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Differentiation of Bovine Bone Marrow and Adipose Tissue Derived Mesenchymal Stem Cells into osteoblasts

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Abstract

Adipose tissue and bone marrow-derived stem cells have been extensively investigated for their applicability in the field of tissue engineering due to their multi-lineage differentiation potential. However, work on bovine-source adipose tissue and bone marrow-derived stem cells have been limited in use. In this work, bovine adipose tissue and bone marrow cells were cultured in osteogenic inductive medium for *in vitro* differentiation into osteoblast cells. Superficial white fat tissue and Bone marrow were isolated from freshly slaughtered 4,6-month-old cal. Cell proliferation as a measure of optical density was measured using absorbance reader at a wavelength of 570nm. Alizarin Red staining for the detection of calcium in the cultured cells was also performed. cDNA was synthesized from 20ng RNA. Using PCR, cDNA was amplified and its size was measured using Gel Electrophoresis. The morphologies of MSCs that proliferated in the *in vitro* culture were similar for cells from both adipose tissue and bone marrow. MTT assay test showed that the proliferation rate was lower for cells cultured in osteogenic medium compared to the cells cultured in the standard cell culture medium. All the gene markers for osteoblast cells were expressed by the MSCs cultured in the osteogenic medium. The culture induced osteoblast cells stained positive on Alizarin Red staining, indicating formation of calcified matrix. Differentiation of MSCs into osteoblast cells was better when combined with osteogenic medium. The study has provided evidence that adipose tissue MSCs can be used instead of embryonic stem cells and bone marrow MSCs as a source of osteoblast cell differentiation *in vitro*.

Key Words: Mesenchymal stem cell, Bovine, Adipose tissue, Bone marrow, Osteogenesis,

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Table of contents

List of Tables

List of Figures

Figures Pages

List of Abbreviations /Acronyms

ATC	Adipose Tissue in the control medium
ATO	Adipose Tissue in osteogenic medium
AT	Adipose Tissue
bASCs	bovine Adipose-derived stem cells
BM	Bone Marrow
BMA	Bone Marrow Aspirates
BMC	Bone Marrow in control medium
BMO	Bone Marrow in osteogenic medium
BMPs	Bone Morphogenetic Proteins
bMSC	Bovine Mesenchymal Stem Cell
BMSCs	Bone Marrow mesenchymal Stem Cells
BMT	Bone Marrow Transplant
BSA	Bovine Serum Albumin
CCM	Cell Culture Medium
cDNA	Bulk first-strand Deoxyribonucleic acid
DEPC	Diethyl Pyro carbonate
DEX	Dexamethasone
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECM	Extracellular Matrix
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium Bromide
HEPE	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic
HUCPVCs	Human Umbilical Cord Perivascular Cells
ips	Induced pluripotent stem cells
L-DMEM	Low Glucose Dulbecco's Modified Eagle's Medium
MSCs	Mesenchymal stem cells

MTT	3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl-tetrazolium bromide
$NaHCO_3$	Sodium Hydrogen Carbonate
O. D	Optical Density
OM	Osteogenic Medium
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RBCs	Red blood cells
SM	Standard Medium
SVF	Stromal Vascular Fraction
TAE	Tris-Acetate

1.Introduction

1.1. General background

Bone is highly dynamic in its turnover and its functional capabilities decline as a result of increase in age, trauma, tumor resection, osteogenesis imperfecta, and osteoporosis (Ferdous *et al.,* 2016). Osteoporosis is an abnormal loss of bone mass and disintegration of bone structures in older adults, aging with the combination of intrinsic and extrinsic factors and causes decline in bone mass that would be exposed to fractures. Intrinsic factors include individual genetic makeup, peak bone mass accrual in youth, alterations in cellular components, hormonal, biochemical and vasculature status (Demontiero *et al*., 2012). When these incidences or other large bone defects occur, different strategies of treatment exist, mainly involving surgery and autologous transplantation. The bone for the grafts is preferably taken from the iliac crest which is considered to be the safest and best strategy for bone repair. But this strategy has many drawbacks: the bone mass one can take from iliac bone is limited for it is not easy to spare and accomplish its normal functions. The surgical procedure for bone extraction is often characterized by donor site morbidities such as long-lasting pain (Sutherland & Bostrom, 2005) . The alternative sources are allogeneic bones which have the disadvantages of having diseased tissue and the immuno-histocompatibility differences that lead to rejection of the grafted bone and other numerous complications as a consequence (Gutiérrez *et al*., 2013).

Tissue engineering, which is a major component of regenerative medicine, can solve the problems associated with treatment of bone pathologies (Kim and Evans, 2005).Tissue engineering is a science that deals with humans and animals that have injured tissues or organ. This new technology can promote biological repair or regeneration of broken bones and

accelerates the healing process, in defective or diseased bones without the formation of scars and to form the normal bone size with its pre-existing properties (Corselli *et al*.,2010). The newly formed bone by osteoblast and osteoclast cells would be indistinguishable from the adjacent uninjured bone. The osteoblasts, which synthesize the bone and mineralize it; and the osteoclasts, which secret an enzyme that breaks down the bone tissue and also facilitate the absorption of the bone debris inside the cell, are the mains cell types that play a major role in bone healing process. Bone mass can be regulated by the bone-forming activity of osteoblasts, which are bone-specific mesenchymal stem cells. Osteoblasts have three functions: bone formation, i.e., the synthesis and secretion of most proteins of the bone's extracellular matrix (ECM) and to express genes that are necessary and sufficient to induce mineralization for this ECM (Huang *et al*., 2007). Once active, osteoblasts secrete collagen and other matrix proteins, which would be used in the construction of scaffolds. Osteocalcin is a protein that has an important role in pro-osteoblastic, or bone-building activities by nature. Osteocalcin is often produced by osteoblasts and is used as a marker for the bone formation process (Komori, 2009).

The healing process in regenerative medicine compared to surgery has many advantages (Kim and Evans, 2005); it takes much less time, does the repairing to the original size and shape without producing a scar;it rarely leaves a hall in the repaired bone; it does not require taking bone tissue from the patient or his relatives thus does not creat morbidity on donor's organ or tissue, instead uses the patient's own unwanted, adipose tissue and differentiate it in an *in vitro* system and deliver it back to the damaged part of the body in degradable scaffolds (Petite *et al*.,2000). The scaffold provides an efficient way to deliver the cells into the host and allow the cells to survive. It produces osteoclast cells that clean or absorb the bone debris while the osteoblast cells that produce new cells that synthesize new bone replace the old and dying cells.

The scaffold is designed to mimic, as much as possible, the extracellular matrix in native tissue and its relationship with the cell population; and in the process of healing it would be disintegrated and absorbed by the nearby normal or innate cells (Dunn *et al*.,2006).

