THE EFFECTS OF BIRD'S NEST AS A CRYOPROTECTIVE AGENT ON HUMAN ADIPOSE DERIVED STEM CELLS

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ABSTRACT

One of the most popular concepts currently is the use of stem cells in the regenerative therapy which replaces the lost or dysfunctional part in a human body. In order for a patient to benefit from such therapies, the stem cell source should be always made available. Therefore, it is important to preserve these cells so that they are always available for treatments. Cryopreservation is one of the important methods to preserve cells and tissues where it can be used for the recovery of damaged tissues and organs. Cryopreservation of stem cells is mainly to minimize any form of damage to biological materials at very low temperatures while freezing and also during storage. This issue can be addressed with the addition of a cryoprotective agent. Dimethyl sulfoxide (DMSO) is a common cryoprotective agent which is a polar compound originally created for the use as a solvent. However the use of DMSO may result in the toxicity of cells. The bird's nest or also known as edible bird's nest (EBN) is a health food with many traditional medicinal properties. Therefore, the objectives of this study are to evaluate the efficiency of EBN to act as a cryoprotective agent, to evaluate the differentiation ability to express the following lineages; osteogenic, adipogenic and chondrogenic after cryopreservation with the respective cryoprotectants (10% DMSO+90% FBS, 5% DMSO+1% EBN+94% FBS and 1% EBN+99% FBS) and to evaluate the proliferative rate of the cryopreserved human adipose derived stem cells with the respective cryoprotectants (10% DMSO+90% FBS, 5% DMSO+1% EBN+94% FBS and 1% EBN+99% FBS). This study is designed to evaluate the ability of EBN to either act as a cryoprotectant to substitute DMSO or to act as a supplement to DMSO. The human adipose tissue were collected from the PPUM was isolated and cultured up to second passage. Then, the cells were cryopreserved in the

respective cryoprotectant; 10% DMSO+90% FBS (Control), 5% DMSO+1% EBN+94% FBS and 1% EBN+99% FBS for a month. After a month, the cells were thawed and cultured for the following tests; cell viability test, resazurin test and differentiation into three lineages (adipogenic, chondrogenic and osteogenic). Then, the differentiated lineages were stained and the respective surface areas were determined. It can be concluded that the human adipose derived stem cells preserved in 10% DMSO+99%FBS had exhibited a higher result as compared to cells preserved in 5% DMSO+1% EBN+94%FBS and 1% EBN+99% FBS. Hence making DMSO still a good and superior cryoprotective agent. DMSO on its own can support the growth of the cells which can be seen in 10% DMSO+99% FBS and does not require the presence of EBN. Therefore, it can be concluded that EBN cannot act as a cryoprotective agent. EBN is better to be used in in vitro cell culture experiments instead of it as a cryoprotective agent.

ABSTRAK

Salah satu konsep yang paling popular pada masa ini ialah penggunaan sel stem dalam terapi regeneratif yang menggantikan bahagian yang hilang atau tidak berfungsi dalam badan manusia. Sumber sel stem harus sentiasa disediakan supaya pesakit dapat memanfaat daripada terapi ini. Oleh itu, adalah penting untuk memelihara sel-sel ini supaya ia sentiasa disediakan untuk rawatan. Krioawetan merupakan salah satu kaedah penting untuk memelihara sel dan tisu di mana ia boleh digunakan untuk pemulihan tisu dan organ yang rosak. Krioawetan sel stem adalah penting terutamanya untuk mengurangkan sebarang bentuk kerosakan pada bahan biologi pada suhu yang rendah ketika proses pembekuan dan penyimpanan. Isu ini boleh ditangani dengan penambahan agen cryoprotektif. Dimetil sulfoksida (DMSO) adalah ejen krioprotektif yang biasa digunakan, asalnya ia dicipta untuk digunakan sebagai pelarut. Walau bagaimanapun, penggunaan DMSO boleh menyebabkan ketoksikan sel. Sarang burung (EBN) adalah makanan kesihatan dengan pelbagai ciri-ciri perubatan tradisional. Oleh itu, objektif kajian ini adalah untuk menilai kecekapan EBN untuk bertindak sebagai ejen krioprotektif, bagi menilai kemampuan pembezaan untuk menyatakan keturunan berikut; *adipogenik*, *osteogenik*, *dan kondrogenik* setelah dikrioawet dengan krioprotektif masing-masing (10 % DMSO+90 % FBS, 5 % DMSO+1 % EBN+94 % FBS dan 1 % EBN+99 % FBS) dan juga untuk menilai kadar proliferative sel stem yang dikrioawet dengan krioprotektif masing-masing (10 % DMSO+90 % FBS, 5 % DMSO+1 % EBN+94 % FBS dan 1 % EBN+99 % FBS). Kajian ini bertujuan untuk menilai keupayaan EBN sama ada bertindak sebagai ejen krioprotektif untuk menggantikan DMSO atau untuk bertindak sebagai tambahan kepada DMSO. Tisu lemak yang dikumpul daripada PPUM telah diasingkan dan diternak sehingga laluan kedua.

Kemudian, sel dikrioawet dalam krioprotektif masing-masing selama sebulan; 10 % DMSO+90 % FBS (Kawalan), 5 % DMSO+1 % EBN+94 % FBS dan 1 % EBN+99 % FBS. Selepas sebulan, sel telah dicairkan dan dikembangkan untuk ujian berikut; sel ujian daya maju, ujian resazurin dan pembezaan kepada tiga keturunan (*adipogenik*, *osteogenik*, *dan kondrogenik*). Sel yang dibezakan telah dinoda dan kawasan permukaan masing-masing telah ditentukan. Kesimpulannya adalah sel stem yang diperolehi daripada tisu lemak manusia yang dikrioawet dalam 10 % DMSO + 99 % FBS telah memaparkan hasil yang lebih tinggi berbanding dengan sel-sel yang dikrioawet dalam 5 % DMSO + 1 % EBN + 94 % FBS dan 1 % EBN + 99 % FBS. Maka, boleh disimpulkan bahawa DMSO adalah ejen krioprotektif yang baik dan unggul. DMSO juga boleh menyokong pertumbuhan sel-sel tanpa memerlukan kehadiran EBN seperti yang dilihat dalam 10 % DMSO + 99 % FBS. Oleh itu, boleh disimpulkan bahawa EBN tidak boleh bertindak sebagai ejen krioprotektif dan ia adalah lebih baik untuk digunakan dalam eksperimen in vitro dan bukannya sebagai ejen krioprotektif.

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LIST OF SYMBOLS AND ABBREVIATIONS

DMSO	Dimethyl sulfoxide
EBN	Edible bird's nest
%	Percentage
ASC	Adipose derived stem cell
MSC	Mesenchymal stem cell
EGF	Epidermal growth factor
ppm	Parts per million
mg/kg	Milligram per kilogram
Con A	Concanavalin A
PHA	Phytohaemagglutinin
°C	Degree Celsius
pH	Power of hydrogen
FBS	Fetal bovine serum
°C/minute	Degree Celsius per minute
HES	Hydroxyethyl Starch
IUPAC	International Union of Pure and Applied Chemists
mV	Millivolts
TGF-β3	Transforming growth factor beta 3
NADPH	Nicotinamide adenine dinucleotide phosphate
FADH	Flavin adenine dinucleotide
FMNH	Flavin mononucleotide
NADH	Nicotinamide adenine dinucleotide

nm	Nanometer
ATP	Adenosine triphosphate
PBS	Phosphate Buffered Saline
rpm	Revolutions per minute
mL	Millilitre
P_o	Primary culture
P_1	Passage One
P_2	Passage Two
P_3	Passage Three
Hrs	Hours
μL	microlitre
g	Gram
mg	Milligram
mm	Millimeter

CHAPTER I

INTRODUCTION

Stem cells are cells with the ability of self renewal and contain the ability to produce multi-lineage differentiation. Currently, to be useful in tissue engineering, stem cells should be safe for implantation, possess ability to differentiate into multi-lineages and can be easily obtained in large numbers. There are several types of stem cells which are generally useful in tissue engineering which are as follows; hematopoietic stem cells, adipose stem cells, mesenchymal stem cells and skin stem cells. All these types can easily be accessed and to be obtained in large numbers. At the same time, they are safe and do not cause immune-rejection since the samples are autologous (Pirraco et al., 2011; Vacanti et al., 2014). One of the most popular concepts used currently is the regenerative therapy which replaces the lost or dysfunctional part in a human body. Using this technique, a lot of focus has been given on the cardiovascular diseases (Rosen, Myerburg, Francis, Cole, & Marbán, 2014).

Adipose tissue which is found throughout the human body and is commonly discarded during procedures such as liposuction and cosmetic surgery can be a good source of stem cells. Their ability to proliferate at high rate and also to produce multi-lineage differentiation has gain interest in both tissue engineering and regenerative medicine to treat various diseases and disorders. A lot of these diseases can be treated with stem cells which are taken from the patient's body itself. Since adipose tissue gives rise to mesenchymal stem cells, therefore it is possible for autologous transplantation. In order for

a patient to benefit from such therapies, the stem cell source should be always made available. Therefore, it is important to preserve these cells so that they are always available for treatments. Cryopreservation is one of the important methods to preserve cells and tissues where it can be used for the recovery of damaged tissues and organs. One of the best options for stem cell preservation is the stem cells banks which will maintain cells in a viable state for a long period of time. However, donors are suppose to pay for these banks. It is also said that a mesenchymal stem cells isolated from an adipose tissue which are cryopreserved are equally the same as the freshly isolated mesenchymal stem cells (Choudhery, Badowski, Muise, Pierce, & Harris, 2014).

Cryopreservation of stem cells is mainly to minimize any form of damage to biological materials at very low temperatures while freezing and also during storage. This issue can be addressed with the addition of a cryoprotective agent. There are two types of cryoprotectant used for cell preservation which are as follows; intracellular and extracellular cryoprotectant. One of the most commonly used cryoprotectant is the dimethyl sulfoxide (DMSO). DMSO is a polar compound which was originally created for the use as a solvent. However the use of DMSO may result in the toxicity of cells. The ability of DMSO to induce non lamellar structures in phospholipids and to enhance membrane permeability may also damage biological membrane at physiological temperature. Moreover, DMSO also has the ability to induce cell differentiation indicates that it might exert an influence at the genetic regulation level (Yu & Quinn, 1994). There are also some natural cryoprotectants which are as follows; sucrose and trehalose which are extracellular cryoprotectant. The main usage of these sugars is because the cells do not produce or synthesizes these sugars. At the same time, the sugar molecules are too big to enter the cell permeable membrane, hence making them an extracellular cryoprotectant. However,

research has concluded that extracellular cryoprotectant is not as efficient as the intracellular cryoprotectant (Motta, Paraguassú-Braga, Bouzas, & Porto, 2014).

The bird's nest or also known as edible bird's nest (EBN) is a health food with many traditional medicinal properties. It is said to maintain the youthful complexion of an individual, does speedy recovery from illness, improves appetite, aid in digestion issue and also boost the immune system. In the traditional Chinese medicine, it is used for curing tuberculosis, dry coughs, suppressing cough and phlegm, improving ulcer, relieving gastric troubles and weakness of bronchial ailments (Chan, 2006; Hobbs, 2004; Marcone, 2005). The extract of EBN is found to be anti-inflammatory in nature where it has suppressed the production of tumor necrosis factor-alpha. Either than that, it is also able to inhibit the infections by influenza virus which is facilitated by the *N*-Acetylneuraminic acid residues which are present in the EBN. EBN is made up of 62-63% of protein, 25.62-27.26% of carbohydrates, bioactive mucoid and epidermal growth factor which stimulates cell division and growth of many epidermal and epithelial tissues (Ma & Liu, 2012; Yang, Cheung, Li, & Cheung, 2014).

Although many research have been carried out using EBN but nothing would related to EBN as a cryoprotectant. Therefore, this study was designed to study the ability of EBN to act as a cryoprotective agent on human adipose derived stem cells. The objectives of this study are as follows:

1. To evaluate the efficiency of EBN to act as a cryoprotective agent.
2. To evaluate the differentiation ability to express the following lineages; osteogenic, adipogenic and chondrogenic after cryopreservation with the respective cryoprotectants (10% DMSO+90% FBS, 5% DMSO+1% EBN+94% FBS and 1% EBN+99% FBS).

3. To evaluate the proliferative rate of the cryopreserved human adipose derived stem cells with the respective cryoprotectants (10% DMSO+90% FBS, 5% DMSO+1% EBN+94% FBS and 1% EBN+99% FBS).

Problem statement: DMSO is a common cryoprotective agent that is known to cause toxic to the cells. Since EBN has exhibited some good properties, therefore, this study is designed to evaluate the ability of EBN to either act as a cryoprotectant to substitute DMSO or to act as a supplement to DMSO.

CHAPTER II

LITERATURE REVIEW

2.1 Stem Cells

2.1.1 Properties of Stem Cells

Stem cells are unspecialized cells that are able to self renew and contain the ability to produce multilineage differentiation. Two of their properties are as follows; firstly, stem cells contain the ability to divide many times while retaining the undifferentiated state. This situation is termed as self-renew. Secondly, under certain or proper conditions these cells can be induced to produce specialized cells via differentiation. For example; heart muscle cells, blood cells or nerve cells. Some of the sources of stem cells includes; bone marrow, embryonic stem cells from inner cell mass, various tissues of the adult body such as fats and skin, fertilized and unfertilized eggs, gestational tissues such as umbilical cord, york sac and amniotic fluid and embryonic germ cells from developing gonads of the fetus. Therefore, stem cells are divided into two main types which are as follows; adult and embryonic stem cells. A stem cell can be totipotent cells, pluripotent cells, multipotent cells, unipotent cells or differentiated cells depending on the source of cells obtained. Traditionally, adult stem cells were thought to give rise to one particular cell type in which the tissue resides. For example if a blood forming cell is obtained from bone marrow (hematopoietic stem cell), it will generate red blood cells, white blood cells and platelets. However further studies suggest that stem cells obtained from tissues may give rise to

several different types of cells which are completely different from where they reside which is termed as plasticity. For example, hematopoietic stem cell which produces heart muscles or blood cells producing neurons. Therefore, extensive studies on using adult stem cells for cell based therapies have become very active areas. Stem cells has many benefits, some of them are as follows; for gene therapy, to produce tissues and organs which genetically matches the patient to replace damaged or diseased parts, to test effects of drugs on a specific lineages, for example cardiac muscle cells, helps develop cures for certain diseases such as Parkinson, Alzheimer, stroke, arthritis, burns, spinal injury, heart disease, certain form of cancer and etc and also can be used to study the development of tissue (Chamberlain, Fox, Ashton, & Middleton, 2007; Mani et al., 2008; Stocchero & Stocchero, 2011).

2.1.2 Embryonic Stem Cells

Embryonic stem cells are obtained from embryos that are produced from eggs via in vitro fertilization with the permission of the donors and these kinds of stem cells are never obtained from a pregnant mother. The embryos that are produced are usually four to five days old and are a hollow microscopic ball of cells which is termed as blastocyst. A blastocyst contains three structures which are as follows; trophoblast; a layer of cells surrounding the blastocyst, blastocoels; the hollow cavity inside the blastocyst and the inner cell mass which is made of a group of 30 cells at one end of the blastcoel. Some of the advantages of embryonic stem cells are as follows; they are able to proliferate indefinitely, both totipotent (ability to produce all types of cells) and pluripotent (ability to produce many types of cells) abilities and they are easily grown in culture. However there are some disadvantages which are as follows; there is a tendency for these types of cells to be malignant or to produce tumors since their gene expression is unstable. Secondly, the moral

and human right issues that are involved makes this stem cells least preferred (Biswas & Hutchins, 2007; A. Smith, 2001).

