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Three-Dimensional Matrix Derived from Tissue Decellularized of Bone Scaffold Rats Treated with Mixed HA and GAG on Mesenchymal Stem Cells Behavior

Abstract

Scaffolds provide a suitable environment for growing living cells in vitro and body. Biodegradable and biochemical properties of scaffolds affect the ability of osteogenic cultured cells. In this study, ECM derived from cancellous bone treated with mixed hydroxyapatite and gelatin was used as a three-dimensional environment for investigating the interactions of mesenchymal stem cells with bone matrix. To remove cells from the cancellous bone, physicochemical methods including snap freeze-thaw and sodium dodecyl sulfate (SDS) as an ionic detergent were used. Then scaffolds were treated with mixed hydroxyapatite and gelatin and mesenchymal stem cells cultured on a 3D matrix on different days in vitro. The removals of the cells, treatment, and induction rate of ossification have been confirmed by histotechniques. This study showed that the co-culture of mesenchymal stem cells and 3D scaffolds might be a suitable model to study cell behavior such as migration and polarity in vitro.

Keywords: *Bone tissue, Stem cell, Hydroxyapatite, Gelatin, Metachromatic Matrix*

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Introduction

Matrix Biology (Biology of Extracellular field) is a relatively new branch of medical science is the branch of cellular and molecular biology, genetics, and clinical sciences localized disease or impact matrix in connective tissues such as bone, cartilage, and lining of blood vessels, skin, and eye .... it covers. Robert, 2001)) is a network of extracellular matrix proteins and proteoglycans, which is an environment for the development of the cell, providing homeostasis and Reconstruction (Soucy et al.2009., Goh et al.1997). To study the interactions of cells and a scaffold made of a matrix, the scaffold must be interconnected with porous network operation, supplying the cells, cellular waste disposal outside the scaffold, extracellular matrix formation, and angiogenesis. Porosity and pore size are important characteristics of tissue engineering scaffolds (Hollister et al.2005). Tissues and organs are 3D environments. Removing 3D cell scaffolds as extracellular matrix is exploited as a model for the reconstruction of all organs used. Signaling and cellular function in a 3D matrix with a 2D matrix is different. Considerably in morphology, cell-cell, and cell-matrix interaction, and their differentiation to different 3D environments. (Griffith et al.2006., Narayanan et al.2009. , Birgersdotter et al.2005)....

Increased use of 3D models that are obtained from other tissues due to advances in tissue engineering and also facilitate the development of new therapeutic methods (Yamada et al.2007). The method is usually applied to tissues obtained from several organisms (microscopic embryonic organs or tissue blocks) separately cultured in the laboratory and are often arranged in three-dimensional culture to retain their original (Gahwiler et al.1997).

So can the extracellular matrix tissues of living organisms use for the study of cell behavior during interaction in vitro. For this task used of decellularization for to remove the effect of the cells from the extracellular matrix cells, As the only remaining extracellular matrix and it can be used as a scaffold (Hazgui et al.2005., Yang et al.2008., Cukierman et al.2002) Bone tissue engineering is a promising field of cell culture matrix and osteogenic inducing signals that are used in bone reconstruction (Kagami, et al.2011). Bone tissue contains large vessels and dynamics in a rehabilitated life. This tissue a crucial role in the movement to provide the appropriate structure, creating a protective coating for the internal organs and the body is vulnerable. In addition, structural function, in the homeostasis of calcium and phosphate ions through the store with the concentration of electrolytes in the blood (Stevens.2008). Bone cells, including osteoblasts, which are cells that are located on bone surfaces, Osteocytic and osteoclastic and osteocytic are each enclosed within the mineralized Lakvna (Sharma et al.2007). In many tissues, the extracellular matrix molecule family of several different evolutionary origins is made. In many tissues, are the