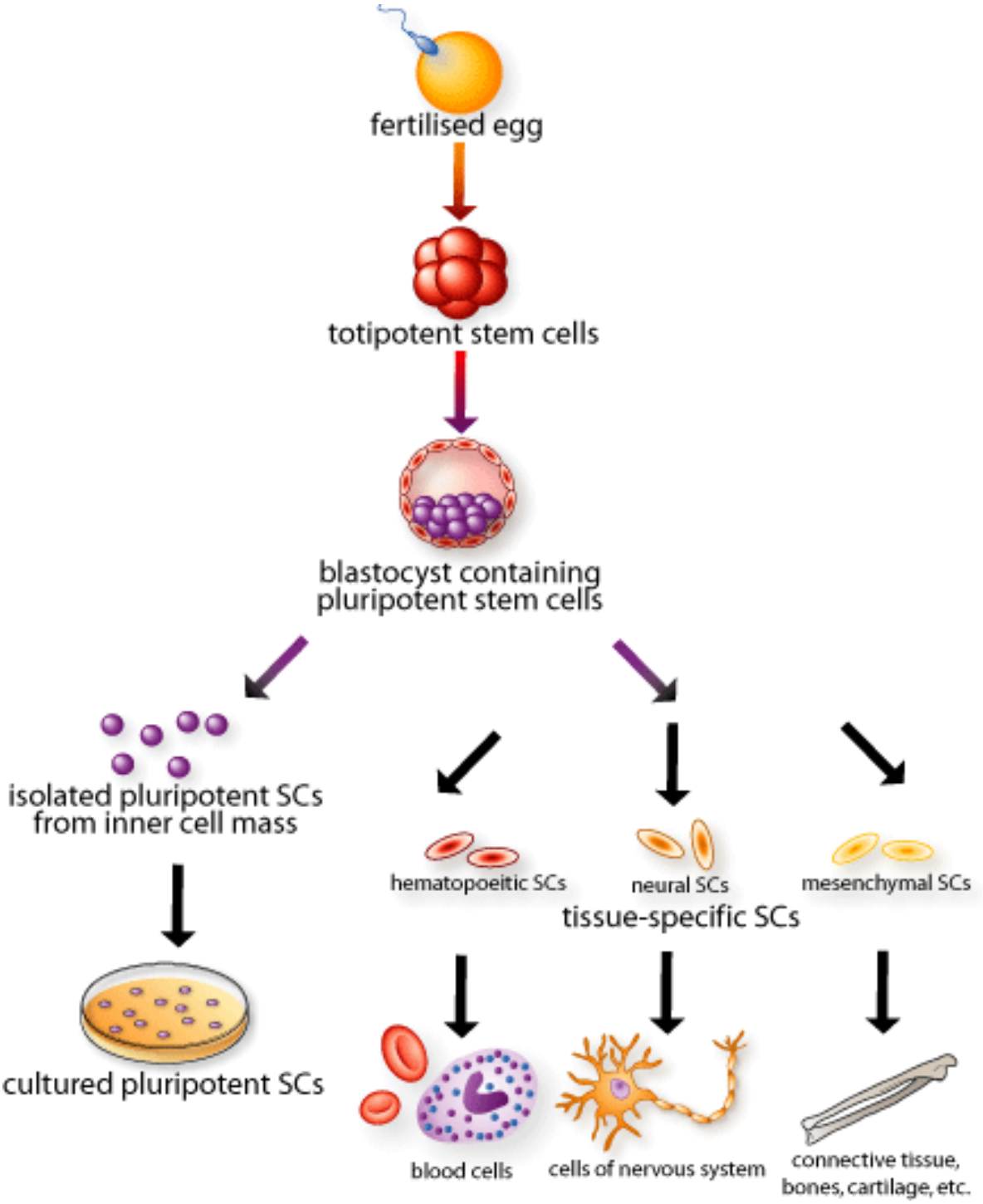
Initially regenerative therapy was practiced by using embryonic stem cells that were isolated from invertebrates and vertebrates. However, later developments led to the use of very simple and most accessible adult somatic cells from adipose tissues, which are easy for isolation and accessibility instead of embryonic stem cells. Mesenchymal stem cells (MSCs) are non-hematopoietic stromal stem cells that have been used mainly in stem cell transplantation, tissue engineering, and immunotherapy. Historically, the definitive presence of MSCs was discovered by Friedenstein *et al* (1976) in bone marrow tissue. These are mononuclear non-phagocytic cells with fibroblast-like phenotype and have a capacity of adherence to the culture surface in a monolayer culture. These cells are known as multipotent stem cells because they have the ability to self-renew and differentiate into more than one cell type specific to the tissue in which they exist. Mesenchymal stem cells have been isolated and shown to self-renew and differentiate into cells in their niche or in a limited fashion to other cell lineages (Huang *et al*., 2007).In the tissue-specific homing of Chemokine receptors with their ligands the adhesion molecules play an important role in transporting hematopoietic precursors into and through the tissues (Wang *et al*., 2013). Successful bone-inducing therapy requires best sources of mesenchymal stem cells from adipose tissue and bone marrow. In experimental settings, these cells can be taken from animals like bovines for bone tissue engineering repair experiments, and other therapeutic interventions such as tendon regeneration. This is because they have the potential to expand *in vitro* and to differentiate into multiple cell types such as osteoblasts, when properly and safely stimulated (Giovannini *et al.,* 2008).

Among bone extracellular matrix components, collagen is the most commonly used in both *in vivo and in vitro* bone tissue engineering experiments. Type I collagen, which is the most common protein in animals, is abundant in the skin, tendon, ligaments, bone, teeth, and in between organs. The most frequently used markers for osteoblast cell differentiation during osteoblastogenesis are Type I collagen, osteocalcin, and *RUNX2.* Osteoblastogenesis has three stages- proliferation, matrix maturation, and mineralization. *RUNX2* gene provides instruction for making a protein that is involved in the development and maintenance of the teeth, bones, and cartilage. Cartilage is a tough and flexible tissue that helps to make the skeleton during its early development. Most cartilage is later converted to bone through the process of ossification (Huang *et al*., 2007).

1.2. Stem Cells

Stem cells are cells that have a capacity of self-renewal and can differentiate into multiple cell lineages (Grove *et al*.,2004). In many medical therapies, adult stem cells are frequently used, autologous stem cells can be harvested from umbilical cord blood just after birth and these are cells that have been obtained from one's own body, just as one may bank his or her own blood for elective surgical procedures (Grove *et al*.,2004).

Embryonic cell lines and autologous embryonic stem cells can be generated through somatic cell nuclear transfer or differentiation and the procedure has been proposed as a promising source of stem cells in medical therapies (Grove *et al*.,2004). Stem cells can also be artificially differentiated into specialized cell types with characteristics consistent with cells of various tissues such as muscles or nerves cells (Grove *et al*.,2004). Stem Cells can be found in three different forms ;- totipotent stem cells, pluripotent stem cells and multipotent stem cells (MSc) (Hemmat *et al*., 2010) (Figure 1).



(www.**stem-cell**-centre.in)

Figure 1: The three types of stem cells and their generation

i. Totipotent stem cells

Totipotency can be defined as the ability of a single cell to divide and produce all the differentiated cells in an organism, including extraembryonic tissues. Zygote is a totipotent cell, that has the capacity to develop into an embryo with all the specialized cells that make up a living being (Pei *et al*., 2010).

ii. Pluripotent stem cells

In pluripotent stem cell each cell has a capacity to form any type of cell and has a potential to differentiate into any of the three germ layers; - endoderm, mesoderm or ectoderm. Embryonic stem cells are an example of pluripotent stem cells. Induced pluripotent stem cells (iPS) can be made directly from somatic cells. Pluripotent stem cells have an application in therapy for the correction of gene by homologous recombination, which is made by reprogramming of anemic mouse fibroblast cells (Makhani *et al*.,2015).

iii. Multipotent stem cells

Cells are differentiated and can form a number of tissue types such as adult cells. Mesenchymal stem cells (MSCs) are an example of multipotent stem cells. These have the capacity to self-renew by dividing and to develop into multiple specialized cell types present in a specific tissue or organ (Sandhaanam *et al*., 2013).

1.3. Embryonic stem cells

The embryonic stem cells come from embryos at a developmental stage before the time implantation would normally occur in the uterus and transform into blastocysts. Blastocyst are pluripotent stem cells, that can be divided into more stem cells or can become any type of cells in

the body. This versatility allows embryonic stem cells to be used to regenerate or repair diseased tissues and organs (Wang *et al*.,2013).

1.4. Mesenchymal Stem Cells and their Source

Mesenchymal stem cells can be found throughout adult body of living organisms and are also referred to as mesenchymal stromal cells. The ability to use adult MSCs is more favorable because it reduces ethical concerns of using embryonic stem cells. The best source of adult MSCs, is not clearly known. Different tissues have been explored including bone marrow, adipose tissue, and umbilical cord (Kim *et al*., 2013). Human umbilical cord perivascular cells (HUCPVCs), otherwise known as Wharton's Jelly, are rich source of mesenchymal stem cells. The chosen source of MSCs is dependent upon ease of harvest and the differentiation capacity towards a chosen purpose.

