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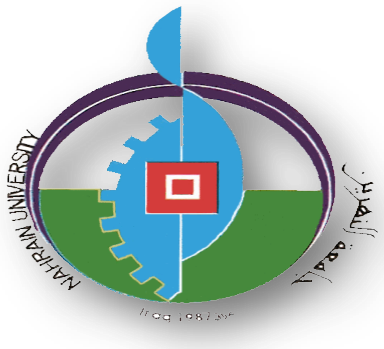

Transplantation of Mesenchymal Stem Cells into Experimental Injured Mice Liver

A Dissertation

Submitted to the Council of Science College, University of Al-Nahrain, In Partial Fulfillment of the Requirements for the Degree of Doctorate in Philosophy/ Biotechnology.

By

Zahraa Kamel Zedan

B.SC. Biotechnology, College of Science, University of Al-Nahrain, 2005.

M.SC. Biotechnology, College of Science, University of Al-Nahrain, 2008.

Supervised by

Dr.Shahlaa M. Salih
Assistant Professor

Dr. Shalal M. Hussien
Professor

May 2015 **Shawal 1436**

Dedication

To: who teaches me the means of personal strength the one who supports and encourage me throughout the study… my dear teacher and Sister **Dr. Shahlaa M. Salih** Allah May bless and guard her…

TO: who raise me to become a strong intelligent girl with their prayers to Allah for me… **(My father and mother).**

To: the one who supports me and gives me the strength to proceed my beloved husband.... (**Osama**)**.**

To: Who ever stands by me in the life troubles and back me at every time… my beloved sisters **(Sabreen, Aseel and Hadeel)**and brother **(Mohammed).**

Finally not last to my beloved sons…

****Hasan and Fahad****…

May Allah bless and guard them all

Zahraa

Supervisors Certification

We, certify that this dissertation entitled **''Transplantation of mesenchymal stem cells into experimental injured mice liver''** was prepared by the student **''Zahraa Kamel Zedan''** under our supervision at the Biotechnology Department/ College of Science/ Al-Nahrain University as a partial fulfillment of the requirements for the degree of **Doctor of philosophy in Biotechnology.**

Signature:
Supervisor: **Dr. Shahlaa M. Salih**
Scientific degree: Assistant Professor.
Date: / / 2015

Signature:
Supervisor: **Dr. Shalal M. Hussien**
Scientific degree: Professor.
Date: / / 2015

In view of available recommendations, I forward this dissertation for debate by the examining committee.

Name**: Dr. Hameed M. Jasim**
Scientific degree: Professor.
Title: Head of Biotechnology Department
Date: / / 2015

Committee Certification

We, the examining committee certify that we have read this dissertation entitled **''Transplantation of Mesenchymal Stem Cells into Experimental Injured Mice Liver''** and examined the student **'' Zahraa Kamel Zedan''** in its contents and that in our opinion is accepted as a dissertation for the degree of **Doctor of philosophy in Biotechnology.**

Signature:
Name: **Dr. Waleed H. Yousif**
Scientific degree: Professor
Date: / / 2015
(Chairman)

Signature:
Name: **Dr. Salim R. Hamoudi**
Scientific degree: Professor
Date: / /2015
(Member)

Signature:
Name: **Dr. Rajwa H. Essa**
Scientific degree: Professor
Date: / / 2015
(Member)

Signature:
Name: **Dr. Jebar H. Yansel**
Scientific degree: Assistant Professor
Date: / / 2015
(Member)

Signature:
Name: **Dr. Omer F. Abdul-Rasheed**
Scientific degree: Assistant Professor
Date: / / 2015
(Member)

Signature:
Name: **Dr. Shahlaa M. Salih**
Scientific degree:
Date: / / 2015
(Member/ Supervisor)

Signature:
Name: **Dr. Shalal M. Hussien**
Scientific degree:
Date: / / 2015
(Member/ Supervisor)

I, hereby certify the decision of the examining committee.

Signature:
Name: **Dr. Hadi M.A. Abood**
Scientific Degree: Professor
Title: Dean of College of Science:
Date: / /2015

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Summary

This study was designed to investigate the hepatogenic potential of bone marrow derived mesenchymal stem cells (BM-MSCs) and to evaluate the ability of differentiated hepatocytes to repair surgically induced liver damaged mice. In the first experimental work; The mesenchymal stem cells were isolated from the bone marrow of the thigh bones of 4-8 weeks old male mice based on the ability of adherence to plastic surfaces. The reactivity of isolated mesenchymal stem cells was tested immunocytochemically for MCSs markers. Results showed a positive reactivity towards the MSC markers (CD 105 and CD 90), and a negative reactivity towards the hematopoietic stem cells markers (CD 34 and CD45). Differentiation of BM-MSCs to hepatocytes was induced by adding different growth factors including hepatocyte growth factor (HGF), fibroblast growth factor-4 (FGF4), oncostatin-M and dexamethasone to Minimal Essential Medium (MEM tissue culture medium) supplemented with 10% of fetal bovine serum. Results revealed that the differentiation medium used was very efficient in directing the BM-MSCs to the hepatocytes, which immunocytochemically showed positive reactivity to specific hepatocyte markers including albumin (ALB) and alpha-fetoprotien (AFP) as well as to cytochrome oxidase p450 (CYP3A4). The quantitative Elisa assay of CYP3A4 exhibited a significant increase in enzyme levels during the three weeks of differentiation. The level was significantly higher after 21 days of differentiation (65.2$^{\vee}$ng/ml) in comparison with the first and second weeks of differentiation (16.73 and 33.00 ng/ml) respectively.

In the second experimental work; the ability of the differentiated hepatocytes to be used as a cellular therapy was studied after induction of subtotal hepatectomy surgical operation in mice which were subjected to a severe injury after removing of most of the liver parts of. Mice were divided into four groups as follows: Group A contained healthy mice served as control group. Group B contained induced liver damage mice. Group C contained mice with induced liver damage and transplanted with 0.7ml of

5×10^5 of differentiated hepatocytes by intrasplenic injection, Group D contained mice with induced liver damage and transplanted with 0.7 ml of 5×10^5 of BM-MSCs by intrasplenic injection. After 10 days of transplantation, results of the liver/ body weight ratio of each mice group showed significant differences as compared to the control group (0.26 g). Mice transplanted with hepatocytes and BM-MSCs showed a significant increase in liver/ body weight ratio (0.20 and 0.19) g respectively in comparison with liver damaged mice (0.17g). Serum Alanine Amino Transferase (ALT), Aspartate Amino Transferase (AST) and Alkaline Phosphatase (ALP) showed a significant increase hepatectomized mice (403, 226 and 36.21 IU/L) respectively in comparison with the healthy control (218, 125 and 16 IU/L) respectively Mice transplanted with differentiated hepatocytes showed a significant improvement in liver function enzymes (ALT, AST and ALP), (263, 146 and 17 IU/L) respectively in comparison with mice transplanted with bone marrow mesenchymal stem cells (280, 183 and 22 IU/L) respectively. Histological sections of liver in mice with damaged liver showed a severe injury in liver tissue characterized by inflammatory and cellular infiltration as well as cytoplasmic vacuolation and degeneration of hepatocytes. Liver sections of mice treated with hepatocytes revealed regeneration of the damaged liver represented by healing of cellular infiltration and reduction of tissue damage. liver sections of mice treated with BM-MSCs showed slight regeneration with a fewer lesions. In conclusion, differentiated hepatocytes were successfully able to repair the liver damage resulting from surgical liver injury in addition to the improvement of serum level of liver function enzymes which were more effective than bone marrow mesenchymal stem cells used for the same goal.

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IPS	Induced pluripotent stem cells
Es	Embryonic stem cells
FGF	Fibroblast growth factor
TGF	Transforming growth factor
IGF	Insulin-Like growth factor
LIF	Leukemic inhibitor factor
EGFR	Epidermis growth factor receptor
EPCs	Endothelial progenitor cells
HPCs	Hepatic progenitor cells
OCs	Oval cells
FAH	Fumaryl acetate hydrolase
UCB	Umbilical cord blood
NASH	Non-alcoholic steatohepatitis
HBV	Hepatitis B virus
VEGF	Vascular endothelial growth factor
MSC	Mesenchymal stem cells
BMP	Bone morphogenetic protein
NaOH	Sodium Hydroxide
ALb	Albumin
AFP	Alpha Feto Protein
CYP3A4	Cytocrome Oxidase p450
PBS	Phosphate Buffer Saline
HGF	Hepatocyte Growth factor
FGF4	Fibroblast growth factor 4
ONCM	Oncostatin M
HCC	Hepato cellular carcinoma
MEM	Minimal Essential Media
HSCs	Hematopoitic stem cells
BM-MSCs	Bone Marrow Derived Mesenchymal Stem cells.
BMT	Bone Marrow Transplantation
PBSCT	peripheral blood stem cell transplantation
ISCT	The International Society of cell therapy
HLA	Human Leukocytes Antigen
BMP	Bone morphogenetic protein
EGF	Epidermal cell growth factor

bFGF	Basic fibroblast growth factor
IL6	Interleukin 6
HRP	Horse radish peroxidase
Dexa	Dexamethasone
NE	Nor epinephrine
PKc	protein kinase C
MAPCs	Multipotent adult progenitor cells
ALT	Alanine Amino-Transferase
AST	Aspartate Amino-Transferase
ALP	Alkaline Phosphatase

Chapter One

Introduction and Literature review

Introduction

1.1 Introduction:

The growing problem of liver disease in Worldwide, due to infections, autoimmune disease, toxins and metabolic diseases are a common cause of death and morbidity. Whatever the aetiological cause of chronic liver disease, liver injury usually results in a form of excess scarring termed liver cirrhosis where the livers synthetic and metabolic function is compromised and there is also an increased risk of developing liver cancer. Within the world growing countries like the United Kingdom, for example, liver disease is the 5th commonest cause of death, and the number of deaths occurring as a result of liver cirrhosis is rapidly rising (Stuart *et al*., 2010).

End stage liver diseases, in particular liver cirrhosis, represent a worldwide health problem. In addition to patients regularly undergo partial hepatectomy to treat benign and malignant hepatic tumors. In most cases, liver undergoes hyperplasia until the normal hepatic mass is re-established (Hadjis, and Blumgart, 2000).

Currently, liver transplantation is the only effective treatment, but it is associated with many problems, including a shortage of donors, operative damage, risk of immune rejection and high costs. Furthermore, liver transplantation comes with considerable long term side effects, such as chronic renal failure, post-transplantation lympho proliferative disorder and cardiovascular complications (Chung *et al*., 2007).

The emerging field of stem cell therapy has raised great hope for improving the treatment of liver diseases, because it has the potential to promote liver repair and regeneration with fewer complications (Tamsel *et al*., 2007).

Stem cells are capable of self-renewal and can differentiate into specialized cell types. They can be classified as embryonic stem (ES) and adult stem cells (Cheng, 2008). The natural repair of liver is mainly dependent on endogenous stem/progenitor cell pools, including hepatocytes, hepatic progenitor cells (HPCs) and oval cells (OCs) (Fausto and Campbell, 2011; Faris *et al* ., 2011).

Sources of exogenous stem/progenitor cells that are currently under investigation in the context of repair of liver injury include ES cells, BM (bone marrow)- Mesenchymal stem cells (MSC) which had been received widespread attention because of their potential use in tissue engineering applications (Ooi,*et al*., 2013).

Mesenchymal stem cells are defined as non-hematopoietic having the capacity of self-renewable, multipotent progenitor cells with potentials to differentiate into several distinct cell lineages (Fuchs and Segre, 2011). MSCs are able to replicate for a long time while maintaining their multilineage differentiation potential. These cells were first recognized with the capacity to generate three cell lineages of osteoblastic, chondroblastic and adipocytic lineages (Woodbury *et al*., 2000).

Many research studies have demonstrated that MSCs may possess more extensive differentiation potentials than expected. These cells are able to differentiate into many other specialized phenotypes other than the skeletal lineages (osteocytes, chondrocytes and adipocytes) including neural cell, pancreatic cell, cardiomyocyte, renal epithelial cell, intestinal cell, hepatocytes and keratinocyte, by using the right type and formulation of growth factors which is specific for directing the MSCs towards the goal cells types (Woodbury *et al*., 2000; Campagnoli *et al*., 2003).

Bone marrow derived mesenchymal stem cells were first isolated using their plastic adherent properties and till now this property is utilized as current method for MSCs isolation from variety of species including human, mouse, rat, by many researchers like (Colter *et al*., 2000; Eslaminejad *et al*., 2006) .

Most liver diseases lead to hepatocyte dysfunction with the possibility of eventual organ failure. The replacement of diseased hepatocytes and the stimulation of endogenous or exogenous regeneration by stem cells are the main aims of liver-directed cell therapy. There is growing evidence to suggest that reservoirs of stem cells may reside in several types of adult tissue, these cells may retain the potential to transdifferentiate from one phenotype to another, presenting exciting possibilities for cellular therapies (David and Hay, 2012).

The field of regenerative medicine opens a wide of new and efficient treatment for liver in acute liver injury cases, thus using stem cells as a new model tool for liver repair process. The regenerative capacity of the liver has been recognized for centuries, but when it is overwhelmed by insulting stimuli or is chronically damaged, its regenerative capability is substantially reduced or lost. Researchers have been working to find solutions to cure failing human liver function. Given the ability of stem cells to self- renew and differentiate into specialized cell liver types, they represent an attractive strategy to replace lost liver function (David and Hay, 2012).

Aims of Study

- Investigation of the ability of mouse bone marrow derive mesenchymal stem cells (BM-MSCs) to differentiate *in vitro* into hepatocyte by:
  1. Isolation and identification of mesenchymal stem cells from mouse bone marrow.
  2. Induction of BM-MSCs differentiation into hepatocytes by using hepatogenic medium.
- Evaluation of the ability of differentiated hepatocytes to repair liver damage in mice by transplantation of hepatocyte and mesenchymal stem cells and assessment of:
  - Liver enzyme function (Alanine Amino-Transferase (ALT), Aspartate Amino-Transferase (AST) and Alkaline Phosphatase (ALP) activity.
  - Histological changes in liver sections before and after transplantation.

2. Literature review

2.1: Liver Microanatomy and Zonation of Hepatic histological features:

The liver is a large, red-brown, multi-lobed, vascular organ that takes up the entire rostral right of abdomen, and consists of a continuous parenchymal mass covered by a capsule. The liver is almost entirely comprised of hepatic cells, which secrete bile into the small intestine directly through the hepatic duct (rodents have no gall bladder), store sugars as glycogen, manufacture certain blood proteins, and remove toxins from systemic circulation (Chung *et al*., 2007).

The liver is structurally organized into polygon-shaped lobules surrounded by connective tissue. This lobular organization is most easily appreciated in sections of liver. The center of the lobule is the central vein with cords of hepatocytes and the intervening sinusoids extend radially between the central vein and the periphery of the lobule (Figure 1-1) (Francoz *et al*., 2007).

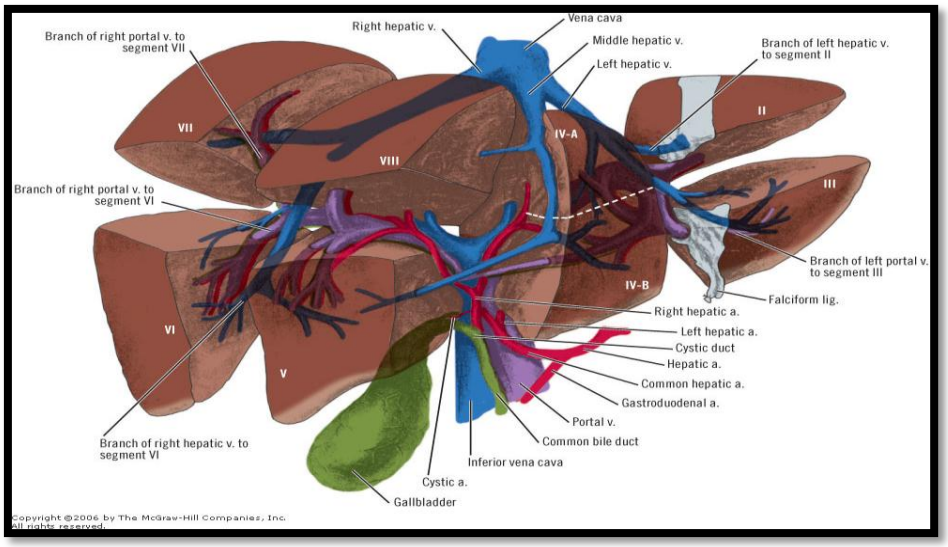


Figure (1-1): Anatomy of liver organ (Francoz *et al*., 2007).

The liver consists of a vast interanastomosing network of hepatocytes arranged in single-cell thick plates separated by vascular sinusoids (figure 1-2). The hepatocytes along with vascular channels form organized micro structures which serve as structural and functional units. In the classic terminology, the liver is composed of innumerable lobules, each of which is a hexagonal structure consisting of a central vein surrounded by radiating hepatocyte plates. However, another concept of a functional unit defines an acinus as the functional unit in relation to terminal portal branches and terminal hepatic venule. Portal tracts surround the classical lobules. An interlobular portal vein is also shown Portal triads consisting of a portal vein, a hepatic artery and bile ducts are found at the apices of the lobule (Gray and Lewis, 2000).

