator clips. The stimulation chamber was placed inside the co2 incubator to maintain optimal conditions of the cells. A measurement instrument namely Oscilloscope () was connected across the platinum wires to monitor the voltage across the Graphite rods (Cathode, Anode) [30]. Figure 4.1 shows the experimental setup.

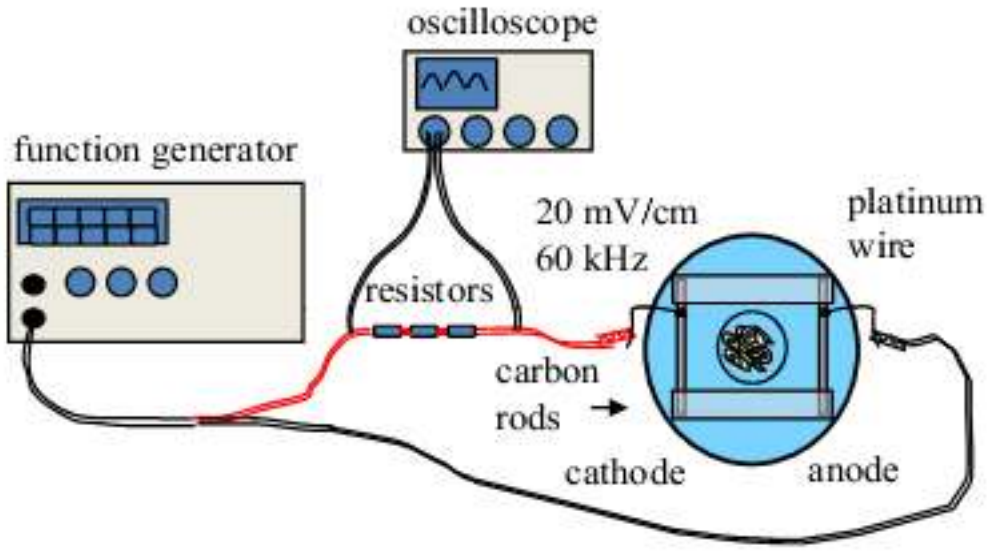

Figure 4.1: Block diagram of experimental setup.

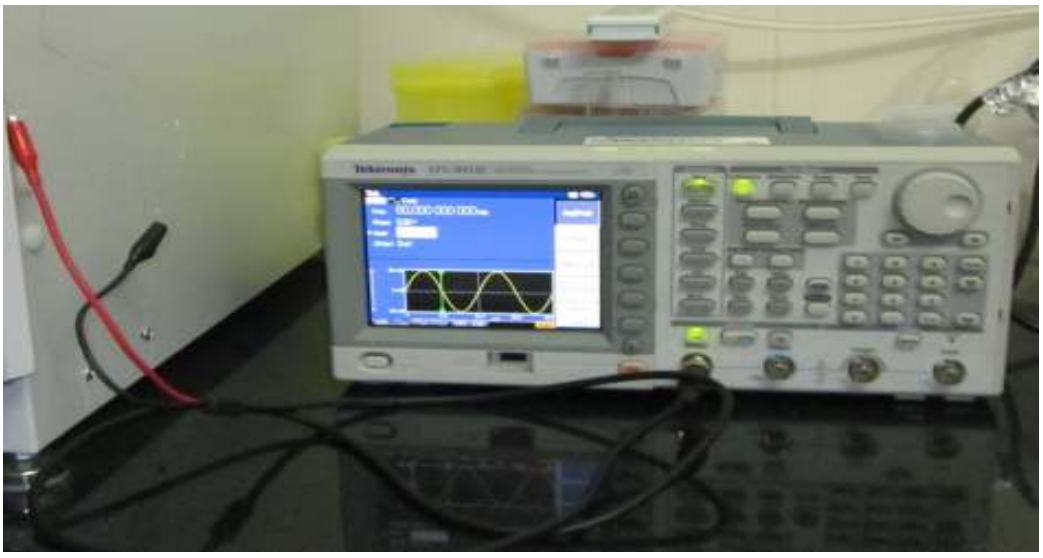

Figure 4.2: Function generator connected to the bioreactor in incubator

4.2 Experiment 0: Stimulation of bAECs

Stimulation Chamber Design 1 is chosen for conducting the experiment on bAECs. 40mV and 60 kHz are set as voltage and frequency respectively. This is considered as a pilot experiment in order to set the optimal voltage & design for the hMSCs to achieve Osteoblastic differentiation. This experiment was run only for 4 days applying AC Voltage for 40min daily.

4.2.1 Procedure

Graphite rods and platinum wires were autoclaved before one day prior to the start of the experiment. Pair of graphite rods were placed in the 2nd dish. Pair of graphite rods and platinum wires were placed in the 3rd dish which is considered as the stimulation chamber. Around 10,000 bAECs of passage 1 were seeded at the center of the petri dish and between the graphite rods with EGCM containing 20% FBS and 1% L-Glutamine. The 3 petri dishes were labeled as

(i) C0 - which contains the bAECs

(ii) G0 - which contains bAECs and Graphite Rods

(iii) E0 - (Stimulation Chamber) which contains bAECs, Graphite Rods and Platinum Wires.

All the petri dishes were kept inside the incubator. Connections were made according to the experimental setup. AC Voltage was applied continuously for 40min daily. Images were taken on alternative days. On day 4, Petri dishes were taken out for observing under microscope.

4.2.2 Observations

Day 1: bAECs in C0 expanded towards the cover-slip periphery in normal phenotype. BAECs in G0 expanded in vertical layer parallel to the graphite rod maintaining their cobblestone morphology. bAECs in E0 expanded in circular layer maintaining their cobblestone morphology. Figure shows the day 1 images.