The MSCs, which also are known as adult stem cells, are found in small numbers in most adult tissues, such as bone marrow and fat (Hwang *et al.*, 2009). When compared with embryonic stem cells, adult stem cells have a more limited ability to give rise to various cell types. Up to very recent times, it was thought that adult stem cells could develop only into similar types of cells for example, stem cells that reside in the bone marrow could only give rise to blood cells. However, at present, there is an emerging evidence that suggests adult stem cells may also develop into unrelated types of cells. For instance, bone marrow stem cells may have a capacity to develop into bone or heart muscle cells. Thus, adult tissues that are more developed and "committed" into non-embryonic stem cell populations exist and are known as adult stem cells. These cells are multipotent because they have retained their ability to self-renew and differentiate into more than one cell type, (Sandhaanam *et al*., 2013).

All MSCs have a perivascular origin and may be regarded as a subset of pericytes (sub-endothelial cells that lie on the abluminal side of blood vessel lining). Cultured pericytes derived from various human fetal and adult tissues have adherence characteristics that display phenotypes similar to MSC. They constitute a heterogeneous population of cells in terms of their morphology, physiology, and expression of surface antigens (Bobis *et al*.,2006). Activated MSCs are present at sites of inflammation or tissue damage, so that pericytes would be released from their endothelial interactions in vascularized locations (Bianco *et al*., 2013).

The identification of progenitors and stem cells in adult tissues started about 40 years ago when hematopoietic stem cells and bone marrow stem cells were identified and shown to be able to differentiate into all cellular lineages exhibiting multipotentiality. However, these cells showed limited stem-like characteristics because they lacked the ability to differentiate into tissue types different from blood and bone. It was suspected that these cells originated from the original germ line cells. However, it is now well established that multipotent stem cells are present in many adult tissues, including skin, muscle, liver, heart, hair follicle, and retina, in addition to the bone marrow these cells have been isolated and shown to self-renew and differentiate into cells in their niche or in a limited fashion to other cell lineages (Huang *et al*.,2007).

On the other hand, progenitor cells are found in many organs and are unipotent or multipotent with limited plasticity. Their ability to self-renewal is highly limited or totally restricted to repairing the injury in tissues where they exist. In fact, it is considered that the adult stem cells are transit-amplifying progenitor cells with varying ability to differentiate into cellular lineages. Examples of progenitor cells are satellite cells in muscles, osteoblasts and chondrocytes, bone marrow stromal cells, angioblasts, and the intermediate progenitor cells in the subventricular

zone of the brain. Other types of adult progenitor cells are primordial germ cells, which are multipotent and give rise to the gametes in the testis. Hence, the difference between adult stem cells, germ cells, and progenitor cells appears to be dependent on the signals from the niche in which they reside have a major role in translational biology (Hwang *et al.*, 2009 ;Parolini and Soncini,2006).

1.5. Adipose Tissue MSCs

Mesenchymal stem cells derived from human adipose tissue have been more in use than those from other animal sources over the past, due to their rapid growth kinetics and overall ease of use and tissue isolation (Izadpanah *et al*,.2006). The human adipose tissue-derived MSCs as multipotent cell source, were later termed "adipose-derived stem cells" (ASC). Adipose stem cells can be found in different type of white adipose tissue, including subcutaneous and omental fat (Locke *et al*., 2009). ASCs derived from human tissue, are characterized by their plastic adherence and by stem cell-specific marker expression this is similar with ASCs isolated from species relevant in veterinary medicine (Arnhold and Wenisch, 2015).

The size and morphology of ASCs are similar to fibroblasts and are difficult to identify within histological sections. More specifically, ASCs have long spindle cell bodies and contain a large, round nucleus, with a prominent nucleolus. These cells are widely dispersed throughout the mature adipose tissue, embedded within the basement membrane of the adipose tissue. ASCs are usually surrounded by a network of reticular fibrils, collagen Type IV, and laminin.

As a stem cell source, fat is ideal in many aspects, in particular the ASC can be easily harvested from the human body by non-invasive liposuction or biopsy methods. The morphological distinction between mature adipocytes and ASCs are favorable for ASC isolation *in vitro*.

Furthermore, adipocytes have a high volume to mass ratio and are easily separated from the stromal vascular fraction (SVF), including the ASCs, based on their density and high intracellular lipid content (Orth *et al*., 2014).

1.6. Bone Marrow MSCs

Bone marrow consists of precursors of stem cells. These are primitive cells that have a capacity to produce all cell types. i.e. blood cells like RBCs, WBCs and platelets. The origins of these are the immature hematopoietic stem cells. Stem cells are found in the bone marrow where they divide to make new blood cells, which up on maturation into adult cells leave the bone marrow and move into the bloodstream. Under continuation when the bone marrow is destroyed by disease, using radiation, the peripheral blood stem cells may be transplanted and the bone marrow normal function can be restored. Bone marrow consists of a hematopoietic and stromal cell compartment that supports proliferation of the hematopoietic cells (Trewhitt,2001).

1.7. Factors that affect the proliferation rate and differentiation capacity of MSCs

There are factors that control the yield and proliferation rate of MSCs such as the seeding density, type of media, age and growth factors. It has been reported that MSCs seeded at lower densities have a faster proliferation capacity than higher densities (Fossett *et al*.,2012). In addition, optimum seeding density of 10^3 or 10^4cells/60 cm^2or$10^3/10^4$cells per cm^2 and donor age have been shown to affect MSC proliferation; with MSCs from older patients showing less change in population doubling time than younger patients with increasing seeding density (Fossett *et al*., 2012).When the growth and proliferation rate of adipose tissue and bone marrow

MSCs are compared, AT-MSCs had more proliferation rate than BM-MSCs in Guinea pig AT-MSCs (Aliborzi *et al*.,2016).

Effects of substrate elasticity and cell seeding densities on MSC lineage differentiation have been studied (Xue *et al*., 2013). Inducing MSC differentiation by osteogenic medium had resulted in osteogenesis on day 6, with increase on day 11 and 21, while the control MSCs showed no differentiation even after passing 21 days (Birmingham *et al*.,2012). Osteoblast cells had shown clusters with high cell density and large amounts of extracellular matrix after two weeks of induction in osteogenic medium; and after four weeks of induction, it formed dense extracellular matrix. Furthermore, the extracellular matrix stained positive on Alizaren Red staining, indicating formation of calcified matrix, and positive for osteocalcin, Runx2 and osteonectin.

2. Statement of the problem

Road traffic injuries are a major health problem in Ethiopia. The treatment for bon injuries used is rely on traditional practitioners but this have lack of knowledge on human anatomy and physiology (Admassie *et al*.,2010). The replacement and repair of bone damage and loss due to traffic and other accidents is a major challenge both in human and veterinary clinical practice and many die in spite of surgical interventions. The effective treatment available is not affordable by many and exposed to death. Three hundred twenty-six patients with maxillofacial trauma were reported in Ethiopia (Admassie *et al*.,2010). The present work intends to optimize the methods of isolation and culture of MSCs to differentiate them into osteoblast cells to solve the complications of fractures treatment.

3. Research objectives

3.1. General objective

The general objective of this study is to isolate MSCs from, bovine adipose tissue, from gluteal muscle, and the bone marrow from bovine tibia and femur from the butcher and to optimize inductive culture media for *in vitro* differentiation of MSCs into osteoblast cells.

3.2. Specific objectives

- To isolate MSCs from adipose tissue and bone marrow;
- To induce formation of bone-like tissue by growing bovine-derived adipose and bone marrow MSCs *in vitro* under osteogenic condition;
- To optimize methods for the isolation and culture of adipose and bone marrow MSCs from bovine source.

4. Hypothesis

The hypothesis to be tested can be stated as:

Bovine-derived adipose and bone marrow MSCs grown under osteogenic medium would be differentiated into osteoblast cells; and the two sources of cells; -adipose tissue and bone marrow have similar rate of proliferation and differentiation into osteoblast cells.

5. Materials and Methods

5.1. Materials, consumables, reagents, sources of cells and equipment

All reagents, materials, cell culture supplements and disposable tissue culture supplies used unless noted otherwise, were from *Gibco Life-Technologies* (UK) and prepared by Pathology laboratory in Freiburg, Germany.

