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IN VITRO Effect of Differentiation Factors on Accumulation of COL1A1, COL2A1 and CRTAC1 for Chondrogenesis of Mice Bone Marrow Mesenchymal Stem Cells

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Abstract : *In tissue engineering fields, recent interest has been focused on stem cell therapy to replace or repair damaged tissues. In particular, the repair of articular cartilage degeneration by stem cell-based tissue engineering could be of potential therapeutic. Bone marrow mesenchymal stem cells (BM-MSCs) therapy has offered new treatment for cartilage damaged; however, we must first understand the interaction of growth factor and receptors that initiate chondrogenic differentiation and the exact expression required for this differentiation. In this research, we focused on identifying the best combination of MSCs and functional extracellular matrices that provides essential elements for successful chondrogenesis. We experimentation mixture of growth factor, transforming growth factor beta1 (TGF-ß1), insulin like growth factor 1 (IGF-1), fibroblast growth factor, addition to dexamethasone, and ascorbic acid. We assessed the chondrogenic capacity by histology using H&E, alcian blue staining and immunohisto chemical analysis with chondrocyte markers which were positive for Collagen I, II proteins responsible for the chondrogenesis while advantageous cartilage acidic protein 1 (CRTAC1),exclusive proteins contribute to differentiation more than to proliferation. And use 3D (matrix) culture system prove to create cartilage like tissue that may use for damaged cartilage treatments.*

Keywords: *isolation of MSCs, chondrogenic differentiation factors, cell therapy, 3D culture system, chondrogenesis,*

1. INTRODUCTION

Cartilage defect or degeneration caused by trauma, inherited abnormalities, injuries or posttraumatic Osteoarthritis are of great consequence give human tissue limited intrinsic potential for healing[1]. One strategy for repairing articular cartlage degeneration via cell therapy technologies is to create constructs of cells injected on to or with scaffold [2].Stem cell therapy aims to provide complete hyaline repair tissues for articular cartilage repair due to their multipotent capacity, bone marrow mesenchymal stem cells (BM-MSCs) are a promising candidates for use in new cell-based regenerative therapies [3]. Chondrogenesis involves mesenchymal cell condensation, chondrocyte differentiation, proliferation, hypertrophy and ossification, resulting in the formation of cartilage [4]. Pellet culture (micro-mass) system was used to induce the differentiation potential of MSCs to chondrocyte; this culture system allows cell – cell interaction, this type of culture led to the generation of 3D structures that are directly reminiscent of true hyaline cartilage [5].Three-dimensional (3D) environment provides Stem cells an environment that more closely mimics chondrogenesis *in vivo* [6]. Medium supplemented with ascorbic acid , dexamethasone and TGF-β3 for up to 46 days found to induce differentiation of MSCs into chondrocyte-like[7].Under appropriate culture conditions with stimulation of growth factors, MSCs can be induced to undergo chondrogenic differentiation. Transforming growth factor-β1 (TGF-β1) stimulates MSCs chondrogenesis and prevents fully differentiated

chondrocytes from hypertrophy [8-9].TGF-β1 and related bone morphogenic proteins can induce the differentiation of cartilage from primitive mesenchyme, and together with basic fibroblast growth factor and insulin-like growth factors promote cartilage growth [10] Insulin-like growth factors (IGFs) are able to stimulate chondrogenesis and regulate cartilaginous tissue synthesis. IGF-1 has the ability to stimulate chondrogenesis*in vitro* and *in vivo* [11].These growth factors can be used during in vitro chondrogenesis, prior to implantation, or following implantation of chondrogenic cells, to drive the requisite phenotype and matrix biosynthesis.

Prior to *in vitro* chondrogenesis, the addition of basic fibroblastic growth factor (bFGF) to culture medium during expansion of MSCs monolayer stimulates cell proliferation, reducing the time required for progenitor population expansion and retaining subsequent differentiation potential [12]. Each growth factor binds to its own specific receptor, which activates a unique set of signaling molecules, this is the reason that some growth factors function primarily like mitogens (stimulating cell division, but not necessarily differentiation), while others stimulate differentiation as well as proliferation [13].

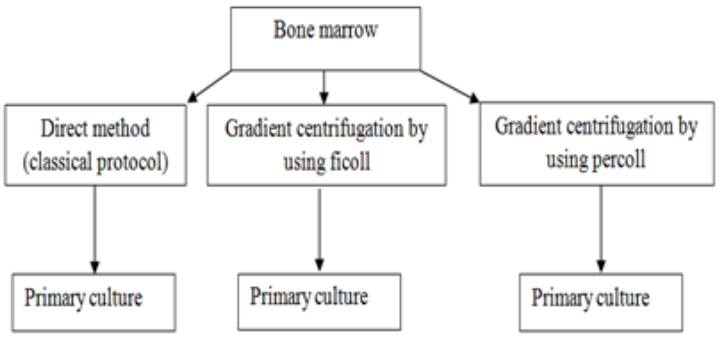
The objective of this research work aimed to evaluating BM-MSCs yield to obtain sufficient quantity and functional characteristic by estimation the differentiation capacity of these cells to determine the stage of chondrogenesis and to define the molecular markers involved in this process. We recognized the morphology of developmentally regulated process of chondrogenesis as well as identified the time lapsing factor indispensable for produce specific marker of chondrocyte. Moreover study the effect of mixture growth factors to produce COL1A1, COL2A1 and CRTAC1 (protein that is found in the interterritorial matrix of articular deep zone cartilage) via 3D culture system. Finally we assessment chondrogenic potential as a result of using scaffold (PCL) to be use in tissue engineering to create cartilage for clinical utilize in future work.