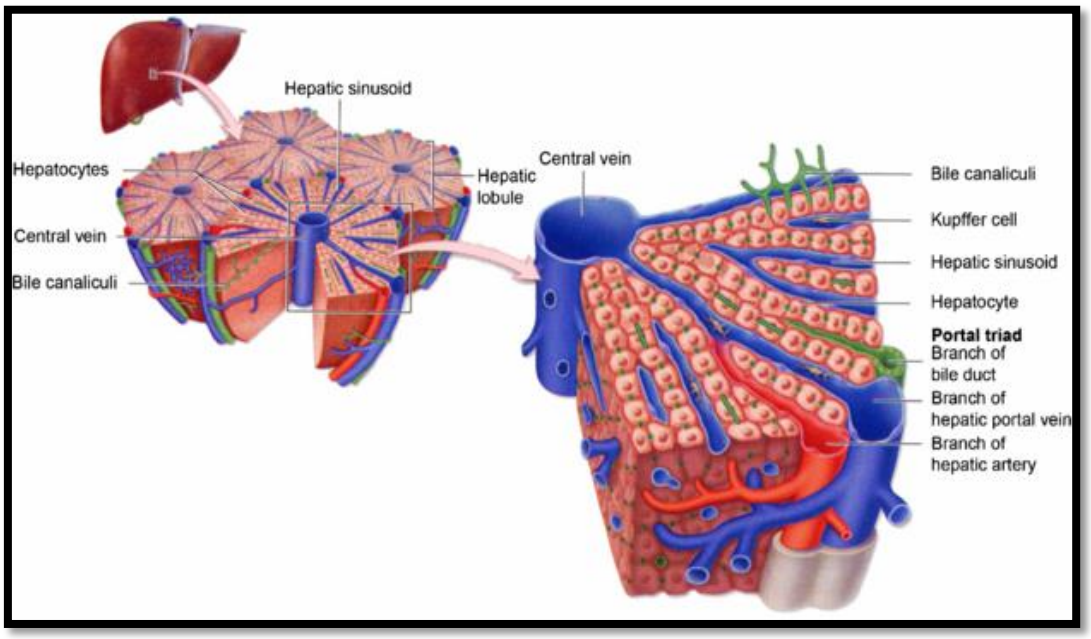


Figure (1-2): The histological features of liver tissue (Gray and Lewis, 2000).

2.2 Liver diseases and possible functional impairments:

The liver is the vital metabolic organ that regulates the body's energy supply, secretes several essential compounds and clears toxic substances by several methods, including recycling, inactivation and excretion. Chronic viral hepatitis

and alcohol abuse are the main causes of liver cirrhosis (Grant *et al.,* 2010). Hepatitis B (HBV) virus infection and its residual effects are now recognized as serious global problems. Worldwide, over 350 million people are chronically infected with HBV and 15–25% of them are at risk of developing and dying from HBV-related chronic liver disease, including cirrhosis and HCC hepatocellular carcinoma (HCC) as reported by(Kane, 2010).

Generally most of the estimated 278000 HBV-related deaths in the Western Pacific Region each year, over 90% are due to chronic infection acquired decades earlier at birth or during early childhood (Clements *et al*., 2006). In addition, other areas with high risk of HBV-related chronic liver disease include sub-Saharan Africa, South and East Asia, the Eastern Mediterranean, the Pacific Islands, the Amazon interior and parts of the Caribbean (Hsu and Murray, 2008). In China, approximately 120 million people are carriers of HBV and 30 million people are chronically infected. During a 5-year period, 10–20% of patients with chronic hepatitis will develop cirrhosis, and 20–30% of the cases with compensated cirrhosis progress to decompensated cirrhosis. Most of them with cirrhosis and chronic hepatitis, 6–15% progress to HCC. Although the 5-year survival rate for compensated cirrhosis is 55%, it is 14% for decompensated cirrhosis and less than 5% for HCC (Liu and Fan, 2007).

The prospective study by Chen *et al* (2013) estimated that 10–15% of the adults infected with HCV will develop cirrhosis. Moreover, in the chronic alcohol consumption is the leading cause of liver fibrosis. Generally, there is a significant increase of death from liver chronic disease and cancer and in great percentage among younger adults due to unfavorable lifestyles, such as alcohol abuse (Leyland *et al*., 2007).

Many studies of alcohol-related liver diseases are making headway, but they have yet to produce safe and effective therapies for alcoholic hepatitis and reversing cirrhosis (Reuben, 2008). Hepatic fibrosis could also be induced by

autoimmune disorders, drugs, helminthic infection, iron or copper overload, biliary obstruction and mutagens, such as afla-toxins (Friedman, 2003). In addition, there is growing evidence that non-alcoholic steatohepatitis (NASH) is another important cause of fibrosis (Jansen, 2004).

If not effectively treated, fibrosis can progress to cirrhosis, ultimately leading to liver failure and possible death. The loss of the liver function results in metabolic instability and destruction of essential functions, such as the acid–base balance. If not reversed rapidly, many complications, such as uncontrolled bleeding occur, and dependent organs, such as the brain and kidneys, begin to fail, reducing the chance of recovery even further. At present, liver transplantation is often the only therapeutic option for patients with deteriorating liver function (William *et al.*, 2011).

Morbidity and mortality from cirrhosis is increasing rapidly in the world. Currently, orthotopic liver transplantation is the only definitive therapeutic option. However, its clinical use is limited, because of poor long-term graft survival donor organ, shortage and high costs associated with the procedure. Stem cell replacement strategies are therefore being investigated as an attractive alternative approach to liver repair and regeneration (Francoz *et al.*, 2007).

2.3 Hepatocytes and their identification markers:

The typical hepatocyte is cubical with sides of 20-30 µm. Hepatocytes display an eosinophilic cytoplasm, reflecting numerous mitochondria, and basophilic stippling due to large amounts of rough endoplasmic reticulum and free ribosomes. Brown lipofuscin granules are also observed (with increasing age) together with irregular unstained areas of cytoplasm; these correspond to cytoplasmic glycogen and lipid stores removed during histological preparation. The average life span of the hepatocyte is 5 months. Hepatocyte nuclei are round with dispersed chromatin and prominent nucleoli. Anisokaryosis (or

variation in the size of the nuclei) is common and often reflects tetraploidy and other degrees of polyploidy (Gumucio, 2000).

The hepatocyte is a cell in the body that manufactures serum albumin, fibrinogen, and the prothrombin group of clotting factors (except for Factors 3 and 4), It is the main site for the synthesis of lipoproteins, ceruloplasmin, transferrin, complement, and glycoproteins. Hepatocytes manufacture their own structural proteins and intracellular enzymes (Romanes, 2000).

Synthesis of proteins is by the rough endoplasmic reticulum (RER), and both the rough and smooth endoplasmic reticulum (SER) is involved in secretion of the proteins formed. The endoplasmic reticulum (ER) is involved in conjugation of proteins to lipid and carbohydrate moieties synthesized by, or modified within, the hepatocytes (Fausto, 2010).

Hepatocytes are organized into plates separated by vascular channels (sinusoids), an arrangement supported by a reticulin (collagen type III) network. The hepatocyte plates are one cell thick in mammals and two cells thick in the chicken. Sinusoids display a discontinuous, fenestrated endothelial cell lining. The endothelial cells have no basement membrane and are separated from the hepatocytes by the space of Disse, which drains lymph into the portal tract lymphatics (Grisham, 2005)

Kupffer cells are scattered between endothelial cells; they are part of the reticuloendothelial system. Stellate cells store vitamin A and produce extracellular matrix and collagen; they are also distributed amongst endothelial cells but are difficult to visualize by light microscopy. Hepatocytes have the ability to metabolize, detoxify, and inactivate exogenous compounds such as drugs, (drug metabolism), and insecticides, and endogenous compounds such as steroids (Gumucio, 2013).

Alpha-fetoprotein (AFP, α-fetoprotein; also sometimes called alpha-1-fetoprotein, alpha-fetoglobulin, or alpha fetal protein) is a glycoprotein of 591 amino acids and a carbohydrate moiety that is encoded by the AFP gene. AFP is a major plasma protein produced by the yolk sac and the liver during fetal development. It is thought to be the fetal form of serum albumin. AFP binds to copper, nickel, fatty acids and bilirubin and is found in monomeric, dimeric and trimeric forms (Jansen, 2004).

AFP is the most abundant plasma protein found in the human fetus. Plasma levels decrease rapidly after birth but begin decreasing prenatally starting at the end of the first trimester. Normal adult levels are usually achieved by the age of 8 to 12 months. The function of AFP in adult humans is unknown; however, in rodents it binds estradiol to prevent the transport of this hormone across the placenta to the fetus. The main function of this is to prevent the virilization of female fetuses. As human AFP does not bind estrogen, its function in humans is less clear (Grant *et al.,* 2000).

Cytochrome P450 3A4 (abbreviated CYP3A4) is an important enzyme in the body, mainly found in the liver and in the intestine. Its purpose is to oxidize small foreign organic molecules (xenobiotics), such as toxins or drugs, so that they can be removed from the body (Qiu *et al.,* 2010).

While many drugs are deactivated by CYP3A4, there are also some drugs which are activated by the enzyme. Some substances, such as grape fruit juice and some drugs, interfere with the action of CYP3A4. These substances will therefore either amplify or weaken the action of those drugs that are modified by CYP3A4 (Sevrioukova *et al.,* 2012).

The enzyme CYP3A4 is a member of the cytochrome P450 family of oxidizing enzymes. Several other members of this family are also involved in drug metabolism, but CYP3A4 is the most common and the most versatile one.

Like all members of this family, it is a hemoprotein, i.e. a protein containing a heme group with an iron atom. In humans, the CYP3A4 protein is encoded by the CYP3A4 gene, this gene is part of a cluster of cytochrome P450 genes on chromosome 7. (Hashimoto *et al.,* 2011).

Cytochrome P450 enzymes are heme-thiolate proteins that catalyze a wide variety of monooxygenation reactions including hydroxylation, epoxidation, and heteroatom dealkylations , Among 57 human P450s, the 3A4 isoform (CYP3A4) is one of the most abundant and important because, in addition to oxidation of various endogenous molecules and xenobiotics, it contributes to the clearance of over 50% of administered pharmaceuticals. The potential ability of CYP3A4 to oxidize molecules with widely diverse in size and chemical structure is due to its large and malleable active site of the detoxitative molecule (Kumar *et al.,* 2009). Another intriguing feature of CYP3A4 is the ability to accommodate more than one molecule in the substrate-binding pocket, where one molecule serves as a substrate while another acts as a modulator of substrate metabolism. Among the substrates that exhibit binding cooperativity with CYP3A4 are testosterone, progesterone, diazepam, α-naphthoflavone, and others (Meunier *et al.,* 2014).

2.4 Stem Cells: Definition, Plasticity، Heterogeneity and types:

Stem cells are unspecialized cells with the ability to renewal themselves for long periods without significant changes in their general properties. They can differentiate into various specialized cell types under certain physiological or experimental conditions (Aznar *et al.,* 2011).Stem cells are certain biological cells found in all multicellular organisms. They are in small portion in body mass, but can divide through mitosis to produce more stem cells. Different types of stem cells vary in their degree of plasticity, or developmental versatility.

Stem cells can be classified according to their plasticity and sources. (Table 1-1) (Vergano, 2010).

Table (1-1): Classification of stem cells according to source (Vergano, 2010).

Classification category: According to source	
Types	**Characteristics**
Embryonic stem cells	**Are pluripotent stem cells derived from the inner cell mass of the blastocyst, an early-stage embryo.**
Adult stem cells	**Include stem cells derived from the adults tissues, which had a multipotent potential to differentiates in to many different cell lineages, are mainly found in the bone marrow and adipose tissue and other tissues according to the type of their origin are:** **Mesodermal Origin: Hematopoietic SCs, Mesenchymal Stroma SCs,** **Endodermal Origin: Pulmonary Epithelial SCs ،Gastrointestinal Tract SCs, Pancreatic SCs, Hepatic Oval Cells, Mammary and Prostatic Gland SCs, Ovarian and Testicular SCs .** **Ectodermal Origin : Neural SCs, Skin SCs, Ocular SCs**
Cancer stem cells	**have been identified in almost all caner/tumor, such as Acute Myeloid leukemic SCs (CD34+/CD38-), Brain tumor SCs (CD133+), Breast cancer SCs (CD44+/CD24 , Multiple Myeloma SCs (CD138+), Colon cancer SCs (CD133+), Liver cancer SCs (CD133+), Pancreatic cancer SCs (CD44+/CD24+), Lung cancer SCs (CD133+), Ovary cancer SCs (CD44+/CD117+), Prostate cancer SCs) CD133+/CD44+)**

Cell therapy is a sub-type of regenerative medicine. Cell therapy based on stem cells describes the process of introducing stem cells into tissue to treat a disease with or without the addition of gene therapy. Hematopoietic stem cells

(HSCs) have been widely used for allogeneic cell therapy (Bianco *et al.*, 2008). The successful isolation of pluripotent embryonic stem (ES) cells from the inner cell mass of early embryos has provided a powerful tool for biological research. ES cells can give rise to almost all cell lineages and are the most promising cells for regenerative medicine (Blau *et al.*, 2011).

Table (2-1): Classification of stem cells according to cell potency. (Vergano, 2010).

Classification category: According to cell potency	
Types	**characteristics**
Induced pluripotent	**a type of pluripotent stem s artificially derived from a non-pluripotent cell, typically an adult somatic cell, by Inducing a "forced" expression of specific genes.**
Totipotent cells	**Zygote, Spore, Morula; It has the potential to give rise to any and all human cells, such as brain, liver, blood or heart cells. It can even give rise to an entire functional organism.**
Pluripotent cells	**Embryonic stem cell, Callus; They can give rise to all tissue types, but cannot give rise to an entire organism.**
Unipotent cells	**Precursor cell**
Multipotent cells	**Progenitor cell, such as hematopoietic stem cell and mesenchymal stem cell; They give rise to a limited range of cells within a tissue type.**

The ethical issues related to their isolation have promoted the development of induced pluripotent stem (iPS) cells, which share many properties with ES cells without ethical concerns. However, one key property of ES cells and iPS cells that may seriously compromise their utility is their potential for teratoma formation. (Steven *et al.*, 2012).

Due to the limitation of using ES and iPS cells in the clinic, great interest has developed in mesenchymal stem cells (MSCs), which are free of both ethical concerns and teratoma formation. These cells were first isolated and characterized by Friedenstein and his colleagues in 1974. MSCs, also called mesenchymal stromal cells, are a subset of non-hematopoietic adult stem cells that originate from the mesoderm. They possess self-renewal ability and multilineage differentiation into not only mesoderm lineages, such as chondrocytes, osteocytes and adipocytes, but also ectodermic cells.(Konno *et al.*, 2013).

2.5 Bone marrow derived mesenchymal stem cells (BM-MSCs):

Adult BM mainly comprises two populations of precursor cells, hematopoietic stem cells (HSCs) and marrow stromal cells (MSCs) (Lagasse, *et al*., 2000). HSC and MSC are both multipotent stem cells, HSCs are present in circulating blood and umbilical cord blood (UCB) and are able to sustain production of all blood cells throughout life. MSCs can be isolated from several other tissues, including adipose tissue, placenta, amniotic fluid, UCB and fetal tissues are able to differentiate into osteocytes, adipocytes, chondrocytes, smooth muscle cells and haematopoietic supportive stroma (Yagi, *et al*., 2010;Herzog, *et al*., 2013).

The BM is a convenient and rich source of stem cells. The usage of BM-derived stem cells might sidestep many obstacles, such as ethical concerns and risks of rejection. MSCs are a type of multipotent adult stem cells that can be

readily obtained from BM aspirates and expanded into large quantities *in vitro*. Furthermore, MSCs possess potent immunosuppressive activities. Therefore MSCs could be used in patients who are in need of immune modulation, as well as tissue repair, such as organ transplant recipients and patients with severe autoimmune diseases as well as BM-MSCs possess potential to differentiates in to a multiple cell lineages (figure 1-3) (Le Blanc *et al*., 2011).

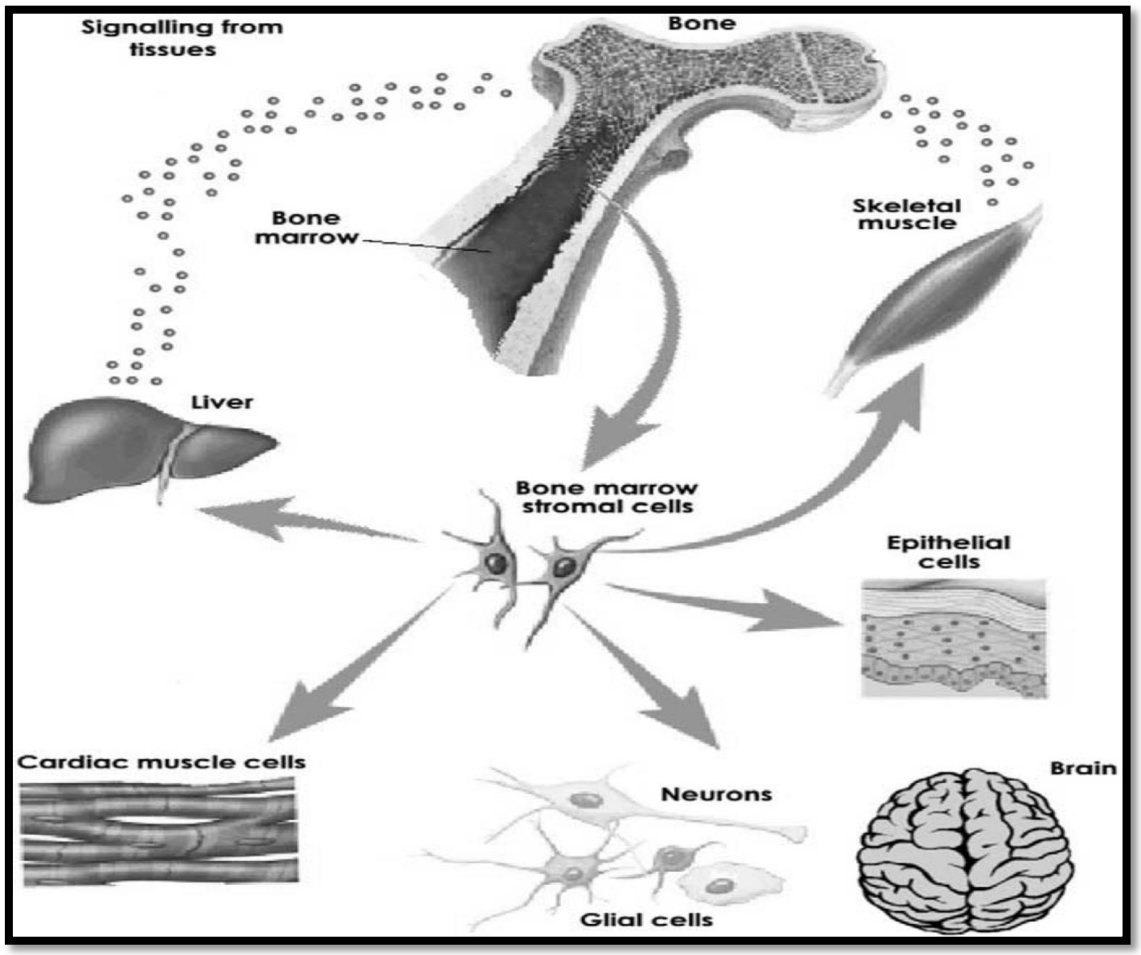


Figure (1-3): Potentials of bone marrow stromal cells to differentiate into a variety of cells lineages (Le Blanc *et al*., 2011).

