Republic of Iraq
Ministry of Higher Education and Scientific Research
Al-Nahrain University
College of Science
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Osteoblast formation from Mesenchymal Stem Cells Derived from mouse Bone Marrow *in vitro*

A Thesis

Submitted to the Council of Science College / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

By

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March 2015 Jumada althani 1436

Dedication

To the women who raised me as a seed in life and irrigated me by her kindness … to her dear and noble soul…….. My Mother

To my late father, God bless his soul.

To those whom I live for their sake, and I owe them my happiness……my Sisters and my husband

To every loving and loyal heart … I dedicate my work.

Sally

Acknowledgments

At the beginning, thanks to great Allah, Lord of the whole creation who gave me the faith and strength to accomplish this work.

My special thanks with respect and appreciation to my supervisors Dr. Shahlaa M. Salih for their continuous efforts, guidance and useful advises during this work.

I would like to express my great full thanks to Dr. Shalal M. Hussein, Dr.khalid J., Dr. Ahmed M. Alshammari and Dr. Yassine I.Omran and to the staff members of Iraqi center for cancer and medical genetic researches for their help.

Grateful thanks are conveyed to Marwa I. and Zahraa K. and for their assistance and to all those whom I forgot to mention for their support.

Deep thanks to the staff members of Alfajer laboratory especially Mohammed, Huda, Sura, Amina and Haider for all kinds of help.

I feel that I am deeply thankful to and my friends and colleagues, Rasha Yasmin, Mina, Mortadha, Albab, Linda, Tara, Naaela, Safa, Sura, Weham, Hesham and Mohammed.

I am sincerely grateful to my mother, sisters and my husband for their help and support asking God to bless them.

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Summary

This study was designed to evaluate osteogenic potential of mesenchymal stem cells (MSCs) isolated from mouse bone marrow *in vitro*.

MSCs were isolated by collecting the thigh bone of 50 male albino mice, both the femur and tibia were collected and cells were flushed from bones and MSCs isolated based on the ability of adherence to plastic surfaces. Reactivity MSCs to CD105 and CD34 were tested by immunocytochemistry. Isolated MSCs exhibited positive reactivity to CD105 and negative for the haematopoietic surface marker CD34.

Differentiation of isolated MSCs into osteoblast was induced by osteogenic medium consisting of high glucose- DMEM supplemented with 50 µg /ml Ascorbic acid, 1nM dexamethasone and10 Mm of beta glycerophosphate disodium salt hydrate after 21 days.

Osteoblast activity was monitored by evaluation alkaline phosphatase (ALP) activity in osteogenic medium by Reflotron at (0, 7, 14 and 21) days of differentiation. Results recorded that a significant ($P \leq 0.05$) increase in ALP activity in osteogenic medium at 7 and 14 day culture in comparison with zero day (32.13±0.46 and 23.33±0.88 *vs*. 5.33±1.76 IU/L) and decreased at 21 day (15.33±1.76 IU/L).

The presence of beta-actin and osteocalcin genes was confirmed in differentiated cells by Reverse transcriptase PCR (RT-PCR) on day 14. Two bands were present with molecular size (200) bp for beta actin and (169) bp for osteocalcin.

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List of abbreviations

ADSCs	Adipose Stem Cells
MEM	Minimum Essential Medium
ASCs	Adult Stem Cells
BM-MSCs	Bone Marrow Mesenchymal Stem Cells
BMPs	Bone Morphogenic Protein

T.D.W	Triple Distil Water
DAB	Di Amino Benzidine
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
ESCs	Embryonic Stem Cell
FBS	Fetal Bovine Serum
FSM	Free Serum Media
GAG	Glucose Amino Glycan
GFs	Growth Factors
HSCs	Hemopoietic Stem Sells
OBCs	Osteoblast Cells
ICC	Immunocytochemistry
MNCs	Mononuclear Cells
MSCs	Mesenchymal Stem Cells
OA	Osteoarthrosis
PBS	Phosphate Buffer Saline
SCs	Stem Cells
TGF-β1	Transforming Growth Factor beta 1
UCB-MSCs	Umbilical Cord Blood - Mesenchymal Stem Cells
OC	Osteocalcin
OI	Osteogenesis Imperfecta

PCR	Polymerase Chain Reaction
ALP	Alkaline Phosphatase
BSP	Bone Sialoprotein
Cbfa1	core-binding factor α1
Osx	Osterix
ASSCs	Adult Skin Stromal Cells
iPSC	Induced Pluripotent Stem Cell
FGF	Fibroblast Growth Factor
IGF	Insulin-like Growth Factor
TNAP	Tissue Non-specific Alkaline Phosphatase
MV	Matrix Vesicles
HRP	Horse Radish Peroxidase
nM	Nano Molar
OD	Optical Density
RPM	Round Per Minute
EB	Ethidium Bromide

Chapter One

Introduction

and

Literatures Review

1 Introduction and Literature review

1.1 Introduction

Bone diseases are disorders and conditions that cause abnormal development and/or impairment in normal bone development such as Osteoporosis which is characterized by an abnormal loss of bone mass and disintegration of bone structure in older adults. This can cause bone fragility and increases the risk of fractures and breaks (Brian *et al.,* 2009), Osteogenesis imperfecta (OI) disease is a genetic disorder that is characterized by brittle bones that break or fracture easily. It is caused by a gene defect in the production of collagen (Steiner *et al.*, 2005) and intervertebral disc degeneration remains a pervasive and intractable disease arising from a combination of aging and stress on the bony and cartilaginous elements of the spinal column (Ciacci *et al*, 2008).

Mesenchymal stem cells have biologic properties that most uniquely identifies is their capacity for tri-lineage mesenchymal differentiation and they are self-renewing, cells must be shown to differentiate into osteoblasts, adipocytes, and chondroblasts (Kim *et.*, 2013). The most successful application of MSCs has been site-directed administration for repair of bone and cartilage. It is estimated that 1,600,000 bone grafts are performed every year to regenerate bone lost to trauma and disease, 6% (96,000) of which are craniomaxillofacial in nature (Einhorn, 2011). Regenerative medicine attempts to repair, regenerate, or replace tissues damaged by factors such as injury or disease (Sundelacruz *et al.*, 2009).

Restoring tissue structure and/or function in the body has largely relied on transplants or grafts (autografts or allografts) and more recently on the use of tissue substitutes like cells, growth factors (GFs) and synthetic devices, in combination or individually, as a therapeutic alternative. A considerable effort has been made, for each specific tissue, to mimic the natural microenvironment in which cells can proliferate and differentiate (Freyria and Mallein-Gerin, 2011).

Tissue engineering with the use and manipulation of MSCs is a novel treatment modality targeting applications in a great variety of pathologies. The advantages of this approach are numerous; they include a high quality repair with regeneration of the injured tissue but without fibrous tissue formation (Pountos *et al*., 2007).

Aims of study:

⇒ Investigate the osteogenic potentials of BM-MSCs in osteogenic medium through:

- Isolation and characterization of mesenchymal stem cells from mice bone marrow.
- Induction of mesenchymal stem cells to differentiate into osteoblast medium.
- Characterization of osteoblast by Reverse transcriptase -PCR.

1.2 Literature Review

1.2.1 Stem cells:

Stem cells are unique populations of undifferentiated biological cells that have the capacity to self-renew and differentiate into different cell types. These cells are important in the field of regenerative medicine, helped to repair and replacement of diseased cells, tissues and organs through the transplantation of healthy cells and tissues in particular, stem cells (Walia *et al*., 2012).

SCs have several different types considered for clinical applications. Embryonic stem cells (ESCs) have the greater generative "potential" being that they are naturally pluripotent and can differentiate into all adult cellular types. The successful isolation and culture of human Embryonic stem cells (hESCs) has allowed investigator stage in a much better understanding of the capabilities of these cells to regenerate different tissue types **(**Thomson *et al.,* 1998**)**.

Ben-David and Benvenisty, (2011) reported that the ESCs research however has been restricted by controversy surrounding the origin and isolation of these cells. Additional obstacles include safety concern severs potential tumorigenicity and immune-compatibility **(**Swijnenburg *et al.,* 2008**)**.

Adult stem cells such as mesenchymal stem cells **(**MSCs**)** circumvent many of the ethical and technical issues associated with ESCs as they can be isolated from developed tissues including bone marrow, fat and skin (bone marrow stromal cells (BMSCs), adipose tissue-derived stem cells (ADSCs), and adult skin stromal cells (ASSCs), respectively. However, these cells are multipotent, and are there for restricted to the cell line again which they reside **(**Al-Nbaheen *et al*., 2013**)**.

Regardless, adult stem cells are a highly useful cell population in regenerative medicine as their ease of isolation, multilineage differentiation, and potential for autologous transplantation makes them a favorable candidate for clinical translation (Takenaka *et al.,* 2010).

The creation of induced pluripotent stem cell (iPSC), or adult somatic cells reprogrammed into pluripotent cells, has allowed researcher subtilize the differentiation capabilities of ESCs, while avoiding the ethical issues associated with ESC isolation of iPSCs share many similar properties with ESCs including expression of certain proteins and chromatin methylation patterns, stem cells genes, potency and differentiability (Takahashi and Yamanaka, 2006).

Phanthong, *et al.* (2013) founded that clinical translations of iPSC the rapids still have noteworthy challenges. Generation of iPSCs has a low reprogramming efficiency and requires the introduction of exogenous transcription factors with viral vectors(Takahashi and Yamanaka , 2006) or through other significant ex vivo manipulations of cells (Okita *et al*,.2008).This process has led to concern sever the stability of these cell lines (Steinemann *et al*., 2013) and the possibility of chromosomal aberrations preventing safe use in human trials currently (Mayshar *et al*., 2010).

Zuk, *et al.* (2002) suggested that ADSCs have recently been investigated as a source of multi-lineage precursor cells, and are particularly promising for regenerative the rapids as they can be easily harvested with minimal donor site morbidity (di Summa *et al.,* 2011). In addition, ADSCs have a differentiation potential similar to other Mesenchymal Stem Cells as well as a higher yield upon isolation and a greater proliferative rate in culture when compared to Bone Marrow Stem Cells (Gimble, 2010; Higuchi *et al*., 2011).

The discovery that ADSCs are not only precursor to adipocytes, but are multipotent progenitors to a variety of cells (Zuk *et al*., 2001) was a miles one that has allowed scientists to utilize the true potential of ADSCs to derive several additional cell types including osteoblasts, chondrocytes, myocytes, epithelial cells and neuronal cells (Brayfield *et al.,* 2010).

Gir, *et al.* (2012) reported that ADSCs are an abundant source of multipotent stem cells that can be easily accessed during many routine procedures. SCs are a promising therapeutic modality for the treatment of tissue defects, malformations and disease, and an attractive tool for the enhancement of aesthetic medicine. However,

scientific evidence on clinical applications is still limited and much is unknown about the safety and efficacy of stem cell therapies. Several key issues must be considered including the:

- Source of stem cells
- Efficiency of transplantation
- Engraftment in host tissue
- Interaction with the surrounding microenvironment
- Long term fate of transplanted cells.

By further elucidating the current strategies for stem cell utilization, this review aims to provide a better understanding of the current state of cellular regenerative techniques in plastic surgery, the progress that remains to be made, and the appropriate direction for future research.

1.2.2 Tissue-specific stem cells

Recent reports have provided substantial new insights into stem cell populations in a variety of adult tissues, In addition to marrow, other sources of stem cells with mesenchymal potential include periosteum (Fukumoto *et al*., 2003), trabecular bone (Tuli *et al.,* 2003), adipose tissue (Wickham *et al*., 2003), Synovium (De Bari *et al.,* 2001), skeletal muscle (Jankowski, Deasy and Huard, 2002), lung (Noort *et al*., 2002).

MSCs isolated from the synovium as an adherent cell population were capable of differentiation into chondrocytes, osteocytes and adipocytes (De Bari *et al*., 2001). They also showed that these cells were capable of contributing to skeletal muscle regeneration in a nude mouse model and restored expression of dystrophic in the sarcolemma in dystrophic muscle of immunosuppressed mdx mice (De Bari *et al*., 2003).

De Ugarte, *et al.* (2003) suggested that there was little difference between cells from marrow and fat in terms of yield, growth kinetics, cell senescence, multi-lineage differentiation capacity, and gene transduction efficiency.

1.2.3 Mesenchymal stem cells

MSCs are defined as adherent cells which possess a proliferative potential and an ability to differentiate *in vitro* into chondrogenic, osteogenic, adipogenic and myogenic lineages. Under proper conditions, MSCs have been demonstrated capable of differentiating into hepatocyte-like(Lee *et al*.,2004) and neuron-like (Lei *et al*.,2007) cells. Apart from bone marrow, MSCs can be isolated from adipose tissue, (Kern *et al*., 2006; Liu *et al.*, 2007) umbilical cord blood (Lee *et al*., 2004) and various fetal tissues such as the placenta, (Miao *et al.*, 2006) amniotic fluid and amniotic membrane (Tsai *et al.*, 2007).

Bone marrow contains stem-like cells that are precursors of non-hematopoietic tissues. These cells were initially referred to as plastic-adherent cells or colony forming-unit fibroblasts and subsequently as either MSCs or marrow stromal cells (Kolf *et al*., 2007; Lindnera *et al*., 2010).

There is much interest in these cells because of their ability to serve as a feeder layer for the growth of hematopoietic stem cells, their multi potentiality for differentiation, and their possible use for both cell and gene therapy (Minguell *et al.*, 2001and Kolf *et al*., 2007).

Friedenstein, *et al.* (1970) initially isolated MSCs by their adherence to tissue culture surfaces, and essentially the same protocol has been used by other investigators. MSCs populations in the bone marrow or those that are isolated and maintained in culture are not homogenous, the heterogeneous precursor cells are morphologically similar to the multipotent MSCs, but differ in their gene transcription range (Baksh *et al*., 2004). It has been proposed that in such populations, cell proliferation, differentiation and maturation are in principle independent; stem cells divide without maturation, while cells close to functional competence may mature, but do not divide (Song *et al.*, 2006).