Day 4: bAECs in C0 proliferated normally maintaining their cobblestone morphology. bAECs in G0 proliferated normally maintaining their cobblestone morphology and their density was a little bit low when compared to the bAECs in C0. BAECs in E0 changed their morphology and are more flattened in shape. Granulations are formed inside the nucleus which clearly states that the cells were affected by electrical stimulation. Figure shows the day 4 images.

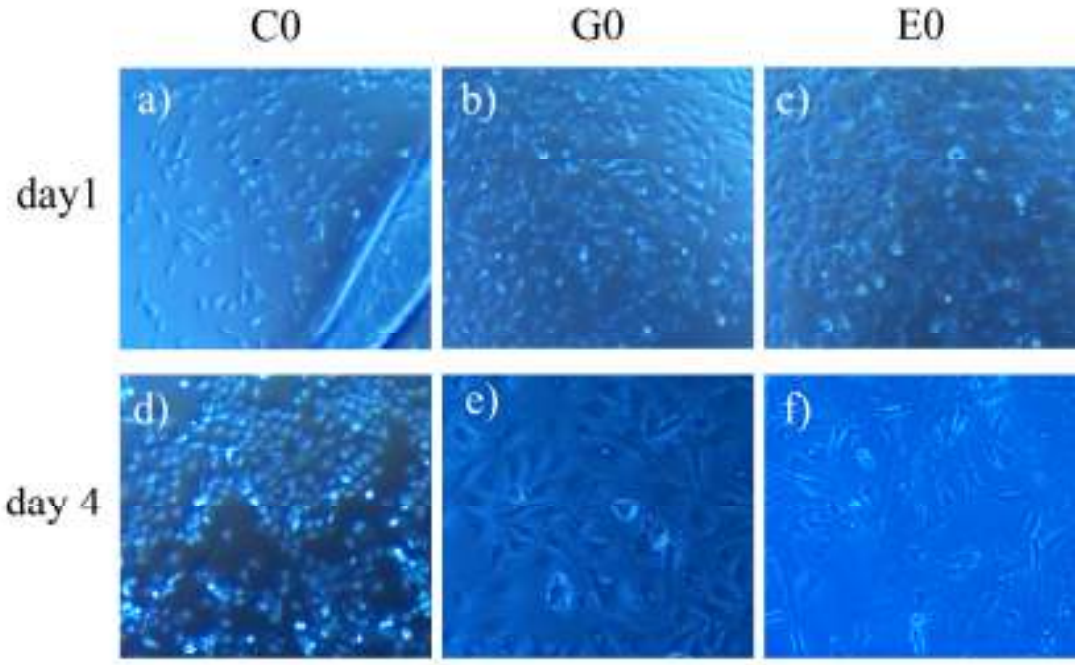

Figure 4.3: a) C0 on day1. b) G0 on day1 c) E0 on day1 d) C0 on day4 e) G0 on day4 f) E0 on day4

4.3 Experiment 1: Stimulation of hUVECs

Stimulation Chamber Design 2 is chosen for conducting the experiment on hUVECs. 20mV and 60 kHz are set as voltage and frequency respectively. This is considered as a pilot experiment in order to set the optimal voltage & design for the hMSCs to achieve Osteoblastic differentiation. This experiment was run only for 17 days applying AC Voltage for 40min daily.

4.3.1 Procedure

Graphite rods and platinum wires were autoclaved before one day prior to the start of the experiment. Pair of graphite rods were placed in the 2nd dish. Pair of graphite rods and platinum wires were placed in the 3rd dish which is considered as the stimulation chamber. Around 10,000 hUVECs of passage 3 were seeded at the center of the petri dish and between the graphite rods with EGCM containing 20% FBS and 1% L-Glutamine. The 3 petri dishes were labeled as

(i) C1 - which contains the hUVECs

(ii) G1 - which contains hUVECs and Graphite Rods

(iii) E1 - (Stimulation Chamber) which contains hUVECs, Graphite Rods and Platinum Wires.

All the petri dishes were kept inside the incubator. Connections were made according to the experimental setup. AC Voltage was applied continuously for 40min daily. Images were

taken on alternative days. On day 17, Petri dishes were taken out for observing under microscope.

4.3.2 Observations

Day 2: hUVECs in C1 expanded towards the cover-slip periphery in normal phenotype. hUVECs in G1 expanded in vertical layer parallel to the graphite rod maintaining their cobblestone morphology. hUVECs in E1 expanded in circular layer maintaining their cobblestone morphology.

Day 17: hUVECs in C1 proliferated normally maintaining their cobblestone morphology. hUVECs in G1 proliferated normally maintaining their cobblestone morphology and their density was a little bit low when compared to the hUVECs in C1. hUVECs in E1 changed their morphology and are more flattened in shape. Granulations are formed inside the nucleus which clearly states that the cells were affected by electrical stimulation. Figure 4.4 shows the day 2 and day 14 images.

