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Research Article

EVALUATION OF GROWTH AND DIFFERENTIATION OF UMBILICAL CORD'S MESENCHYMAL STEM CELLS IN THE PRESENCE OF INSULIN TO OSTEOCYTES

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ABSTRACT
Mesenchymal stem cells are focused by most researchers for planning cell therapy due to their unique features. Human's mesenchymal stem cells were treated for 21 days by bone differentiating culture and some of samples were treated with different concentrations of insulin. Von Kossa and alizarin red staining were performed at day21 for the cells under treatment. Evaluation of cells after staining revealed the precipitation of calcium in extra cellular matrix. The nano scaffold containing differentiated cells were sporadically turned to black in Von Kossa stain and red in alizarin red stain due to calcium precipitates. In the samples treated with different insulin concentrations, Differentiating to bone tissue was poorly seen in high concentrations. According to the results, 4IU of insulin concentration is the proper concentration for differentiation of mesenchymal stem cells to bone.

Keywords: *Mesenchymal Stem Cells, Cell Differentiation, Insulin*

INTRODUCTION
Stem Cells
If a stem cell doesn't receive particular signal in favor of growth and differentiating toward specific or specialized cell, it doesn't basically change. Stem cells have the ability to change into several types of cells in multicellular eukaryotes and act as a repair system by unlimited proliferation without averting other cells. When a stem cell is divided, each new cell whether stays as a stem cell or changes into other types of cells with specific function (Meeker and Coffey, 1998).
Embryonic Stem Cells
Fetal stem cells are the cells derived from inside blastocyst of mammals and in mammals the ovocyte is fertilized (Sobolewski *et al.*, 2000; Smit, 2003).
Adult Stem Cells
These cells exist in different mature tissues with limited numbers. These cells are often inactive and do not proliferate unless there are some factors such as diseases or injury by which they are activated (Greider and Blackbum, 1998).
Umbilical Cord Blood Stem Cells
They are derived from fetal umbilical cord and are the most important source of deriving stem cells due to easy access and low risk of viral infection. Umbilical cord consists of one vein and two arteries surrounded by wharthon's jelly. All parts of umbilical cord are covered by epithelium from fetal amnion. Stem cells can be derived from any part of umbilical cord (Maryam *et al.*, 2005).
Markers of Stromal Stem Cells / Mesenchymal
Flow cytometry analysis showed that, if umbilical cord's mesenchymal stem cells stay in the culture, they express CD44, CD155 adherence molecules and CD51, CD20 integrin markers and also CD77, CD106, CD120, CD13, CD16, CD90 were positive for mesenchymal stem cells (Roa and Matton, 2008).
Characteristics of Mesenchymal Stem Cells
These are known in the time of culturing by the appearance of spindle-like and ability to stick to the bottom of the flask (Bruder *et al.*, 2001). One of the other important features of these cells is the ability of self-renewing for long periods, meaning that they can make a copy of mother cell with mitosis and keep this ability for a long time (Wright *et al.*, 1996). One of the features of mesenchymal stem cells is their colonization from the time of primary culture. Differentiation of mesenchymal stem cells is in a way that,

in addition to bone; they also differentiate to epithelial cells of lungs, liver and neurons (Grove *et al.*, 2004).

Insulin

Is a hormone secreted from Beta cells of langerhans islets in pancreas. A structure made and binded together by disulfide Bounds. The role of this hormone is known as blood sugar regulator. The secretion of insulin to blood is complicated. Insulin secretion is elevated when blood glucose is high, so glucose is stimulator of insulin secretion (Segev *et al.*, 2004).

MATERIALS AND METHODS

Isolation of Umbilical Cord's Mesenchymal Stem Cell

Umbilical cord of a newborn was transferred in a sterile way, in normal saline, to the lab with the blood inside it. The isolation of umbilical cord's mesenchymal stem cells was performed (Yanfang *et al.*, 2008).