Hypothetically, the fate of MSCs appears to be determined during very early stages of cell differentiation "commitment". During this mostly unknown period, both intrinsic genetic and environmental (local and/or systemic)

conditions interplay to outline the cell's fate towards one of the possible lineages. Based on microarray assays comparing gene expression at the stem state and throughout differentiation, it has been proposed that MSCs multilineage differentiation involves a selective mode of gene expression. Several molecular markers identify committed progenitors and the end stage phenotypes, but at present there are no reliable cell markers to identify the uncommitted mesenchymal stem cells and given the difficulty to identify a single marker to evaluate the population of stem cells, various combinations of these markers may be used (Lin *et al*., 2008 and Xu *et al*., 2009).

Therefore, MSCs are mainly defined in terms of their functional capabilities: self-renewal, multipotential differentiation and trans-differentiation (Baksh *et al*., 2004).

1.2.4 Characterization of Mesenchymal Stem Cells

MSCs have been studied for decades and true MSC marker has not yet been identified. The cells are characterized by the expression of numerous surface antigens. Unfortunately, none of them appears to be exclusively expressed on MSCs which makes the definition of MSCs difficult. To better define human MSCs, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy has reached a consensus on minimal criteria (Dominici *et al.*, 2006).

First, MSCs must be plastic-adherent when maintained in standard culture conditions. Second, MSCs must express markers CD105, CD73and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79- alpha or CD19 and HLA-DR surface molecules. Third, MSCs must at least be able to differentiate into lineages of osteoblasts, adipocytes and chondroblasts *in vitro*. Many studies continue to search for novel markers to isolate highly purified MSCs. Recently, CD271 was reported as the most specific marker for BM–derived MSCs (Buhring *et al*., 2007).

Other studies have clearly demonstrated that native-form MSCs are phenotypically and functionally different from cultured MSCs and similar to perivascular cells (Deschaseaux *et al*., 2009).

1.2.5 Osteogenic induction of MSCs

Cultured MSCs are largely used in experimental bone reconstruction *in vivo* and *in vitro*. The cells are generally cultured in basal medium such as Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Meijer *et al.,* 2007; Deschaseaux *et al.,* 2009). Researchers believe that serum components in fetal bovine serum play crucial roles in the attachment and proliferation of MSCs (Lennon *et al.,* 1996). Osteogenic induction requires the presence of:

- Dexamethasone is a synthetic corticosteroid. While it is not found naturally in the body, it imitates the actions of various glucocorticoids located naturally in the body such as cortisol, estradiol, testosterone, vitamin D3, thyroxine and retinoic acid. The effect of the glucocorticoid on cell function eventually depends on cell type, the gene regulatory proteins enclosed within the cell and the regulatory region of the gene (Alberts *et al.,* 2002).

Dexamethasone supports osteogenic differentiation (Peter *et al.,* 1998; Atrnani *et al*., 2002) by binding to some special regulatory proteins in the cell and then activating transcription of osteoblast-specific genes. There are also evidences that dexamethasone functions at multiple points in the differentiation process to stimulate osteoblastic maturation (Porter *et al.,* 2003).

Therefore, a constant dexamethasone presence in culture medium is required to achieve maximal osteoblastic differentiation of MSC cultures (Nuttelman, 2005). however, very long presence of dexamethasone in differentiation media would have toxic effect on osteoblasts and cause lysis hence, they have to be harvested and utilized soon after they are completely differentiated.

- β-glycerophosphate is organic phosphates aids osteogenesis by starting mineralization in cell cultures and is thought to modulate osteoblastic activities by promoting a bone-like mineral phase (Tenenbaum *et al.*, 1992). Robison and Soames (1924) noticed that organic phosphate could be an origin of inorganic phosphate. Despite many studies so far, the exact mechanism by which β-glycerophosphate can induce mineralization is still unclear but it is believed its support is closely related to the ability of alkaline phosphatase to hydrolyze organic phosphate and release inorganic phosphate (Nuttelman, 2005).

  This osteogenic differentiation might be owing to the presence of osteopontin which strongly adsorbs to the charged mineral phases created by presence of β-glycerophosphate and other organic phosphates. Besides, free phosphates are competent that directly influencing the differentiation of MSCs to osteoblasts by inducing the mRNA and protein expression of osteogenic markers likes osteopontin. Furthermore, free phosphates have numerous effects on the construction of the key osteogenesis regulatory transcription factor and core binding factor alpha-1(Cbfa1) (Beck *et al.*, 2000 and Fujita *et al.*, 2001).
- Ascorbic acid: also assists in facilitation of osteoblast proliferation and maintaining the cells in an osteoblastic phenotype by increasing the total protein, collagen synthesis and alkaline phosphatase activity (Hitomi *et al.*, 1992). Ascorbic acid could be substituted with the more stable analog such as ascorbic acid phosphate as the half-life of ascorbic acid in culture is only seven hours whereas ascorbic acid phosphate has a half-life of seven days (Gronthos *et al.*, 1994).

Alternative cellular therapies have turned to progenitor cell populations such as bone marrow stromal cells, which have the ability to differentiate into several connective tissue cells types, including cartilage (Pittenger, *et al.*, 1999).

1.2.6 Osteoblast differentiation and maturation

Bone formation is a prolonged, strictly regulated process that takes place during embryonic development, growth, remodeling and fracture repair (Aubin, 2001). Bone formation is characterized by a sequence of events starting with the commitment of osteo progenitor cells and their differentiation into pre-osteoblasts and then into mature osteoblasts whose function is to synthesize the bone matrix that becomes progressively mineralized. Osteoblasts are derived from pluripotent mesenchymal stem cells (Caplan, 1991), which prior to osteoblast commitment can also differentiate into other mesenchymal cells lineages such as fibroblasts, chondrocytes, myoblasts and bone marrow stromal cells including adipocytes, depending on the activated signaling transcription pathways (Friedenstein *et al.,* 1987 and Yamaguchi *et al.* 2000).

Several specific transcription factors are responsible for the commitment of pluripotent mesenchymal cells into the osteoblast cell lineage. One of the most important of these is represented by Cbfa1 is a transcription factor belonging to the runt-domain gene family, which plays a critical role in osteoblast differentiation, although it is not sufficient alone to support the achievement of the mature osteoblast phenotype (Komori *et al.,* 1997).

Cbfa1 is highly expressed in osteoblast lineage cells and regulates the expression of various osteoblast-specific genes (Tsuji *et al*., 1998 and Harada *et al*., 1999). Cbfa1-deficient mice are completely lacking in bone formation (Hoshi *et al*., 1999), because of the maturational arrest of their osteoblasts, whereas the over-expression of Cbfa1 induces non-osteogenic cells to express osteoblast related genes (Yamaguchi *et al*., 2000).

Another runt-related gene that plays an important role in the commitment of multipotent mesenchymal cells to the osteoblastic lineage and for osteoblast differentiation at a nearly stage is Runx-2 which is involved in the production of bone matrix proteins (Komori *et al*., 1997).

As it is able to up-regulate the expression of major bone matrix protein genes, such as type I collagen, osteopontin, bone sialoprotein and osteocalcin (Ducy *et al*., 1997) leading to an increase of immature osteoblasts from pluripotent stem cells; the immature osteoblasts form immature bone(Komori, 2010).

Runx-2 expression is down-regulated in the late stage of osteoblast maturation, when phenotypically mature osteoblasts form mature bone (Komori, 2010).Runx-2-deficient mice are completely lacking in bone formation, because of an absence of osteoblasts (Komori *et al.,* 1997).

Osterix (Osx) is also an essential transcription factor for osteoblast differentiation at an early stage (Ogawa *et al,* 1993). It inhibits osteoblast differentiation at a late stage (Komori, 2003).

Osteoblast commitment, differentiation and growth are controlled by several local and systemic factors that can also act in a paracrine and/or autocrine way and that can regulate the activity of specific transcription factor (Aubin and Liu, 1996). They include bone morphogenetic proteins(BMPs)(Centrella *et al,* 1994), hedgehog proteins, cell growth factors (Canalis *et al*., 1993) such as fibroblast growth factor (FGF) and insulin-like growth factor (IGF),hormones (Cheng, *et al.,* 1994), cytokine modulators (Goldring, M. and Goldring, S., 1990), canonical Wingless (Wnt) /β-catenin (Ambrosetti *et al.,* 2008) and mechanical physical forces (Buckley, *et al.,* 1990). These factors can exhibit different and often opposite effects in modulating cell metabolism depending on the maturation stage and cell phenotype (Canalis *et al.,* 1988 and MacDonald *et al*., 1993).

BMP-2, BMP4 and BMP-7 have been shown to be able to induce immature cells to differentiate into osteoblasts (Ahrens *et al.,* 1993 and Asahina, *et al.,* 1996). BMP-7 induces the expression of Cbfa1 mRNA (Ducy *et al.,* 1997), indicating that Cbfa1 represent a nuclear target of BMPs signalling during osteoblast differentiation, even if other transcription factors might also be

involved in BMPs signaling. Conversely, many factors can affect Cbfa1expression, such as transforming growth factor-beta (TGF-β), which can up-regulate Cbfa1 (Lee and Lorenzo, 1999).

In this phase, BMP-2 and BMP-5 play a significant role in increasing alkaline phosphatase activity, osteocalcin synthesis (Yamaguchi *et al.*, 1991) and parathyroid hormone (PTH9 responsiveness (Kodama *et al.*, 1982; Takuwa *et al.*, 1991). Immediately after growth arrest, a developmental sequence involving the selective expression of specific genes that characterize the differentiated osteoblast phenotype (alkaline phosphatase, osteocalcin) occurs (Stein *et al*., 1992).

The accumulation of matrix proteins contributes, in part, to the cessation of cell proliferation. The active bone-matrix-secreting osteoblasts are cuboidal cells, with a large Golgi apparatus and an abundant rough endoplasmic reticulum, and are provided with regions of plasma membrane specialized in the trafficking and secretion of vesicles that facilitate the deposition of bone matrix (Anderson, 2003).

During the post proliferative phase, which is characterized by the high synthesis of alkaline phosphatase, the extra-cellular matrix progresses into the mineralization phase in which osteoblasts synthesize several proteins that are associated with them mineralized matrix *in vivo* (Hauschka *et al*., 1989), including sialoprotein (Nagat *et al.*, 1991).

Osteopontin and Osteocalcin (Owen *et al*., 1990). Osteopontin is expressed during the stage of active proliferation 25% of maximal level (Lian and Stein, 1995), decreases immediately after the post-proliferative stage and increases again at the onset of mineralization, achieving the greatest level of expression during mineralization. Osteopontin might be involved in the control of the relationship between the cells and extra-cellular matrix, as its amino acid sequence containing arg-gly-asp can mediate cell attachment (Oldberg *et al.*, 1986). Unlike osteopontin, osteocalcin (bone Glaprotein) is expressed by

osteoblasts only in the post proliferative phase. Osteocalcin is maximally expressed during mineralization *in vivo* (Hauschka *et al*., 1989) and *in vitro* (Owen *et al.,* 1990).

Studies suggest that osteocalcin is involved in the regulation of mineral deposition and that it acts as a bone matrix signal that promotes osteoblast differentiation and activation(DeFranco *et al.,* 1991 and Chenu *et al*., 1994), confirming that osteocalcin is a marker of mature osteoblasts (Lian *et al.*, 1989;1991). Osteocalcin synthesis is regulated by various hormones, Vitamin D, and growth factors (e.g. TGF-β).

The onset and progression of matrix mineralization processes might be responsible for the down-regulation of genes expressed by mature osteoblasts during the same processes of extra-cellular matrix maturation and organization. At the end of the synthesis and mineralization of the extracellular matrix, cellular levels of alkaline phosphatase mRNA decline (Lian and Stein, 1995) and 50%–70% of mature osteoblasts undergo apoptosis, whereas the remainder can differentiate into lining cells or osteocytes or trans differentiate into cells that deposit chondroid bone (Tamara, 2006).

Lining cells remain on the bone surface, regulate the influx and efflux of mineral ions and retain the ability to re-differentiate into secreting osteoblasts upon exposure to various stimuli (hormones, mechanical forces; Clark, 2008).

Osteocytes are metabolically quiescent osteoblasts embedded in bone matrix; they communicate with other bone cells through cell processes and function as strain and stress sensors (Lozupone *et al.,* 1996).

1.2.7 Morphology and function of osteoblasts

Osteoblasts are engaged in bone formation. They are generally round in shape and line on the bone surfaces (Fig.1A). Ultrastructural property of osteoblasts shows typical secretory characteristics, possessing well-developed rough endoplasmic reticulum with dilated cisterna (Baron, 1999).

A large Golgi complex comprises multiple Golgi stacks, vesicles and vacuoles containing fibrillar structures which are considered to represent pro-collagen and proteoglycans (Fig. 1A).

Osteoblasts are responsible for the production of collagen and non-collagenous proteins including osteocalcin, bone sialoprotein, osteopontin, and osteonectin (Aubin *et al*., 2006).