Bone marrow derived MSCs are well defined by function and phenotype that they can be readily induced *in vitro* to differentiate into cells of the mesoderm lineage, such as adipocytes, chondrocytes and osteoblasts. Moreover, transplanted BM-MSCs can generate into multiple lineage cells, including

brain, lung, renal, gastrointestinal tract, skin and pancreatic β-cells (Imai and Ito, 2012; Jiang *et al*., 2012; Yen *et al*., 2013).

A series of investigations have been undertaken to study the hepatic differentiation capacity of BM-derived MSCs exist in almost all tissues. They can be isolated from the bone marrow, adipose tissue, the umbilical cord, fetal liver, muscle, and lung and can be successfully expanded *in vitro*. The number of clinical trials on MSCs has been rising since 2004. Although the "gold rush" to use MSCs in clinical settings began with high enthusiasm in many countries, with China, Europe and USA leading the way for numerous scientific issues remain to be resolved before the establishment of clinical standards and governmental regulations (Kern *et al.,* 2006). The definition of MSCs relies solely on the analysis of *in vitro* culture-expanded cell populations. Despite years of intense investigation, the location and role of the native MSCs within their tissue of origin *in vivo* are not known, mainly because of the lack of specific markers allowing their unambiguous identification (Banas *et al*., 2008; Jones and McGonagle, 2008; Morikawa *et al*., 2009).

Bone marrow transplantation (BMT) and peripheral blood stem cell transplantation (PBSCT) are the current clinical procedures to restore stem cells that have been destroyed by high doses of chemotherapy and/or radiation therapy. The isolation of a large number of potent HSC/MSC sets the basis of new methods for tissue regeneration and cell therapy (Korbling *et al.,* 2011). Nevertheless, the procedure of BM extraction is traumatic and the amount of material extracted is limited. Therefore, exploring new sources and isolation techniques for obtaining such cells is of great interest. Adult stem cells are any stem cells taken from mature tissue. Because of the stage of development of these cells, they have limited potential compared to the stem cells derived from the inner cell mass of the embryonic tissue (Ding, *et al*., 2011).

Most bone marrow derived stem cells are adult stem cells are multipotent and are generally. They play important roles on local tissue repair and regeneration. The application of adult stem cells in research and therapy is not as controversial as embryonic stem cells, because the production of adult stem cells does not require the destruction of an embryo. Additionally, because in some instances adult stem cells can be obtained from the intended recipient (an autograft), the risk of tissue rejection is essentially non-existent in these situations. Consequently, more USA government funding is being provided for adult stem cell research (US Department of Health and Human Services, 2004). (Pittenger *et al*., 2007; Dominici *et al*., 2006).

2.6 Identification Markers of Cultured MSCs:

Before the International Society of cell therapy (ISCT) had been published the positive and negative markers of MSCs, there was a minimal criteria for defining MSCs, variability in the methods used to isolate, expand, and characterize MSCs made it difficult to compare data from different labs. The Immunocytochemistry analysis of CD markers criteria offered a quality control step for researchers to verify MSC identity, which decreased experimental variability and increased the transparency of published data (Sun *et al.*, 2003).

The ISCT had published the positive and negative markers that enabled researchers to distinguish MSCs from other cells in the bone marrow compartment. The negative markers were selected to include surface antigens that are expressed by hematopoietic stem cells, while the positive markers were selected to include surface antigens that are absent from most hematopoietic cells (Table 3-1) (Halfon *et al.*,2011).

However, ISCT acknowledges that the criteria must be met with some flexibility, particularly as they relate to expression of the negative marker, HLA

Class II. Specifically, HLA Class II can be expressed by MSCs under certain conditions, such as cytokine stimulation (Dominici *et al.*, 2006).

Table 3-1: Negative and Positive MSC Markers as proposed by the International Society for Cell Therapy

Negative HSC Marker	Used to Exclude
CD34	**Primitive hematopoietic cells and endothelial cells**
CD45 , CD40	**Leukocytes**
CD11b and CD14	**Monocytes and macrophages**
CD79 alpha and CD19 alpha	**B cells**
HLA Class II	**Antigen presenting cells and lymphocytes**
Positive MSC Markers	**Biological Role**
CD73/5'-Nucleotidase	**Catalyzes production of extracellular adenosine from AMP**
CD90/Thy1	**Wound repair, cell-cell and cell-matrix interactions**
CD105/Endoglin	**Vascular homeostasis; modulates TGF-beta functions via interaction with TGF-beta RI and TGF-beta RII**

The use of markers to verify MSC identity serves as an important quality control step that can save significant time and reduce experimental variability. In an attempt to further decrease experimental variability, several labs have tried to increase the purity of isolated MSCs through positive and negative selection.

For example antibodies against CD 34 or other hematopoietic markers can be used to negatively select MSCs, while antibodies against markers such as CD90 can be used to positively select MSCs. The success of positive and negative

selection varies depending on the specificity of the marker, the starting cell population, and the isolation strategy (Wang *et al*., 2003).

Negative selection of cells is often preferred over positive selection since the isolated cells remain untouched by antibodies, beads, and magnets. In contrast, cells isolated by positive selection are labeled with antibodies and/or beads which may interfere with downstream applications and introduce experimental variability, either by preventing antibody binding or by stimulating signaling pathways.

MSCs can be negatively selected by using a cocktail of antibodies to proteins expressed by hematopoietic cells. This selection step removed endothelial cells and precursors, yielding a cell population that was 97% negative for the hematopoietic marker CD45 (Modder *et al.,* 2012).

The first definitive markers of MSCs were proposed in a pioneering study of Pittenger *et al*.,(2007) who also developed robust and reproducible *in vitro* assays of MSC multipotentiality towards bone, cartilage, and fat lineages. These BM MSC markers included SH2 and SH3، later shown to correspond to CD105 and CD73 molecules، The exact function of the CD90 (Thy1 antigen) is less well defined. It has been proposed to mediate cell-cell interactions involved in adhesion of monocytes and leukocytes to endothelial cells and fibroblasts and may have a role in the stromal adherence of CD34+ cells (Pittenger *et al.*, 2007). Cultured MSCs are uniformly and strongly positive for CD105, CD90, and CD73, regardless of their passage or time in culture , However, CD105 and CD73 are also expressed on skin fibroblasts cells with a much lower ability to proliferate and differentiate, compared to BM MSCs (Jones *et al* ,2004).

The fact that CD105, CD73, and CD90 are expressed at similar levels in early-passage and late-passage of MSCs cultured cells indicates that their value maybe limited only to basic MSC characterization. The limitation of these

markers is further demonstrated by the fact that although CD73 and CD105 are expressed on clonally derived MSCs, only 1/3 of these clones are truly multipotential this suggests that CD73 and CD105 expression may not be directly linked with MSC differentiation capacity. The knowledge of the phenotype and gene expression profile of BM MSCs in their original niche should undoubtedly help to develop new methodologies for expanding these MSCs and Testing of novel purified and expanded MSC based products in large animal models will allow through pre-clinical evaluation of novel products prior to clinicaltrials in humans (Quirici *et al*; 2010).

2.7 MSCs in liver regenerative medicine:

The idea of replacing diseased liver tissue by healthy hepatocytes is being a good idea, in order to provide the metabolic power, which gets lost during liver damage from any kind of challenge – viral, genetic and chemical intoxication (Muraca, 2011).

Many recent preclinical and clinical investigations that explore the therapeutic potential of stem cells in repair of liver injuries is involved the use of several types of stem cells, including embryonic stem cells, haematopoietic stem cells and mesenchymal stem cells, from the fact that each of these cells can be induced to differentiate into hepatocyte-like cells by defined culture conditions *in vitro* (Harn *et al.,* 2012).

Stem cell transplantation has been shown to significantly improve liver function and increase animal survival in experimentally-induced liver-injury models. Moreover, several pilot clinical studies have reported encouraging therapeutic effects in patients treated with stem cells. Although there remain many unresolved issues, the available data support the notion that stem cell technology may lead to the development of effective clinical modalities for human liver diseases (Patel *et al*., 2013).

The emerging field of stem cell therapy has raised great hope for improving the treatment of liver diseases, because it has the potential to promote liver repair and regeneration with fewer complications. The natural repair of liver is mainly dependent on endogenous stem/progenitor cell pools, including hepatocytes, HPCs (hepatic progenitor cells) and OCs (oval cells) which had been clinically showed a remarkable decrease in their regenerative ability in severe liver injury (Faris *et al*., 2011).

Sources of exogenous stem/progenitor cells that are currently under investigation in the context of repair of liver injury include ES cells, BM (bone marrow)- or fat-derived MSCs (mesenchymal stem cells), fetal stem cells and EPCs (endothelial progenitor cells) (Campard *et al*., 2008).

Under normal physiological conditions, mature hepatocytes are able to maintain their number. However, in persistent liver injury, resident HPCs or OCs generate hepatoblasts that differentiate into both hepatocytes and biliary cells may acts very slowly or inefficient enough to repair the damage. If the liver is exposed to retrorsine before partial hepatectomy, liver mass is restored by the emergence and expansion of both HPCs and OCs (Faris *et al*., 2011).

In addition, BM-derived stem cells (BM-SC) may represent another important source of hepatocytes. The hepatocytes restore the liver mass by self-proliferation. The best example of the capacity of adult hepatocytes and bile epithelial cells to proliferate is seen after (partial hepatectomy) in rats and mice. In addition, experiments of hepatocyte transplantation in urokinase plasminogen activator transgenic mice have shown that liver re-population by transplanted hepatocytes involves at least 12 rounds of replication (Rhim *et al*., 2014).

To determine the true regenerative potential of re-populating hepatocytes, Overturf *et al*. (2010) performed serial transplantation of a limited number of unfractionated adult parenchymal hepatocytes into fumaryl acetoacetate

hydrolase (FAH) deficient mice (a model of the metabolic liver disease tyrosinaemia type I). The results testified that hepatocytes could divide without loss of function at least 69 times (Overturf *et al*., 2010).

One of the hypothesis of liver regenerative mechanism was showed by Alison *et al*. (2004) includes that oval cells of the liver appear as a rich population of small round cells spreading from the periportal area into the parenchyma acts along with the hepatocytes when the later are unable to mount a proliferative response to severe liver injury.

Usually, cells are administered to the liver either via injection into the spleen or via the portal vein. Cells then spread with the blood stream over the entire organ and enter the parenchyma after endothelial penetration. They integrate and proliferate and ideally take over the hepatocytes' metabolic functions in the long-term range. (Hughes *et al.*, 2012).

Hepatocyte transplantation has also gained proof-of-concept in clinical trials, which is documented by more than 30 ongoing or published studies. One problem seriously hampers clinical breakthrough of hepatocyte transplantation. There are 30% more patients on the waiting list for liver transplantation than actually receive the life-saving organ both in the United States and in Europe indicating the scarcity of donor livers (Muraca, 2011).

Factors like site of application, mode of action, principles of tissue integration, and finally therapeutic support in the short- and long-term range are the main factors considerable in hepatocytes clinical application. The approach sounds rather straight forward and therefore it is worthy to talk about alternatives, which would aid to provide surgical potential in order to manage the bottleneck of donor liver availability both for organ and hepatocyte transplantation (Giuseppe and Marie, 2013; Hughes *et al.*, 2012).

2.8 Cytokines and growth factors involved in the hepatogenic differentiation:

Induction of cellular differentiation *In vitro*, under the circumstance of being induced by external cytokines such as hepatocyte growth factor (HGF), epidermal cell growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGF), insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF), Oncostatin M (OSM), leukemia inhibitory factory (LIF) and bone morphogenetic protein (BMP), MSCs could be differentiated into hepatocyte like cells (Greenbaum *et al.*,2005).

Since various kinds of cytokines and chemical compounds have shown certain effects on differentiation of mesenchymal stem cells, certain mechanisms of cytokines and combinations of protocols of cytokines have been well explored, while others are remain unclear (Gordon, 2000).

Hepatocyte growth factor (HGF) serves as a starting signal of liver regeneration, thus this cytokine is found to be involved with the endoderm development in the process of embryonic development. It is a potent mitogen for hepatocyte cloning, and has been considered as a pleiotropic cytokine for the mesenchymal origin (Elkasaby *et al.*, 2011).

C-Met is receptor of regulating growth of liver, it is a transmembrane protein, with an intracellular tyrosine kinase domain, and it has stimulating effect in the processes of mitosis and remodeling. When short term exposure to HGF, MSCs can induce its cognate receptor activation of ERK1 / 2, p38, MAPK and PI3K/Akt, while long-term exposure to HGF, MSCs will lead to the cytoskeleton, cell migration, and significantly inhibited in G1-S restriction point multiplication, Which suggest that HGF may play a different role according to cell's proliferation cycle (LeCluyse *et al.*, 2010).

Epidermal cell growth factor (EGF) stimulates ion flow, accelerate glucose transport, glycolysis and increases DNA, RNA and protein synthesis, especially for liver epithelial cells, EGF is a mitogenic factor and combines with membrane receptor EGFR, promoting liver stem cells proliferation. Studies of EGF mRNA sub- family showed that the combination between EGF and its receptor EGFR is mainly focused upon transcriptional factors signaling pathway (Xue-Jun *et al.*, 2010).

Basic fibroblast growth factor (bFGF) belongs to the polypeptide cell growth factors and is a broad-spectrum mitogen, it play an important role in embryonic development and cell proliferation, especially for those who derive from mesoderm and ectoderm ,When bFGF was intravenous injected into rats, their osteogenic precursor cells were significantly increased and new bone formation improved (Duret *et al.*, 2007).

Fibroblast growth factor-4 (FGF-4) can induce the cells to differentiate into hepatocytes and then express their related genes. It shows that FGF-4 effect upon cell initial stage and endoderm parts (Yen *et al.*, 2013).

Interleukin 6 (IL-6) not only serves as important molecule start liver regeneration but also activates cell signal transduction system when mice whose IL-6 gene were knockout were found out that the regeneration ability of its liver were reduced, meanwhile the injection of IL-6 can restore DNA replication and regenaration action. Oncostatin M (OSM) is a subfamily member of IL-6, it restrain the activity of melanoma cells, OSM is capable of stimulating maturation of hepatic parenchymal cells and of terminating embryonic liver function (Gallego *et al.*, 2005).

Leukemia inhibitory factor (LIF) is another member of IL-6, which involve in the acquisition of hepatocyte features in BM-MSCs like protein and gene expression of hepatic markers. Bone morphogenetic protein (BMP) involved in

cell's regulation of growth, reproduction, differentiation, and apoptosis. The BMP plays a key role in inducing MSCs expressing the transcription factors Runx- 2 and Osterix, The process is irreversible, yet no evidence had been showed that BMPs have biological effects on mature cells (Lee *et al.,* 2008).

Another important chemical compound is dexamethasone (Dexa), which induces the expression of nuclear factor of 4 and CCAAT / enhancer -binding protein alpha, these two factors belong to hepatocyte nuclear factors and are the crucial transcription factors for hepatocyte differentiation. Dexa inhibits the expression of hepatocyte growth inhibitory molecules such as CXC chemokine receptor, amphiregulin, cyclooxygenase 2, and hypoxia inducible factor (Mukhopadhyay *et al.,* 2010).

Nor epinephrine (NE) is another important chemical compound, experiment show that NE at 10^{-6} - 10^{-4} mol / L and cultured 8h could promote BM-MSCs cell growth, the proliferation rate increased (5, 37 and 10) %, its mechanism involve protein kinase C (PKC) was excited and then translocated from cytosolic to membrane, there are two types of isomers of PCK, PCKδ and PCKλ, their distribution are various according to different animals, even in a single cell their amount, activation and function are different depend upon cell's physiological stages. It is still unknown who type of PKC are increasingly important (Sanchez *et al.,* 2014).

2.9 Formulation of optimal combinations of hepatogenic growth factors:

Over the last years, various studies have suggested that the effects cytokines and chemical compounds are the major contributor for the differentiation of mesenchymal stem cells towards hepatocyte; current research suggests that most adult tissues and organs are derived from the process of

mesenchymal to epithelial transition and its reversible process (Yoon *et al.,* 2010).

The optimal amount of cytokines and growth factors involved in the hepatogenic differentiation, HGF, EGF, TGF, Dexa, oncostatin M and FGF4 are the most cytokines used for researching. For any certain cytokines or growth factor, it is important to note differences in the different stage of MSCs development and different species/source of MSCs (Hu *et al.,* 2012).

The optimal cytokine stimulation, dosage, time and combination for the differentiation of MSCs should be well organized according to the stem cell sources and types (such as ES cells, bone marrow mesenchymal stem cells or heamopoitic stem cells). While the formulation of the "optimal" cytokine / growth factor combinations is still at an immature stage of development (Isfort *et al.,* 2008).

The search for the best combination of growth factors for directing the stem cells towards hepatocytes cell lineage is still under investigation. While using the combined exposure to FGF, HGF and Dexa to transform multipotent adult progenitor cells (MAPCs) into hepatocytes, yet the result are less ideal. (Ling *et al.,* 2010).

Several researchers added a mixture of FGF and HGF or FGF, HGF and OSM according to distinctive hepatocyte markers such as albumin and urea secretion, glycogen storage, and low-density lipoprotein, while others put emphasis on hepatic function conversion by adding inducing factors, the synergistical effects of Dex and nicotinamide are mainly focus upon driving the singling pathways (Hu *et al.,* 2012).

another consideration is the approach of adding factors, More than 85% of sequentially cultured cells phenotype express highly differentiated hepatocytes, including induction of cytochrome P450 (CYP) - dependent activity, When

compare the approach of sequential exposure and cocktail exposure to liver-specific factors, the later result shows more obvious and homogeneous BMSC differentiation into functional hepatocyte can be gained through sequential differentiation process (Lysy *et al.*, 2011).