2. METHODS

2.1. Animals

Swiss Albino male mice at 4-8 weeks of age and weighing 20-25 g, were supplied by the Animal house unite , experimental therapy department, Iraqi center for cancer and medical genetic research, Mustansiriyah University, Iraq. The animals were housed (4/cage) in a facility with constant temperature and humidity and a 12/12 hours light/dark cycle, and had free access to rodent chow and water. Animal use was in accordance with the standards by Iraqi Center for cancer and medical genetic research (ICCMGR) for the care and use of laboratory animals. All experimental procedures were approved by the Supervisory Committee of ICCMGR Animal Council.

2.2. Isolation and Separation Of Bone Marrow Mesenchymal Stem Cells (BM-Mscs)



We used three procedures to achieve BM-MSCs isolation: first protocol. By using adherence properties of Mesenchymal stem cells briefly isolated whole bone marrow cells were resuspended in growth culture medium MEM[14]second protocol. Mononuclear cell isolation from bone marrow by using lymphocyte separation media (ficoll) at density 1.077(Cellgro, USA)[15].Third protocol. The cell suspension was loaded on 60% of percoll density 1.088 (Santa cruz biotechnology, USA) for isolation of MSCs [16](Flow chart 1).The cells were cultured in plastic tissue culture flasks with expansive medium: Minimum essential media (MEM) (USbiological-USA), 15% fetal bovine serum (Cellgro, USA), Pen/Strep/Amphotericin B (100 U/mL and 100

mg/mL/250 mg/mL) (cellgro, Carlsbad, CA, USA). Cells were maintained at 37°C. The medium was changed every two day and the unattached cells in suspension were discarded, while the adherent cells were further cultured achieved at least 70% of confluence. At that moment the cells were trypsinizated with Trypsin versin (US biological-USA) and chondrogenic differentiation was performed.

2.3. Immunocytochemistry of Mscs

To determine the phenotypic nature of MSCs according to the surface antigens, the following markers CD105+,CD90+,CD44+, CD34¯and CD45¯,were examined by Immunocytochemistry staining technique, according to the manufacturer's instructions. Briefly, monolayer cultures of the cell line were cultured to confluence in Labtek slide chambers (Nunc, Denmark). Then the cells were fixed in 4% neutral buffer formalin for 10 min and rinsed gently with PBS. The cells were incubated in a humid chamber, first with blocking reagents for 10 min and then with different marker antibodies for 60 min. The antibodies (Santa Cruz biotechnology, USA) used were against mesenchymal stem cells surface markers. The standard protocol for biotin-streptavidin technique was followed using a Universal HRP Immunostaining kit (Santa Cruz biotechnology, USA), including the substrate 3', 3' diaminobenzidine as the chromogen for antibody detection.

2.4. Chondrogenic Differentiation of BM- Mscs

we used three different culture systems, in each one used three types of chondrogenic differentiation media as following : type one differentiation media (M1) consisting of DMEM free serum, high glucose(4.5g/L),L-glutamine(Santa cruz biotechnology, USA) supplemented with ,10ng/ml recombinant human transforming growth factor beta1(TGF-β1) 100Nm(US biological-USA) dexamethasone, ascorbate2-phospho(37.5 micrograms per milliliter) , 10ng/ml recombinant murine insulin like growth factor 1(IGF1) (US-biological-USA).10ng/ml recombinant human fibroblast growth factor, basic,(FGFb) (US-biological-USA). Type two of chondrogenic differentiation media (M2) contenting all components in type one except the absence of IGF-1. Type three of chondrogenic differentiation media (M3), contenting all components in type one except the absence of TGF-β1.

2.5. Culture Systems for Differentiation

Three types of systems were used; *2D culture system:* The cell suspension of MSCs was centrifuged at 1000rpm for 10min and the cells were recultured in multi-well tissue culture plates 8-wells at density (1x104 cells/well). The plates were incubated at 37˚C for 24h. The cultured media were discard and replaced with three types of chondrogenic differentiation media to induce the chondrogenic differentiation. Culture condition and media were identical to pellet culture in addition to control media. Chondrogenic differentiation media was changed every 2-3 days until 21 days. This study involved the morphological change of condensation MSCs and modulating to chondrocyte, also the exact time that required differentiating the cells. *micro-mass(pellet) culture system:* Mesenchymal Stem Cells were induced to differentiate into chondrocyte using the pellet technique[17].Adherent cell colonies were trypsinized and counted and resuspended in growth media at density (1.25 X 105 cells/ml) MSCs centrifuge at 1000rpm for 10 min, after 24h the cell seeded in the tubes, the culture media were replaced with 10ml chondrogenic differentiation induction media, thencells centrifuged at 1000rpm for 10min, incubated up right at 37ºC to form a pellet (Fig. 1, B).*3D matrix culture system using scaffold:* We used 3D insert 12-well polycaprolactone plate (PCL) scaffold(sigma-AldrichUSA)(3D biotek).The cell concentration of MSCs were (0.1x106 cell/ml) in volume 270 µl (according to the manufacturer's instructions), every 2-3 days the spent media removed and replaced with (1-2) ml of fresh chondrogenic differentiation media, after 21 days of seeding , scaffolds seeded cells were washed with PBS and fixed with 4% formaldehyde in PBS. The samples were washed with distilled water and dehydrated by using a graded series of ethanol, embedded thescaffoldin paraffin tissue blocks were sectioned at 4 micron using microtome, and amounted on microscopic glass slides used for desired analysis.

