Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science
Department of Biotechnology

Tissue Culture of Mice Bone Marrow Mesenchymal Stem Cells Differentiation to Motor Neural cell

A Dissertation

Submitted to the College of Science / Al – Nahrain University as partial fulfillment of the requirements for the Degree of Doctorate Philosophy of Science in Biotechnology

By

Rafal Hussamildeen Abdullah

B.Sc. Biotechnology/College Science/Al-Nahrain University/2003

M.Sc. Biotechnology/ College Science/Al-Nahrain University/2007

Supervised by

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March 2016

Jamad Al- Alawal 1437

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

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صدق الله العظيم

سورة الانعام/اية 90

Supervisors Certification

We, certify that this dissertation entitled (**Tissue Culture of Mice Bone Marrow Mesenchymal Stem Cells Differentiation to Motor Neural cell)** was prepared by **(Rafal Hussamildeen Abdullah)** under our supervision at the College of Science, Al-Nahrain University as a partial fulfillment of the requirements for the degree of doctorate of philosophy in biotechnology.

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Date: / /2016

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We, the examining committee certify that we have read this dissertation entitled **(Tissue Culture of Mice Bone Marrow Mesenchymal Stem Cells Differentiation to Motor Neural cell)** and examined the student (**Rafal Hussamildeen Abdullah**) in its contents and that in our opinion it is accepted for the degree of doctorate of philosophy in biotechnology.

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Dedication

To the soul of my mother

To all my family members

To all who benefit from this work

I dedicate it

Rafal

Acknowledgement

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Chapter one

Introduction

And

Literature review

1.2 Literature Review

1.2.1 Stem cells

Stem cells are relatively unspecialized cells that have the ability to renew themselves and produce a variety of specialized cells after division without limit, as long as animal is still alive. These criteria were directed to produce many types of differentiated cells to repair damage in the body (Watt , 2011).

It is the original cell of an organism that has the ability to develop into many different types of cells in the body. Stem cells serve as a source of internal repair system; divide mainly without limit to replace other cells as long as the organism is alive. When divided, each cell has the potential to remain a stem cell or become one type of specialized like red blood cell or a brain cell (Hongbao *et al*.,2015).

Stem cells are divided on the base of their potency into totipotent, pluripotent ,multipotent, oligopotent and unipotend regarding their division ability and according to their source where they derive to embryonic, fetal, adult, amoniatic fluid, cord blood and induce pluripotent stem cell (Dubie, *et al*., 2014).

Pluripotent stem cells are distributed into two categories, endogenous pluripotent stem cells found during development that divide into embryonic stem cells (ESCs) and postnatal ("adult") stem cells (ASCs) and reprogrammed Pluripotent stem cells derived by either somatic cell nuclear transfer (SCNT) or insertion of specific genes into terminally differentiated cells (Young and Black, 2014)

Embryonic stem cells (ESC) are pluripotent cell that are used for transplantation therapies as they can be successfully differentiated into central nervous system cell types like neurons, astrocytes, and oligodendrocytes, in higher quantities. Induced pluripotent stem cells (iPSCs), have been recently identified as a potential source for clinical applications. The pluripotent stem cell types also have a high potential for *in vitro* platforms and can be used for

toxicology studies, drug screening, developmental research, and patient-specific drug research and diagnostics (Yla- outinen, 2012).

Stem cells obtained by destroying an embryonic human being in the first week of development, are called “embryonic stem cells” while Stem cells from adult tissues, umbilical cord blood, and placenta (often called adult stem cells) can be obtained without harm to the donor and without any ethical problemsuch cells have already demonstrated a great medical promise (Hongbao and Ma, 2014; Baddoo *et al.*, 2003;).

Stem cell regeneration needed in tissue requires balance and control in order to persist the out growth, transformation and cancer possibility, therefore studies on mice show a relationship between cell cycle progression and the self-renewal versus cell fate decision in embryonic and adult stem cells including modulation by the stem cell niche (Bianco *et al.*, 2001).

A new way for the differentiation of pluripotent stem cells into types of neuropeptidergic hypothalamic neurons using ‘self-patterning’ strategy, yields many cell types or more reproducible directed differentiation approach. These differentiated cells have characteristics and gene expression patterns with their cells *in vivo* (Merkle *et al.*, 2015).

Induced pluripotent stem cells isolated from somatic cells can be differentiated into neural stem cells and treatment of spinal cord injury with a full functionality which shows a therapeutic promise for neurons and glia (Lee-Kubli and Lu, 2015).

1.2.2 Embryonic stem cells Isolation, culturing and detection

(ES) cells are cells derived from the early embryo. They are supposed to be propagated indefinitely in the primitive undifferentiated state. They share these properties with embryonic germ (EG) cells and can differentiate into multiple types of somatic cells (Kumar *et al.*, 2000).

Embryonic stem cells are derived from the inner cell mass of blastocyst and their pluripotency maintained in culture contains some growth factors such as leukemia inhibitor factor. Embryonic stem cells were characterized morphologically as intracellular and extracellular by specific marker, by understanding markers, factors and conditions for the long culture embryonic stem cells without losing their pluripotency and genetic stability (Pauklin *et al*., 2011).

Human blastocyst-derived, pluripotent cell lines have normal karyotypes and express high levels of telomerase activity. Cell surface markers that characterize primate embryonic stem cells do not present in early lineages. After proliferation of cell *in vitro* for 4 to 5 months, these cells keep maintaining the developmental potential to form trophoblast and derivatives of all three embryonic germ layers, including gut epithelium (endoderm); cartilage, bone, smooth muscle, striated muscle (mesoderm); neural epithelium, embryonic ganglia, and started squamous epithelium (ectoderm) (Keller, 2005).

Moues embryonic stem cells from more than 30 of the discovered represent a basis for establishing an *in vitro* mammalian model for the development of cell repair and clinically relevant population of cell therapy. The unlimited capacity of esc to multiplication itself concern the risk of tumor formation, potential immune rejection, and the risk of differentiation into unwanted cell (Tachibana *et. al*., 2013).

Totipotent cells, such as zygote of mammalian have the ability to differentiate to all body cells in human about 200 cell kinds. Therefore human disease results from a defect in the cells of this stage.

Pluripotent cells are located in the inner mass of gastrocyt covered by trophectoderm . These cell isolated and propagated as embryonic stem cells that have the ability to differentiate into ectoderm, endoderm and mesoderm that latterly forms mesenchymal stem cells.

Multipotent stem cells have retracted the capacity of differentiation towards the lineage of related cells such as hematopoitic stem cell which can form blood cell but not brain and liver. Oligopotent cells are able to form few cell type of specific lineage such as lymphocyte stem cells differentiated to basophil, neutrophil, eosinophil, monocyte and thrombocyte. Unipotent cell like progenitor cell of specific tissue cells are able to differentiate into only one kind of cells (Singh *et al*., 2014).

Blastocyst trophectoderm layer are isolated and maintained *in vitro* under the presence of fibroblast growth factor 4, heparin, and a feeder layer of mouse embryonic fibroblast cells, These immortal stem cell have the capacity to be differentiated into multiple cell types *in vitro* (Tanaka,2006).

For studying the ESC blastocyst derived from mouse, it was isolated and cultured for three days until the inner cell mass reached the outgrowth stage, then the cells were disaggregated and cultured every 3 day until the colonies of ES are appeared. The colony positive cells were fixed and stained for alkaline phosphatase. After culturing from 6-9 days, the EC appeared like flat monolayer, have strike boundaries and no distinguish cytoplasm with a few nuclei (Hashemi- Tabar, 2005).

The specific marker of embryonic stem cell was divided into lectines and peptide marker , cluster of differentiation markers, pathway related markers and transduction markers, But unfortunately different cell types, specially tumor cell share similar characteristics, The marker-based flow cytometry (FCM) technique and magnetic cell sorting (MACS) are the most effective cell isolating methods (Yusuf, *et al*. 2013).

pluripotent stem cell technique like flow cytometry, reverse transcription polymerase chain reaction and using immunohistochemistry technique for detection of specific markers such as CD90,CD73,Sca-1,CD44,CD29, Vimentin and absence of CD45. They also express SSEA-1, Oct4 , Nanog, Sox-2,

ABCG2, Nestin, β-III tubuline, OTX-2, MEF-2, Mesp-2,GATA-2,GATA-4, alpha amylase and PDX1. (Hashemi-Tabar, 2005).

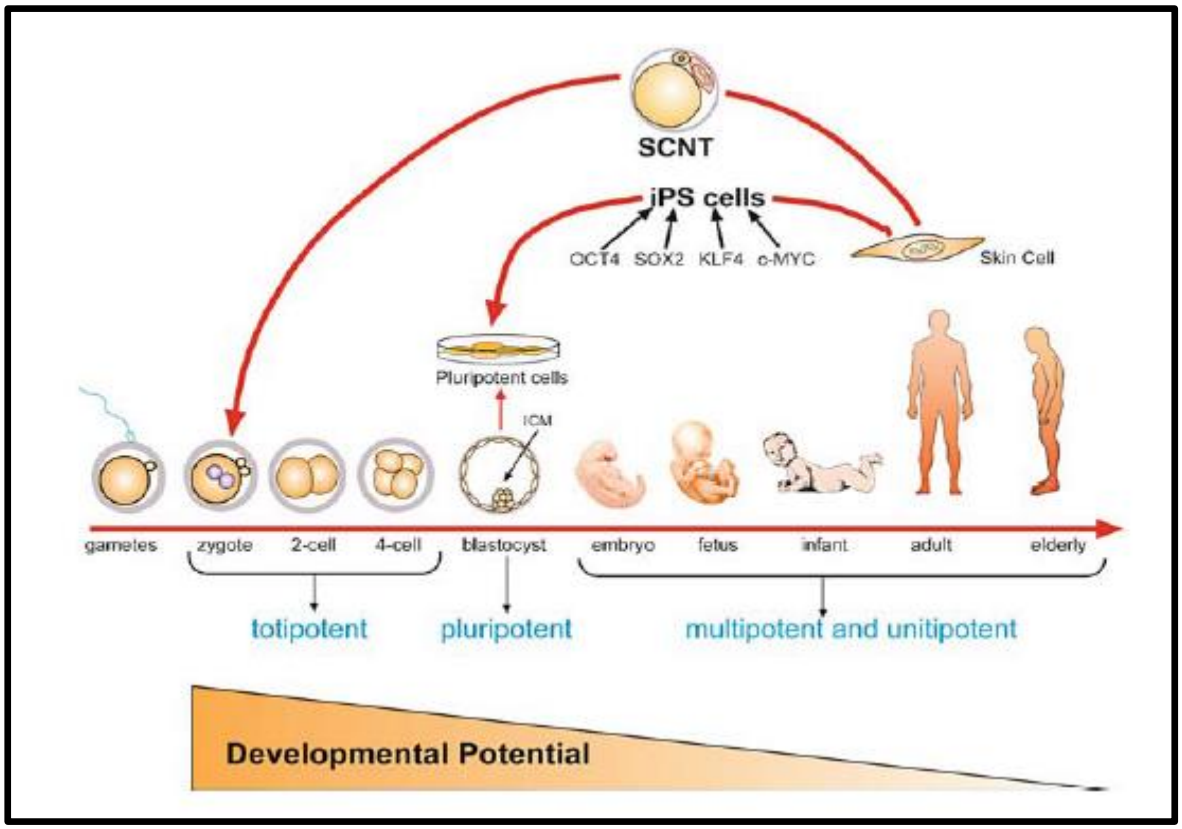


Figure (1-1): Types of stem cell (Mitalipov and Wolf, 2009).

1.2.3 Adult stem cells

Stem cell renewal and differentiation controlled by many factors are divided to intrinsic and extrinsic. The intrinsic cell plasticity is the expression of pluripotency genes and enrichment of biological functions involved in the embryogenesis and organogenesis which is most prominent in human skin derived precursor cell and least in human adipose derived mesenchymal stem cells (Kock *et al*., 2013).

Reprograming differentiated cell into pluripotent cell is a new approach for studying disease and for generating new therapy, a great deal of research failed in this application but some of it succeeded by cloning nucleus of differentiated

cell into ESC, excreted nucleus, then cells were controlled to multiplication and cell were formed homogenous kind. (Mitalipov and Wolf, 2009; Rodolfa and Eggan, 2006).

Extrinsic, or also called the nich, is a three dimensional environment biochemical and mechanical of soluble factors, Adult stem cells protect, control their renewal and differentiation capacity by cells and extracellular matrices, It is located either near or far the recruitment point required cell immigration (Diaz-Flores *et al.*,2006). De cellularrized tissue matrices and synthetic polymer niches are being used to study stem cell attraction homoeostasis and repair and integration *in vivo* for therapeutic benefits (Stanton and Peng, 2010).

1.2.3.1 Mesenchymal stem cells isolation, culturing and detection

Mesenchymal Stem Cells (MSCs) are differentiated mainly from mesoderm embryonic germ layer give rise to skeletal and connective tissues, and also able to differentiate into neural and myogenic cells (Divya *et al.*, 2012).

They are also isolated from many adult tissues like stromal bone marrow, marrow aspirates, periosteum, trabecular bone, synovium, skeletal muscle and deciduous teeth and adipose tissue .These cells have the capacity to differentiate into connective tissues including bone, fat, cartilage and muscle. It has the ability to expand in culture while maintaining their multipotency (Niibe *et al*., 2011; Yang *et al.*, 2011). MSCs have plastic adhesion ability to express CD105 ,CD90 and lack the expression of CD45, CD34,CD14 or CD11b, CD19 and HLA-DR surface molecules and must differentiate to osteoblasts, adipocytes and chondrocytes *in vitro* (Sandhaanam, *et. al*, 2013).

MSCs derived from umbilical cord have a potential to differentiate into multiple lineages of mesoderm in addition to ectodermal and endodermal lineages by crossing germ line barrier so umbilical cord MSC has varying

degrees of neural differentiation from batch to other simple ones. While other batches require an extensive exposure to growth factors, therefore, it is hypothesize that human umbilical cord MSC contain multiple progenitors type with varying neurogenic potentials that vary from one batch to another (Dominic *et al*. 2006).

Yang and Zhang (2009) reported that human adipose derived stem cells *in vitro* differentiated into motor neurons using retinoic acid and sonic hedgehog after 6h induction showed neural morphologically and positive beta-III tubulin, choline acetyletransferase and neuron specific enolase marker. Reverse transcriptase polymerase chain reaction indicates a ventral spinal fate (Nkx2.2, Pax6, Hb9 and Olig2) and a candidate in the cellular therapy for motor neurons**.**

Many studies in Iraq were focused on the differentiation of mesenchymal stem cell into functionally active differentiated cell, differentiation of MSC used in the treatment of diabetic disease like induction of Islet-like functional cells can be differentiated from bone-marrow mesenchymal stem cells (MSCs), which may be a new procedure for clinical diabetes stem -cell therapy, these cells controlled blood glucose level in diabetic rabbits as the effect of insulin (Mohamed, *et.al.*2012). The endogenous regeneration stem cell of the same tissue into functionally active cell like the differentiation of pancreatic cell from pancreatic duct epithelium in rats (Jaumah *et.al*., 2009). Differentiation of multipotent stem cell of organs have potential capability to differentiated into active cell or other organs, like differentiation of hepatic oval stem cell into pancreatic islet cell (jaumah, *et.al.* 2008). Many studies focused on the differentiation of mice mesenchymal stem cell into beta cell of Langerhans islands using different protocols and studying its activity *in vivo* and *in vitro* (Al- Qaisy ,2013) (Majeed, *et.al.*, 2015).

Retinoic acid used in the differentiation of human umbilical cord derived mesenchymal stem cell to form neural cell (Mutlak, 2007).

Rat bone marrow cells differentiated into myocardial cell in vitro and injected into rat body to study its functionality (Mnati, 2007).

Tendon cells were produce from mesenchymal stem cells of lipid tissue in the house in vitro and in vivo, showing promises results for the treatment of tendom diseases in horses (Al-Bayaty,2011).

Al-Shammari, *et.al*. (2015) differentiate mouse bone marrow MSCs into chondrocyte cells in 2D and 3D culture ,which represent a promising approach for treat cartilage disease in human latterly.

Spinal cord injury from wars and bombs and other accident represent a big problem in Iraq, therefore studies on using stem cell for regenerative spinal cord is necessary. Hammadi, *et.al*. 2012 use stem cell isolated from the peripheral blood of spinal cord injured patients and reject it in there spinal canal, 43% of the patients improved; American spinal injury association ASIA score shift the level of injury from A (complete, no motor or sensory function preserved in the sacral segments S3-S5) to B (incomplete, sensory but not motor function is preserved below the neurological level and include the sacral segments S4-S5) in 88 and from A to C (incomplete, motor function is preserved below the neurological level) 32. The best results were achieved in patients treated within one year from the injury.

1.3 Neuron cell

The nerve system is composed of two cell type neurons and gilia. Neurons conduct an electrical current and have a role in relaying information throughout the nerves system while gilia cells regulate the neurons activity, synaptic development as well neuronal migration and outgrowth process (Shaham, 2005). Neurons communicated with chemical transmitter are called synapses. They are composed of billions of cells in the brain which differ in size, length and shapes to determine the different function (Caplan, 2007).

There are three parts in the neurons cell: **first** cell body which contains a single nucleus, extensive rough endoplasmic reticulum, a Golgi apparatus, and mitochondria surround the nucleus, large numbers of neurofilaments and microtubules to organize the cytoplasm. **Second** dendrites are short, highly branching cytoplasmic extensions that are tapered from their bases at the neuron cell body to their tips. Dendrites usually receive information from other neurons or from sensory receptors and transmit the information towards the neuron cell body and **third** is axon which is a long cell projection extending from the neuron cell body (Vanputte, 2010).

1.4 Motor neuron

Motor neurons are specialized nerve cells in the brain and spinal cord that transmit the electrical signals to muscle and generate movement. There are two groups of motor neuron, the upper motor neuron at the top of the brain in motor cortex which is extended down in the spinal cord to connect the lower motor neurons which travel out of the spinal cord and connect the muscle (Talbot and Marsden, 2008).

Neuron are polarize cells that have dendrites and axon extend long distances from the cell body to form synapses that mediate neuronal communication, cell lipid and protein essential for controlling the cell shape and synapses (Ramirez and Couve, 2011).