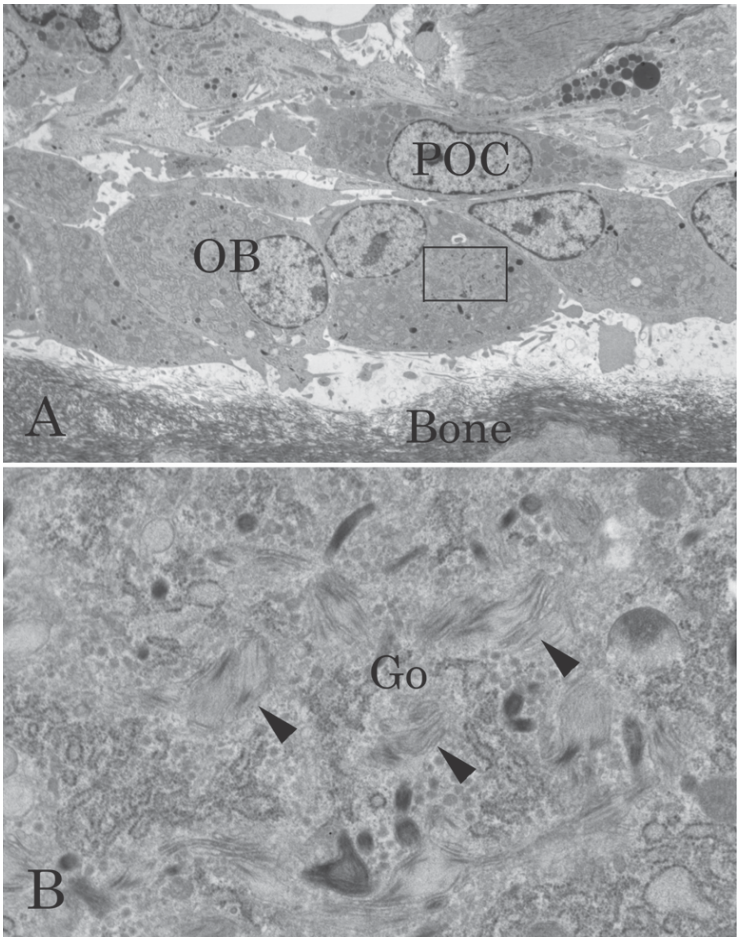

Figure (1): Electron micrographs of osteoblasts. A: Round osteoblasts (OB) are seen on bone matrix (Bone). POC; preosteoclast. B: electron micrograph, at a higher magnification, of square in A. Golgi apparatus (Go) of osteoblasts consists of cistern, vesicles, and vacuoles containing fibrilar structures (arrow head) Baron R. (1999).

Osteoblasts demonstrate intense alkaline phosphatase activity on their plasma membrane. This histochemical feature has been used for a marker of osteoblast-lineage cells. Research of Tissue Non-specific Alkaline Phosphatase (TNAP)-deficient mice revealed that TNAP acts as pyrophosphatase

hydrolyzing pyrophosphate, inhibitor of calcification, and increases the concentration of inorganic phosphates required for calcification (Hessle *et al*., 2002).

1.3 Bone function

The skeletal system of the human body is comprised of bones, cartilage, muscles, ligaments and tendons. Bone is a complex and dynamic vascular mineralized connective tissue with various mechanical, synthetic and metabolic functions. The skeleton acts as a frame for the body giving it support and keeping its shape. In particular, bones provide physical protection and support for internal organs, such as the skull protecting the brain, or ribs protecting the lungs and heart. Bone serves as an attachment site for tendons and muscles which together form a movement apparatus. Moreover, the presence of bone is essential for the sense of hearing. The auditory ossicles (malleus-hammer, incus-anvil, and stapes-stirrup) present in the middle ear space transmit sounds waves from the air to the cochlea (Gray, 1973).

In addition, within the medullar cavity of long bones and the interstices of cancellous bone marrow is found, which is the source of multipotent cells and the Centre of hematopoiesis. Finally, by having a direct effect on energy metabolism, bone acts as an endocrine organ whose action influence the whole body (Schwetz *et al.*, 2012).

Bone is not inert tissue but dynamically metabolized connective tissue throughout life (Baron, 1999). Old bone matrices are always replaced by newly formed matrices. This continual process, named bone remodeling, is important for maintaining bone volume and strength. Bone volume is maintained by the balance of bone resorption and bone formation. Bone cells consist of osteoblast lineage cells (Lian *et al*., 1999 and Aubin *et al.*, 2006) and osteoclast-lineage cells (Ross, 2006).

Their differentiation and function are regulated by osteotropic hormones and cytokines. Research has revealed that osteoblast-lineage cells are not only

involved in bone formation but also in bone resorption via supporting differentiation and activation of osteoclasts (Suda *et al*., 1999).

1.3.1 Bone matrix

1.3.1.1 Inorganic compartment

The main inorganic bone matrix component, which gives bone its hardness and rigidity, is an analogue of naturally occurring hydroxyapatite ($Ca_{10}(PO4)_6(OH)_2$). In hard tissues, such as bone and dentine, the OH group can be substituted with $CO3^2$, Mg^{2+}, F^- or $H2PO^{-4}$ (Boskey, 2008). In general, apatite crystals deposited in bone have a needle-shape measuring 20-40nm in length and 3-6nm in breadth (Gray, 1973).

The deposition of the apatite crystals is oriented in relation to the collagen fibrils. Specifically, in bone, crystals are located within the individual fibers and along the fibril surfaces in the extra-fibrillar space between the fibers (Orgel *et al*., 2011; Veis and Dorvee, 2013).

The process of bio mineralization is initiated and coordinated by the bone-related proteins. Bio mineralization is biphasic, comprising mineral formation within matrix vesicles (MV) and mineral propagation into the extracellular matrix (ECM), which is regulated by enzymes, proteins and phospholipids (Anderson, 2003).

1.3.3.2. Organic compartment

1.3.3.2.1. Collagen matrix

Type I collagen is the most abundant protein in bone comprising about 90% of bone's organic material. It is also abundantly present in the extracellular matrix of tendons, ligaments and skin. In bone it provides tensile strength and torsional stiffness, especially after calcification. Type I collagen belongs to the group of fibrillar collagens, which consist of long triple helical molecules self-assembling into highly organized fibers (Kadler *et al.,* 1996).

1.3.3.2.2. Non-collagenous proteins and their role in matrix mineralization

Alkaline phosphatase (ALP) has an important role in the mineralization and the tissue non-specific isoform of ALP present in bone is bound to the MV within the cell membrane that buds from osteoblasts via phosphatidylinositol-glycan (Hoshi *et al.*, 1997 and Moss, 1997).

Numerous studies have indicated that ALP may act as an early indicator of cellular activity and differentiation. The protein levels have also been shown to be up regulated in response to mechanical force application. Levels of ALP mRNA have been shown to increase as little as 2 days post stimulation with steady increases with the progression of osteoblastic differentiation (Shui *et al*., 2003 and Qi *et al*., 2003). Various types of force and the *in vitro* method of application have also been shown to illicit this response (Qi *et al.*, 2003 and Jagodzinski *et al.*, 2004).

Bone sialoprotein (BSP) and osteonectin belong to the SIBLING (Small Integrin-binding Ligand N-linked Glycoprotein) gene family which contain RGD (Arg-Gly-Asp) motifs in their structure that can bind to various matrix constituents and cell types (Roach, 1994). Studies using a steady-state agarose gel system showed that BSP acts as a nucleation factor for hydroxyapatite formation and deposition (Hunter and Goldberg, 1993), whereas osteonectin is a promoter of hydroxyapatite crystal formation.

Osteopontin is another glycoprotein is believed to act as an inhibitor of mineralization as it hinders hydroxyapatite nucleation de novo in gelatin and agarose gel systems (Hunter *et al.*, 1996), by preventing premature precipitation of calcium phosphate crystals (Roach, 1994). Osteopontin is first expressed in the proliferation phase, where it reaches the level of 25% of maximum that occurs during mineralization (Sodek *et al.*, 2000).

Another non-collagenous protein, osteocalcin is a small bone-specific bone matrix protein produced primarily by osteoblastic cells during the late phases of bone formation (Hauschka *et al.,* 1989). The structure of osteocalcin is characterized by three Gla-residues which provide the protein with a high affinity to bone hydroxyapatite (Hoang *et al.,* 2003).

These residues interact with calcium and hydroxyapatite (Poser and Price, 1979) by regulating the process of mineralization and bone remodeling (Roach, 1994). At the gene expression level, no osteocalcin mRNA was detected in tissues, such as the liver, heart, lung and skin (Fraser and Price, 1988 and Hauschka *et al.,* 1989). Instead, the presence and accumulation of osteocalcin is restricted to the osseous culture systems (Aarden *et al.,* 1996).

Osteocalcin is rapidly degraded in serum and in addition to the intact molecule, osteocalcin fragments of various sizes exist in the circulation (Garnero *et al*., 1994a). There are numerous commercial kits available for the measurement of human osteocalcin and levels of circulating osteocalcin have been used for assessing relative degrees of bone turnover in many clinical studies (Lee *et al.*, 2000).

1.4 Detection of osteoblast cells by Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence (Bartlett and Stirling, 2003). PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications (Saiki *et al.*, 1988).

There are many variants of PCR first, reverse transcription polymerase chain reaction (RT-PCR). This technique is commonly used in molecular biology to detect RNA expression (Freeman *et al.,* 1999). Second, quantitative RT-PCR (Joyce, 2002) or q RT-PCR (Kang, *et al.,* 2010).

In classical PCR, at the end of the amplification, the product can be run on a gel for detection of this specific product. It is used to amplify a specific region of a DNA strand (the DNA target). Most PCR methods typically amplify DNA fragments of between 0.1 and 10 kilo base pairs (kbp), The amount of amplified product is determined by the available substrates in the reaction, which become limiting as the reaction progresses (Carr and Moore, 2012).

Reverse transcriptase is commonly used in research to apply the polymerase chain reaction technique to RNA in a technique called RT-PCR. The classical PCR technique can be applied only to DNA strands, but, with the help of reverse transcriptase (Temin and Mizutani, 1970).

Beta-actin (human gene and protein symbol ACTB/ACTB) is one of six different actin isoforms which have been identified in humans, ACTB gene provides instructions for making a protein called beta (β)-actin, which is a member of the actin multi gene family encodes a non-muscle, cytoskeletal actin protein that is ubiquitously distributed in most animal cell types. Proteins in this family are organized into a network of fibers called the actin cytoskeleton, which makes up the structural framework inside cells (Dugina *et al.*, 2009).

The ß-ACT is highly conserved in the animal kingdom and plays essential roles in maintaining cytoskeletal structure, cellular mobility, cell division, and contractile processes (Reece *et al.*, 1992).

Osteocalcin OC, OCN, the approved gene symbol for this protein is BGLAP (bone gamma-carboxyglutamate protein). Osteocalcin is a small, noncollagenous and highly conserved and secreted protein that is associated with the mineralized matrix of bone. The mouse appears to contain two osteocalcin genes that are referred to as OG1 (osteocalcin gene 1) and OG2 (osteocalcin gene 2) and are expressed at different levels in osteoblasts (Desbois, 1994).

Expression of osteocalcin is considered to be a specific marker for late osteoblast differentiation. The expression of osteocalcin is influenced by various

growth factors, hormones, and vitamins including bFGF (Xiao, 2002), IGF-1 (Davis *et al*, 2006), TGF-beta (Alliston *et al.*, 2001) and glucocorticoids (Meyer *et al.*, 1997).

Chapter Two

Materials

and

Methods

2 Materials and Methods

2.1 Materials:

2.1.1 Apparatus and Equipments:

The following apparatus and equipment were used in this study:

No.	Equipment or Apparatus	Company	Origin
1	Tissue culture plate with 8 wells	Iwaki	Japan
2	Plastic tissue culture tube (15) ml		
3	Plastic tissue culture tube (25) ml		
4	Pap pen	Daido sangyo-tokyo	
5	Inverted microscope	Olympus	
6	Light microscope		
7	Glass culture bottle	Santa Cruze	USA
8	Glass tissue culture Petri dish		
9	Beaker		
10	Graduated cylinder		
11	Nalgene filter units, pore size 0.22 μm	Nalge	
12	Nalgene syringe filters, pore size 0.22 μm		
13	Light microscope digital camera	Scopetek	
14	Liquid nitrogen container	Union Carbide	
15	Whatman filter papers No.1	What man	
16	Magnetic stir bar	Science Lab	
17	Distillator	Running waters	
18	Parafilm	Bemis	
19	Plastic tissue culture flasks (25 cm^2)	Nunc	Denmark
20	Plastic tissue culture Petri dish		
21	Disposable sterile syringes (5 ml)	Medeco	UAE
22	Disposable sterile syringes (1 ml)	Medeco	UAE

23	Cooled Centrifuge	Hettich	Germany
24	Water bath	Memmert	
25	Incubator		
26	Shaker	Cyan	Korea
27	Laminar air flow cabinet	K&K	S. Korea
28	Electrical oven		
29	Magnetic stirrer	Gallenkamp	UK
30	pH-meter		
31	Sensitive balance	Stanton	
32	Cover slips (22*22mm)	Apel	China
33	Microscope glass slides	Afco	
34	Micro- pipette (2-20 µl)	Lypress	Belgium
35	Micro- pipette (10-100 µl)		
36	Micro- pipette (100-1000 µl)		
37	Micro- pipette (1000-5000µl)		
38	Disposable Tips		
39	Deep freeze (-80ºC)	Nuve	Turkey
40	Autoclave	GSL	
41	Improved Double Neubauer Ruling Counting Chamber	Assistant	
42	Polymerase Chain Reaction	Esco	Korea
43	Gel electrophoresis		
44	Refrigerator	-	-
45	Vacuum pump	Franklin elective	-

2.1.2 Chemicals and biological materials

No.	Chemical or biological material	Company	Origin
1	Streptomycin	Troge	Germany
2	Ampicillin		
3	DPX mountant	Fluka	
4	Ethanol alcohol 99%	BDH	UK
5	Di sodium hydrogen phosphate (Na_2HPO_4)		
6	Potassium chloride (KCl)		
7	Sodium bicarbonate(NaHCO3)		
8	Trypan blue stain	US Biological	USA
9	Potassium dihydrogen phosphate (KH_2PO_4)		
10	Dulbecco's Modified Eagle Medium		
11	Fetal Bovine Serum		
12	Ascorbic acid growth factor		
13	Dexamethasone growth factor		
14	Beta-glycerophosphate growth factor		
15	Minimum Essential Medium, MEM		
16	Trypsin EDTA		
17	Formaldehyde 37%	Santa Cruze	
18	Hydrogen peroxide (H_2O_2)	-	Iraq
19	Xylene	Scharlau	Spain
20	glacial acetic acid		
21	Sodium chloride (NaCl)	Thomas baker	India
22	Trise Borate Buffer (TBE)	Bioworld	USA
23	Lysis Buffer	Agilent	USA

24	Beta- Mercaptoethanol	Agilent	USA
25	RNase-free DNase I (lyophilized)		
26	DNase Reconstitution Buffer		
27	DNase Digestion Buffer		
28	High-Salt Wash Buffer		
29	Low-Salt Wash Buffer		

2.1.3 Kits:

No.	Kit	Company	Origin
1	Mouse anti human CD 34	US-biological	USA
2	Mouse anti human CD105		
3	Alkaline phosphatase strip	Roche	
4	Beta –actin primer	Kapa	
5	Osteocalcin primer		
6	RNA Miniprep Kit	Agilent	U S A
7	Immunocruz mouse ABC Staining system:sc-2017(kit)	Santa cruz	U S A

2.1.4 Laboratory animals:

Fifty mice used in this study were obtained from the Iraqi center for cancer and researchs medical genetics / Al- Mustansryia University. The animal age from 4-8 weeks and it weight was from 20 to 30 gram. These animals were subjected to unified condition of temperature, light and feeding.