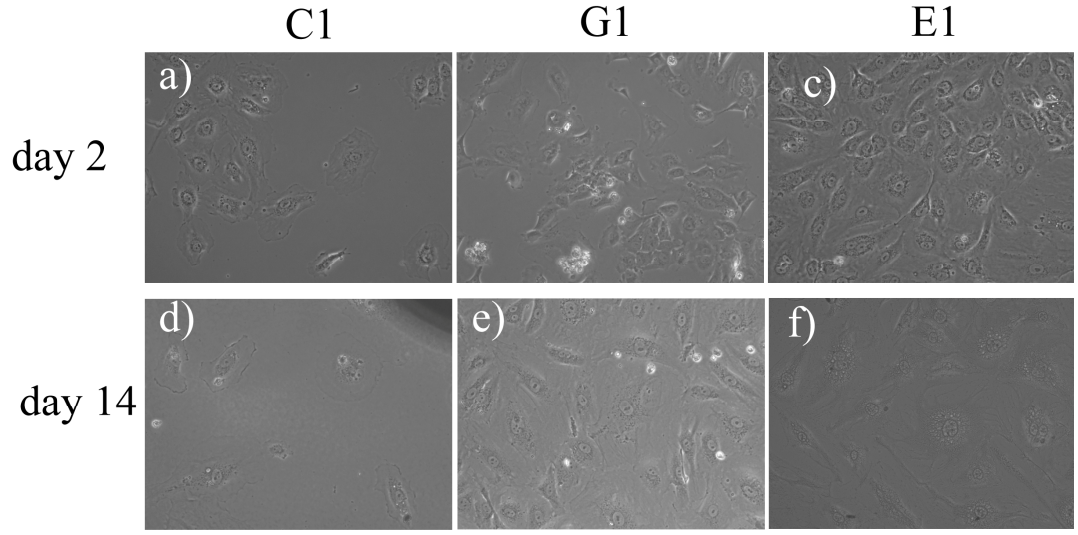

Figure 4.4: a) C1 on day2. b) G1 on day2 c) E1 on day2 d) C1 on day14 e) G1 on day14 f) E1 on day14

4.4 Experiment 2: Stimulation of hUVSCs

Stimulation Chamber Design 3 is chosen for conducting the experiment on hUVSCs. 20mV and 60 kHz are set as voltage and frequency respectively. This experiment was run for 17 days applying AC Voltage for 40min daily.

4.4.1 Procedure

Graphite rods and platinum wires were autoclaved before one day prior to the start of the experiment. Pair of graphite rods were placed in the 2nd dish. Pair of graphite rods and platinum wires were placed in the 3rd dish which is considered as the stimulation chamber.

Around 50,000 hUVSCs of passage 3 were seeded at the center of the petri dish and between the graphite rods with DMEM containing 20% FBS and 1% L-Glutamine. The 3 petri dishes were labeled as

(i) C2 - which contains the hUVSCs

(ii) G2 - which contains hUVSCs and Graphite Rods

(iii) E2 - (Stimulation Chamber) which contains hUVSCs, Graphite Rods and Platinum Wires.

All the petri dishes were kept inside the incubator. On day 4 OIM is added in all the dishes. Connections were made according to the experimental setup. AC Voltage was applied continuously for 40min daily to the E2. Images were taken on alternative days. On day 17, Petri dishes were taken out for observing under microscope.

4.4.2 Observations

Day 2: hUVSCs in C2 expanded towards the cover-slip periphery in normal phenotype. hUVSCs in G2 expanded in vertical layer parallel to the graphite rod maintaining their cobblestone morphology. hUVSCs in E2 expanded in circular layer maintaining their cobblestone morphology.

Day 17: hUVSCs in C2 proliferated normally maintaining their cobblestone morphology. hUVSCs in G2 proliferated normally maintaining their cobblestone morphology and their density was a little bit low when compared to the hUVSCs in C2. hUVSCs in E2 changed their morphology and are more flattened in shape. Granulations are formed inside the nucleus which clearly states that the cells were affected by electrical stimulation. Figure 4.5 shows the day 2 and day 14 images.

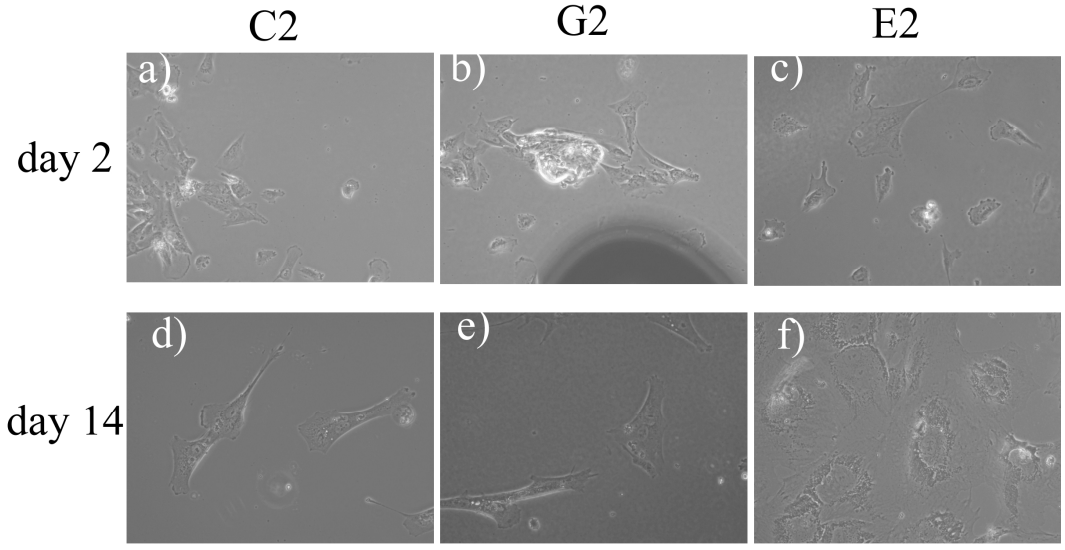

Figure 4.5: a) C2 on day2. b) G2 on day2 c) E2 on day2 d) C2 on day14 e) G2 on day14 f) E2 on day14

4.5 Experiment 3: Stimulation of hASCs

Stimulation Chamber Design 3 is chosen for conducting the experiment on hASCs. 20mV and 60 kHz are set as voltage and frequency respectively. This experiment was run for 20 days applying AC Voltage for 40min on alternative days.