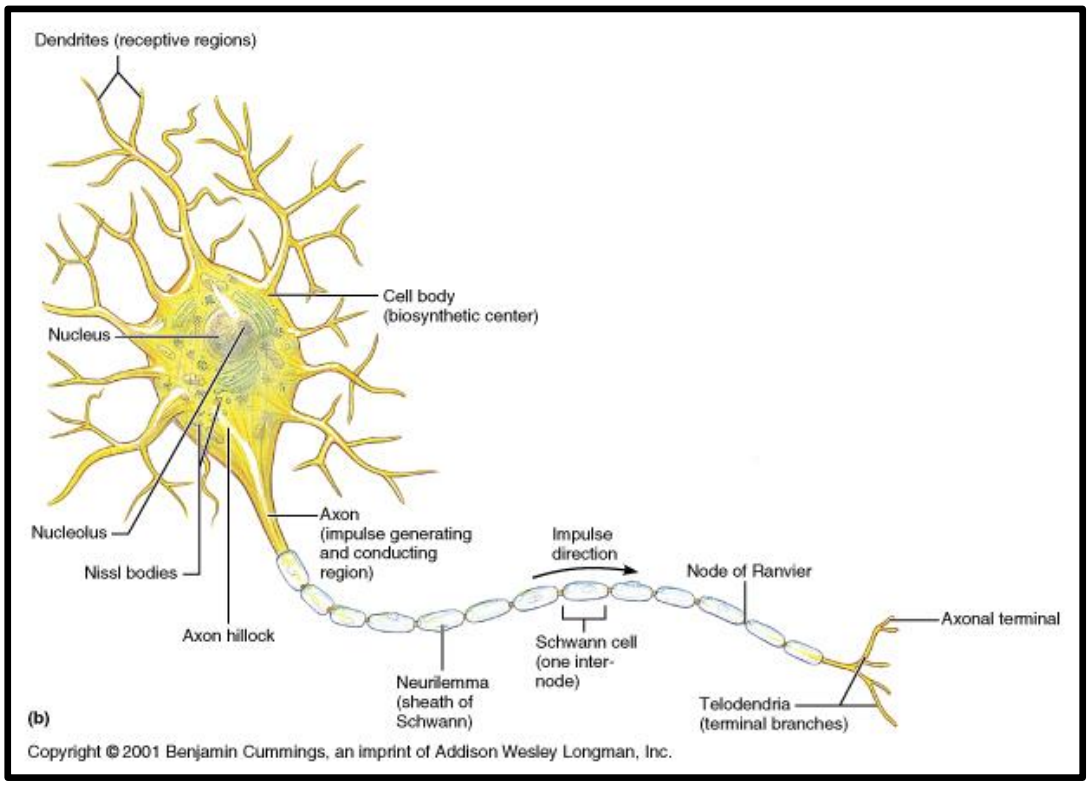


Figure (2-1): Atypical motor neuron cell (Cummings, 2001).

1.5 Differentiation Mesenchymal stem cell into neuron

Mesenchymal stem cells were found in the amniotic fluid of mammalian and readily used in cell therapy, regenerative medicine and tissue engineering. Amniotic fluid mesenchymal stem cells have the potential ability of differentiation into neural cells using differentiation reagents and, therefore, they are suitable alternative source of stem cells in neurodegenerative disease (Kim *et al.*, 2013).

Bone marrow mesenchymal stem cells are differentiated into neural cells, hepatocytes and myocytes, by expanding their differentiation potential. The differentiation into neural cells require either tissue plasminogen activator TPA, forskolin, 3- isobulet-1-1-methel xanthin IBMX, fibroblast growth factor FGF-1, or retinoic acid and 2-mercaptoethanol and cytokines (Zeng *et al.*,2011; Scintu *et al.*, 2006).

Mesenchymal stem cells are differentiated into dopamine neurons cell using a cocktail of factors such as sonic hedgehog, fibroblast growth factor 8, and basic

fibroblast growth factor. The addition of brain derive neurotropic factor to the culture media was increase the electrophysiological properties of these cell (Trzaska and Rameshwan, 2011; Guo *et al*., 2005).

1.5.1. Motor neurons differentiation *in vitro*

- **Sonic hedgehog**

Sonic hedgehog (Shh) is one of the three hedgehog homologs in vertebrate, and it is essential for the embryonic development. Shh binds to Patched (Ptch1) receptor that activates Smoothened (Smo) leading to the activation of Gli2, which regulates the transcription of target genes that include Gli1 and Ptch1. GANT58 and GANT61 molecules have been reported to inhibit Shh signaling (Stanton *et al*.,2009). Shh is blocked by a small molecule called 'robotnikinin' and rescued by it is agonist of Smo which is immediately downstream Shh receptor Patch (Wu *et al*., 2004).

Purmorphamine is (Shh analoguse) a small molecule agonist of hedgehog signaling resulting in the up and down regulation of its downstream target genes. It is used in treatment of bone related disease by inducing the osteogenesis in multipotent mesenchymal progenitor cells and neurodegeneration diseases (Stahles, 2008).

- **Retinoic acid**

Retinoic acid (RA) is a lipophilic, biologically active metabolite of vitamin A with a low molecular weight. Its function in nucleus is to induce gene transcription, and to induce regeneration of several tissue and organs (Maden and Hind, 2003). RA is non peptidic small lipophilic molecule that acts as a ligand for nuclear RA receptors (RARs) converting them from transcriptional receptors to activators (Rhinn and Dolle, 2012). RA is a morphogen and its embryonic distribution correlates with the neural differentiation and positional specification in the developing central nerve systems. RA promoted both neural differentiation and caudalization in a concentration dependent manner. The high

concentration of retinoic acid induces a dorsal phenotype while a low concentration induces a more ventral phenotype, this is due to a higher expression level of the N- terminus of sonic hedgehog protein, and these confirm that RA concentrations strictly and simultaneously regulate neutralization and positional specification during the differentiation of mouse embryonic stem cells (Okada *et al.*, 2004).

RA is frequently used to differentiate stem cells to neural cell using a wide variety of culture conditions in prolonging exposure and intracellular communication of high level cells in culture. These conditions increase the neural gene expression and appearance of neuron. RA ability to direct the differentiation to a neural lineage is blocked by GSK3β activity which regulates WNT signaling. These indicate that the RA differentiation of stem cells to neural cells is not auto mouse program but multi stage program that requires an intercellular input (Tonge and Andrews, 2010).

- **Nerve growth factor**

Nerve growth factor (NGF) is a cluster of three cysteine disulfides and two very extended but distorted β-hairpins and a prototype of a large family of neurotrophins. NGF binds as dimer to two cell surface receptor types expressed in neural and non- neural cells. Its first discovering was in 1950 in the sensory and autonomic nervous system, then in central nervous, endocrine and immune systems. NGF plays a role during development and adulthood ensuring maintenance of phenotypic and functional characteristics of neuron and immune system (Aloe *et al.*, 2012).

- **Purmorphamine**

It is a purine derivative that has the ability to activate the hedgehog pathway which is an important regulator of embryonic patterning tissue regeneration, stem cell renewal and cancer growth, and it affects the osteoblast differentiation (Sinha and Chen, 2005).

1.5.2 Motor neuron detection markers

- Microtubule associated protein 2 (MAP-2) was defined as static, structural protein, with sensitizing to many inputs, It has dynamic functions in the growth, differentiation, plasticity of neurons, neuronal responses to growth factors, neurotransmitters, synaptic activity, and neurotoxins (Johnson and Jope, 1992).

MAP-2 is a neuronal phosphoprotein and it functions as a net of microtubule growth and actin cross-linking and bundling *in vitro,* while *in vivo* MAP-2 interacts with cytoskeleton and its function disrupted by cAMP-dependent protein kinase leading to MAP2 localization to the actin cytoskeleton. A single mutation in its sequence gene disrupts MAP-2 function while a double or triple point mutation promotes MAP2 localization to the actin cytoskeleton. There are specific phosphorylation states that may enhance the interaction of MAP-2 with the actin cytoskeleton, thereby, providing a regulated mechanism for MAP-2 function within distinct cytoskeletal domains (Ozer and Halpan, 2000).

MAP-2 was present in axons of spinal motor neurons, but it was not detected in axons of the white matter tracts of spinal cords and in the majority of axons of the dorsal root. It was present in the dendrites, cell bodies and axons of the cell (Papasozomenos *et al*., 1985).

Lepski *et al.*, (2010) reported that differentiation of MCSs into a mature neuron induces expression MAP-2 and also neuronal-specific nuclear protein (NeuN), and neurofilament 200 and it produces neurotransmitters.

- Acetyl choline transferase is a single stranded globular protein, synthesized in the perikaryon of cholinergic neuron and transported to the nerve terminals as solution and non- ironically membrane bounded. CHAT is the enzyme responsible for the biosynthesis of acetylcholine in the cholinergic neurons of the central and peripheral nerves system (Houser *et al*., 2004; Oda, 1999).

CHAT function is to transfer an acetyl group from acetyl-coenzyme A to choline to form the neurotransmitter acetylcholine. It is essential for the development and neuronal activities of cholinergic systems involved in many fundamental brain functions (Govindasamy *et al.,* 2004).

It is used as a marker in the detection of motor neuron and used in the detection of differentiated stem cells into motor neuron spinal cord precursor cells and electrophysiological studies or ion exchange when co cultured with muscle cell (Macdonald, *et. al* .2003).

1.5.3 Measurement of motor neuron activity

- **Patch clump technique** it is a high-resolution recording technique of an ionic flowing through a cell's plasma membrane. Motor neuron cell differentiated from stem cells were functionally studied using a patch clamp technique to proof the activity of the cell after the addition of differentiation media containing RA and Shh (Lee *et al*., 2014).

The plasma cell membrane contains phospholipid which is lipophilic and hydrophilic residue. The concentration gradient induces the diffusion of the particles from a high to low concentration. The imbalanced charge on either side of the membrane causes an electrical potential over the membrane.

Electrophysiological technique records ion fluxes across membrane indirectly using extracellular electrode (Molleman, 2003).

- **FM dye**

FM1-43, a styryl dye is widely used to study endocytosis, vesicle trafficking and organelle organization in living eukaryotic cells and plant cells (Bolte *et al.,* 2004). This dye appears to label the membranes of recycled synaptic vesicles by being trapped during endocytosis. The distaining evidently reflects an escape of dye into the bathing medium from membranes of exocytosing synaptic vesicles (Henkl *et al*., 1996).

1.5.4 HPLC for Acetylcholine detection in culture.

Acetylcholine is a neurotransmitter at neuromuscular junctions responsible for synapses in the ganglia of the visceral motor system, and at a variety of sites within the central nervous system. Acetylcholine is synthesized in nerve terminals from acetyl coenzyme A (acetyl CoA, which is synthesized from glucose) and choline, in a reaction catalyzed by acetylcholine transferas. Choline is present in plasma at a concentration of about 10 m*M* and is taken up into cholinergic neurons by a high-affinity Na^+/choline transporter (Purves *et al.*, 2001).

Acetylcholine receptor is a cell surface receptor involved in cell to cell signaling due to a nicotinic acetylcholine which is a component of vertebrate skeletal neuromuscular junction and it provides signaling between the nerve and the muscle (Stroud and Finer- Moor, 1985).

High-performance liquid chromatography is a technique in analytical chemistry used to separate the components in a mixture, to identify each component, and to quantify each component. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly and differently with the adsorbent material causing different flow rates for the different components and leading to the separation of the components as they flow out the column (Kupiec, 2004).

Liscovitch *et al.* in 1985 developed a new method for the separation of acetylcholine from crude solution containing it using HPLC. The gradient system with normal phase silica column is stationary phase, with a mobile phase of acetonitrile, water, ethyl alcohol, acetic acid and sodium acetate.

Choline is separated from acetylcholine using phosphorylcholine chromatography, electrochemically quantified from post column reactor by covalently bonded acetylcholine esterase and choline oxidase, This is an

effective method for recovering 72% choline and 79% acetylcholine (Bertrand *et al*., 2006).

In vitro kinetic study of acetylcholine and acetylthiocholin uses choline esterases hydrolysis dependent on the substrate concentration and time and also production of choline and thiocholine and acetic acid (Stepankova *et al.,* 2005).

1.6 The motor neuron axon elongation

Each neuron has a single axon that extends from the cell body. The area where the axon leaves the neuron cell body is called 'the axon hillock'**.** Each axon has a uniform diameter and may vary in length from a few millimeters to more than a meter. Axons of motor neurons conduct action potentials away from the CNS, and axons of sensory neurons conduct action potentials toward the CNS. Axons also conduct action potentials from one part of the brain or spinal cord to another part. An axon may remain un branched branch to form collateral axons**.** Axons can be surrounded by a highly specialized insulating layer of cells called 'the myelin sheath' (Shaham , 2005).

Actin destabilization is an early step in specifying axon identity in young neurons. Once neuritis were becomes differentiated, it will form the axon in neural during development. Plasma membrane ganglioside sialidase (PMGS) and other associated molecules are required for axon formation but they do not affect dendrite formation (Jiang and Roa, 2005).

Axon is a cable for transforming action potential once it is generated. Many inherited and acquired neurological disorders caused by axon dysfunction. Several complex operations that not only control signal processing in brain circuits but also neuronal timing and synaptic efficacy are determined by intrinsic ion voltage and geometrical properties (Debanne *et al*.,2011).

Axon initial segment is a specialized compartment enriched with Na (+) and K (+) channels which initiate the action potential and also T and R type Ca+ are concentrated, contribute to local sub threshold membrane depolarization and thereby influence action potential initiation. T-type Ca (2+) channels were

down regulated by dopamine receptor activation acting via protein kinase C, and reduced neuronal output without altering AIS Na (+) or somato dendritic T-type channel activity and could be mediated by endogenous dopamine sources present in the auditory brainstem (Bender *et al.*,2010).

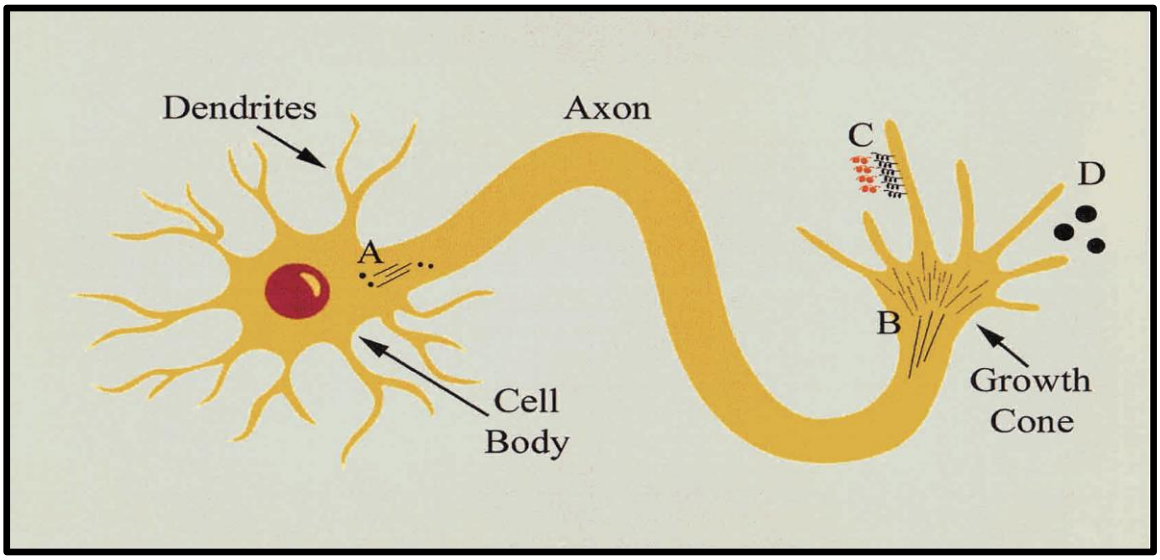


Figure (3-1): Structure of axon growth cone in neuron cell (Vanputte, 2010)

Neuronal elongation cone growth of axon is occurs through cytoskeletal dynamics involving the polymerization of actin and tubulin subunits at the tip of the axon. The axons and growth cones are generated forces through cytoskeletal dynamics (kinesin, dynein, and myosin) forces inducing axonal elongation, and axons lengthened by stretching (Suter and Miller, 2011). The formula for determining the rates of directed axonal growth was calculated (elongation or retraction) using measurements of growth cone movements. The axonal length measured between elongation and non-elongation growth cone movements reflects the detailed cellular growth mechanisms. An axonal elongation during development includes sending out long cellular projections longer than the cell diameters that will eventually form synapses. This process involves the *en masse* movement of the cytoskeleton in the distal axon, whereas

the cytoskeletal network near the cell body remains constant. The microtubule-based motor dynein has a role in axons elongation (Roossien *et al*., 2014).

Studying immunostaining showed a significant increase in the length of neurite axon after the addition of 50 ng /ml nerve growth factor and the neuritis expressed active TrkA receptors which is S-100-positive unsheathing cells were also present (Martin *et al*., 2002).

The axon growth rates are regulated to control over path finding and this regulation was a key step in the formation of functional synapses and the proper patterning of the nervous system. The rate of axonal elongation is increased by factors such as netrin-1 and nerve growth factor, which stimulate axon outgrowth using incompletely defined pathways. To clarify the mechanism of netrin -1 and NGF stimulated axon growth, the intra– axonal protein translation is required for stimulation, but not basal axon outgrowth . Par3 mRNA is localized to develop axon, and NGF and netrin-1 trigger its local translation (Hengst *et al.,* 2009).

1.7 Spinal cord

The spinal cord is a cylinder of nervous tissue that begin at the foramen magnum and pass through the vertebral canal as far as the inferior margin of the first lumbar vertebra (L1) is concerned . In adults, it averages about 1.8 cm thick and 45 cm long. It occupies only the upper two-thirds of the vertebral canal; the lower one third is described shortly. The cord gives rise to 31 pairs of spinal nerves that pass through the intervertebral foramina. The function of spinal cord is conduction information up and down the cord, locomotion and reflexes (Saladin, 2004).

The spinal cord is composed of gray matters and white mater. Horizontal sections of the spinal cord show the gray matter being arranged in a butterfly shape or H shape depending on the level of differentiation and enclose the central canal, while the white matter (consisting of mainly the axon and glial cells) surrounds the gray matter except in some spaces (Watson *et al.,* 2009).