Also the dosage of and growth factors and cytokines should be fine-tuned *in vitro*, usually 0-60ng/mL for EGF and FGF, 0-30ng/mL for HGF and OSM, according to type of combination, that seems that the optimized differentiation is gained from the best combination of hepatogenic factors as well as the proper differentiation media (Ling *et al.*,2010).

In which the signaling pathways play an important role in switching on the differentiation regulatory program. Some of the important cytokines and chemical compounds such as FGFs, BMP and norepinephrin are involve in this pathway. The major cytokines like HGF, EGF and Dexamethasone are involved in DNA modification. The HGF, EGF, TGF and FGF4 used for combination, while the formulation of the "optimal" cytokine / growth factor combinations is still at an immature stage of development depend upon sources and types of MSCs (Michalopoulos *et al.*, 2013).

2.10 Factors influencing stem cell differentiation into the hepatic lineage *in vitro*:

A major area of research in transplantation medicine is the potential application of stem cells in liver regeneration. This would require well-defined and efficient protocols for directing the differentiation of stem cells into the hepatic lineage, followed by their selective purification and proliferation *in vitro*. (Kim *et al.*, 2005).

The development of such protocols would reduce the likelihood of spontaneous differentiation of stem cells into divergent lineages upon transplantation, as well as reduce the risk of teratoma formation in the case of

embryonic stem cells. Additionally, such protocols could provide useful *in vitro* models for studying hepatogenesis and liver metabolism (Banas *et al.,* 2007).

The development of pharmokinetic and cytotoxicity/genotoxicity screening tests for newly developed biomaterials and drugs, could also utilize protocols developed for the hepatic differentiation of stem cells. The cellular signaling pathway for hepatic differentiation could be given a 'kick-start' through temporary expression of liver-enriched transcription factors coupled to light-inducible promoters (Newsome *et al.,* 2013).

After that, it is possible that the pathway for hepatic differentiation could carry on independently of the recombinant expression of these transcription factors, since the entire array of hepatocyte-specific genes would have already been activated. The advantage of this approach is that there is no constitutive over-expression of any one particular transcription factor (Sulpice *et al.,* 2011).

Also, the natural cellular pathway for hepatic differentiation could carry on physiologically upon switching off the recombinant expression of these transcription factors, through removal of light stimulus. Upon transplantation *in vivo,* it is extremely unlikely that light-inducible promoters would again be activated, since light stimulus would be completely absent *in situ*. Genetically modified stem cells may also run the risk of becoming malignant within the transplanted recipient (Shimizu-Sato *et al.,* 2010).

Moreover, there are overriding safety concerns with regards to the use of recombinant viral-based vectors in the genetic manipulation of stem cells. It remains uncertain as to whether legislation would ultimately permit the use of genetically modified stem cells for human clinical therapy. At present, the potential detrimental effects of transplanting genetically modified stem cells *in vivo* are not well-studied. More research needs to be carried out on animal models to address the safety aspects of such an approach (Kim *et al.,* 2005).

There is emerging evidence that some transcription factors (which are commonly thought of as cytosolic proteins) have the ability to function as paracine cell to cell signaling molecules. This is based on intercellular transfer of transcription factors through atypical secretion and internalization processes. Hence, there is an exciting possibility that liver enriched transcription factors implicated in hepatic differentiation, may in the future be genetically engineered to incorporate domains that enable them to participate in novel paracrine signaling mechanisms. This in turn would have tremendous potential for directing the differentiation of stem cells into the hepatic lineage (Evarts *et al.*, 2009).

Chapter two

Materials and Methods

2.1 Materials:

2.1.1 Apparatus and equipment:

Table (2-1): The main Apparatus and equipments were used in this study.

Apparatus	Company and origin
Autoclave	K&K – Korea
Centrifuge	Hettich-Germany
Cooling centrifuge	Hettich-Germany
Digital camera	Canon-Japan
Distillator	Running waters, USA
Electric oven	Binder-Germany
ELISA microplates reader	Asyshitech- Austria
Incubator	Binder-Germany
Inverted microscope	Olympus-Japan
Laminar air flow cabinet	K&K-Korea
Light microscope	Olympus-Japan
Magnetic stirrer	IKA-USA
Microtome	Leitz, Germany
Nanodrop	USA
pH-meter	Fisher-USA
Sensitive balance	Ohaus-USA
Vortex mixer	Stuart Scientific, UK

	Company and origin
Cover slips	Marienfeld-Germany
Disposable insulin syringes (1ml)	Medico inject –Abu Dhabi
Disposable Petri dishes	Sterilin LTD-England
Disposable syringes (5ml)	Medico inject –Abu Dhabi
Disposable tissue culture dishes	IWAKI-Japan
Eppendrof tubes	Eppendrof-Germany
Forceps	Hide-Germany
Glass tissue culture bottle	Duran-Germany
Haemocytometer	Nauber-Germany
Micropipettes and tips	Islamed-Germany
Millipore filters, size 0.20µm	Asahi glass-Japan
Nalgene filters unite, size 0.20 µm	Nalgen-USA
Pap pen	USAGE- Japan
Parafilm	Pechiney-USA
Pasteur pipettes	Fortunr-Germany
Plastic tissue culture flask (250cm^2)	Nunc-Denmark
Plastic tissue culture flask (50cm^2)	Nunc-Denmark
Plus charged microscope slides	Santa Cruz
Polypropylene centrifuge tube 15&50ml	IWAKI-Japan
Scissors	Hide-Germany
Slides	Sarlbrand-China

2.1.2 Chemicals and biological materials:

Table (2-2): The main chemicals and biological materials were used in this study.

Chemical or biological materials	Company /Origin
Absolute alcohol	Pharmacia-Sweden
Absolutely RNA miniprep Kit	Agilent – USA
Antibiotics (Ampicillin & streptomycin) vials	TROGE-Germany
Antifungal (Amphotericin B)	US-Biological-USA
Dexamethasone	Sigma-USA
Dimethylsulfoxide (DMSO)	Santa Cruz, USA
DMEM medium	US-Biological-USA
DPX	Fluka-Germany
Eosin stain solution	Sorachim-Switzerland
Fetal bovine serum (FBS)	Cellgro- USA
Fibroblast growth factors 4(FGF4)	US-Biological-USA
Glucose	Sigma-Germany
Hank's Balanced Salt Solution (HBSS)	US-biological-USA
Hematotoxylin stain	Sorachim-Switzerland
Hepatocyte growth factor (HGF)	US-Biological-USA
Hepes buffer	Sigma-Germany
Hydrochloric acid (HcL)	Fluka-Germany
Minimum essential media (MEM) medium	US-Biological-USA
Mouse Albumin-primers (228)	BioCorp-USA
Mouse Alpha feto protein-primers (242)	BioCorp-USA
Oncostatin M	US-Biological-USA
Paraffin wax	HistoLine-Italy
Percoll (density 1.130 g/ml)	Sigma-Alderch-USA

Phosphate buffered saline (PBS)	US-Biological-USA
potassium chloride (KCl)	BDH –England
Sodium bicarbonate ($NaCo_3$)	Sigma-USA
Sodium chloride (NaCl)	BDH –England
Sodium hydroxide (NaoH)	BDH-England
Sodium hydroxide (NaoH)	BDH-England
Streptomycin vial	TROGE-Germany
Trypan blue	Fluka-Germany
Trypsin-EDTA	US-Biological-USA
Xylene	BDH –England

2.1.3 Kits:

Table (2-3): The kits had been used in this study.

Kit	Company/Origin
CD105 Kit CD90 Kit CD34 Kit CD45 Kit Anti-albumin primary Ab Anti-cytochrome oxidase p450 primary Ab Anti-alpha fetoprotein primary Ab Mouse cytochrome oxidase p450 Elisa detection kit.	US-biological/USA
Mouse ALT, AST and ALP kits	Randox Company
Anti-rabbit biotenylated secondary antibody Immunocruz mouse ABC Staining system.	Santa cruze biotechnology /USA

2.1.4 Laboratory animals

Four to eight weeks Swiss albino male mice obtained from the Laboratory Animal Unit of the Iraqi center for cancer and medical genetics researches Baghdad, Iraq were used in this study. An average weight of mice was (25-30 g) were used and maintained in a plastic cages under controled conditions of temperature (23 C^o), water and food were given ad libitum.

2.2 Methods:

2.2.1 Preparations of solutions for culture media:

2.2.1.1 Antibiotics solutions: 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
- Amphotericin B (Freshney, 2000): it was a ready-made solution.

2.2.1.2 Phosphate Buffer saline (PBS) (pH 7.2):

This solution was prepared by dissolving the 10.8 g of PBS powder in one liter of triple deionized water (triple DW). The solution was filtered through a Nalgene filter 0.20 µm, and then stored at 4ºC, prior to use PBS was warmed to 37C°.

2.2.1.3 Fetal Bovine serum (FBS):

Frozen Fetal calf serum was first warmed at 45 Cº in a water bath, Then serum was sterilized by Nalgen filter 0.22µ m unit and used directly for tissue culture media (Freshney, 2000).

2.2.1.4 Sodium Bicarbonate:

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

2.2.1.5 Trypsin-EDTA (pH7.6):

Adherent cell dissociation solution was prepared according to the US-

Biological-USA manufacturer instruction, by dissolving 10.1 g of trypsin-EDTA powder in 1 liter of triple D.W. and stirred constantly on a magnetic stirrer at room temperature. Then the solution was sterilized by filtration through a Nalgen filter 0.20 µm and stored at 4C°.

2.2.2 Preparation of stains:

2.2.2.1 Trypan blue stain (1%):

This stain was prepared at a concentration of 1% by dissolving 1 g of trypan blue stain powder in 100 ml of PBS. Solid residue was filtered by filtration through Whatman No.1 filter paper. Then stored at 4 C° until use. Prior to use, 10% dilution in PBS was made of the stock solution.

2.2.2.6.2 Eosin stain: it was readymade solution by diluting eosin at a ratio 1:1 with ethanol and then aliquot of 2-3 drops of glacial acetic acid was added.

2.2.2.3 Harris Haematoxylin stain: it was readymade to use.

2.2.3 Preparation of ABC staining system working solution

- **Blocking serum:** It was prepared by mixing of 75 µl normal blocking serum stock with 5 ml of PBS.
- **Primary antibody:** It was prepared as mentioned by manufacture instruction.
- **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), aliquot of 50 µl from reagent B (biotinylated Horse radish peroxidase (HRP)) then 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 10x substrate buffer, 1 drop of 50x DAB chromogen and 1 drop of 50x peroxidase substrate.
  - **1 % of Hydrogen peroxide H_2O_2:** To prepare 1% H_2O_2 1ml of H_2O_2 stock solution was mixed with 24ml 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 96ml PBS.

2.2.4 Preparation of growth factors: Growth factors were prepared according to Ayatollahi *et al* (2011).

2.2.4.1 Hepatocyte growth factor (HGF) (20 ng/ml): It was prepared by dissolving 0.1 mg powder in 1 ml of sterilized PBS, then 2 µl of HGF was added to 10 ml of media.

2.2.4.2 Fibroblast growth factor 4 (20 ng/ml): It was prepared by dissolving 100 mg powder in 1m of sterilized PBS, then 2 µl of FBS was added as recommended by the manufacturer for decreasing the product instability. Then 2 µl of FGF was added to 10 ml of media.

2.2.4.3 Dexamethasone (10^{-7} nM/ml): It was prepared by dissolving 1 mg powder in 250 of sterilized PBS, then 100 µl of dexamethasone was added to 10 ml of media

2.2.4.4 Oncostatin M (OSM) (20 ng/ml): It was prepared by mixing 0.2 µl of OSM solution with 1 ml of sterilized PBS containing about 0.1 % of FBS to increase the stability of the product as recommended by the manufacture. Then 4 µl was added to 10 ml of media

2.2.5 Preparation of media:

2.2.5.1 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). A quantity 2.2 g of sodium bicarbonate powder, 1 ml of Ampicillin, 0.5 ml of Streptomycin and 100 ml of Fetal Bovine Serum (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).free media was prepared as the previous method except without adding FBS.

2.2.5.2 Hepatogenic medium:

Minimum essential medium supplemented with 10% FBS media was used for differentiation protocol the medium was prepared by the same method as mentioned in item (2.2.5.1) with the addition of growth factors including, 20 ng/ml HGF, 20 ng/ml FGF4, 10^{-7} M/L dexamethasone and 10 ng/ml of oncostatin M (Ayatollahi*et al,* 2011).

2.2.6 Primary culture of bone marrow:

A bone marrow cell was isolated from the femur of 4-8 week-old male albino mouse. The mouse was killed by cervical dislocation, placed the mouse on its back on a cutting board and soaks 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 MEM free serum. A flushing method was used to remove the bone marrow cells from bones using a 1ml syringe containing 1ml of growth culture media (MEM) (Igura *et al*., 2004).

2.2.6.1 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 incubation 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.

2.2.6.2 MSCs passaging:-

Passaging of the cells were done in suspension culture at cell density is greater than ($>10^6$ cells /ml). Typically ,the first time to passage occurs between 5 and 7 days after cultured. 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. Pellet were resuspended in 1ml of culture medium

MEM containing 15% FBS, the cells were counted using a hemocytometer. Cells were detached from the surface with trypsin-versene and counted.

2.2.6.3 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 viable 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 heamocytometer 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.
- 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).

2.2.7 Immunocytochemistry analysis of MSCs:

In this study the following CD markers were used (primary antibody) for detection of MSCs :(mouse anti human-CD 105, CD90 Mouse anti human CD 34 and CD45).

2.2.7.1 Immunoperoxidase staining procedure for detection MSCs

2.2.7.2 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.

2.2.7.3 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.

2.2.7.4 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 over night 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 2.2.3) and washed with three changes of PBS for 5 minutes.

- A liquot of 650 µl of AB enzyme reagent (which prepared in 2.2.3) was added to cell section. And washed two times with PBS for 2 min.
- Three drops of peroxidase substrate (which prepared in 2.2.8.1) 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 at magnification powers 40x and 100 x.

2.2.8 Induction of hepatogenic differentiation of MSCs according to (Ayatollahi *et al*, 2011):

In hepatogenic differentiation the 3rd passage of (2×10^4) of mBM-MSCs were used. The differentiation strategy involved two main stages in first stage which lasted for a week the cells were cultured in a MEM medium supplemented with 10% FBS, 20 ng/ml HGF, 20 ng/ml FGF4 and 10^{-7} M/L dexamethasone. At the second stage which lasted for two weeks the same media where used as the first stage with the addition of 20 ng/ml of oncostatin. As a negative control, mBM-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 3 days. The cell morphology was observed under inverted microscope.

2.2.9 Characterization of induced hepatocytes by immunocytochemistry analysis:

After 3 weeks of differentiation, the cultured cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30-45 min at room temperature and permeabilized with 0.4% Triton X-100 for 10 min. After blocking with bovine serum albumin, the washed cells were incubated overnight at 4 °C with

primary antibodies, including mouse anti-human albumin (1:1000) , mouse anti-human α-feto protein (AFP) (1:500) and mouse antihuman cytochrome oxidase p450 (1: 500). The procedure of immunocytochemical analysis is the same as mentioned in the immunocytochemistry analysis of BM-MSCs accept for the type of the primary antibodies here was for ALB and AFP.

2.2.10 Estimation of cytochrome oxidase p450 by ELISA kit:

Cytochrome P450 3A4 (CYP3A4) BioAssay™ ELISA Kit (Mouse) utilizes the sandwich Enzyme Immunoassay technique for *in vitro* quantative measurement of CYP3A4 in mouse, the microtiter plate provided in the detection kit has been pre-coated with an antibody specific to CYP 3A4.Standers or samples are added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to CYP3A4. Next, avidin conjugated to horseradish peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain CYP3A4, biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulfuric acid solution and the color change is measured spectrophotometrically at a wave length of 450nm ± 10nm. The concentration of CYP3A4 in samples is then determined by comparing the OD of the samples to the standard curve.

B. Assay procedure

- Adherent cells were detached with trypsin and and centrifuged for 15 min at 3000 rpm at 2-8°C and washed three times in PBS. Cells were resuspended in PBS and subjected to ultrasonication for 3 times (50 Hz per min). Alternatively, cells frozed at -20 °C, then cells were thawed with gentle mixing. The freeze/thaw cycle was repeated for 3 times and centrifuged at (or 3000rpm) for 15 minutes at 2-8 °C to remove cellular debris. Samples were stored at -20°C or -80°C till the day of analysis.

- All Reagent was Prepared as mentioned in the preparations above before starting assay procedure, it was recommended that all Standards and Samples being added in duplicate to the microtiter plate.
- The desired numbers of coated wells were secured in the holder then 100 µL of Standards ,blank and samples were added into appropriate wells in the antibody pre-coated Microtiter Plate. Aliquot of 100 µL of PBS (pH 7.0-7.2) was added in the blank control well.then plate was covered with plate sealer and inocubate for 2hrs at 37 C^{o}.
- The liguid was removed from each well (without washing).
- One hundred µL of detection reagent A was added to each well (NOT blank control well) and mixed well. The plate was covered and incubated for 1 hour at 37°C, and then the plate was washed three times via adding 350 µL of wash solution to each well.
- One hundred µL of detection reagent B was added to each well (NOT blank control well) and mixed well. The plate was covered and incubated for 30 min at 37°C, and then the plate was washed three times.
- Ninety µL of TMB substrate were added to each well including blank control well, subsequently. Then plate was covered and incubated for 25 minutes at 20-25°C (in dark).
- An aliquot 50 µL of Stop Solution was added to each well including blank control well and mixed well.
- The Optical Density (O.D.) was determined at 450 nm using a microplate reader immediately. The amount of colored product was determined for each well. The amount of color produced was proportional to the amount of primary antibody bound to the proteins on the bottom of the wells.