2.2 Methods:

2.2.1 Preparation of solutions for cell cultures:

2.2.1.1 Antibiotics:

- Streptomycin: 1 g of streptomycin was dissolved in 5ml triple distill water, and 0.5ml of it was added to 1 litter of culture media.

- Ampicillin: 1000000 IU of ampicillin was dissolved in 5ml triple distill water, and 1ml of it was added to 1 litter of culture media (Freshney, 2000).

2.2.1.2 Sodium Bicarbonate:

The solution was prepared by dissolving 2.2g of Sodium bicarbonate in one liter of culture medium or as recommended by manufacturing company.

2.2.1.3 Phosphate Buffer Saline PBS (pH 7.2):

It was prepared by dissolving 8 g NaCl, 0.2g KCl, 0.92 g Na_2HPO_4 and 0.2 g KH_2PO_4 in 1L triple distilled water and stirred constantly on a magnetic stirrer at room temperature; the pH was adjusted to 7.2 and autoclaved at 121^{o}C for 15 min and stored at 4^{o}C until used.

2.2.1.4 Fetal Bovine Serum (FBS):

The serum was ready to use, kept at sterile conditions in -20˚C until it used.

2.2.1.5 Trypsin-EDTA Solution:

Trypsin-EDTA Solution was prepared by dissolving 10.1g of trypsin-EDTA powder and 1g sodium bicarbonate in one liter of triple distal water. One ml of ampicillin and 0.5 ml of streptomycin were added. The solution was stirred constantly on a magnetic stirrer at room temperature and sterilized by Nalgen filter 0.22μm unit. Finally the solution was stored in refrigerator and used within a short period of time.

2.2.2. Preparation of stains solutions:

2.2.2.1 Trypan Blue Solution:

A concentration of 1% was prepared as described by Yaseen (1990) by dissolving 1g of trypan blue in 100 ml PBS. Excess solid residue was filtered off using Whatman No.1 filter paper. The clarified dye was autoclaved at 121°C for 10 min. and stored at 4°C till use. Prior to use, a 10% dilution in PBS was prepared by mixing 10 ml of stock solution with 100ml PBS.

2.2.2.2 Hematoxylin:

Hematoxylin stock solution was ready to use.

2.2.3 Preparation of Tissue Culture Media:

2.2.3.1 Minimum Essential Medium (MEM):

MEM culture medium was prepared by dissolving 16.65 g MEM powder with HEPES buffer and L-glutamine in approximately 600 ml of Triple Distilled Water (TDW). 2.2 g of sodium bicarbonate powder, 1 ml of Ampicillin, 0.5 ml of Streptomycin and 100 ml of FBS were added. The volume was completed to one liter with TDW and the medium was sterilized using Nalgen filter using 0.22 μm filter unit. Finally, the media was aliquot into sterile containers (Freshney, 2000).

2.2.4 Preparation of ABC staining system working solution:

Reagents *were prepared according to Santa Cruz leaflets:

- **Blocking serum:** It was prepared by mixing of 75 μl normal blocking serum stock with 5 ml of PBS.
- **Biotinylated secondary antibody:** Seventy five μl of normal blocking serum stock was mixing with 5 ml of PBS and 25 μl biotinylated secondary antibody stock.
- **AB enzyme reagent:** It was prepared by mixing of 50 μl of reagent A (avidin), 50 μl of reagent B (biotinylated HRP) and 2.5 ml of PBS and left to stand for approximately 30 minutes.
- **Peroxidase substrate:** It was prepared by mixing of 1.6 ml of distilled water, 5 drops of 10 x substrate buffers, 1 drop of 50x Diamine Benzidine (DAB) chromogen and 1 drop of 50x peroxidase substrate.
    - **1 % of Hydrogen peroxide H_2O_2:** To prepare 1% H_2O_2 16.6 ml of H_2O_2 stock solution (6%) was mixed with 83.4 ml PBS.
    - **Counter stain:** Hematoxylin ready to use.
    - **Mounting medium:** PDX mounting ready to use.

- **Fixing reagent:** Formalin solution (4%) was prepared by mixing 4 ml of stock Formaldehyde 37% with 96 ml PBS.

2.2.5 Preparation of differentiation growth factors:

2.2.5.1 Beta glycerophosphate disodium salt hydrate (10mM/ml)

A quantity of 0.0216 g of Beta glycerophosphate disodium salt hydrate powder was dissolved in 10 ml of media.

2.2.5.2 Ascorbic acid (50 µg /ml)

A quantity of 0.0005 g of Ascorbic acid was dissolved in 10 ml of media.

2.2.5.3 Dexamethasone (1nM/ml)

One ml of Dexamethasone was mixed with 9 ml of media.

2.2.6 Preparation of PCR solutions:

Reagents *were prepared according to Agilent leaflets:

- **RNase-Free DNase I:** the lyophilized RNase-Free DNase was dissolved by adding 290 µl of DNase Reconstitution Buffer to the vial. The contents were mixed gently but thoroughly to ensure all the powder (including powder on the cap) dissolves into solution without introduce air bubbles into the solution and Stored at –20°C.
- **High-Salt Wash Buffer 1×:** Prepared by adding 16 ml of absolute ethanol to the bottle of 1.67× High-Salt Wash Buffer. After adding the ethanol, the container marked as follows: [√] 1× (Ethanol added) and stored at room temperature.
- **Low-Salt Wash Buffer 1×:** Prepared by adding 68 ml of absolute ethanol to the bottle of 5× Low-Salt Wash Buffer. After adding the ethanol, the container marked as follows: [√] 1× (Ethanol added). Then stored at room temperature
- **Ethanol 70%:** ethanol 70% (v/v) Prepared by diluting 95% or absolute ethanol with RNase free water.
- **DNase solution**: was prepared by gently mixing 50 µl of DNase

Digestion Buffer with 5 µl of reconstituted RNase-Free DNase I.

- **Di Ethyl Pyro Carbonate (DEPC).**

2.2.7 Primary culture of bone marrow:

A bone marrow cell was isolated from the femur of 4-8 week-old male albino mouse with weights ranging from 20-30g.The mouse was killed by cervical dislocation, placed the mouse on its back on a dissecting board and soak it with 70% ethanol and 10% iodine solution, followed by making a long transverse cut through the skin in the middle of the abdominal area. Reflected skin from the hindquarters and the hind legs, then remove the muscles and placed the bone in a Petri dish containing medium Minimum Essential Medium (MEM) free serum. A flushing method was used to flush the bone marrow cells from bones using a 1ml syringe containing 1ml of growth culture media (MEM) (Ishaug *et al*., 1997).

2.2.8 Mesenchymal stem cells isolation by using adherence properties:

The first and simplest method used implies the adherence properties of MSCs which were first identified by the pioneer work of Friedenstein group (Friedenstein *et al*., 1976) freshly isolated whole bone marrow cells were resuspended in growth culture medium MEM supplemented with 15 % FBS, 1 % Ampicllin/Streptomycin. The BM samples were centrifuged at 1000 rpm for 10 min at 18°C the supernatant was aspirated and the pellet was washed twice with PBS (modified from Fortier *et al*., 1998). Cells obtained from one mouse (2 femurs and 2 tibias) were seeded in 6 ml in tissue culture flasks. The cultured cells were incubated at 37°C and left to adhere 24 hours, non-adherent cells were removed, Mesenchymal stem cells were selected by adherence during first 24 h and maintained in growth culture media (Hui *et al*., 2008). Media changed 3 times a week and when the cultures reached 80 - 100 % confluence.

2.2.9 MSCs passaging:

Passaging of the cells were done in suspension culture at cell density is greater than (>10^6 cells /ml). Cells were detached from the surface with trypsin-versene and counted 5 squares by using a haemocytometer. The MSCs are subcultured at approximately 80% confluence to prevent contact inhibition of growth and spontaneous differentiation (solchaga *et al*., 2004), the culture medium was aspirated and the cells were washed three times with MEM free serum and detached by incubation with 1ml of trypsin-versene (were prepared in 2.2.1.5) for 5-10 minutes at 37ºC temperature. In order to dislodge the cells, the flask gently rocked, then 5ml of culture media containing 15% FBS was added by using a sterile Pasteur pipette and mixed to obtain a single cell suspension. The cell suspension was centrifuged at 1000 rpm for 10 minutes in 18 ºC then the supernatant was aspirated and the cells pellet were resuspended in 1ml of culture medium MEM containing 15% FBS.

2.2.10 Viable Cell Count:

Method of Daring and Morgan (1994) was used to measure the cell viability by using trypan blue dye which allows distinguishing between healthy cells with uncompromised membrane integrity (unstained) and dead ones (stained blue) as below:

- Cell suspension was prepared by trypsinization and resuspension in culture medium.
- Cover slip was fixed on a clean haemocytometer Improved Double Naubauer Ruling Counting Chamber.
- Two hundreds µl of cell suspensions were mixed with 200µl of trypan blue solution and 1600 µl of PBS, twenty µl of the diluted cell suspension was transferred to the edge of the cover slip, along running into the counting chamber.

- A light microscope was used to count the cells under magnification powers 100X and 40X (Yaseen, 1990).
- The following equation was then used to calculate the number of cells per unit volume (cells/ml):

$$\mathbf{C = N \times D \times 10^4}$$

Where C is the number of viable cells per milliliter, N is the number of viable cells counted, and D is the dilution factor (= 10) (Freshney, 1994).

2.2.11 Immunocytochemistry analysis of MSCs:

A. Principle: The Principles of the test is that the primary antibody binds to the corresponding antigen in the tissue section, and the secondary antibody binds to determinants on the primary antibody. Then the avidin-biotin complex containing the horseradish peroxidase enzyme was allowed to bind to the biotin molecule attached to the secondary antibody.

B. Assay procedure: After MSCs were dispersed with trypsin-versene, and suspended in MEM growth media the cell were re-cultured in multi-well tissue culture plates (8 well) in MEM supplemented with 15% FBS, the plates were incubated at 37˚C to allow the cells for development a monolayer of adherent cells within 1-3 days, after that the media was aspirated and the cell were fixed by 4% paraformaldehyde for 10 min.

C. Staining procedure:

In all steps the sections were placed in the humid chamber and at room temperature (20-25 ºC)

- To inhibit endogenous peroxidise, cells were incubated with 1% hydrogen peroxide for 10-15 minutes and wash with PBS for 5 min three times.
- Aliquot of 1.5% blocking serum was added to cell section for one hour and then washed with three changes of PBS for 5 minutes. To decrease background staining.

- Cells sections were incubated with 125 µl of diluted primary antibody at a ratio(1:50) for one hour at room temperature or overnight at 4ºC then washed with three changes of PBS for 5 minutes .
- Cell sections were incubated for 30 min with 1.2 ml biotinylated secondary antibody (which prepared in 2.2.4) and washed with three changes of PBS for 5 minutes.
- A liquot of 650 µl of AB enzyme reagent (which prepared in 2.2.4) was added to cell section. And washed two times with PBS for 2 min.
- Three drops of peroxidase substrate (which prepared in 2.2.4) was added to cells for 10min, or until desired stain intensity develops and washed with distilled water for 5 minutes.
- Hematoxylin stain was added to cell section for 5-10 seconds and immediately washed with distilled water. Finally 1-2 drops of permanent mounting medium was added and examined by light microscopy.

2.2.12 Induction osteogenic differentiation of MSCs:

In osteoblast differentiation from the 0-1passage of MSCs were used. The cells were cultured in L-Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 10mM Beta glycerophosphate ,50 µg /ml of Ascorbic acid and 1nM of Dexamethasone. As a negative control, MSCs were cultured in medium without differentiation stimuli along with the differentiation experiments in the same conditions. Cells were cultured in a humidified atmosphere of 5% CO2 and 95% air at 37℃. Cultures were maintained by medium exchange every 2-3 days. The cell morphology was observed under inverted microscope (Zhou, *et al*., 2008).

2.2.13 Evaluation Alkaline phosphatase activity (ALP) in osteogenic media:

A. Principle: Dye formation is determined kinetically at 37ºc after adding sample into reaction zone. The ALP hydrolyzes O-cresolphthalein phosphate

to O-cresolphthalein and transfers the phosphate group to the acceptor molecule methylglucamine. The colored hydrolysis product O-cresolphthalein that is produced per unit of time under alkaline conditions is directly proportional to alkaline phosphatase activity (Heins, 1995; Haenseler, 1997 and Abicht, 2001).

B. Assay procedure:

Thirty two µ from osteogenic media at (0, 7, 14 and 21) days was added to test strip, and then the sample allowed to flow into the reaction zone. The result was displayed after 135 seconds in IU/L by using Reflotron instrument.

2.2.14 Detection of β-actin and Osteocalcin by Reverse Transcriptase PCR:

2.2.14.1 Total RNA isolation kit:

Total RNA was extracted from differentiated cells after 14 day using absolutely RNA Miniprep Kit (Agilent- USA) which contains the following materials.