4.5.1 Procedure

Graphite rods and platinum wires were autoclaved before one day prior to the start of the experiment. Pairs of graphite rods were placed in the 2nd, 5th and 8th dishes. Pairs of graphite rods and platinum wires were placed in the 3rd, 6th and 9th dishes which are considered as the stimulation chambers. Totally 9 dishes were considered for this experiment. Around 50,000 hASCs of passage 3 were seeded randomly all across the plates and between the graphite rods with DMEM containing 20% FBS and 1% L-Glutamine. The 9 petri dishes were divided as 3 sets each containing 3 plates labeled as

1. AR-C3 - which contains the hASCs
2. AR-G3 - which contains hASCs and Graphite Rods
3. AR-E3 - (Stimulation Chamber) which contains hASCs, Graphite Rods and Platinum Wires.
4. ALP-C3 - which contains the hASCs
5. ALP-G3 - which contains hASCs and Graphite Rods
6. ALP-E3 - (Stimulation Chamber) which contains hASCs, Graphite Rods and Platinum Wires.
7. PG-C3 - which contains the hASCs
8. PG-G3 - which contains hASCs and Graphite Rods
9. PG-E3 - (Stimulation Chamber) which contains hASCs, Graphite Rods and Platinum Wires.

The 1st set of plates is for doing ALP staining. The 2nd set of plates is for AR staining. The 3rd set of plates is for PG assay. All the petri dishes were kept inside the incubator. On day 7 OIM is added in plates 2nd, 3rd, 5th, 6th, 8th and 9th plates. On day 7 Connections were made according to the experimental setup. AC Voltage was applied continuously for 40min on alternate days (for 7 days). Images were taken on alternative days. On day 20, Petri dishes were taken out for observing under microscope and for staining.

4.5.2 Observations

Day 1: BAECs in C0 expanded towards the cover-slip periphery in normal phenotype. BAECs in G0 expanded in vertical layer parallel to the graphite rod maintaining their cobblestone morphology. BAECs in E0 expanded in circular layer maintaining their cobblestone morphology. Figure shows the day 1 images.

Day 7: BAECs in C0 proliferated normally maintaining their cobblestone morphology. BAECs in G0 proliferated normally maintaining their cobblestone morphology and their density was a little bit low when compared to the BAECs in C0. BAECs in E0 changed their morphology and are more flattened in shape. Granulations are formed inside the nucleus which clearly states that the cells were affected by electrical stimulation.

Day 20: BAECs in C0 proliferated normally maintaining their cobblestone morphology. BAECs in G0 proliferated normally maintaining their cobblestone morphology and their density was a little bit low when compared to the BAECs in C0. BAECs in E0 changed their morphology and are more flattened in shape.

4.5.3 Staining

The differentiated hMSCs have elevated levels of alkaline phosphatase on their cell membrane. Therefore alkaline phosphatase staining is used to detect the ostoblastic differentiation of hMSCs. The differentiated hMSCs will also have calcium deposits. Alizarin red staining is used to detect to calcium mineralization during the process of osteoblastic differentiation. Each petriplate is divided into different quadrants so as to observe the amount of differentiation in each quadrant. Figure4.6 shows the division of quadrants.

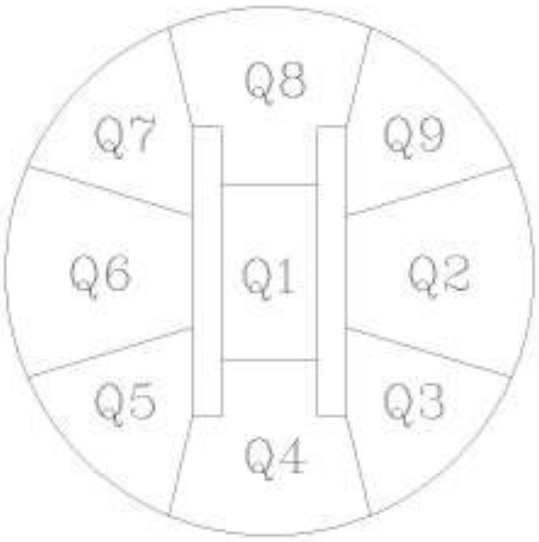

Figure 4.6:: Division of quadrants between and around the two graphite rods

4.5.3.1. Alizarin Red Staining (AR)

AR staining is done to the 1st, 2nd and 3rd plates. The medium is taken out from all the plates and are washed with PBS 1x for 2 to 3 times. $1ml$ of 10% formalin is added in the plates and allowed for fixation for 10min. formalin is taken out and $1ml$ of Alizarin Red stain is added in all the 3 plates. Waited for 5 min to allow the cells to absorb the stain and washed with sterile water after 5 min. all the plates were observed under microscope and images were taken. Figure shows AR staining across different quadrants (Figure 4.7 Figure 4.8 Figure 4.9) of the petriplate.