5.2. Isolation and harvesting of Bovine MSCs

5.2.1. Adipose Tissue collection

Bovine adipose tissue-derived stem cell samples (b-ASCs) were obtained from 4, 6 months old calfe in accordance with the Human Adipose Stem cell (Human-ASCs) extraction protocol described by (Izadpanah *et al*., 2006). Superficial white fat tissue was isolated and collected from gluteal muscles of the freshly slaughtered calve in sterile 50ml tubes and rinsed with phosphate buffered saline (10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl pH of 7.4) (Sigma -Aldrich). It then was minced into small pieces of approximately 5–10 µg size using sterile scalpel on petri-dishes in Class II laminar flow hood. The minced tissue was transferred into 50 ml tubes containing 3 mM glucose (150µl), 25 mM HEPES (25ml), 20 mg/ml bovine serum albumin (BSA) and collagenase (Type VIII) solution(1gm) incubated for 3 hours at 37°C water bath with shaking at 100 rpm and swirling it at 5- minutes intervals until the mixture is homogenously "soupy". The digested tissue was passed through 300-µm pore size sterile nylon mesh filter

affixed on top of the funnel and collected into another 50ml tube. The b-ASC-enriched cell fraction was pelleted by centrifugation at 1200 x g for 5 min. The supernatant was discarded and $2.5x10^4$ MSC was re-suspended in low glucose Dulbecco Modified Eagle Medium (L-DMEM) supplemented with 100 U/ml Penicillin G, 100µg/ml Streptomycin, 0.25µg/ml Amphotericin B, 2.4 mg/ml HEPES, 3.7 mg/ml NaHCO3 and 10% fetal bovine serum (FBS).

Isolated b-ASCs were plated in 75 cm^3s culture flasks and kept in at 37°C, 5% CO_2 incubator. Twenty-four hours later the old medium and non-adherent cells were removed and washed 3 times with 10 ml of Phosphate-buffered saline (10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl pH of 7.4.) Ten ml of fresh CCM was re-incubated and for 2-3 days per week was observed daily under phase contrast microscope. Every third day, the media was removed, rinsed with 10 ml of PBS, and refreshed with 10 ml of the CCM and left to grow until the **fibroblast tissue grew** at **95% confluence. The adhering cells were then** trypsin-released, **(**0.25% trypsin/0.1% EDTA, Gibco, Burlington, Canada), from the **flask** and washed several times with PBS and re-plated at $4x10^6$ cells/cm^3 density.

5.2.2. Bone Marrow

Bovine bone marrow aspirate was taken from the femoral bone and processed in accordance with Fujinaga (2005) protocol. Briefly, using 30ml PBS (10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl pH of 7.4), bone marrow was washed out from the bone by passing through heparinized needle (figure 2). The aspirated bone marrow sample was further diluted with equal volume of Phosphate-buffered saline (10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl pH of 7.4). From the diluted aspirate sample, 30 ml was gently overlaid on 15ml ficoll in 50 ml centrifuge tube and centrifuged at 1,800 g for 30 min at room temperature in a swinging bucket rotor without break.

The “buffy coat” separated at the Ficoll-PBS interface was collected with sterile pipette and placed into a clean 50 ml conical tube. Each sample was washed twice in 20 ml in sterile Phosphate-buffered saline (10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl pH of 7.4) and centrifuged at 1,500/rpm for 10 min in a swinging bucket rotor. The supernatant was discarded and reconstituted into 10 ml low glucose enriched DMEM (100 U/ml Penicillin G, 100µg/ml Streptomycin, 0.25µg/ml Amphotericin B, 2.4 mg/ml HEPES, 3.7 mg/ml NaHCO3 and 10% FBS). $1.8x10^4$ MSC was then diluted in 20ml of DMEM cell culture medium (CCM) and incubated at 37 °C, 5% humidified CO2 for 24 hours to allow the cells adhere to the plate. Twenty-four hours later the old medium and non-adherent cells were removed and washed 3 times with 10 ml Phosphate-buffered saline (10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl. pH of 7.4). Ten ml fresh CCM was added and cell culture re-incubated for 2-3 days and observed daily under phase contrast microscope. Every third day, the culture medium was removed, rinsed with 10 ml Phosphate-buffered saline (10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl. and pH of 7.4,) and refreshed with 10 ml CCM and left to grow until the fibroblast tissue reached 95% confluence. The adhering cells were trypsin-released **(**0.25% trypsin/0.1% EDTA, Gibco, Burlington, Canada), from the flask and washed several times with PBS and re-plated at $4x10^6$ cells/cm^3 density. The number of cells was calculated using the follow formula;

Concentration (cells/ml) = $\frac{\text{counting \#of cells*Dilution factor } *10^4}{\text{4 squares}}$

A

B

Figure 2: (A) Bone marrow was harvested from femur and tibia that were cut the bon in to small pieces and carefully cleaned of adherent soft tissue; (B): Adipose tissue was collected from dorsal region of the gluteal muscle at the base of the tail, where relatively clean mass of adipose tissue without large veins is easily accessible.

5.3. Osteogenic differentiation of Mesenchymal stem cell

In the standard culture medium (SM), which is enriched with L-Glucose DMEM supplemented with 100 U/ml Penicillin G, 100µg/ml Streptomycin, 0.25µg/ml Amphotericin B, 2.4 mg/ml HEPES, 3.7 mg/ml NaHCO3, and 10% FBS, $3x10^4$ MSCs were seeded in 6 cm diameter Petri-dishes in duplicate in 75 cm^3 culture flasks in triplicate and 2.5 x 10^3cells were seeded in 96 well microtiter plates in triplicate. The three culture sets were incubated in a humidified 5% CO_2 atmosphere at 37°C for 24 hours. The spent SM was removed except from the control group and replaced with osteogenic medium (OM), which is composed of low glucose DMEM supplemented with 100 nM Dexamethasone (DEX), 10 mM β-glycerophosphate and 50 µM Ascorbate-2-phosphate. The experimental cell cultures were incubated for additional 7,14 and 21 days. The controls were incubated for the same period of time in the SM. The OM and the SM were changed Every third day. After incubation for 7, 14 and 19 days, Alizarin red staining (for detecting Ca^{2+} ion deposition) was done. Furthermore, PCR-based molecular biological analysis for osteogenic differentiation was also done.

a b c

Figure 3: (a) Cell culture in 75 cm3 flasks used for RNA isolation sample (b) 60cm petri-dish for alizarin red staining (c) 96 well microtiter plates for MTT cell proliferation test.

5.3.1.MTT Assay Test for Cell Viability

Both bone marrow and adipose tissue samples in the osteogenic medium (ATO and BMO) and as control in the standard medium (ATC and BMC) were cultured in triplicate 96 wells microtiter plates. Having osteogenic medium and SM (media was changed every third day). For 3 consecutive weeks (day 7,14 and 21) the old osteogenic medium and Standard medium (control group) were removed and a 150 µl tetrazolium bromide (MTT) (Sigma Aldrich, St. Louis, MO, USA) in Phosphate-buffered saline (10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl pH of 7.4) was added to the cell cultures in 96 well microtiter plates and the culture incubated at 37ºC in humidified 5% CO2 condition overnight. Then after, MTT solution was removed and the wells washed three times with PBS. This was followed by addition of 100 µl DMSO (ROTH, Germany) to each well. Intensity of the cell culture was measured using absorbance reader (Sunrise, Tecan, Germany) at a wavelength of 570nm.All experiments were performed in triplicate, and the relative cell viability was expressed as (mean ± standard error) relative to the untreated control cells.

5.3.2. Alizarin Red Staining for Calcium Detection

The following procedure was used for alizarin red staining for both 14 and 21 days old cell culture. The spent medium was removed from each Petri dish and the cells were washed gently 3 times with Phosphate-buffered saline (10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl. and pH of 7.4). After decanting the PBS, the cells were fixed in 4% formaldehyde for 15 minutes at room temperature. The fixative was removed and the cells were washed 3 times with PBS. After removing the PBS, 1 mL of 40 mM ARS was added to the per petri-dish and the cell culture

incubated at room temperature for 30 min. After removing the dye by washing the cells 6 times with PBS, photographic images were taken by using inverted microscope at 100x magnification power (China, SGS) (Freiburg, Germany).