2.1.3 Adult Stem Cells

Adult stem cells are cells that are undifferentiated found among the differentiated cells in an organ or tissue. These cells can self-renew and also has ability to differentiate to produce many types of cells of organs and tissues. The main function of an adult stem cell in a living organism is to maintain and repair tissues at sites they are found. Some of the sources of adult stem cells are bone marrow, skin, fats, blood vessels, liver, gut and etc.

Some of the advantages of adult stem cells are as follows; does not involve moral issues, therefore not controversial, contain pluripotent abilities, ability to differentiate into a narrow set of cell types hence it is easier to direct then to a particular lineage, can be clinically used for many diseases, tumour formation can be avoided, ability to differentiate into different tissue types which is termed as plasticity or transdifferentiation and lastly immunorejection avoided by obtaining autologous stem cells; which may protect donor from contamination, viral or bacterial infections. However the disadvantages are as follows; firstly, the proliferation capacity is limited, short life as compared to embryonic stem cells, there is a risk of infection to the recipient if the source is allogenic (cells taken from others), risk to the donor during isolation if autologous source is preferred and culturing adult stem cells in vitro is difficult and almost impossible for some types of cells as compared to embryonic stem cells (Palsson & Bhatia; Young & Black, 2004). Table 2.1 summarizes the difference between adult and embryonic stem cells.

Table 2.1: Difference between Adult and Embryonic Stem Cells (Palsson & Bhatia)

Embryonic Stem Cells	Adult Stem Cells
Pluripotent (ability to produce all types of cells).	Limited capacity to differentiate into different types of cells. However, some adult stem cells contain plasticity ability.
Large number of cells can be easily grown in culture.	Culturing adult stem cells in vitro is difficult and almost impossible.
Embryonic stem cells from a donor introduced into a patient cause transplant rejection.	Autologous source can be obtained from the patient, expended in culture and reintroduced into the patient once again. This avoids immunorejection. Hence, avoiding the immunosuppressive drugs.

2.1.4 Adipose Derived Stem Cells

Adipose-derived stem cells are important for many therapeutic applications, multipotent and are plastic adherent isolated which are obtained through collagenase digestion of adipose tissue. Multipotent means ability to produce limited number of cell types. Therefore, adipose-derived stem cells can be differentiated into several lineages which are as follows; chondrogenic, neurogenic, adipogenic and also osteogenic. Some stromal cells exhibit adipose-derived stem cells characteristics and these cells can be isolated from the adipose tissue of an adult and grown in vitro and differentiated into adipocytes. Stromal cells can be isolated in either large or small amounts from the human adipose tissue. In human, the mesenchymal cells will undergo a complex process of transcription and non-transcription to produce adipocytes which occurs throughout the human life. Adipocyte differentiation will result in changes in cell morphology, hormone sensitivity and also gene expression (Bunnell, Flaat, Gagliardi, Patel, & Ripoll, 2008).

There are several terms used to identify an adipose tissue, which are as follows; adipose-derived stem cells, adipose-derived adult stem cells, adipose-derived adult stromal cells, adipose-derived stromal cells, adipose stromal cells, adipose mesenchymal stem cells, lipoblast, pericyte, pre-adipocyte and processed lipoaspirate cells. Since too many terms

leads to confusion, therefore the International Fat Applied Technology Society has come up with the term adipose-derived stem cells (ASCs) to identify the isolated, multipotent, plastic adherent cells. This important as to differentiate it from the plastic adherent adult stem cells of bone marrow which is termed as multipotent mesenchymal stromal cells (MSCs) (J. M. Gimble, Katz, & Bunnell, 2007).

2.1.5 Differentiation

Differentiation is a process of treating the unspecialized stem cells under proper conditions to produce specialized cells. Differentiated cells are cells with a specific function; they can’t be changed into another cell type or can’t produce stem cells. Since adipose-derived stem cells are multipotent, therefore their therapeutic values have created interest for regenerative medicine. The differentiation can be initiated with the addition of specific induction medium with chemical inducers or cytokines. It is also suggested that differentiation is age dependent, where the differentiation capacity is higher in culture for younger patients as compared to the older ones.

Adipogenic differentiation: As the stem cell proliferation takes place, some the cells will differentiate into preadipocytes. These preadipocytes will undergo a second differentiation where it will begin to be filled with lipid. The lipid accumulation within cell vacuoles will result in the droplet appearance.

Osteogenic differentiation: Osteogenesis can be determined via several methods which are as follows; Alizarin Red staining to demonstrate mineralization, measurement of calcium and Alkaline Phosphatase (AP) activity and also detection of lineage specific gene and protein regulations. During osteogenic differentiation, the stem cell proliferates rapidly and will form tightly packed colonies. Sometimes these colonies will result in dense nodules which highly elongated spindle shaped cells with large nuclei.

Chondrogenic differentiation: Here, differentiation takes places in three steps. Proliferation of stem cells will express extracellular matrix called protenin. The protenin produced will tell the cells to condense into nodules. Cells in these nodules will become chondrocytes which will secrete proteoglycan and collagen which are necessary for the formation of cartilage (Bunnell et al., 2008).

2.2 Bird's Nest or Edible Bird's Nest (EBN)

2.2.1 Description

The EBN are mainly swiflets, tiny birds about the size of sparrow that feed on insects from the following order; *Hymenoptera* (winged ants, fig wasps and bees), *Diptera* (flies), *Coleoptera* (small beetles), *Homoptera* (leafhoppers) and *Ephemenoptera* (mayflies) (Lim et al., 2002). They are found in the following areas; Indian Ocean, Southeast Asia, North Australia and also the Pacific. The EBN used for human consumption in the Southeast Asian region is mainly from the genus Collocalia and there are 4 main species that are consumed which are as follows; *Collocalia fuciphaga*, *Collocalia germanis*, *Collocalia maxima*, and *Collocalia unicolor* (Sankaran, 2001).

However in Malaysia the main EBN source comes from the swiflet species of *Aerodromus fuciphagus.* These birds construct their nests with their saliva which is described as glutinous strands of starch. The saliva comes from a pair of large salivary glands under the tongue and is produced after mating and breeding their young ones in the nest. The breeding seasons are usually from December to March, April to July and August to November (Deraman, 2012). Some of the main areas in Malaysia where EBN is source is found are Sitiawan, Teluk Intan, Kota Bharu, Kuala Terengganu, Parit Buntar, Bukit Mertajam, Nibong Tebal, Kuantan, Muar, Segamat and many other old townships. EBN can be mainly divided into two groups which are house nest and cave nest. In Malaysia, the colour of the EBN is used to grade them accordingly. Usually the red coloured EBN which is also known as red blood nest is graded as high quality nest; therefore, it is priced expensive as compared to others (Norhayati Jr, Azman, & Wan, 2010).

2.2.2 Traditional Uses of EBN

Some of the main traditional uses of EBN that are associated with enhancing the health are as follows; improves the immune system and digestive system which hence improves appetite, improves complexion and skin tone and prevents flu, cough, phlegm and asthma. Other uses which have been stated are the ability to enhance the renewal of cells and tissues and also improving blood circulation and strengthen lungs and kidneys(Lim et al., 2002).

In the traditional Chinese medicine, the EBN is used for the following; for treating consumptive diseases, curing tuberculosis, dry coughs, suppressing cough and phlegm-dyspnea (difficulty in breathing), alleviating asthma, hemoptysis (coughing blood), improving the voice, asthenia, stomach ulcer, relieving gastric troubles, and general weakness of bronchial ailments. Either than that, it is also used to nourish the kidneys, lungs heart and stomach to aid renal functions, raise libido, strengthen the immune system, promote growth, enhance the immune system, improve concentration, increase energy and metabolism, and regulate circulation (Chan, 2006; Hobbs, 2004; Kong et al., 1987).

It is also believed that consuming EBN often can improve physical and mental strength and at the same time slows down aging effect hence restoring one’s youthfulness. Even though there many health benefits that have been claimed, yet the mechanism of action are still unknown and there is no evidence to support the claims (Norhayati Jr et al., 2010).

2.2.3 Biological Compounds

The nests that are built by the male swiflets during breeding seasons are entirely from their saliva. Some of the birds will include feathers while constructing their nests and this amount is always within 10% of the dry weight. The average content of protein that has been reported are as follows; (HYDROLYSATES, 2012) at 53.26%, (Marcone, 2005)at 62-63% and (Kathan & Weeks, 1969) at 32.3%. All 18 types of amino acids that are needed by a human body are present in the nest with aspartic acid and serine as the major amino acids present. These amino acids are water soluble in nature and aids in skin healing, cell energy production, regulates cell function produces antibodies and immunoglobulin which enhances immune system .

The other common group that is found in the nest is carbohydrate in the form of glycoprotein. The nest is mainly composed of glycoprotein with the mucin type glycoprotein which allows it to serve as lubricant and protective agent. The carbohydrate content is found to be at 27.26% (Marcone, 2005). The carbohydrate components are as follows; 9% of sialic acid, 7.2% of galactosamine, 5.3% of glucosamine, 16.9% of galactose and 0.7% of fucose. However, it is stated that sialic acid is the major substitute of carbohydrate in the EBN (Oda, Ohta, Suga, & Aoki, 1998).

Processed EBN contains a reasonable amount of minerals which are as follows; sodium (650 ppm), potassium (110 ppm), calcium (1298 ppm), magnesium (330 ppm), phosphorous (40 ppm) and iron (30 ppm). Hence, sodium, potassium, calcium and magnesium are said to be the major minerals present in the nest and are mainly important to activate enzyme reactions in the body (Marcone, 2005). Table 2.2 explains the properties of EBN.

Table 2.2: Properties of EBN (Chan, 2006; Guo et al., 2006; Ibrahim, Sugimoto, & Aoki, 2000; Kong et al., 1987; MARNI et al.; Matsukawa et al., 2011; Ng, Chan, & Kong, 1986; Yida, Imam, & Ismail, 2014).

EBN properties	Discussion
Antioxidant activity	Some of the amino acids for instance cysteine, methionine, histidine, tryptophan and lysine that are found in the EBN have been proven for antioxidant activity. It is said that the hydroxyl and carboxyl groups in the amino acids are responsible for this for this activity.
Epidermal growth factor (EGF)-like activity and mitogenic effects	The function of EGF is to stimulate the proliferation of epidermal and epithelial tissues, in cell culture it stimulates thymidine and amino acid incorporation into human fibroblasts and also initiates 3T3 fibroblast cell division. Partially purified swiftlet nest extracts possess the first known EGF which induces cell division, tissue growth, cellular regeneration and cell-mediated immunity. It has been demonstrated that the glycoprotein is capable of promoting cell growth. The EBN extract that contain glycoprotein are responsible for the mitogenic response of human peripheral blood monocytes to stimulate the Concanavalin A (Con A) or Phytohaemagglutinin (PHA).
Inhibits influenza virus	EBN extract has been reported to contain haemogglutination which possess an inhibiting action against the influenza virus.
Bacteriostatic and bacteriocidal activites	EBN contains ovortransferrin-like protein which are both bacteriostatic and bacteriocidal and is effective in fighting against infections.
Neurological properties	One of the functions of sialic acid is for mediating gaglioside distribution and structure of the brain. It is reported that a good amount of sialic acid contributes to neurological and intellectual functions in infants.
Immunomodulating properties	It is said that the glucosamine that is found in the EBN contain some immunomodulating effects.
Improves skin texture	Showed that rats' bone strength and skin thickness were increased when administered with 100 mg/kg of EBN extract.

2.3 Cryopreservation

Cryopreservation is a method of freezing cells or tissues at low temperatures of -196°C in liquid nitrogen where the metabolic activities of the cells are arrested. Generally, water is important for living cells to function and therefore we can conclude that freezing of water can lead to death of cells. Yet, it is said that freezing can preserve cells for a long period of time without disturbing its viability, where it slows or stops certain biochemical reactions but accelerates others. The main objective of cryopreservation is to minimize the amount of damage to the biological material during freezing at low temperature and storage. The cryopreservation can be achieved at -196°C in liquid nitrogen or above -196°C in conventional freezer, freeze drying or vitrification. However, it is stated that freezing biological material at above -196°C (-130°C to be exact) has lower viability in long terms as compared to storage in liquid nitrogen. Therefore, the conventional freezer is only used for microbial suspension and for mammalian tissue culture with large number of cells. During freezing, the cells are exposed continuously to hypertonic solution, therefore it is necessary to remove a certain amount of water from the cells osmotically to avoid intracellular freezing. At a slow cooling rate, there is equilibrium between the cells and it's external environment. However, the external environment freezes before the internal medium due to the protective cell membrane layer. This will make the external environment more hypertonic and thus there forcing the water to flow out from the cells. Removal of too much of water from the cells may result in the increase of solutes within the cells; hence the environment is not in equilibrium. At a higher cooling rate there is less time for the water to flow out of cells resulting in the formation of intracellular ice and hence causing the cells to be lethal. Therefore, an optimum cooling rate is needed to be achieved between these two situations. A summary; at slow rate of cooling, cells will die

due to exposure to hypertonic condition for a long period of time whereas, for high rate of cooling, the cells die due to the formation of intercellular ice. Other challenges that maybe encountered by the cells are as follows; increase in ionic concentration, extreme viscous of solution, pH changes, cells becomes closed packed, dehydration and also precipitation of salts (Anchordoguy, Cecchini, Crowe, & Crowe, 1991; Ozkavukcu & Erdemli, 2002).

2.4 Cryoprotective Agent

The main goal of cryopreservation is to replace some of the water content within the cell with other components that will not result in large ice crystals. Some of the examples of these components are DMSO and glycerol. They are generally non-toxic, contains low molecular weight and also able to permeate cells. Usually cells that are to be freezed in liquid nitrogen are suspended in this component which is mixed with a medium and serum. As the medium begins to freeze, the salt concentration in the external environment becomes greater as compared to the inner of the cells hence forcing water out of the cells and to be replaced by this cryoprotectant. Thus, reducing cellular damage during freezing process. Some of other functions of a cryoprotectant are as follows; protects cells by reducing cell shrinkage, reduces the fraction of solution that is to be frozen and minimizes the formation of intracellular ice. A cryopreservation medium will contain the following; cryoprotectant, base medium and protein source. The cryoprotectant is used to protect cells from mechanical and physical stress and also reducing cellular damage by reducing the formation of ice crystals. As for the protein source, the most common one used is the fetal bovine serum (FBS) which serves to protect the cells from any freeze-thawing process stress. Cells are usually frozen slowly at 1°C/minute (Ozkavukcu & Erdemli, 2002).

Generally, DMSO will permeate into the cell membrane and reduces the amount of ice crystal formed both internally and externally. However, osmosis and dehydration will

still occur but at a very much lower temperature, hence allowing for the absence of formation of intracellular ice crystals. Another less commonly used cryoprotectant which is a high molecular weight polymer which does not permeate the cell membrane yet contributes to the same effect is the Hydroxyethyl Starch (HES). HES is preferable used with DMSO. Cryoprotectant such as glycerol is able to form hydrogen bonds with the head of the lipid which are present in the membrane, hence stabilizing the lipid membrane which is important for extreme dehydration situation (Arakawa, Carpenter, Kita, & Crowe, 1990). The amount of cell shrinking and also re-swelling depends on cryoprotectant concentration as well as the permeability of the cell membrane to both water and cryoprotectant used. As the cells are thawed, a reverse process will take place; the cells will first swell and then shrink. If the cell expands too much than it may lead to cell damage and hence it can be prevented by stepwise removal of the cryoprotectant (Gilmore, Liu, Woods, Peter, & Critser, 2000).