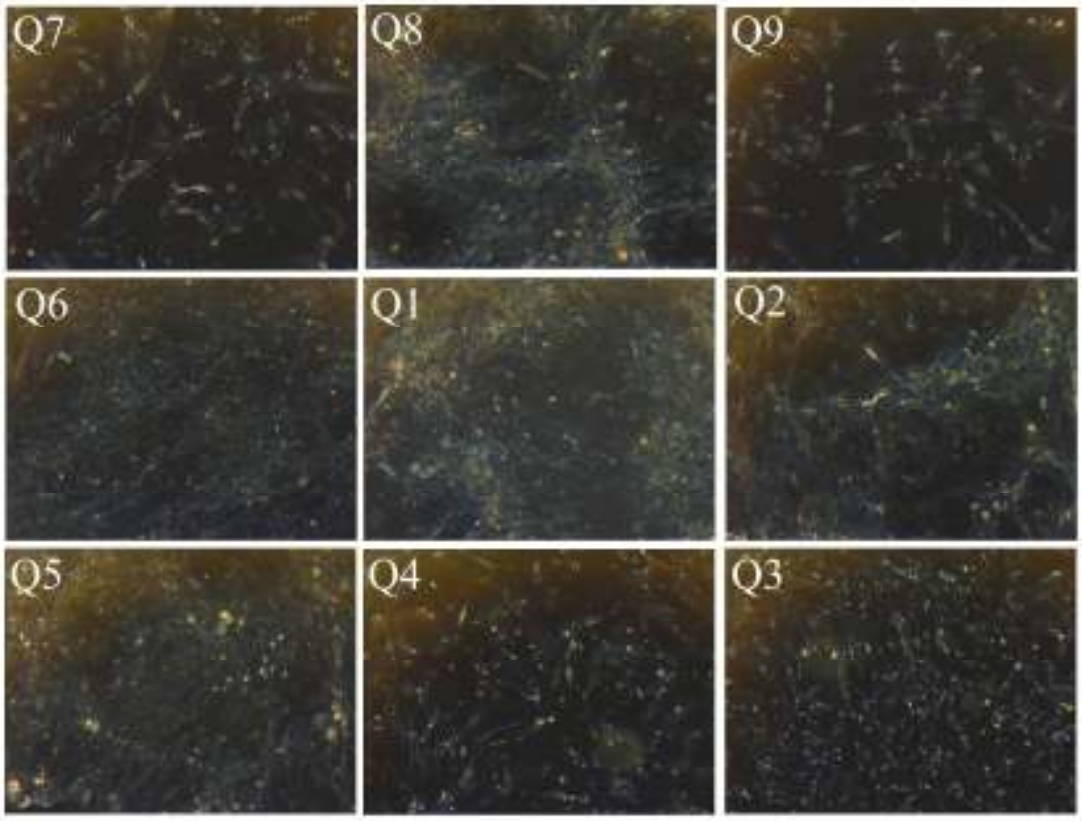

Figure 4.7: AR staining of C3 across different quadrants

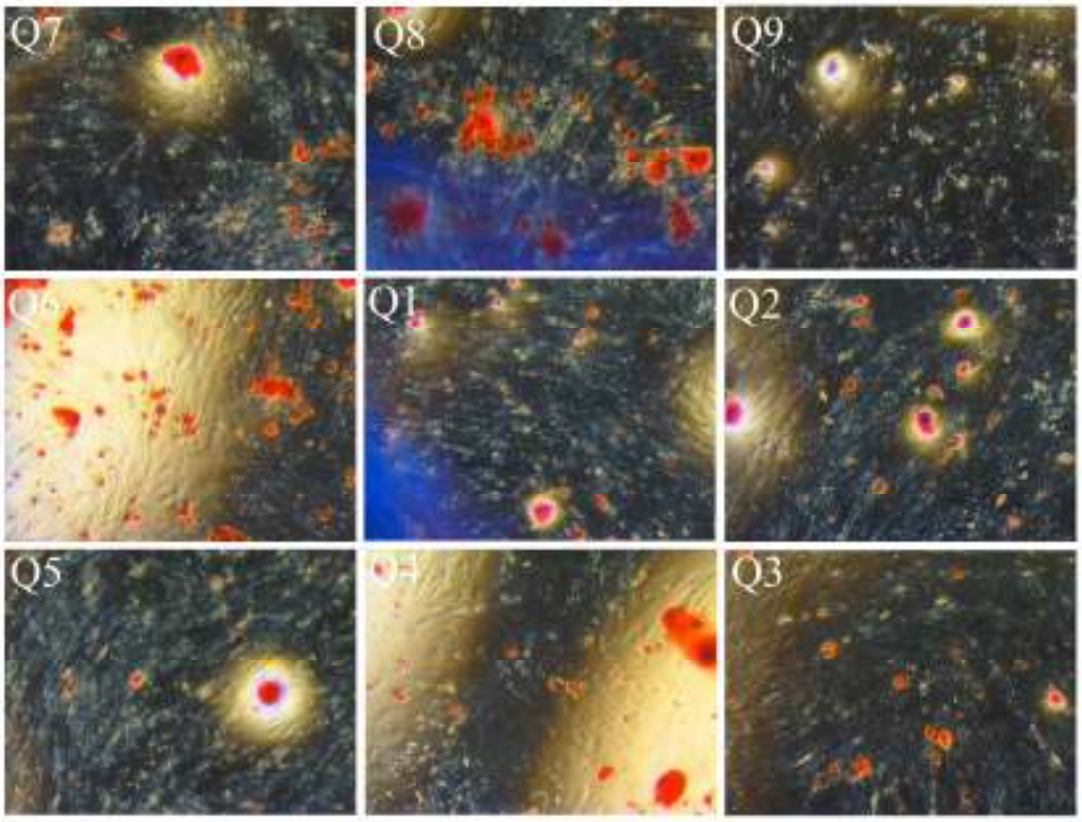

Figure 4.8: AR staining of G3 across different quadrants

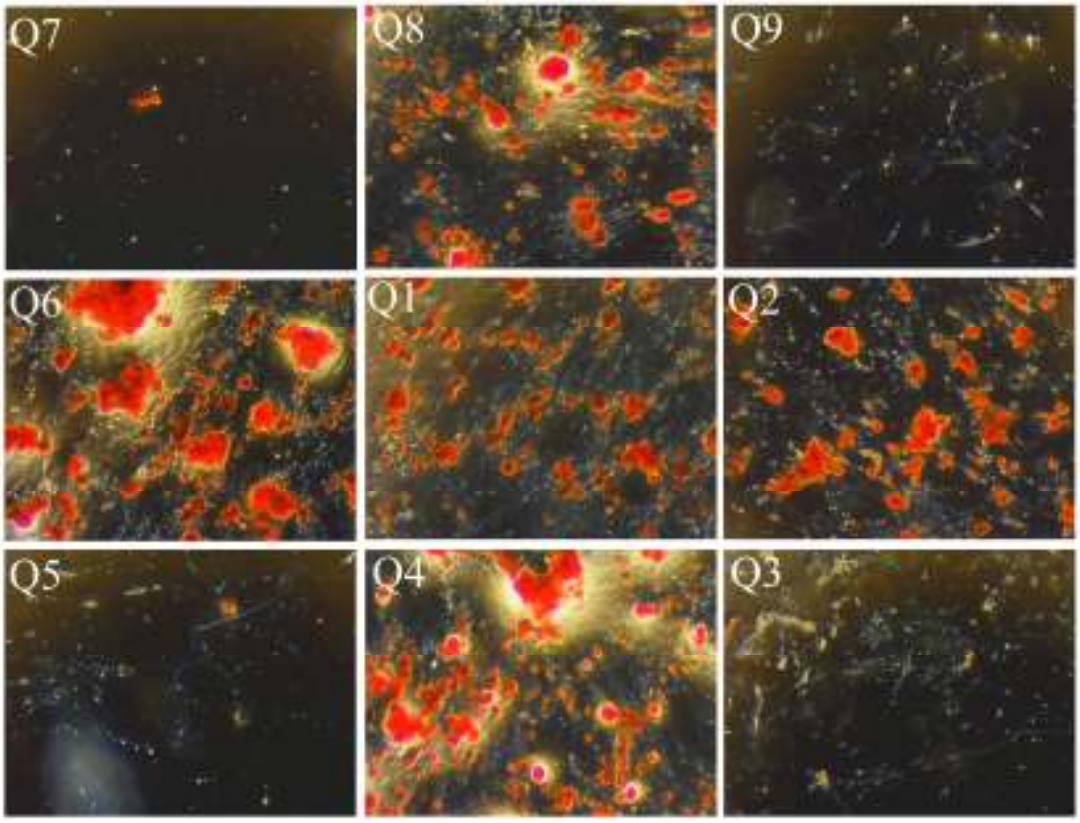

Figure 4.9: AR staining of E3 across different quadrants

AR staining was observed more in plate E3 when compared to plate G3. AR staining was completely negative in the control group C3.AR staining was approximately uniform across the different quadrants of the plate G3. But heavy staining was observed in plate E3 across quadrants Q2, Q4, Q6 and Q8. In plate E3 the staining was almost zero in Q3, Q5, Q7 and Q9. These staining results clearly show that Osteoblastic differentiation is more in plate E3 to which A.C is applied.