C. Calculation of results

The standard curve was drawn using curve expert 3.1 computer software by plotting on the horizontal axis the cytochrome p450 concentrations of the standards and on the vertical axis the corresponding average absorbance. To locate the concentration of cytochrome p450 in the samples, the average absorbance for each sample on the vertical axis was located and the corresponding cytochrome p450 concentration was located on the horizontal axis. (figure 1- 2).

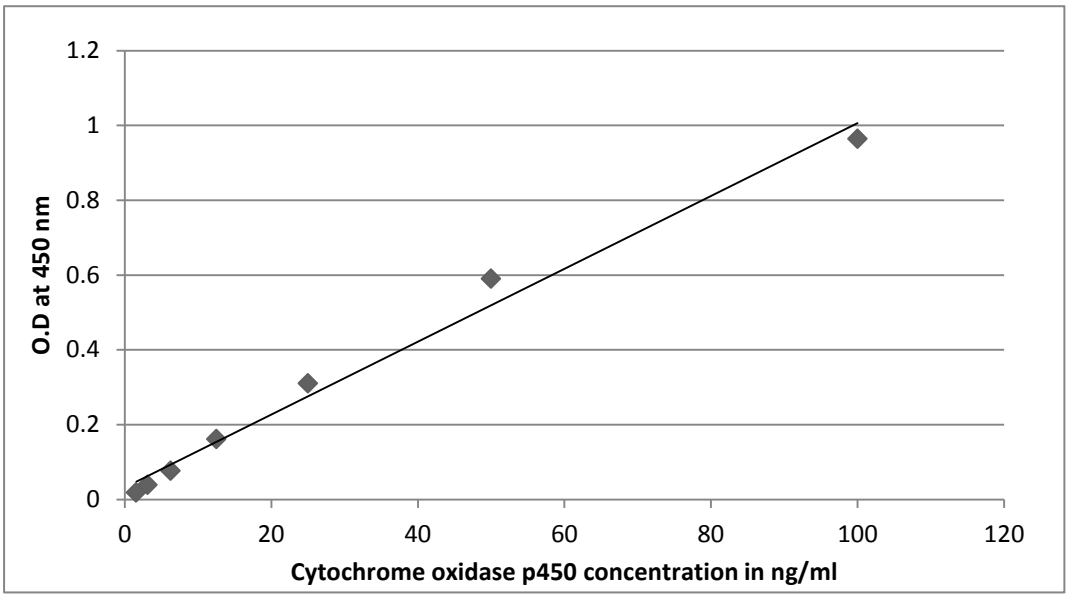


Figure (1-2): Standard curve for mouse cytochrome p450

2.2.11 Induction of liver damage:

Induction of acute liver failure in mice was achieved via subtotal liver resection based on a modified method described by Higgins and Anderson 2001, which include the removal of left and median lobes of the liver were removed after central ligature with a 4.0 absorbable synthetic, braided thread. Both the right upper and lower lobes were removed rendered necrotic by ligation of the common right liver lobe pedicles using a braided silk thread. Then both omental liver lobes and parts of liver tissue surroundings the intrahepatic portion of the inferior vena cava remained, together

approximately represent 10 % of the whole mass of the liver tissue (Eguchi *et al*, 1996). Using this method a highly and reproducible signs of fatal hepatic failure is achieved including a severely impaired ability of the residual liver tissue generation ability. After surgical procedure mice daily monitored and injected with antibiotic solutions to prevent the infection of the operation scar figure (2-2).

2.2.12 Transplantation of hepatocytes and mesenchymal stem cells in induced damaged liver mice:

Hepatocyte and BM-MSCs implantation were performed by slow injection of about 0.7 ml of 5×10^5 differentiated hepatocytes, as well as the BM MSCs as a positive control cells were suspended in phosphate-buffered saline using a 25-gauge needle connected to a 1-ml syringe. Intrasplenic injection method was used in cell transplantation, figure (2-2) in this approach the blood flow in both splenic arteries and veins was clamped before injection and remained occluded for a further 5–7 min to avoid direct cells passaging into the portal vein. Then the area of injection was ligated to prevent bleeding and leakage of injected cells. Instead of hepatocytes, a saline solution (0.9% sodium chloride of analogous volume) was used in each series for control group (Ohashi *et al*; 2007).

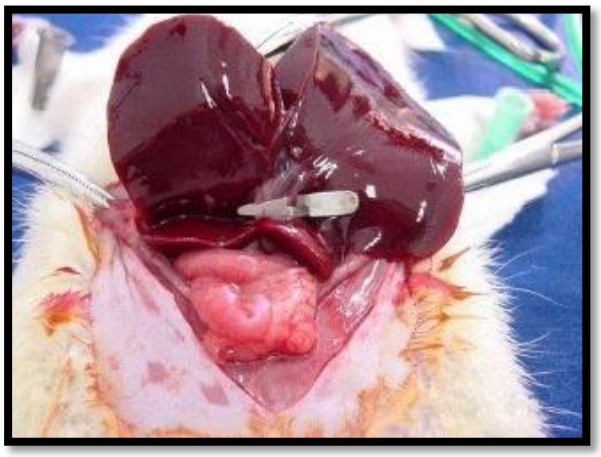
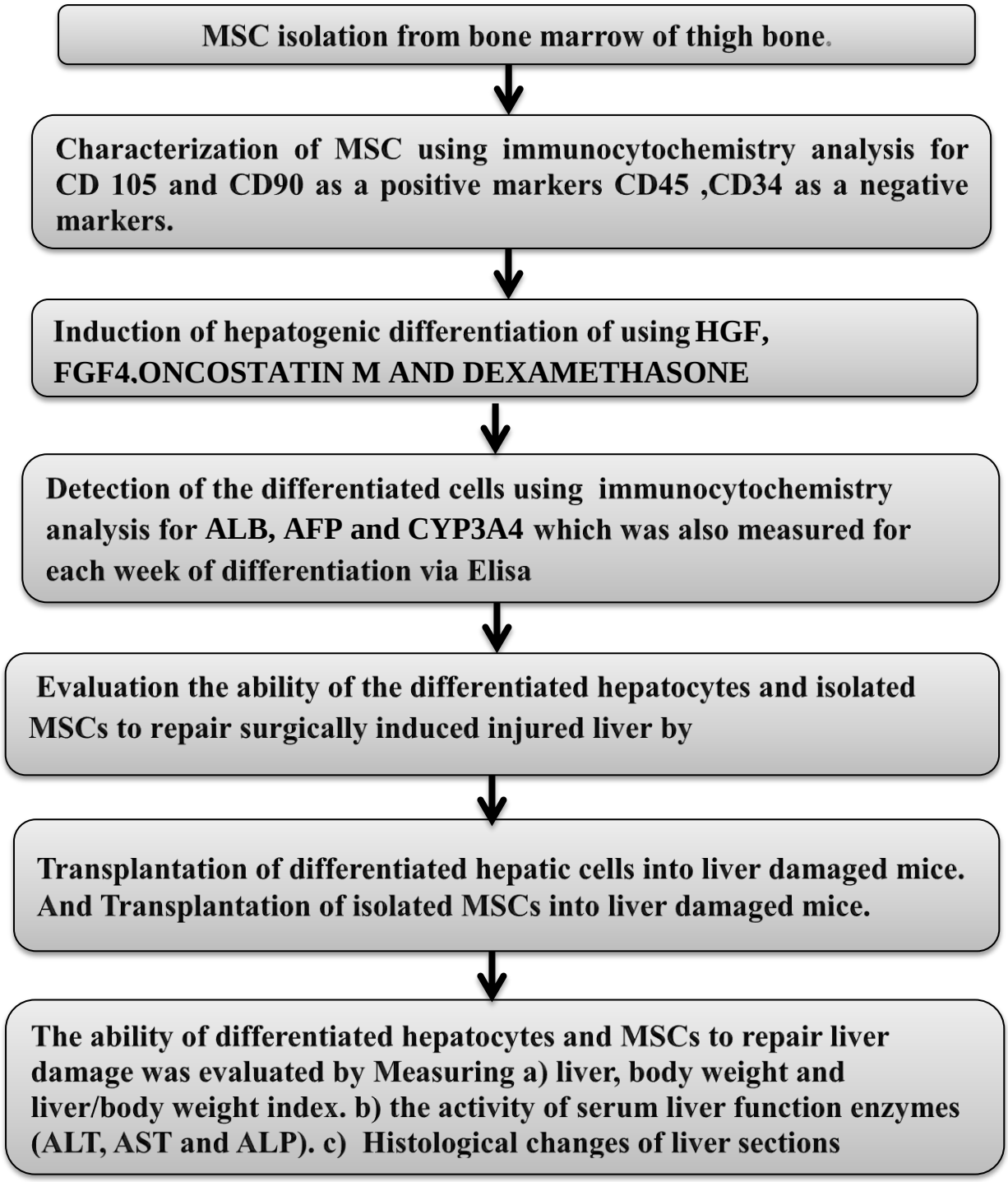


Figure (2-2): Mouse sub-total hepatectomy surgical operation under complete anesthesia using 0.1 ml of lidocaine injectable anesthetic solution of 10 mg/ml.

2.2.13 Experimental design: the experimental work of this study can be summerized in the following diagram:



Although, in the *In vivo* part of the study: 24 healthy male mice were divided into four groups as follows:

- Group A contained (6) healthy mice regarded as control group.
- Group B contained (6) induced liver damage mice via subtotal hepatoctomy method, not received any treatment.
- Group C contained (6) induced liver damage mice, received 0.7 ml of 5 $\times 10^5$ hepatocytes as a treatment per mouse as a single dose).
- Group D contained (6) liver damage induced mice, received 0.7 ml of autologous bone marrow – derived mesenchymal stem cells about 5 $\times$ 10^5 as a treatment per mouse (As a single dose).

All mice were weighted before and after experiment, then at the end of the experiment (10 days post transplantation) mice were sacrificed via cervical dislocation. Blood and liver sections were collected from all groups of mice for further study.

2.2.14 Liver Index:

After scarifice of mice by cervical dislocation of all groups, a mid ventral incision that extends laterally to the abdomen area to expose the liver which was isolated and cleaned from excess adipose tissue then immediately weight by sensitive balance (Ishii *et al*; 2008).

2.2.15 Collection of blood samples for liver function analysis:

Blood samples were taken under a light anesthesia by heart puncture using either two or three ml syringes and put in eppendorf tubes and left for 10 minutes, then serum was separated and stored under -20 C^o until use for liver

functions analysis including both liver ALT (Alanine Transferase), AST(Aspartate amino transferase) and ALP (Alkaline Phosphatase).

A- Aspartate Amino-Transferase (AST)

The enzyme activity of AST was evaluated in the mouse serum following the enzymatic colorimetric method of Reitman and Frankel (1957). For this purpose a commercial kit (Randox Company) was used.

Procedure: Two test tubes (blank and sample) were used and the above solutions were added as shown in table (2-4).

Table 2-4: Method of measuring AST activity

Reagents	**Tubes**	
	Blank (ml)	**Sample (ml)**
Serum	-	0.1
R1	0.5	0.5
Distilled H_2O	0.1	-
The tubes were mixed well and incubated in a water bath (37°C) for 30 minutes		
R2	0.5	0.5
The tubes were mixed well and incubated at room temperature for 20 minutes		
R3	5	5
The tubes were mixed well and left at room temperature for 5 min, and then the absorbency was measured at 546 nm		
The activity of the enzyme AST (Unit/L) was calculated from the standard curve of the kit		

B- Alanine Amino-Transferase (ALT)

The enzyme activity ALT was evaluated in mouse serum following the enzymatic colorimetric method of Reitman and Frankel (1957). For this purpose a commercial kit (Randox Company) was used.

- **Procedure**: Two test tubes (blank and sample) were used and the above solutions were added as shown in table (2-4).

C- Alkaline Phosphatase (ALP)

The enzyme ALP was evaluated in mouse serum using a commercial kit produced by Randox Company and the most commonly used method is that of King and Armstrong (2003), in which di-sodium phenyl phosphate is hydrolyzed with liberation of phenol and formation of sodium phosphate. The amount of phenol formed is estimated colorimetrically.

- **Procedure**: Four test tubes (sample, control, standard and blank) were used, and the forthcoming reagents were added as shown in table (2-5).

Table 2-5: Method of measuring ALP activity

Reagents	Tubes			
	Serum sample	**Serum blank**	**standard**	**Reagent blank**
Reagent 1	2 ml	2 ml	2 ml	2 ml
The tubes were incubated in a water bath (37°C) for 5 minutes				
Serum Reagent 2	50 µl -	- -	- 50 µl	- -
The tubes were mixed well and incubated in a water bath (37°C) for 15 minutes				
Reagent 3	0.5 ml	0.5 ml	0.5 ml	0.5 ml
The tubes were mixed well and vortexed				
Reagent 4 Serum Distilled water	0.5 ml - -	0.5 ml 50 µl -	0.5 ml - -	0.5 ml - 50 µl
The tubes were well-mixed and left to stand for 10 minutes in dark place. Then the optical density (O.D.) was measured at a wave length of 510 nm.				

- **Calculation:** The following equation was employed to assess the serum level of ALP.

$$\text{Serum Level of ALP (I.U./ml} = \left(\frac{\text{O.D. Serum Sample - O.D. Serum Blank}}{\text{O.D. Standard}} \right) \times 142$$

2.2.16 Histological analysis of liver sections:

Liver sections were first isolated and cleaned from access adipose tissues in a petridish containing normal saline then the liver sections were weighted by an electronic precision balance then the organs were put in tubes containing 10% formalin for about 16-18 hours for fixation purpose, then they were transferred in to tubes containing 70% ethanol alcohol in which they preserved till the time of final preparation. In the final preparation the samples were transferred in 99% alcohol for about 6 hours then put in xylol for 2 hours (Bancroft, 1980). Then sections were put in a paraffin wax for blocks preparation. Then a 5 µ paraffin sections were optained using a sharp knife of a handling laboratory microtome. Paraffin sections of liver tissue were put in a water bath of 43 ºC then were stick in a glass slide in a proper angled manner to obtain the best result, then glass slides containg liver tissue section were dried using hot plate. The staining method was performed using heamatotoxilin and eosin stain (Junqueria *et al*., 2000; Humson, 2001). For all histological slides pictures were taken using a digital camera under light microscope at a magnification power of 200X and 400X.

2.2.17 Statistical analysis:

Data were analyzed statistically 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 at $P \leq 0.05$.

Chapter three

Results and Discussion

3. Results and Discussion

3.1 Morphology of bone marrow mesenchymal stem cells

Mesenchymal stem cells were isolated based on their ability to form adherent monolayer in culture and the lack of adherence of other cells in the bone marrow stroma such as hematopoietic stem cells, erythrocyte, adipocytes and macrophages.

Hence, change media performed after 24 h. and many of MSCs had attached to the base of the tissue culture flask (Figure 3-1 A), but the cells didn't yet form the spindle elongated shape of the mesenchymal stem cells. These adherent cells were remained attached to the bottom of the flask, formed high numbers of scattered plastic adherent colonies (figure 3-1 B).

Some of adherent cells assumed spindle morphology, a characteristic oval body with tapered shape ends. When they slowly elongate, others appeared as individual small size spindle-shaped cells (Figure 3-1 C, D). The media MEM was reported as the most appropriate media for mesenchymal stem cells isolation and proliferation since a high density of viable attached cells expanded rapidly to lift from primary culture (Peister *et al*., 2004).

Moreover, Some reports suggested that MSCs secrete cytokines and growth factors such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and interleukins as well as numerous extracellular matrix components (Liu and Hwang, 2005; Kolf *et al*., 2007) and these factors contribute to the proliferative potential of MSCs *in vitro.* Colter *et al*(2010) ;Ja ckson *et al* (2013) and Isern *et al* (2013) proposed that BM-MSCs cells represent particularly multipotent progenitors with the potential to differentiate into many lineages. It seems likely that the BM- MSCs cells described in these studies may not represent a pure progenitor fraction but represent a variation in

the morphology of MSCs that have the potential to rapid expansion in culture (Andrietti, 2012).

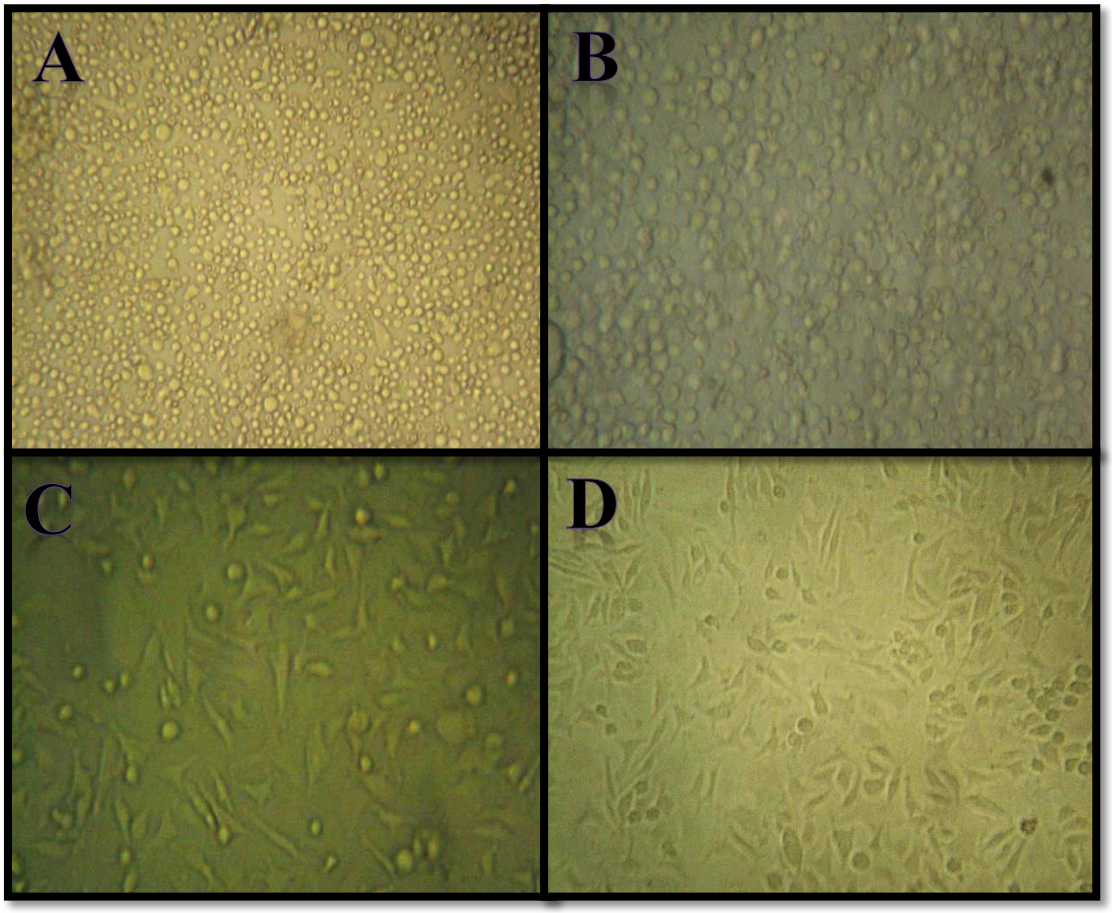


Figure (3-1): MSCs Isolated from mouse bone marrow cultured on a falcon containing DMEM 10% FBS viewed by inverted microscope (100X) (A) After 24 h. (B), after 48 h. (C) after 3 days and (D) after 5 days showing the (80% confluence of isolated BM MSCs with their distinct spindle elongated ends morphological characteristic.