2.5 Research Assays

2.5.1 Morphology of Human Adipose Derived Stem Cells

After 24 hours of cell isolation, the ASCs would adhered to the respective culture flask, where as the non adherent cells for example; red blood cells would be floating in the culture. MSCs are plastic-adherent and posses a spindle shaped cells, which develops into visible colonies after approximately three days. After the second passage, human adipose derived stem cells will apprear more uniform with fibroblast-like shape which is similar to a bone marrow stromal cell.

Adipogenic differentiation: According to (Zhu et al., 2008), no lipid droplets would be observed in an undeffientiated ASCs, where as ASCs differentiated into adipocytes via mutiple passage showed intracellular lipid filled droplets in almost 80-90% of the cells in adipogenic media. The cells contain lipid vesicles with the majority of intracellular space occupied by droplets and lipid vesicles which gives an expanded morphology. However, at higher passage number, the differentiation potential decreased with only 40-55% ASCs differentiated into adipocytes (Csaki, Matis, Mobasheri, Ye, & Shakibaei, 2007).

Osteogenic differentiation: The culture that was incubated for about 3 weeks, showed multiple layers of cells which could also give rise to dense nodules from which radiated highly elongated spindle-shaped cells with large nuclei. The ASCs that undergo osteogenic differentiation is about 90% at passage 4 where the mineralization nodules were visible to the naked eye. As the passage number increases, the differentiation capacity decreases.

Chondrogenic differentiation: ASCs cultured (micromass pellet culture or high density culture) in chondrogenic induction medium contains a round, nodule like morphology (Csaki et al., 2007).

2.5.2 Induction Medium and Staining

Adipogenesis: Isobutyl-methylxanthine, insulin and dexamethasone are added to the adipogenic induction medium. Insulin promotes the differentiation of human adipose-derived stem cells into adipocytes. It is also proven that glucocorticoids in the presence of insulin are able to promote differentiation of MSCs towards adipogenic lineage. Oil red O staining can be done to show the presence of intracellular lipid vacuoles (Rampersad, 2012).

Osteogenesis: Ascorbic acid, β-glycerophosphate, and dexamethasone are added to the osteogenic induction medium. Dexamethasone is needed for osteogenesis of stem cells as well as for the formation of bone nodule. Ascorbic acid on the other hand functions as a cofactor in hydroxylation of proline and lysine residues in collagen and also increases the synthesis of non-collagenous bone matrix proteins. β-glycerophosphate is important for the calcification of the extracellular matrix. Alizarin red staining can be done to show the presence of a calcified extracellular matrix (Rampersad, 2012).

Chondrogenesis: Dexamethasone and TGF-β3 are added to the chondrogenic induction medium promotes chondrogenesis. Glucocorticoids promote chondrogenic differentiation by enhancing the expression of the cartilage extracellular matrix gene (Derfoul, Perkins, Hall, & Tuan, 2006). Alcian blue staining can be done to show the presence of proteoglycans (Guzzo, Scanlon, Sanjay, Xu, & Drissi, 2014).The average percentage of mineralization or lipid vacuoles or proteoglycans area against the surface area can be determined via images taken after their respective staining.

2.5.3 Resazurin Reduction Test

Alamar blue is used to monitor the reducing environment of a living cell. The active ingredient, resazurin is water soluble, non-toxic, stable in medium and possesses cell membrane permeability. Resazurin (IUPAC name: 7-hydroxy-10-oxidophenoxazin-10-ium-3-one) is also known as diazo-resorcinol, azoresorcin, resazoin and resazurine. This test was first used to assess bacterial and yeast contamination in milk in the year 1929. Resazurin; a highly dichromatic solution is a blue non-fluorescent dye that will be reduced into resorufin, which is highly fluorescent and pink in color. The function of the dye is to act as an intermediate to accept electron in the electron transport chain without interfering the normal transfer of electrons. The oxidation-reduction potential of Alamar blue is +380 mV at pH 7.0, 25 °C. Alamar blue can be reduced by the following; NADPH, FADH, $FMNH_2$, NADH and cytochromes. Once the indicator dye accepts the electrons, the non-fluorescent blue color is reduced to pink color. Other enzymes such as diaphorases, NAD(P)H:quinone oxidoreductase, dihydrolipoamine dehydrogenase, flavin reductase within the cytoplasm and the mitochondria reductases may also reduce the Alamar blue. Therefore, the Alamar blue reduction is not solely dependent on the electron transport where as it can be also the cellular metabolism. The oxidation-reduction state allows the measurement to be either quantitative; colorimetric and fluorometric readings or qualitative; changes in color indicating the presence and absence of viable cells. Spectrophotometric absorbance will be taken at two wavelengths which are as follows; 570 and 600 nm or 540 and 630 nm. Absorbance values will differ according to the microtiter plate. The fluorescence signals are measured at an excitation wavelength of 530–560 nm and an emission wavelength of 590 nm (Rampersad, 2012).

2.5.4 Cell Viability Assays

Cell viability assays are commonly used for drug screening. There are various cell functions to determine this which are as follows; ATP and coenzyme production, nucleotide uptake an enzyme activity, cell adherence and cell membrane permeability. Generally, viability assays measures the percentage of viable cells in a suspension which can be done via dye exclusion stain. Here, cells with intact membrane will exclude the dye while the cells without an intact membrane with uptake the dye. Examples of dye exclusion stain are as follows; Trypan Blue, erythrosine and naphthalene black. Trypan blue is commonly used for measure viability by staining the dead cells (Freshney). It is a rapid, simple and inexpensive method to assess cell viability (Longo-Sorbello, Say, m, Banerjee, & Bertino, 2006). In this method, the viability of the cells is determined by counting the unstained cells with the aid of a microscope (Freshney). According to (Longo-Sorbello et al., 2006) the dye exclusion test is the ability of the viable cells to be impermeable to the following dyes; Trypan Blue, erythrosine, naphthalene black and other dyes. When the security membrane is compromised, there is an uptake of dye by the non viable cells which will appear as dark blue color where as the viable cells which was not stained will appear as clear with refractile ring around them. One of the down points of trypan blue staining is that, the dye can’t be used to differentiate between healthy cells and the viable cells that are losing their cell functions.

CHAPTER III

METHODOLOGY

Figure 3.1 shows the overview of the experimental process. Subcutaneous adipose tissue that was collected was isolated within 24 hours and was cultured up to second passage. Then, the cells were subjected to cryopreservation for a month in liquid nitrogen. After a month, the cells were thawed and were grown to sufficient amount to carry out the following research assays: cell viability test, resazurin test, morphology observation and differentiation assay. The cells were differentiated into the following lineages: adipogenic, chondrogenic and osteogenic and the respective stains were carried out.

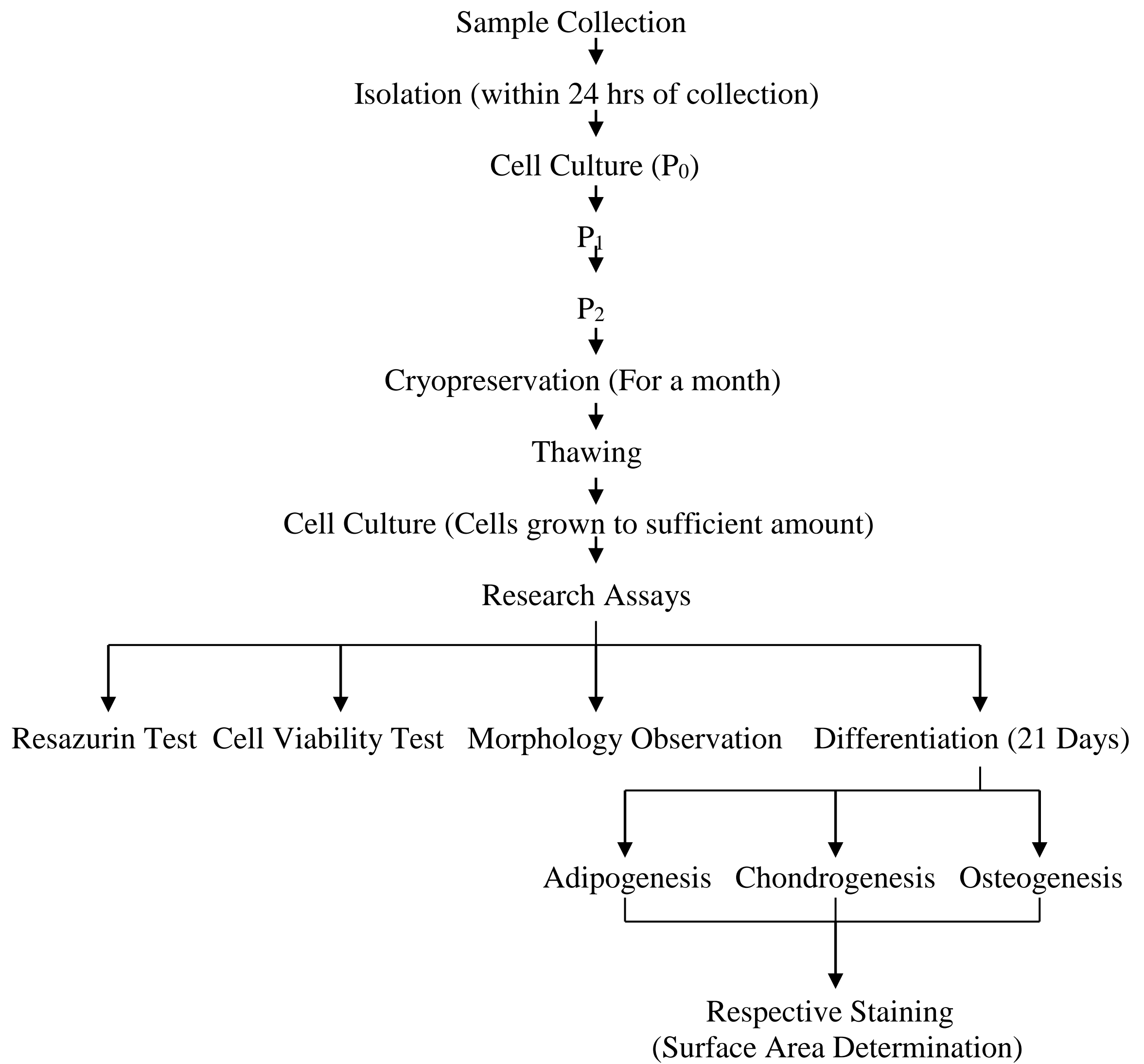


Figure 3.1: The overview of the experimental process

3.1 Sample Collection

The subcutaneous adipose tissue was collected from the C-section patients from Dewan Bedah, Pusat Perubatan Universiti Malaya. The collected sample was placed in a container containing saline solution. Prior to collection, the patient's were presented with a consent form. This is mainly to protect the human rights. After collection, the sample was brought back to the laboratory for isolation. The container containing the sample was wrapped with tissue to prevent any form of exposure to the sun.

3.2 Isolation

Under the laminar air flow, the subcutaneous adipose tissue that was collected was removed from the container and was placed on a sterile petri dish and was washed twice with Phosphate Buffered Solution (PBS). Than, with the aid of a sterile tweezer and scissor, the blood vessels were removed and the remaining tissue was finely chopped. The resulting tissue was place into a sterile Falcon tube and 6 mL of Collagenase Type I was added to it. Than, the tube was placed into the shaking incubator for 10 minutes, 37°C and 250 rpm and was later centrifuged at 10 minutes and 1500 rpm. After centrifuging, the supernatent was removed with the aid of a pastuer pipette and the remaining solution was washed with PBS. 10 mL of PBS was added and was vortexed and was centrifuged for 10 minutes and 1500 rpm This step is to remove all the oily substances. Than, the supernatent was removed with the aid of a pastuer pipette. 2 mL of complete medium was added to the pellet that remained in the tube and was dispersed with the aid of a pipette. The medium containing the dispersed pellet was divided into two T 25 Flasks. Finally, 5 mL of complete medium was added to both the flasks (P_o) and was incubated at normoxia condition; 21% O_2, 5% CO_2 for 24 hours before viewing under the microscope (Zuk et al., 2002).

3.3 Trypsinization

After 24 hours, the cells were viewed under the microscope and the culture medium was replaced to remove the non-adherent cells. The culture medium was renewed every 2 to 3 days. The human adipose derived stem cells primary culture (P_o) was grown to 90% confluence. Once it has reached 90% confluence, the cells were detached from the culture flasks with trypsin-EDTA (Gibco). The cell culture was grown up to passage 3 (P_3) which was used for further experiment. Once the cell culture has reached confluence, the cell culture was trypsinised. The cells were washed with 5 mL PBS. Then, 4 mL of trypsin was added to the culture and was incubated for 5 minutes. After 5 minutes, the cells were viewed under the microscope to ensure the cells were detached from the flask. Once the cells were detached, 5 mL of complete medium was added to the flask to deactivate the function of trypsin. All the solution was then transferred into a Falcon tube and was centrifuged for 10 minutes, 1500 rpm. The supernatant was then removed with the aid of a Pasteur pipette leaving behind the pellet. 4 mL of complete medium was added to the pellet and was suspended with the aid of the pipette and was divided into four T 25 Flasks. Finally, complete medium was added to all the flasks and was incubated at normoxia condition for 24 hours before viewing under the microscope. This process was repeated until it reaches P_3 (Zhu et al., 2008).

3.4 Cryopreservation

In this research, cells were preserved in the respective cryoprotectant which are as follows; 10% DMSO+90% FBS (Serves as Control), 5% DMSO+1% EBN+94% FBS and 1% EBN+99% FBS.

For all the three groups above, the cells were grown up to P_2 and once it reaches confluence, the cells were cryropreserved. Once the cell culture has reached confluence, the

cell culture was trypsinised. The cells were washed with 5 mL PBS. Then, 4 mL of trypsin was added to the culture and was incubated for 5 minutes. After 5 minutes, the cells were viewed under the microscope to ensure the cells were detached from the flask. Once the cells were detached, 5 mL of complete medium was added to the flask to deactivate the function of trypsin. All the solution was then transferred into a Falcon tube and was centrifuged for 10 minutes, 1500 rpm. The supernatant was then removed with the aid of a Pasteur pipette leaving behind the pellet. 1 mL of the respective cryoprotectant was added to the pellet and was suspended with the aid of a pipette and was transferred into a cryovial. The cryovials were placed into Mr. Frosty Freezing Container. The system is designed to achieve a rate of cooling very close to -1°C/minute, the optimal rate for cell preservation. The container was placed in -80°C overnight. This step is important because the cells must be frozen slowly which done by the Mr. Frosty Freezing Container. Then, the vials were transferred into the liquid nitrogen tank the following day. The cells were cryopreserved for a month (Liu et al., 2008).

3.5 Thawing Cells

After one month, the cells that were cryopreserved were thawed. The vials were placed in the water bath at 37°C. Once it has been thawed, under the laminar air flow, 10 mL of complete medium was added to the cells and was centrifuged at 2000 rpm for 10 minutes. Then, the supernatant was removed with the aid of a pastuer pipette leaving the pellet behind where 3 mL of complete medium was added to it. The resulting solution were suspended. Separately, 90 µL was taken out with aid of a pipette and were transferred into the centrifuge tube for cell counting. The remaining solution was transferred into a T 25 flask and appropriate amount of complete medium was added to it (Koch, Heerkens, Thomsen, & Betts, 2007).