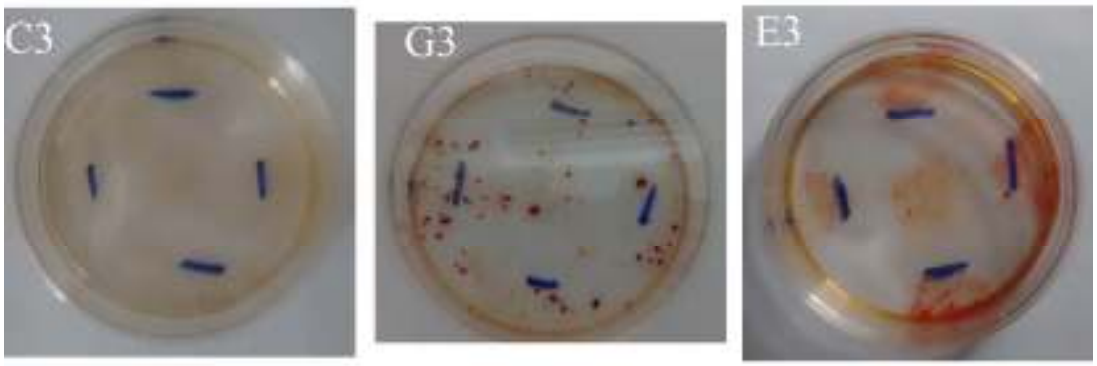

Figure 4.10: AR staining gross picture of C3, G3 and E3 plates

4.5.3.2. ALP

ALP AR staining is done to the 1st, 2nd and 3rd plates. The medium is taken out from all the plates and are washed with PBS 1x for 2 to 3 times. $1ml$ of 10% formalin is added in the plates and allowed for fixation for 10min. formalin is taken out and $1ml$ of Alizarin Red stain is added in all the 3 plates. Waited for 5 min to allow the cells to absorb the stain and washed with sterile water after 5 min. all the plates were observed under microscope and images were taken. Figure shows AR staining across different quadrants (Figure 4.11 Figure 4.12 Figure 4.13) of the petriplate.