2.6. Evaluation of Chondrogenic Differentiation

Analysis of differentiation by histochemical staining was performed using standard methods to detect cartilage-associated proteoglycans. Samples were fixed with 4% PBS-buffered

paraformaldehyde and cut into paraffin and frozen sections. For histological analyses the sections were stained with hematoxylin and eosin (H&E), and stained with Alcian blue, nuclear fast red (Santa Cruz Biotechnologies, USA) to visualize any glycosaminoglycan (GAG) deposit. COL1A1, COL2A1 and CRTAC1 were immunolocalized with mouse monoclonal antibodies against COL1A1, COL2A1 and CRTAC1 (Santa Cruz Biotechnologies, USA) according to the manufacturer's instructions. Negative control was performed without primary antibodies under identical conditions, mounting and qualitative light microscopic evaluation.

3. RESULTS AND DISCUSSIONS

3.1. Primary Culture of Bone Marrow

The results of present study demonstrated that the first protocol resulting in a much higher cells yield than obtained by another two methods of gradient centrifugations density by using Lymphocyte separation media (Ficoll or percoll).Erythrocyte lysis by using lysing buffer, this method yielded more MSCs and that explained by this type of culture that will provide wide range of cell population from bone marrow which work as a feeder cells that will support the normal growth of the MSCs which supply the cells with growth factors and other needed factors, thus providing well-defined culture conditions for expanding of MSCs. Moreover, the results showed that cells isolated by Ficoll gradient media allowed recovery of more nucleated cells from BM than percoll gradient media. Furthermore, the recovery (self –renewal capacity) of MSCs with percoll protocol was considerably lower than that of MSCs isolated with the classic protocol and Ficoll protocol. Cell isolation with whole bone marrow transplantation requires less time and fewer steps and uses less costly equipment. MSCs easily expanded *in vitro* at day 2 the cells in all cultures were adherent, at day 6 in all cultures cells with spindle-shaped morphology (fibroblast-like morphology) was visible, characteristic of MSCs and at day7th of primary cultures achieved 70% of confluence (Fig. 1 A).

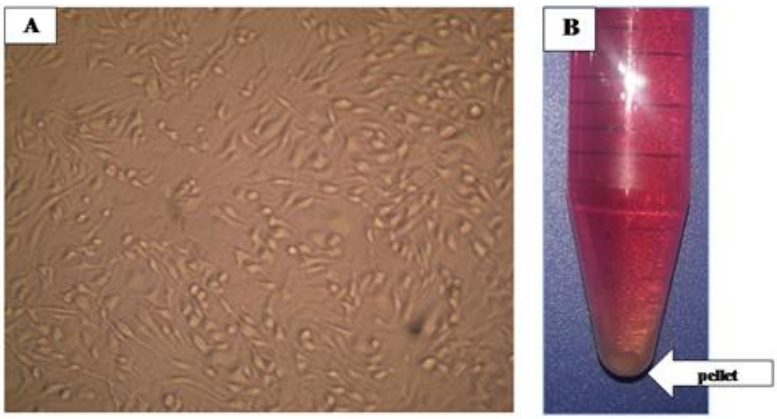
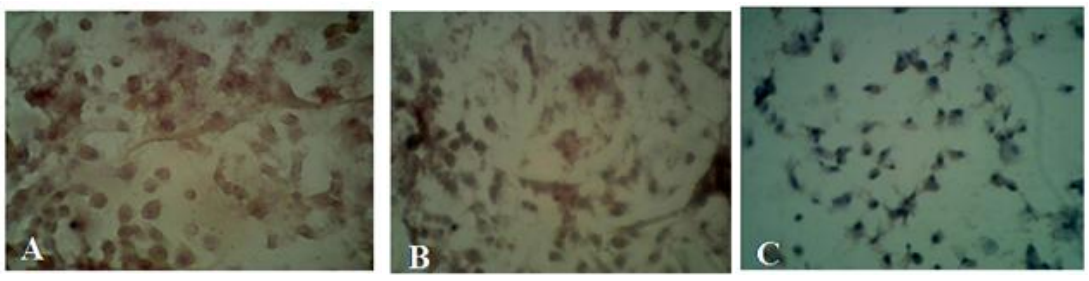


Fig1. *(A) Cells in primary cultures of mouse BM-MSCs growing in a monolayer under in vitro conditions (X20) under inverted microscope. (b) Chondrogenic differentiation (pellet culture) of mouse BM-MSCs forming a spherical pellet in differentiation media containing growth factors that stimulate chondrogenisis*

3.2. Immunocytochemistry of MSC

To determine the phenotypic nature of MSCs we examined the cells according to the surface antigens CD105^{+}, CD90^{+}, CD44^{+}, CD34^{-}& CD45^{-}, by Immunohistochemistry staining technique (imunophenotypic characterization of MSCs) The results of phenotypic characterization from 0-1 passage cells showed that almost all of the BM-MSCs are positive for CD105,CD44 and CD90,but was negative for CD45 and CD34(Fig.2).