extracellular matrix molecule family of several different evolutionary origins made. Glycosaminoglycans are linear polymers of repeating disaccharide hexosamine acids and Uric acid. The most impressive feature of the carboxyl and hydroxyl molecules and sulfate groups attributed. The poly-anionic molecules are properties of their osmotic activity. Except for hyaluronan, all GAGs and proteoglycans form covalent bonds with proteins to make Hara (Rojario, et al.2010). Hydroxyapatite is the main component of bone mineral with the chemical formula Ca10 (po4) 6 (OH) 2 (Hemmerle, et al.1995). Hydroxyapatite by Albee was first introduced in 1920 and was used in various forms. The natural bone substitute material is typically as filler or as a coating material used to promote bone growth on artificial. Crystalline phase Electrostatic is similar to natural bone and the most favorable for bone replacement, making use of HA makes it possible to build bone (Kim, et al.2011) In the extracellular matrix of bone tissue of the epiphysis of mice treated with the combined use And its effect on inducing differentiation of human mesenchymal stem cells into bone tissue is examined. Non-hematopoietic precursor cells are mesenchymal cells that are found in adult tissues And can transform into various lineages of mesenchymal origin in response to stimulation, respectively. These cells were discovered in the late 1960s. MSCs are easily isolated from adult tissues and differentiate into desired cells of mesenchymal lineage (Chen, et al.2005). The purpose of this study was to prepare a scaffold for bone cells to a mixture of glycosaminoglycan and relieved that hydroxyapatite crystals were stained this experience is intended to induce bone formation.

In this study, Wistar rats, weighing approximately 200-300 g were prepared from the Razi Research institute, was used. They were kept in the animal house of the Faculty of Sciences, Islamic Azad University of Mashhad, and fed individually in cages under controlled temperature and lighting at C ° 2 ± 20 and 12 hours, respectively. On the other hand, their health status was controlled to prevent infection. To prepare samples of cancellous bone scaffold mice were killed with chloroform and then the long thigh bone was separated Cancellous bone from the epiphysis was separated into parts 4mm in diameter.(fig 1-B) The samples were washed with saline and were stored at 4 ° C for one week and then removed from the freezer and thawed at the laboratory.

Figure 1: Image A shows an example of a Wistar rat femoral and image B cancellous bone sample bone fragments epiphysis.

To remove fat cells from bone samples, they were placed 5 cycles of 30 seconds in boiling water treatments. The bone fragments were washed several times with saline next to Freeze-rapid melting. The samples were for 2 min, in liquid nitrogen (-196°C) then to melt quickly in distilled water for 5 min, and then melted in saline at 37^0C . This step was repeated 5 times and the last part were washed with phosphate-buffered saline (PBS) . The chemical step of decellularization, scaffolds were placed in 10% sodium dodecyl sulfate for 48 hours. Samples of SDS solution were removed and washed with PBS buffer for half- hour. Scaffolds in asolution involved 0.3 g glycosaminoglycan and 1 g powder oyster shell eggs in 100 ml of distilled water and 0.5 g of gelatin to adhere more for overnight. To sterilize the scaffolds were washed with 70% ethanol and then sterile water was used to remove the effects of ethanol. The next step is to remove the effect of distilled water used saline . The last step is to prepare samples for culture placed into a sterile Petri dish and was added 5 ml of culture medium to the and were incubated for 10 min. To prepare . To prevent contamination were added. 500 macrolitter of antibiotics (penicillin/streptomycin) .It was used of human mesenchymal stem cells. Mesenchymal stem cells were bought from the Institute of Technology, Ferdowsi University of Mashhad. These stem cells were human adipose tissue-derived mesenchymal stem cells (ADMSC). cells cultured on rat cancellous bone scaffolds, to separate cells from the flask trypsinized. After separating the cells from the flask, the contents were spilled into Falcon 20 ml. After