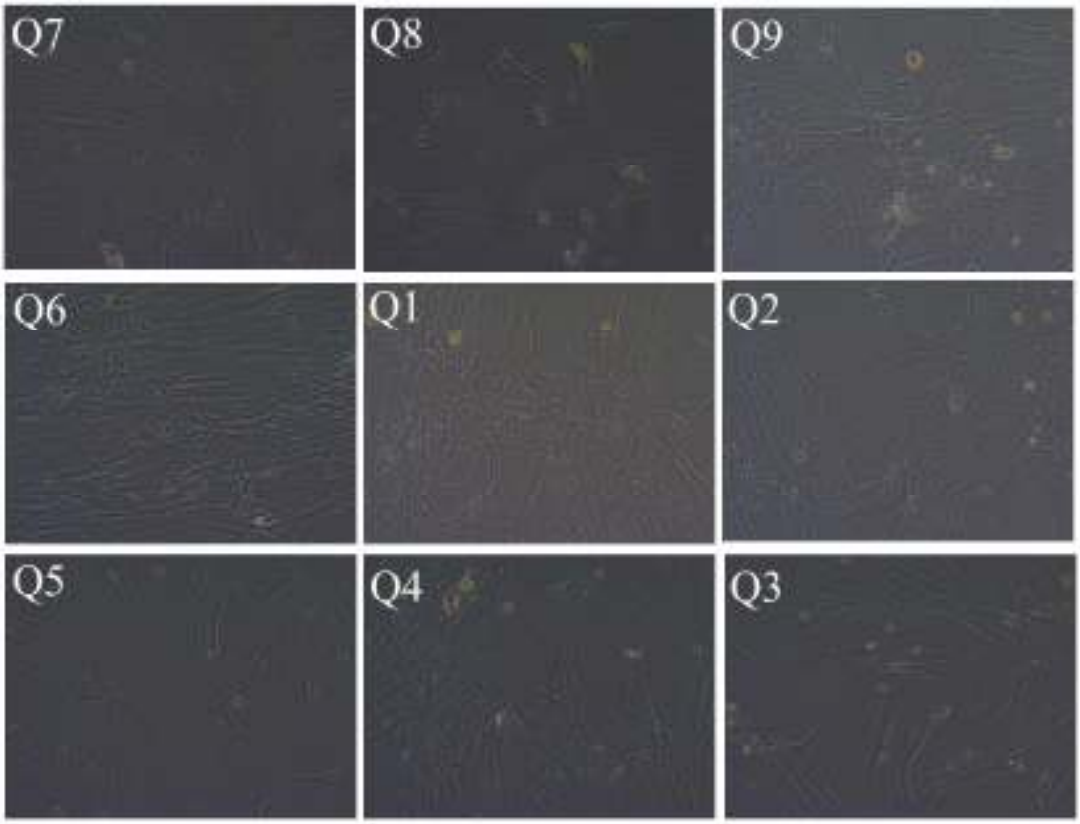

Figure 4.11: AR staining of C3 across different quadrants

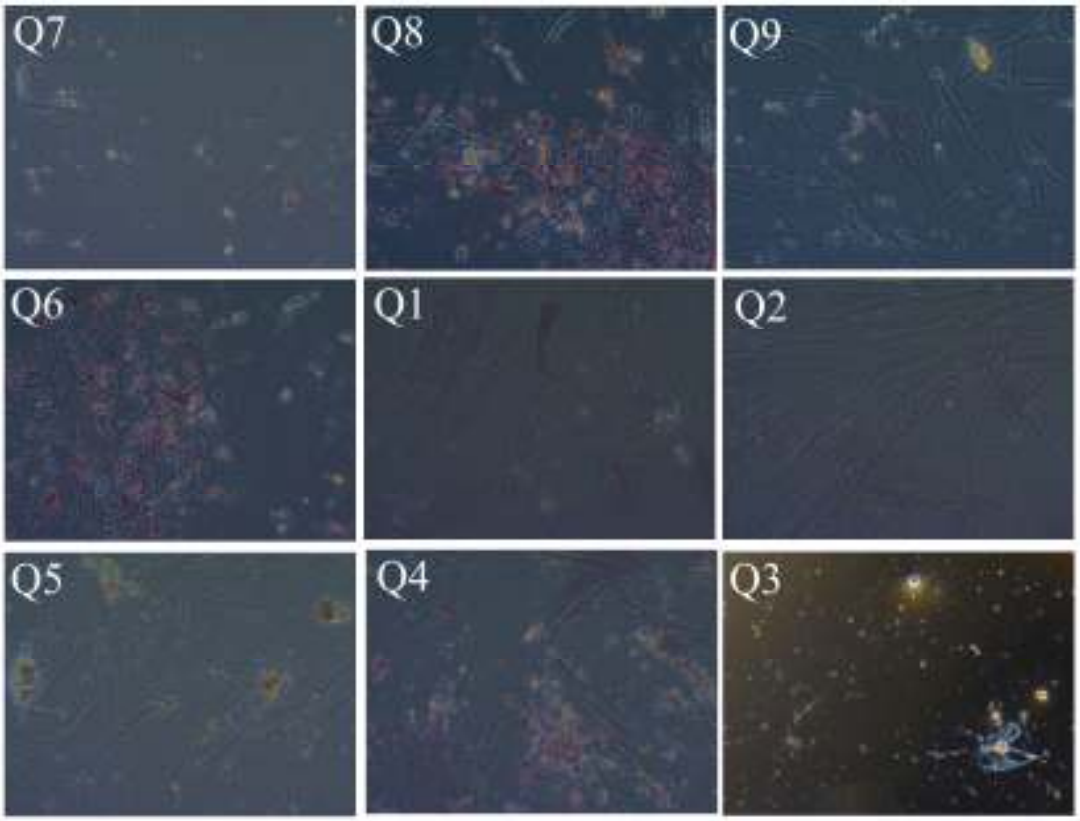

Figure 4.12: AR staining of G3 across different quadrants

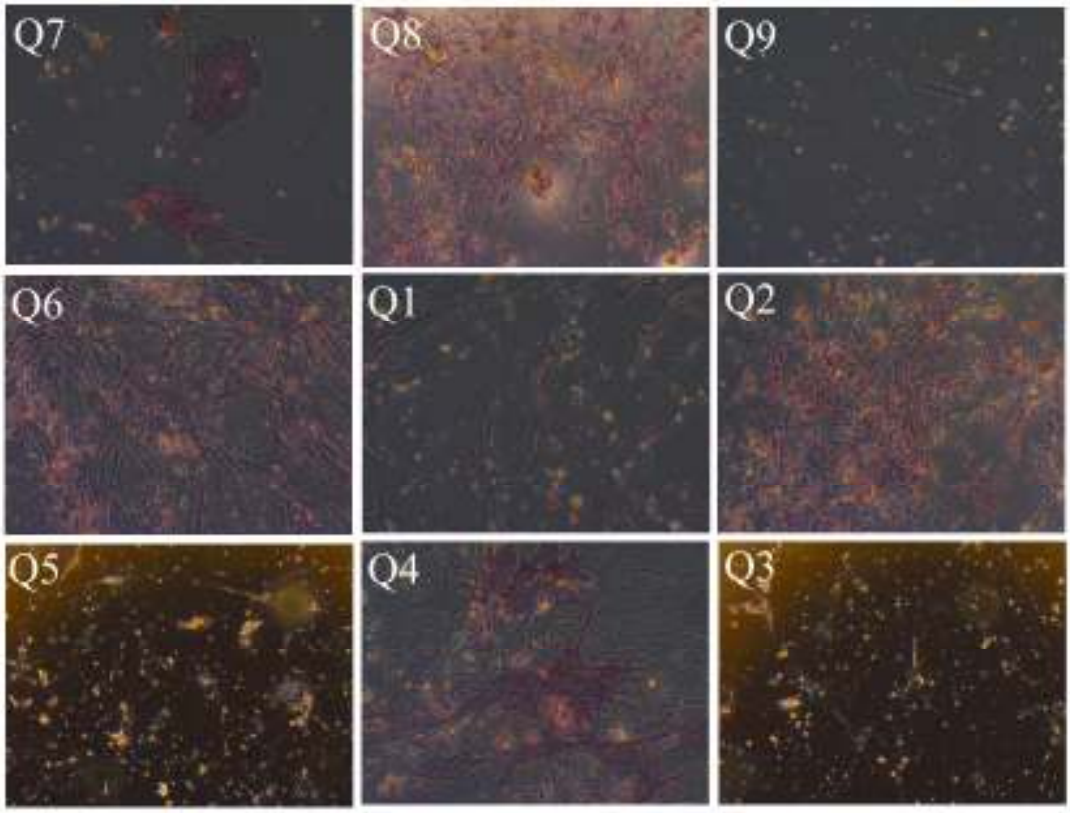

Figure 4.13: AR staining of E3 across different quadrants

AR staining was observed more in plate E3 when compared to plate G3. AR staining was completely negative in the control group C3.AR staining was approximately uniform across the different quadrants of the plate G3. But heavy staining was observed in plate E3 across quadrants Q2, Q4, Q6 and Q8. In plate E3 the staining was almost zero in Q3, Q5, Q7 and Q9. These staining results clearly show that Osteoblastic differentiation is more in plate E3 to which A.C is applied.

4.5.4 PG Assay

PG Assay is done to the 7^{th}, 8^{th} and 9^{th} plates. The medium is taken out from all the plates and are washed with PBS 1x for 2 to 3 times. $1ml$ of cell lysis buffer is added in the plates and incubated for 10min and is collected in an eppendorf tube. Figure shows DNA quantification of PG assay across different plates.

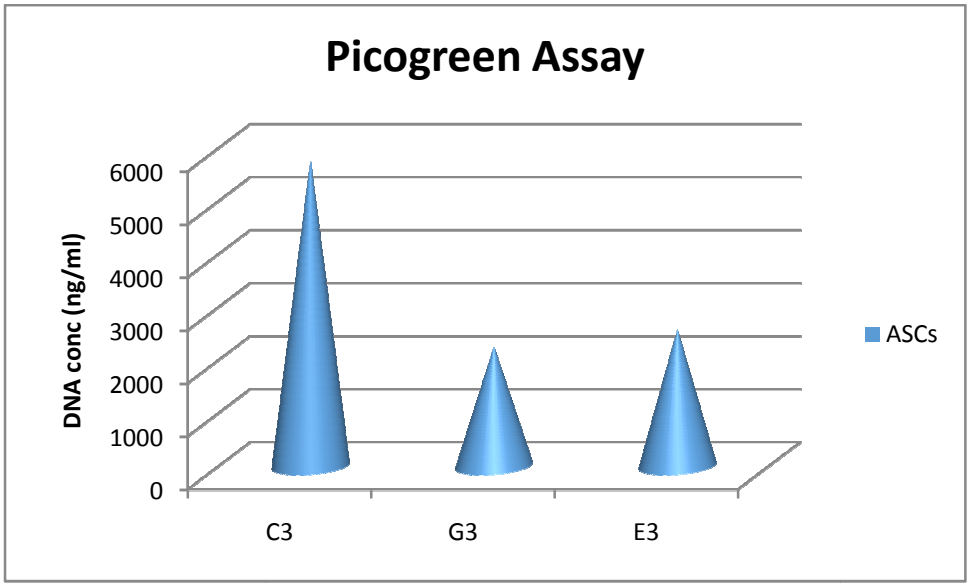

Figure 4.14: AR staining of C3 across different quadrants

DNA concentration was observed more in plate C3 when compared to plates G3 and E3. This clearly shows that cells are more in C3 because they are completely confluent on day 20. In G3 and E3 the concentration is low due to osteoblastic differentiation which stops proliferation. In E3 the concentration is a little bit high as the cell number is high. Electrical stimulation promotes proliferation and differentiation and that was clearly proved in this case by PG assay.

Chapter 5