5.3.3. Isolation of RNA using RNeasy Mini Kit

After the cells were cultured in Osteogenic medium (OM) for 14 and 21 days, the spent media was aspirated and the cells washed with PBS (10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl. pH of 7.4) and 0.1–0.25% trypsin / EDTA in PBS added. The cell culture was then incubated in a humidified 5% CO_2 atmosphere at 37°C for 5 min to detach cells from the Flask. Trypsin was neutralized by adding an equal volume of medium (containing serum to inactivate the Trypsin) and the osteoblast cells detached from the flask were transferred to *Eppendorf* tube and centrifuged at 300xg for 5min.The supernatant was discarded and 10 ml PBS was added to the pellet and the process repeated. The supernatant was discarded and the cell pellet transferred into a 2-ml small *Eppendorf* tube. The cells were Frozen at -80^0 C and stored. The cells were taken out of the deep freezer and disrupted by adding 600 µL RLT buffer (Quanigi, Hilden, Germany) and the lysate pipetted directly into a QIAshredder spin column was placed in a 2-ml collection *Eppendorf* tube, and centrifuged for 1min at full speed ($1x10^{14}$ rpm). An additional 600 µL 70% ethanol was added to the homogenized lysate and mixed well by pipetting and were taken 700µl of the sample including any precipitate that may have formed were transferred to a RNeasy spin column placed in a 2-ml collection tube and centrifuged for 15s at (≥10,000rpm). The flow-through was discarded and add 700µl RW1 buffer to the RNeasy spin column and centrifuged for 15s at ≥8000g (≥10,000rpm) to wash the spin column membrane. The flow-through was discarded again and 500µl RPE buffer added to the RNeasy spin column, and centrifugation repeated for 2-min at≥8000g(≥10,000rpm). The spin column membrane was washed by placing

the RNeasy spin column in a new 1.5ml collection *Eppendorf* tube, 30 µl RNase-free water was added directly to the spin column membrane and centrifuge for 1min at ≥8000xg (≥10,000rpm) to elute the RNA. Volume=20 µl

C1V1 = C2V2 90ng/ µl =1 µl $\frac{69ng/\ \mu l * 1\ \mu l}{}$ *10 =7.7 µl RNA +2.3 µl H2O

69ng/ µl? 90ng/ µl

$\frac{69\ ng/\ \mu l*1\ \mu l*10}{130}$ =5.3 µl RNA+4.7 µl H2O

All RNA samples were equalized by adding RNase-free water treated with diethyl pyro carbonate (DEPC) to 20 µl RNA These samples were incubated at 65^{0C} for 10 min and then chilled on ice. Then after 11 µl bulk first-strand cDNA reaction mixes, 1 µl DTT solution and 1 µl primer were added to each 20 µl RNA samples then incubated at 37°C for 1 hour and placed in -20^{oC} Refrigerator until processed by PCR.

PCR reaction mixtures for 25µl final volume containing:5 µl cDNA, 2.5µl PCR buffer, 2.5µl Mgcl2, 2.5µl dNTPs, 5 µl Q-solution, 1.5µl Forward primer (F-primer),1.5Reverese primer (R-primer), 0.2Tag polymerase and 4.3H2O

Three genes were amplified using specific primers. The PCR conditions used were indicated in Table 1.

Table 1 Bovine-specific primer pairs used for PCR amplification and the cycling conditions.

Markers	Gene	Primer sequence (5'->3')	Expected product size	PCR cycling conditions.
Osteoblast	Collagen Type I	Forward: ATGGAACTCCAGGTCAAACG Reverse: CACCAACACGTCCTCTCTCA	60	Initial denaturation: 95°C for 2 min Denaturation:95°C for 30 sec. Annealing: 58.7°c for 30 sec. Elongation: 72°C for 30 sec. Cycles:40 Final elongation: 72°C for 10 min Hold at 4°c
	Osteocalcin	Forward: CATGGGAGCTGGGAGAGTAA Reverse: AAGGGGCAAATGGATTAAG	113	Initial denaturation: 95°C for 2 min; Denaturation:95°C for 30 sec. Annealing: 54°c for 30 sec. Elongation: 72°C for 30 sec. Cycles:40 Final elongation: 72°C for 10 min Hold at 4°c
	RUNX2	Forward: CAGATGACTCCTCCGTCCAT Reverse: ACTGAGAGTGGAAGGCCAGA	147	Initial denaturation: 95°C for 2 min; Denaturation:95°C for 30 sec. Annealing: 60.3°c for 30 sec. Elongation: 72°C for 30 sec. Cycles:40 Final elongation: 72°C for 10 min Hold at 4°c

The PCR products were detected on gel electrophoresis. First, the gel was prepared using 2% agarose (2g universal agarose powder dissolved in 100 ml TAE (a mixture of 242 g Tris free base ,18.61 g, Disodium EDTA and 57.1 ml Glacial Acetic Acid buffer) (Freibrg, Germeny) and boiled for 5 minutes under microwave oven. The gel was then stained with 10 µl Ethidium Bromide (EtBr) prior to casting on gel tray. Prior to loading, PCR products were mixed with 5µl of loading dye (LB). Then, 20µl of the amplified PCR product samples and a molecular DNA ladder of 100bp were loaded in to the casted gel lane. Finally, the gel was run for 1 hour at 80-150 V until the dye line reached approximately 75-80% of the way down the gel. Hence, the PCR products were visualized under UV-transilluminator connected to gel picture documentation computer (Cole-Parmer, Germen).

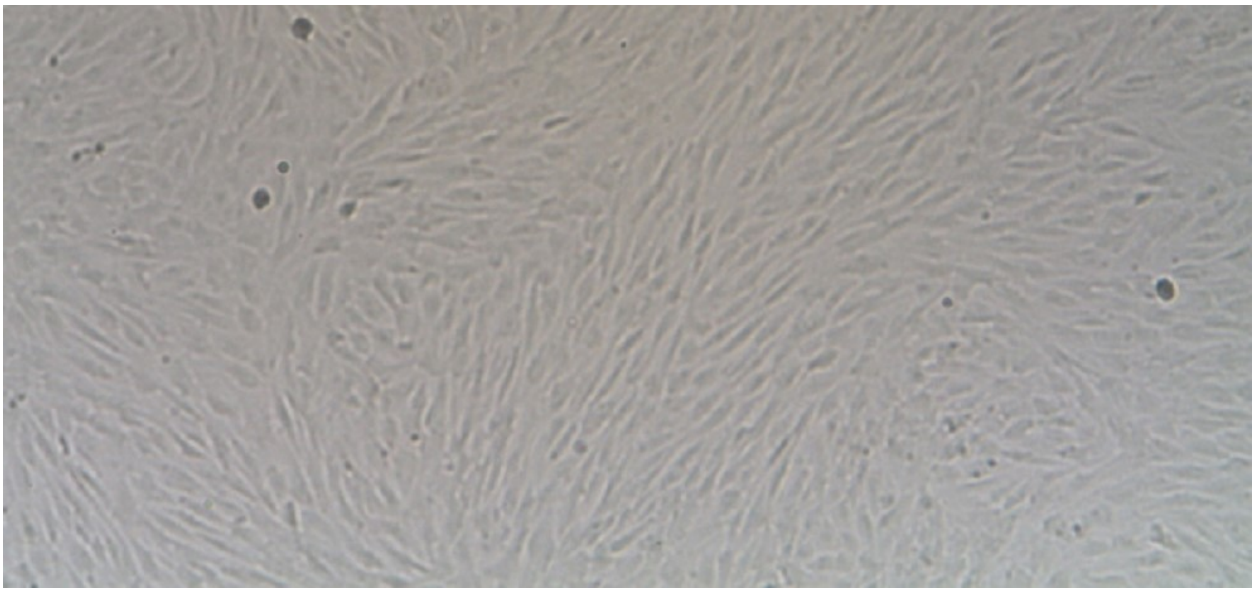
5.4. Statistical Analysis

Statistical analyses were performed with SPSS Software version 20. The data were analyzed by Paired Samples t-test statistics and significance level was considered at $p<0.05$ to express possible difference between durations of incubation days in culturing MSCs in osteogenic medium and standard medium in terms of cell viability (i.e. MTT experiment).