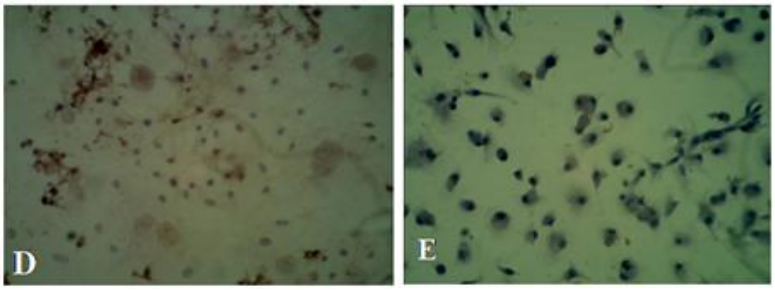


Fig2. *immunophenotypic analysis of MSCs on the second day on the first passage of culturing revealed by microscope.(A) (B) (C) The most of adherent cells MSCs were positive for CD105,CD90,CD44respectively markers and stained with brown color of DAB stain(X40).(D)(E)The majority of adherent cells MSCs were stained negative for CD45 and CD34 markers respectively (X40).*

3.3. Morphology of 2D Culture System

In general, cell morphology changed from spindle-shape to spherical shape and in additional to increasing cells density. At first two days of culturing in 2D culture system, dissociated cells with chondrogenic capacity interact to first form small aggregates , then bigger clusters after one week, and finally cartilage nodules, subsequent 21 days of chondrogenic differentiation, cartilage nodules containing round-shaped chondrocytes were observed this corresponding to lose mesenchymal tissue (Fig.3), as illustrated by the distinct structure comprising of round-shaped cells separated by extracellular matrix and surrounded by a circular arranged fibrillar matrix. In general, differences were observed in the frequency of cartilage nodules between the three types of chondrogenic differentiation media. During the first week of cells cultured in media 1 we showed obvious morphological change than what showed of media 2 or media3. Some of adherent cells died (in all three types of media) whereas the surviving cells began to proliferate and differentiate during the second week. At the end of second week, 70% of all remaining adherent cells had increased in size and had formed a ball-like appearance in one direction; this cell began to connect with adjoining cells. After 21 days of cells culture in differentiation media we noticed that most of the cells are mononuclear and the differentiated cells can be distinguished by the presence of a number of spherical cells (Fig.3).

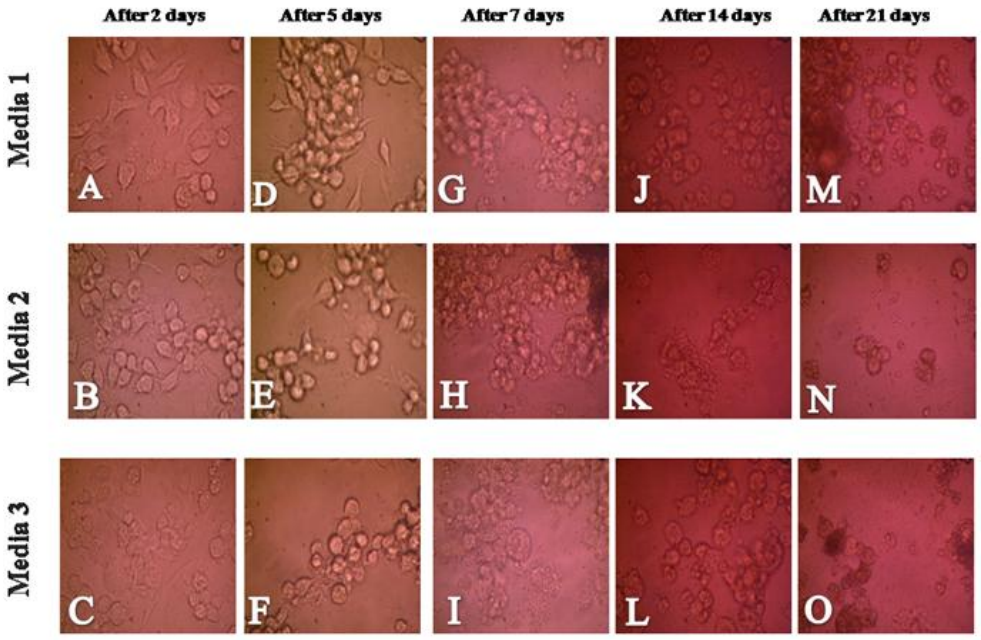


Fig3. *the morphology of differentiated MSCs in vitro in 2D culture system at first passage after 2,5,7,14 and 21 days of treatment with differentiation media under inverted microscope.(A-C) the cells after 2 days after treatment with differentiation M 1,M2,M3 respectively (X40). (D-F) the cells at day 5th after treatment with differentiation M1, M2, M3 respectively (X40). (G-I) the cells in 7th days after treatment with differentiation M1, M2, M3 respectively (X40). (J-L) the cells at day 14 after treatment with differentiation media 1, 2, 3(X40). (M-O) the cells atday21 after treatment with differentiation media1, 2, 3 (X40)*

3.4. Collagen Accumulation & Immunohistochemical Characterization of Chondrogenic Differentiation in Monolayer (2D) Culture System

