Original Research

Comparison of bone cell viability and proliferation in 3D scaffold to Monolayer cell culture

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Abstract: **Introduction:** Today, due to high rates of accidents and fractures leading to bone defects and due to the limited possibility of bone graft bonding, using the patient's cell culture on appropriate scaffolds and transferring it to the defect area is suggested as one of the treatment plans.

Materials and methods: Bone samples of 8 male subjects that were under craniotomy surgery in the hospital were collected. First, the samples were cut into smaller pieces and then, transferred to incubator culture dishes. Two weeks later, the osteoblast activity on the bone matrix began and on average, the cells covered the dishes within two weeks. The first generation of the cells was removed by Trypsin_EDTA method from the opaltes, then were divided into two parts, one was added to alginate gel and the other to monolayer culture. In order to prove the osteoblast activity on the bone matrix and investigate these activities, Van Kossa staining method was used, and also to investigate the cell viability, MTT method was employed.

Results: There was a significant difference in the number of the cells created in alginate gel and those created in monolayer after two weeks ($P < 0.001$). Moreover, the difference between mean cell counts in alginate gel and monolayer was statistically significant ($P < 0.001$). The results of the MTT test in second week showed that the number of alive cells is significantly higher in alginate gel ($P < 0.001$). Finally, the result of the Van Kossa method proved extracellular matrix in both experimental groups.

Conclusion: Results showed that alginate gel better can support duplication and survival of osteoblasts compared to monolayer culture. This may be attributed to the biological properties of this gel; alginate gel porosity provides conditions under which cellular and metabolic activities are accelerated.

Keyword: Alginate, Bone Graft, Monolayer Culture, Osteoblast, Three-Dimensional Scaffold

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1. Introduction

Today, due to high rates of accidents and fractures leading to bone defects and due to the limitation of the possibility of bone graft bonding, researchers are looking for new solutions to repair damaged bones. Bone regeneration involves a collection of regular biologic events and bone conduction. Today, different methods are used to reconstruct the bone and autogenous bone has been used as the golden standard of comparison and the success of new methods (1). Although bone fractures improve with orthopaedic and supportive therapies due to the high repair power in this tissue, in many cases the crushed bone cannot be restored, and patients suffer from problems such as organ shortages and psychosocial problems (2, 3). For this reason, the necessity of providing a source of bone tissue, is important for restoring damaged areas. Since, cell culture requires appropriate supportive factors such as

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proper environment, time and scaffold, it is necessary to separate the osteoblastic cells from the bone and plant them on a suitable scaffold to achieve the maximum number of osteoblasts (at a shorter interval) (4, 5). The faster transfer of these cells to the fracture site could provide faster bone repair. Most studies have shown that scaffolds are essential for accelerating the process of repairing and renovating tissues in living creatures and laboratory conditions (5, 6). Scaffolds act as a suitable platform for migration and hanging, membrane dams, delivery carriers, as well as for the development of new tissue form and its binding to the living body (7). The structure of the scaffold must have appropriate pores with controlled porosity to facilitate the transfer of regulating molecules and nutrients between cells and the medium. In addition, the scaffold must have appropriate mechanical properties with the tissue texture (8, 9).

The simplest cell culture is the single-layer culture in which the cells grow on the plate. Typically, in this culture, in addition to the main cells, several other cells, such as fibroblasts grow, which then are purified by performing several cell passages (10). Monolayer of cultured bone cells for preliminary investigation is considered (11).

In a scaffold or three-dimensional crop, the cells are transferred onto the scaffold and then grafted into the target site. This scaffold facilitates the adhesion of the cells and promotes tissue development (9). In addition, it facilitates the transfer of nutrients and metabolites to cells, facilitates the removal of waste materials from cells, while the scaffold must be biodegradable and also stable in the patient's body and not immediately degraded, and decomposed at a suitable speed (12).