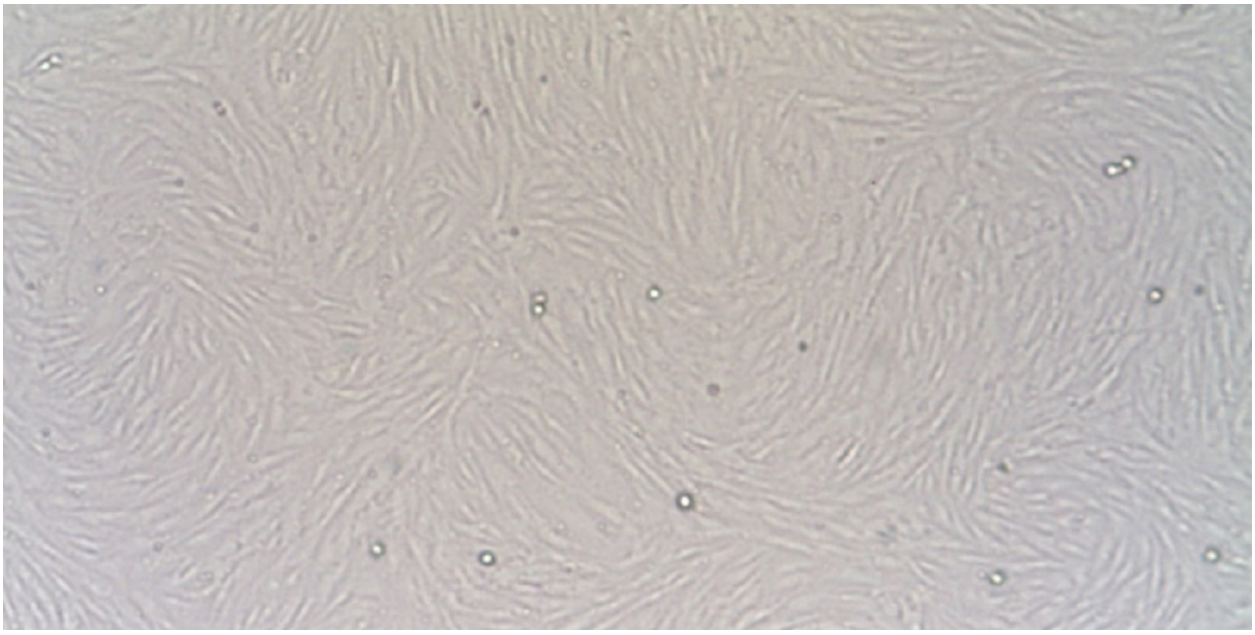
6.Results

6.1. Morphology of Adipose Tissue and Bone Marrow-derived MSCs

Active fibroblast cells appeared plump and spindle-shaped or stellate-shaped and circular non-fibroblast cells are also seen. The non-adherent cells and also the non- MSCs floated freely within the culture flask and were eliminated by decanting (Fig 4).



a



b

Figure 4: Representative cell morphology of bovine MSC in three weeks old culture after seeding in SM, observed using an inverted microscope at 100x magnification power (a) From adipose tissue MSC; (b) From bone marrow MSC, showing similar morphology (fibroblast-like appearance).

6.2. Detection of calcium with Alizarin Red staining

Alizarin red staining of cells cultured in osteogenic differentiation medium and standard medium (SM) after culturing for 14 and 21 days, the treatment groups were turned red. A stronger red color developed in cells incubated for 21 days than for 14 days. The cells incubated in SM (control groups) did not stain red showing lack of calcium deposition (Fig 5).

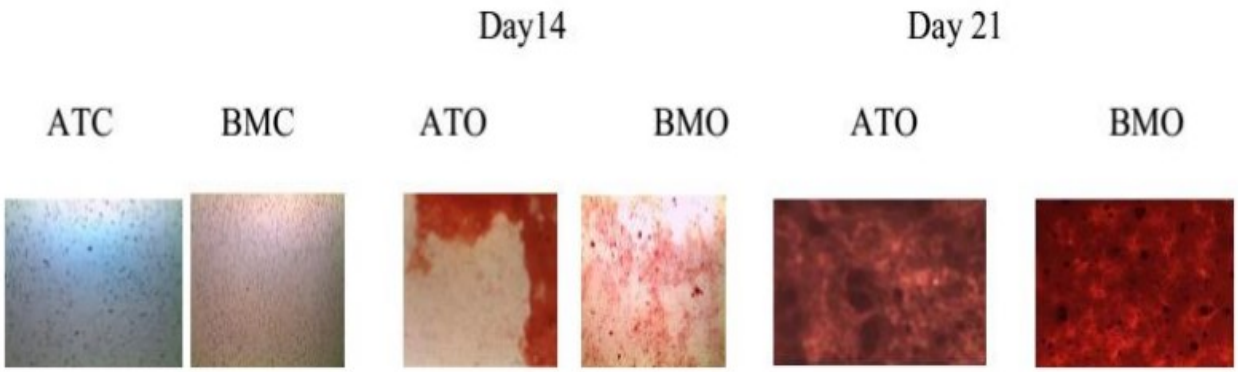


Figure 5: MSC culture - Adipose Tissue (ATO) and Bone Marrow (BMO) in osteogenic medium. Control MSC culture in SM – (ATC & BMC). Cultured MSCs stained with Alizarin Red. Calcium deposits stained orange-red in the osteogenic cultures (ATO & BMO) and no staining in the SM cultures (ATC & BMC).

6.3. Cell Viability and Proliferation Assay

The Cell proliferation rate for incubation periods, days 7,14 and 21 in the osteogenic medium, for both ATO and BMO showed statistically significant difference ($P<0.05$) (Table 3).

Table 2. MTT assay test for measured in an average of osteoblast development from MSCs; - adipose tissues and bone marrow in osteogenic medium and standard medium.

Day	ATO	ATC	BMO	BMC
7	0.4223	0.8419	0.27536	0.54687
	0.4814	0.7419	0.25112	0.26604
	0.6391	0.8532	0.34669	0.56163
Average ± SD	0.514 ±.112	0.812 ±.061	0.291 ±.051	0.458 ±.163
14	0.33558	0.59792	0.23516	0.74869
	0.38678	0.73373	0.15589	0.63688
	0.31616	0.69051	0.20884	0.75018
Average	0.346 ±.036	0.674 ±.067	0.199 ±.038	0.711 ±.063
21	0.23076	0.5613	0.17136	0.59366
	0.43853	0.3442	0.34019	0.72301
	0.39273	0.4301	0.31155	0.72301
Average	0.354 ±.109	0.445 ±.109	0.274 ±.090	0.627 ±.074

Table 3. MTT assay for quantification of osteocyte development from MSCs from adipose and bone marrow tissues in osteogenic medium.

Pair samples	No. of days	Mean	Std. Deviation	Std. Error Mean	T value	Degree of freedom	Sig. (2-tailed)	95% Confidence Interval of the Difference
ATO-BMO	7	514, .291	0.07	0.042	5.36	2	0.033*	(.044,.400)
ATO-ATC		514, .810	0.11	0.063	-4.69	2	0.043*	(-.569, -.025)
BMO-BMC		291,.459	0.13	0.075	-2.21	2	0.158	(-.492,.159)
ATO-BMO	14	348,.201	0.07	0.043	3.52	2	0.072*	(-.033,.326)
ATO-ATC		348,.672	0.06	0.032	-10.1	2	0.010*	(-.462, -.186)
BMO-BMC		201,.712	0.03	0.017	-29.4	2	0.001*	(-.586, -.437)
ATO-BMO	21	354,.274	0.02	0.011	7.06	2	0.019*	(.031,.128)
ATO-ATC		354,.445	0.22	0.126	-.726	2	0.543	(-.631,.449)
BMO-BMC		274,.679	0.02	0.011	-34.4	2	0.001*	(-.456, -.354)

NB: *Represents significant difference at 95% CI**, P-value <0.05***

6.4. Comparison of proliferation rate for adipose tissue and bone marrow

When the proliferation rate was compared at day 7, adipose tissue in the control group (ATC) was the highest and bone marrow cultured in osteogenic medium (BMO) had the lowest rate of proliferation. Also, adipose tissue in the osteogenic medium (ATO) had less proliferation rate than the control (ATC). Similarly, bone marrow in the control group (BMC) had higher proliferation rate than bone marrow in the osteogenic medium BMO (Figure 6). The same pattern of proliferation rates observed for day 7 were also true for the osteogenic medium and the SM at days 14 and 21 (Figure 6, 7&8).