Differentiation of Stem Cells to Bone Cell

In differentiation of stem cells to bone cells, differentiation medium containing DMEM with low glucose, ascorbic acid, dexamethasone, beta glycerolphosphate and FBS was added. Treatment time was 21 days, and the medium was replaced every 3 days. Simultaneously, by adding the differentiation medium, insulin was added in 3 different concentration of 40 micro liter (4 IU) 100 micro liter (10 IU) and 16 micro liter (16 IU) (Tu *et al.*, 2003; Do *et al.*, 2004)

Alizarin Red Staining

Alizarin red staining was used to prove the differentiation of stem cells to bone cells. This staining was done on 21st day of culturing. Cells were fixed by paraformalin 4% at room temperature. Alizarin red staining 1% was added and incubated. Then it was washed several times again and observed by microscope (Wang and Yu, 2010; Eslaminejad *et al.*, 2007).

Von Kossa Staining

A proof of differentiation of stem cells to bone cells was done by von kossa staining. This staining was done on 21st day of culturing. Cells were fixed and washed. AgNo3 (argentine nitrate) 2/5 % and sodium thiosulfate were added. Finally they were washed by distilled water and observed under invert microscope (Eslaminejad *et al.*, 2007).

MTT Test and Viability of Cells

Viability tests were performed in a week, on the first, third and seventh days. RPMI medium and MTT staining were added and incubated. After that, DMSO was added and put into incubator. Samples were transformed to cuvettes after taking out from incubator and light absorption was measured by ELISA reader with 570 λ wave length (Falak *et al.*, 2004).

RESULTS

Isolation of Umbilical Cord's Mesenchymal Stem Cells

in this method, cell's growth started after 10 days and were easily detectable under microscope (Figure 1A). Cell colony were made on day 12 and on 14th day, 80% of flux was filled with attached cells which had spindle like shapes (Figure 1B). Then venous tissue was separated from medium and cell culturing was performed again. The ability of colonization was greatly seen in the isolation of umbilical cord's stem cells and these cells were attached to the bottom of flux and were spindle like from the beginning.

Stem Cell's Isolation by Flowcytometry Techniques

Flowcytometry machine was used to record and analyze the intensity of gained fluorescence from single cells, in which for examining a cell we should use fluoro-chromes attached to antibodies. CD45 is a specific marker of blood cells and main index of leukocytes and mesenchymal stem cell don't have it. 1/83% expression of it shows low percentage of blood cells in this population. CD90 marker was expressed at 95/27%. CD105 marker was expressed at 90/28% rate. This high expression rates were like specific markers of mesenchymal stem cells.

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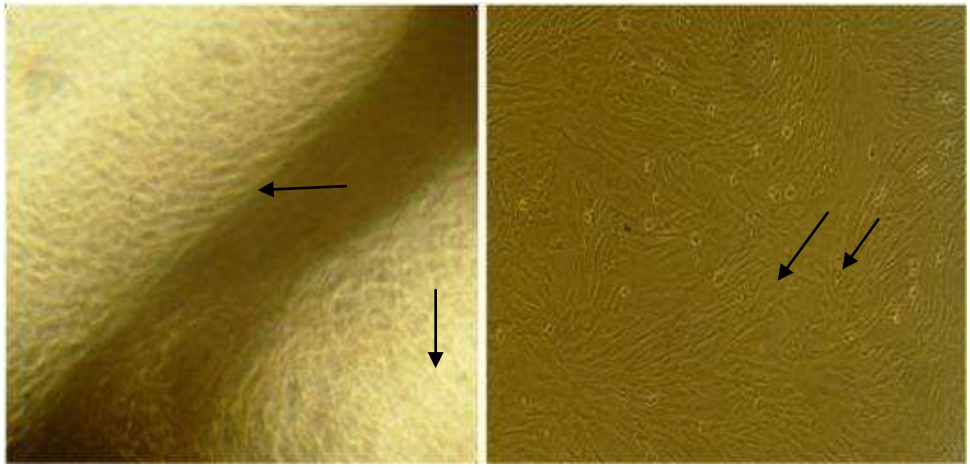

A **B**

Figure 1: Mesenchymal stem cells (20x); (A) Exclusion of germ cell; (B) Formation of a cell layer