The results of Chondrogenesis-related proteins for cartilage formation were observed using immunohistochemical staining for collagen type I & collagen type II, early in chondrogenic 2D culture (day7), there was no considerable difference in the collagen content, all cells in three types of chondrogenic differentiation media, were revealed negative results for (COL1A1) and (COL2A1) (data not show). At day 14, a weak deposition staining of COL1A1 in cells cultivated in media 1, whereas no staining throughout the cells cultivated in the media 2 and 3. Weak Positive staining with COL2A1 can be observed on cell cultivated in media 1, very low staining can be observed on certain area for COL2A1 in cells cultivated in media 2 ,but negative stained in cells cultured in medai3 (Fig.4) at day 21 of MSCs chondrogenic in 2D culture , small islands with positive stain of type I collage matrix (brown color) were observed by immunohistochemical analysis in cells cultivated in media 1, a weak positive stain of type I collage matrix in media 2. While no staining in media 3. Cells stained weak positive for type II collagen matrix in media 1&2, but was negative stained in cells at media 3 (Fig.4). Undifferentiated cells (negative control) sections showed no staining for COL1A1 and COL2A1 throughout the cells cultivated in MEM media with 10% FBS (no growth factor). Growth factors such as TGF-β1 and IGF-1 have been shown to be capable of modulating the Proliferation and differentiation potentials of mesenchymal stem cells. In the hyaline cartilage, growth factors regulate homeostasis and integrity, as well as development[18] .We were able to characterize a differentiation stage from mouse MSCs by using different 3 types of chondrogenic differentiation media followed by mature chondrocytes organized in nodules. We showed that IGF-1 have no stimulating effect on initiatechondrogenic differentiation this indict when used M3, nevertheless considered necessary to proliferation after condensation of MSCs by effect of TGF_β1. While another study suggested, IGF-1 (also known as somatomedin C or mechano growth factor) is a hormone similar in structure to insulin plays an essential role in the growth of the organism and has significant anabolic effect [19]. we recognized that product of COL1A1 occur during earliest stage of chonrogenisis, the condensation of MSCs expressed by accumulation of COL1A1while as a mesenchyme differentiation into chondrocyte interconnected with the cell began to produce an ECM rich in COL2A1.

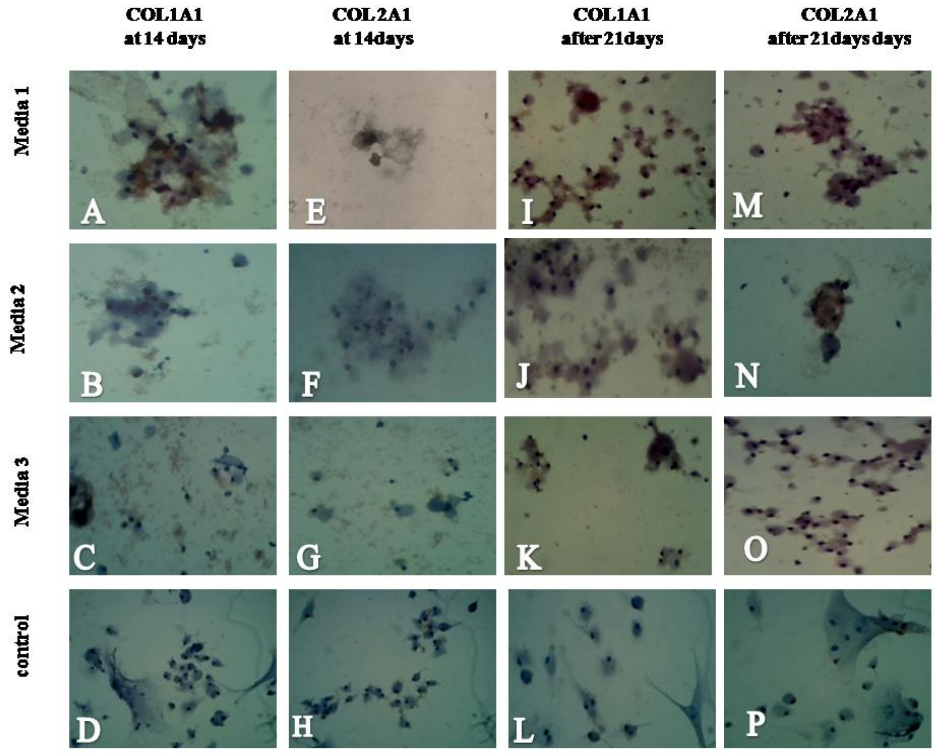


Fig4. *Identification cell surface markers expressed immunostaining of cartilage nodules in the 2D chondrogenic culture of MSCs (A-H) at day 14, (I-P) at 21 day. (A-D) immunostaining for COL1A1 at 14 day (A) weak positive for COL1A1 in media 1 , (B)(C) no expression of COL1A1 in media 2&3*

respectively,(E) weak positive staining of COL2A1 in media 1 at 14 day (F) very low staining of COL2A1 in media 2 at 14 day ,(G) no stained for COL2A1 in media 3 at 14 day .(D)(H) the negative controls accounting for Non specific binding of the primary antibody in MEM media 10% (FBS). (I) positive stained for COL1A1 in media 1 at day21 , (j) weak positive staining of COL1A1in media2 at day21 (k) no expression of COL1A1in media3 at day21 ,(M)(N) weak positive staining of COL2A1 in media 1&2 respectively at day21 ,(O) no stained for COL2A1in media 3 at day21 .(L)(P) The insets show the negative controls accounting for Nonspecific binding of the primary antibody .X40

3.5. Evaluation of Chondrogenic Differentiation for Pellet Culture System

3.5.1. Histological Analysis

3.5.1.1. Hematoxyl in And Eosin (H and E)

Our experiment results showed that MSCs pellets treated with media 1and 2, were larger when compared to pellets cultured with media 3, this explained by action of TGF-β1 in combination with other factors which typically act on target cells as signaling molecules, promoting cell differentiation and chondrocytic proliferation.