1.7.1 Spinal cord disease

- **Spinal cord injury**

Spinal cord injury is a loss of nervous tissue and consequently loss of motor and sensory functions. The loss of function is induced to a degree that an independent life can be guaranteed. Stem cell represents a promising way for treatment of this injury because of theself-renewal and their ability to become any cell in an organism like neuron and glia cell. Neuroprotective and axon regeneration promoting effects have also been credited to transplanted stem cells (Tewarie *et al.*, 2009).

Spinal injury tissue loss when occurs in the white matter and myelinated fiber tracts that carry sensation and motor signals to and from the brain is called "myelopathy" while if it occurs in the gray matter, it causes a segmental loss of interneuron and motor neuron and restricts therapeutic options (Ronaghi *et al.*, 2010). Pluripotent stem cells were used in the treatment of SCJ isolated from different sources and evaluation of their differentiation potentials and tumorigenic activities in different contexts are very important and effective for cell transplantation therapies. Pluripotent stem cell is used to treatment with a remyelination and induction of the axonal regrowth of host $5HT^{+}$ serotonergic fibers and promoting loco motor function recovery (Tsuji *et al.*, 2010).

Mesenchymal stem cells treatment for spinal cord injury improves functional recovery and enhances astrogliosis and levels of inflammatory cytokines in rats using balloon-induced spinal cord compression lesions. Transplantation of mesenchymal stem cells is limited by poor survival of the cells in the damaged nervous tissues. A number of studies have tried to improve MSC transplant survival with limited or short-term effects. The survival enhancing strategies include optimizing timing of transplantation, suppressing the immune response, transplantation within a scaffold to limit a noikis, reducing reactive oxygen

species and macrophages, genetically modifying MSCs, and electrical stimulation of the spinal cord (Ritfeld and Oudega , 2014; Cui *et al*., 2014).

- **Amyotrophic lateral sclerosis (ALS)**

It is a neurodegenerative diseases are a growing public health challenge, fatal and an incurable disease. It affects many cell phenotypes besides motor neuron and outside nerve system. Pluripotent stem cells derived from patient provide a new approach in the treatment of ALS with the discoveries of new genes and animal models (Cova and Silani , 2010). MSCs treatments are efficient in generating neuronal and non-neuronal cell replacement, trophic factor delivery, and modulation of the immune system (Lewis and Suzuki, 2014).

MSCs were isolated from bone marrow expanded in vitro containing the autologous cerebrospinal fluid (CSF) and directly transplanted into the spinal cord at a high thoracic level in a surgical procedure. Patients were monitored before and after transplantation by clinical, psychological, neuroradiological and neurophysiological assessments. There were no transplant-related toxicity and no structural changes in either the brain or the spinal cord and no tumor was formed (Mazzini *et al.*, 2010).

1.8 **Matrix three dimension**:

The development of 3D scaffolds, supporting structures or matrices that induce cells to form functional tissues, is one of the main objects of tissue engineering and regenerative medicine research (Langer and Vacanti, 1993).

Scaffold plays a unique role in tissue regeneration and repair. During the past two decades, many works have been done to develop potentially applicable scaffold materials for tissue engineering. Scaffolds are defined as three-dimension porous solid biomaterials designed to perform some or all of the following functions: (i) promote cell-biomaterial interactions, cell adhesion, and

extracellular matrix deposition, (ii) permit sufficient transport of gases, nutrients, and regulatory factors to allow cell survival, proliferation, and differentiation, (iii) biodegrade at a controllable rate that approximates the rate of tissue regeneration under the culture conditions of interest, and (iv) provoke a minimal degree of inflammation or toxicity *in vivo* (Langer and Tirrell, 2004).

The developing scaffolds with the optimal characteristics, such as their strength, rate of degradation, porosity, and microstructure, as well as their shapes and sizes, are more readily and reproducibly controlled in polymeric scaffolds (Fuchs, *et.al*, 2001)

Mechanical support against compressive or tensile forces present inside the physiological environmental system of the human body should be granted depending on the tissue. Then scaffold is designed for enough mechanical strength and stiffness needed to initially contrast wound contraction forces and later to guarantee a perfect reconstruction of the tissue. For this reason scaffold degradation profile must be designed so that it can support the tissue formation until neotissue (cells and extracellular matrix without vascularisation) is developed (Ghosh *et al*., 2008)

Injecting cell suspension in vivo without scaffold has been sometime utilized as a technique for replacement (Mikos *et.al*., 1994; Mooney, *et.al*. 1996 ;Yang *et. al*., 2006) even if it presents the disadvantage that it is quite complicated to control the placement of transplanted cells and most of the mammalian cells reveal an anchorage-dependent behavior and they can handily survive without a proper adhesion support. Therefore, the primary function of a scaffold is tissue conduction and , thus, it must allow cells attachment, migration onto or within the scaffold, cells proliferation and differentiation.

The few scaffolds that displayed biological activity have induced regeneration of tissues and organs that do not regenerate spontaneously have been referred to as “regeneration templates”. Biological scaffolds are derived from human, animal tissues and synthetic scaffolds from polymers. The first

biologically active scaffold was synthesized in 1974; its degradation behavior exceptionally low antigenicity *in vivo*, as well as its thrombo resistant behavior *in vitro,* were described (Yannas *et al*., 1975).

The initial patent describing these scaffolds was granted in 1977 (Yannas *et al*., 1977). Principles for synthesizing a biologically active scaffold, including the critical importance of the degradation rate, were described in detail in 1980 (Yannas and Burke, 1980). The first reports of induced regeneration of tissue were in an adult (dermis) by a scaffold in animals (Yannas and Burke, 1982) and humans (Burke *et.al.,* 1981), the peripheral nerve regeneration across a gap of unprecedented length (Yannas *et.al*., 1985).

It is important to underline that the ideal scaffold design does not exist but each tissue requires a specific matrix design with defined material properties. Finally scaffolds should be manufactured in a reproducible, controlled and cost effective fashion with the possibility to include biological components, such as cells and grow factors.

Scaffolds are porous, ideally degradable structures fabricated from various materials, which can be divided in four groups: (1) biogenic (collagen, alginate, glycosaminoglycans, fibrin), (2) semi synthetic, (3) synthetic (polyglycolide (PGA), polylactide (PLA), polylactide coglycolide (PLGA) and (4) inorganic (hydroxyapatite, tricalcium phosphate, titanium alloys) (Griffith and Naughton, 2002; Knight and Evans, 2004).

Biomaterials can be manipulated to direct the growth of cells *in vitro* and *in vivo* (Sittinger *et al*., 1996). The ideal biomaterial for a scaffold would selectively interact with the specific adhesion and growth factor receptors expressed by target cells required for the repair of damaged tissues (Griffith and Naughton, 2002). This “smart” scaffold could guide migration of the target cells and stimulate their growth and differentiation and extracellular matrix production (Knight and Evans, 2004).

Biogenic materials may most closely simulate the native cellular milieu, but their limitations include large batch-to batch variations upon isolation from biological tissues, as well as restricted biomechanical properties, not to mention the risk of viral infection and antigenicity (Rosso *et al* .,2005), Additionally, matrix scaffolds based completely on synthetic materials such as poly (lactic-coglycolic acid), poly (ethylene glycol), poly (epsilon-caprolactone) have also been fabricated (Lee *et al*.,2008) .

1.8.1. Collagen

Collagens are a large family of proteins that are widespread in the body and are important for a broad range of functions, including cell adhesion, cell migration, tissue scaffolding, cancer, angiogenesis, tissue morphogenesis and tissue repair.

It is the principal tensile element of vertebrate tissues such as tendon, cartilage, bone and skin. It is located in basement membranes and in the kidney glomerulus where it functions in molecular filtration and it occurs in the extracellular matrix of vertebrate cell serving to store elastic energy during muscular deformation, transmit stored energy into joint movement, and transfer excess energy from the joint back to the attached muscles for dissipation, transfer stress in order to either up - or down - regulate tissue metabolism as a result of changes in mechanical loading and prevent premature mechanical failure of tissues by limiting deformation of most ECMs and organs (Silver ,2009).

Collagen is a super coiled three poly peptide chain of proline formed in helical structure of collage. These coiling make cell attach to the surface of collagen matrix (Bella *et al.*, 1992).

Collagen families of proteins contain 19 formally defined collagens and an additional ten proteins that have collagen-like domains. The most important

collagens form extracellular fibrils or network-like structures, but the others fulfill a variety of biological functions (Prockop and Kivirikko, 1995).

Collagen fibrils range from a highly disordered arrangement in bone, the networks around blood vessels to a highly ordered orthogonal array in the cornea, and the parallel bundles in tendons. Its fibril is composed of highly ordered and packed single molecules which are necessary for cell interaction and other tissue and it is removed during cutting (Piez and Miller, 1974).

The major fibrils of extracellular matrix collagen are type I,II and III, These molecules are packed together to form long thin fibrils of similar molecule while type IV forms two dimensional reticulum while other types are linked to them. Collagen is produced by the fibroblast in connective tissues but recently scientists have found that epithelial cell also produce it (Lodish *et al.,* 2000).

Type I and III provide the tissue with tensile strength, control cell attachment and migration. Type I and II fibril metabolically change during diseases, like impaired fibril formation and increased susceptibility of collagen to photolytic attack. Collagen type III was studied in human atherosclerotic plaques, abdominal aortic aneurysms, colon and ovarian cancer, and finally, colon diverticulosis, Type I and III maturely cross-linked in colon diverticulosis indicating a slightly increased metabolic activity of type III collagen (Bode, 2000).

Collagen type I is acid solubilized extraction and form highly organized a three dimensional scaffold is used for bioengineering and cell culture application because the conventional method requires a long time isolation and nonhuman animal tissues used as a source . The new method use rabbit, lamb and human skin in a three hour isolation method which is in sufficient for fabrication using rapid agitating technique and incorporating centrifugal filtration units (Pacak *et al*., 2011).

The other method for the extraction of collagen is pepsin-solubilized collagens. The collagen was characterized to be type I with no disulfide bond.

Electrophoretic study revealed slight differences in molecular weight between acid-solubilised collagen and all pepsin-solubilized collagens. The molecular weight of acid extraction collagen alpha I and alpha II is higher than that of pepsin extraction which mean pepsin solubilize collagen may undergo partial cleavage in the telopeptide region by pepsin treatment. All collagens were highly solubilized in the pH range of 2–5 and sharply decreased at the neutral pH. No changes in solubility were observed in the presence of NaCl up to 3% (w/v) and the decrease was more pronounced with an increasing NaCl concentration (Nalinanona *et al.,* 2006).

Collagen type I is used to form a three dimensional cell culture matrix composed of collagen fibers which are able to support short- and long-term growth of various cell types, including cancer cell lines, endothelial cells, endometrial cells, hepatocytes, osteoblasts and fibroblasts and to sustain or even enhance cell differentiation, *in vitro*. 3-D culture systems have been successfully used in the investigation of complex biological processes, such as angiogenesis, wound healing, tumor invasion and metastasis. The latter suggested that 3-D culture systems have the potential to simulate cell-cell interactions which take place in tissues under physiological and pathophysiological conditions (Themistocleous *et al*., 2004).

1.9 Other three dimensional scaffold

Poly (lactide-co-glycolidea (PLGA) is very popular and an important biodegradable polyester because of its degradation rates, good mechanical properties and processibility. Recently PLGA fabricated at room temperature, with long time for biodegradable and a three-stage model was established; even the effects of pore size and porosity on *in vitro* biodegradation. The PLGA scaffolds have also been implanted into animals. It is used in the regeneration of some tissues using stem cells (Pan *et al*. 2012).

Polymers are derived from high internal phase emulsions. They are a three dimensional scaffold used for cell culture because of their properties such as high porosity, controllable morphology, and suitable mechanical strength (Hayward *et al*., 2013).

Three dimensional scaffolds utilized a micro mold made from hydrogel agarose. A cell suspension is settled in recesses inside the micro mold and grows as spheroid. This technique is used in some fields such as stem cell research, tissue engineering, and cancer biology. But its use was reduced because of the caveats involving scale; expense, geometry, and practicality have hindered the widespread adoption of these techniques (Napolitano *et al*., 2007).

Hydrogel is a group of polymeric materials. Its hydrophilic structure renders them being capable of holding large amounts of water in their three-dimensional networks. Natural hydrogels were replaced by synthetic types due to their higher water absorption capacity, long service life, and wide varieties of raw chemical resources (Ahmed, 2015). Besides, their compositional structure is unique and it is structural similar to the natural extracellular matrix which is a desirable framework for cellular proliferation and survival (Elsherbiny and Yacoub, 2013).

Culturing cells in 3d gave the researcher more physiological environment to study neurological phenomena than normal 2d culture. However, the density of 3d culture in about 500 µm is not of the same thickness as central nerves system because a limitation in diffusion and mass transport necessary. Therefore many studies increase the thickness of matrix and increase cell density more than 500 µm and high cell density but they need more cell media change per day (*Cullen et al.,* 2006).

Chapter two

Materials

And

Methods

2.1 Materials:

2.1.1 Apparatus and equipment

The following apparatus and equipment were used in this study:

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

No	Equipment or Apparatus	Company	Origin
23	Disposable sterile syringes (1 ml) and(5 ml)	Medeco	UAE
24	Cooled Centrifuge	Hettich	Germany
25	Water bath	Memmert	
26	Incubator		
27	Shaker	Cyan	Korea
28	Laminar air flow cabinet	K&K	S. Korea
39	Electrical oven		
30	Magnetic stirrer	Gallenkamp	UK
31	pH-meter		
32	Sensitive balance	Stanton	
33	Cover slips (22*22mm)	Apel	China
34	Microscope glass slides	Afco	
35	Micro- pipette (2-20 µl)	Lypress	Belgium
36	Micro- pipette (10-100 µl)		
37	Micro- pipette (100-1000 µl)		
38	Micro- pipette (1000-5000µl)		
39	Disposable Tips		
40	Deep freeze (-80ºC)	Nuve	Turkey
41	Autoclave	GSL	
42	Improved Double Neubauer Ruling Counting Chamber	Assistant	
43	Scanning electron microscope	Phenom	Austria

44	Vacuum pump	Franklin elective	-

2.1.2 Chemicals and biological materials

No.	Chemical or biological material	Company	Origin
1	Streptomycin	TROGE	Germany
2	Ampicillin		
3	PDX mountant	Fluka	
4	Ethanol alcohol 99%	BDH	UK
5	Di sodium hydrogen phosphate (Na_2HPO_4)		
	Potassium chloride (KCl)		
6	Retinoic acid		
7	Sodium bicarbonate(NaHCO3)		
8	Trypan blue stain	US Biological	USA
9	Potassium dihydrogen phosphate (KH_2PO_4)		
10	Fetal bovin serum		
11	Nerve growth factor		
12	Purmorphamine		
13	Sonic hedgehog		
14	Minimum Essential Medium, MEM		
15	Trypsin EDTA		
16	Formaldehyde 37%	Santa Cruze	
17	Collagen		
18	Gluteraldehyde		

19	Osmium		
20	Hydrogen peroxide (H_2O_2)	-	Iraq
21	Hematoxylin	Syrbio	SAR
22	Eosin		
23	Xylene	Scharlau	Spain
24	glacial acetic acid		
25	Sodium chloride (NaCl)	Thomas baker	India

2.1.3 Kits:

No.	Kit	Company	Origin
1	Anti-got biotenylated secondary antibody	Santacruze biotechnology USA	
2	Anti-rabbit biotenylated secondary antibody		
3	Anti-MAP-2 antibody		
4	Got anti human CD 34		
5	Immunocruz mouse ABC Staining system:sc-2017(kit)		
6	Rabbit anti mice CD 44	US-biological/USA	
7	Anti-choline acetyle transferase antibody		
8	Mouse anti human CD 90		
9	Mouse anti human CD105		

2.1.4 Laboratory animals:

Mice used in this study were obtained from the Iraqi Center for Cancer and Medical Genetics Research/ Al- Mustansryia University. The mice age was from 4-8 weeks. The mice were subjected to unified conditions of temperature, light and feeding.

2.1.5 Culture Conditions

The experimental work of this study was done at the Tissue Culture Unit of the Experimental Therapy Department which belongs to the ICCMGR.

All the experiments of the present study were carried out under sterile conditions including apparatus, tools, solutions and culture medium, and all laboratory manipulations were carried out in laminar flow. The metallic/stainless steel, glass tools and solutions were sterilized by oven for 2hr at 120c°. The equipment was autoclaved at 121°C temperature for 30 minutes under 15 P / SI pressure. As for the culture media and serum, were sterilized by using Nalgen filter with a porosity of (0.22) microns.

2.2 Methods:

2.2.1 Preparation of solutions for cell culture:

2.2.1.1 Antibiotics:

- Streptomycin: 1 g of streptomycin was dissolved in 5ml triple distill water, and 0.5ml of it was added to 1 litter of culture media.
- Ampicillin: A 1000000 IU of ampicillin was dissolved in 5ml triple distill water, then 1ml was added to 1 litter of culture media (Freshney, 2000).

2.2.1.2 Sodium Bicarbonate:

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

2.2.1.3 Phosphate buffer Saline PBS (pH 7.2):

It was prepared by dissolving 8 g NaCl, 0.2g KCl, 0.92 g Na_2HPO_4 and 0.2 g KH_2PO_4 in 1L triple distilled water and stirred constantly on a magnetic stirrer at room temperature; the pH was adjusted to 7.2 and autoclaved at 121^{o}C for 15 min and stored at 4^{o}C until use (Bates, 1964).

2.2.1.4 Fetal bovine serum:

Ready to use serum from US biological company.

2.2.1.5 Trypsin-EDTA Solution:

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

2.2.2. Preparation of stain solutions:

2.2.2.1 Trypan Blue Solution:

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

2.2.2.2 Scanning electron microscope solutions (4% gluteral dehyde**).** It was prepared by mixing 4 ml of stock gluteral dehyde with 94 ml D.W. **(**Eisenbach, 2015).

2.2.2.3 Osimum solution

It was prepared by dissolving 1mg of osmium tetroxide in 10 ml D.W. , then stored in a dark container at -20C°.

2.2.2.4 Hematoxylin and Eosin stain solutions

- **Hematoxylin solution**: ready to use.
- **Eosin solution**: ready to use.
- **Differentiating solution** it was prepared by transferring 2.5 ml HCL solution in container containing 1 L of 70% alcohol.
- **Bluing solution** about 0.1% NH_4OH was prepared by dissolving 1g of NH_4OH in 1 litter of DW.

