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# Effect of Insulin on Umbilical Cord's Mesenchymal Stem Cells on PLLA Polymer Scaffold

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## ABSTRACT

Tissue engineering started a new field of study which has made great view in renewal, repairing and keeping the function of different tissue especially bone tissue, by the use of cells, scaffold and biomolecules. Nano scaffold of PLLA polymer is made by means of electro-spinning method. Nanoscaffold was examined for surface feature, biodegradability, percentage of porosities and Size of pores. To prove the stem cell character of detached cells from venous tissue, cells were examined by flowcytometry for cell makers by which they were positive on CD90, CD105 and negative CD45 marker. Humane mesenchymal stem cells were cultured on PLLA nano scaffold. Nano scaffold having these cells, where treated with bone differentiation medium for 21 days and some samples were treated with different dosage of insulin. On day 21 alizarin red and von kossa staining was performed for treated cells. Observation of cells after getting stained, showed the precipitation of calcium in extra cellular matrix .The nano scaffold having differentiated cells spread black in von kossa and red in alizarin red Because of calcium precipitates. Differentiating to bone in high concentration was hardly seen in samples containing different insulin dosages and low glucose.SEM supported the calcium precipitation in nano scaffolds having cells differentiated to bone tissue. Observations of SEM showed that lower insulin concentration has better effect on differentiation of bone cells. According to gained results, concentration as 4IU of insulin suggested as proper concentration for bone cells differentiation on PLLA nano scaffolds.

**KEY WORDS:** polymer, nano scaffold, poly L -lactic Acid (PLLA), cell differentiation, insulin.

## INTRODUCTION

### Tissue engineering:

The technology of tree-dimensional network growth is developed for replacing the damaged tissue. The main goal of tissue engineering is to synthesize or heal tissues which are damaged due to several reasons. But the technology of 3D network growth is developed for replacing the damaged tissue[1,2] . for making a tissue by means of engineering methods, it is needed to design a scaffold with proper physical structure and ability of adhesion of cells to it, then having Cell migration, proliferation and differentiation and finally replacing the new tissue .in tissue engineering, first a porous material is prepared as extra cellular matrix or a scaffold for cell growth, then growth factors are placed on it. After cell's growth in porosities, scaffold is transferred to the body from the lab[3,4].

### Fiber bonding:

Electro-spinning is used to prepare the nano scaffold. This technique is a simple tool for making nano fiber scaffold which are gained from biodegradable polymers. The mechanism is so that there is a high voltage, having a container in which the polymer solution making nano scaffold is poured[5]. This material is spread out, And makes some fiber in nano the result are fibers, instead of sprays of drops and as the time passes, the solvent is vaporized and at the end they are gathered by collector[6].

### Bone tissue engineering:

Bone tissue engineering is usually performed by the help of scaffold having bone making inducers.an increase is bone cell's growth was seen in comparison to 2D culturing. Mechanical actions also caused an increase in bone cell making 3 to 4 times better in comparison to stationary cultures[1,7].

### Stem cells:

Stem cell is non-differentiated somatic cells which can differentiate to many types of cell by having proper condition. They have the features of self renewal, multiplication ability, high level of cell proliferation, differentiating to several mature cells and replacement ability in treatment of some disease[8].

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**Umbilical cord's Mesenchymal stem cells:**

Isolation of umbilical cord's mesenchymal stem cells is the most relevant source of mesenchymal stem cells. Umbilical cord is affordable and easy to get. Umbilical cord is consists of a vein and two arteries which are surrounded by Wharton jelly. Umbilical cord is covered by an epithelium from amnion. Collagen fibers are intertwined and the produced clusters are as a skeleton covering umbilical cord 's vessels[9].

**Insulin**

Is a hormone secreted from Beta cells of Langerhans islets in pancreas .a structure made and bended 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[10].