3.6 Cell Counting

10 µL of trypan blue was added to 90 µL of the solution containing cells which was taken out earlier and was suspended with the aid of a pipette. 20 µL of the resulting solution was taken and was placed onto hemocytometer. Cell counting was done under the microscope (4x objective). The non-viable cells was stained blue, viable cells remained opaque. Cell counting was done in triplicates to obtain average (Louis & Siegel, 2011). Equation I below shows the formula to calculate the percentage of cell viability.

Equation I

$$\text{Percentage of Cell Viability} = \frac{\text{Total number viable cells}}{\text{Total number of cells}} \times 100\%$$

3.7 Cell Morphology

The cell morphology was determined by observing the morphological characteristics of the MSCs. The cell culture flasks were examined at all medium re-feeding intervals at two to three days to detect abnormalities in cell morphology. All MSCs should possess fibroblast-like morphology and at the same time must be plastic-adherent. Once the cells have reached confluence at passage 3, the cell culture was viewed at 4x and 10x objectives under the microscope and images were taken (J. Gimble & Guilak, 2003; Zhu et al., 2008).

3.8 Differentiation Assay

Once the cells have reached confluence at P_3, the ASCs were seeded into 6 well plates and were expended to 90% confluence. Osteogenic differentiation was carried out for 21 days. ASCs were maintained at normoxia condition and the culture medium (osteogenic induction medium) will be replaced every 2 days. After 21 days, the cells were stained with Alizarin Red solution to determine the calcium deposits (Zhu et al., 2008).

At the confluence of P_3, ASCs were seeded into 6 well plates and were expended to 70% confluence. Adipogenic differentiation was carried out for 21 days. ASCs were cultured in adipogenic induction medium and were maintained at normoxia condition. After 2 days, adipogenic induction medium was removed and was replaced with complete medium for the next 2 days. Cyclic treatment was continued for 21 days. After 21 days, the cells were stained with Oil-red-O Solution. Lipid droplet formation was detected using this stain to assess MSCs differentiation towards adipocytic phenotype (Zhu et al., 2008).

As for chondrogenic differentiation, at P_3, cells were seeded into the T 75 flask and were expended to 90% confluence. Once it has reached confluence, the cells were detached and were placed into the 50 mL Falcon tube and were centrifuged for 10 minutes, 2500 rpm. The pellet formed was cultured in the chondrogenic induction medium and was maintained at normoxia condition. The culture medium was replaced every 2 days for 21 days. After 21 days, the pellet was fixed, dehydrated, cleared and infiltrated and embedded in paraffin wax. The specimen was sectioned and fished on slides. Then, Alcian Blue staining was carried out and the resulting slide was viewed under the microscope (Sudo et al., 2007; Zhu et al., 2008). All the three lineages were stained and were viewed under the microscope at appropriate objective. Multiple images were taken which were used for surface area determination.

3.9 Staining Protocol

3.9.1 Alizarin Red

To prepare the Alizarin Red solution, 2g of Alizarin Red powder was mixed with 100 mL of deionized water in a beaker with the aid of a stirrer. Under the laminar air flow, the solution was filtered through a 0.25 mm syringe filter (De Bari, Dell'Accio, Tylzanowski, & Luyten, 2001).

Staining Procedure

Under the laminar air flow, the osteogenic induction medium was removed from the 6 well plates and the cells were fixed with 2 mL of 10% Formalin and were incubated at 4°C for 10 minutes. After 10 minutes, formalin was removed and was washed with deionized water. Then, the water was removed from each well and 2 mL of deionized water was added to each and was left for incubation between two to five minutes at room temperature. After the incubation period, the deionized water was removed and 2.5 mL of Alizarin Red solution (pH 4.1-4.3) was added to each well and was incubated at room temperature for 10 minutes. The stained cells were then extensively washed with PBS to remove excess stain and any nonspecific precipitation. Then, the plates were viewed under the microscope (10x objectives). The calcium deposition on the differentiated cells was represented by the positive red staining (De Bari et al., 2001; Ovchinnikov, 2009).

3.9.2 Oil-Red-O

Under the laminar air flow, a fresh 60% Oil-red-O working solution was prepared from a stock solution (300 mg Oil-red-O powder in 100 mL isopropanol), was left for 10 minutes and was filtered through a 0.25 mm syringe filter (De Bari et al., 2001).

Staining Procedure

Under the laminar air flow, the medium was removed from the 6 well plates and the cells were fixed with 2 mL of 10% Formalin and were incubated at 4°C for 10 minutes. After 10 minutes, formalin was removed and was washed with deionized water. Then, 60% isopropanol working solution was prepared. The water was removed from each well and 1 mL of 60% isopropanol was added to each and was left for incubation between two to five minutes at room temperature. After the incubation period, the , 60% isopropanol was removed and 2.5 mL of Oil-Red-O working solution was added to each well and was incubated at room temperature for 10 minutes. The stained cells were then washed with PBS to remove the residual Oil-Red-O. However, the stained cells were not washed extensively to maintain the hydration for clear viewing. Then, the plates were viewed under the microscope (4x objective). The lipid formation that was detected using this stain represented the differentiation of MSCs towards adipocytic phenotype (De Bari et al., 2001).

3.9.3 Alcian Blue Staining

After 21 days, the medium was removed and the micromass culture pellet was washed with PBS. For histological analysis, the pellet was first fixed in 10% Formalin for 24 hours. Then, the pellet was dehydrated in a graded ethanol series, cleared with xylene, infiltrated with paraffin wax and was embedded in paraffin wax. Next, the specimens were cut into 5 mm thick sections with the aid of a microtome and the paraffin sections were fished onto slides which were dried on a hot plate at 37°C (De Bari et al., 2001). Figure 3.2 shows the overview process of embedding the cells into the paraffin wax.

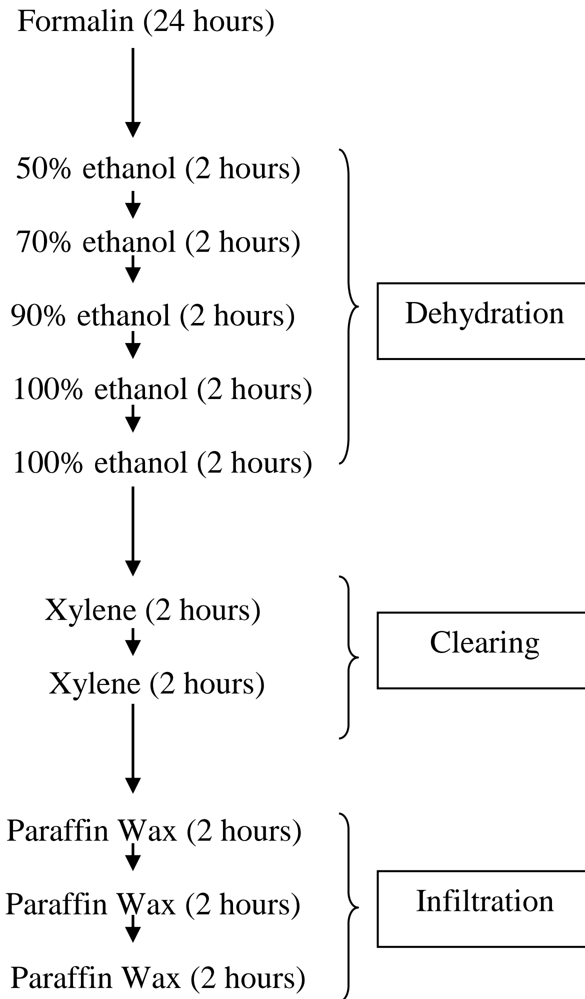


Figure 3.2: Process of Embedding the Cells into the Paraffin Wax

The slides with the paraffin sections were deparaffinised in xylene, rehydrated through a serious of ethanol, rinsed in distilled water and was stained with Alcian Blue to assess the proteoglycan content. Then, the stained tissue sections were mounted with DPX mounting and were visualized under the light microscope (10x objective). The differentiated cells were stained in light blue color (De Bari et al., 2001). Figure 3.3 shows the steps involved in the Alcian Blue staining.

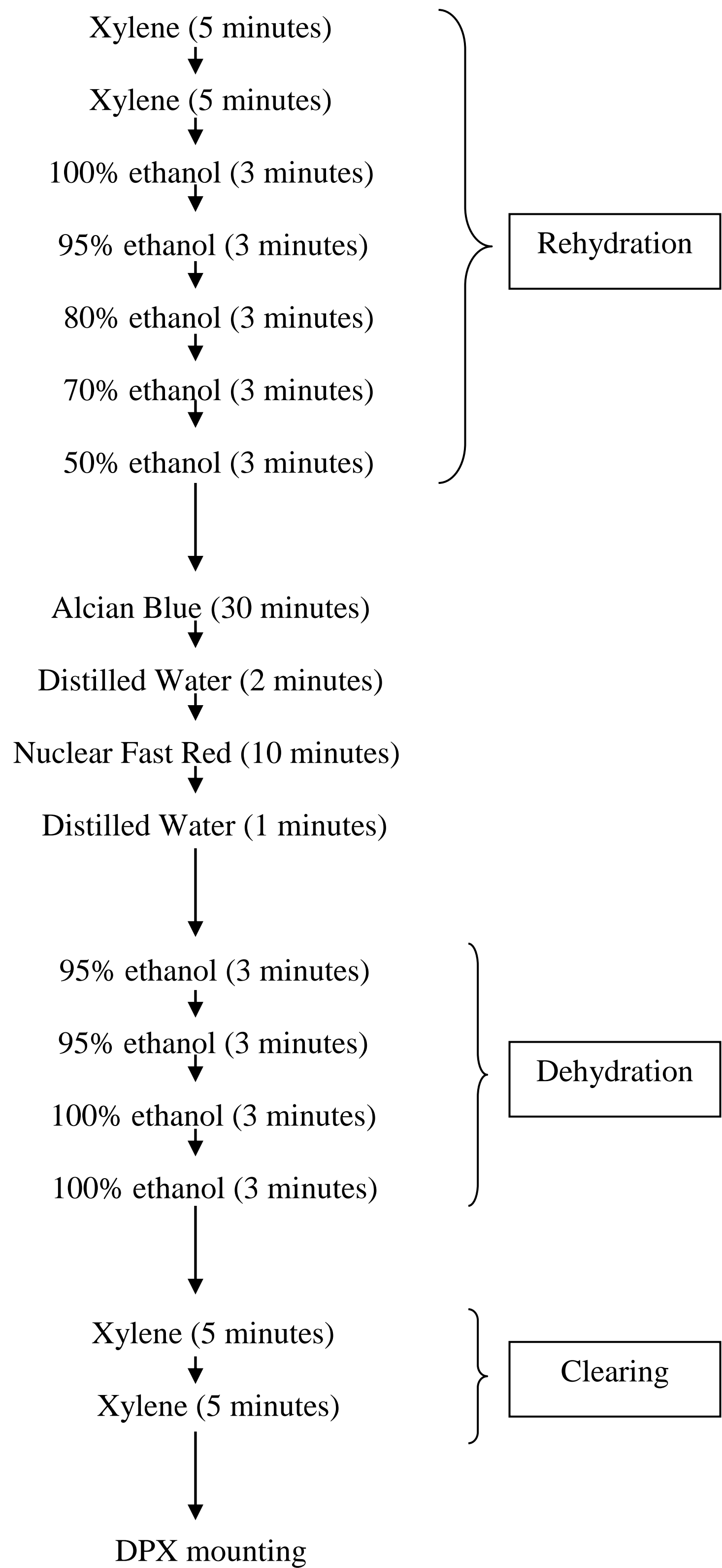


Figure 3.3: Alcian Blue Staining

3.10 Resazurin Reduction Test

As for resazurin test, the cells were seeded into the 24 well plate based on Equation I and II below.

Equation I

Cells per mL = the average count per square x the dilution factor x 10^4 (count 10 squares)

Equation II

$$\text{Cells per } \mu L = \frac{40\,000}{\text{Cells per mL}} \times \text{the dilution factor} \times 100$$

After seeding, the plate was incubated for 24 hours under normoxia condition. The resazurin reduction test was carried out on day 1, day 3, day 7, day 10 and day 14. Under the laminar air flow, after 24 hours of seeding, the medium was removed and was washed with PBS. Then, in the dark, 1000 µL (1 mL) of resazurin blue dye was added to the wells containing cells and two empty wells which serve as control. Once the dye has been added, the plate was wrapped with aluminium foil and was incubated for three hours under normoxia condition. After 3 hours, the plate was placed onto the shaking incubator for 10 minutes, 30 rpm. Next, under the laminar air flow, in the dark, 100 µL was taken from each well and were transferred into the 96 well plate. Triplicates from each well were done. The 96 well plate was wrapped with aluminium foil and was subjected to the microplate reader. The data obtained from the microplate reader will show the proliferation rate of the samples (O'Brien, Wilson, Orton, & Pognan, 2000). Using the data obtained, the following results were calculated using Equation III and IV below to plot both the standard and growth curve.

Equation III

$$\text{Percentage of Resazurin Reduction} = \frac{(\varepsilon OX)\ \lambda 2 A\lambda 1 - (\varepsilon OX)\ \lambda 1 A\lambda 2}{(\varepsilon RED)\ \lambda 1 A'\lambda 2 - (\varepsilon RED)\ \lambda 2 A'\lambda 1} \times 100$$

$\lambda 1 = 570$

$\lambda 2 = 600$

$(\varepsilon OX)\lambda 2 = 117{,}216$

$(\varepsilon OX)\lambda 1 = 80{,}586$

$(\varepsilon RED)\lambda 1 = 155{,}677$

$(\varepsilon RED)\lambda 2 = 14{,}652$

$A\lambda 1$ = Observed absorbance reading for test well

$A\lambda 2$ = Observed absorbance reading for test well

$A'\lambda 2$ = Observed absorbance reading for negative control well

$A'\lambda 1$ = Observed absorbance reading for negative control well

Equation IV

$y = 0.0004x$

y = Percentage of Resazurin Reduction

x = Number of cells

3.11 Medium Preparation

3.11.1 Basal Medium

Basal medium is a cell culture medium which is a complex mixture of salts, carbohydrates, vitamins, amino acids, metabolic precursors and trace elements. One packet of F12: DMEM powder (Commercial) and 1.2g of sodium bicarbonate was added to 1000 mL of distilled water in a beaker and were mixed with the aid of a stirrer. Then, the pH was adjusted to 7.2 with the aid of a pH meter. Under the laminar air flow, the medium was filtered with the aid of a vacuum pump (A. G. Smith, 1991).

3.11.2 Complete Medium

To enhance cell growth, growth factors, hormones and other proteins are added to the basal medium. Often this is accomplished by supplementing the basal medium with serum. The resulting medium is referred to as complete medium. Under the laminar air flow the ingredients in Table 3.1 were mixed together and were filtered.

Table 3.1: Ingredients of Complete Medium (A. G. Smith, 1991)

Ingredients	Volume (mL)
Basal medium (F12: DMEM)	435
10% FBS	50
1% antibiotic-antimycotic solution	5
1% glutamate	5
1% ascorbic Acid	5
Total	500

3.11.3 Phosphate Buffered Saline (PBS) Solution

PBS is buffer solution commonly used in biological research mainly to wash tissues and cells. It is a water-based salt solution containing sodium phosphate, sodium chloride and in some formulations, potassium chloride and potassium phosphate. Five PBS tablets were added to 1000 mL of distilled water in a beaker and were mixed with the aid of a stirrer.

Then, the pH was adjusted to 7.2 with the aid of a pH meter. The PBS solution was autoclaved (A. G. Smith, 1991).