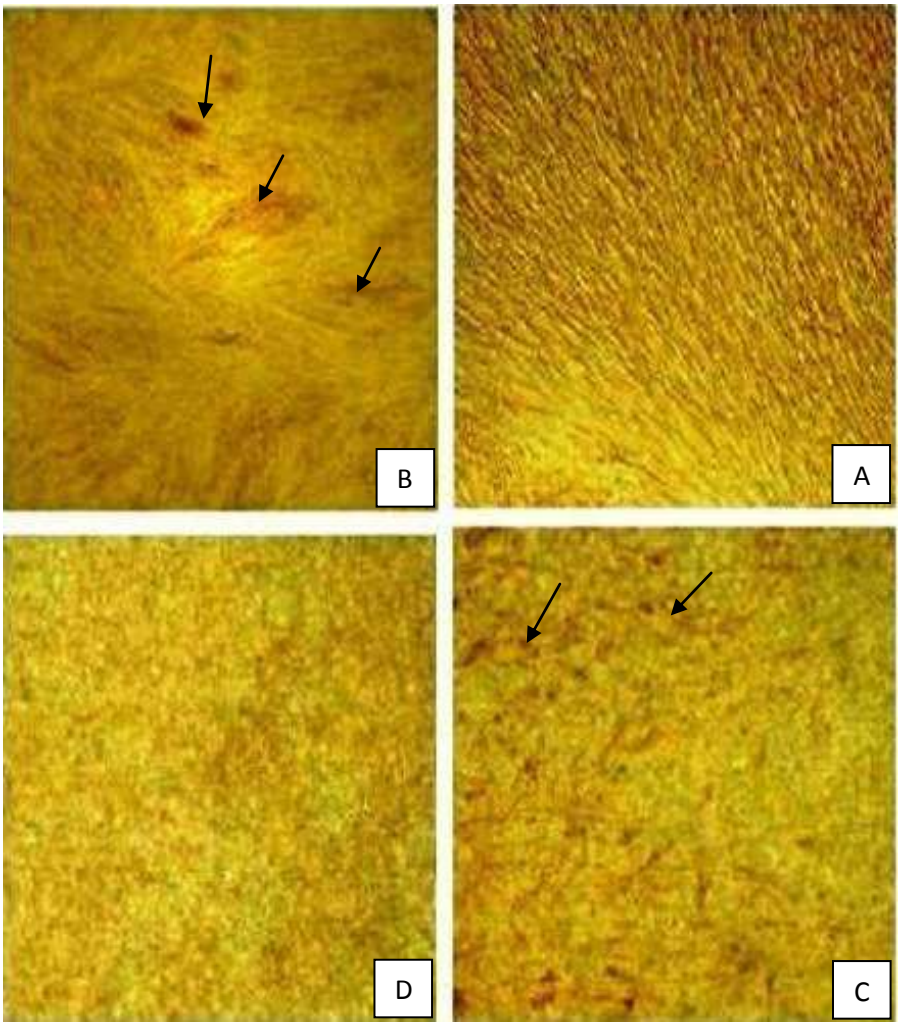

Figure 2: Alizarin red staining (20x); (A) Control group stained (Stem Cells); (B) Differentiated cells to the bone cell, the concentration of insulin in the presence 4IU; (C) Differentiated cells to the bone cell, the concentration of insulin in the presence 10IU; (D) Differentiated cells to the bone cell, the concentration of insulin in the presence 16IU

Staining Alizarin Red and Von Kossa

Red calcium precipitate with alizarin red (Figure 2) and black precipitates in von kossa stain was proved (Figure 3). Staining of cells under differentiating treatment was done on 21th day for nano scaffold in the presence or absence of insulin. Full calcium precipitate was seen in medium containing, 4IU concentration. Less precipitate was detected for 10IU and in 16IU no precipitate was seen.