## MATERIALS AND METHODS

**Isolation of umbilical cord's mesenchymal stem cell**

Umbilical cord of a newborn was transferred inasterile way in normal saline to the lab with the blood inside it. The isolation of umbilical cord's mesrnchymal stem cells was performed. For the isolation, umbilical cord was cut in to 1cm pieces and put in HBSS .for removing the left blood transverse section was done on umbilical cord pieces and arteries were detached and eventually the left part which was the vein was detached and put in to HBSS for preparation. For culturing, first, low glucose DMEM with FBS and antibiotic was transferred to culturing flux, then the pieces of vein were added and the culture flux was put in an incubator with humidity, 0.5 % of CO2 and temperature of 37°c. When 80% of flux is filled by cell's growth, we transferred some cells to new flux for preventing food starvation and lack of space, to make them grow by preparing former medium. For re -culturing and also detachment of adhered cells in flux, EDTA trypsin was used, then the cell suspension was centrifuged for 5minuteson 2000 RPM And was transferred to two fluxes[11,12].

**PLLA nanoscaffold and features of scaffold**

Nanotechnology techniques were used in making Nano scaffolds. after the synthesis of nano scaffold the biodegradability, biocompatibility and porosities of nano scaffold was examined and pore sizes and microscopic studies(electron microscope) of nano scaffold was observed[13,6].

**Viability of Cells And MTT test**

For examination Of biocompatibility of nano scaffold they were sterilized and put in a plate .nano scaffolds were incubated for 2 hours is 37°c with PBS buffer.a concentration of $10^5$ Cells in one ml Cell was placed on nano scaffold and put into incubator for 3 hours to stick to de nano scaffold. after the required time, DMEM medium with FBS antibiotic were added to samples. biocompatibility 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 cuvettesafter taking out from incubator and light absorption was measured by ELISA reader with 570 ƛ wave length[14].

**Umbilical cord mesenchymal stem cells cultured on nano scaffolds**

Nano scaffolds were transferred to 24 well plate with PBS and incubated for 2 hours in 37 °c .cells of passage 3 were detached by trypsin from flux and made ready to get transferred to nano scaffold with concentration of $2*10^5$ cells in ml .then the nano scaffold was incubated for 3 hours in 37 °c[15].

**Differentiation of stem cells attached in nano scaffold to bone cells under different insulin concentration**

In differentiation of stem cells, attached to nano scaffold, 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)[16] .

**Alizarin red staining**

Alizarin red staining was used to prove the differentiation of stem cells to bone cells .this staining was done on $27^{th}$ 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[17,19].

**Von kossa staining**

A proof of differentiation of stem cells to bone cells is von kossastaining. This stain was done on 21th 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[17].

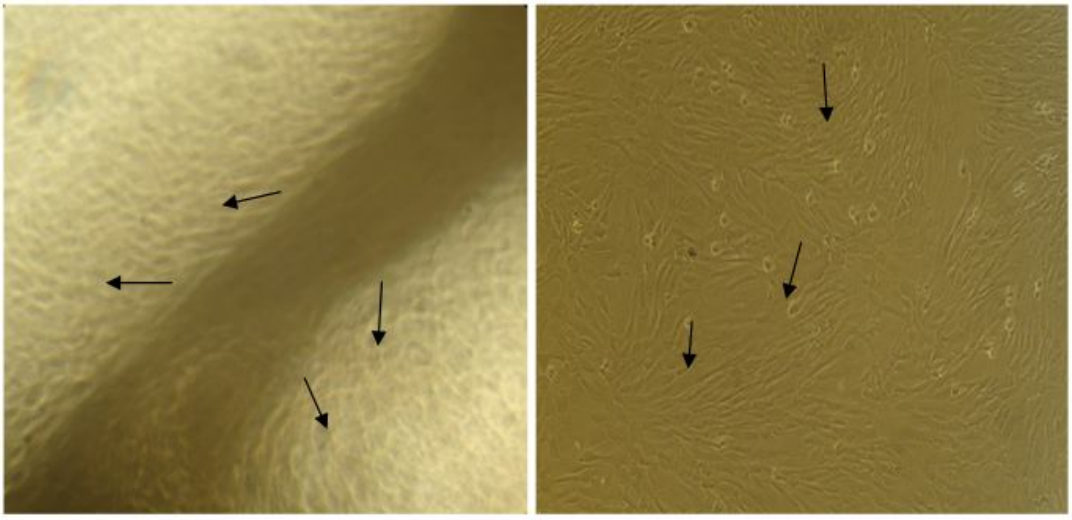
**Examination of SEM images of nano scaffold having differentiated bone cells**

Nano scaffold containing differentiated bone cells were fixed on day 21 and SEM imaging was performed for examining the classification of nano scaffold surface.