3.11.4 Osteogenic Induction Medium

Induction medium is a medium that facilitate the differentiation of cells into the specific lineage. Under the laminar air flow, in the dark, the ingredients in Table 3.2 were mixed together and were filtered.

Table 3.2: Ingredients of Osteogenic Induction Medium (Peister et al., 2004)

Ingredients	Volume
Complete medium	98 mL
β-glycerophosphate	1.2 mL
Ascorbate-2-Phosphate	1 mL
Dexamethasone	100 μL

3.11.5 Adipogenic Induction Medium

Under the laminar air flow, in the dark, the ingredients in Table 3.3 were mixed together and were filtered.

Table 3.3: Ingredients of Adipogenic Induction Medium (Peister et al., 2004)

Ingredients	Volume
Complete medium	97 mL
Indomethacin	1 mL
Insulin Transferrin Selenium (ITS)	588 μL
3-Isobutyl-1-methylxanthine (IBMX)	2.2 mL
Dexamethasone	1 mL

3.11.6 Chondrogenic Induction Medium

Under the laminar air flow, in the dark, the ingredients in Table 3.4 were mixed together and were filtered.

Table 3.4: Ingredients of Chondrogenic Induction Medium (Peister et al., 2004)

Ingredients	Volume
Complete medium	97.5 mL
Insulin Transferrin Selenium (ITS)	1 mL
Insulin Growth Factor-1 (IGF-1)	50 µL
Ascorbate-2-Phosphate	1.2 mL
Dexamethasone	100 µL
L-proline	100 µL
Transforming growth factor beta 3 (TGF-β_3)	10 µL

CHAPTER IV

RESULTS

4.1 Cell Viability Test

The cell viability was carried out after thawing the cells and growing it to a confluence of 90% in a T25 flask. Table 4.1, 4.2 and 4.3 shows the percentage of cell viability for cells preserved in the 10% DMSO+90% FBS, 5% DMSO+1% EBN+94% FBS and 1% EBN+99% FBS respectively. The bar graph depicted in Figure 4.1 shows the comparison for the percentage of cell viability (using the mean value) for cells preserved in 10% DMSO+90% FBS, 5% DMSO+1% EBN+94% FBS and 1% EBN+99% FBS.

Table 4.1: Percentage of Cell Viability for Cells Preserved in 10% DMSO+90% FBS

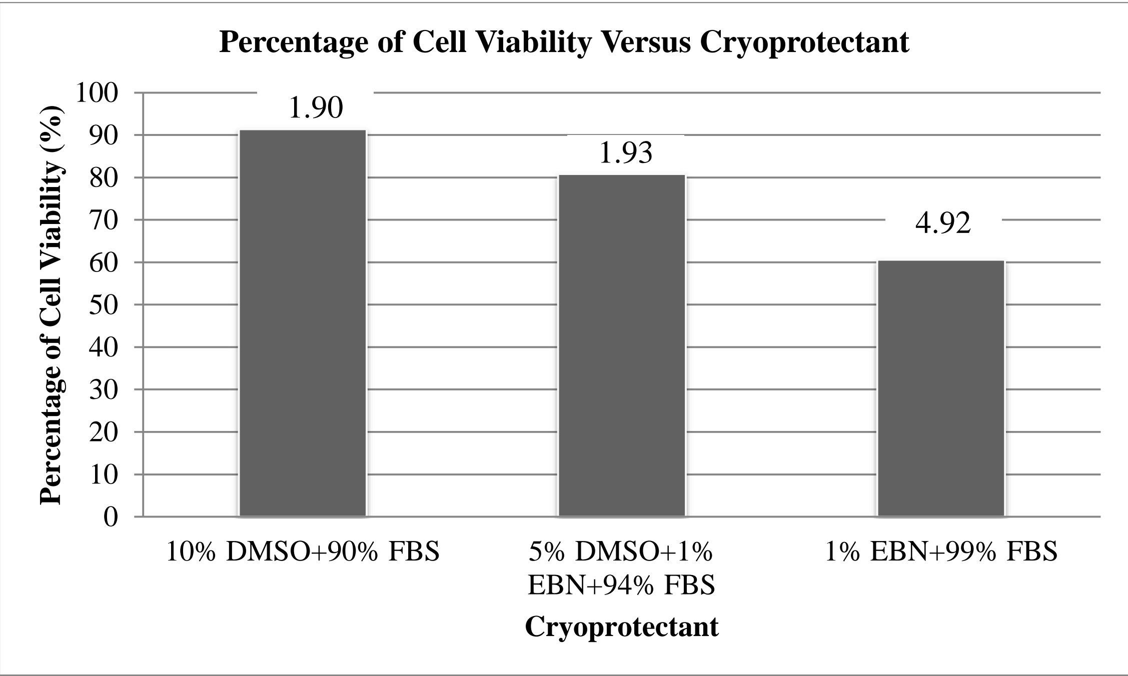
10% DMSO+90% FBS			
Non-viable cells	Total cells	Viable cells	Viability (%)
4	135	131	97.04
14	112	98	87.50
15	120	105	87.50
17	131	114	87.02
4	115	111	96.50
9	119	110	92.44
Mean			91.33
Standard Deviation			4.66
Standard Error			1.90

Table 4.2: Percentage of Cell Viability for Cells Preserved in 5% DMSO+1% EBN+94% FBS

5% DMSO+1% EBN+94% FBS			
Non-viable cells	Total cells	Viable cells	Viability (%)
29	136	107	78.68
19	145	126	86.90
15	107	92	85.98
21	110	89	80.91
21	85	64	75.29
24	105	81	77.14
Mean			80.82
Standard Deviation			4.74
Standard Error			1.93

Table 4.3: Percentage of Cell Viability for Cells Preserved in 1% EBN+99% FBS

1% EBN+99% FBS			
Non-viable cells	Total cells	Viable cells	Viability (%)
35	72	37	51.39
46	84	38	45.24
16	81	65	80.25
27	75	48	64.00
25	62	37	59.68
27	73	46	63.01
Mean			60.59
Standard Deviation			12.04
Standard Error			4.92



There is a significant difference ($P<0.05$) between all the three groups of the cryoprotectant.

Figure 4.1: Percentage of Cell Viability versus Cryoprotectant

4.2 Resazurin Test

Table 4.4 below shows the percentage of resazurin reduction and the respective number of cells for the cells preserved in 10% DMSO+90% FBS, 5% DMSO+1% EBN+94% FBS and 1% EBN+99% FBS. The number of cells was calculated based on the percentage of resazurin reduction for day 1, 3, 7, 10 and 14. Figure 4.2, 4.4 and 4.6 shows the standard curve (percentage of resazurin reduction versus the respective days) for 10% DMSO+90% FBS, 5% DMSO+1% EBN+94% FBS and 1% EBN+99% FBS respectively. Whereas, Figure 4.3, 4.5 and 4.7 shows the growth curve (cell number versus the respective days) for 10% DMSO+90% FBS, 5% DMSO+1% EBN+94% FBS and 1% EBN+99% FBS respectively.

Table 4.4: Percentage of Resazurin Reduction and Cell Number for the Respective Cryoprotectant

Day	10% DMSO+90% FBS		5% DMSO+1% EBN+94% FBS		1% EBN+99% FBS	
	Percentage of resazurin reduction (%)	Number of cells	Percentage of resazurin reduction (%)	Number of cells	Percentage of resazurin reduction (%)	Number of cells
1	19.59	48975	14.32	35800	14.72	36800
3	25.20	63000	15.98	39950	16.35	40875
7	26.02	65050	28.30	70750	16.74	41850
10	28.41	71025	31.47	78675	23.48	58700
14	40.72	101800	45.12	112800	12.01	30025