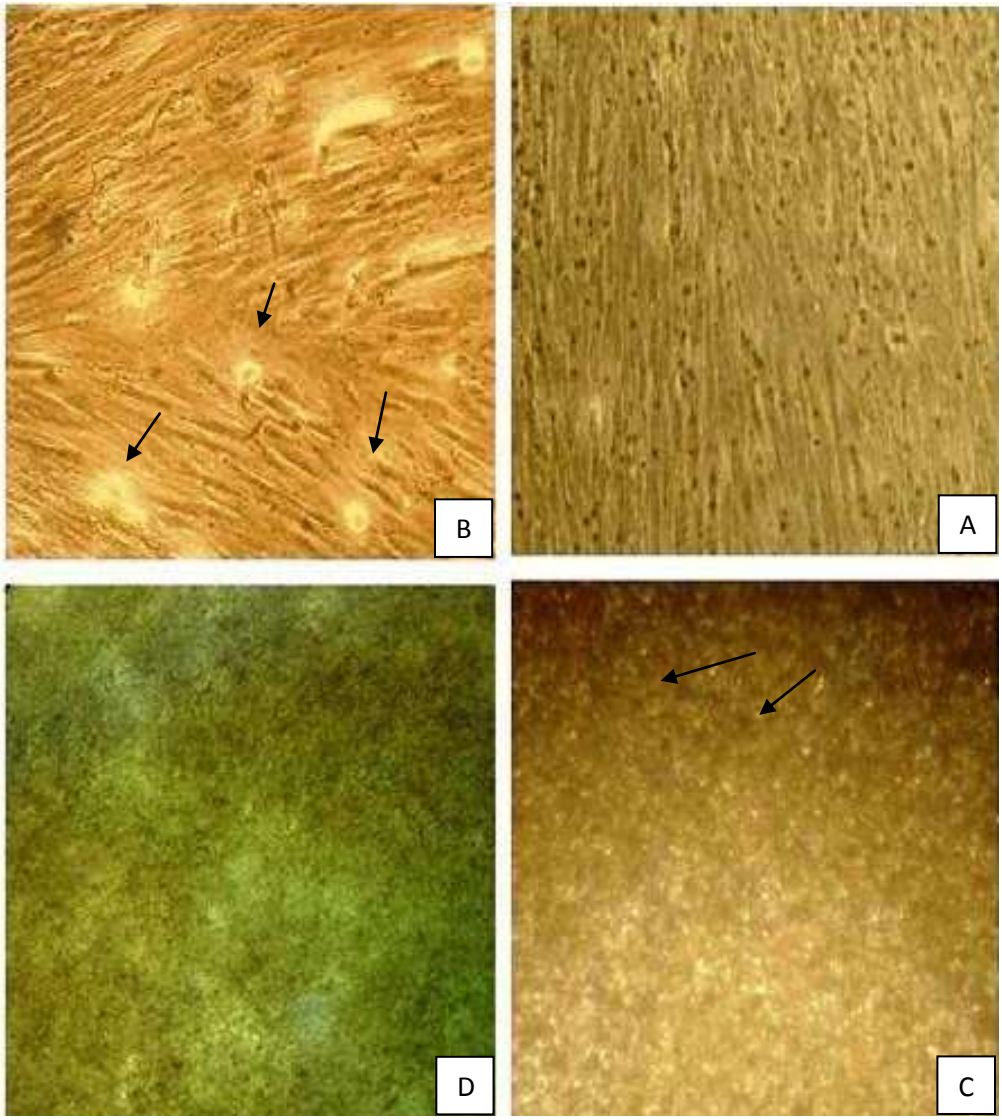

Figure 3: Von kossa staining in (20x); (A) Control group stained (Stem Cells); (B) Differentiated cells to the bone cell, the concentration of insulin in the presence 4IU; (C) Differentiated cells to the bone cell, the concentration of insulin in the presence 10IU; (D) Differentiated cells to the bone cell, the concentration of insulin in the presence 16IU

Result of MTT Test

When samples were treated by different concentration of insulin, various results come out. Cell's growth was increased with the passage of time. However, after observing cells treated with 10IU insulin, a different result came out, which showed highest growth rate on seventh day and the growth level was increased with time (Figure 4).