## RESULT

**Isolation of umbilical cord's mesenchymal stem cells**

In this method, cell's growth started after 10 days and were easily detectable under microscope. cell colony were made on day 12 and on 14$^{th}$ day , 80% of flux was filled with attached cells. which had spindle like shapes. 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.



A B

Figure 1. Images derived mesenchymal stem cells derived from human umbilical vein (20x)
(A) Tenth day of culture: Exclusion of germ cell
(B) Fourteenth day of culture: 80% of the number of cells and the formation of a cell layer

**Proof of stem cell's isolation by flowcytometry techniques**

Flowcytometry machine is 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, andmesenchymal stem cell don't have it .1/83% expression of it showes low percentage of blood cells in this population. CD90 marker was expressed at95/27% . CD105 marker was expressed at 90/28% rate. this high expression rates are as specific markers of mesenchymal stem cells.

**Features of nano scaffold**

Biodegradability examination of nano scaffold was done which showed 80%of degradability and observation of SEM images from nano scaffold surface, showed that, nano scaffold has high porosities and supported the intercommunication of pores. Size of pores are20-60 micro meter and examination of porosities revealed that nano scaffold has 90/15% of porosity.

**Viability of cells in the presence of insulin on PLLA nano scaffold**

When samples were treated by different concentration of insulin, various results come out. When the growth of cells attached to nano scaffold was observation presence of 4IU insulin. It was observed that, cell's growth was increased by the pass of time. However, after observing cells treated with 10IU insulin, a different result come out , that showed highest growth rate on first day and the growth level was decreased as time

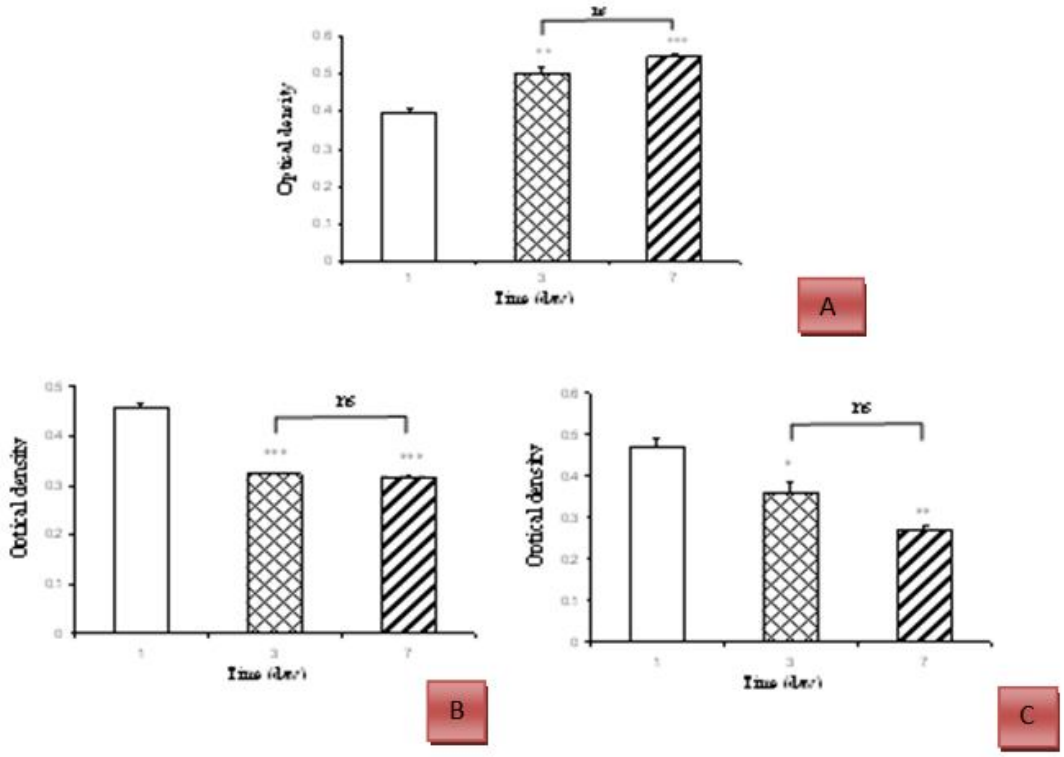



Figure 2. Cell growth charts

(A) Cell growth charts attached to a concentration of 4 IU of insulin in the presence of nano-scaffolding on the first, third and seventh

(B) Cell growth charts attached to a concentration of 10 IU of insulin in the presence of nano-scaffolding on the first, third and seventh

(C) Cell growth charts attached to a concentration of 16 IU of insulin in the presence of nano-scaffolding

**mesenchymal stem cells on nano scaffold**

Observation of SEM images and studying the rough surface is due to attachment of cells to nano scaffold.