centrifugation, the cells were determined by counting the number of cells, and the cell suspension was prepared with 2×10^5 levels. Scaffolds after maintenance for 1hour in the culture medium, were placed in 24 well plate. After filled with 3 ml of medium were transferred to the incubation medium and were controlled every day. If the color of the medium changed from red to orange or yellow, replaced the culture medium. Otherwise, every two or three days, the cell culture medium was replaced. Sampled on days 5, 10, 15, and 20 days after culture were studied. Bouin's solution for fixing the samples used for 2 days. In order to the cutting should tissue placed in softener solution. This solution also leads to the decacification from the bone tissue. Samples in solution contain 100 mL of alcohol, 10 ml of formaldehyde, and 4 ml of nitric acid for 5 days, and this solution was changed every day for more effect. Then placed in 60% alcohol for 24 hours. To remove the effect of nitric acid ,samples were placed for a day in 5% sodium sulfate solution includes 5 grams of sodium sulfate in 100 ml of distilled water. To remove the effect of sodium sulfate, the samples were placed for one day in the current water and then for dehydrated through ascending grades of alcohol and water samples to be taken slowly.so sample molding with paraffin, sections cut with 5 microns in diameter. The sections deparaphin and hydration. Then stained with hematoxylin and eosin (H & E), toluidine blue .H & E to demonstrate decellularization of cancellous bone tissue. Toluidine blue to show generated of cartilage cells in cartilage and demonstrate metachromatic mode. Matrix staining intensity studied according to Gong et al. (Gong et al.1997)Thus, the (+)low staining, (++) moderate staining (+++)high staining so small amount of glycosaminoglycan takes less color With (+) and showed more GAG levels at day 10 and stained with moderate (++), shown at the twentieth day and reached maximum levels of staining (+++) are shown. By graphical methods based on staining intensity and days of culture is shown and was categorized into three levels:: Exhibited metachromatic mild, moderate, and exhibited metachromatic exhibited metachromatic was considered extreme for any special privileges so that exhibited metachromatic mild (+), 10 were attributed ,Exhibited metachromatic moderate (++) exhibited metachromatic number 20, and severe (+++) 30 was considered. Therefore, based on the results of the culture were studied microscopically on different days.(chart1 , table1)

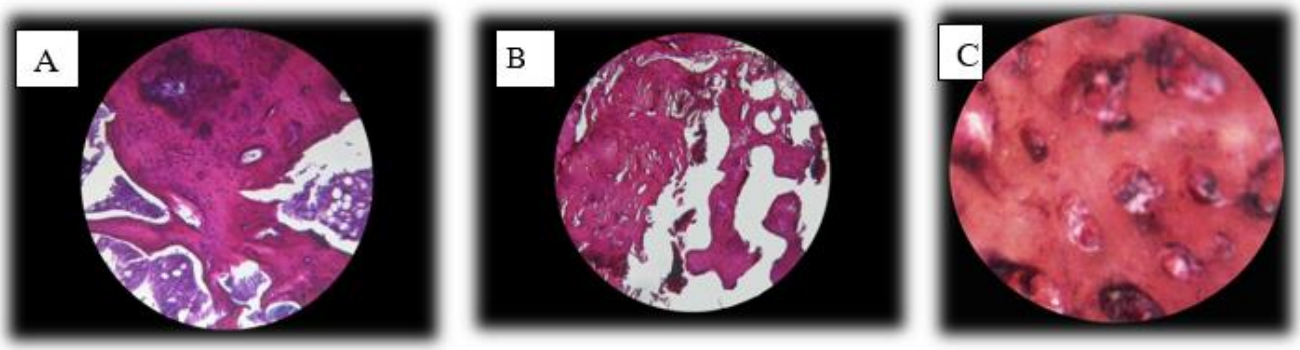

Figure 2: The cancellous bone of rats before and after removal of cells stained by H & E.A) cancellous bone tissue cells with osteoblast cells scattered throughout the trabeculae and bone marrow cells in the space between the blades of trabeculae, The tip of the arrow (magnification 40x) B) relieved cancellous bone tissue cells .C) impregnated with glycosaminoglycan rat cancellous bone and hydroxyapatite were stained by ink. Black areas indicate that the material is a bone scaffold.