2.2.3 Preparation of Tissue Culture Media:

2.2.3.1 Minimum Essential Medium (MEM):

MEM culture medium was prepared by dissolving 16.65 g MEM powder with HEPES buffer and L-glutamine in approximately 600 ml of triple distilled water (TDW). 2.2 g of sodium bicarbonate powder, 1 ml of Ampicillin, 0.5 ml of Streptomycin and 200 ml of Fetal Calf 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).

2.2.4 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.
- **Biotinylated secondary antibody:** Seventy five μl of normal blocking serum stock was mixed with 5 ml of PBS and 25 μl biotinylated secondary antibody stock.
- **AB enzyme reagent:** It was prepared by mixing of 50 μl of reagent A (avidin), 50 μl of reagent B (biotinylated HRP) and 2.5 ml of PBS and left to stand for approximately 30 minutes.

- **Peroxidase substrate:** It was prepared by mixing of 1.6 ml of distilled water, 5 drops of 10x substrate buffer, 1 drop of 50x DAB chromogen and 1 drop of 50X peroxidase substrate.
- **1 % of Hydrogen peroxide H_2O_2:** To prepared 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.

2.2.5 Preparation of differentiation and elongation factors

2.2.5.1 Betamercaptoethanol (1mM /ml)

It was prepared by transferring 7 µl from stock solution (14 M) to container containing 100ml of MEM media with 20 % FCS (Woodbury *et al*., 2000).

2.2.5.2 Betamercaptoethanol (2 mM/ml)

It was prepared by transferring 14 µl from stock solution (14 M) to a container containing 100 ml of MEM media with 20 % FCS (Liu *et al.*, 2011).

2.2.5.3 Retinoic acid (1 mM/ml)

It was prepared by dissolving 0.001g of retinoic acid powder in 1000 ml MEM free serum (Hu and Zhang, 2009)

2.2.5.4 Sonic hedgehog (0.1ng/ml)

The concentration was prepared by dissolving 25µg in 250µl of DDW containing 0.1% BSA (Abdanipour and Tiraihi, 2012).

2.2.5.5 Nerve growth factor (160 ng/ml)

It was prepared by dissolving of 160 ng in 1ml free serum media.

Then different concentrations (10, 20, 40 and 80) ng/ml were prepared (Tonge *et al*., 1997).

2.2.6 Slandered Acetylcholine:

It was prepared by dissolving 1 mg acetylcholine chloride powder >98% purity in 1ml D.W.

2.2.7 Preparation of three dimensional culture media solutions

2.2.7.1 Collagen solution Ready to use**.**

2.2.7.2 0.01M NaOH

It was prepared by dissolving 0.04 g of NaOH powder in 100ml DDW, sterilized by 0.22µm filter and stored -12C°.

2.2.7.3 Buffered serum free cell cultured medium (10X)

The medium was prepared by dissolving (16.7g) of MEM media according to manufacturer Instruction Company in 0.1 volume of DDW, filtered in 0.22µm and stored at -20^0C.

2.2.7.4 Preparation of neutralized collagen solution

Neutralized collagen solution was prepared by mixing 8 parts of chilled collagen solution to 1 part 0.01M NaOH and 1 part 10X buffered serum free cell cultured medium. Then, pH was adjusted to 7.4±0.2 and poured on 3D seeding plate with 8 wells (the thickness of collagen gel should be 1-2mm) and incubated at 37 ^{0}C for 20 min. The plate left over night to dry and rinsed with DDW to remove excess salt and to rehydrate collagen gel and used immediately or dried again and stored for weeks at 2-8^0 C as manufacturer instruction (Wuensch, *et.al*. 2013).

2.2.7.5 0.5%Triton X-100

The solution was prepared by transferring (0.5) ml of stock solution triton x-100 in container containing (100) ml PBS while stirring, then filtered through 0.22 µm and stored for 2 week at room temperature (Artym and Matsumoto,2010).

2.2.7.6. Three culture fixation solution

It was prepared by mixing 4 ml of paraformaldehyde with 96 ml PBS, then 5g sucrose was added to prepare 4% paraformaldehyde with 5%sucrose (Artym and Matsumoto, 2010).

2.2.8 Primary culture of bone marrow

A bone marrow cell was isolated from the femur of 4-8 week-old male albino mouse with weights ranging from 10-15g. The mouse was killed by cervical dislocation, placed on its back on a cutting board and soaking it with 70% ethanol, followed by making a long transverse cut through the skin in the middle of the abdominal area. The skin reflected from the hindquarters and the hind legs then, the muscles were removed and the bone placed in a Petri dish containing medium MEM free serum. A flushing method was used to flush the bone marrow cells from bones using a 1ml syringe containing 1ml of growth culture media (MEM) (Ishaug *et al*.,1997).

2.2.9 Mesenchymal stem cells isolation by using adherence properties

The simplest method used implies the adherence properties of MSCs on plastic surface in which freshly isolated bone marrow cells which were resuspended in growth culture medium MEM supplemented with 20 % FBS, 1 % Ampicllin/Streptomycin. Cells obtained from three mouse (2 femurs and 2 tibias) were seeded in 6 ml in tissue culture flasks as Soleimani and Nadri (2009). The cultured cells were incubated at 37°C and left to adhere 24 hours, as for the non-adherent cells, they were removed, Mesenchymal stem cells were selected by adherence during first 24 h and maintained in growth culture media. Media changed 3 times a week and until the cultures reached 70-80 % confluence.

2.2.10 MSCs passaging

Passaging of the cells was 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 culture. The MSCs are sub-cultured 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 2.2.3) for 5-10 minutes at 37ºC temperature .in order to dislodge the cells, the flask gently rocked, then 5ml of culture media containing 20% FBS was added by using a sterile Pasteur pipette and mixed to obtain a single cell suspension. The cell suspension was centrifuged at 1000 rpm for 10 minutes in 18 ºC then the supernatant was aspirated and the cells pellet were suspended in 1ml of culture medium MEM containing 20% FBS, Then, the cells were counted using a hemocytometer. The cells were detached from the surface with trypsin-versene and counted.

2.2.11 Viable Cell Count:

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

- Cell suspension was prepared by trypsinization and resuspension in culture medium.
- Cover slip was fixed on a clean heamocytometer Improved Double Naubauer Ruling Counting Chamber.
- Two hundreds µl of cell suspensions was mixed with 200µl of trypan blue solution prepared in(2.2.2.1)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):

$$C = N \times D \times 10^4$$

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).

2.2.12 Immunocytochemistry analysis of MSCs:

In this study, the following CD markers (primary antibody) were used for the detection of MSCs :(mouse anti human-CD 105, mouse anti human CD90, Mouse anti goat CD 34, Mouse anti human CD 44 (Lin, *et al*,2013).

2.2.12.1 Immunoperoxidase staining procedure for detection MSCs

- **Principle:** The principle of the test is that the primary antibody binds to the corresponding antigen in the tissue cells, and the secondary antibody binds to determinants on the primary antibody. Then, the avidin-biotin complex containing the horseradish peroxidase enzyme is allowed to bind to the biotin molecule attached to the secondary antibody (Davies, 2003).

2.2.12.2 Assay procedure

After MSCs were dispersed with trypsin-versene, and suspended in MEM growth media, the cells were re-cultured in multi-well tissue culture plates (8 well) in MEM supplemented with 20% 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 cells were fixed by 4% paraformaldehyde for 10 min.

2.2.12.3 Staining procedure:

In all steps, the sections were placed in the humid chamber and at room temperature (20-25ºC) according to manufacturer procedure (Moral-Sanz, *et.al*.2012)

- To inhibit endogenous peroxidise, cells were incubated with 1% hydrogen peroxide for 10-15 minutes and washed 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 min to decrease the background staining.
- Cells sections were incubated with 125 µl of diluted primary antibody at a ratio(1:50) for one hour at room temperature or overnight at 4ºC then washed with three changes of PBS for 5 minutes .
- Cell sections were incubated for 30 min with 1.2 ml biotinylated secondary antibody (which prepared in 2.2.4) and washed with three changes of PBS for 5 minutes.
- A liquot of 650 µl of AB enzyme reagent (which prepared in 2.2.4) was added to cell section, and washed two times with PBS for 2 min.
- Three drops of peroxidase substrate (which prepared in 2.2.8.1) was added to cells for 10 min, 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 200X and 400 x.

2.2 .13 Induction motor neuron differentiation of MSCs

In motor neuron differentiation, the 1st passage of (2×10^4) MSCs were used. The differentiation strategy involved two main steps (pre induction step) and the cells were cultured in a MEM medium supplemented with 20% FBS

and 1 mM Betamercaptoethanol. After 24 h of incubation, the media was discarded and MEM free serum media containing 2 mM BME was added and incubated for 1h. Then, media was discarded and cell washed with free serum media. At the second step (induction step) which lasted for four days, MEM free serum media with 1mM retinoic acid, 10ng/ml nerve growth factor and 0.1 ng/ml sonic hedgehog were added and incubated at 37℃ for 4 days. As a negative control, MSCs were cultured in medium without differentiation stimuli along with the differentiation experiments in the same conditions. Cells were cultured in a humidified atmosphere of 5% CO2 and 95% air at 37℃. Cultures were maintained by a medium exchange every 2 days. Beside the cell morphology was observed under inverted microscope according modification to methods of (Hu and Zhang, 2009).

2.2.14 Motor neuron detection by immunocytochemisty

After that the media was aspirated and the cell was fixed by 4% paraformaldehyde for 10 min.

- To inhibit endogenous peroxidise, cells were incubated with 1% hydrogen peroxide for 10-15 minutes and washed with PBS for 5 min three times.
- Aliquot of 1.5% blocking serum was added to the cell section for one hour and then washed with three changes of PBS for 5 minutes. So as to decrease the background staining.
- Cells sections were incubated with diluted acetyle transferase antibody at a ratio of (1:50 vol/vol) overnight according to the production company instruction and diluted in microtubule associated protein antibody at a ratio of (1:100vol/vol) for 1 h then washed with three changes of PBS for 5 minutes .
- Cell sections were incubated for 30 min with 1.2 ml biotinylated secondary antibody and washed with three changes of PBS for 5 minutes.

- A liquot of 650 µl of AB enzyme reagent (which prepared in 2.2.8.1) was added to the 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 developed and washed with distilled water for 5 minutes.

Hematoxylin stain was added to the 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 a light microscopy at magnification powers 200X and 400 x.

2.2.15 Motor neuron activity

The activity of motor neuron was detected using HPLC by the estimation of acetylcholine concentration in media aspirated from cell culture at different periods of differentiation.

The test was done in Ibin Sena Center /Ministry of Higher Education and ScientificR , using shimachzi lc-2012 AHT machine.

- **HPLC conditions**

Column: 250*4.6 mm colume 5micron lava

Injection volume 0.7 ml/min.

Column temperature: 18 °C

Detection wavelength: 210 nm

Mobile phase: Acetone, water, methanol

80%, 14%, 6%

According to Landgraf, *et .al(*,2010):

- **Calculation of acetylcholine concentration**

Acetylcholine concentration was calculated according to the following equation (Chen and Yun, 2009).

$$\text{Concentration of compound (ppm)} = \frac{\text{area of sample} \times \text{conc. of standard}}{\text{area of standard}}$$

2.2.16 Elongation of Moto neuron cell axon

Mesenchymal stem cell was cultured in five tissue culture flask 25cm^3 at 37^{o}C in CO2 incubator until monolayer formed. Media was discarded and pre induction media was added to each flask as mentioned in section (2.2.13). At the end of pre induction time, the pre-induction media was discarded and induction media containing different concentrations of nerve growth factor started from 10 to160 ng/ml as prepared in (2.2.5.5) was added to each flask (Lentz, *et al.* 1999).

2.2.16.1 Axon length measurement program

The axon length was measured by using MiniDV camera and compared to different lengths by Axivision program. (Qiang *et al.*,2010 ; Olsson-Carter et *al*., 2010 and Cregg *et al.*, 2010).

2.2.17 3D collagen culture

Mesenchymal stem cell was cultured on neutralized collagen seeding plate with 8 wells according to Artym and Matsumoto, (2010) with modification:

- Mesenchymal stem cell was suspended at a concentration of 6 X10^6 cell\20 ml of 20% FCS culture media.
- Aliquot 400µl of cell suspension was added to each well of the neutralized collagen seeding plate and incubated in CO2 incubator at 37^{0}C until monolayer formed.

- Every 2-3 day, the slide media was aspirated carefully without contact to the collagen to prevent its shrinking or damage from the side wall and new culture media was added carefully. Motor neuron differentiation was induced as in section (2.2.13).

2.2.18 . Immocytochemistry of motor neuron cell after differentiation in 3D culture

- The media was aspirated gently and slide washed with warm PBS at the end of differentiation.
- Section was fixed with 4% paraformaldehyde sucrose solution for 20 min at room temperature and washed with PBS. Triton X-100 was added for 10 min and aspirated and washed with PBS. Blocking serum solution, primary and secondary antibody was added as in item 2.2.14.

2.2.19. Hematoxylin and Eosin study according (Chen, *et.al*.2012).

- 3D slide was immersed in Hematoxylin solution for 2-10 min, and then rinsed in running tap water.
- Slide was dipped in differentiation solution for three times and stain was checked under microscope, and then rinsed in running tap water.
- Slide was immersed in bluing solution for 1 min and then rinsed in running tap water.
- Slide was immersed in 95% alcohol for 30 sec and eosin stain was added for 3 min and then rinsed in running tap water
- Slide was dehydrated in 80% alcohol, 95% alcohol for 1 min each, and two changes were made in 100% alcohol for 3 min each.
- Clearing was made by adding xylene for 1min. Then absolute alcohol was added for 2 min to remove xylene. Finally cell sections were mounted with xylene based mounting medium (DPX).

2.2.20. Scanning by electron microscope

The slides were washed by PBS and fixed with gluteraldehyde 4% for 24h at 4^0C then Osmium solution was added for 2 h in dark. At the end of two hour, slides were washed with PBS two times. A serial dehydration for the sections were carried out starting from (50, 70, 95, and 100) % for 30 min, dried and examined under the electron microscope (Eisenbach, 2015).

2.2.21. Statistical analysis

Data were analyzed statistically using SPSS program version 20. The results were expressed using simple statistical parameters such as mean and standard error. Differences between means were assessed by ANOVA. The difference was considered significant when the probability value ($P \leq 0.05$).

Chapter three

Results

And

Discussion

3. Results and discussion:

3.1. Morphology of mesenchymal stem cells:

Mice bone marrow was isolated from the femurs and tibias of young albino mice by flushing. Bone marrow mesenchymal stem cells are multipotency, easy to purify and amplify, therefore, they are an ideal stem cell source for cell therapies.

After 24 hours, the mesenchymal stem cells have a round shape or quasi-circular shapes attached to the surface of flask and other cell like hematopoitic stem cell, still suspended in media. The media was discarded and changed with fresh 20% serum MEM media without any additional growth factors.

Some researchers isolated and maintained MSC in media without additional growth factors together with passage-dependent reseeding following trypsinization (Sreejit *et al.*, 2012).

Attached cells represent 80% in the few days after culturing which gives rise to mesenchymal stem cells and the other 20% still floating in culture, Increasing the serum percentage in culture increases the number of cells significantly from 5%,10%,15% and 20%(Eslaminejad, *et al.*,2006).

The cultured media changed every 3-4 days and in the third day of culture, the cells have small spindle like shapes starting propagation. At the end of 5^{th} day of cultures the number of cell increases forming colonies distributed in flask 70-80% field forming, Cells have a short fibroblast like structure with two polar cell shapes and some cells have a triangular shape in a homogenized monolayer, Figure (3-1).

Mutlak (2007), stats that at the end of 5^{th} day of cultivation, a numerous fibroblast like cells are formed and gradually grow to form small individual colonies like elongated morphologically. When the cells reached confluent

percentage about 70 - 80% in the flask about 2.5X10^{6}cell/ml, they should be sub-cultured (Hatlapatka *et al.*, 2011).

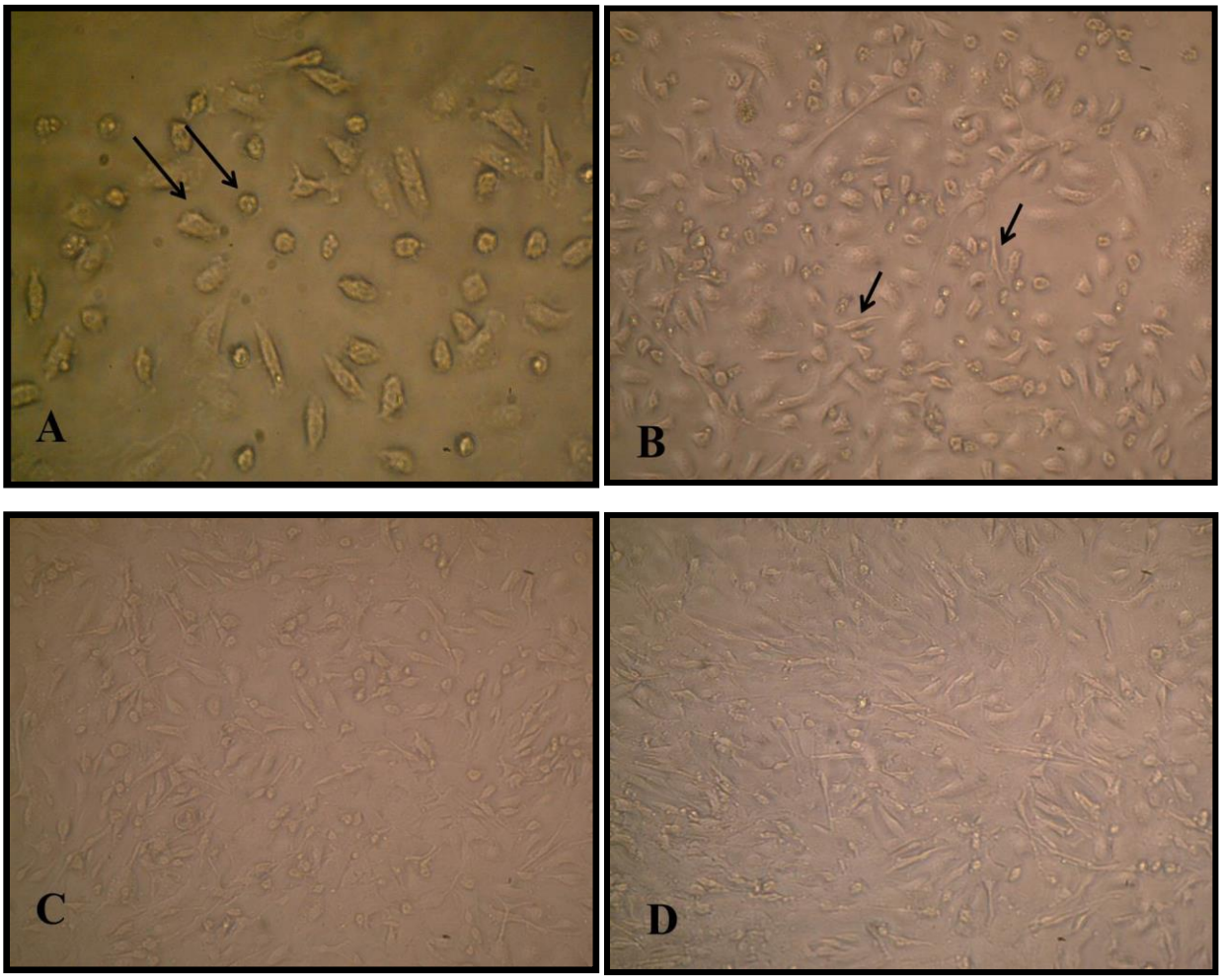


Figure (3-1): Morphological study of MSCs cultured in MEM media. A- Primary culture MSCs after 24 h showed a round attached cell (as showed by pointer arrows) (10X20). B- After 48 h culturing MSCs have a small spindle like shape or triangular structure (as showed by pointer arrows) (10X10). C & D- monolayer primary MSCs culture after 5-7 days (10X10).