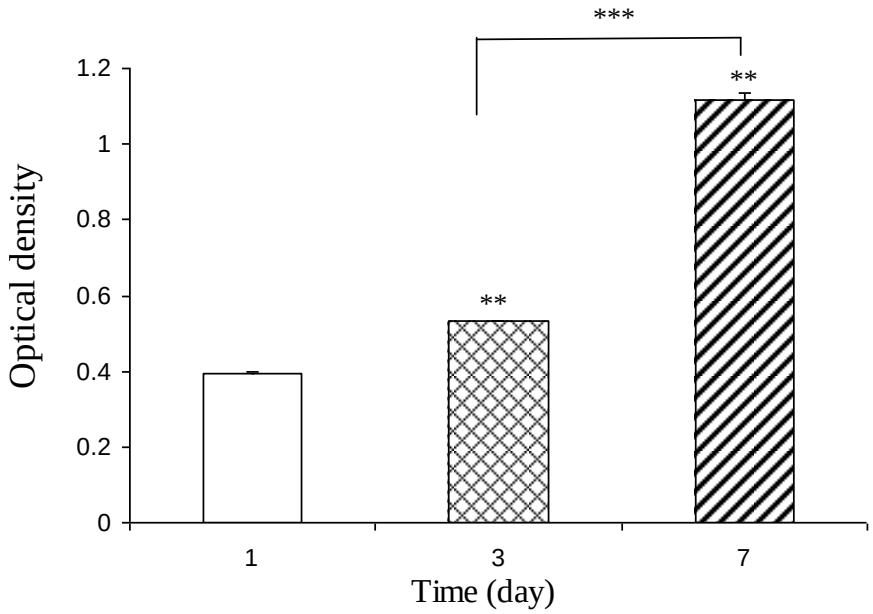

Figure 4: Cell growth chart on the first, third and seventh

DISCUSSION

These days the easy access of stem cells and their effect on several types of cell therapy such as ossification and bone repair defects are confirmed. Mesenchymal stem cells are derived from 3 separate parts of umbilical cord including pre-vascular, inter-vascular and sub-amniotic regions (Meeker and Coffey, 1998). In this research, umbilical cord's mesenchymal stem cells were derived by culturing a part of umbilical cord's tissue (Ayuzawa *et al.*, 2009).

The study by flow cytometry method revealed that umbilical cord's tissue stem cells express CD68, CD13, CD95, CD73, CD29, CD51, CD105 and CD44 markers. This is why these cells are negative for blood stem cell markers of CD38, CD71, CD34 and Cd45 (Roa and Matton, 2008).

In this research different concentrations of insulin were used during differentiation of cells to bone cells. Differentiation was observed in samples containing 4IU insulin in which precipitates sere confirmed by alizarin red and Von Kossa staining. But differentiation wasn't seen for the other two concentrations of insulin meaning in samples containing 10 and 10 IU. In the samples containing 10, 16 IU concentrations, very little precipitates were observed at 7th day of differentiation by alizarin red staining. But in 14th and 21st day of differentiation, precipitates weren't observed in Von Kossa or alizarin red staining. In a sample of differentiation of mesenchymal stem cells to bone cells which was exposed to concentration of 10IU insulin after t days of starting the differentiation meaning that the treatment was performed from day10 to 21, little precipitates were seen by staining at 21st day. The elevation of blood glucose is the most important physiologic factor controlling insulin secretion. The overrunning from glucose concentration's threshold is accompanied by secretion of insulin. A group of poly peptide hormones are related to insulin which are usually called insulin like growth factors.

MMT test was performed to evaluate the life of cells. Evaluation of cells' growth was performed by MMT and the rate of growth was determined by measuring light absorption. Tests were done on first, third and seventh days among which cells had significant growth in 3rd and 7th days than the first day.

Growth was evaluated in the presence of 4IU insulin and revealed that cells' growth was increasing by the pass of time. Although, this growth had high speed till 3rd day and from 3rd to 7th days the speed was average, in a way that there wasn't any significant difference between 3rd and 7th day. However, when these cells were evaluated in the presence of 10IU insulin, different result came out. In a way that, maximum growth was at the first day and growth decreased by pass of time, so that, no significant difference was seen in comparison of 3rd and 7th days.

Growth increased at first day in high concentrations, but different results were seen at 3rd and 7th days. The overall conclusion gained by evaluation of cells' growth is that, cells' growth increases by the pass of time and the growth of cells with 4 IU insulin was nearly half of cells without insulin. The growth of cells containing 10 and 16 IU concentrations decreased gradually. Thus, insulin couldn't increase the growth speed. Off course the growth speed increased a little in low concentrations.

Therefore, differentiation occurs in cells taking adequate insulin concentration proportional to environmental glucose. According to this, the most important physiologic factor controlling insulin is environmental glucose.

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