H&E staining showed that the section of cells in media 1 were flattened round cells. Moreover, stained sections showed a various homogenous cells distribution in all pellets and the rounded cells darkly stained nuclei, but the intensity of H&E staining slightly decreased from the sections of cells cultivated through media 1 to media 3 (Fig. 5 A,B,C). A homogenous cell distribution in the section of pellet cultivated in media 1 showed formation of compact cartilage tissue , while in the section of pellet cultivated in media 2 Showing semi homogenous cell distribution with less compact tissue which may somehow resemble cartilage tissue.

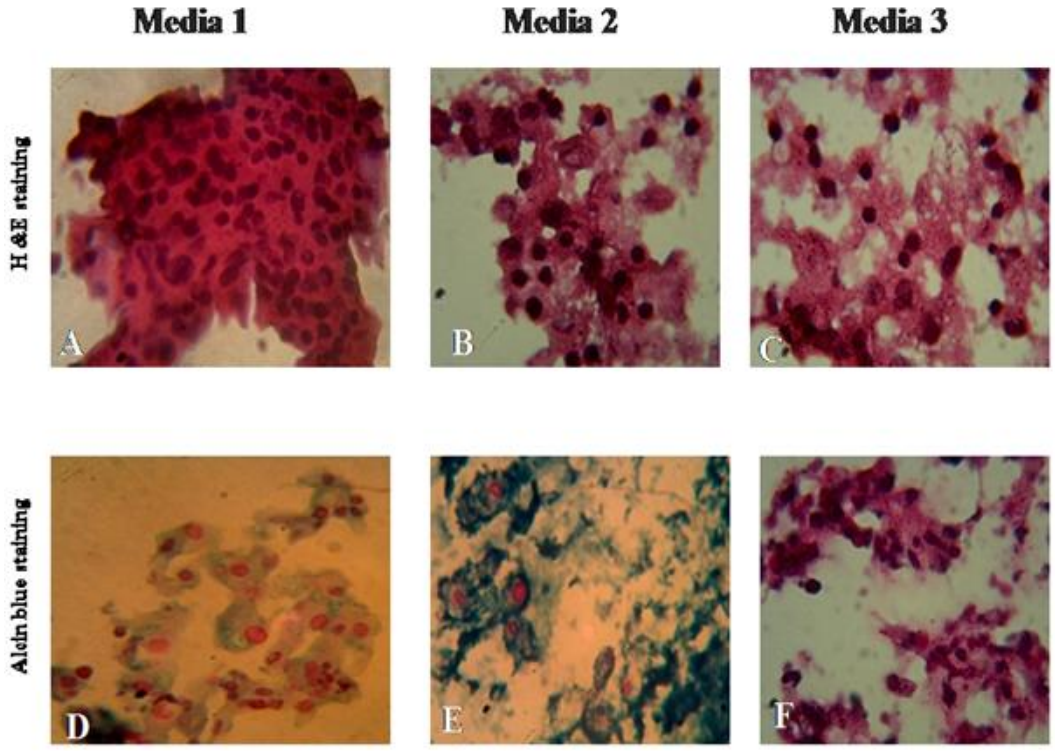


Fig5. *Histological analysis of mouse MSCs in pellet culture after 3 weeks of differentiation (hematoxilin and eosin staining) (X100). (A) Displayed a homogenous cell distribution in the section of pellet cultivated in media1 where compact cartilage tissue was evident. (B) Showing semi homogenous cell distribution in the section of pellet cultivated in media 2. (C) Cell cultivated in media 3 no cartilage formation. (D) Alcian blue staining of the pellets in media 1, the cell nuclei are stained with Nuclear fast red. (X100).(E) positive stained nodules of chondrocyte in media 2 the cell nuclei are stained with Nuclear fast red (X100) .(F) alcian blue stain disappeared indicating a change in composition of the extracellular matrix.*

3.5.1.2. AlcianBlue Staining

Special staining with alcian blue was used for checking the presence of extracellular matrix in the hyaline cartilage, this staining showed deposit of acid mucopolysaccharides in the proximity of

the cells in pellets of media 1 & media 2. The pellets consisted of rounded cells stained positive and the cells surrounded by extracellular matrix which were similar to chondrocyte as observed from the magnified view with lightly pink stained nuclei corresponding to nuclear fast red stain and loose of MSCs, no positive staining present in the pellets that cultivated with media 3(Fig.5D, E,F). In the pellets that cultured in media 1 which contain TGF-*ß*1 together with other growth factors, the chondrocytes expressed the mature cartilage matrix protein collagen II and stained positive for Alcian blue indicating the presence of cartilage proteoglycans. We found the importance of TGF-*ß*1 on MSCs condensation during chondrogenesis. These results are in accordance with the other findings reported by Havlas [20]. As they have an additive effect, which leads in a significant increase of cartilage matrix synthesis. Using TGF-β1 growth factor enhance both collagen and GAGs production and their biomechanical properties [21-22]

3.5.2. Immunohistochemistry Analysis In Pellet (Micro-Mass) Culture System

The results of immunohistochemistry for differentiated MSCs into chondrocyte after 21 day of pellet (micro-mass) culture system in three type of chondrogenic differentiation media (M1, M2, M3).Type I collagen (COL1A1) immunoreactivity was positive throughout the extracellular matrix in cells cultured in media 1, weak deposition of COL1A1 observed in cells cultivated in media 2, while it was negative in pellet cultivated in media 3 (Fig. 6 A, B, C).