- Lysis Buffer : 35 ml
- β-Mercaptoethanol (β-ME) (14.2 M) 0.3 ml
- RNase-free DNase I (lyophilized) 2600 U
- DNase Reconstitution Buffer 0.3 ml
- DNase Digestion Buffer 2 × 1.5 ml
- High-Salt Wash Buffer (1.67×) 24 ml
- Low-Salt Wash Buffer (5×) 17 ml

2.2.14.2 RNA isolation:

1- The Lysis Buffer–β-ME mixture was added to cell pellet (it was prepared freshly by mixing 600 µl of lysis buffer with 4.2 µl of β-ME) and the

samples was homogenized by vortexing or repeated pipetting to obtain low viscosity of the lysate.

2- Up to 700 µl of homogenate was transferred to a Prefilter Spin Cup that is seated in a 2-ml receptacle tube and the cap snapped of the receptacle tube onto the spin cup.

3- The tubes were spanned in a micro centrifuge at maximum speed for 5 minutes.

4- The spin cup was removed from the receptacle tube and discarded. The filtrate was retained. The cap firmly seated in the tube, to reduce the chance of leakage during vortexing.

5- An equal volume of 70% ethanol was added to the filtrate and the tube vortexes for 5 seconds until the filtrate and ethanol are mixed thoroughly.

6- Up to 700 µl of this mixture was transferred to an RNA Binding Spin Cup that is seated in a fresh 2-ml receptacle tube and the spin cup was capped.

7- The mixture was spanned in a micro centrifuge at maximum speed for 30–60 seconds.

8- The spin cup was retained and the filtrate was discarded. The spin cup replaced in the receptacle tube. Steps 6–8 repeated with the remaining mixture.

9 - High-Salt Wash Buffer (1×) 600µl was added to the spin cup and capped. The tube was spin in a micro centrifuge at maximum speed for30–60 seconds.

10- The spin cup was removed and retained and the filtrate discarded. The spin cup Replaced in the receptacle tube.

11- Low-Salt Wash Buffer (1×) 600µl was added and the spin cup capped. The tube spinned in a micro centrifuge at maximum speed for 30–60 seconds.

12- The spin cup was removed and retained and the filtrate was discarded.

The spin cup replaced in the receptacle tube.

13- Low-Salt Wash Buffer (1×) 300µl was added and the spin cup capped and spanned in a micro centrifuge at maximum speed for 2 minutes to dry the matrix.

14- The spin cup was transferred to a 1.5-ml micro centrifuge tube and the 2-ml receptacle tube discarded.

15- The Elution Buffer 30–100µl directly was added onto the center of the matrix inside the spin cup and was incubated for 2 minutes at room temperature. The tube was spin in a micro centrifuge at maximum speed for 1 minute.

This elution step was repeated to maximize the yield of RNA. The optical density (OD) was measured at 260 nm and 280 nm to quantitate and qualify the RNA.

2.2.14.3 Primers of β-actin and osteocalcin:

Sequences for beta-actin of forward primer 5’ GGT GGG AAT GGG TCA GAA GG -3’and reverse primer 5’AAT GCC TGG GTA CAT GGT GG -3’, sequences for osteocalcin of forward primer 5’ GTC CAA GCA GGA GGG CAA TA -3’ and reverse primer 5’ TCG TCA CAA GCA GGG TCA AG -3’.

2.2.14.4 First-Strand cDNA Synthesis:

- The following 20 µl reaction volume was added to a nuclease – free microfuge tube based on the following order:

Sterile distilled water	9 µl
5X First-strand buffer	4 µl
(40 Units/ µl) Recombinants RNAase Inhibitor	1 µl
10mM dNTPs mix	1 µl
0.1 M DTT	1 µl

2 µM forward primer	1 µl
2 µM Reverse primers	1 µl
(200 Units/ µl) SuperScript™ III RT	1 µl
5 µg of total RNA	1 µl

- The mixture was mixed by pipetting gently up to down and incubated at 55 ºC for 60 min. Then the reaction was inactivated by heating at 70 ºC for 15 min.

2.2.14.5 PCR Reaction Setup:

Before preparing PCR reactions, all kit components and all reactions assembled on iced rack to avoid premature cDNA synthesis. The required volumes of each component were added according to the following order:

Sterile distilled water	18 µl
10X PCR Buffer	2.5µl
50mM $MgCl_2$	0.75µl
10mM dNTPs	0.5µl
10µM Forward primer	0.5µl
10µM Reverse Primer	0.5µl
5 Units/µl Taq Polymerase	0.25 µl
cDNA (50 ng)	2 µl

2.2.14.6 Run the PCR Reaction:

The PCR instrument was programed for the following cycling protocol:

Step	Temperature	Duration	Cycles
Denaturation	94 ºC	2 min	35 cycles
Annealing	55 ºC	1 min	
Extension	72 ºC	1 min	

2.2.14.7 Gel electrophoresis:

Protocol of Gel Electrophoresis was carried out according to (Sambrook *et al*., 2000).

- **Agarose Gel Electrophoresis materials:**
    - Agarose (medEEO, US Biological, USA)
    - TBE Buffer (Tris-Borate-EDTA, Bioworld, USA)
    - Loading Dye blue 6× 1ml (US Biological –USA)
    - Ethidium Bromide (EB) 10 mg/ml solution, 25ml, (US-biological- USA)
    - Ladder (50-800 bp) (US-biological-USA)

2.2.14.8 Run the Gel:

Electrophoresis was carried out on a horizontal electrophoresis apparatus. Agarose gel (1.5%) was prepared in 1X TBE buffer, and ethidium bromide was added to a final concentration of 0.5µg/mL in melted agarose gel. Electrophoresis was performed at 5 V/cm for approximately 90 min. The DNA bands were visualized on a UV transilluminator and photographed. DNA Ladder was used to determine the molecular weights of DNA bands.

2.2.15 Statistical Analysis:

Differences in ALP were analyzed using SPSS program version 20. Results were expressed using simple statistical parameters such as mean and

standard error. Differences between means were assessed by ANOVA, followed by either LSD or Duncan test. Acceptable level of significance was considered to be $P \leq 0.05$.

Chapter Three

Results

and

Discussion

3 Results and Discussion

3.1 Morphology of bone marrow mesenchymal stem cells (BM-MSCs):

Results showed after 24 h of primary culture most of MSCs are rounded and the cells attached in the tissue culture flask *as* reported by Friedenstein, *et al.* (1979*)* MSCs isolated by adherent on plastic culture flasks during first 24 hr and Non-adherent cells were removed by exchange media with a new fresh media as Figure (3.1).

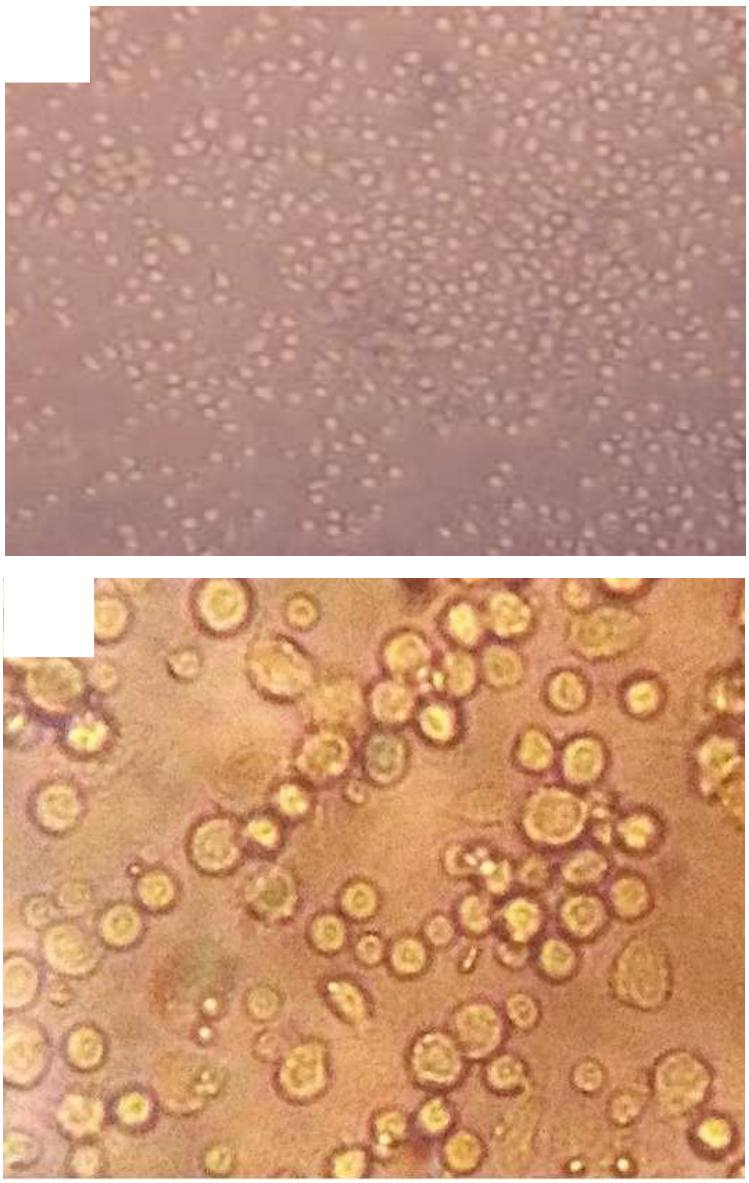

Figure (3.1): Morphology of BM-MSCs in primary culture after 24 h. (rounded cells) as seen under inverted microscope A(X10) and B(40X).

After two days of primary culture, the MSCs were attached to the tissue culture flask sparsely, single adhered cells could be observed, some of which might have begun forming colonies already. Bone marrow cell seeding density was high enough to enable the cells to enhance each other's proliferation, resulting in rushed fibroblast-colony formation and cell proliferation. At this stage, the cells are displayed a spindle-like shaped (fibroblast-like morphology) with one nucleus, then these cells began to proliferate in culture growth medium (Fig3.2).

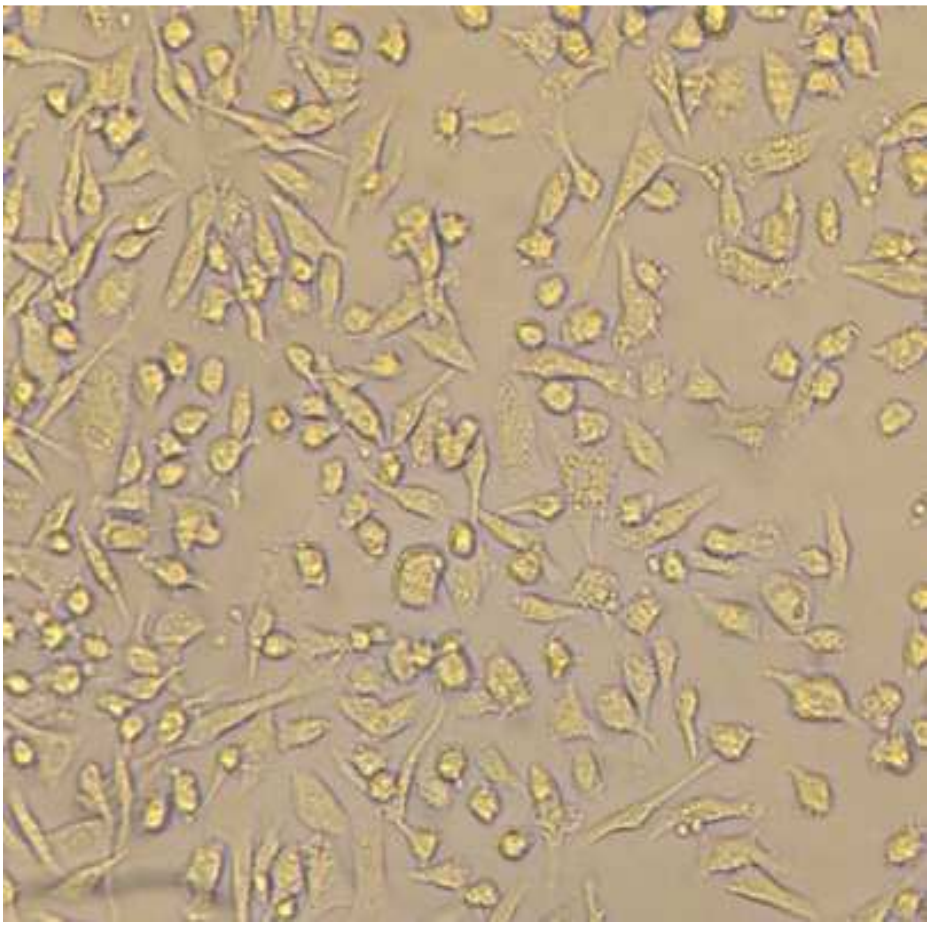

Figure (3.2): Morphology of BM- MSCs in primary culture after 2 days (spindle cells) as seen under inverted microscope (X40).

The results shown, the number of cellular colonies with different size growth had obviously increased through 4 days as shown in Figure (3.3). The isolated adherent cells were observed as heterogeneous groups of cells. However they became homogeneous as the cells continued to proliferate, and

were more homogeneous after subsequent passaging. The initial heterogeneous morphology may be due to presence of other types of adherent cells such as macrophages, lymphocytes, and endothelial cells (Rickard *el al.*, 1996).