3.2 Immunocytochemistry of isolated BM-MSCs:

Isolated MSCs were identified from the other bone marrow cells haematopoietic stem cells using immunocytochemical analysis, which based on the cell nature of expressing specific cell markers on their surface. The BM-MSCs showed positive reactivity to CD105 and CD90 (brown color) and negative reactivity to haematopoietic stem cells markers CD34 and CD45 Figure (3-2).

These techniques are based on the immunoreactivity of antibodies and the chemical properties of enzymes or enzyme complexes which react with colorless substrate- chromogens to produce a colored end product. (Herrera *et al*; 2006). Initial immuno-enzymatic stains utilized the direct method, which conjugates enzymes directly to an antibody with known antigenic specificity (primary antibody).

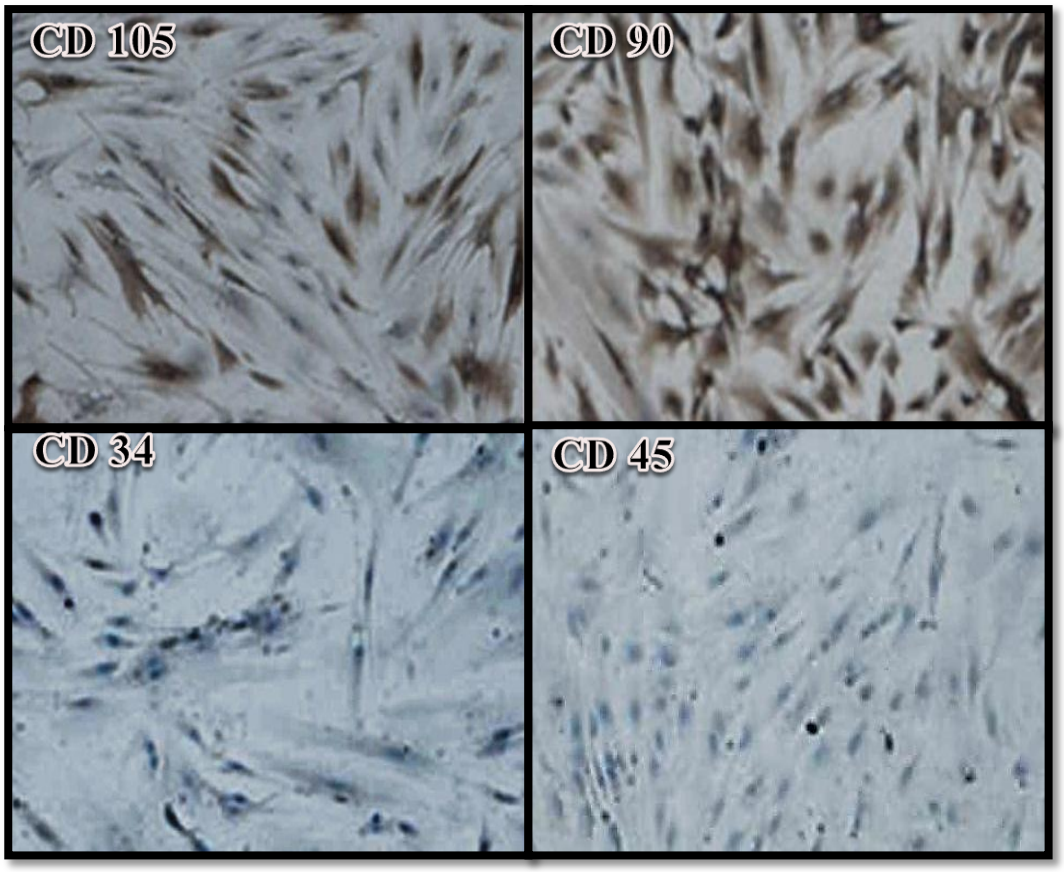


Figure (3-2): Immunocytochemistry analysis of isolated BM MScs viewed under light microscope at (400X): showing the positive reactions of cells towards CD 105 and CD 90 markers of cell surface and the negative reactions for CD 34 and CD 45 cell surface markers.

In 1976, Friedenstein and colleagues described a method for isolating fibroblast-like colonies from rodent bone marrow. These clonogenic cells were isolated based on their relatively high adhesion to plastic compared to other cells in the bone marrow compartment. The method used by Friedenstein is still

widely used today for isolation of MSCs from unpurified bone marrow or Ficoll-purified bone marrow mononuclear cells.

While isolation by plastic adherence is efficient, it does not yield a homogeneous population of cells. The isolated cells demonstrate different growth kinetics and differentiation capacities and include a small population of contaminating hematopoietic cells. Cell homogeneity can be increased over several weeks by culturing under conditions that preferentially expand MSCs and select against expansion of hematopoietic cells (Manca *et al*, 2008).

However, the remaining MSCs can still demonstrate a range of proliferation rates and differentiation potentials. This heterogeneity may contribute to experimental variability, contradictory data, and poor pre-clinical outcomes (Barry and Murphy, 2009). The results of the present study agreed with Minguell *et al* (2001) and Pittenger *et al* (2007) who showed the positive expression of CD90 and CD 105 of MSCs and negative expression for CD 34 and CD 45 surface markers using flow cytometry analysis system. The cells surface markers used in the positive and negative selection of MSCs are officially described and published by the international society of cell therapy (ISCT).

3.3 Hepatogenic differentiation of BM-MSCs:

During the differentiation experiment the cultured cells were monitored via inverted microscope for morphological changes of MSCs during the stages of differentiation of BM-MSCs into hepatocyte like cells Figure (3-3) (A, B,C,D,E and F). After treatment with hepatic cell differentiation medium, BM- MSCs was monitoring during the whole three weeks of differentiation protocol, many morphological changes had been reported under inverted microscope examination, thus, after the first two week, the cells were gradually begin to lose their fibroblastic morphology and expanded slowly to become spindle-shaped (Fig 3-3 B, C).

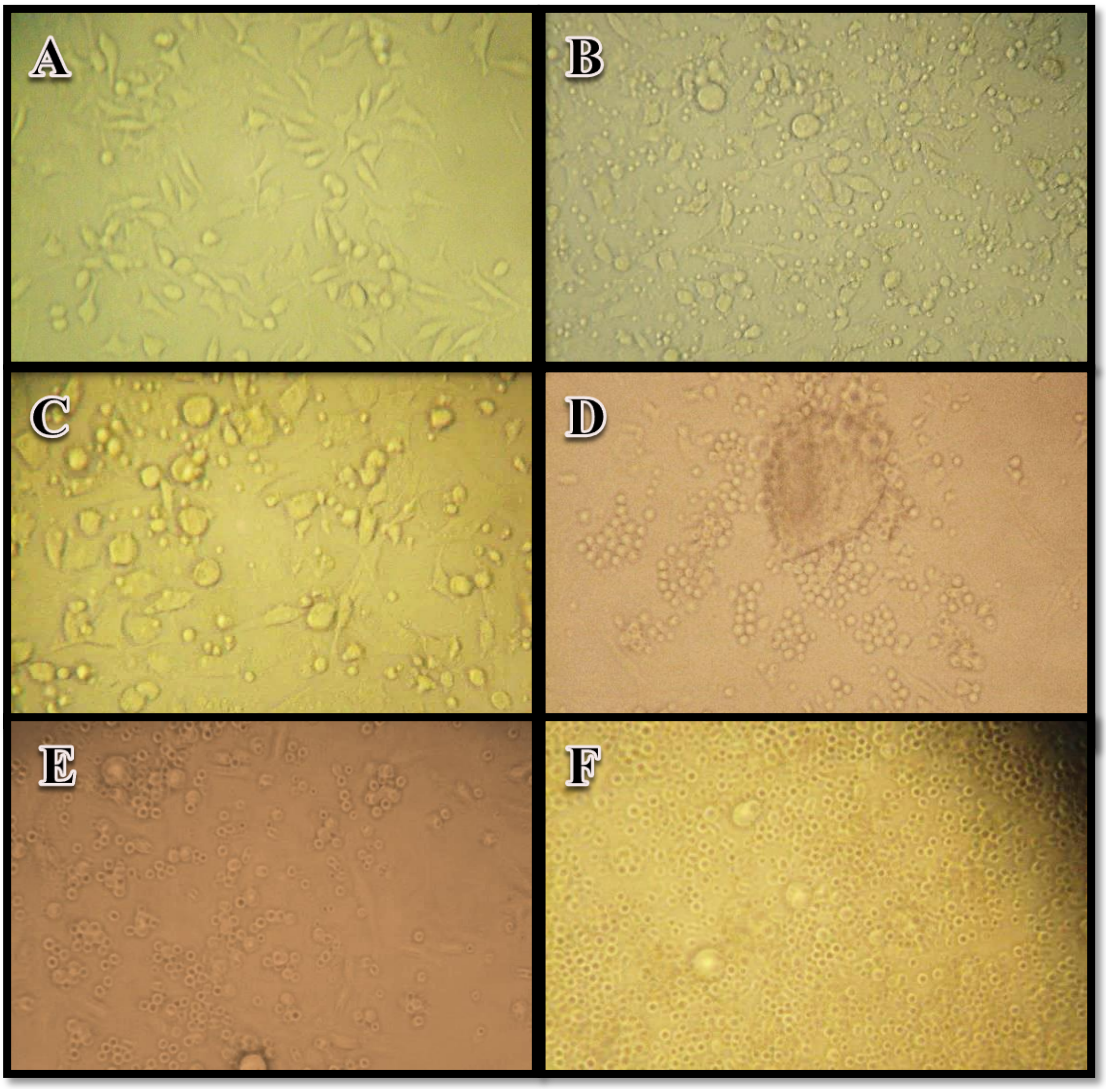


Figure (3-3): Different stages of morphological patterns of hepatogenic differentiation of Mouse MSCs. A: the mesenchymal stem cells isolated from mouse bone marrow; B: MSCs under differentiation condition at day 10; C: MSCs under differentiation condition at day 16; D, E: Differentiated cells at day 19 and 23 F: cells at the end of differentiation.

Then in the second two week cells were started to convert in to small, and round cells (Figure 3-3 D) and finally at the third week cells appeared round or polygonal and appeared to look like the hepatocyte morphological characteristics (Figure 3-3 E, F).

For each stage of differentiation and growth cells are strictly regulated by cell autonomous mechanisms and extracellular signals including cytokines and growth factors (Anjos, *et al*., 2004).

Although the stem cell differentiation mechanism remains unclear, transdifferentiation might either be induced by stimulating with suitable media/substrates/factors, or by genetic reprogramming *in vitro* (Safford *et al*., 2002; Tosh and Strain, 2005). Liver development is known to proceed via several distinct steps in which growth factors and cytokines are involved. Among the factors implied in the embryonic liver development, fibroblast growth factors (FGFs), produced by cardiac mesodermal cells, are involved at an initial stage of endodermal patterning to induce hepatic fate (Wang *et al* 2003). Oncostatin M, a member of the interleukin-6 cytokine family produced by hematopoietic cells, is required from the mid-fetal to the neonatal stages and apparently coordinates liver development and hematopoiesis in the fetus (Duret *et al*., 2007). Finally, several extracellular signals including EGF, HGF, OSM, FGFs, glucocorticoids and insulin are involved in the late maturation stage leading to an increase in liver-specific gene expressions, and their effects on differentiation vary as a function of gestation age (Herrera *et al*; 2010). Corticosteroids, HGF and EGF play important roles in hepatic biology thus, HGF is a more potent proliferating factor for hepatocytes in culture than other growth factors involved in *in vitro* hepatic generation. The differentiation of BM-MSCs into hepatocyte-like phenotypes *in vitro* by induction with HGF has been reported also by both Ohsh *et al*. (2010) and zhang *et al.* (2007) in their *in vitro* studies on Rat bone marrow derived msenchymal stem cells lineage. Other reports showed differentiation of bone marrow-derived MSCs toward hepatocyte-like cells induced by FGF-4. However the degree of differentiation was higher when cells were also treated with HGF (Schwartz *et al.,* 2012). This is consistent with the fact that FGF-4

may play a role in endoderm specification (Wells *et al*, 2011), and that HGF induces differentiation of hepatocytes that are not actively proliferating (Gomez *et al.* 2004). Bone marrow cells cultured with HGF and FGF showed morphologic and phenotypic characteristics of mature hepatocytes (Michalopoulos *et al.*,2013), dexamethasone has been shown to have a specific differentiation-inducing effect on primary fetal hepatic cells towards mature hepatocytes (Seo *et al*., 2005).

In this study the induction to the hepatogenic differentiation of mouse bone marrow mesenchymal stem cells showed that BM-MSCs can be differentiated into hepatocyte-like cells by treatment with cytokine mixtures (HGF and FGF4, dexamethasone and oncostatin M) in 10% serum medium. However, used a 2-step differentiation protocol with a sequential addition of growth factors (HGF, FGF4 and dexamethasone at the first stage then the same growth factors in addition to Oncostatin M were added in the second stage of differentiation protocol (Maryam *et al*., 2011). The choice of exogenous factors and the time course to induce hepatogenic transdifferentiation are based on previous reports on BM-MSCs differentiation (Suzuki *et al*., 2004). As previously mentioned, HGF plays an essential role in the development and regeneration of the liver in addition to FGF4 also is required to induce a hepatic fate in the foregut endoderm (Seo *et al*., 2005). Dexamethasone increases hepatocyte size and enhances hepatic differentiation (Lázaro *et al*, 2011). As well as recent studies demonstrated that oncostatin M helps significantly in the maturation of fetal hepatic tissues (Sakai *et al.*, 2012).

The morphologic and phenotypic features and the role of key hepatic differentiation factors in the regulation of the transdifferentiation process had been investigated to show, that mouse BM- MSCs are capable of giving rise to a hepatogenic transdifferentiation in response to a sequential addition of growth factors, assessed by an examination of morphology and hepatocyte-specific

marker (Sakai et al., 2012). The results of the current work showed that the differentiated cells displayed the hepatocyte like morphology which was agreed with the results showed by both Ayatollahi *et al*. (2007) and Pan *et al.,* (2008).

3.4 Immunocytochemical analysis of induced hepatocytes:

The immunocytochemistry analysis of hepatocytes like proteins produced by hepatocytes cells is shown in figure (3-4). The cells were positively reacted to mouse antihuman albumin and alpha-fetoprotein (AFP), confirming by the brown color of the DAP stain in the ABC staining kit (Figure 3-4 A and B).

There were some cells in the culture was stained with the purple stain of the hematotoxylin which was used as a counter stain in the staining procedure after incubation with the mouse antihuman primary antibody, these cells are found to be negatively reacted toward mouse antihuman albumin and alpha-fetoprotein primary antibody suggesting that these cells were undifferentiated cells which means they are still in their original lineage BM-MSCs or they were not completely finished their differentiation towards hepatocytes lineage (Figure 3-4 C and D).

These results agreed with both Min Jeong (2010) and Yamamoto *et al*. (2012) which are found that Hepatocyte like Cells differentiated from Adipose Derived Stem Cells were expressed both Albumin and alpha-fetoprotein proteins when tested cytochemically. The dynamics of synthesis of alpha-fetoprotein and albumin have been studied in rat and man by immunocytochemical localization of each protein in the liver and its quantitation in serum at different periods was measured (Kew *et al*., 2003). The proteins are present only in the cytoplasm of hepatocytes. During physiologic development, an inverse relationship exists between the serum concentrations of the two proteins, the latter for each appearing to be directly related to the number of hepatocytes synthesizing it.

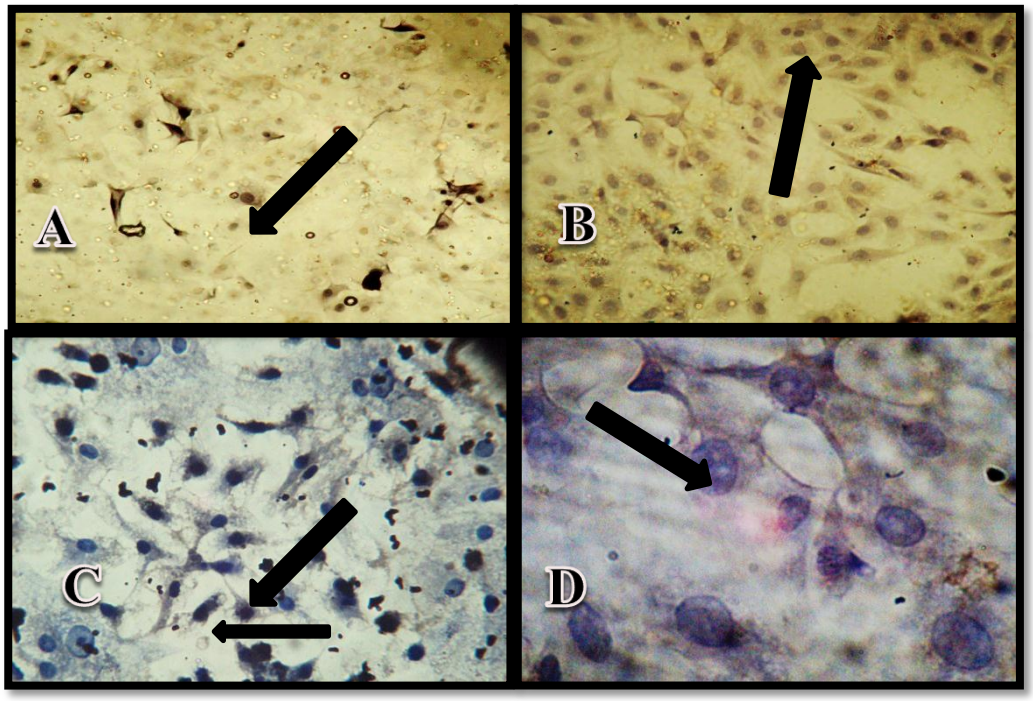


Figure (3-4): Immunocytochemical analysis of albumin and alpha-fetoprotein in hepatocytes after 21 days of differentiation. Cells were underlight microscope at (100X for A, B, C and 400X for D). Homogeneous expression of albumin (A) and AFP (B) showed positive reactivity (brown color). Staining for both albumin and AFP was negative in undifferentiated cells (Cand D).