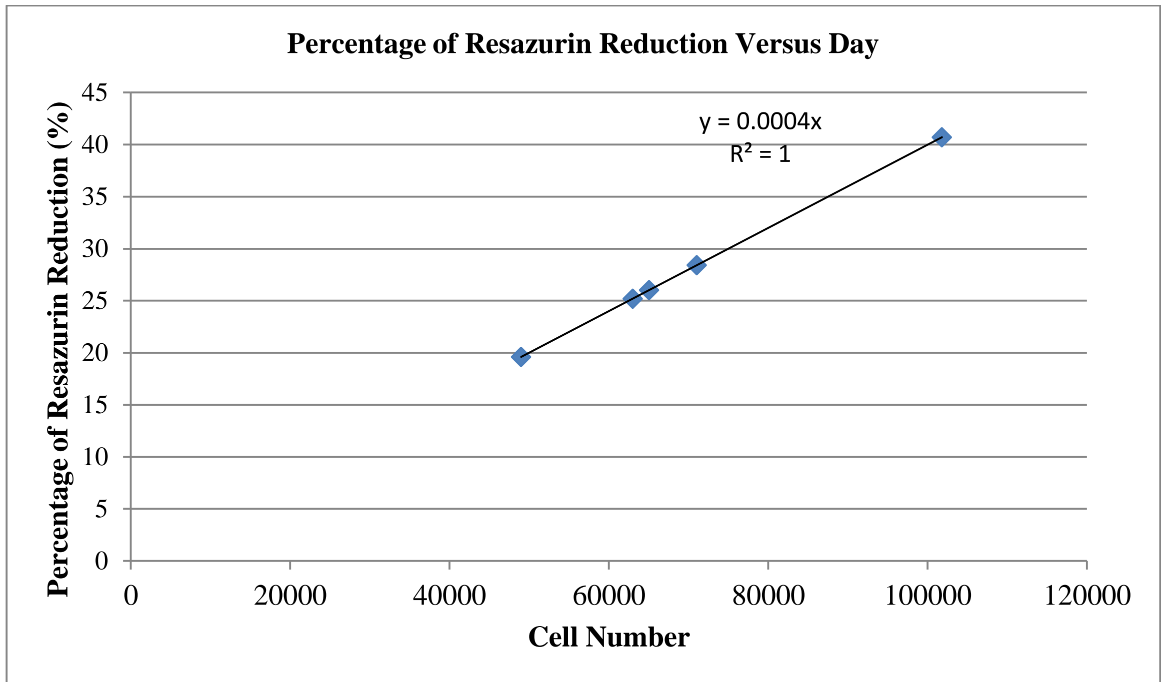


Figure 4.2: Standard Curve for 10% DMSO+90% FBS

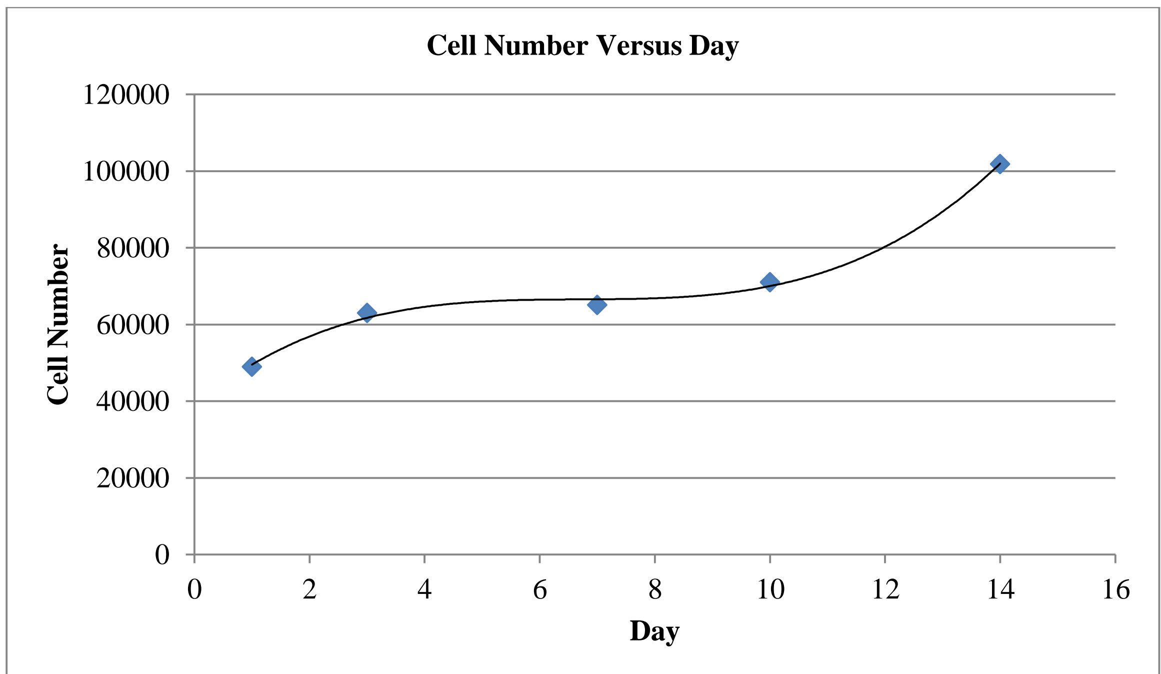


Figure 4.3: Growth Curve for 10% DMSO+90% FBS

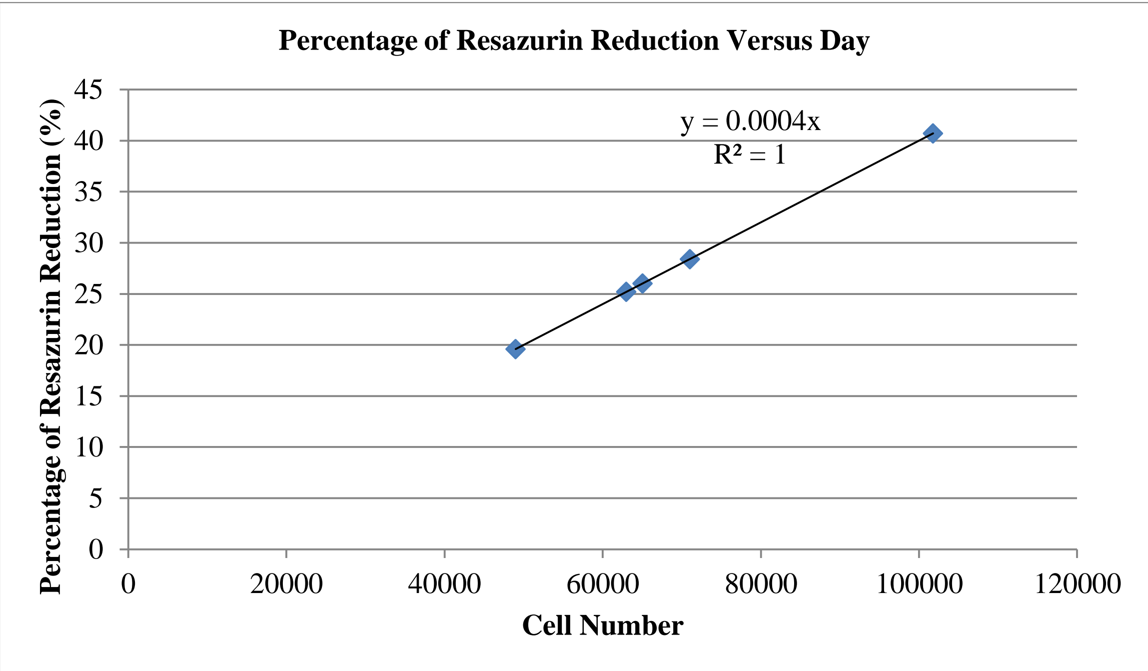


Figure 4.4: Standard Curve for 5% DMSO+1% EBN+94% FBS

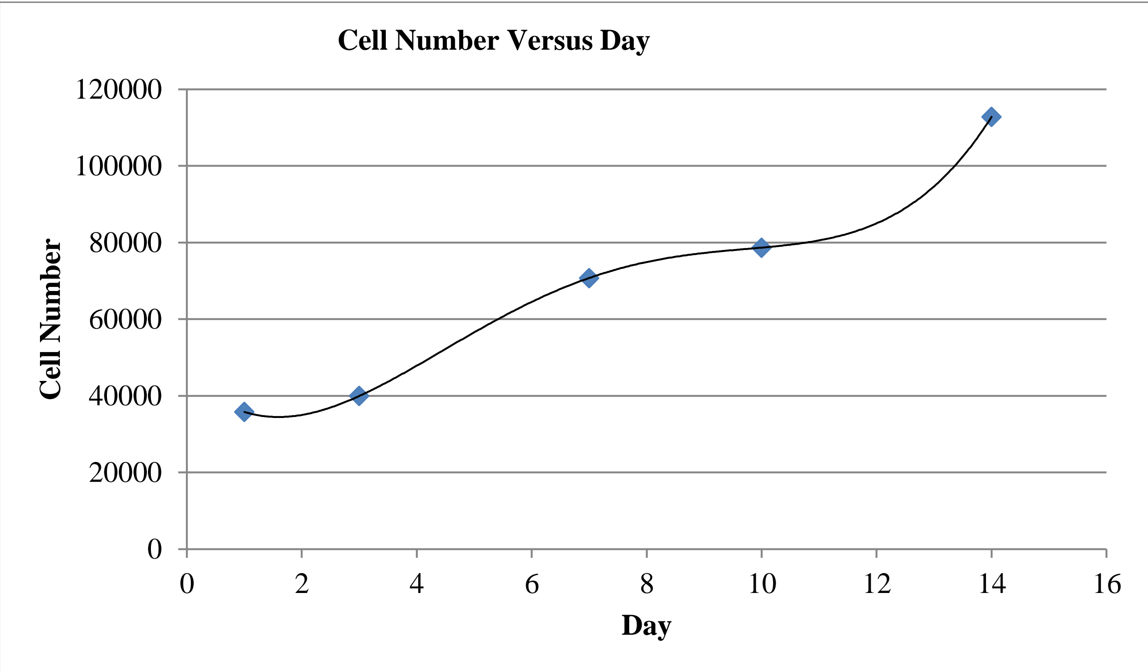


Figure 4.5: Growth Curve for 5% DMSO+1% EBN+94% FBS

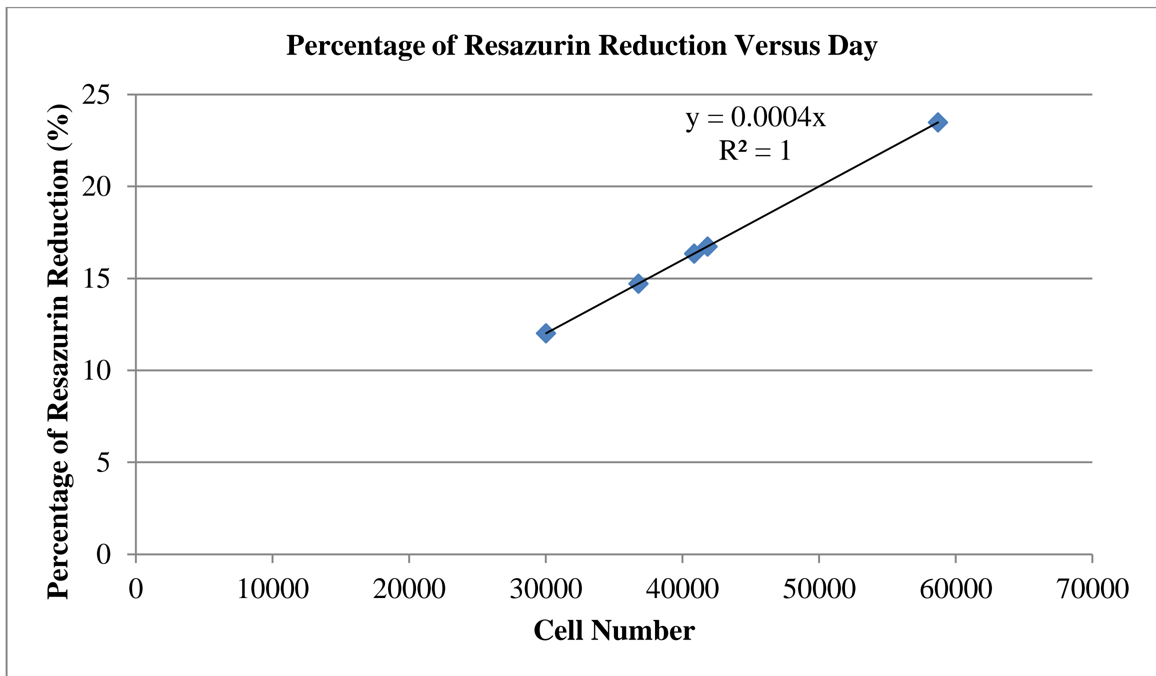


Figure 4.6: Standard Curve for 1% EBN+99% FBS

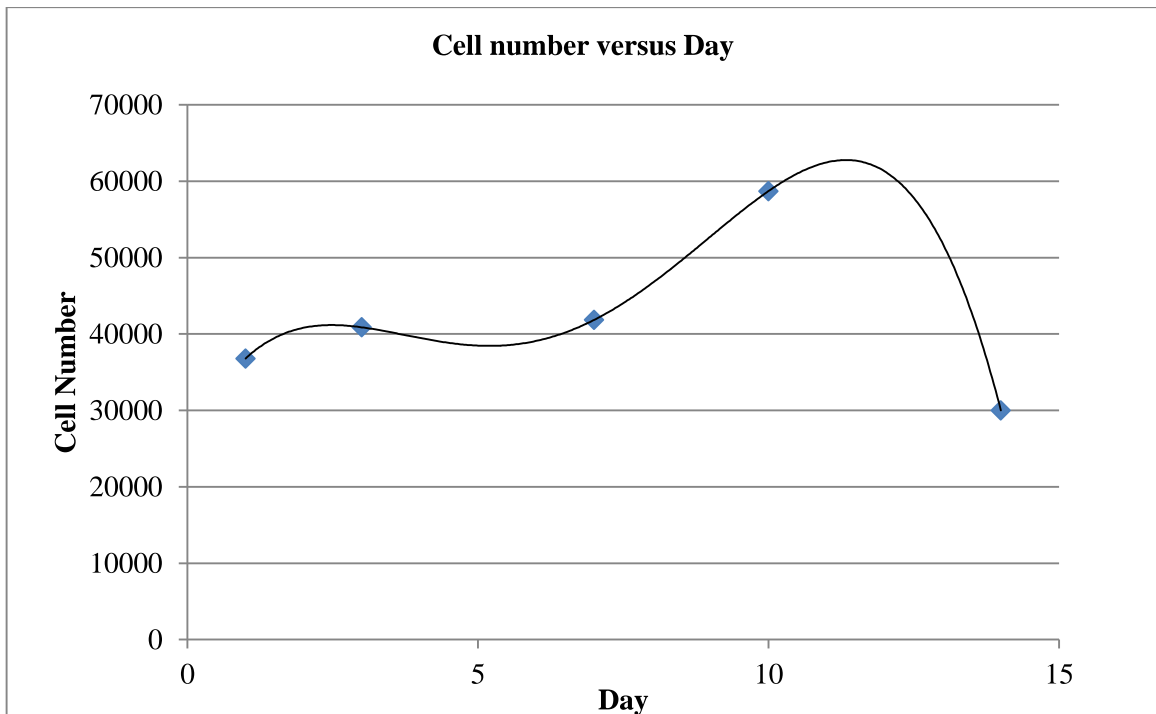
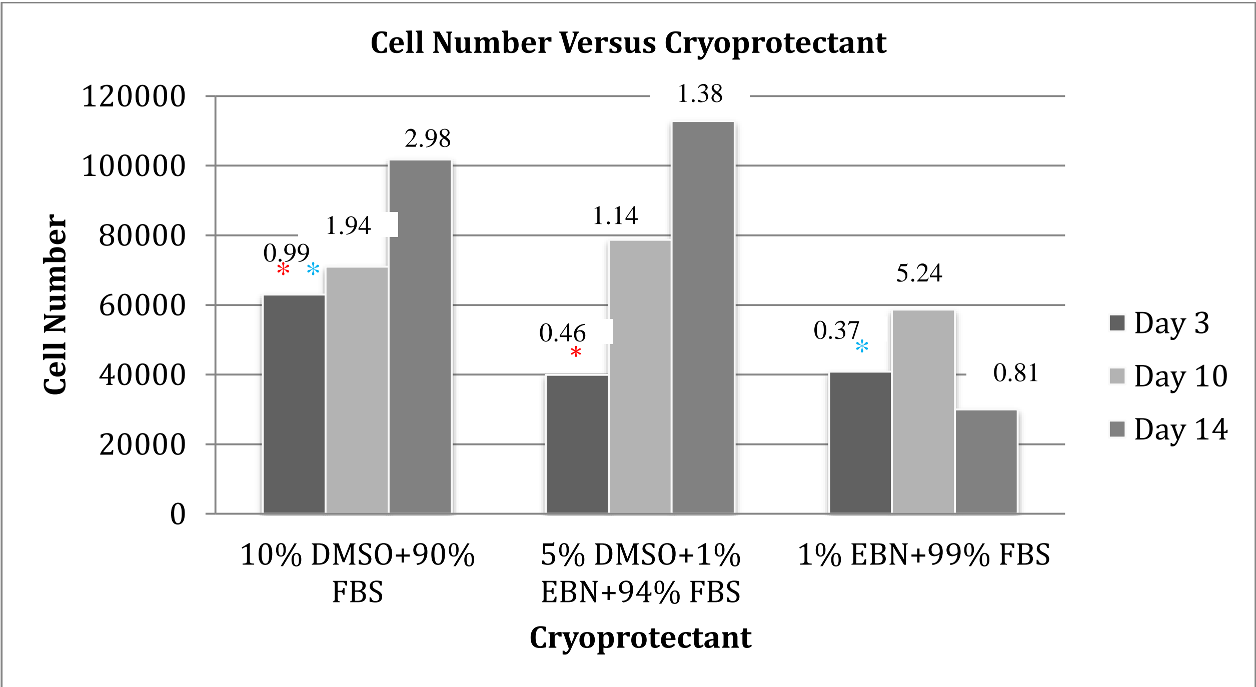


Figure 4.7: Growth Curve for 1% EBN+99% FBS

Figure 4.5 below shows the comparison the comparison for number of cells for day 3, 10 and 14 for cells preserved in the 10% DMSO+90% FBS, 5% DMSO+1% EBN+94% FBS and 1% EBN+99% FBS respectively. The standard error of mean is presented above each bar.



Day 3: There is a significant difference (P<0.05) which is indicated by * * between the groups of the cryoprotectant. Day 10: There is no significant difference (P>0.05) between all the three groups of the cryoprotectant. Day 14: There is a significant difference (P<0.05) between all the three groups of the cryoprotectant.

Figure 4.8: Comparison of Cell Number for Two Respective Days for the Respective Cryoprotectant

4.3. Differentiation Ability

4.3.1 Cryoprotectant: 10% DMSO+90% FBS

Figure 4.9 and 4.10 shows the morphology for cells preserved in 10% DMSO+90% FBS at a magnification of 4x and 10x objective, before staining.

I. Before Staining

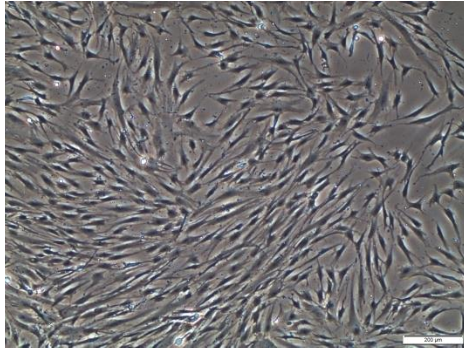


Figure 4.9: Stem Cells Preserved in 10% DMSO+90% FBS (Magnification: 4x objective)

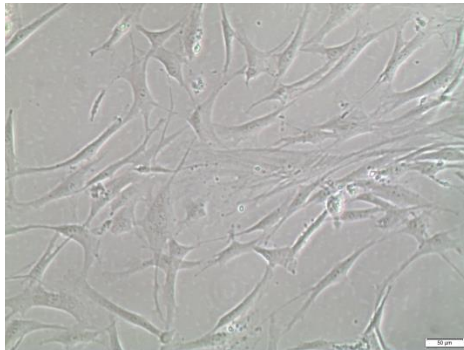


Figure 4.10: Stem Cells Preserved in 10% DMSO+90% FBS (Magnification: 10x objective)

Figure 4.11, 4.12 and 4.13 shows cells stained with the respective stains for the differentiation assay for cells preserved in 10% DMSO+90% FBS. Adipogenic differentiation was stained with Oil Red-O stain, whereas chondrogenic and Osteogenic were stained with Alcian blue and Alizarin red respectively.

II. After Staining

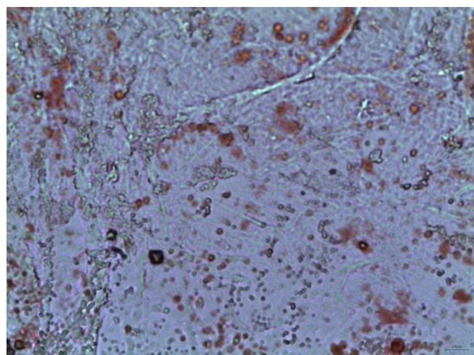


Figure 4.11: Adipogenic (Magnification: 4x objective)

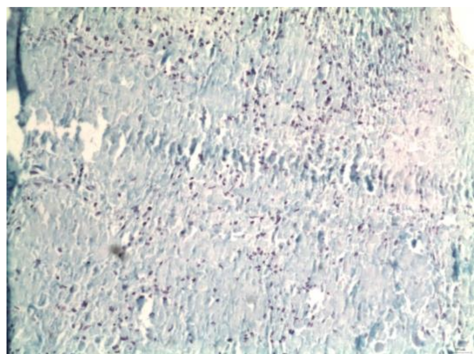


Figure 4.12: Chondrogenic (Magnification: 10x objective)

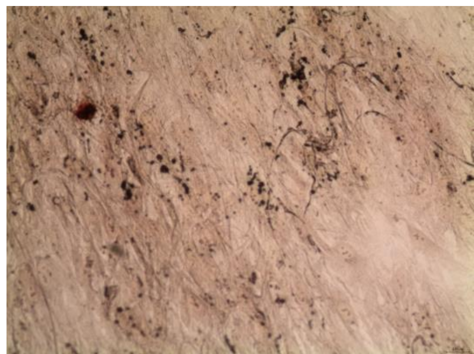


Figure 4.13: Osteogenic (Magnification: 10x objective)

4.3.2 Cryoprotectant: 5% DMSO+1% EBN+94% FBS

Figure 4.14 and 4.15 shows the morphology for cells preserved in 5% DMSO+1% EBN+94% FBS at a magnification of 4x and 10x objective, before staining.