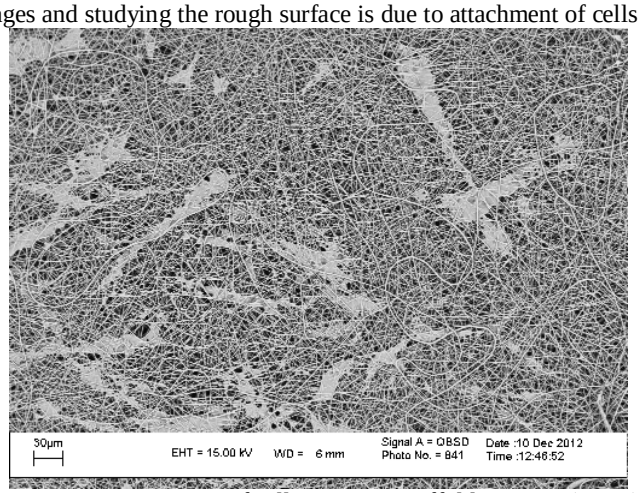



Figure 3. SEM images of cells on nano-scaffolds, PLLA (500X)

**Alizarin red and von kossa staining**

Red calcium precipitate with alizarin red and black precipitates in von kossa stain was proved. Staining of cells under differentiating treatment was done on 21th day for nano scaffold in the presence or absence of insulin. SEM image differentiation of umbilical cord mesenchymal stem cells to bone cells in the presence 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.

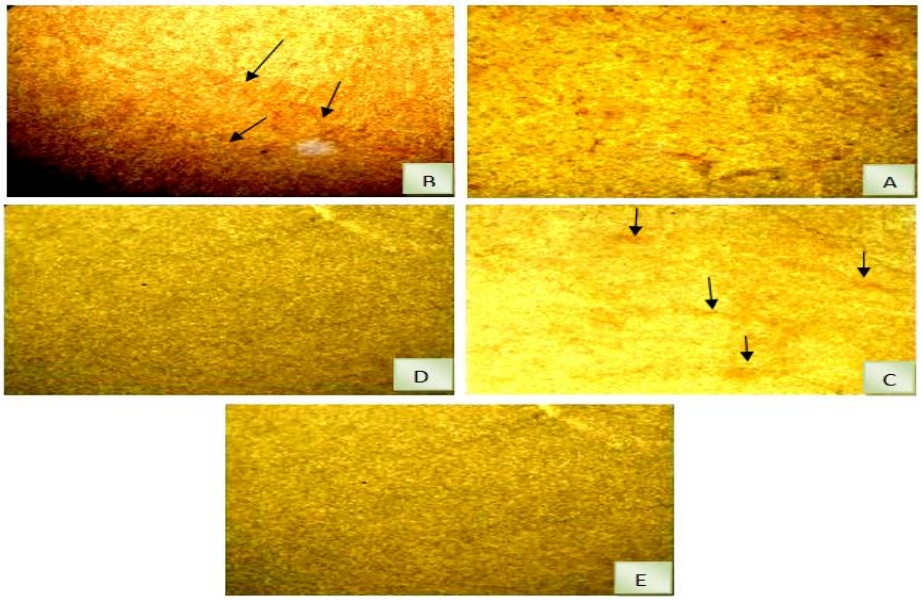



Figure 4. Alizarin red staining in the twenty-first day of differentiation (20x)
(A) Control group stained (nano-scaffold)
(B) Differentiated cells to the bone cells on the nano- scaffold at day 21 of differentiation
(C) Differentiated cells to the bone cell, the concentration of insulin in the presence 4IU on nano-scaffold
(D) Differentiated cells to the bone cell, the concentration of insulin in the presence 10IU on nano-scaffold
(E) Differentiated cells to the bone cell, the concentration of insulin in the presence 16IU on nano-scaffold
(F)