AFP-containing cells are randomly distributed, while albumin-containing cells are more uniformly spread out. Both, however, are often preferentially located around venous channels. In the case of AFP-containing cells, the last to disappear with advancing postnatal age are seen around the hepatic veins (Nayak, 2001).When both proteins are present in serum, several hepatocytes seem to synthesize the two simultaneously, though others contain only one of them. It is unlikely that in the physiologic state different populations of hepatocytes are assigned to synthesize AFP and albumin separately (Abelev *et al*, 2000).

At the end of the differentiation experiments the resulting hepatocytes like cells were tested for Cytochrome oxidase p450 (CYP3A4 p450) expression, results showed that the majority of the differentiated cells (about 70%) were

stained with the brown DAP stain (Figure 3-5 A), which mean that these cells are positively reacted towards CYP3A4 p450 mouse anti human primary antibody explain that the cells are positive for CYP3A4 enzyme expression, as well as some of these cells were shown to be negatively reacted to the CYP3A4 p450 suggesting that these cells may be undifferentiated cells or are not completely differentiated to express the CYP3A4 p450 enzyme as a mature differentiated hepatocytes.

The above results are approved by Masaru Tsutsui and his collagenous in 2005 that they were characterized the cytochrome oxidase p450 expression in murine embryonic stem cells-derived hepatic tissue system. The enzyme CYP3A4 P450 is a member of the cytochrome p450 super family of enzymes. The cytochrome p450 proteins are monooxigenase which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. This protein is localized in the endoplasmic reticulum and its expression is induced by glucocorticoids and some pharmacological agents. This enzyme is involved in the metabolism of approximately half the drugs which are used today, including acetaminophen, codeine and erythromycin. The enzyme also metabolizes some steroids and carcinogens (Yamashita *et al*., 2010).

3.5 Cytochrome oxidase p450 (CYP3A4 p450):

The results revealed that a significant increase ($p \leq 0.05$) in CYT3A4 concentration in hepatocyte lysate at 21 day (65.27± 0.52) ng/ml in comparison with 14 and 7 33.0 ±0.0.72 and 16.73±0.92) ng/ml respectively (Figure 3-6). The cytochrome P450 (P450) superfamily consists of a large number of haem-containing mono-oxygenases that play a pivotal role in the metabolism of many drugs and carcinogens (Volker *et al*., 2014).

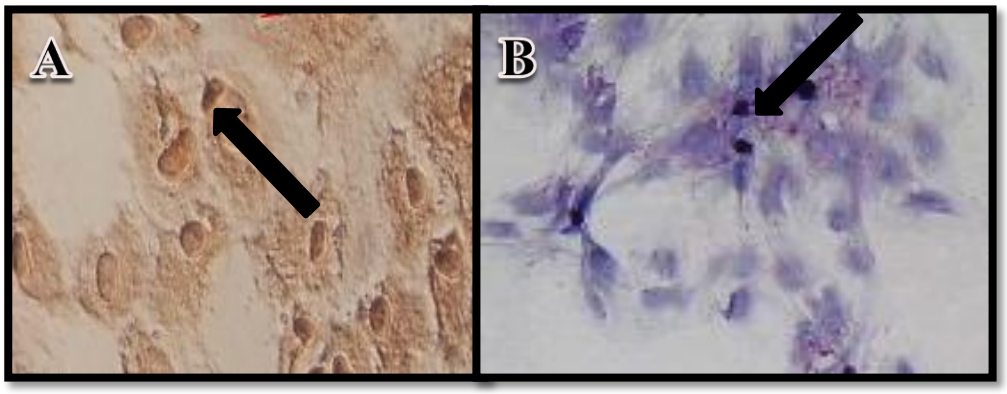


Figure (3-5): Immunocytochemical analysis of cytochrome oxidase p450 (CYP3A44 p450) in hepatocytes like cells. Positive reaction in (A) in induced hepatocytes like cells, while (B) shows the negative reaction of cells against mouse antihuman CYP3A4 p450.

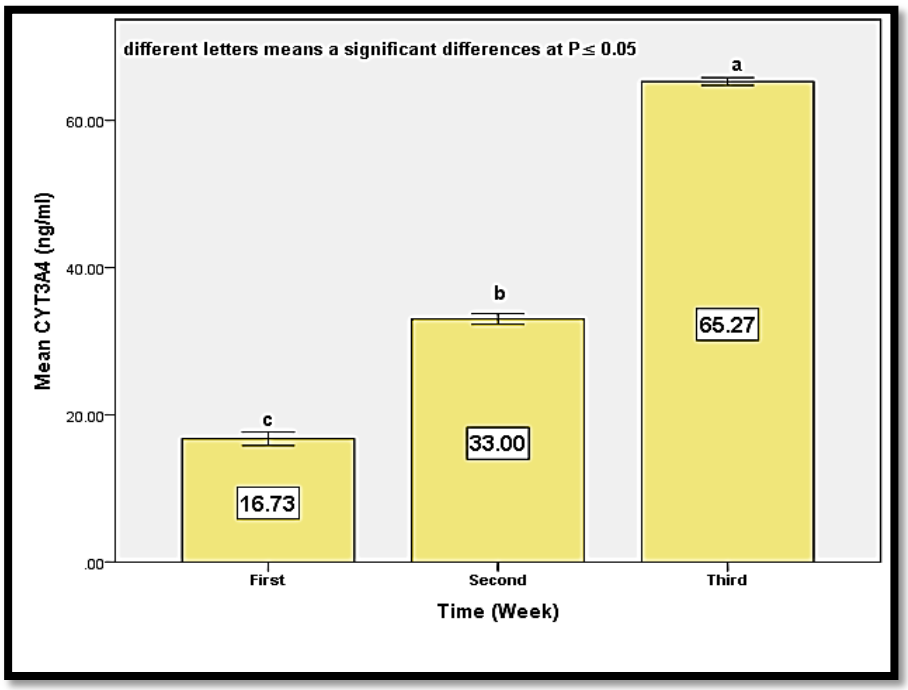


Figure (3-6): Level of CYT3A4 (ng/ml) in hepatocyte lysate after different differentiation period.

The results agreed with results obtained by Masaru *et al*. (2006) who used real time analysis showing an increase in CYP3A4 gene expression by the time of

differentiation strategy of murine embryonic stem cells derived hepatic tissue system. Ochiya *et al.* (2010), studied the whole profile of CYPs enzymes in the hepatic cells obtained from mesenchymal stem cells for both mouse and human.

3.6 Mice body and liver weight index:

The results of body and liver weight for each group of the experimental animals were listed in table (3-1).

Table (3-1): Body/ liver weight ratio (Mean±SE) in liver damage mice treated with hepatocyte and BM-MSCS and controls.

Group	Body weight (g)	Liver weight (g)	Liver/body weight ratio
Group A (healthy control group).	25.7±0.61**a**	6.75 ±0.32 **a**	0.26±0.02 **a**
Group B (liver damage mice).	19.93 ±1.0 **d**	3.57±0.33 **d**	0.17±0.20 **d**
Group C (liver damaged mice treated with hepatocytes).	24.31±1.0 **b**	5.0±0.42 **b**	0.20±0.14 **b**
Group D (liver damaged mice treated BM-MSCs).	20.94 ±0.50 **c**	4.11 ±0.33 **c**	0.19 ±0.18 **c**

Different letter represents significant differences (P≤0.05) between the means.

Results showed that there was a significant decrease in the body and liver weight in group B which was exposed to subtotal hepatectomy operation as compared with the normal healthy control group (A). Similar results were described by Giorgio *et al*. (2015), who explained that there was a significant

decrease in both body weight and liver weight after partial hepatectomy of rat liver.

The results showed a remarkable significant increase of the body and liver weight which was recorded in mice treated with differentiated hepatocytes as a as compared to group B of partially hepatectomised mice. The possible explanation for these results that the injectable hepatocytes transfer gradually from the splenic artery to the site of hepatic injury where they may function as recovered healing cells to treat the damage caused by the induced subtotal hepatectomy which leads to a massive liver injury.

Group D showed a little increase in the liver and body weight since BM-MSCs which may migrates to the site of injury and get a potential signaling growth factors emerged from the damaged surroundings hepatocytes cells and interfered with the BM-MSCs programing and directed them towards hepatogenic differentiation making use of these cells as a possible substitution along with the normal liver regeneration strategy to overcome the damage process.

3.7 Serum liver function assay:

The results of serum liver enzymes activity of (ALT, AST and ALP) for each group of the experimental animals were listed in table (3-2). An induced hepatectomised mice (Group B) which were subjected to a severe liver injury**,** showed a significant ($P \leq 0.05$) increase in levels of serum ALT (403 IU/L) as compared to healthy (218 IU/L). Results revealed that treatment with hepatocyte was more effective in reducing ALT activity (263 IU/L) in comparison with BM-MSCs (280 IU/L) with significant differences ($P \leq 0.05$).

Similarly, the results of AST of Group B were also showed a significant($P \leq 0.05$) increase (226 IU/L) as compared to the healthy control group A (125 IU/L), while in group C, the transplanted hepatocytes participated in the

significant decrease (183 IU/L), helping in regulating the enzyme level nearly back to the normal more efficiently than the MSCs treated group D (146 IU/L).

The serum level of ALP were also revealed a significant ($P \leq 0.05$) increase in group B (36.21IU/L) of induced liver hepatoctomy, as compared to the healthy control group A (16 IU/L), a significant decrease in the ALP serum level of group C (17 IU/L) as compared to group B which was more significantly decreased as in the group D (22 IU/L).

Table (3-2): Serum ALT, AST and ALP activity (Mean±SE) in liver damaged mice treated with hepatocyte and BM-MSCs and controls.

Group	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
Group A (healthy control group).	218 ±3.6 **d**	125±1.0 **d**	16 ± 1.3 **d**
Group B (liver damaged mice).	403±1.8 **a**	226 ±1.5 **a**	36.21± 1.2 **a**
Group C (liver damaged mice treated with hepatocyte).	263±1.3 **c**	183± 2.3 **b**	17 ±1.0 **c**
Group D (liver damaged mice treated BM-MSCs).	280 ±2.8 **b**	146 ±0.19 **c**	22 ±1.6 **b**

***Different letters represents a significant (P≤0.05) differences between means.**

The above results were matched the biochemical studies of liver explained by Franklin *et al*, (2010) , who reported a remarkable increase in the ALT and ALS post major hepatic resections in rat indicating Liver damage effect

Since serum levels of AST also called serum glutamic oxaloacetic transaminase or aspartate aminotransferase, is similar to ALT (Alanine Amino-Transferase) are groups of blood tests that give information about the state of a patient's liver (Lee, 2009). Liver transaminases (AST or SGOT and ALT or SGPT) are useful biomarkers of liver injury in a patient with some degree of intact liver function. Both enzymes are associated with liver parenchymal cells. they raised in acute liver damage. The ratio of AST to ALT is sometimes useful in differentiating between causes of liver damage. (Nyblom *et al*., 2004).

Otherwise the injection of cells plays an important role in healing of liver and interferes with the improvement and regulation of liver function enzyme secretion. Since the group C of hepatocytes transplantation showed the best regulation of the liver function enzyme regulation via recording a significant decrease in the elevated enzyme levels after induced hepatoctomy, which had been more efficient than transplantation with bone marrow mesenchymal stem cells group.

In addition to Xiaodan *et al.* (2003), who published by the American society of clinical investigation a scientific finding on the ability of hepatic stem cells progenitors to repair damaged liver damaged tissue and regulates the liver functional enzymes after 70% hepatoctomy operation on mouse liver.

3.8: Histological analysis of liver sections

Results in Figures (3-7), (3-8), (3-9) and (3-10) are showing the histological changes in liver sections for each group of mice. In comparison with figure (3-7) A, from the healthy control group which represents the normal histology of liver of scattered hepatocytes and a central vein. In figure (3-8), which displayed the microscopic histological investigations of liver sections from mice subjected to liver damage induction. The main histological changes which observed are characterized by a massive liver injury, resulting in severe

granulomas marked by concentric fibrosis surrounded by a cuff of aggregated lymphocyets. Also a presence of a high amount of granulomas which leads to disorganization of the hepatic tissue strands and lobular structure.

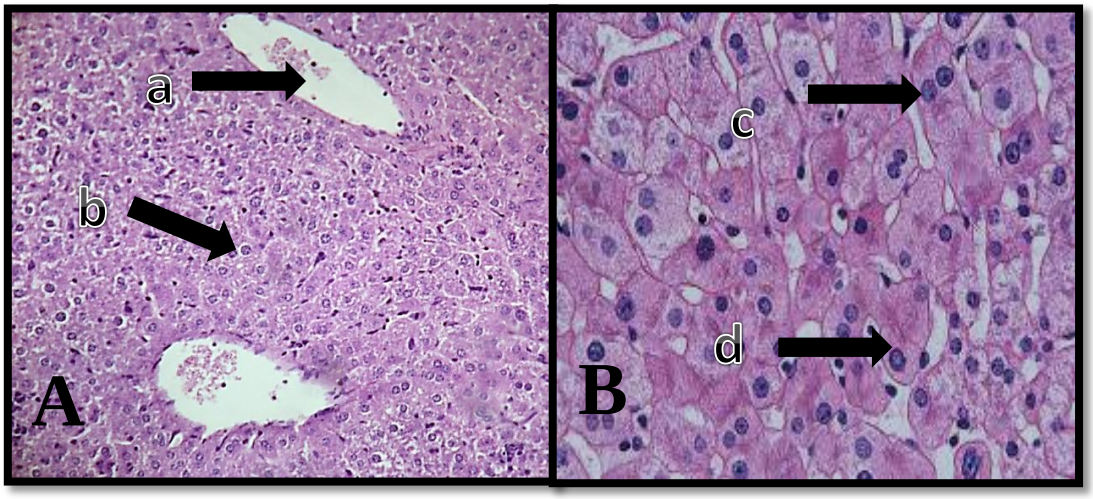


Figure (3-7): histological sections of group A (healthy control group) showing the normal histological features of liver, the hepatocytes are scattered along with a central hepatic vein (arrows a- central hepatic vein, b- hepatocyte cells, c-hepatocyte cell with two nucleuses and d- hepatocyte cell with mononucleus), (A: section under low power 100X; B: sections under high power 400X). (H and E staining).

As well as there was a dileation or extends of the hepatic sinusoids to became apperantly having much more of kupffer cells. (Toderke *et al*,2014).

The above results were agreed with the histological findings observed by Kwon *et al*, (2000), who showed that experimental mouse hepatoctomy would leads to a massive liver tissue damage characterized by different alterations in tissue histological feauters and losing the lobular trait of liver tissue.

The reasonable explanation of the damage is due to the effect of the hepatoctomy operation since a lot of liver mass being loosed in addition of injuring the remaining mass of tissue. The severed granulomas resulting from increasing of lymphocytes number being transported to the side of injury by the hepatic vein (Gama *et al*, 2010).

On the other hand, the main histological recovery for liver sections of the group C which was receives the hepatocytes cell therapy by intrasplenic transplantation of cells, were reported in figure (3-9) which shows a remarkable regeneration of liver damaged tissue a long with the decreasing of cells infiltration and cells vacuolization resulting from the induced liver injury.

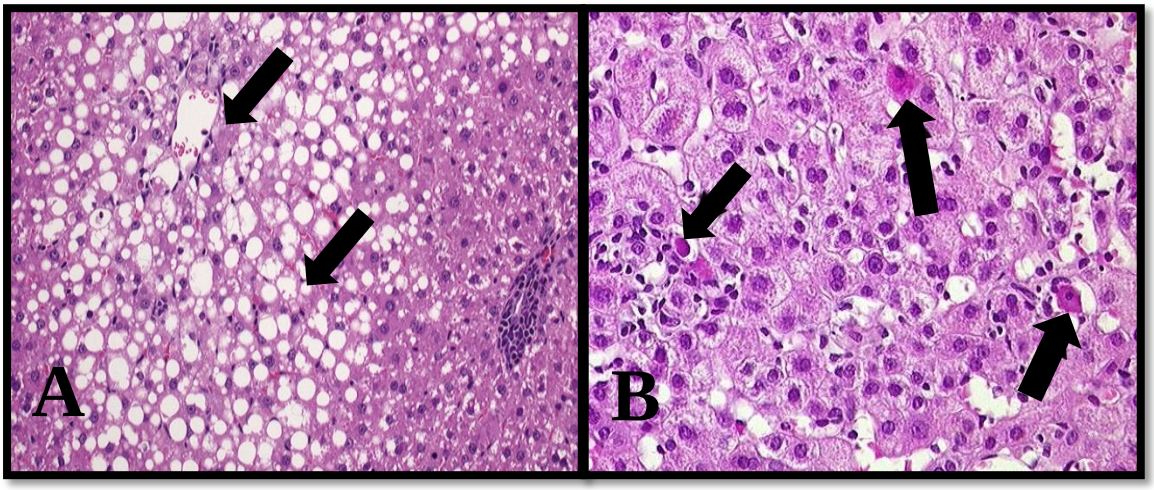


Figure (3-8): Histological changes of group B (induced hepatectomized group) showing the severe injury of liver tissue represented by inflammatory cellularinfiletration as well as cytoplasmic vaculation and degeneration of hepatocytes (arrows a- Inflammatory cells) b- Cytoplasmic vaculation, c-hepatocyte cell with two nucleuses and d- degeneration of hepatocytes) (A: section under low power 100X; B: sections under high power 400X). (H and E staining).