I. Before Staining

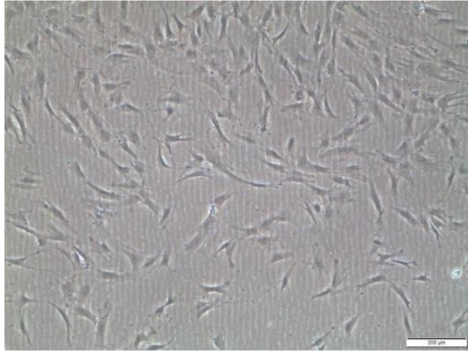


Figure 4.14: Stem Cells Preserved in 5% DMSO+1% EBN+94% FBS (Magnification: 4x objective)

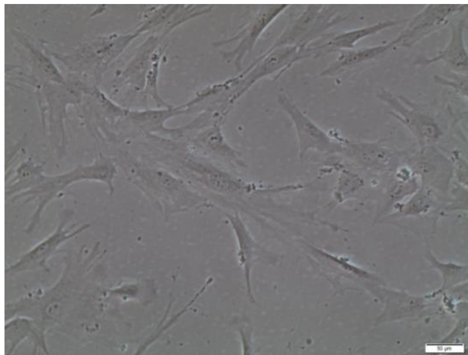


Figure 4.15: Stem Cells Preserved in 5% DMSO+1% EBN+94% FBS (Magnification: 10x objective)

Figure 4.16, 4.17 and 4.18 shows cells stained with the respective stains for the differentiation assay for cells preserved in 5% DMSO+1% EBN+94% FBS.

II. After Staining

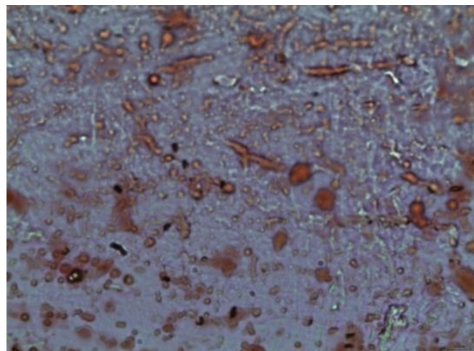


Figure 4.16: Adipogenic (Magnification: 4x objective)

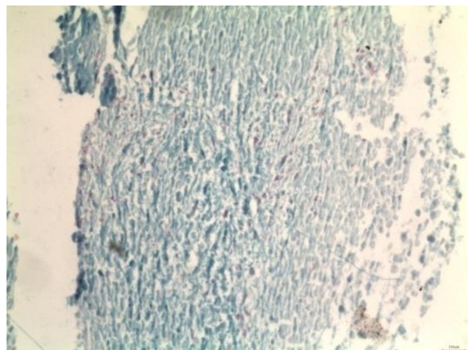


Figure 4.17: Chondrogenic (Magnification: 10x objective)

Figure 4.18: Osteogenic (Magnification: 10x objective)

4.3.3 Cryoprotectant: 1% EBN+99% FBS

Figure 4.19 and 4.20 shows the morphology for cells preserved in 1% EBN+99% FBS at a magnification of 4x and 10x objective, before staining.

I. Before Staining

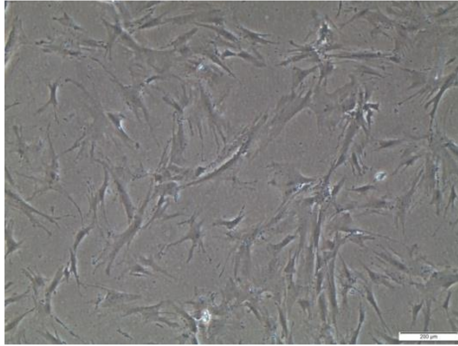


Figure 4.19: Stem Cells Preserved in 1% EBN+99% FBS (Magnification: 4x objective)

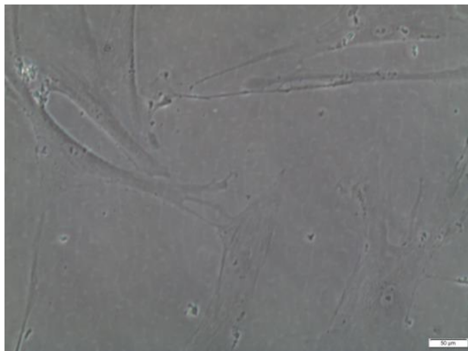


Figure 4.20: Stem Cells Preserved in 1% EBN+99% FBS (Magnification: 10x objective)

Figure 4.21, 4.22 and 4.23 shows cells stained with the respective stains for the differentiation assay for cells preserved in 1%EBN+99% FBS.

II. After Staining

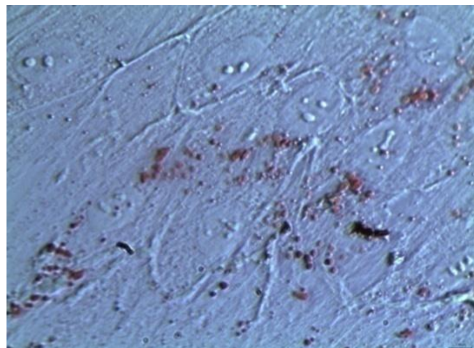


Figure 4.21: Adipogenic (Magnification: 4x objective)

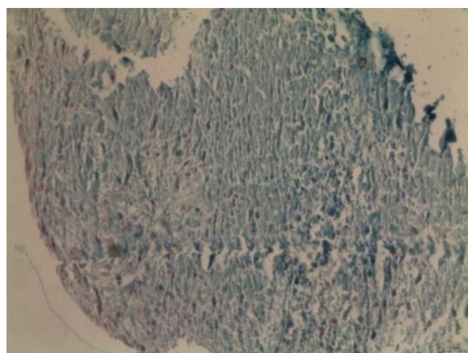


Figure 4.22: Chondrogenic (Magnification: 10x objective)

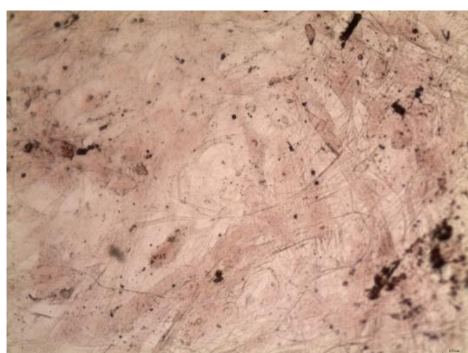


Figure 4.23: Osteogenic (Magnification: 10x objective)

4.4 Surface Area

Table 4.5, 4.6 and 4.7 shows the percentage of surface area of adipogenesis, chondrogenesis and osteogenesis for cells preserved in the 10% DMSO+90% FBS, 5% DMSO+1% EBN+94% FBS and 1% EBN+99% FBS respectively.

Table 4.5: Percentage of Surface Area of Cells Preserved in 10% DMSO+90% FBS

Percentage of Surface Area of 10% DMSO+90% FBS (%)			
	Adipogenesis	Chondrogenesis	Osteogenesis
	8.09	42.04	5.53
	17.73	60.19	9.22
	19.17	66.17	7.63
Mean	15.00	56.13	7.46
Standard Deviation	6.02	12.57	1.85
Standard Error	3.48	7.26	1.07

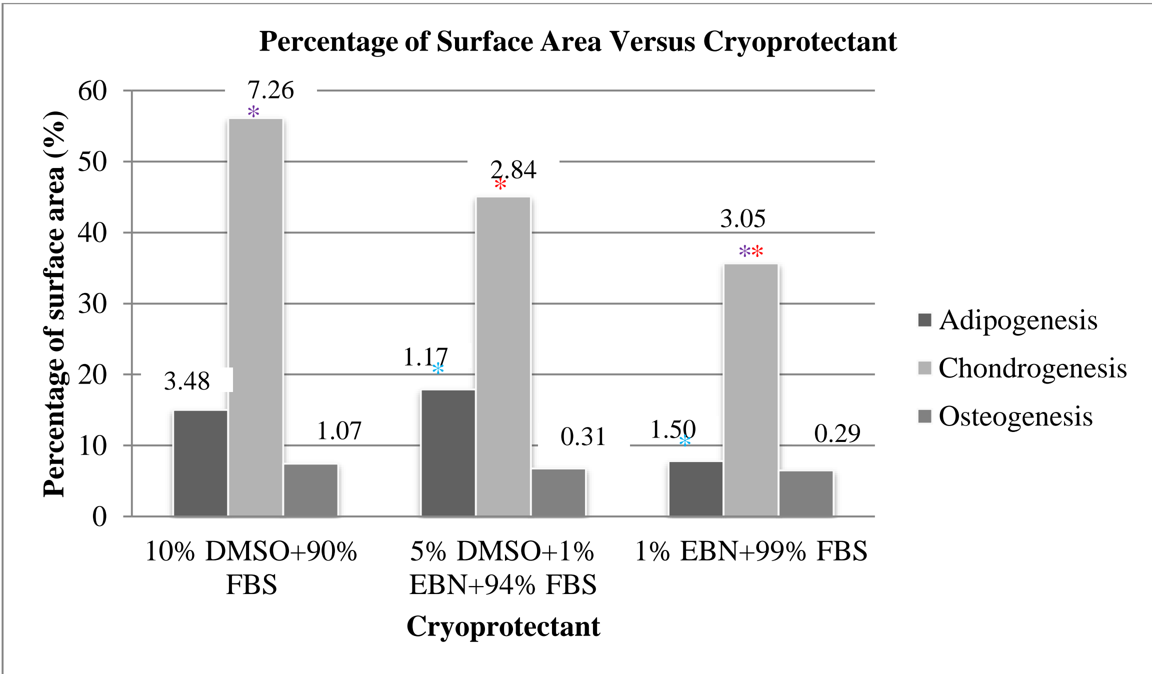
Table 4.6: Percentage of Surface Area of Cells Preserved in 5% DMSO+1% EBN+94% FBS

Percentage of Surface Area of 5% DMSO+1% EBN+94% FBS (%)			
	Adipogenesis	Chondrogenesis	Osteogenesis
	15.85	44.03	6.63
	19.91	50.43	7.34
	17.92	40.76	6.29
Mean	17.89	45.07	6.75
Standard Deviation	2.03	4.92	0.54
Standard Error	1.17	2.84	0.31

Table 4.7: Percentage of Surface Area of Cells Preserved in 1% EBN+99% FBS

Percentage of surface area of 1% EBN+99% FBS (%)			
	Adipogenesis	Chondrogenesis	Osteogenesis
	5.25	33.26	6.36
	7.77	32.00	7.05
	10.45	41.70	6.08
Mean	7.82	35.65	6.50
Standard Deviation	2.60	5.27	0.50
Standard Error	1.50	3.05	0.29

Figure 4.24 shows the comparison between the cryoprotectant for the percentage of surface area (using the mean value) for all the three lineages.



Adipogenesis: There is a significant difference (P<0.05) which is indicated by * between the groups of the cryoprotectant.

Chondrogenesis: There is a significant difference (P<0.05) which is indicated by * * between the groups of the cryoprotectant.

Osteogenesis: There is no significant difference (P>0.05) between all the three groups of the cryoprotectant.

Figure 4.24: Percentage of Surface Area for the Respective Differentiated Lineages versus Cryoprotectant

CHAPTER V

DISCUSSION

Based on Figure 4.1, the cells preserved in 10% DMSO+90% FBS has exhibited the highest percentage of cell viability with a mean of 91.33%. Generally, when a tissue or cells is subjected to a low temperature, ice crystals will be eventually formed. These crystals may disrupt the cell membrane leading to death of the cell and hence reducing cell viability. Therefore, according to (Ozkavukcu & Erdemli, 2002), a cryoprotectant is used to protect cells from mechanical and physical stress and also reduces the cellular damage by reducing the formation of ice crystals. Since, the cells preserved in 1% EBN+99% FBS had the lowest percentage of cell viability with a mean of 60.59%, hence it can be concluded that EBN perhaps does not have the mechanism to protect cells from mechanical and physical stress unlike DMSO. Research has concluded that extracellular cryoprotectant is not as efficient as the intracellular cryoprotectant (Motta et al., 2014). DMSO is an example of intracellular cryoprotectant which has its own mechanism to protect the cells. Perhaps EBN does not have the mechanism unlike DMSO to protect the cells. Hence, the viability of cells is low. Based on the One-way ANOVA shown in Appendix A, there is a significant difference ($P<0.05$) between all the three groups of cryoprotectant tested.

Based on Figure 4.3 and 4.5, the growth curve shows a continuous increase in the number of cell for the cells preserved in both 10% DMSO+90% FBS and 5% DMSO+1% EBN+94%FBS from Day 1 to Day 14 of the resazurin reduction test. Based on Table 4.4,

the cells preserved in 1% EBN+99% FBS had relatively lower number of cells as compared to the cells preserved in 10% DMSO+90% FBS and 5% DMSO+1% EBN+94%FBS. The number of cells for cells preserved in 1% EBN+99% FBS for Day 1, 10 and 14 are as follows: 36 800, 58 700 and 30 025. This further confirms the cell viability assay. Since the percentage of cell viability for the cells preserved in 1% EBN+99% FBS was initially low, therefore, this indicates a low number of cells in the culture and hence resulting in a lower proliferation rate. According to (Kong et al., 1987), EBN was proven to show an epidermal growth factor (EGF)-like activity which functions to induce cell division, tissue growth, cellular regeneration and cell-mediated immunity. It also contains glycoprotein which is responsible for promoting cell growth and gives them the ability to proliferate. According to (Roh et al., 2011), EBN strongly promotes proliferation of human adipose derived stem cells. However, these claims were not reflected on the results. Based on observation on the research papers above, all these studies were carried out in in vitro cell culture. Hence, it can be concluded that EBN promotes proliferation when it is in the in vitro cell culture environment whereas EBN as a cryoprotectant, it does not support the proliferation of the human adipose derived stem cells. Based on Figure 4.8, on Day 3, there is a significant difference ($P<0.05$) between 10% DMSO+90% FBS with 5% DMSO+1% EBN+94%FBS and 10% DMSO+90% FBS with 1% EBN+99% FBS. On Day 10, there is no significant difference ($P>0.05$) between all the three groups of cryoprotectant. Whereas on Day 14, there is a significant difference ($P<0.05$) between all the three groups of cryoprotectant tested.

Based on Figure 4.24, the average percentage of surface area for adipogenic, chondrogenic and osteogenic lineage for the cells preserved in 1% EBN+99% FBS are relatively lower as compared to the cells preserved in 5% DMSO+1% EBN+94% FBS and

10% DMSO+99% FBS. The average percentage of surface area for adipogenic, chondrogenic and osteogenic for cells preserved in 1% EBN+99% FBS are as follows: 7.82%, 35.65% and 6.50% respectively. Some of the studies that are related to the lineages studied and EBN extracts are as follows: according to (Chua et al., 2013), EBN extracts can act as a chondro-protective agent for human chondrocytes. Studies have also shown that rats' bone strength and skin thickness were increased when administered with EBN extract (Matsukawa et al., 2011). Both these research papers have shown good results that were not reflected in the result of this research. Based on observation, again, these papers were carried out in in vitro cell culture. Hence, it can be concluded that EBN can support differentiation when it is in an in vitro cell culture environment. In terms of cryoprotectant, once the cells are thawed and cultured back, EBN does not support differentiation. Therefore, resulting in poor results. In conclusion, this confirms the statement earlier that EBN can promote proliferation and differentiation when it is in an in vitro cell culture but as a cryoprotectant it fails to exhibit the same results. Based on Figure 4.24, there is no significant difference ($P>0.05$) between all the three groups of cryoprotectant for osteogenic lineage. There is a significant difference ($P<0.05$) seen for both adipogenic and chondrogenic lineage.