Summary, Conclusion and Future Work

5.1 Summary & Conclusion

Bone healing, bone regeneration, osteoblastic differentiation, MSCs and their role in bone healing process were discussed. Stimulating MSCs to achieve osteoblastic differentiation by electric current was taken as the thesis and developed bioreactor. Bioreactor models were designed and are simulated as mathematical models. Using finite element analysis the parameter electric field intensity/electric potential field distribution inside the bioreactor was observed using comsol multi-physics software. Different models were implemented and experimented with different types of cells to optimize the parameters. hMSCs were exposed to A.C fields to achieve osteoblastic differentiation. Different staining methods were done to notice the calcium and ALP deposits. PG assay was done to notice the DNA concentration levels during Osteoblastic Differentiation of hASCs.

Significant differences in control groups and experimented groups shows that A.C field with 20mV potential and 60kHz frequency can promote Osteoblastic Differentiation of MSCs.

5.2 Future Work

Further work can be done to find out the exact parameter which was influencing the cells to differentiate. Whether it was the electromagnetic flux that develops around the graphite rods or whether it was the cell culture medium that undergoes electrolysis is yet to be known. This work can be done by varying different parameters like voltage, medium, frequency and materials. Gene expression procedures can be done for further studies. This work can be implemented in 3D environment to enhance the bone healing process in practical.

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