Under the same conditions, the cell began to propagate and form homogenize layer of mesenchymal stem cell in continuous passaging; starting from passage 1 used in the differentiation into motor neuron cells.

The work on MSCs morphology and size indicate that the cells changed from small and spindle-like cells in the early passage to large and polygonal types in later passages. Their growth was the highest in the third passage, followed by a gradual decrease. There were no special modifications of cell surface antigens or the karyotype of the MSCs from the first to the tenth passage (Choi *et al.*, 2010).

Bonab *et al*., (2005) showed that multiple MSC passaging more than 9 passages will reduce the population doubling of cell from 7.7 to 1.2 in the 10th passage and telomere length decreased from 9.19 Kbp to 8.7 kbp in the 9th passage resulting in a drop differentiation potential from the 6th passage on.

3.2. Phenotypic detection of mesenchymal stem cell

Mesenchymal stem cells phenotypic characteristic was detected in the culture of passage one by culturing passage 0 cell on multi well tissue culture slides when reached monolayer, it was fixed with 4% paraformaldehyde and immunocytochemistry test was used for detection specific CD markers for MSCs, These CD markers were anti CD105,anti CD90 and anti CD44 as positive markers and anti CD34 as negative marker and the results are shown in the Figure (3-2)and (3-3). Mice bone marrow MSCs were detected using chromogen accumulation on the secondary antibody of the primary antibody of CD105, CD90 and CD44 showing dark brown color while cells for negative CD34 was still blue, the color of hematoxylin , with no brown stain .

The results match with the results of the International Society for Cellular Therapy in assuring the identity of MSCs using CD70, CD90, and CD105 as positive markers and CD34 as a negative marker (Lin, *et al*., 2013). Bone marrow, adipose tissue and umbilical cord MSC have CD markers positive to CD105, CD90 and CD44 using flow cytometry analysis while negative CD34 for all type sources in more than 95% of cells (Jin et al., 2013).

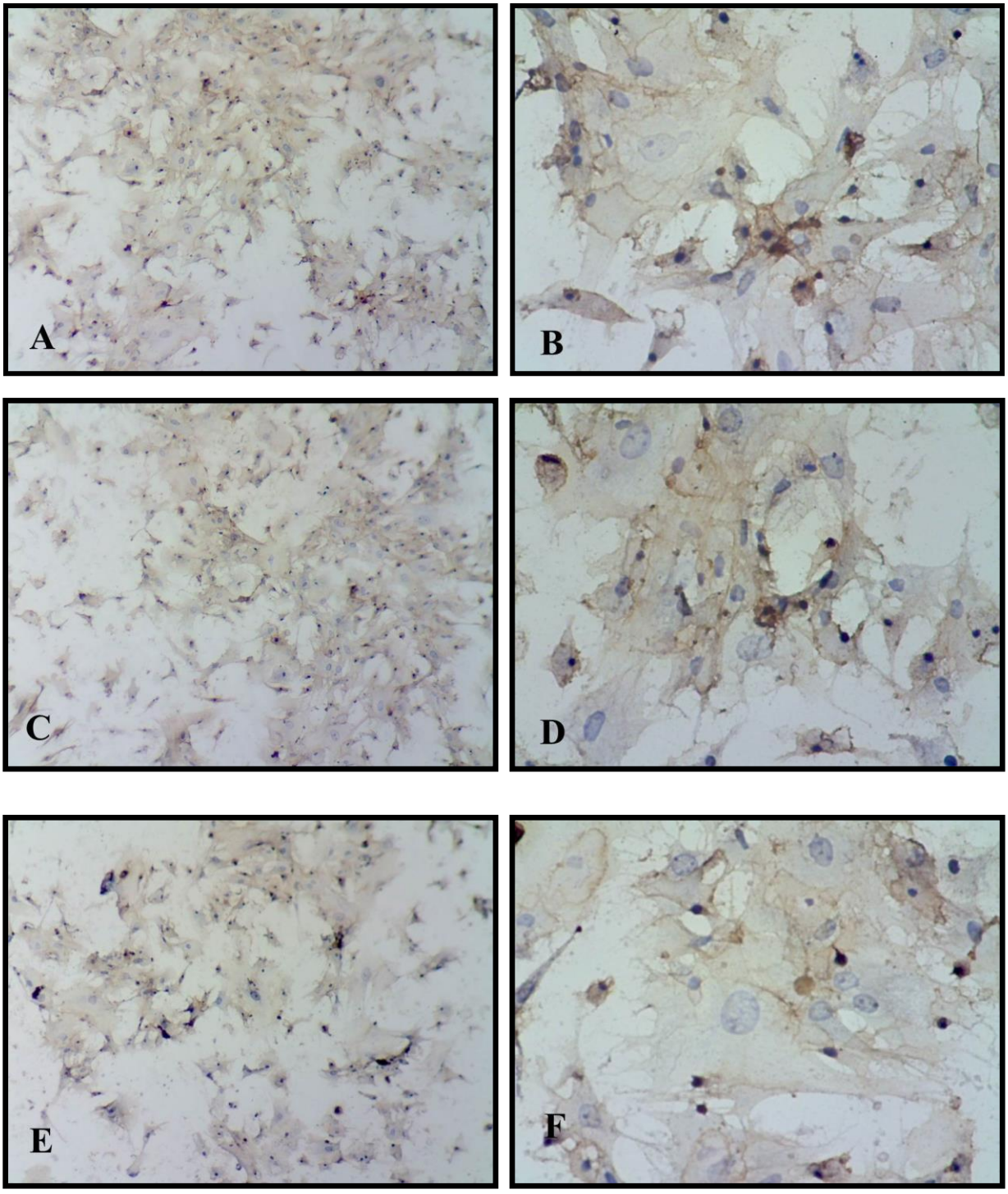


Figure (3-2): ICC study of MSCs after induced differentiation. A & B- ICC of MSCs for CD 105 (10X20)(10X40). C- Positive CD90 (10X20)(10X40). E and F- positive for CD 44 (10X20)(10X40) brown of chromogen accumulated they are cellular membrain localization .

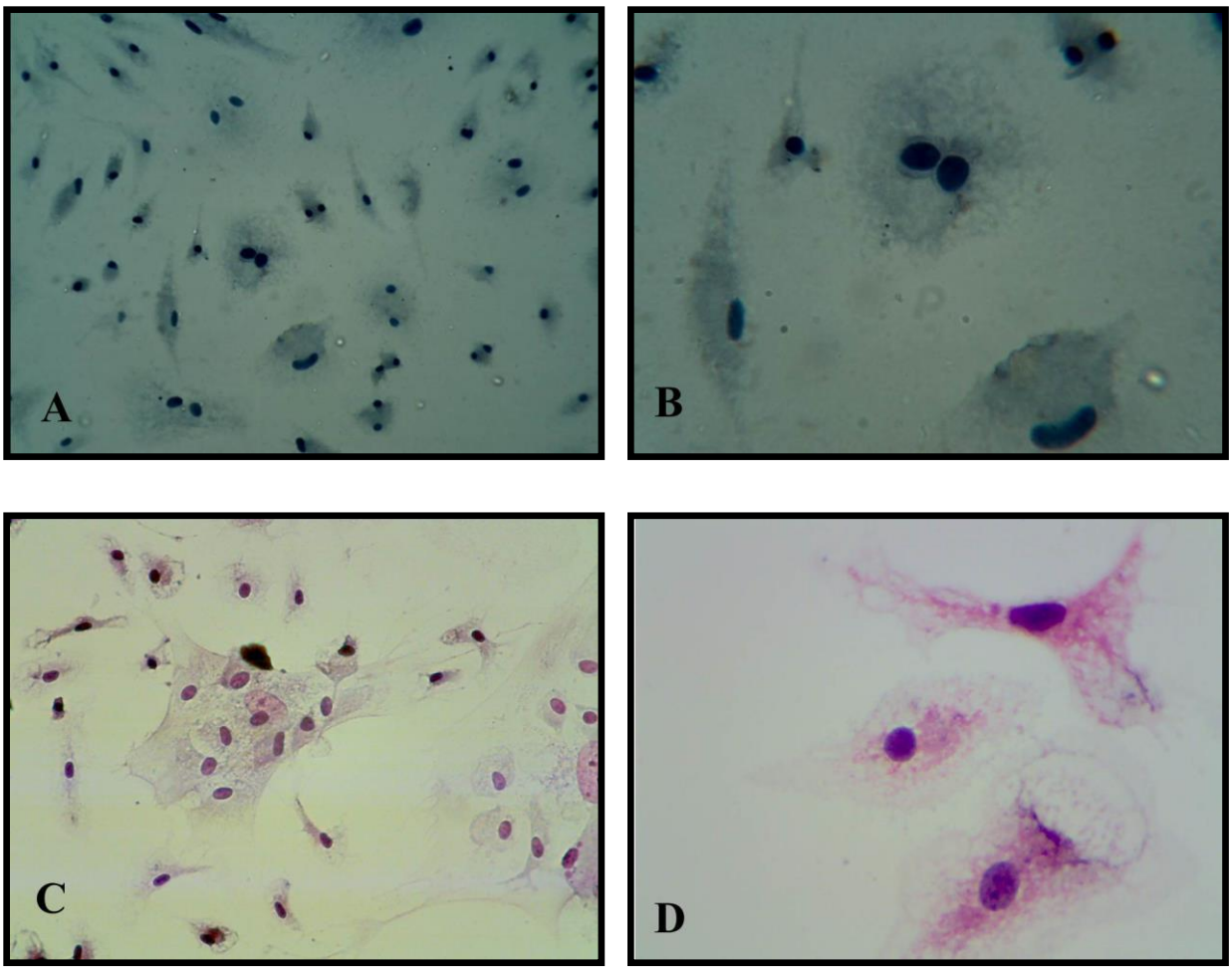


Figure (3-3): ICC study of mesenchymal stem cells A & B- that showed a negative result for CD 34 (10X20) (10X40). C & D- A morphological study by hematoxylin and eosin study for MSC (10X20) (10X40).

3.3. Differentiation of mesenchymal stem cell into motor neuron

Mesenchymal stem cell have the ability to differentiate into neuron like cell and more specialized cells such as motor neuron when using differentiation factors in two stages of differentiation using monolayer of passage one MSCs. The first stage of differentiation, a pre induction stage, the cells were cultured in β-mercaptoethanol in two concentrations. About 1mM β-mercaptoethanol contained MEM 20 % Fetal bovine serum for 24 h showed that the fibroblast- like cell MSC cell membrane withdrew to the middle of the cell, extension became

apparent and the cell still viable with account 1.9 X10 ^{6} and attached to the flask surface Figure (3-4). The media discarded and incubated for 1hour in 2mM βME of free serum MEM, the cell became more radical in shape, multipolar, and extension increased in number of viable count to about 1.40 X10^{6}.

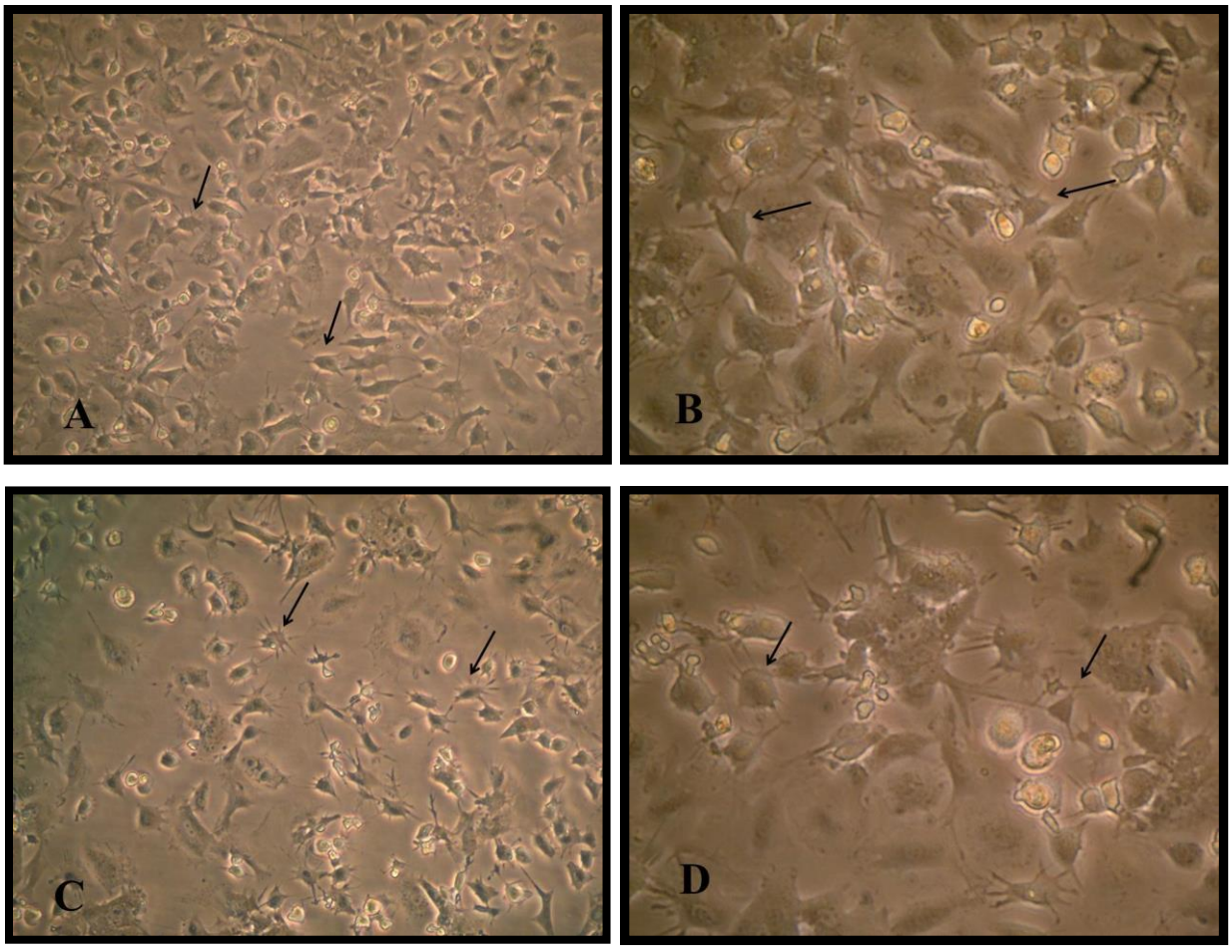


Figure (3-4) : BM-MSCs differentiation A and B pre induction stage after 24 h in 1M BME in MEM 20% FBS (as showed in by pointer arrows) viewed by inverted microscope (10X10) (10X20), which showed that MSCs membrane withdraw to the middle of the cell. C and D a pre induction stage after1hour in 2.mM βME of free serum MEM showed that the cell became more radical in shape multipolar.

Scientists works on bone marrow cultures in 1mM BME showed that cell cytoplasm retracted and when exposed to 5mM BME for 6 hours, the cell had a

multipolar body structure (liu et al., 2011) and also cells incubated in BME for few hours were elongated and beard process- like extensions and were immunoactive for neuron specific enolase, nestin, glial fibrillaey acidic protein (Mareschi et al, 2006).

The second stage of differentiation was the induction stage in which used a combination of Retinoic acid and a sonic hedgehog factors was used for just four days. The cell body became typical like motor neuron cell, shiny nuclease and with the cell soma having many dendrites and long axon like structure ended with small extensions. The MSCs, under a three thousand increase size using scanning microscope, appeared as circular to spindle shape with no extended extension from the cell while the differentiated cell into motor neuron showed cell body contain dendrites and a long extended axon ended with small dendrite under a two thousand increase in size, Figures (3-5) and (3-6).

The first and second stages of induction represent the most effective, efficient and shortest induction time method for the differentiation of mouse bone marrow MSC into motor neuron cells when compared to Shetty and his workers (2015) who cultured cells in BME as pre induction, induction in RA and forskolin for 24 h and finally 6-8 days in RA and SHH but they started culturing forming a neurospher from MSC on an unattached surface flask.

Other workers induced differentiation of MSCs into motor neuron cells MNCs for longer time up to two weeks using a large amount of growth factor and specific culture conditions (Ebrahimin- Barough, *et al* .2014).