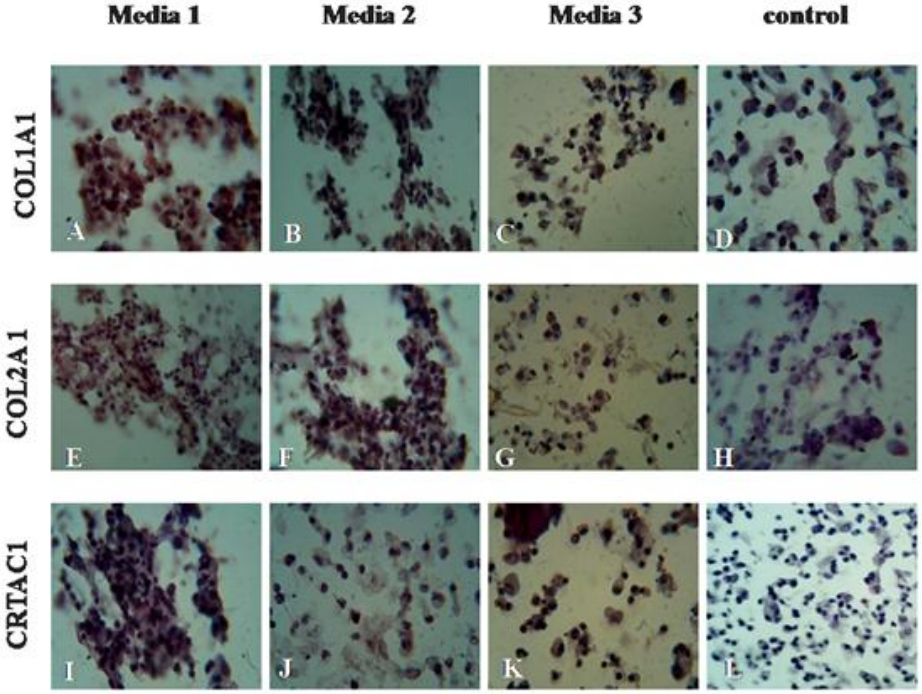


Fig6. *Immunohistochemical analysis of chondrogenic differentiation of mouse MSCs in vitro (A-D) Immunohistochemical for type I collagen on pellets (A) in media 1 positive stain,(B) in media 2weak positive stain,(C) negative stain in media 3(D) negative in media without growth factors. Sections (E-H) stained for type II collagen on pellets (E)type II collagen was mostly localized at the most region of the pellet in media,(F) weak staining in medai 2 .(G) negative stain in media 3 (H) negative in media without growth factors (I-L) Immunohistochemical for CRTAC1 (I) positive stain in media 1,(J) slight positive stain in media 2 .(k) no stained in media3 (L) negative in media without growth factors.*

Type II collagen (COL2A1) stained strong positive in cells cultured in media 1, weak deposition of collagen II observed in cells cultivated in media 2. No positive staining for type II collagen in pellet of cells that cultivated in media 3(Fig. 6 E, F and G). positive staining of cartilage acidic protein 1 (CRTAC1) can be observed of pellets cultivated in media 1, Slight positive stained in media 2, negative CRTAC1 staining was visible in sections of cells cultivated in media 3 (Fig. 6 I, J and K). Chondrogenic assessment by means of immunohistochemistry indicate no type I collagen, type II collagen, CRTAC1 in pellet of media3. Several different collagen types are found in articular cartilage; however, 90–95% of the collagen present is in the form of collagen type II fibrils [23]. Later, in pellet cultured in media 2 absence of IGF-1, the chondrocytes expressed collagen I, a marker for hypertrophic chondrocyte. Our study showed that IGF-1 improved proliferation and chondrogenic differentiation of MSCs. Furthermore, TGF-*β*1

stimulates synthetic activity of chondrocytes and acts against catabolic activity of inflammatory mediator interleukin 1 *in vivo*[24] Moreover, TGF-β1 treatment of MSCs leads to up regulation of collagen type II and aggrecan gene expression typical for hyaline cartilage formation[25]Likewise (TGF-β1) seems to have several regulatory activities to stimulate the production of extracellular matrix and collagen type II by meniscus cells[26] In the pellet cultured in media3 absence of TGF-β1 the nodules lost their stain ability for Alcian blue and no expression of collagen I, II and CRTAC1. Histological analysis demonstrated that IGF-1 treatment did not have any effect on cell morphology which was also provide by immunohistochemistry analysis [27].

3.6. Scaffold 3D Matrix Culture System

Histological sections of matrix polycaprolacton (PCL) scaffold culture demonstrated after 21 days the formation of cell layer on the surface of the cultured meshes. The sections were stained with hematoxylin and eosin for histological analyses. H&E staining revealed cell attachment and distribution within each scaffold pore/layer suggesting growth and proliferation (Fig.7 A, B, C) showed staining all the section of the scaffold cultured with H&E under static conditions .

Sections of scaffold constructs were stained with alcian blue to compare their capacity for glycosaminoglycan (GAG) and proteoglycan accumulation (Fig. 7 D, E, F). Generally For Alcian blue staining, indicated glycosaminoglycan (GAG) accumulation in the scaffolds that varied with the type of differentiation media, chondrocytes showed intense blue color in media 1 suggesting the presence of abundant GAG and this results indicate formation of an approximately continues cartilaginous extracellular matrix rich in proteoglycan. The chondrocytes were able to synthesize and accumulate extracellular matrix typical of articular cartilage in 3D culture when used scaffolds compared with that of cell-free scaffolds. Our current experiment found that scaffold produced a higher level of GAG when compared to pellet culture , and pellet culture measured higher GAG production than 2D culture, this result indicated by alcian blue staining which showed positive stain in the cells cultivated in media 3 of scaffold cultures while negative stained in cells cultivated in media 3 of pellet cultures.