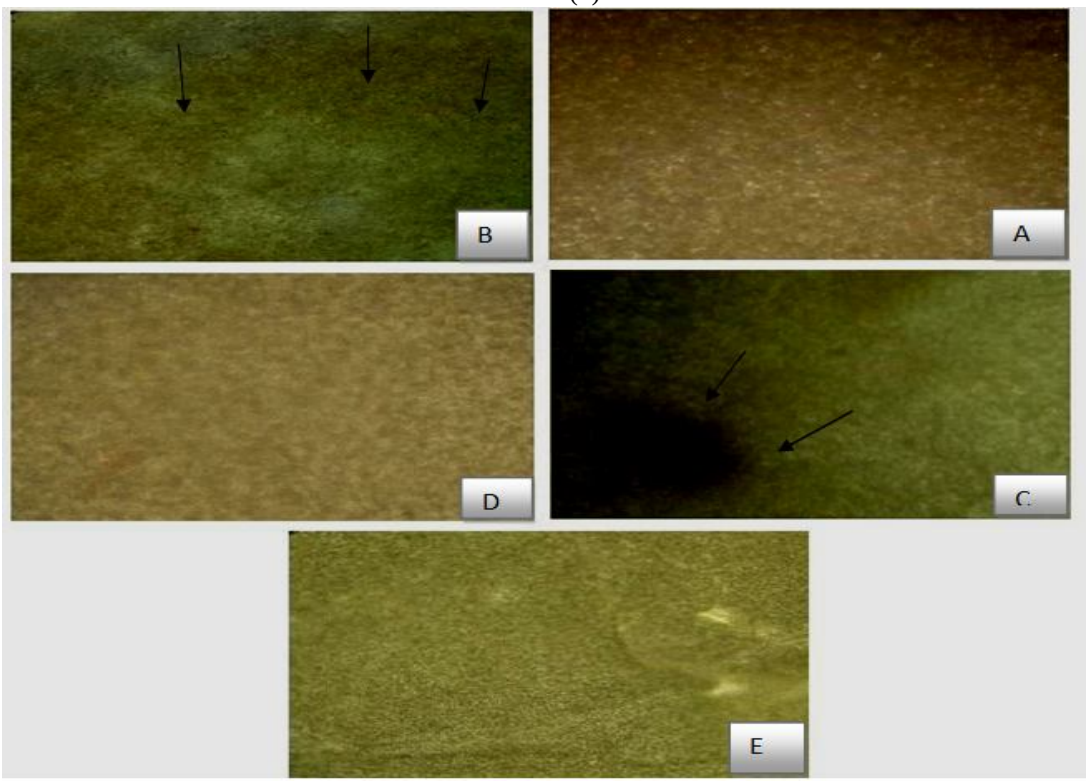



Figure 5. Von kossa staining in the twenty-first day of differentiation (20x)
(A) Control group stained (nano-scaffold)
(B) Differentiated cells to the bone cells on the nano- scaffold at day 21 of differentiation
(C) Differentiated cells to the bone cell, the concentration of insulin in the presence 4IU on nano-scaffold

(D) Differentiated cells to the bone cell, the concentration of insulin in the presence 10IU on nano-scaffold
(E) Differentiated cells to the bone cell, the concentration of insulin in the presence 16IU on nano-scaffold