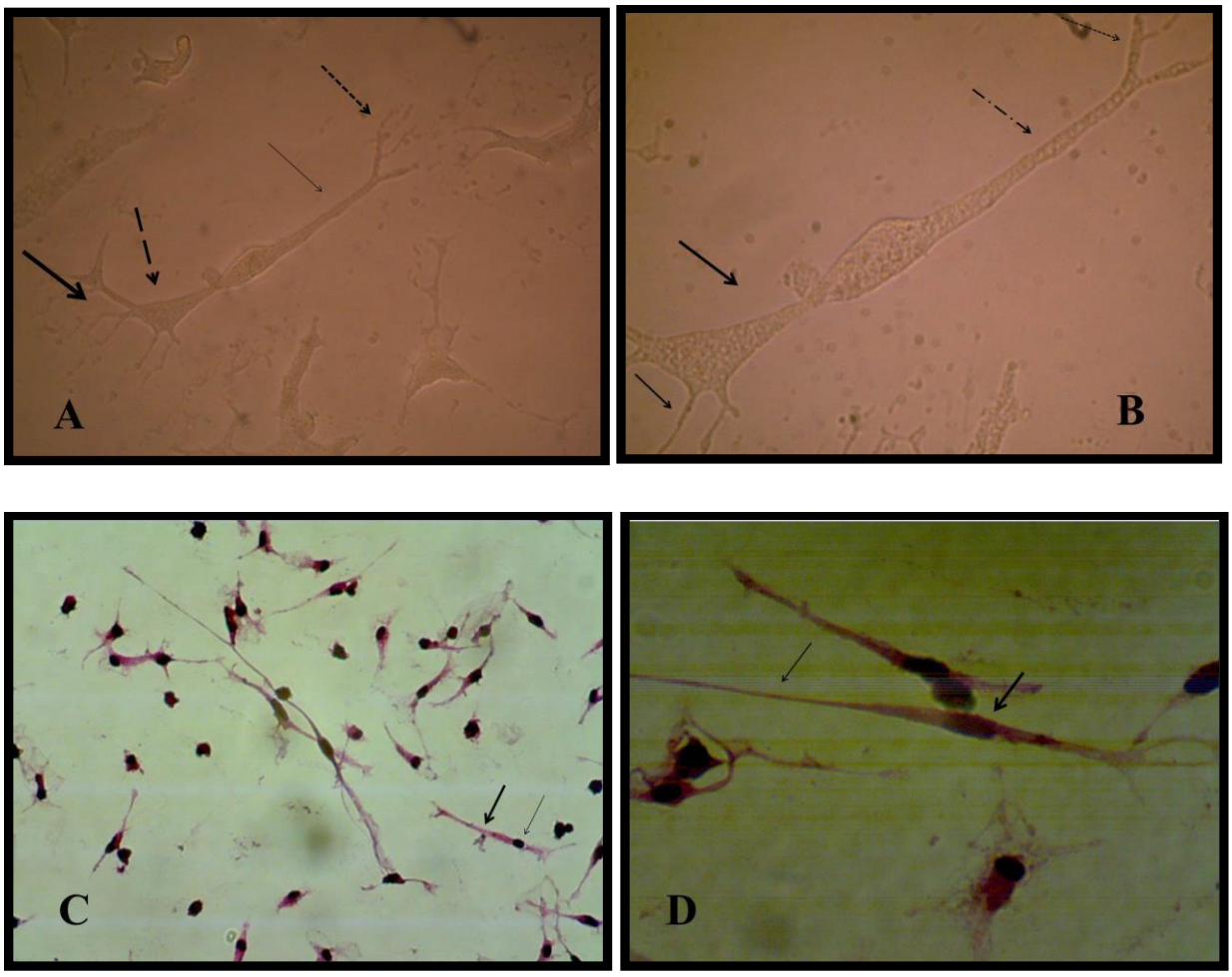


Figure (3-5): Motor neuron cells, A & B- Microscopic study after differentiation which showed formation of soma with dendrite and extended of axon as a viewed (10X10)(10X20) under inverted microscope at the end of induction differentiation stage, C & D- Hematoxylin and eosin study that showed the differentiated motor neuron which take the red color of cytoplasm and blue color for nucleus (10X20)(10X100).

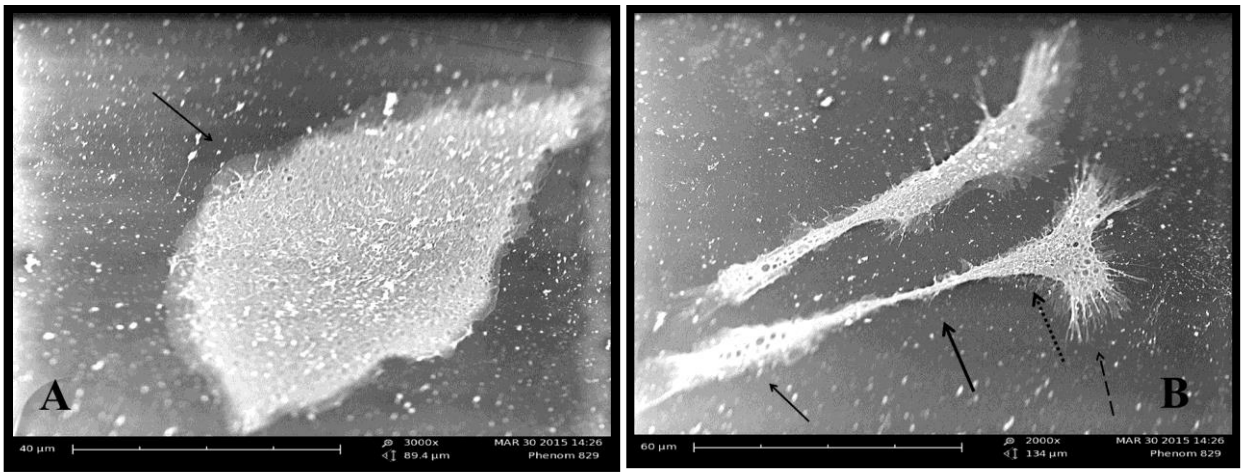


Figure (3-6): A MSC using scanning electron microscope 3000 X magnification in size showing dipolar structure of cell. B. Differentiated motor neuron under scanning electron microscope 2000 X magnification in size cell soma appeared less in size with many dendrites and extended of cell axon.

3.4. Motor neuron cell detection using immunocytochemistry

Bone marrow mesenchymal stem cell differentiation into motor neuron cell was detected by an immunocytochemistry study. Motor neuron cell are mature differentiated cells having a microtubule associated protein 2 (MAP2) and cholinacetyl transferase (CHAT) as specific marker. The results revealed that 90% of the differentiated motor neuron was positive for MAP2 and about 85 % for CHAT Figure (3-7).

Differentiation indication markers MAP-2 were used in this study and the results proved that mature spinal cord neuron, MAP-2 a cytoskeletal protein, present in the axon and dendrite of the cell body revealed by mRNA in situ hybridization (Hirokawa *et al.*, 1996).

Bi, *et al.* (2010) found that the cells have three neural related marker expressions: increased nestin, NES and MAP-2 with an increasing concentration

of Retinoic acid by Real time PCR, These factors determined the differentiation into neuron.

The other marker that improved the differentiation into motor neuron cells is acetyle choline transferase (CHAT), the enzyme responsible for the biosynthesis of acetylcholine, and it is present in the functional cholinergic neurons of the central and peripheral nervous system indicating motor neuron cell (Oda ,1999) Other studies on embryonic stem cell differentiation into motor neuron using sequence of culturing procedures detected immunocytochemistry using Quantification of HB9 and CHAT cells which showed just 30% of the total cells are mature motor neurons (Hester, 2011).

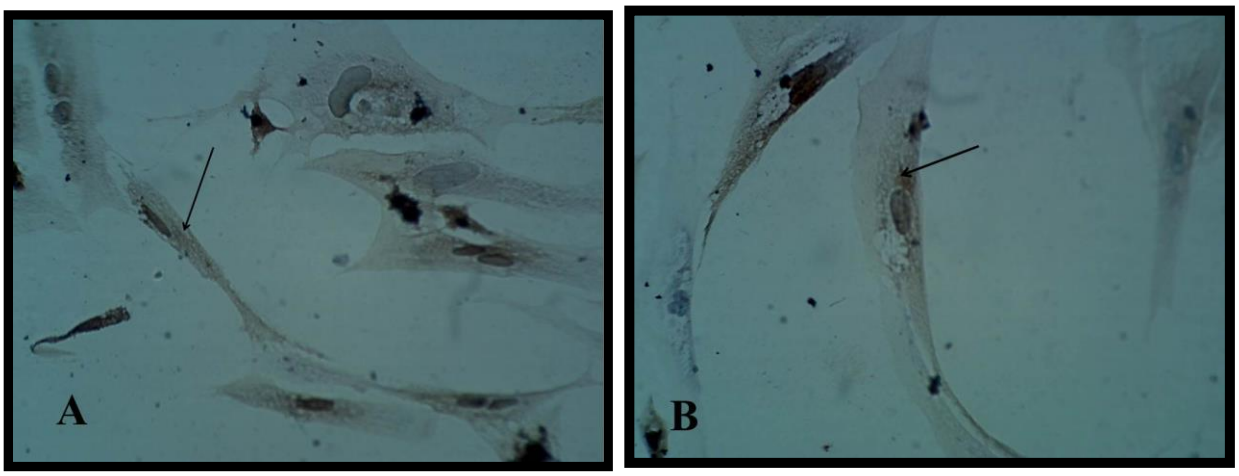


Figure (3-7): A & B- Immunocytochemical analysis for MAP-2 and CHAT in motor neuron after induction stage showed positive reactivity and brown color in the cytoplasm of the cell (10X40).

3.5. Level of Acetyl choline by HPLC

The activity of motor neuron by measuring the level of acetylcholine produced by the differentiated cells in the culture media. The increase level of neurotransmitter acetyl choline produced in culture media estimated functionality of motor neuron.

The results revealed that a high increase in acetylcholine (75.0±0.57 ppm) was produced by motor neuron in differentiation medium at 4th day after the induction stage in comparison to zero day as (MSC) and after 24h (pre induction stage (15.40±0.43 and 15.36±0.44ppm) Figure (3-8).

Motor neuron cell produced acetylcholine as a cholinergic compound from choline and acetyl co A metabolized by enzyme acetyl choline transferase which was expressed strongly in differentiated cell immuonocytochemicaly by CHAT primary antibody as shown in figure (3-7), therefore, acetylcholine present in the media indicated that the motor neuron was a functionally active cell .

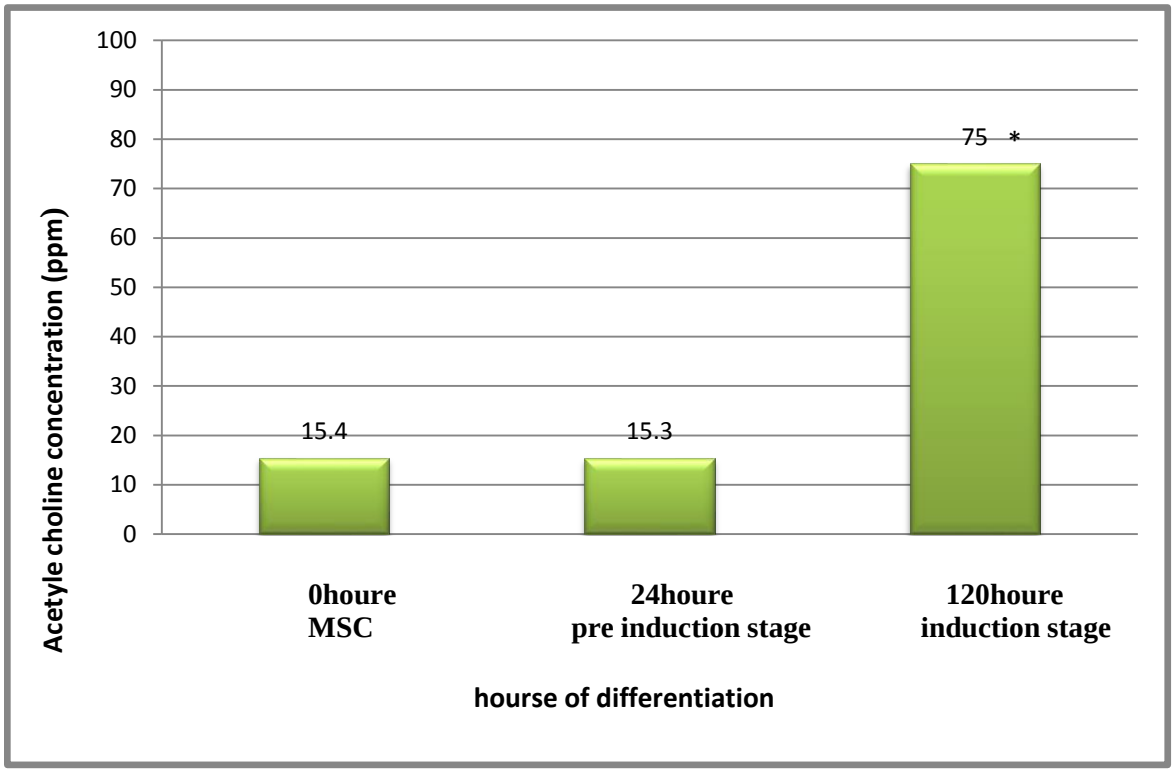


Figure (3-8): Level of Acetylcholine produced by motor neuron after different differentiation periods (*) significant difference.

Liu, *et al*. (2013) showed the production and secretion of acetylcholine from motor neuron cells derived from umbilical cord mesenchymal stem cell a positive

result for acetylcholine transfarase and increased during the period of differentiation from very little in the 9th day differentiation days from about 2.8 ng/ml to 5.7 ng/ml at the end of differentiation using heparin end fibroblast growth factor compare to mesenchymal stem cells control which did not produce any acetylcholine.

Murine and human embryonic stem cells synthesize acetyl choline and express acetyl choline transferase in the regulation of embryonic stem cells as a calcium dynamics, cell survival and proliferation; it's increased the viability, but decreased the proliferation of embryonic stem cells. (Landgraf, *et .al* 2010; Takahashi , 2015).

3.6. Elongation of differentiated motor neuron cells using NGF

The results showed the highest elongation of motor neuron cell axon with a significant increase was (1.93 ±0.185) mm noticed at a concentration 160 ng/ when a nerve growth factor was added to motor neuron differentiation media in the induction stage in addition to RA and SHH compared to control Figure (3-9 A). The results showed a significant grated increase in axon length observed (0.833± 0.145, 0.86 ±0.145, 1.3± 0.01 and 1.76 ±0.317 *vs* 0.433± 0.145) mm respectively in comparison to the control when serial concentrations of NGF added to culture media (10, 20, 40 and 80) ng/ml respectively ($P<0.05$) figure (3-9) and (3-10).

This indicates that NGF induced axon elongation when added to the differentiation culture media into motor neuron cell. The nerve growth factor which has an elongation capability for motor neuron cell axon with increasing concentration represents promising tools for producing spinal cord motor neuron cell with long axons.

NGF is able to precisely control neurtie outgrowth via local action on distinct cellular segments. Studies showed that NGF foster sensory axon elongation and arborization. In culture media, NGF induces outgrowth of dorsal root ganglion neurons (Lemke, 2009).

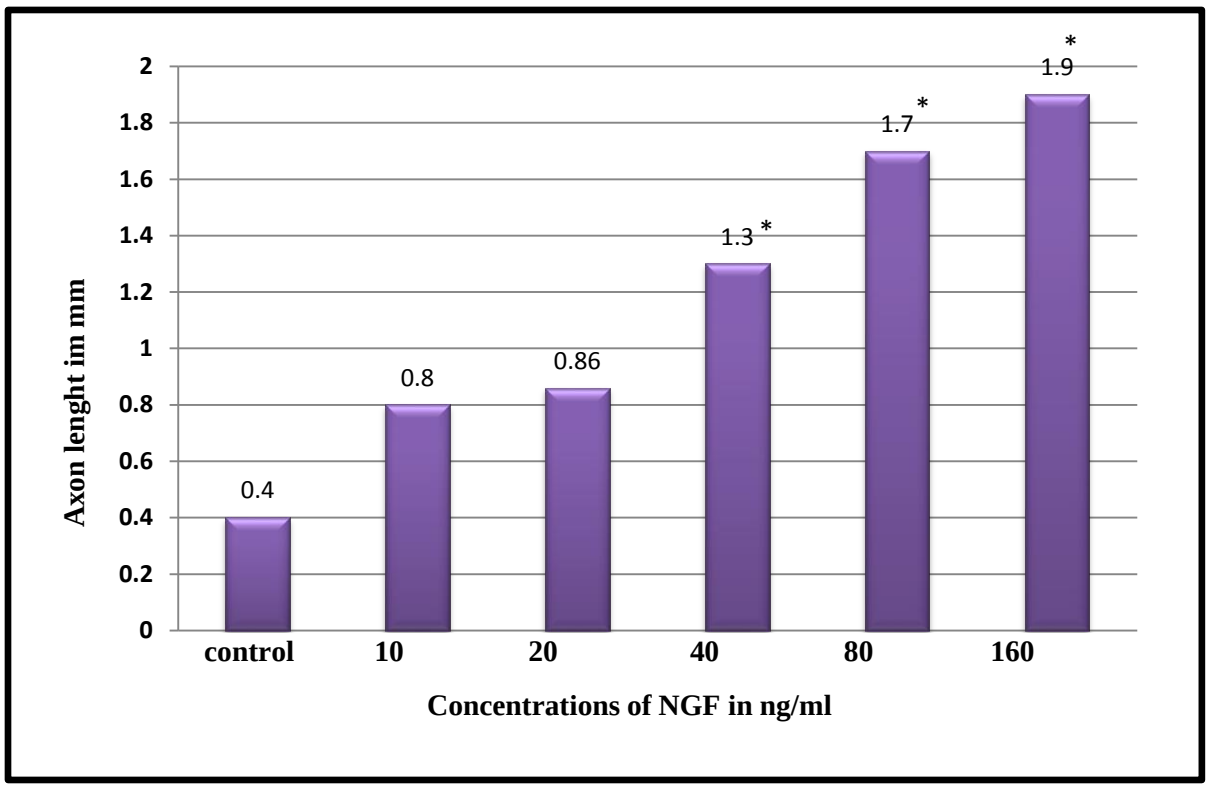


Figure (3-9): Axon length after adding different concentrations of nerve growth factor in differentiation media(*)mean a significant difference in length compare with control p(<0.05).

Lentz, *et al.* (1999) studied the effect of NGF on the sensory nerve cell axon during embryogenesis showed that different concentrations of NGF had different effects on the length of axon in which culture without NGF produce cell with small extended axons.