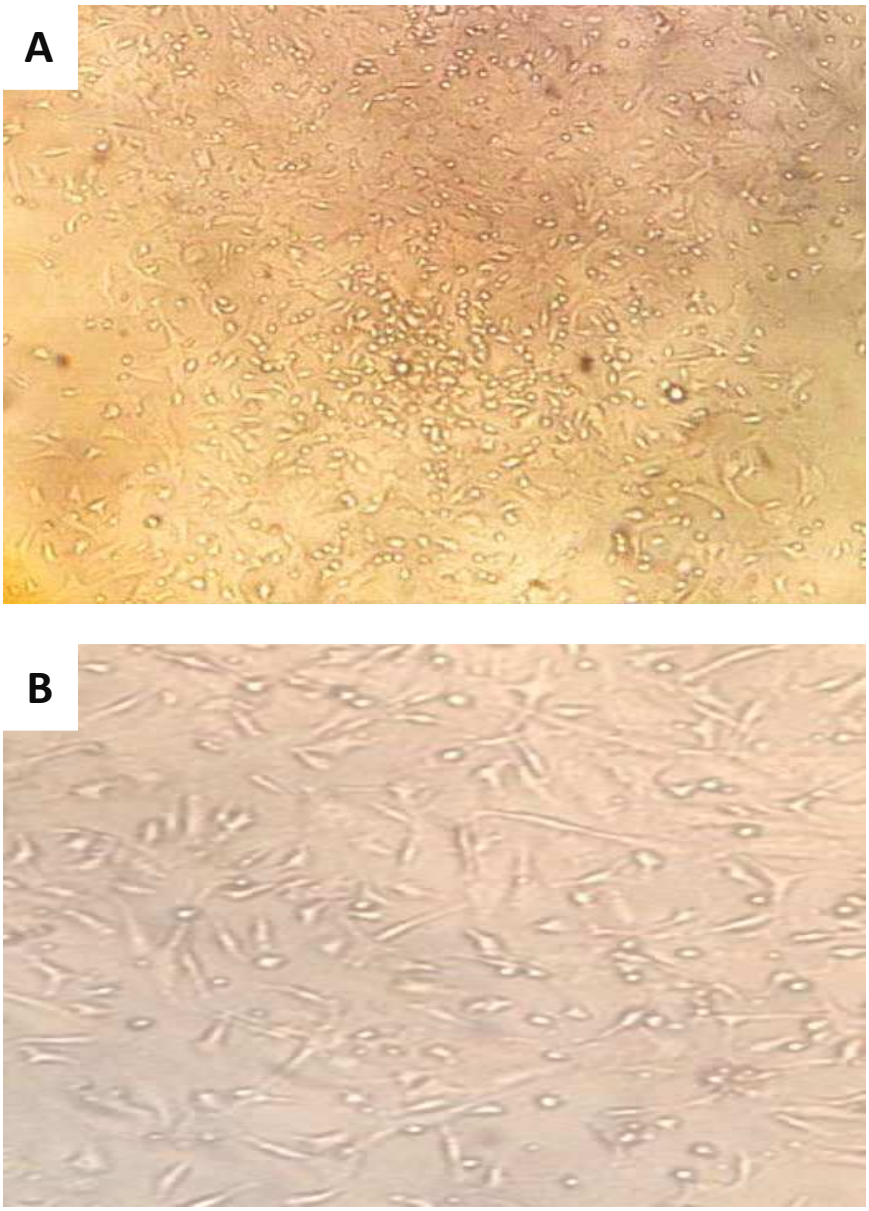

Figure (3.3): Morphology of BM- MSCs in primary culture after 4 days (cellular colonies) as seen under inverted microscope A(X10) and B(20X).

After six days of primary culture the adherent cells were nearly 80-90% confluence and formed monolayer of adherent cells Figure (3.4). The best candidate cells for this purpose are MSCs, because they are multi-potent and have a high proliferative capacity. Because there is no definitive marker to

identify MSCs, the gold standard procedure to prove their stem cell identify is their adherence on cell culture plates after isolation, their expression of specific marker, and their differentiation potential to osteoblasts, adipocytes and chondrocytes *in vitro* (Dominici *et al*., 2006).

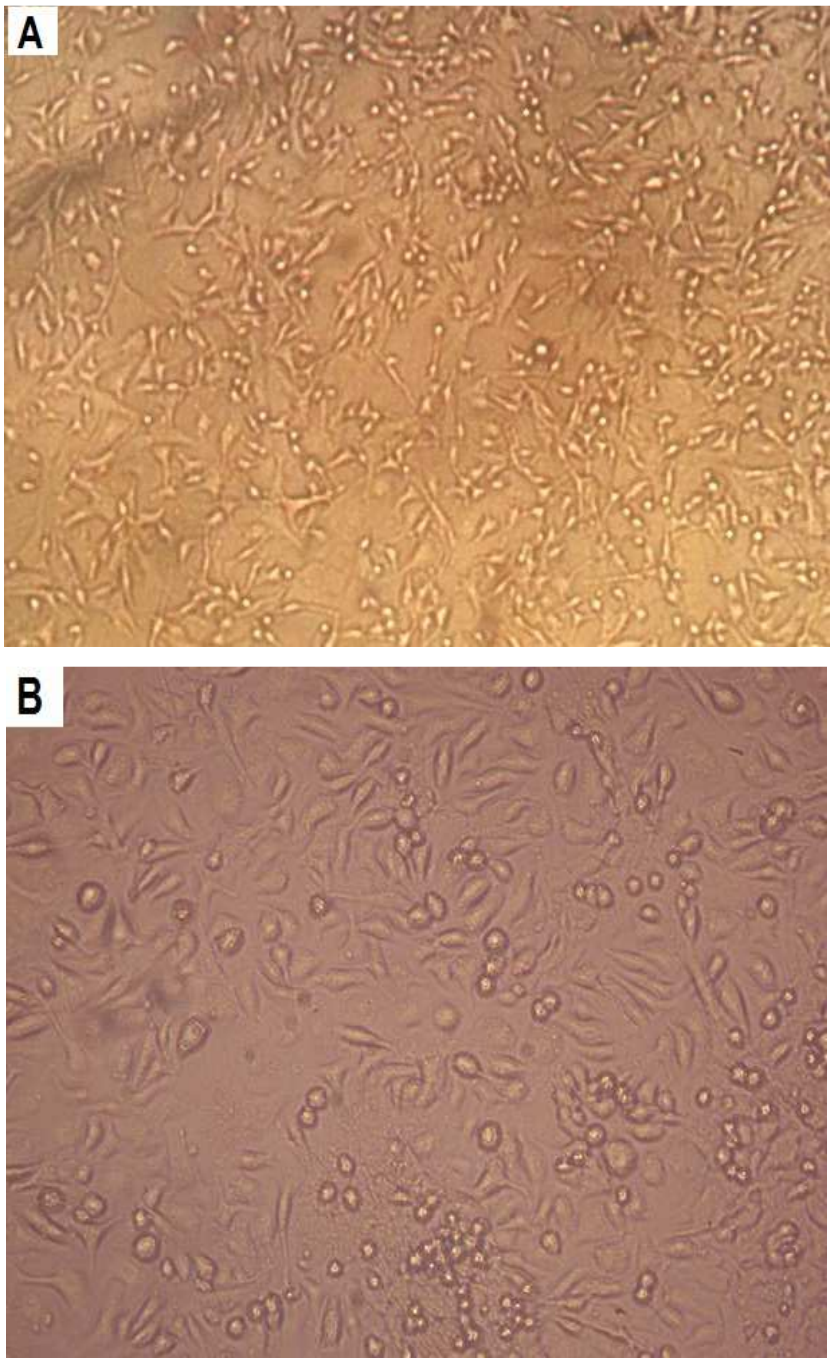

Figure (3.4): Morphology of monolayer of adherent cells in primary culture after 6 days as seen under inverted microscope A(X10) and B(X20).

3.2. Immunocytochemistry of MSCs:

MSCs were characterized by the surface antigens CD, these CD (CD+105,-34) markers were found on MSCs surface using to examine phenotypic properties by immunocytochemistry staining technique. Figure (3.5) (A) showed MSCs from first passage positive to CD+105 because adherent cells were stained with DAB stain (brown color). This result seems agree with Harvanova *et al*. (2011), who reported MSCs were positive for CD105.The brown granular stain in the cytoplasm of the cells produced by DAB reaction was considered positive for each surface antigen CD105. MSCs were negative to CD-34 because it was stained with hematoxylin (counter stain) with blue color and it found on Hematopoietic Stem Cells (HSCs) surface in figure 3.4 (B).This result is in agreement with Bühring *et al*. (2007) and Uccelli and Moretta, (2008) who reported that mesenchymal stem cells (MSCs) express cell marker negative for CD34.

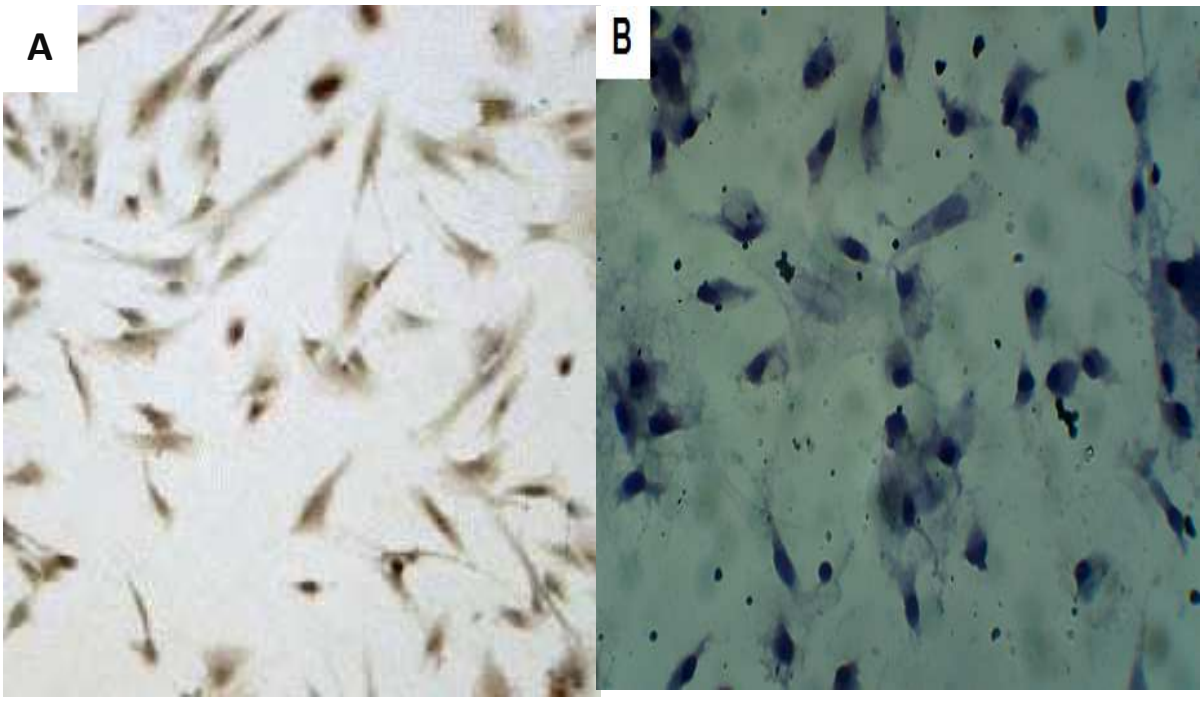

Figure (3.5): Immunocytochemistry analysis of MSCs. (A) MSCs were positive for CD105 marker and stained with brown color DAB stain(X40).(B) MSCs were stained negative for CD34 marker (X40).

3.3. Differentiation BM-MSCs to osteoblast cells:

Bone marrow mesenchymal cell cultures have been used as *in vitro* models for studying several aspects of osteogenesis (Long, 2001). The induction of MSCs to osteoblast cells were done by using osteogenic medium included growth factors previously described 10mM beta glycerophosphate, 50µg/ml ascorbic acid and 1nM of dexamethasone, the cells showed changes in cell morphology after 3 days of the supplementation of the culture media the cells under osteogenic conditions exhibited morphological changes typical into round shape as shown in figure (3.6).

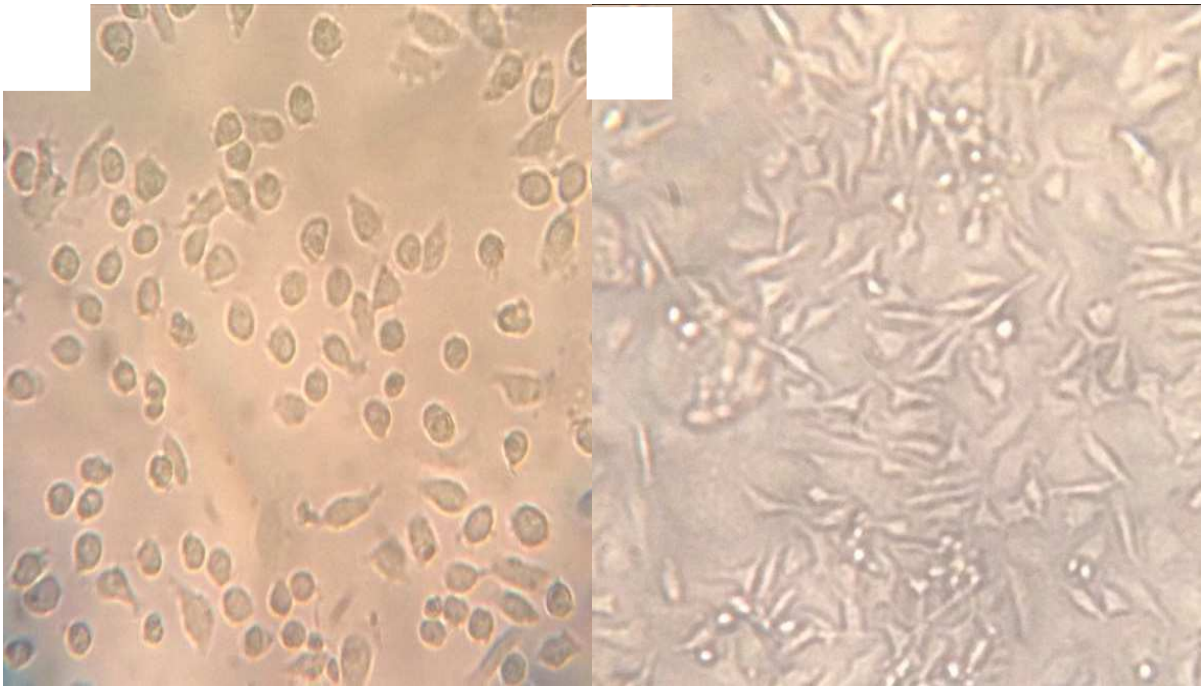

Figure (3.6): MSCs differentiation in osteogenic medium after 3 days (round cells) (A) as seen under inverted microscope (X20). (B) Non-differentiated MSCs as seen under inverted microscope (X20).