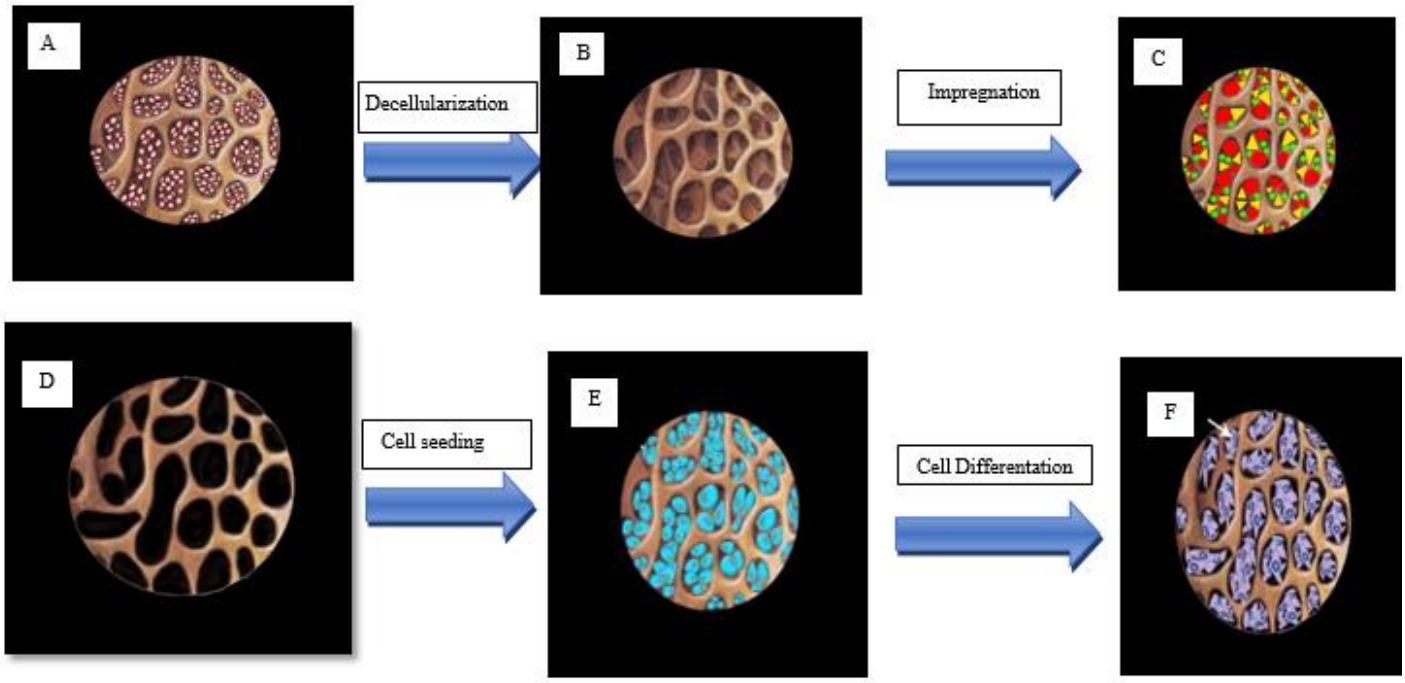

Figure 3: Schematic of cancellous bone scaffold epiphysis. A) cancellous bone scaffold in bone cells shows. B) Schematic of removing cells by sodium dodecyl sulfate bone scaffold that shows. C) Schematic picture shows the two glycosaminoglycan and hydroxyapatite scaffolds impregnated. The black areas indicate the existence of the material D) stained by ink shows the scaffold. E) shows the location of mesenchymal stem cells on scaffolds and Figure F) creates cartilage cells cultured on the twentieth day of the show. That might affect bone scaffolds that have been indoctrinated in the matter.