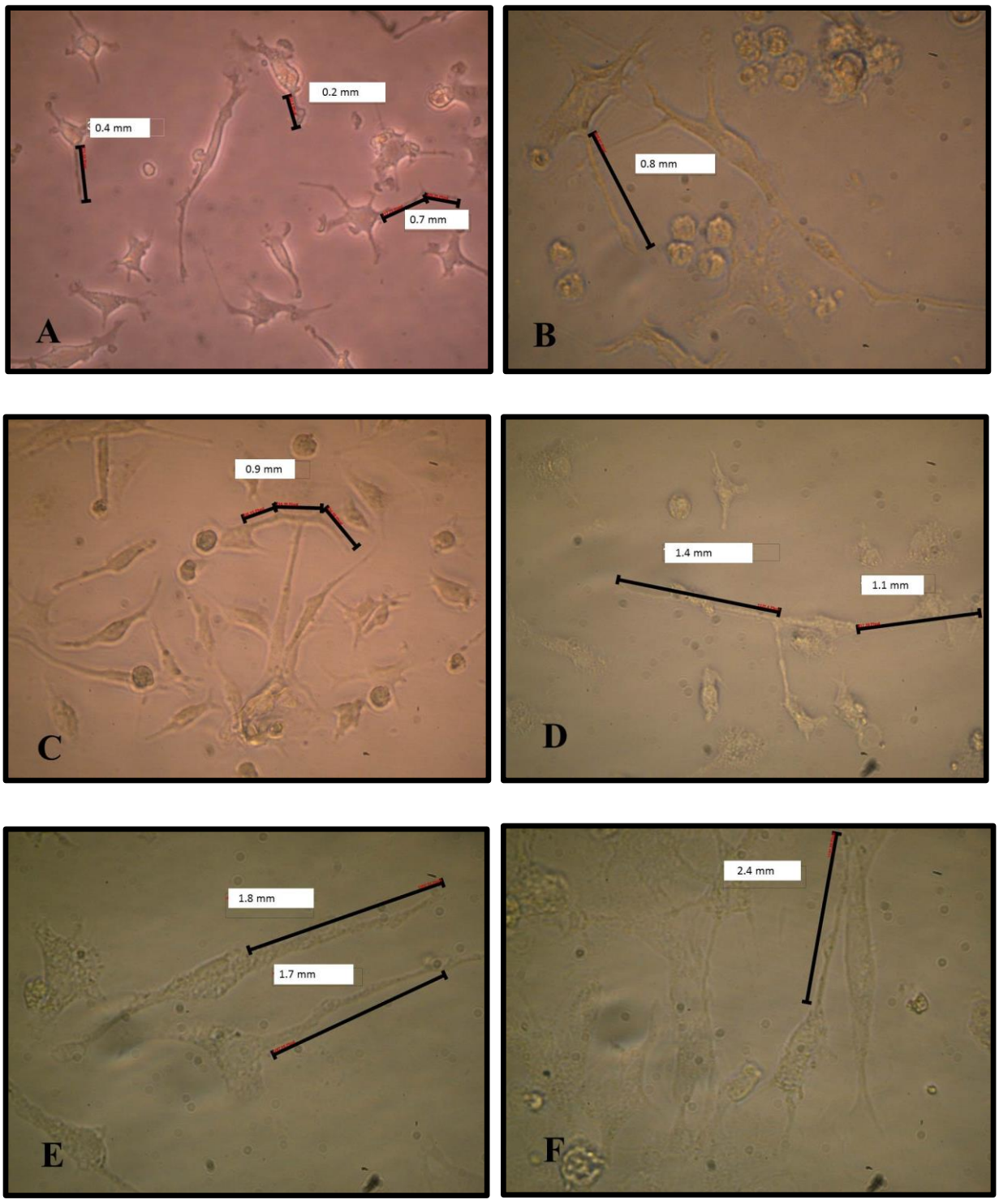


Figure (3-10): Motor neuron elongation. A- Motor neuron cell without NGF. (B-F)- Motor neuron cells with different concentrations of NGF (10, 20, 40, 80 and 160 ng/ml) added to culture media, an increase in axon length was noticed.

Also, significant increases in axon length were shown in the study of Martin *et al.*, (2002) on olfactory neuron axon when 50 ng/ ml of NGF was added to the culture of cells. One of the GTP-superfamily binding proteins was inactivated by NGF through the prevention of axon extension via the actin cytoskeletal so that differentiation culture media containing NGF increased the axon length (Stocum, 2013).

3.7. Differentiation of mesenchymal stem cells in three dimensional cultures:

Neutralized collagen matrix in 8well tissue culture slides had a smooth surface area.Then mouse bone marrow MSCs was cultured on the surface of matrix using the previous differentiation media for the same period of time successfully to mimic *in vivo* structure of brain and spinal code tissue. The cell and matrix after fixation and staining showed something like a thin lens layer .

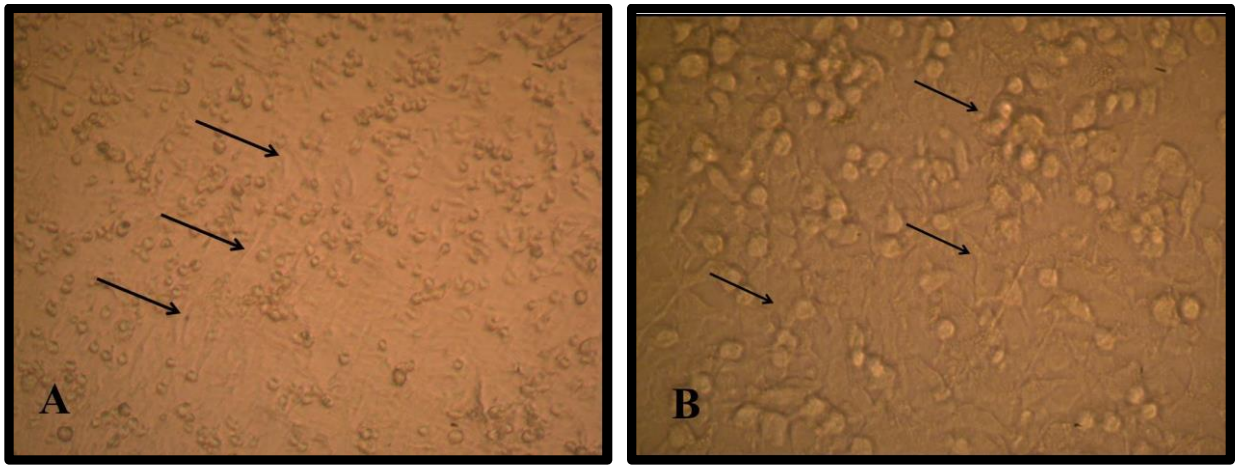
The cellular microenvironment engineering has great potential to create a platform technology toward engineering tissue and organs, a neural microenvironment through fabrication of three-dimensional (3D) engineered collagen matrixes mimicking in-vivo-like conditions. The cell culture of dorsal root ganglion (DRG) cells in 3D collagen matrix was greatly influenced by 3D culture method and significantly enhanced with engineered collagen matrix conjugated with IKVAV peptide. It may be concluded that an appropriate 3D culture of neurons enables DRG to positively improve the cellular fate toward further acceleration in tissue regeneration (Hosseinkhani, *et al* .2013).

Culturing cells by Artym and Matsumoto (2011) inside three-dimensional (3D) collagen matrices indicated the increasingly popular and enlarged understanding of cellular processes and cell - ECM interactions

Mesenchymal stem cells in collagen matrix in CO2 incubator showed either a spindle shape with two or three polar cytoplasmic processes and extended within a matrix or semicircular structure on three-dimensional culture conditions. The cells had accumulat together on the matrix and some are overlaped as shown on the surface of matrix in Figure (3-11).

The pre induction stage of differentiation of MSCs into motor neuron cell on collagen matrix in CO2 incubator showed that the cells became penetrating in matrix and had more cytoplasmic extension forming a neural- like structure figure (3-12).

This phenomena was also show in other study of MSCs on collagen gel matrix containing fibronectin. Cells were adjacent in many parts, in direct contact and lined up in a row while and in 3D collagen–laminin gel, cells exhibited a highly elongated star shape and three-dimensional cultured cells appeared to present directional growth, a form of neuritis outgrowth found in neuronal cells (Lee, *et al*., 2011).



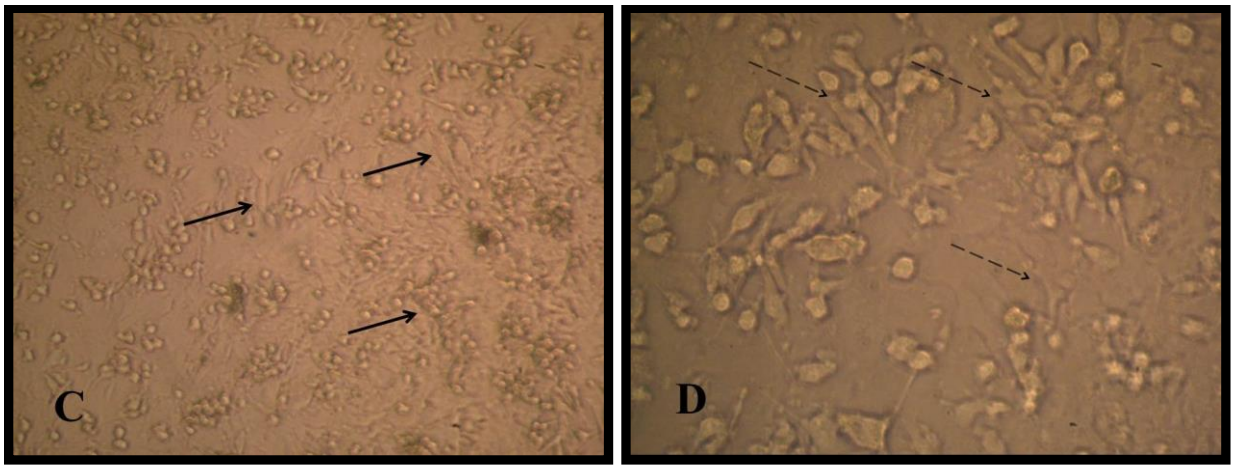


Figure (3-11): MSCs cultured on collagen matrix. A & B- BM-MSCs cultured on collagen matrix after 24h which showed a circular like shape attached to the matrix (as showed by pointer arrows) under inverted microscope (10X10) (10X20). C & D- Cultured on collagen matrix after 48h in which, the cell extended on matrix and have a MSC structure(under inverted microscope) (10X10)(10X20).

At the end of the induction stage, the cell had a big soma structure containing many extensions of dendrites and on the other side, a long extended axis like axon ended with small dendrites like in the structure the motor neuron cell Figure (3-13).

Stem cells differentiated on Hydrogel with culture media containing 1µM RA added regulated formation of neural and astrocyte lineages .When RA and Shh were added to the culture, either alone or together, 3D collagen-1 scaffolds enhanced a significant motor neuron formation, while 3D matrigel stimulated a dopaminergic neuron differentiation. These indicate the synergistic role of micro environmental cues ESC differentiation and maturation, with potential applications in cell transplantation therapy (Kothapalli and Kamm, 2013).

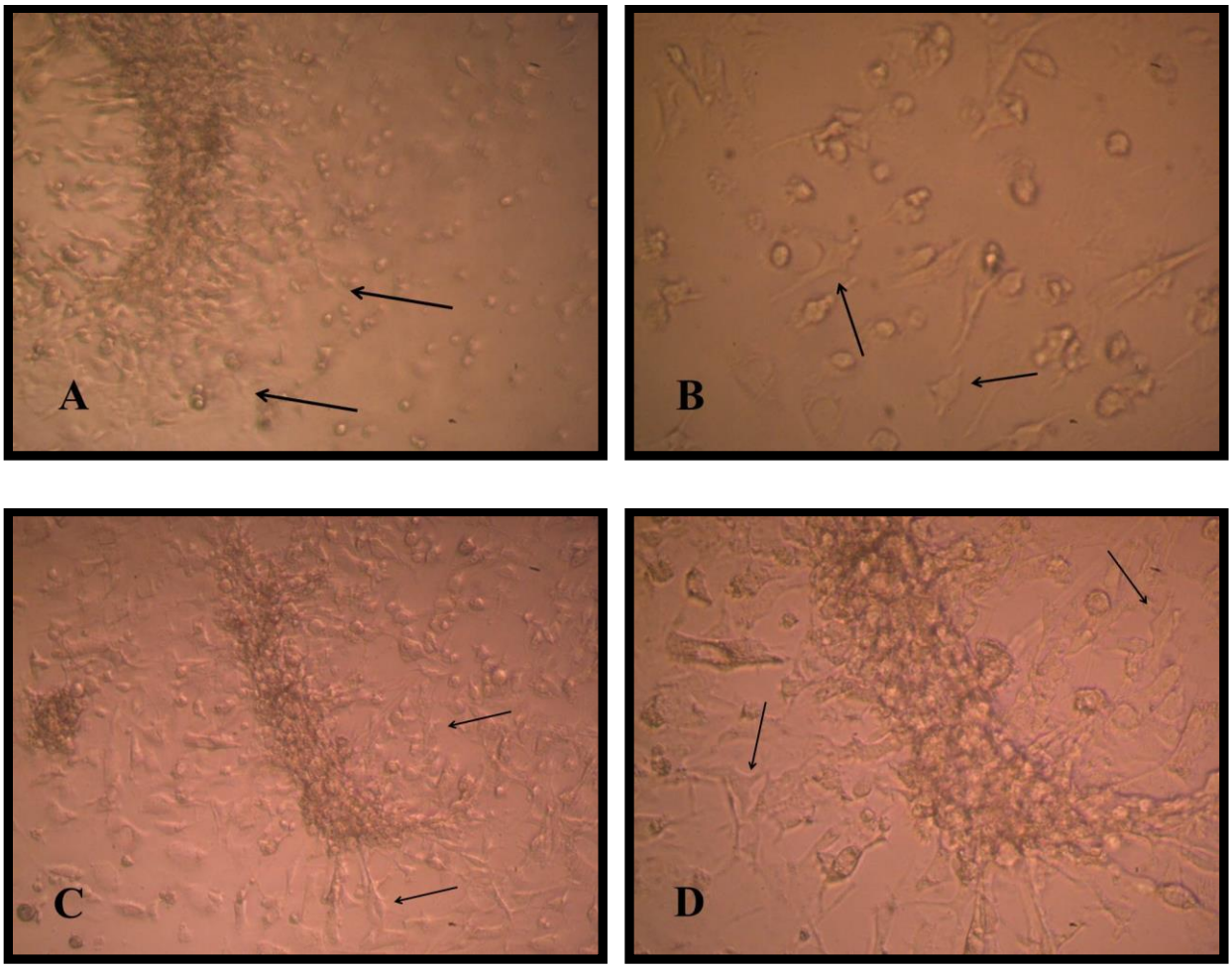


Figure (3-12): Differentiated MSCs on collagen matrix. A & B- BM-MSC cultured on collagen matrix in pre-induction stage in 1mM BME for 24h that showed a cell round structure with cellular extend (under inverted microscope) (10X10) (10X20). C & D- cultured on collagen matrix in a pre-induction stage after 1h 2.5 mM BME in which cell have more extended and more neuron in structure (under inverted microscope) (10X10)(10X20).

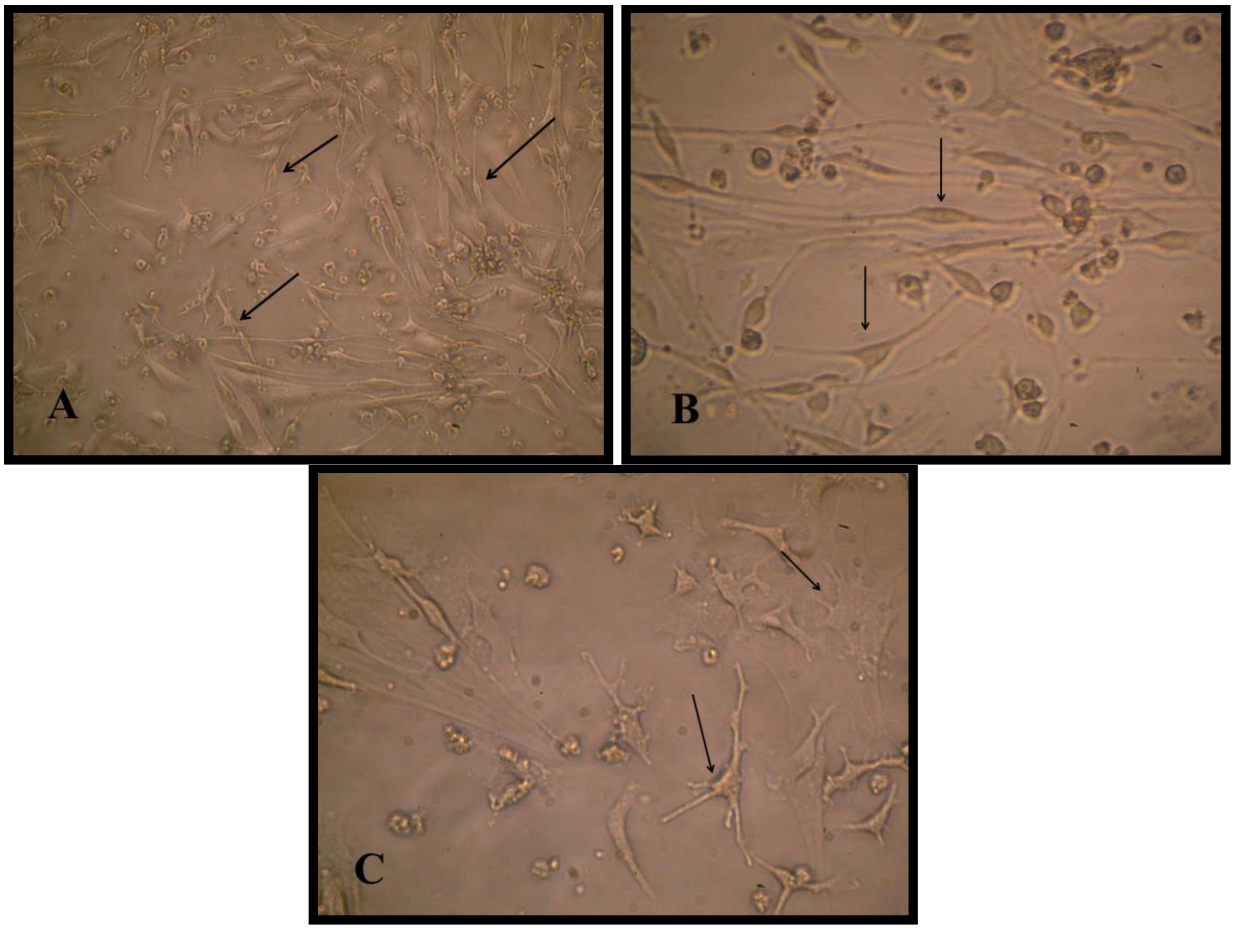


Figure (3-13): Morphological study of MNC on collagen matrix. A- Differentiated motor neuron cell on collagen matrix showing cell on the surface and other inserted into the collagen under inverted microscope (10X10) B- under inverted microscope (10X20) the neural cell structure appears clearly and other long extended cell. C- Motor neuron cell differentiated on collagen matrix, small dendritic soma with long extended axon (10X20).