The regeneration of the severe damaged tissue was happened in a superior time since the histological investigation after 10 days post cells transplantation, while many studies showed that the normal autoregeneration of liver for such a massive tissue injury may takes much longer time depending on the liver natural ability of regeneration strategy, such results were reported in the clinical studies of patients with severe liver injury resulting post-surgical removal of liver carcinoma mass of tissue (Rubbia *et al*,2004).

As well as, the route of transplantation, may also played an important role in fasting and improvement of liver damage repair, thus the route of entry of cells facilitate the fast reaching of transplanted cells towards the site of injury. In

addition to increasing the amount of cells being used as a precursor of regeneration mechanism and repair the damaged tissue. This conclusion were also reported by Xuan *et al* (2013), who mentioned the relationship between intratrasplenic injuction route and liver metastases in nude mice.

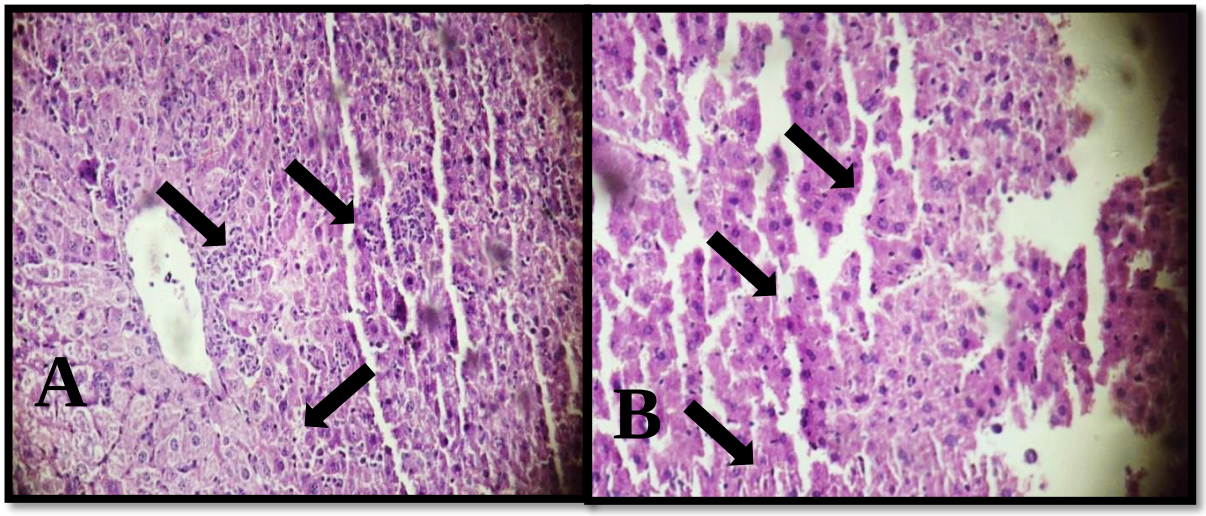


Figure (3-9): histological changes of group C (hepatocytes transplantation group) showing the regeneration of liver tissue represented by healing of cellular infiletration and reduction of tissue damage (arrows). (A: section under low power 100X; B: sections under high power 400X). (H and E staining).

Results in Figure (3-10) illustrates the histological changes of group D which subjected to BM-MSCs transplantation, revealing a slightly regenerative changes and reduction in tissue damage, this indicates that the injectable msesnchymal stem cells were act as a precursor of hepatocytes helping in the damaged tissue regeneration process. The possible analysis of this results is that the system of both the reaming undamaged hepatocytes and the injected bone marrow mesenchymal stem cells acts together as an *in vivo* co culture a system that's leading to induced the hepatic differentiation of BM-MSCSs, by the growth signaling hormones and growth factors secreted from the undamaged hepatocytes in the remaining intact hepatic tissue, leading to directed the undifferentiated injected cells towards hepatogenic cell lineage and the later

participates in acceleration of liver regeneration and to overcome `the liver severe injury being induced by the subtotal hepatoctomy process.

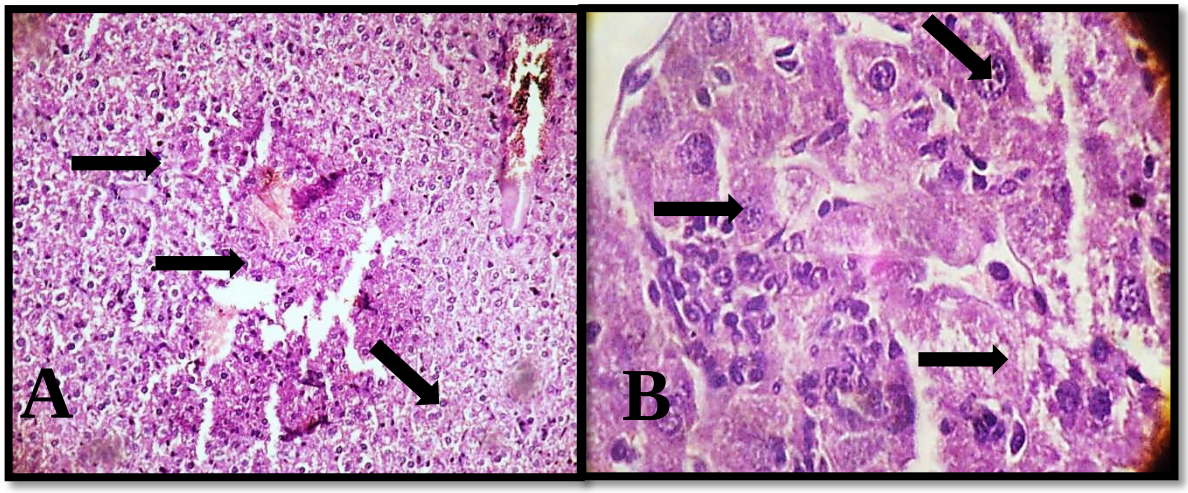


Figure (3-10): histological changes of group D (BM-MSCs transplantation group) showing the slight regeneration of liver tissue with a fewer lesions (arrows). (A: section under low power 100X; B: sections under high power 400X). (H and E staining).

Other possible explanation is that the hepatic damaged tissue leads to leakage of a vast number of growth factors and signaling hormones from the cytoplasm of damaged hepatocytes which may interfere directly with the differentiation of the mesenchymal stem cells to function as hepatocytes and participate in tissue regeneration, noting that there is a huge difference in the differentiation in vivo which regards is in this case and the in vitro differentiation strategy in both the environmental condition and in the amount and types of differentiation factors which may interfere with the process which may explained the difference in the time needs to BM-MSCs for hepatogenic differentiation *in vitro* which was recording as three weeks during which different sets of growth factors were used as mentioned before, and the *in vivo* hepatogenic differentiation which observed after ten days from the transplantation of the undifferentiated stem cells strategy. (Xiaodan et al, 2003).

The above results were also reported by Patrisia *et al* (2012) whow showed that the systemic administration of a novel human umbilical cored mesenchymal

stem cells population accelerates the resolution of acute liver injury. Thus the histological studies on the liver sections of transplanted mice with the human umbilical cord mesenchymal stem cells were shown much more rapid resolution of damaged liver tissue and less inflammation as compared to the undamaged liver mice group.

Hepatocytes or BM-MSCs treatment post to hepatectomy presents a unique, beneficial, dual-function strategy for hepatic protection and regeneration in the immediate post-subtotal hepatectomy period in mice. This strategy may offer potential treatment benefits for the enhancement of liver regeneration and recovery after large-volume hepatectomy for benign and malignant conditions and for living donor graft transplantation

Conclusions and Recommendations

Conclusions:

- It is possible to isolate and culturing mouse bone marrow mesenchymal stem cells (BM-MSCs) using adherent property of MSCs**.**
- Minimal essential medium (MEM) can be used successfully for isolation and maintenance of mouse BM-MSCs.
- The BM-MSCs can be differentiated in to hepatic cells via using combinations of specific formula of hepatogenic growth factors like (Hepatocyte growth factor (HGF), fibroblast growth factor 4 (FGF4), oncostatim M and dexamethasone).
- Three weeks period were the optimum time for the isolated BM-MSCs to differentiate in to hepatic cells line.
- Isolated BM-MSCs showed a positive reactivity toward the immunocytochemical analysis using positive MSCs markers (CD90 and CD106) and a negative reactivity towards MSCs negative markers (CD34 and CD45).
- Differentiated hepatocytes showed a positive reactivity by immuno-cytochemical analysis to albumin, alpha fetoprotein and cytochrome oxidase p 450 (CYP3A4).
- Transplanted hepatocytes were succeeded to improve the liver enzymes activity more efficiently than the transplanted BM-MSCs.
- Intrasplenic injection of hepatocytes were very helpful in accelerating the liver regeneration process as compared with BM-MSCs liver repair.

Recommendations:

- Investigation of the possible human Mesenchymal stem cells differentiation into hepatogenic cells lineage which may open a new field of first clinical trials of using stem in damaged tissue treatments in Iraq.
- Using matrix 3D (scaffolds) to provide a compatible environment for three dimensional tissue engineered liver formation.
- Studying the possible role for the use of co- culture or conditioned media methodologies for tissue engineering applications.
- Using other new cells source to study the hepatogenic capacity of stem cells like using the adipose tissue as a source of human mesenchymal stem cells.
- Studying the possibility of differentiation of MSCs toward other lineage such us osteoblasts using osteogenic differentiation media.
- Stem cell research offers unprecedented opportunities for developing new treatments for debilitating diseases for which there are few or no cures. Stem cells also present a new way to explore fundamental questions of biology, such as determining the basic mechanisms of tissue development and specialization, which will be required for the development of therapies.

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الخلاصة

صممت الدراسة للتحري عن قابلية الخلايا الجذعية المزنشيمية لنقي العظم على التحول الى خلايا كبدية وتقييم امكانية استعمال الخلايا الكبدية المتمايزة في علاج التلف الكبدي الناتج من عملية القطع الجراحي شبه الكامل لكبد الفأر. عزلت الخلايا الجذعية المزنشيمية في التجربة الاولى من نقي العظم من عظم الفخذ لذكور الفئران البالغ عمرها٤-٨ اسابيع باستعمال طريقة قابلية الخلايا المعزولة على الالتصاق. اظهرت الخلايا المعزولة تفاعلا ايجابيا لواسمات الخلايا الخاصة (CD markers) لل MSCs لكل من (CD105 , CD 90) بينما اظهرت الخلايا المعزولة تفاعلا سلبيا تجاه واسمات الخلايا الخاصة بالخلايا الجذعية المولدة لخلايا الدم (Hematopoietic stem cells) والتي تضمنت (CD 34, CD 45) عن طريق التشخيص المناعي الخلوي. بعدها عرضت الخلايا الجذعية المزنشيمية المشخصة لثلاثة اسابيع من بروتوكول التمايز باستعمال عدة انواع من عوامل النمو الخاصة بخلايا الكبد والتي تضمنت: عامل نمو الخلايا الكبدية (HGF) ,عامل نمو الخلايا الليفية نوع ٤ (FGF4) , Oncostatin M والديكساميثازون. اظهرت الخلايا المتمايزة تفاعلا ايجابيا لكل من الواسمات الخاصة بخلايا الكبد والتي شملت (الالبومين (ALB) والالفا فيتوبروتين (AFP) والسايتوكروم اوكسيدايز p450 (CYP3A4) عن طريق التشخيص المناعي الخلوي. اظهرالتحليل المناعي باستعمال تقنية الاليزا (للCYP3A4) وجود فروقات معنوية ($p\leq0.05$) في كميات انزيم السايتوكروم اوكسيدايز خلال الاسابيع الثلاثة من تجربة التمايز حيث سجلت النتائج اعلى كمية حوالي (65.27ng/ml) في نهاية الاسبوع الثالث من التجربة بينما القراءات الاقل كانت قد سجلت في الاسبوع الاول والثاني من تجربة التمايز (16.73) و (٣٣.٠٠) ng/ml بالتتابع.

تضمن الجزء الثاني من البحث دراسة امكانية استعمال الخلايا الكبدية المتمايزة كعلاج خلوي لتلف النسيج الكبدي المحرض من خلال الازالة الجراحية شبه الكاملة للكبد والتي عرضت فيها الحيوانات الى تلف بالغ في الكبد نتج عن ازالة معظم الكبد. وقسمت الفئران (اربع وعشرون فأرة) إلى أربع مجاميع على النحو التالي:.

المجموعه (A) كمجموعة السيطرة غير المعاملة. المجموعة الثانية (B) احتوت على فئران عرضت للتلف الكبدي الناتج من الازالة الجراحية شبه الكاملة للكبد وبدون استعمال اي علاج. المجموعه (C) تضمنت فئران عرضت لعملية التلف الكبدي وعولجت بحقن 0.7 ml من 5×10^5 من الخلايا المتمايزة للكبد عن طريق الطحال (intrasplenic) . المجموعة (D) تحتوي على الفئران التي عرضت لعملية التلف الكبدي و عولجت بحقن 0.7 ml من 5×10^5 من الخلايا الجذعية المزنشيمية المعزولة من نقي عظم ا لفأر. تم قياس كلا من وزن الجسم ووزن الكبد بعد مرور عشرة ايام على التلف الكبدي المحفز وزراعة الخلايا حيث اظهرت النتائج بأ ن نسبة وزن الكبد / وزن الجسم بمجموعة السيطرة A مقدارها ٠.٢٦ غرام ، في حين أظهرت مجموعة B

انخفاضا معنوياً($p\leq0.05$) مقارنة مع مجموعة (A) (٠.١٧غرام) ,أظهرت نتائج المجموعة C و D (٠.٢ و٠.١٩ غرام) على التوالي مقارنة مع المجموعة (ب) من تلف الكبد الناجم بدون خلايا الزرع. أظهرت نتائج فعاليات إنزيمات وظائف الكبد في المصل (ALT، AST و ALP) زيادة معنوية ($p\leq0.05$) 403، 226 و 36.21 IU /L على التوالي في مصل مجموعة (B) من الفئران التي تعرضت للتلف الكبدي المحفز، مقارنة مع مجموعة السيطرة (218، 125، و 16 IU / L) على التوالي. وكذلك أظهرت النتائج أن زرع خلايا الكبد المتمايزة في مجموعة C قد ادى الى تحسين فعالية الانزيمات الوظيفية للكبد (263، 146 و 17 IU / L) على التوالي بعد ارتفاعها عن طريق التلف الكبدي المحفز، وكان هذا التأثير أكثر فعالية من استعمال الخلايا الجذعية المزنشيمية لنقي العظم كعلاج في المجموعة (D) (280 ، 183 و 22 IU / L) على التوالي . اظهرت نتائج دراسة المقاطع النسيجية للكبد لكل من حيوانات التجارب من المجموعة (B) اللتي تعرضت لعملية التلف الكبدي المحفز،ظهورتلف كبدي بليغ تميز بظهور التهابات وارتشاح خلوي بالاضافة الى التفجج السايتوبلازمي وانحلال الخلايا الكبدية. بينما اظهرت المقاطع النسيجية للمجموعة (C) ان زراعة الخلايا المتمايزة الكبدية قد نجحت في تسريع عملية الاصلاح لنسيج الكبد التالف بالتحفيز، تميز الاصلاح بتقليل الارتشاح الخلوي وتقليل التلف النسيجي. وكانت أكثر فعالية من استعمال الخلايا الجذعية المزنشيمية لنقي العظم في علاج التلف الكبد المحفز حيث اظهرت المقاطع النسيجية وجود تجديد طفيف في أنسجة الكبد مع عدد أقل من الآفات. اظهرت النتائج ان لخلايا الكبد المتمايزة قدرةً على إصلاح التلف الكبدي المحفز جراحيا، بالإضافة إلى تحسين اوزان كلا من الكبد والجسم فضلا على ان هذه الخلايا عملت على تحسين فعالية إنزيمات وظائف الكبد في المصل وبشكل أكثر فعالية بكثير من الخلايا الجذعية المزنشيمية لنقي العظم المزروعة ، والتي وظفت للغرض نفسه.

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

سَنُرِيهِمْ آيَاتِنَا فِي الْآفَاقِ وَفِي أَنفُسِهِمْ حَتَّىٰ يَتَبَيَّنَ لَهُمْ أَنَّهُ الْحَقُّ ۗ أَوَلَمْ يَكْفِ بِرَبِّكَ أَنَّهُ عَلَىٰ كُلِّ شَيْءٍ شَهِيدٌ ﴿٥٣﴾

صدق الله العظيم

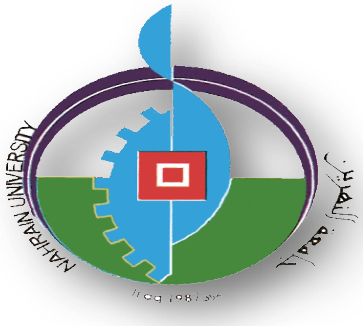
سورة فصلت الاية (٥٣)

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين

كلية العلوم



زراعة الخلايا الجذعية المزنشيمية في الفئران المصابة بتلف الكبد التجريبي

اطروحة مقدمة الى مجلس كلية العلوم جامعة النهرين كجزء من متطلبات نيل درجة

دكتوراة في فلسفة / التقانة الاحيائية

من قبل

زهراء كامل زيدان

بكالوريوس تقانة احيائية/كلية العلوم/جامعة النهرين/٢٠٠٥

ماجستير تقانة احيائية /كلية العلوم/جامعة النهرين/٢٠٠٨

إشراف

الاستاذ المساعد الدكتور

شهلاء مهدي صالح

الاستاذ الدكتور

شلال مراد حسين

٢٠١٥ آب

١٤٣٦ شوال