Osteoprogenitor cells differentiate into osteoblasts, which produce extracellular matrices that are then mineralized (Kasugai *et al.*, 1991). After 5 days of differentiation cells were spherical in osteogenic media as showed in figure (3.7).

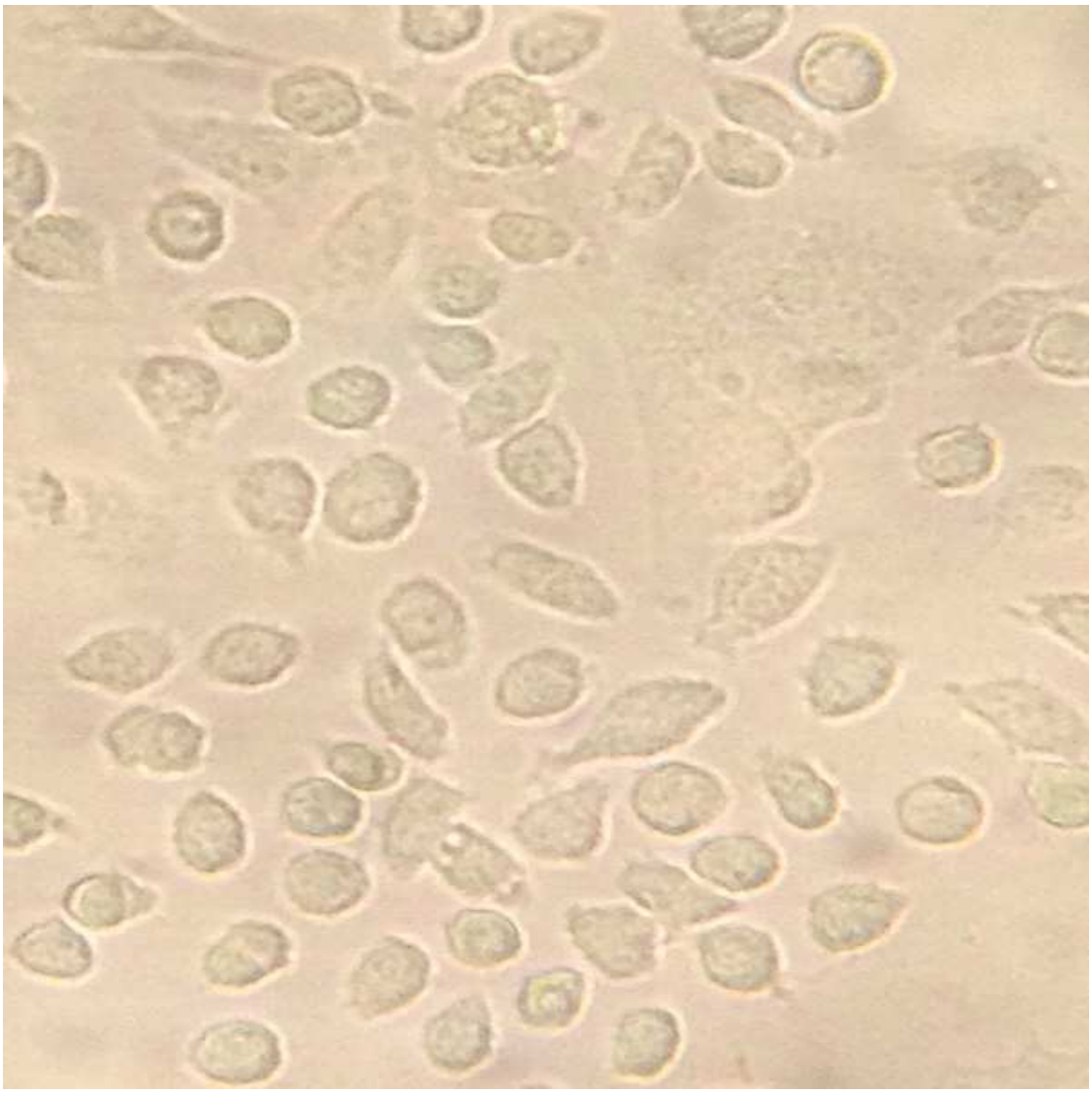

Figure (3.7): MSCs differentiation in osteogenic medium after 5 days (osteoprogenitor cells) as seen under inverted microscope (X40).

Figure (3.8A) shows MSCs after 1weeks of differentiation in osteogenic conditions converted into pre-osteoblast cells in shape, but Cells in non-osteogenic conditions maintained an undifferentiated phenotype with a fibroblast-like morphology as figure (3.8B).

Preosteoblasts are heterogeneous since they comprise all cells differentiating from progenitors to mature osteoblasts (Long, 2012).

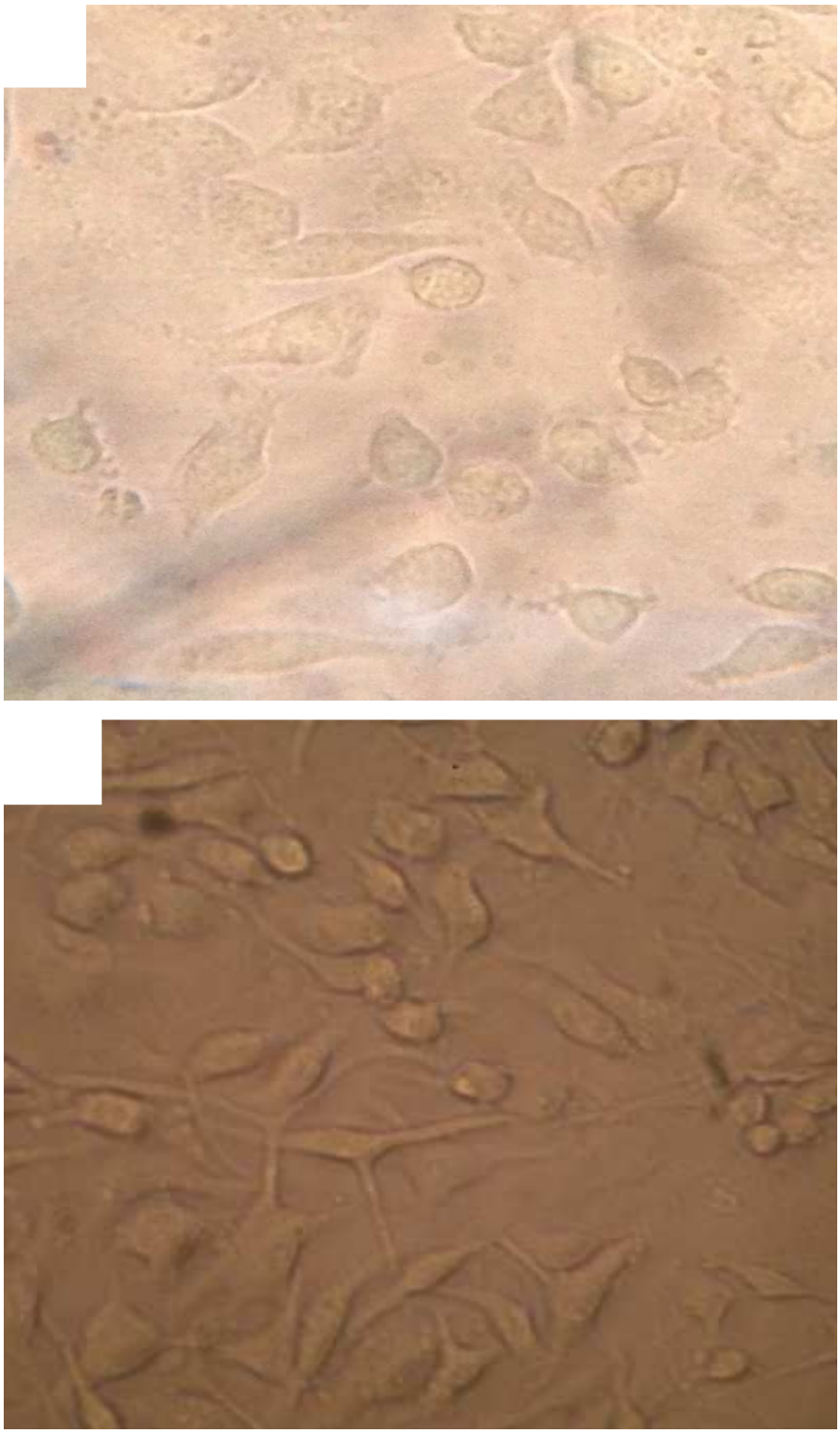

Figure (3.8):MSCs differentiation in osteogenic medium after 7 days. (pre-osteoblast cells) (A) as seen under inverted microscope (X40). (B) Non-differentiated MSCs as seen under inverted microscope (X40).

After 2 weeks of differentiation in osteogenic conditions most of cells are converted into osteoblast like cells as shown in figure (3.9).

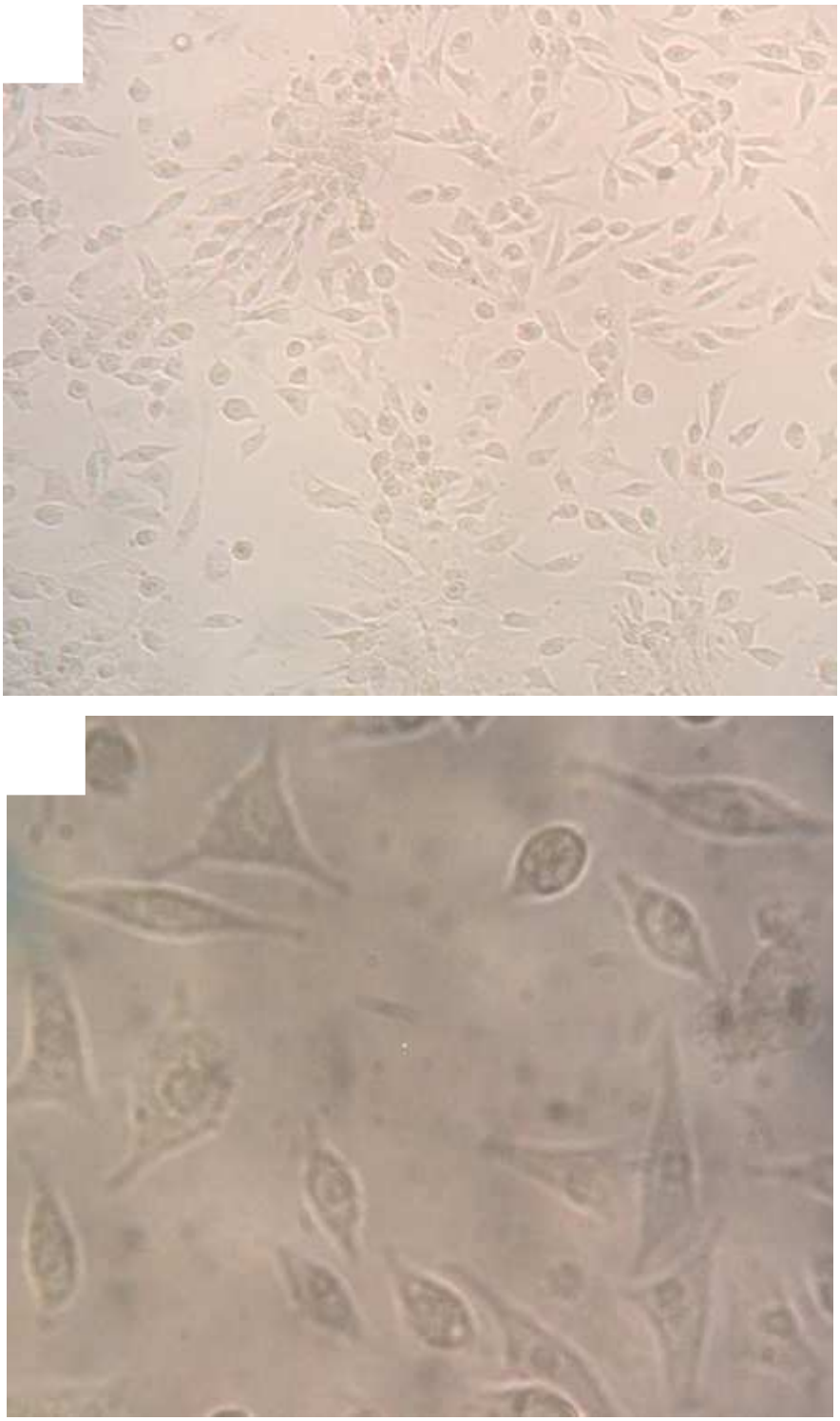

Figure (3.9):MSCs differentiation in osteogenic medium after 14 days (osteoblast like cells) as seen under inverted microscope A (10X) and B (40X).

Pittenger *et al.* (1999) reported that treatment with the osteogenic induction medium led to a morphological change of the MSCs from an elongated fibroblast-like cell type to shorter, cuboidal or polygonal cells, this agreement with results after 21 days, most of MSCs are transferred to osteoblasts as shown in figure (3.10).