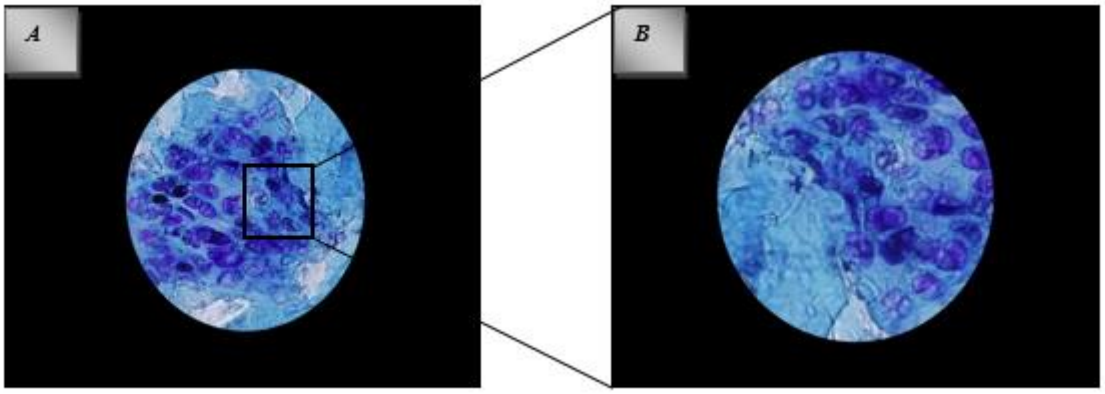

Figure 4: The newly formed cartilage within the cancellous bone scaffold impregnated with GAG and HA by toluidine blue staining shows. A) purple chondrocytes can be seen in the blue matrix. Magnification 10 x. B) 40x magnification of chondrocytes has been created.

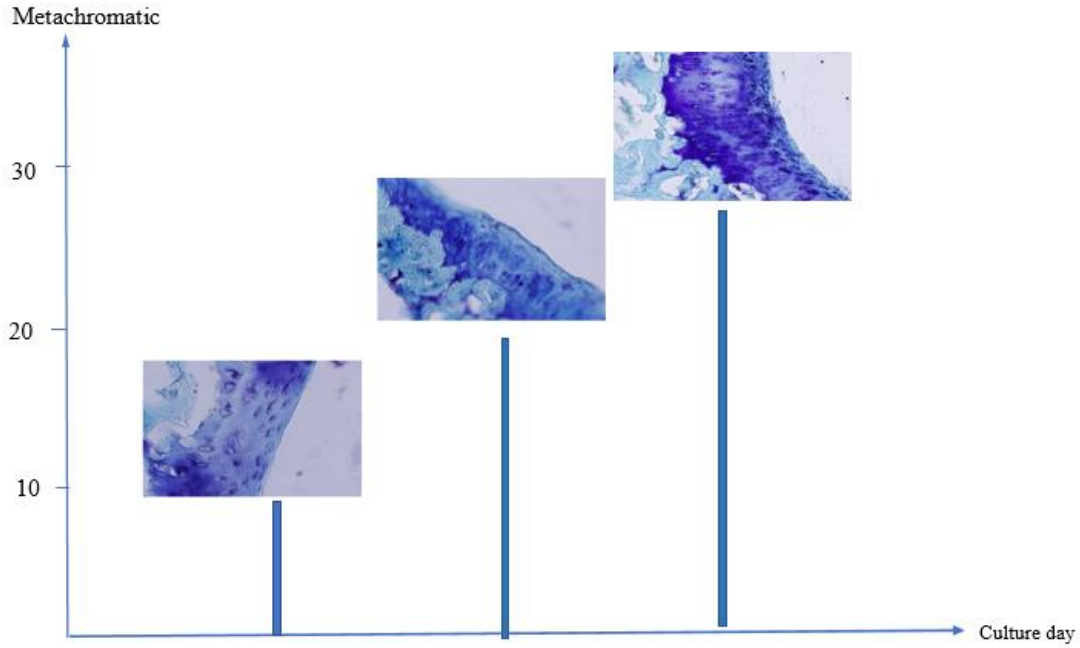

Chart 1: create a show that is based on the technique exhibited metachromatic cartilage Gong was categorized into three levels: mild exhibited metachromatic, exhibited metachromatic for each license was considered medium and extreme exhibited metachromatic This is exhibited metachromatic mild (+) 10 was attributed exhibited metachromatic moderate (++) exhibited metachromatic number 20 and extreme (+++), 30 were considered. Therefore, based on culture results were studied microscopically.

Table 1: GAG staining intensity Gong.