CHAPTER VI

CONCLUSION

Based on the results for the following tests; cell viability test, resazurin reduction test and differentiation assay, it can be concluded that the human adipose derived stem cells preserved in 10% DMSO+99%FBS had exhibited a higher result as compared to cells preserved in 5% DMSO+1% EBN+94%FBS and 1% EBN+99% FBS. Hence making DMSO still a good and superior cryoprotective agent. DMSO on its own can support the growth of the cells which can be seen in 10% DMSO+99% FBS and does not require the presence of EBN. Therefore, it can be concluded that EBN cannot act as a cryoprotective agent. EBN is better to be used in in vitro cell culture experiments instead of it as a cryoprotective agent. There has been no study done on EBN as a cryoprotective agent which is the main limitation of this study. DMSO is known to cause toxicity to cells. The ability of DMSO to induce non lamellar structures in phospholipids and to enhance membrane permeability may also damage biological membrane at physiological temperature. Moreover, DMSO also has the ability to induce cell differentiation indicates that it might exert an influence at the genetic regulation level (Yu & Quinn, 1994). Some of the suggestions for future studies are as follows: to study on higher percentage of EBN extracts with lower concentration of DMSO on the human adipose derived stem cells, to study EBN extracts with other cryoprotective agents (e.g. trehalose) and also to evaluate the biosafety of the EBN extracts to be used on human adipose derived stem cells. Any positive

results from these studies can introduce a natural cryoprotectant for cryopreservation of stem cells which will limit the usage of DMSO on whole, hence, reducing toxicity of DMSO to the cells.

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APPENDIX A

Statistical Analysis for Cell Viability Test

One-way ANOVA

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
10% DMSO+90% FBS	6	548.00	91.33	21.68
5% DMSO+1% EBN+94% FBS	6	484.90	80.82	22.45
1% EBN+99% FBS	6	363.57	60.60	145.02

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2928.81	2	1464.41	23.23	0.00	3.68
Within Groups	945.72	15	63.05			
Total	3874.53	17				

APPENDIX B

Statistical Analysis for Resazurin Test

(a) One-Way ANOVA for Day 3 Resazurin Test for the Respective Cryoprotectant

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
1% EBN+99% FBS	7.00	130.80	16.35	1.10
5% DMSO+1% EBN+94% FBS	7.00	111.86	15.98	1.45
10% DMSO+90% FBS	7.00	176.42	25.20	6.91

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	389.31	2.00	194.65	63.93	0.00	3.52
Within Groups	57.86	19.00	3.05			
Total	447.16	21.00				

(b) One-way ANOVA with post hoc t test for Day 3 Resazurin Test for the Respective Cryoprotectant

I. Between 1% EBN+99% FBS and 5% DMSO+1% EBN+94% FBS

t-Test: Two-Sample Assuming Equal Variances

	1% EBN+99%FBS	*5% DMSO+1% EBN+94% FBS*
Mean	16.35	15.98
Variance	1.10	1.45
Observations	7.00	7.00
Pooled Variance	1.26	
Hypothesized Mean Difference	0.00	
df	13.00	
t Stat	0.64	
P(T<=t) one-tail	0.27	
t Critical one-tail	1.77	
P(T<=t) two-tail	0.54	
t Critical two-tail	2.16	

II. Between 1% EBN+99% FBS and 10% DMSO+90% FBS

t-Test: Two-Sample Assuming Equal Variances

	1% EBN+99%FBS	*10% DMSO+90% FBS*
Mean	16.35	25.20
Variance	1.10	6.91
Observations	7.00	7.00
Pooled Variance	3.78	
Hypothesized Mean Difference	0.00	
df	13.00	
t Stat	-8.80	
P(T<=t) one-tail	0.00	
t Critical one-tail	1.77	
P(T<=t) two-tail	0.00	
t Critical two-tail	2.16	

III. Between 5% DMSO+1% EBN+94% FBS and 10% DMSO+90% FBS

t-Test: Two-Sample Assuming Equal Variances

	5% DMSO+1% EBN+94% FBS	*10% DMSO+90% FBS*
Mean	15.98	25.20
Variance	1.45	6.91
Observations	7.00	7.00
Pooled Variance	4.18	
Hypothesized Mean Difference	0.00	
df	12.00	
t Stat	-8.44	
P(T<=t) one-tail	0.00	
t Critical one-tail	1.78	
P(T<=t) two-tail	0.00	
t Critical two-tail	2.18	

(c) One-Way ANOVA for Day 10 Resazurin Test for the Respective Cryoprotectant

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
10% DMSO+90% FBS	7.00	198.86	28.41	26.45
5% DMSO+1% EBN+94% FBS	7.00	220.26	31.47	9.15
1% EBN+99% FBS	7.00	187.86	23.48	219.90

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	244.82	2.00	122.41	1.33	0.29	3.52
Within Groups	1752.94	19.00	92.26			
Total	1997.75	21.00				

(d) One-way ANOVA with post hoc t test for Day 10 Resazurin Test for the Respective Cryoprotectant

I. Between 10% DMSO+90% FBS and 5% DMSO+1% EBN+99% FBS

t-Test: Two-Sample Assuming Equal Variances

	10% DMSO+90% FBS	*5% DMSO+1% EBN+94% FBS*
Mean	28.41	31.47
Variance	26.45	9.15
Observations	7.00	7.00
Pooled Variance	17.80	
Hypothesized Mean Difference	0.00	
df	12.00	
t Stat	-1.36	
P(T<=t) one-tail	0.10	
t Critical one-tail	1.78	
P(T<=t) two-tail	0.20	
t Critical two-tail	2.18	

II. Between 10% DMSO+90% FBS and 1% EBN+99% FBS

t-Test: Two-Sample Assuming Equal Variances

	10% DMSO+90% FBS	*1% EBN+99% FBS*
Mean	28.41	23.48
Variance	26.45	219.90
Observations	7.00	7.00
Pooled Variance	130.62	
Hypothesized Mean Difference	0.00	
df	13.00	
t Stat	0.83	
P(T<=t) one-tail	0.21	
t Critical one-tail	1.77	
P(T<=t) two-tail	0.42	
t Critical two-tail	2.16	

III. Between 5% DMSO+1% EBN+94% FBS and 1% EBN+99% FBS

t-Test: Two-Sample Assuming Equal Variances

	5% DMSO+1% EBN+94% FBS	*1% EBN+99% FBS*
Mean	31.47	23.48
Variance	9.15	219.90
Observations	7.00	7.00
Pooled Variance	122.63	
Hypothesized Mean Difference	0.00	
df	13.00	
t Stat	1.39	
P(T<=t) one-tail	0.09	
t Critical one-tail	1.77	
P(T<=t) two-tail	0.19	
t Critical two-tail	2.16	

(e) One-Way ANOVA for Day 14 Resazurin Test for the Respective Cryoprotectant

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
10% DMSO+90% FBS	7.00	265.07	37.87	62.26
5% DMSO+1% EBN+94% FBS	7.00	315.81	45.12	13.39
1% EBN+99% FBS	7.00	96.06	12.01	5.22

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4609.21	2.00	2304.60	89.29	0.00	3.52
Within Groups	490.42	19.00	25.81			
Total	5099.63	21.00				

(f) One-way ANOVA with post hoc t test for Day 14 Resazurin Test for the Respective Cryoprotectant

I. Between 10% DMSO+90% FBS and 5% DMSO+1% EBN+99% FBS

t-Test: Two-Sample Assuming Equal Variances

	10% DMSO+90% FBS	*5% DMSO+1% EBN+94% FBS*
Mean	37.87	45.12
Variance	62.26	13.39
Observations	7.00	7.00
Pooled Variance	37.82	
Hypothesized Mean Difference	0.00	
df	12.00	
t Stat	-2.21	
P(T<=t) one-tail	0.02	
t Critical one-tail	1.78	
P(T<=t) two-tail	0.05	
t Critical two-tail	2.18	

II. Between 10% DMSO+90% FBS and 1% EBN+99% FBS

t-Test: Two-Sample Assuming Equal Variances

	10% DMSO+90% FBS	*1% EBN+99% FBS*
Mean	37.87	12.01
Variance	62.26	5.22
Observations	7.00	7.00
Pooled Variance	31.55	
Hypothesized Mean Difference	0.00	
df	13.00	
t Stat	8.90	
P(T<=t) one-tail	0.00	
t Critical one-tail	1.77	
P(T<=t) two-tail	0.00	
t Critical two-tail	2.16	

III. Between 5% DMSO+1% EBN+94% FBS and 1% EBN+99% FBS

t-Test: Two-Sample Assuming Equal Variances

	5% DMSO+1% EBN+94% FBS	*1% EBN+99% FBS*
Mean	45.12	12.01
Variance	13.39	5.22
Observations	7.00	7.00
Pooled Variance	8.99	
Hypothesized Mean Difference	0.00	
df	13.00	
t Stat	21.34	
P(T<=t) one-tail	0.00	
t Critical one-tail	1.77	
P(T<=t) two-tail	0.00	
t Critical two-tail	2.16	

APPENDIX C

Statistical Analysis for Surface Area of Differentiated Lineages

(a) One-Way ANOVA for the Percentage of Surface area of Adipogenesis for the Respective Cryoprotectant

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
10% DMSO+90% FBS	3	44.99	15.00	36.30
5% DMSO+1% EBN+94% FBS	3	53.68	17.90	4.12
1% EBN+99% FBS	3	23.47	7.82	6.76

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	161.25	2	80.63	5.13	0.05	5.14
Within Groups	94.36	6	15.73			
Total	255.61	8				

(b) One-way ANOVA with post hoc t test for Percentage of Surface area of Adipogenesis for the Respective Cryoprotectant

I. Between 10% DMSO+90% FBS and 5% DMSO+1% EBN+94% FBS

t-Test: Two-Sample Assuming Equal Variances

	10% DMSO+90% FBS	*5% DMSO+1% EBN+94% FBS*
Mean	15.00	17.89
Variance	36.29	4.12
Observations	3.00	3.00
Pooled Variance	20.21	
Hypothesized Mean Difference	0.00	
df	4.00	
t Stat	-0.79	
P(T<=t) one-tail	0.24	
t Critical one-tail	2.13	
P(T<=t) two-tail	0.47	
t Critical two-tail	2.78	

II. Between 10% DMSO+90% FBS and 1% EBN+99% FBS

t-Test: Two-Sample Assuming Equal Variances

	10% DMSO+90% FBS	*1% EBN+99% FBS*
Mean	15.00	7.82
Variance	36.29	6.76
Observations	3.00	3.00
Pooled Variance	21.53	
Hypothesized Mean Difference	0.00	
df	4.00	
t Stat	1.89	
P(T<=t) one-tail	0.07	
t Critical one-tail	2.13	
P(T<=t) two-tail	0.13	
t Critical two-tail	2.78	

III. Between 5% DMSO+1% EBN and 1% EBN+99% FBS

t-Test: Two-Sample Assuming Equal Variances

	5% DMSO + 1% EBN+94% FBS	*1% EBN+99% FBS*
Mean	17.89	7.82
Variance	4.12	6.76
Observations	3.00	3.00
Pooled Variance	5.44	
Hypothesized Mean Difference	0.00	
df	4.00	
t Stat	5.29	
P(T<=t) one-tail	0.00	
t Critical one-tail	2.13	
P(T<=t) two-tail	0.01	
t Critical two-tail	2.78	

(c) One-Way ANOVA for the Percentage of Surface area of Chondrogenesis for the Respective Cryoprotectant

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
10% DMSO+90% FBS	3	168.4	56.13	157.91
5% DMSO+1% EBN+94% FBS	3	135.22	45.07	24.19
1% EBN+99% FBS	3	106.96	35.65	27.82

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	630.49	2	315.25	4.51	0.06	5.14
Within Groups	419.84	6	69.97			
Total	1050.33	8				

(d) One-way ANOVA with post hoc t test for Percentage of Surface area of Chondrogenesis for the Respective Cryoprotectant

I. Between 10% DMSO+90% FBS and 5% DMSO+1% EBN+94% FBS

t-Test: Two-Sample Assuming Equal Variances

	10% DMSO+90% FBS	*5% DMSO+1% EBN+94% FBS*
Mean	56.13	45.07
Variance	157.91	24.19
Observations	3.00	3.00
Pooled Variance	91.05	
Hypothesized Mean Difference	0.00	
df	4.00	
t Stat	1.42	
P(T<=t) one-tail	0.11	
t Critical one-tail	2.13	
P(T<=t) two-tail	0.23	
t Critical two-tail	2.78	

II. Between 10% DMSO+90% FBS and 1% EBN+99% FBS

t-Test: Two-Sample Assuming Equal Variances

	10% DMSO+90% FBS	*1% EBN+99% FBS*
Mean	56.13	35.65
Variance	157.91	27.82
Observations	3.00	3.00
Pooled Variance	92.86	
Hypothesized Mean Difference	0.00	
df	4.00	
t Stat	2.60	
P(T<=t) one-tail	0.03	
t Critical one-tail	2.13	
P(T<=t) two-tail	0.06	
t Critical two-tail	2.78	

III. Between 5% DMSO+1% EBN+94% FBS and 1% EBN+99% FBS

t-Test: Two-Sample Assuming Equal Variances

	5% DMSO+1% EBN+94% FBS	*1% EBN+99% FBS*
Mean	45.07	35.65
Variance	24.19	27.82
Observations	3.00	3.00
Pooled Variance	26.01	
Hypothesized Mean Difference	0.00	
df	4.00	
t Stat	2.26	
P(T<=t) one-tail	0.04	
t Critical one-tail	2.13	
P(T<=t) two-tail	0.09	
t Critical two-tail	2.78	

(e) One-Way ANOVA for the Percentage of Surface area of Osteogenesis for the Respective Cryoprotectant

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
10% DMSO+90% FBS	3	22.38	7.46	3.43
5% DMSO+1% EBN+94% FBS	3	20.26	6.75	0.29
1% EBN+99% FBS	3	19.49	6.50	0.25

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.49	2	0.75	0.57	0.60	5.14
Within Groups	7.92	6	1.32			
Total	9.42	8				

(f) One-way ANOVA with post hoc t test for Percentage of Surface area of Osteogenesis for the Respective Cryoprotectant

I. Between 10% DMSO+90% FBS and 5% DMSO+1% EBN+94% FBS

t-Test: Two-Sample Assuming Equal Variances

	10% DMSO+90% FBS	*5% DMSO+1% EBN+94% FBS*
Mean	7.46	6.75
Variance	3.43	0.29
Observations	3.00	3.00
Pooled Variance	1.86	
Hypothesized Mean Difference	0.00	
df	4.00	
t Stat	0.64	
P(T<=t) one-tail	0.28	
t Critical one-tail	2.13	
P(T<=t) two-tail	0.56	
t Critical two-tail	2.78	

II. Between 10% DMSO+90% FBS and 1% EBN+99% FBS

t-Test: Two-Sample Assuming Equal Variances

	10% DMSO+90% FBS	*1% EBN+99% FBS*
Mean	7.46	6.50
Variance	3.43	0.25
Observations	3.00	3.00
Pooled Variance	1.84	
Hypothesized Mean Difference	0.00	
df	4.00	
t Stat	0.87	
P(T<=t) one-tail	0.22	
t Critical one-tail	2.13	
P(T<=t) two-tail	0.43	
t Critical two-tail	2.78	

III. Between 5% DMSO+1% EBN+94% FBS and 1% EBN+99% FBS

t-Test: Two-Sample Assuming Equal Variances

	5% DMSO+1% EBN+94% FBS	*1% EBN+99% FBS*
Mean	6.75	6.50
Variance	0.29	0.25
Observations	3.00	3.00
Pooled Variance	0.27	
Hypothesized Mean Difference	0.00	
df	4.00	
t Stat	0.61	
P(T<=t) one-tail	0.29	
t Critical one-tail	2.13	
P(T<=t) two-tail	0.58	
t Critical two-tail	2.78	