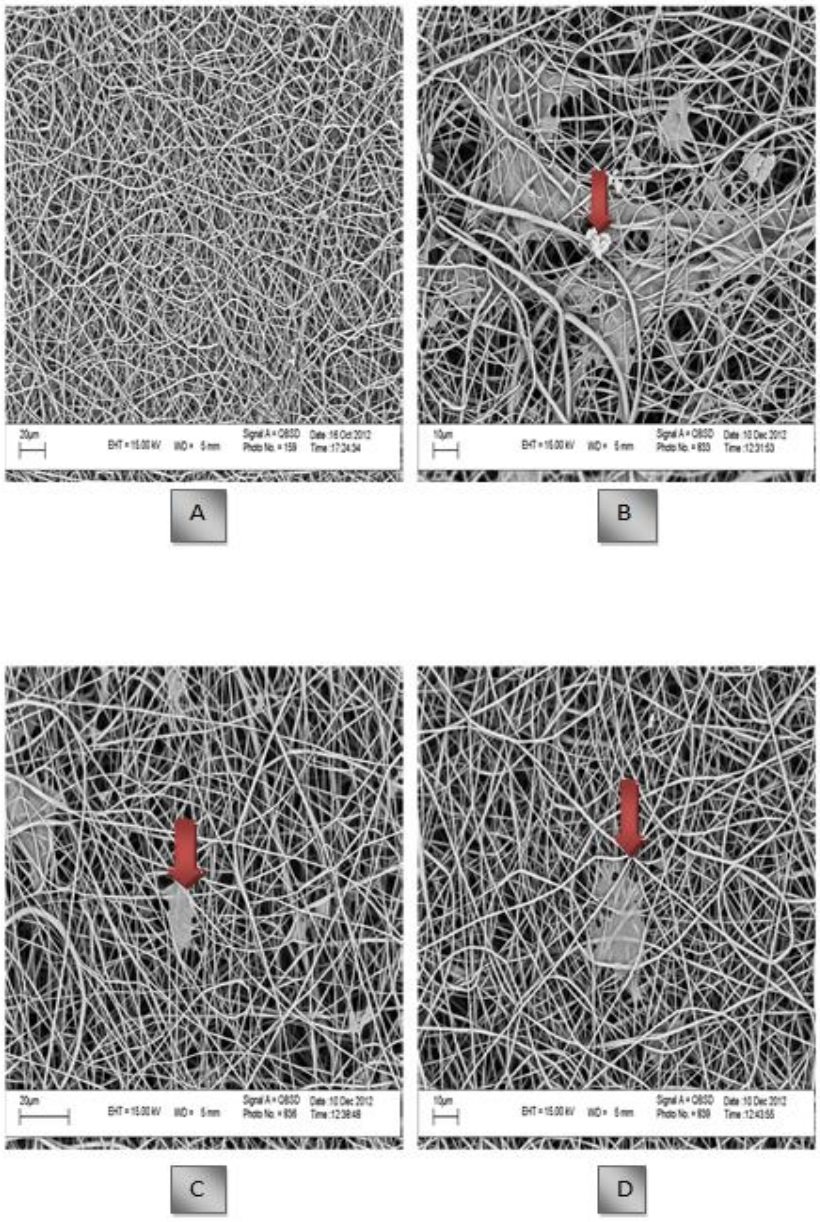



Figure 6. SEM images of nano scaffold with cells differentiated bone cells in the presence of various concentrations of insulin (2kx)

(A) Without cell
(B) At day 21 of differentiation in the presence of 4IU of insulin concentration
(C) At day 21 of differentiation in the presence of 10IU of insulin concentration
(D) At day 21 of differentiation in the presence of 16IU of insulin concentration

## DISCUSSION

Performed tests, supported the biodegradability of PLLA nano scaffold. According to result, we can say that this nano scaffold has a high degradability speed because of existence of small pores[18]. Speed of degradation is related to time and causes cell growth, food and deposit transfer 80 % of nano scaffold was degraded on day 40. An important factor in nano scaffold degradation is the straight relation between rate of nano scaffold degradation and its water absorption. Infact there is a straight relation between water absorption and biodegradability. The reason is that, massive water absorption can facilitate nano scaffold's hydrolysis, by

interactions between molecules of water and polymer's matrix.at the end of 40 day period , PH degradation was at lowest rate. PH decrease is due to nano scaffold destruction which shows the existence of lactic acid in synthesizedpolymers .results revealed that, nano scaffold has 90/15 % of porosities which elevates cell growth and affects food and oxygen transfer inside 3D matrix[20].

For the isolation of mesenchymal stem cells from human newborn's umbilical cord, non-enzymatic techniques were performed. In pervious studies they could detach umbilical cords mesenchymal stem cells by culturing a piece of umbilical cord.after putting venous tissue on culture, cell germination started on day 10 , so that in 14$^{th}$ day cell count reached 100%. Umbilical cord's stem cells secrete cytokines, same as bone marrow stem cells. However, umbilical cord's stem cells, secrete macrophage-granulocyte stimulating factor and granulocyte colony inducing factor. bone marrow stem cells don't secrete these factor[21].