GAG staining intensity in the matrix	Day of study
+	5
++	١٠
+++	٢٠

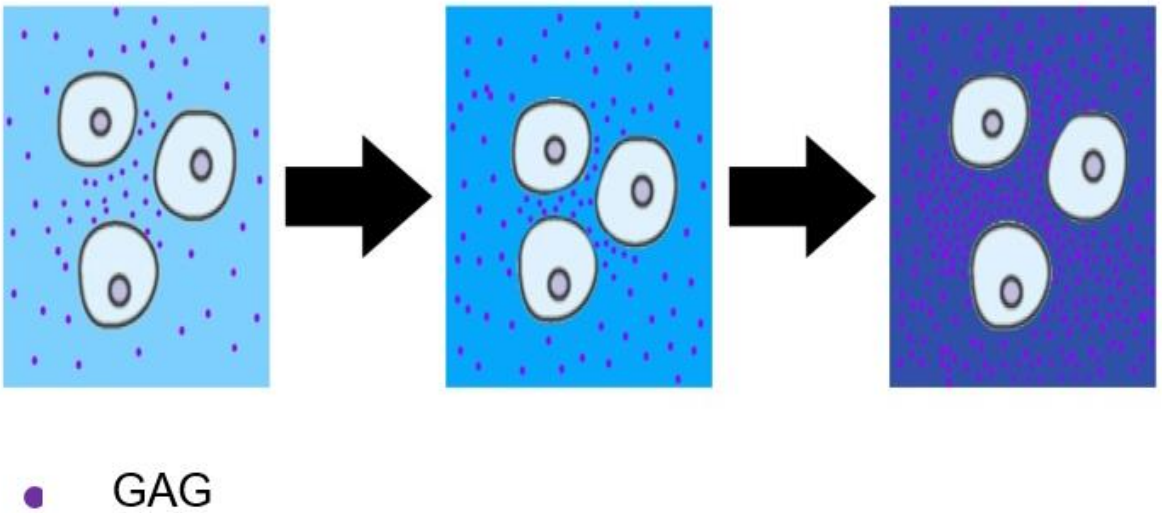

Figure 5: Schematic of expression exhibited metachromatic extracellular matrix of cartilage in the show. Gradually the amount of glycosaminoglycan and proteoglycans secreted by cells in the cartilage matrix increases more and more mature cartilage matrix color intensity of toluidine blue to show.

Discussion:

The first part of the study is to develop a natural scaffold for bone tissue that can be used to study the behavior of mesenchymal stem cells. Stained hematoxylin-eosin showed complete removal of osteocytic fat cells and the bone marrow. A natural extracellular matrix scaffold was prepared from rat cancellous bone. So based on the above provision of scaffolding, the scaffold was prepared which is visible in Figure 1. The behavior of mesenchymal stem cells with normal bone scaffolds was examined. Mesenchymal stem cells on rat bone scaffolding cells were relieved 5,10,15,20 days after the culture medium was removed and the samples were fixed with Bouin fixation. Enter histological samples and these steps until the contamination was performed with paraffin during molding samples were embedded in paraffin. At this stage to assess the changes in the chemical structure of the matrix and mesenchymal cells cartilage cells were stained with toluidine blue. This stained for histological examination by glycosaminoglycan-induced bone and eggshell powder (containing hydroxyapatite) was used. Merkx and his studies in 2000 showed that cancellous bone due to its ease of use and the ability of the bone to heal, bone grafts have been used in Furthermore, the process of angiogenesis and cellular features with the growth and differentiation of bone graft makes accelerating (Merkx et al.2000). Therefore, in this study of rat cancellous bone as the scaffold was used. Criteria for cancellous bone, ease of access, and porosity were appropriate. Grayson and his studies in 2010. In this research, to provide a natural scaffold for bone tissue spongy bone tissue is initially placed at 4- C ° The temperature of the ice crystals forms slowly and causes damage to the membranes of cells. The next step is to remove the fat and bone marrow cells, the samples were placed in boiling water (Grayson et al. 2010). Based on the research by Omae and colleagues in 2009 Freeze-the rapid melting method in liquid nitrogen at C ° 196- cycle was used And then stored in saline solution and distilled water to arrive at the laboratory, the process can effectively be killed cells (Omae et al. 2009) Yamasaki et al (2005) have shown that the frequency of Fryz- melt in liquid nitrogen, resulting in the loss of all the cells in the meniscus tissue (Yamasaki et al. 2005). Sandmann and his studies showed bone matrix is very hard and cannot be destroyed by Freeze-melt quickly in liquid nitrogen (Sandmann et al. 2009) Gilbert and his colleagues reported in 2006 that the complete removal of cell debris should be different detergents. In this phase, the ionic detergent SDS and washing to remove cell debris (nucleus and nuclear membrane) was used (Gilbert et al. 2006) Therefore, in this study the use of physical methods for removing cells from a 10% SDS-ionic detergent for 48 hours As can be seen in Figure 2, the extracellular matrix of bone remains. Swetha and colleagues in 2010 stated hydroxyapatite as a bone mineral natural substance used in orthopedic and dental building. Due to the biocompatible hydroxyapatite, bio-activity, induction of bone formation, non-toxic and non-inflamed known. Consequently, as an alternative to bone hydroxyapatite is an ideal material due to its biocompatibility and mechanical strength (Swetha et al.2010). Ramakrishna and his research in 2008 showed that osteoblasts on the scaffold made of collagen and hydroxyapatite mineral were attached to more than control (Venugopal et al.2008). Zhao and colleagues in 2006, two types of compounds gelatin-chitosan and HA / gelatin-chitosan scaffold, and demonstrated that the combination of chitosan and gelatin which contains 3D porous hydroxyapatite Protein adsorption to hydroxyapatite and calcium ions increases, which causes the initial adhesion and long-term growth and osteogenic cells can be induced after (Zhao et al.2006). Tang and colleagues showed that natural hydroxyapatite and chitosan biocompatibility have a hard tissue and the ability to induce bone formation as well (Tang et al.2008). Madhumathi and his studies in 2009 showed that chitosan hydrogel membranes and membranes containing hydroxyapatite and hydroxyapatite β- catenin are suitable for the connection of osteoblast cells and multiply (Madhumathi et al.2009). Zhao and colleagues showed that studies of hydroxyapatite and chitosan and gelatin biodegradable composition are similar to natural bone composition and provide a 3D environment that is suitable for tissue engineering. (Zhao et al.2002) Matthew and his colleagues in 2009 reported that chitosan-gelatin HA scaffolds cell binding, and distribution and increases cell proliferation (Matthew et al.2009). Duckeyene studies and his colleagues showed that the mineral calcium phosphate (CaP) effect induced bone formation, increased migration of mesenchymal stem cells and bone tissue, and the consistency of tissue (Duckeyene et al.1987,1999). The importance of glycosaminoglycans (GAG) in the extracellular matrix of the effects on cell migration, and cell adhesion to the matrix to determine the polarity of cell proliferation, differentiation, and repair has been demonstrated (Templon, 1992).

Friedl's studies have shown that adhesion and cell movement occur at the same time. Matrix molecules such as collagen, fibronectin, and proteoglycans are involved in cell adhesion (Friedl and Brocker 2000). Also, Murphy and colleagues 2011 showed that the glycosaminoglycan, and hyaluronic acid on the differentiation of mesenchymal stem cells affects Chondrogenesis increases while chondroitin sulfate increases osteogenic differentiation of stem cells (Murphy et al.2011). Therefore, in this study, the scaffold of chondroitin sulfate glycosaminoglycan composed of calcium phosphate and hydroxyapatite was soaked overnight And ink painting that can be seen in Figure 3. Black areas indicate that the compound is in the scaffolds. Tscheadschilsuren and colleagues candidate

cells that is considered important for cell therapy, announced mesenchymal stem cells (Tscheadschilsuren et al.2006).