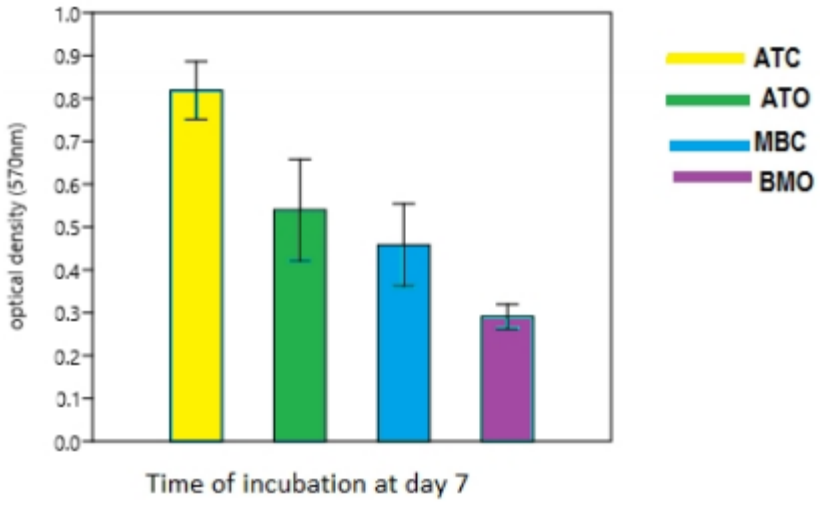


Figure 6: Mean comparison (mean ± standard error) of cell proliferation from adipose tissue in osteogenic medium (ATO), adipose tissue in SM (ATC); from bone marrow cells in osteogenic medium (BMO) and bone marrow cells in SM (BMC) at day 7.

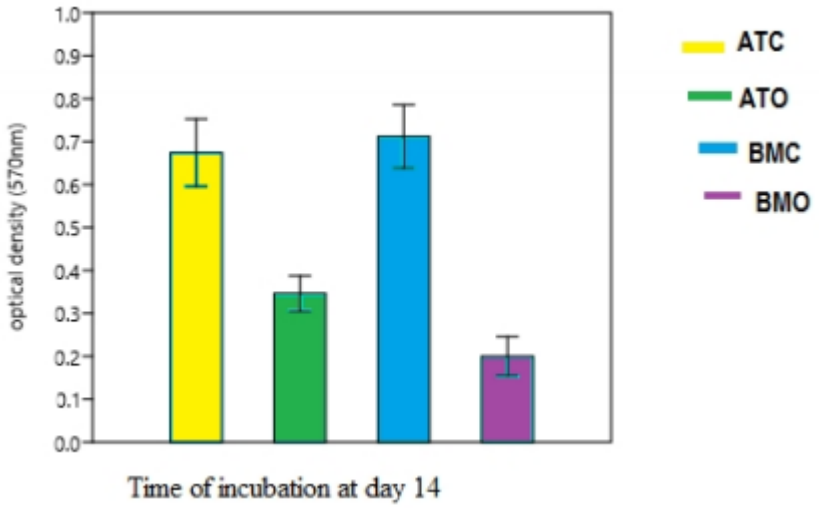


Figure 7: Mean comparison (mean ± standard error) of cell proliferation for in ATO, ATC, BMO and BMC at day 14.

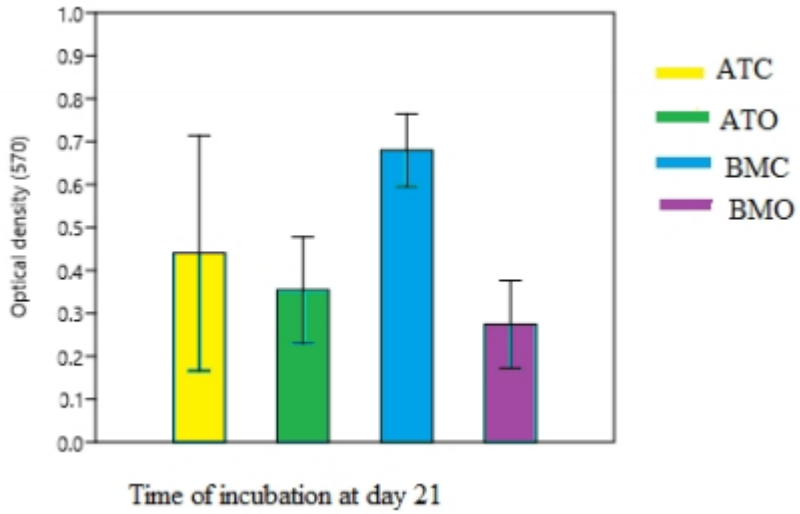


Figure 8: Mean comparison (mean ± standard error) of cell proliferation in ATO, ATC, BMO and BMC at day 21.

6.5. PCR analysis of osteogenesis

Using Nanodrop, 69 ng RNA from adipose tissue and 59ng RNA from bone marrow cultured in the osteogenic medium; 130ng from adipose tissue and 90ng from bone marrow cultured in the SM (control groups) were measured in day 21. After PCR was carried out to verify specific marker expression for osteoblasts (Collagen Type I, RUNX2, and osteocalcin). Gel Electrophoresis was performed after incubation time for 21 day, measured were taken as a specific marker of osteoblast cell expressions. Adipose Tissue (ATO) and bone marrow (BMO) in the treatment groups were expressed, Collagen Type I, RUNX2, and osteocalcin. For the positive control ATC and BMC the expression of collagen I, RUNX2, and osteocalcin didn't show clear bands .