Umbilical cord's Matrix and blood stem cells have lower proliferation time in comparison to bone marrow stem cells which may indicate their initial phase. these cells pass more passages to get old. Also, umbilical cords blood and tissue stem cells cause expantion of hematopoietic stem cells. These are benefits in comparison to other sources[22,23]. Flowcytometry studies revealed that umbilical cord stem cells express CD29 , CD51, CD105 and CD44 marker. Although, these cells are negative on blood's stem cell markers (CD45, CD34). Surface markers were examined to prove that they are stem cells , in which CD105 and CD90 had positive , and CD45 had negative observation. Mesenchymal stem cells which has been exposed to bone differentiating inducer medium , got observed by alizarin red stain. After 21 days of treatment by differentiating medium, nano scaffold having cells was stained and turned into red because of calcium precipitates. Von kossa stain turns calcium precipitates to black. Different insulin concentration were used .4, 10 and 16 IU of insulin was added to mesenchymal stem cells under treatment of differentiation medium. Cells differentiation in nano scaffold condition having cells, was seen and proved by alizarin red and von kossa staining in samples containing 4IU of insulin. But, in 2 other concentration -10 and 16 IU- no differentiation was seen.

No calcium precipitate was seen in samples having 10 or 16 IU at day 21 in alizarin red and von kossa staining. Elevation of blood glucose is followed by an increase in insulin secretion. In former studies, co-effects of insulin sensitive pathways on differentiation of mice's fetal stem cells to bone cells was examined. Insulin induces the differentiation to fat. In this research , insulin compounds along with insulin like growth factor caused differentiation to bone cells so that activated Sirt1 intermediate gene pathway and speeded up the differentiation process. But, having this pathway blocked, causes fetal stem cells with animal origin to differentiate to fat cells. In this research , differentiating in low insulin concentration and non-differentiating in high concentrations , is a reason for existence of insulin receptors on bone cells. In the presence of glucose, insulin can bind to receptors and high level of insulin can cause ossification and differentiation of stem cells to bone cells. Therefore, the differentiation happens in cells having proper insulin around, according to surrounding glucose. Noting that, the most important physiologic regulator of insulin is the glucose. SEM imaging of nano scaffolds having cells under differentiating treatment in the presence or absence of insulin after 21 days showed that, after cell culturing and differentiation, the diameter of pores was reduced and nano scaffolds' surface were more rough in comparison to the condition without cells. Bone cells were detectable on nano scaffold. Calcium precipitation was occurred at surface of insulin nano scaffold. Comparison of differentiation in the presence of different insulin concentrations showed that calcium precipitate was fully seen in 4 IU, but in 10 IU , cells were round shaped meaning no calcium precipitate was seen. Cells remodeling to round shaped, shows the initiation of differentiation to bone cells but with low speed due to high insulin concentration. In 16 IU, cells were seen unchanged, that was because of imbalances between glucose and insulin concentration. For biocompatibility and growth rate, light absorption was measured with MTT test. This test is a light measuring test. Unlike other methods, all steps, from culturing to reading the result from photometer were done in one plate , so the sensitivity of experiment was high. Cells growth observation was done by MTT test and growth rate was revealed by measuring light absorption. When samples were exposed to treatment of different insulin concentrations, different results came out. When cell growth was observed in nano scaffolds with 4 IU of insulin, it was shown that cell growth was elevated by the pass of time, however this growth was at high rate until 3$^{rd}$ day and after that from 3$^{rd}$ to 7$^{th}$day was at average speed , in a way that there was no meaningful difference between 3$^{rd}$ and 7$^{th}$ day. Although, when these cells attached to nano scaffold, were observed in presence of 10 IU insulin, a different result came out, saying that, maximum growth rate was at the first day and decreased by pass of time in a way that there was no meaningful difference between 3$^{rd}$ and 7$^{th}$ day. We conclude that this concentration causes limited cell growth in samples. And also a similar result came out of 16 IU, showing that these two last concentrations had nearly the same effect.

Results revealed that, PLLA polymer nano scaffold synthesized by electro-spinning method, is a proper choice for bone tissue engineering. Isolation and culturing of humane umbilical cord cells is an appropriate source of mesenchymal cells for lots of researches especially in the field of differentiation. The result of this research can be used for diabetic patients with broken bone. Because bone reconstruction is blocked, due to absence of insulin receptors, causing high blood glucose level. In fact glucose provides the required energy for

insulin function. In the absence of proper glucose concentration, insulin can't connect to insulin receptors and eventually the growth and differentiation is blocked.

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