Collagen matrix forming thin layer with cells collony distributed on the surface as showen in figure (A 3-14) while increasing the microscope lence to(10X40) the cells showed anetwork of cells with motorneuron like structure. H and E study of motor neural cell on collagen matrix indicate atypical cell structure at (10X100).

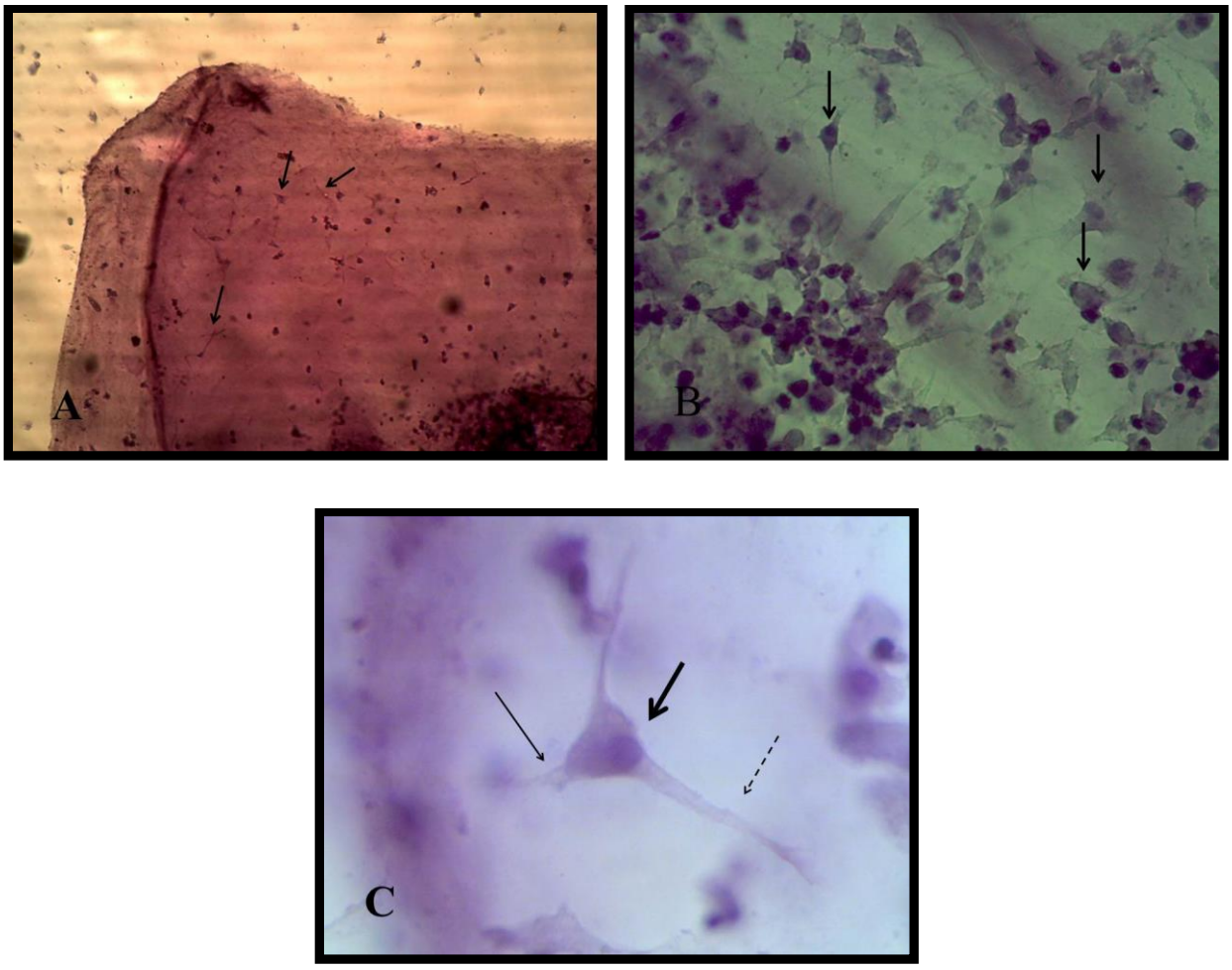


Figure (3-14): Morphological study of MNC culture on colagen matrix. A & B- Motor neuron cell differentiated on collagen matrix stained with H & E under the inverted microscope (10X10) (10X40). C- H & E study showed a red color of cytoplasm with extended axon and a blue color of nucleous (10X100).

Another scientific work on MSCs cultured on extracellular matrix and Matrigel immobilize gell showed a greater differentiation into neural cells than 2D culture indicating a special interaction between cell and extracelullar matrix. Cells cultured on extracellular-grafted also showed higher neural differentiation ratios than those on extracellular 2Dculture .This strenghten the resulted cells after differentiation with motor neuron structure (Higuchi,*et al*,2011).

Motor neuron cells differentiated from MSC in this study showed positive results for the differentiation marker MAP-2 and CHAT when immuonocy-tochemicaly was done on the surface of the matrix, Figure (3-15).

The cells had dark brown color of accumulated chromogenic material on the cell, Single cell as figure (A,B3-15) had soma with long axon extended while figure (C,D 3-15)shoe cells with network like accumulation and also had brown color of chromogen.

Human embryonic stem cells hEnSCs differentiation into motor neuron using in (polylactic-co-glycolic acid) PLGA showed that PLGA scaffolds enhances more the differentiation of hEnSCs into motor neuron-like cells in comparison to the tissue culture plats. So, PLGA nanofibers provide a suitable topography for motor neuron differentiation of hEnSCs. The expression of Islet-1 (80%), Chat (89 %), NF-H (50%), and beta-tubulin III (40 %) in PLGA group were higher than the expression of Islet-1 (70 %), Chat (79 %), NF-H (40 %), and beta-tubulin III (40 %) in tissue culture plats (Ebrahimi-Barough *et al*.2014) .

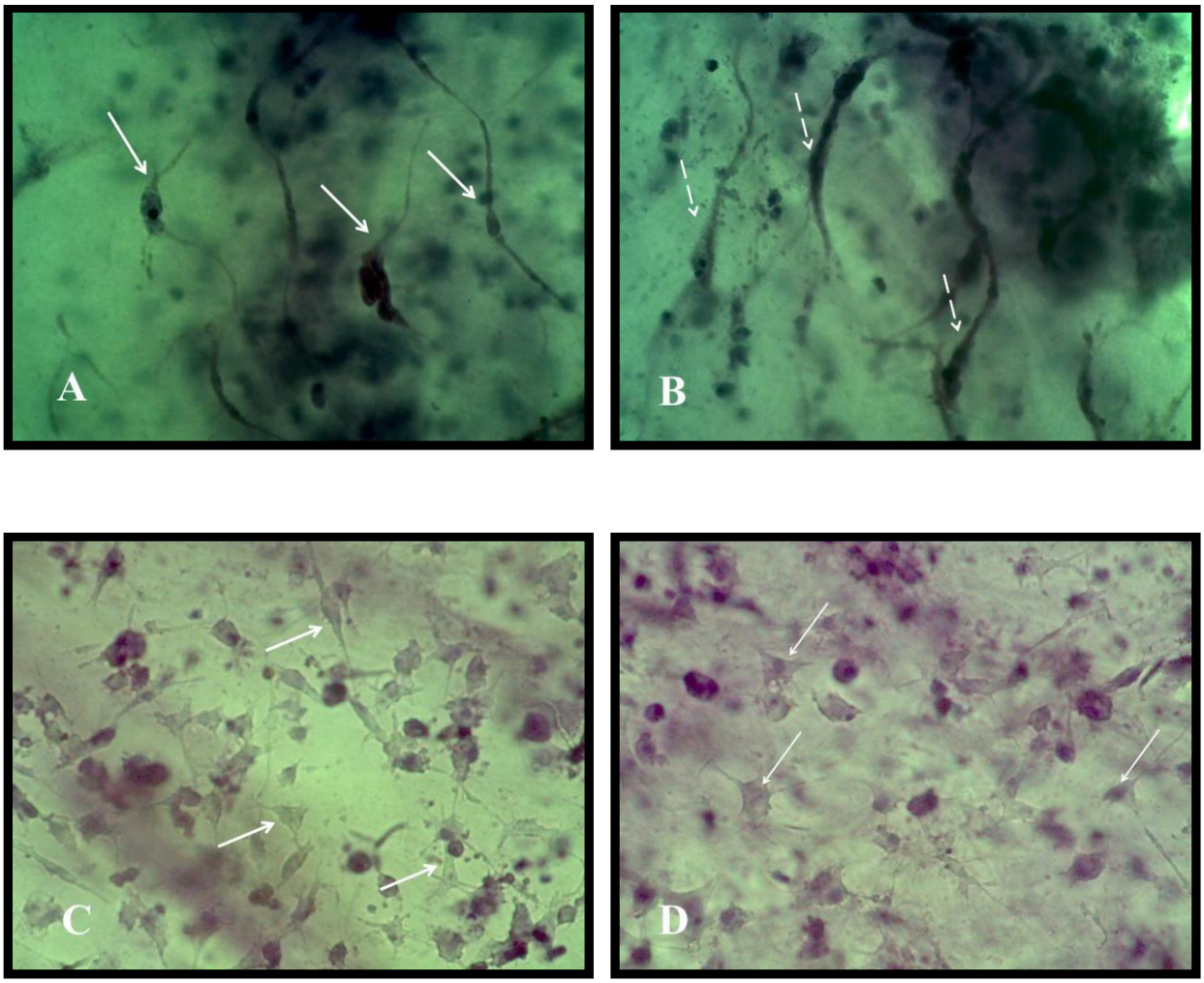


Figure (3-15): ICC of motor neuron on colagen matrix. A & C- ICC study of motor neuron differentiated cell on the surface of collager matrix for cytoplasmic anti -MAP-2 antibody, which showed (positive results) cell body with extended axon (10X40). B & D- ICC study of motor neuron differentiated cell (positive results) on the sufrace of collagen matrix for cytoplasmic anti-CHAT antibodyand cell showed inserted cell structure in the matrix.

3.8. Formation of aggregated motor neuron cell structure

The other indicator for differentiation of MSC into motor neuron in addition to acetylcholine concentration is the formation of aggregation cell phenomena, in which motor neuron cell aggregates to form a long spindle of cell body and many other soma cell accumulated and extend their axon outside, Under the scanning

microscope, a number of accumulated cells appeared like single long cells with edges, Figure (3-16).

Natural aggregates arise from the dissociated cortical neuronal cells in culture from 2-7 days *in vitro* depending on the concentration of the cells. At high concentrations (> 10^6 cells/ml), large aggregates will develop and make interconnections of grouped cellular protrusions, which constitute bundles. The cellular knots and the bundles of protrusions interconnecting cables in the network, occupied with single cells or more forming mushroom like appearance, and their cells are placed at the exterior part of the head in order to get amaximum exchange with the cultured fluid. Phosphorylated forms of neurofilament characterize the development of immature neurons and axons to mature ones and help growing axons to accommodate the demands for plasticity and stability by modulating the structure of the axonal cytoskeleton (Marani *et.al*, 2003).

Beside the structure similar to the present study on culturing septa, cortical or all brain dissociated cells was cultured for 2-5 days in free culture media with some growth factors .The cells aggregates tended to attach and grow neuritis after their transfer to poly-*l*-lysine- or Matrigel-coated dishes under stationary conditions. Early aggregates transplanted to the hippocampus of adult rats developed into identifiable grafts, with fluorescent-labeled cells showing that young neural cell aggregates, maintain their ability to undergo two basic phenomena for cellular interaction, i.e., attachment and neuritic growth (Colombo,*et.al*, 1987).

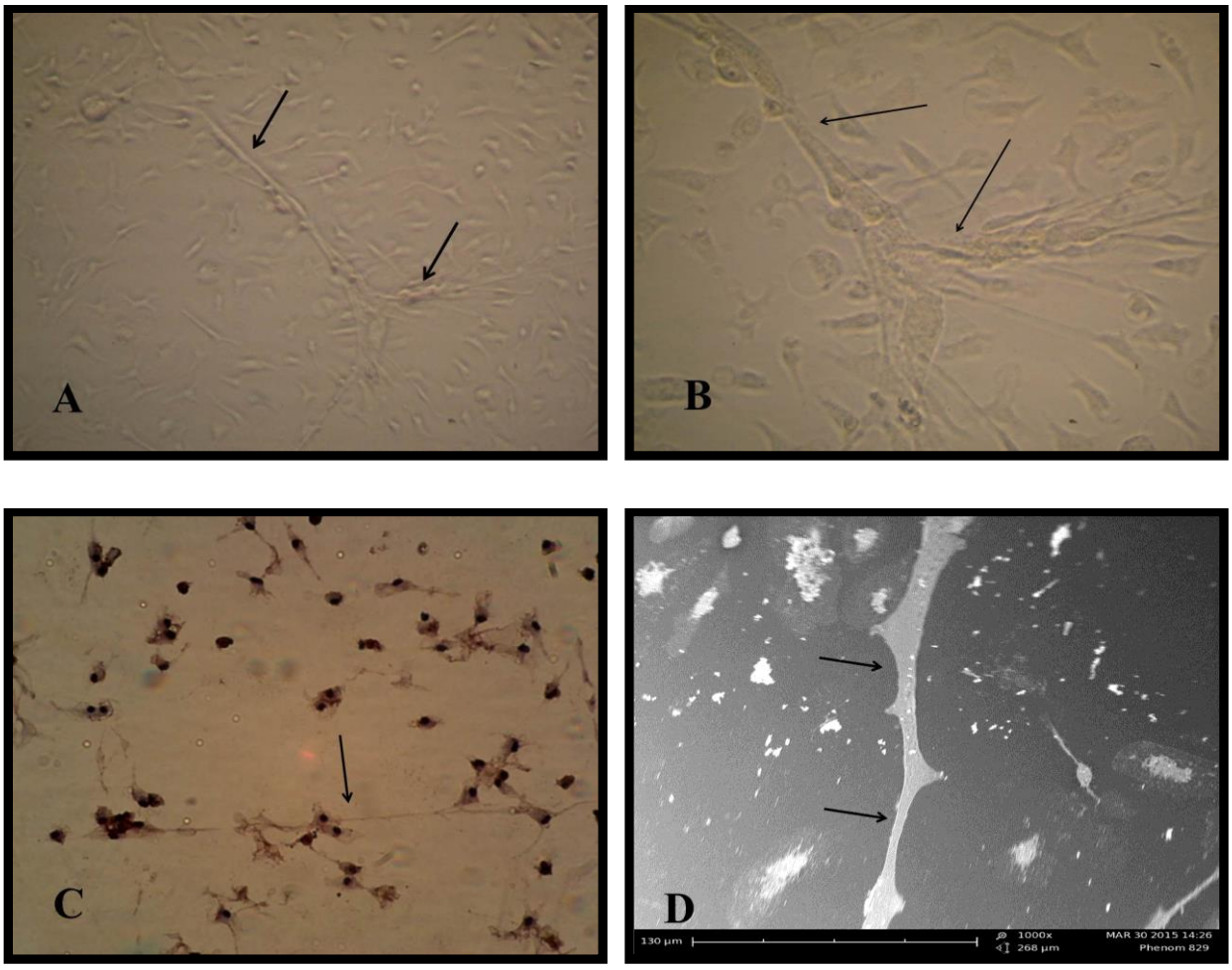


Figure (3-16): Motor neuron cell aggregation. A & B - Aggregated motor neuron cell after differentiation from MSCs which showed aggregate of many motor neuron cells in line like structure (under the inverted microscope). C- ICC study of these aggregated motor neuron cells using CHAT antibody for detection it's as differentiated cell. D- Aggregated motor cell under electronic scanning microscope for the aggregated cell.

Conclusions
And
Recommendations

Conclusions:

The research of the present study has shown the following conclusions:

1- MSCs were successfully differentiated into motor neuron using a specific combination of growth factors including Retinoic acid m sonic hedgehog after 4 days.

2- Differentiated motor neuron showed a positive reactivity by immuno-cytochemical analysis to marker MAP-2 and CHAT.

3- MSC differentiated into active motor neuron by measuring the production of acetyl choline as significantly expressed after 4 days of differentiation.

5- Nerve growth factor according to concentration dependency showed a significant increase in the length of Axon.

6- Collagen three dimensional matrix differentiated MSC to motor neuron show positive results to CHAT and MAP-2 marker indicating a useful tissue method for culturing and differentiation of MSC into motor neuron when using the differentiation factors RA and SHH.

Recommendations

The research of the present study has shown the following recommendations:

1- Inducting Spinal cord injury in mice and using differentiated motor neuron cells to repair the defect.
2- Investigating the possible human Mesenchymal stem cells differentiation into motor neuron lineage which may open a new field of first clinical trials of using stems in the damaged tissue treatments in Iraq.
3- Studying the isolation of mesenchymal stem cells from other adipose tissue which represents an easy and useful way form differentiation.
4- Using more specific methods for the isolation of MSCs like negative isolation by magnetic beads.
5- Studying the benefits of are different matrixes and scaffolds in the differentiation of stem cell into motor neuron other than collagen.
6- Induction differentiation and elongation of MSCs *in vivo*.

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قسم التقانة الاحيائية

تمايز المزرعة النسيجية للخلايا الجذعية المزنشيمية لنقي العظم للفئران لخلية عصبية حركية

اطروحة

مقدمة الى كلية العلوم /جامعة النهرين

كجزء من متطلبات نيل درجة الدكتوراه فلسفة في علوم التقانة الاحيائية

من قبل

رفل حسام الدين عبد الله

بكلوريوس 2003

ماجستير 2007

اشراف

الاستاذ المساعد الدكتورة
شهلاء مهدي صالح

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