Hui and colleagues 2007, according to research, mesenchymal stem cells have the ability differentiating into various cells, including chondrocytes, osteocytic, myocytes and adipocytes possess (Hui, et al.2007).However, the differentiation potential of mesenchymal stem cells to bone strength characteristics and to introduce it so much promise in the treatment of bone tissue has occurred. . Previous work on the reconstruction of large defects in bone cell function has been shown to be good. (Khojasteh et al, 2008., Jafarian et al, 2008) In addition to bone marrow mesenchymal stem cells from other sources, including ,periosteum, cancellous bone, adipose tissue, lungs and teeth used to be. In all cases shown to differentiate along multiple paths defined done (Barry et al.2004). Therefore, based on these studies, the study of mesenchymal stem cells derived from human adipose tissue was used. Chi Heng and his colleagues reported in 2004 in perfect condition (presence of growth factors, appropriate scaffolding, environmental conditions) for the differentiation of mesenchymal stem cells into cartilage cells require time (Heng et al.2004). Pountous and colleagues in 2005 during the differentiation of mesenchymal stem cells into chondrocytes on scaffold hydroxyapatite with BMP-2 to 6 week announced (Pountos et al.2005). The studies in this thesis cartilage formation induced differentiation of mesenchymal stem cells in about 3 weeks. As can be seen in Figure 5, after 20 days of culturing cells on scaffolds in bone cells, cartilage cells, they were relieved. Kruyt review and colleagues showed new bone formation is dependent on the internal structure of materials. Small pores to allow the phagocytes cells to adhere to scaffold and attract While the larger pores and growth of new blood vessels to strengthen bone tissue (Kruyt et al.2003). Fukui et al in 2005 showed that the main issues in bone tissue engineering environment suitable vessel and scaffold potential to support and enhance the differentiation of stem cells for bone building (Fukui et al.2005). in the study used of natural cancellous bone, probably provide more favorable conditions for the adhesion and attach stem cell . Also on the twentieth day cartilage formation was observed because the vessel is required for bone formation and limited time and cells cannot survive so many in the laboratory ,bone cells was not observed . (Figure 5). Ovchinikov showed that the growth and differentiation of chondrocytes and cartilage septum, alcian blue staining intensity around chondrocytes and is also more ECM . If the addition of a catalyst for the repair of cartilage, due to the increased amount of glycosaminoglycan matrix, a large number of molecules adsorbed alcian in this area, the intensity of the colors around chondrocytes and later increases in ECM (Ovchinikov et al 2003). Due to the presence of acidic sugar components in cartilage ECM looks sharp and fast response acidic glycosaminoglycan in cartilage ECM toluidine blue to growth during treatment with GAG and HA show. As can be seen in Figure 1. The color intensity toluidine (exhibited metachromatic mode) increasing duration of culture is increase. This is probably due to the release of proteoglycans and GAGs by cells for cartilage matrix, followed by the mineral matrix of cartilage gradually transformed mesenchymal stem cells on the matrix to be osteocytic.

In this study, likely because time was limited and the cells cultured in vitro can not survive on the other hand, the lack of large blood vessels, bone cells was observed.

Conclusion:

This study aimed to investigate the effect of glycosaminoglycan and hydroxyapatite scaffold with cells in the spongy bone decellularization male wistar rats. The first phase of this study were to develop a spongy bone tissue-derived extracellular matrix. Thus, for the removal of cells from the tissue cells, aids physical and chemical methods And H & E staining was used as shown in Figure 2. The results showed that the extracellular matrix of bone tissue spongy trabecular blades have remained the same. The ink stained black areas indicate that picture 3 However, two are female. The mesenchymal stem cells were placed on scaffolds And on 20,15,10,5 were analyzed. The results showed that in the twentieth day after they were cultured chondrocytes in bone tissue with staining was toluidine blue probably because need more time to create osteocytic matrix were observed. Also part of the epiphysis cartilage and cancellous bone mesenchymal stem cells on scaffolds were then stained with toluidine blue in this area exhibited metachromatic mode gradually increases On the fifth day after culturing mesenchymal stem cells exhibited metachromatic low and twentieth day after the culture reaches its maximum That the amount of glycosaminoglycan and proteoglycans secreted by chondroblast is more mature than the matrix and staining intensity increases. (Figure 1) The staining intensity was assessed by gangs, so that (+) diffuse staining (++) moderate staining (+++) can be intense coloration . Accordingly, a small amount of glycosaminoglycan is less color and with (+) and shown on 10 more GAG levels and stained with moderate (++) shown And twentieth day and reached a maximum level of staining (+++) is shown. So we can say that probably Glycosaminoglycan and hydroxyapatite can induce mesenchymal stem cells to chondrocytes and to have osteocytic.

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Conflict of interest:
Matrix Biology

Financial support:
All the project costs have been paid by me.
Ethics statement:
The present study followed international, national, and/or institutional guidelines for animal treatment.

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