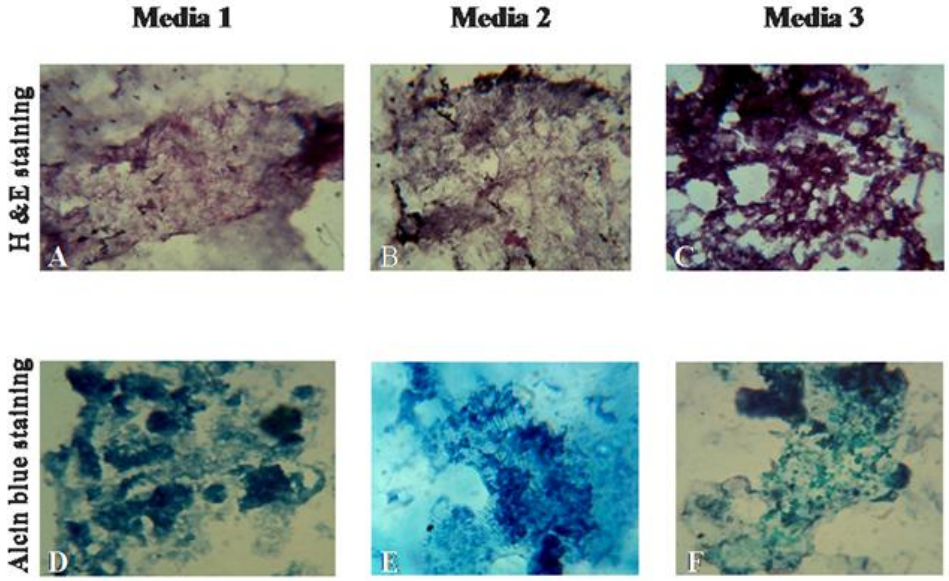


Fig7. *Identification of Histological staining within the 3D matrix (PCL) scaffold,(A-C)The scaffolds show positive staining for (H& E) expansion of modulated growth of MSCs in to chondrocyte showing sample extracellular matrix formation, positive staining for H&E of all groups. (D-F),the scaffolds show positive staining for alcian blue, expansion of modulated growth of MSCs in to chondrocyteshowing sample extracellular matrix formation, positive staining for alcian blue staining to determine the proteoglycan content of the cell clusters, positive staining was detected within scaffolds in all samples (X100).*

During mesenchymal cell aggregation and condensation, cell–cell interactions promote cell adhesion and the release of paracrine factors [28-29]. We hypothesis that the exact molecular events that occur in chondrogenesis Thus, a direct in vitro 3D culture system could more accurately determine the stages of chondrogenesis via IHC characterizations.

As a result, reduced cell-cell interaction and absence of cellular condensation may lead to the inhibition of chondrogenic differentiation in 2D culture, So this study suggest that the cartilage tissue stimulate with 3D (scaffolds) was a more apropreate tissue model for induce stem cells to chondrocyte. This result corresponding with shen [30]. who propose to promote chondrogenic differentiation and expanded MSCs need to be subsequently cultured in micro-mass or in scaffold materials, such as polymers, alginate beads, collagen sponges or hydrogels and microspheres. Stem cells that receive signals from their surrounding environment galvanize intracellular transduction pathways, which deliver information to the genome in the form of activated transcription factors. These factors recognize specific sequence motifs in the genome, where they associate with different transcription factors, coactivators, and chromatin remodelers to exert their effects [31].Genome-wide analyses of Smad proteins bound to chromatin have greatly advanced our understanding of TGF- β superfamily signaling. On their own, Smads bind with low affinity to DNA, and require interactions with other transcription factors to stabilize their binding to chromatin [32]. In response to TGF-β super family signaling increases upon differentiation, the master regulators ultimately dominate the competition for interactions with Smad2/3 [33]. the majority of TGF-b signaling routes go through phosphorylated R-Smads, not all responses involve Smad4.An interesting case in point is the catalytic subunit of a well known transactivator of nuclear factor kB (NF-kB), IkB kinase (IKKa). A Smad4-independent role for pR-Smads has also been implicated in microRNA maturationin the nucleus [34]. Activated TGF-b receptors can also phosphorylate Shc, thereby recruiting Grb and Sos to the membrane, activating Ras GTPase and ERK MAPK signaling [35].Weiss and colleagues [36] showed that parathyroid hormone-like peptide and basic fibroblast growth factor (FGF) play a critical role in regulating terminal differentiation of BMSCs by suppressing collagen X while maintaining the expression of other matrix protein, thus preventing hypertrophic differentiation of BMSCs by *invitro* pellet cultures.

4. CONCLUSIONS

Our results have demonstrated that direct culture of BM-MSCs is the best for expansion and chondrogenic differentiation. IGF1 and TGF mediates hypertrophic differentiation these indicate by accumulation and produce of mature type I, II collagen and CRTAC1 markers of mature chondorocyts .as well as the use of IGF1 and TGF induce high level of CRTAC expression which indicate the importance of this marker as chondrocyte differentiation marker which represents a promising tool for regenerative medicine and these cells can be used for cartilage repair.

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Conflict of Interest

"The authors declare that there is no conflict of interests regarding the publication of this article."

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