Day.21

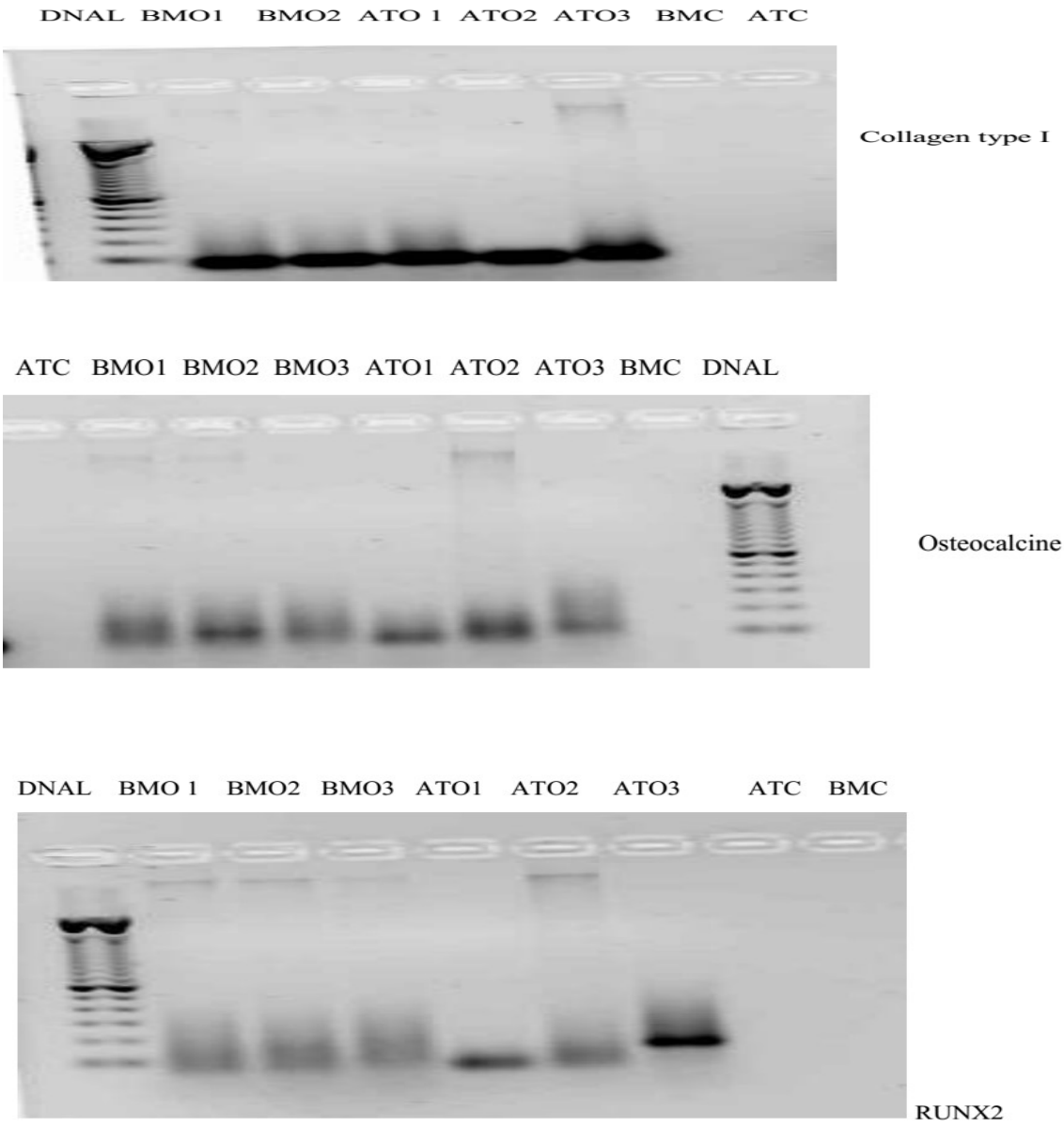


Figure 9: RNA was isolated from the cells on day 21 and expression of (collagen Type I, RUNX2, and osteocalcin) were measured by gel electrophoresis. Triplicate samples for ATO and BMO in osteogenic medium; ATC and BMC, in standard medium and DNAL, DNA Lader.

7.Discussion

Abdominal adipose tissue, which is abundantly accessible for isolation of MSCs and is often applied in tissue engineering is used in the present study. Bovine MSC (bMSC) were isolated from bone marrow and adipose tissue based on their capacity to adhere to plastic flasks (Grove *et al*.,2004). This study was necessary because of absence of reports on the use of MSCs from bovine adipose tissue and bone marrow for differentiation into osteoblast cells. Demonstration of such differentiation has value to develop a better understanding of bovine adipose tissue and bone marrow for their applications in tissue-engineering. The demonstration in the present study that both bovine derived bone marrow MSC (BM-MSC) and adipose tissue MSC (AT-MSC) showed similar morphology (fibroblast cells) and also were able to differentiate into osteoblast cells when cultured in osteogenic medium is consistent with the study by Melief *et al*.(2013) who stated that both BM-MSC and AT-MSC have similar phenotype and capacity for multilineage *in vitro* differentiation toward osteogenic and adipogenic lineages.

The proliferation rate of the treatment and control group, adipose tissue (ATO) and bone marrow (BMO) cultured in the osteogenic medium; adipose tissue (ATC) and bone marrow (BMC) cultured in the standard medium (SM) showed statistically significant in all days (7,14 and 21) that means the cells proliferated equally. When the proliferation rate was compared between adipose tissue (AT) and bone marrow (BM) culture in standard medium (ATC and BMC), and osteogenic medium (ATO and BMO), those cultured in osteogenic medium (ATO and BMO) in MTT Cell Proliferation Assay showed reduction in proliferation rate for all days (7,14 and 21). The reason for the reduction of cell proliferation rate is due to cell death, this may be due to the development of MSC to osteoblasts cells. The culture medium (osteogenic medium) is new to the cells so some of the cells are not adapted to this media and lead to apoptosis.During

morphological change from MSC to osteoblast cells all MSC are not changed to osteoblast cells,some MSC stem cells may have short lifespan due to inadaptability to the new osteogenic medium during this event it may cause an apoptosis to the cells.Mesenchymal stem cells obtained from bovine drived adipose tissue and bone marrow were not homogenous, the cells were not purely MSC,as other few nonfibroblast circular cells grew which did not differentiate into osteoblast cells as aresult it exposted to apoptosis similar with the findings of Aliborzi *et al*, (2016) on mesenchymal stem cells of Guinea Pigs, adipose tissue MSCs (ATO-MSCs) and bone marrow MSC (BMO-MSC) cultured in the osteogenic medium, the proliferation rate of AT-MSCs was higher than BM-MSCs. The explanation suggested for this is that adipose tissue MSC adaptes to the new Osteogenic medium and proliferates much more rapidly in culture and reaches saturation level earlier than BM-MSCs (Aliborzi *et al*.,2016)).According to Zaminy *et al*,(2008) the reason for cell growth reduction is due to a decrease in the cells entering the S phase of the cell cycle.

Within the osteogenic medium, on day 21, the bone-forming cells (Osteoblasts), synthesized a macromolecule of the bone matrix including extracellular matrix rich in Collagen Type I, Osteocalcin and *RUNX2* (Huang *et al*.,2007).The first stage for bone formation begins with the deposition of osteoid, an organic matrix consisting primarily of Collagen type I. Collagen Type I showed the expected expression profiles of the osteoblast phenotype it expressed during the commitment to the osteoblastic phenotype at day 21 incubation time. The transcription factor genes Runt-related transcription factor (*RUNX2*) activates osteocalcin, an osteoblast-specific gene, which is expressed by fully differentiated osteoblasts and is an essential gene for osteoblast differentiation, and also determines the lineage of osteoblastic cells from multipotent mesenchymal stem cells (Meyer *et al*.,2014). Runx2 protein is expressed in pre-osteoblasts

development, induces the differentiation of mesenchymal stem cells into immature osteoblasts, formation of immature bone. During bone development the protein level of Runx2 in osteoblasts have been reduced (Komori, 2009).

Osteocalcin was expressed in cells when mineralized tissues was formed and expressed only after cells reached the mineralized tissue-formation stage during osteoblastic differentiation this is similar with the finding by Komori (2009) who suggested that Osteocalcin is expressed in mature osteoblasts and which expressed strongly than Runx2. Osteocalcin was poorly expressed when compared with collagen Type I for 21day incubation. The reason for this is that osteocalcin is one of the limited osteoblast-specific genes.

Initiation of bone mineralization is an extracellular, biochemical process that means the presence of collagens, certain lipids and glycoproteins could be triggered for the initiation of calcification. The Mitochondria of bone-forming cells (osteoblast cells) produced mineral ions, matrix and accumulate calcium. Osteoblast cell also synthesized large amounts of extracellular matrix. The cells in day 21 were stained by Alizarin red staining more than day 14 because osteoblast cells completed their mineralization stage at late incubation time and also closest to the bone forming stage this were the highest Ca^{2+} content appears in the cell membrane and mitochondria. From these results it can be suggested that osteoblasts cells processed more Ca^{2+} when closer to the mineralization site,on the other hand, the control (SM) cells did not stain positive for Alizarine red staining.

8. Conclusion and Recommendations

8.1. Conclusion

- Bovine-derived adipose tissue and bone marrow mesenchymal stem cells grow well (i.e. proliferate, adhere, live, and differentiate) *in vitro*. These phenotypic characteristics of isolated bovine adipose tissue and bone marrow cells in association with their gene expression profile and their differentiation potential confirmed that the isolated cells are actually MSCs. MSCs can be used as a source of cell therapies in regenerative medicine. Adipose tissue-derived MSCs are easily harvested than bone marrow MSCs. This makes them an attractive tool for veterinary and medical applications.
- Osteogenic differentiation of cells into osteoblast cells is better when combined with 100 nM Dexamethasone (DEX) in growth medium, 10 mM β-glycerophosphate and 50 μM ascorbate-2-phosphate.
- Late incubation (21, days) of bovine-derived adipose tissue and bone marrow mesenchymal stem cells with osteogenic medium could yield better calcification than early incubation. Furthermore, late incubation also results in higher expression of osteogenic marker genes and would allow complete calcification.

8.2. Recommendations

- Bovine experimental animal model should be practiced in the laboratory of Veterinary medicine in Ethiopia.
- In adult stem cell technology, adipose tissue mesenchymal stem cells (AT-MSC) should be used instead of embryonic stem cells and bone marrow cell.

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