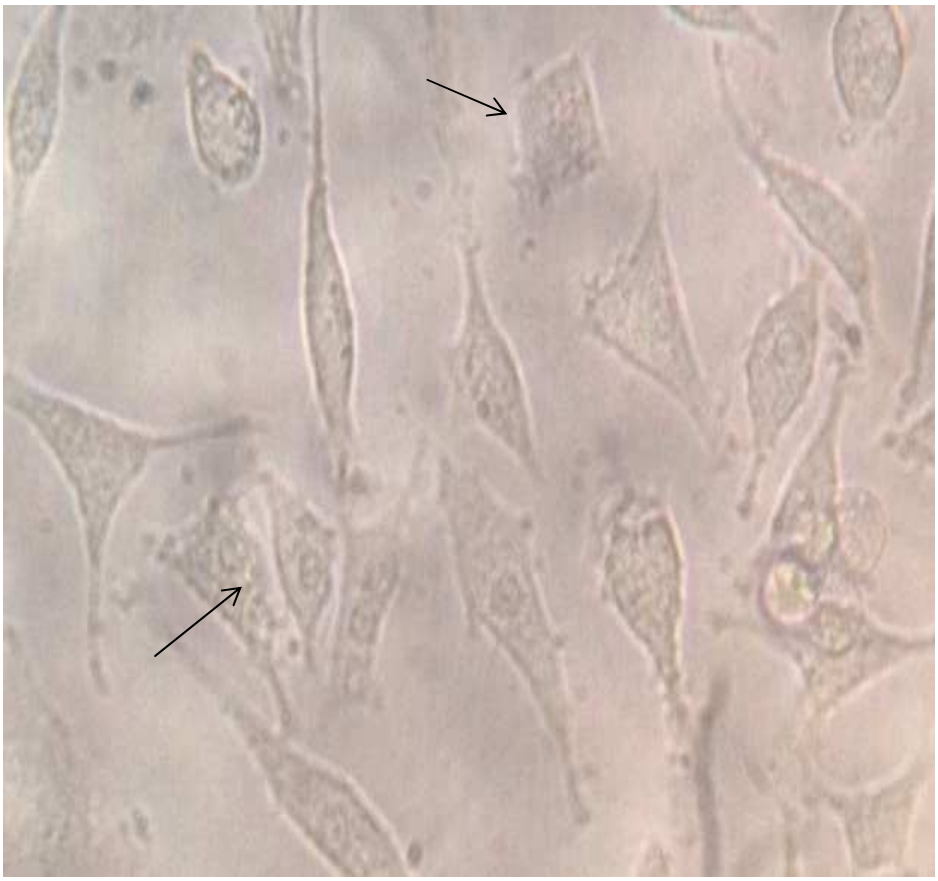

Figure (3.10): MSCs differentiation in osteogenic medium after 21 days (osteoblast) as seen under inverted microscope (40X) (Arrows).

Bonewald (2011) reported that some populations of osteoblasts become osteocytes when they are trapped in the bone matrix that they produced. Osteogenic lineage cells are a population of cells that include mesenchymal progenitors, preosteoblasts, osteoblasts, osteocytes, and bone- lining cells. Others can undergo apoptosis or become inactive bone-lining cells.

Osteocytes are important in responding to mechanical and hormonal signals, playing major roles in bone remodeling (Chen *et al.* 2010).

3.4. Alkaline phosphatase activity (ALP):

Results revealed that a significant increase in ALP activity in osteogenic medium at 7 and 14 days of differentiation in comparison with zero day (32.13±0.46 and 23.33±0.88 *vs*. 5.33±1.76 IU/L) and decreased at 21 day (15.33±1.76 IU/L) as in figure (3.11).

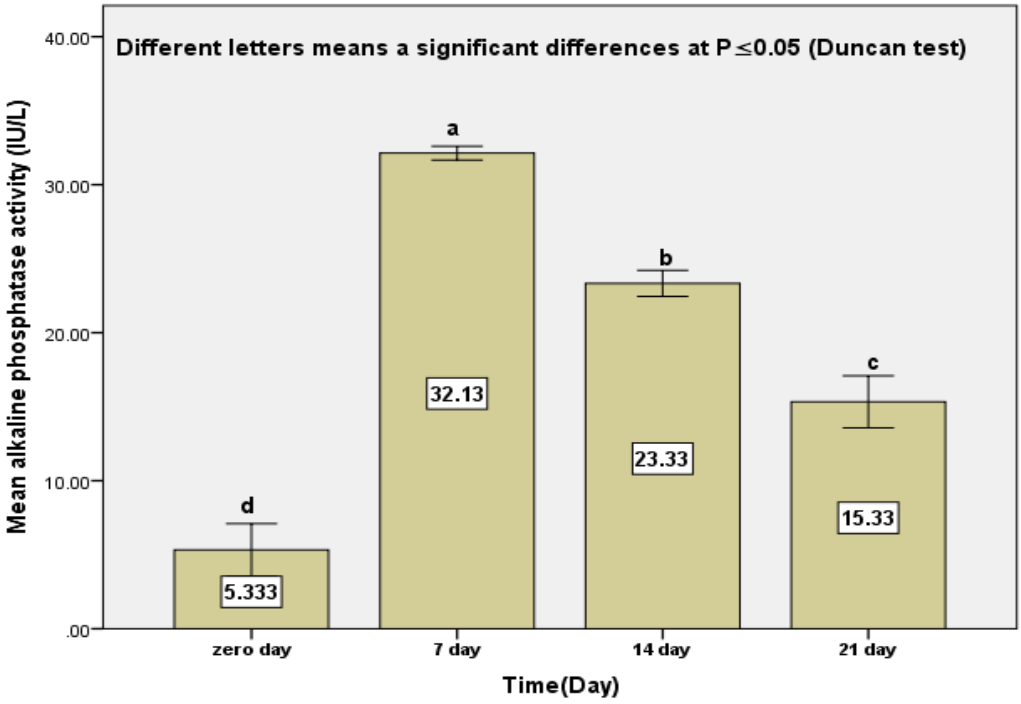

Figure (3.11): Alkaline phosphatase activity (IU/L) in osteogenic medium at different Differentiation period.

This result pointed to cells transform to osteoblast cells because each constituent is found to support in osteogenic differentiation *in vitro.* The presence of dexamethasone in combination with ascorbic acid and ß-glycerophosphate resulted in significantly increased cell growth and ALP activity (Coelho and Fernandes, 2000). Increase in ALP activity is a marker of MSCs differentiation into osteoblasts as expression of ALP is less in MSCs as compared to mature osteoblasts (Karsenty and Wagner, 2002).

The osteoblast cells produce ALP during normal osteogenic differentiation, but when exposure to oxidative stress normally secrets cytokines, these cytokines induce the activity of osteoclast that secretes acid and enzyme

protease which causes lysis of collagen and ALP production followed by a subsequent decrease as the cells mature and lay down minerals (Aubin, 2001; Huang *et al.,* 2007; Thibault *et al.,* 2010).

3.5 Identification of Beta-actin and osteocalcin in osteoblast using by PCR:

Gel electrophoresis showed clear two bands at the expected sizes of 200 bp for Beta-actin and 169 bp for osteocalcin , there was increasing band intensity for both genes, these result demonstrated that the differentiated osteoblasts from MSCs have the characteristics of normal osteoblasts and that can be used as the *in vitro* model of osteogensis as in figure (3.12).

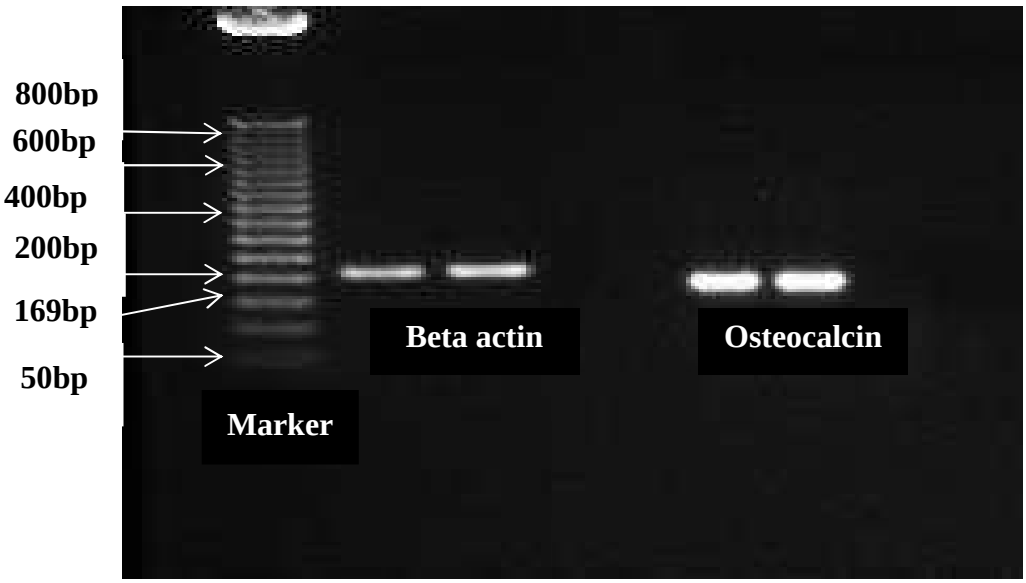

Figure (3.12): Gel electrophoresis of beta-actin and osteocalcin of differentiated osteogenic cells. Bands were fractionated by electrophoresis on 2% agarose (30 min, 5 V/cm) and visualized under UV light after staining with ethidium bromide dye. Lane M (50 bp/ladder).

PCR Reaction was conducted for osteocalcin and beta-actin to confirm the osteoblastic phenotype of the cells. Since beta-actin and osteocalcin could be detected by PCR, mRNA could be sufficiently isolated and reverse transcribed to cDNA for analysis with PCR methods, Osteocalcin and Beta-actin genes expression, an osteoblast marker gradually increased in osteogenic

differentiation during proliferation, and between days14–21, during mineralization. Osteocalcin was described as a late marker of developing osteoblasts appearing with matrix mineralization (Aubin, 1998) and was maximally expressed on day 21, and beta actin is produced by all cells, It is an isoform of actin and one of two non cytoskeletal actins, which is involved in cell movement and structural integrity. Extracellular matrix proteins play an important role in the organization, architecture and differentiated function of bone (Boudreaux and Towler, 1996; Ganss *et al.*, 1999).

Chapter four

Conclusions

and

Recommendations

4 Conclusions and Recommendations

4.1 Conclusions:

1. Bone marrow derived mesenchymal stem cells had osteogenic potential and could be a suitable option for cell-based tissue engineering therapies.
2. Osteogenic medium of dexamethasone, beta-glycerophophate and ascorbic acid induced MSCs osteogenesis *in vitro* after 21 day.
3. The alkaline phosphatase activity is a specific for osteogenic cell in osteogenic medium was found to reach a peak at 7 day of differentiation.
4. The presence of beta-actin and osteocalcin genes was confirmed by RT-PCR in differentiated osteogenic cells on day 14.

4.2 Recommendations:

1. Studying the possible role for the use of co- culture or conditioned media methodologies for tissue engineering applications.
2. Investigation the osteogenic potential of human bone marrow derived mesenchymal stem cells.
3. Studying the osteogenic capacity of new source of cells.
4. Using matrix 3D (scaffolds) to create environment for three dimensional tissue engineering therapies.
5. Studying the therapeutic role of MSCs and osteoblast for bone fracture *in vivo.*

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الملخص

صممت الدراسة لتقييم إمكانية تحول الخلايا الجذعية المزنشيمية المعزولة من نخاع عظم الفأر الى خلايا مولدة لخلايا العظم في المختبر. عزلت الخلايا من عظم الفخذ ل 50 فأر على أساس قدرة التصاقها بالسطوح البلاستيكية.

اختبر الخلايا الجذعية المزنشيمية للمعلما ل CD105 الخاص بالخلايا الجذعية المزنشيمية و CD34 الخاص بالخلايا الجذعية المولدة لخلايا الدم واسطة كيمياء الخلوية المناعية. أظهر الخلايا الجذعية المزنشيمية المعزولة تفاعلا ايجابيا ل CD105 وسلبيا للمعلم على سطح الخلايا الجذعية المولدة لخلايا الدم CD34.

حفز الخلايا الجذعية المزنشيمية المعزولة على التمايز لخلايا مولدة لخلايا العظم واسطة وسط كو يتألف من Dulbecco's Modified Eagle's Medium(DMEM) مع 50 ميكروغرام / مل حمض الأسكوربيك، 1 نانو مولاري ديكساميثازون و 10 ملي مولاري بيتا كليسروفوسفيت هيدرات الصوديوم بعد 21 يوما.

تم قياس فعالية الخلايا المولدة لخلايا العظم عن طريق تقييم فعالية انزيم الفوسفاتيز القاعدي (ALP) في الوسط المكون لخلايا العظم واسطة Reflotron بعد فترات مختلفة من التمايز (0، 7، 14، 21) يوما. سجلت النتائج زيادة معنوية $P \leq 0.05$ في فعالية ALP في وسط التمايز في الايام 7 و 14 مقارنة مع اليوم صفر (32.13 ± 0.46 و 23.33 ± 0.88 مقابل 5.33 ± 1.76 وحدة دولية / لتر) وانخفضت في اليوم 21 (15.33±1.76 وحدة دولية/لتر.

تم التحقق من وجود جينات بيتا الأكتين وأوستيوكالسين في الخلايا المتمايزة من قبل (RT-PCR) بعد 14 يوما من التمايز. اكدت النتائج ظهور حزمتين بحجم الجزيئي (200) زوج قاعدي لبيتا الأكتين و (169) زوج قاعدي لأوستيوكالسين.

فَتَعَالَى اللَّهُ الْمَلِكُ الْحَقُّ وَلَا تَعْجَلْ بِالْقُرْآنِ مِن قَبْلِ أَن يُقْضَى إِلَيْكَ وَحْيُهُ وَقُل رَّبِّ زِدْنِي عِلْمًا

صدق الله العظيم

سورة طه الاية "١١٤"

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

تكوين الخلايا بانية العظم من الخلايا الجذعية المزنشيمية المأخوذة من نخاع عظم الفأر في الزجاج

رسالة

مقدمة الى مجلس كلية العلوم/جامعة النهرين

كجزء من متطلبات نيل درجة الماجستير علوم/تقانة احيائية

من قبل

سالي طارق يونس

بكلوريو علوم/تقانة احيائية/كلية العلوم/ جامعة النهرين,2012

باشراف

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أذار 2015