Alginate is a natural biopolymer, which is mainly extracted from brown algae not from bacteria. It accounts for 40% of the dry weight of algae and is combined with calcium, magnesium and sodium cations in extracellular matrix of algae (9) . Alginate can be regarded as an extracellular matrix material, suitable to provide cell growth. This gel creates a three-dimensional scaffold that, on the one hand, provides more space for cell proliferation and, on the other hand, facilitates the release of food in the medium and cell growth (13). These features make this material suitable for texture design. Porosity facilitates the release of macromolecules. It should be noted that the release of food in Alginate scaffolds depends on the alginate density, pH, temperature and gel preparation methods (14). Monomeric compounds, construction sequences and the rate of formation of alginate gel affect the amount of material release, porosity, infiltration, and strength and biocompatibility of this gel. As stated earlier, alginate gel and monolayer cultures can provide conditions for bone cell culture (15). Therefore, this study dealt with the characteristics of osteoblast cells obtained from human skull bones, cultured by two methods of the monolayer and 3D in order to determine the most appropriate culture method.

2. Method

2.1. Sampling:

Out of patients referring to the AL Zahra hospital, eight bone samples were taken during craniotomy by obtaining the informed consent from 18 - 45 years old male patients who were under surgery with no history of any bone disease. was approved independently by the Institutional Ethics Committee for this clinical study. Extra bone fragments (1 × 1 cm) with patient satisfaction was removed by a surgeon under general anaesthesia and complete sterile conditions, then transferred to the culture laboratory in 50cc Falcon tubes containing PBS and 1% antibiotic.

2.2. Sample preparation:

First, the samples were washed several times with phosphate-buffered saline (PBS) to remove blood and tissue. Then, the bone periosteum was mechanically separated by a surgical blade. Finally, the samples were cut into 1 mm pieces by bone cutter and rewashed by PBS.

Then, bone fragments were transferred to a 10 cm petri dish containing 10 ml Dulbecco's Modified Eagle's Medium (DMEM) and 10% Fetal bovine serum (FBS), 50 µg / ml ascorbic acid, 10 mM beta-glycerol phosphate and 1% penicillin-streptomycin, and petri dish (NEST) contents of the samples were transferred to an incubator with humid atmosphere containing 5% carbon dioxide at 37 °C. After two days, the culture supernatant was removed and replaced with fresh DMEM medium. The medium was changed every three to four days for two weeks. The culture dishes were examined for signs of bone cells on pieces as well as any multiplication. When the cells (spindle shape) completely covered the dish surface, the culture medium removed with PBS then using 25% EDTA trypsin mixes, the cells were separated from the dish. After counting, cells were separated equally (counting with Neubar Lam) and transferred to two culture dishes.

Daily microscopic examination of the cultures: dishes were examined every day by inverse microscopy for any cell migration in bone fragments, cell growth and environmental contamination.

2.3. Cell culture in alginate scaffold:

For the culture of osteoblast on alginate scaffold, about 0.4 millilitres of the alginate (Sigma, USA) solution were added to the cell deposition from the second passage.

After mixing the cells with the alginate, the resulting solution was added drop by drop to a calcium chloride solution of 105 mM within 15 minutes, alginate-cell droplets change into gel form in calcium chloride solution. After drainage of calcium chloride, 0.9% sodium chloride solution was added to the alginate granules for 10 minutes, this was repeated twice. Each granule contains 10,000 cells (counting with Neubauer), which was placed in 3 cm dishes. The dishes were transferred to the incubator (Memert, Germany), and their medium was replaced every three to four days.

2.4. Monolayer cell culture:

2.4.1 Van Kossa staining

For monolayer cell culture, petri dishes were transferred from the incubators to the hood and washed twice with PBS. After fixing the cells with 4% paraffin aldehyde, the Van Kossa staining stages were performed on the petri dishes. For this purpose, the samples were first placed in 5% silver nitrate for 1 hour, followed by 1% sodium peroxide for 3 minutes, sodium thiosulfate 2.5% for 5 minutes, and fast forward nucleotide. After washing, they were observed by a light microscope.

For alginate samples, petri dishes were first transferred from the incubator under the hood and washed twice with PBS. Dehydration, molding and preparation samples with 5 micrometre thickness were performed. These slides were stained with Van Kossa stains.

2.4.2 MTT

For monolayer culture of single cells: petri dishes were first washed twice with PBS solution and then the mediums were added to the cells in the plate and then, the solution of Methyl Thiazolyl Diphenyl-Tetrazolium bromide (MTT) (Sigma, USA) was added to the cells. Plates were then placed inside the incubator for 4 hours. After incubation time, the culture medium was slowly removed. Then, 10% Dimethyl Sulfoxide (DMSO)(Gibco, USA) solution was added to each plate of the house and after pipetting was read using reader ELISA (read off at 540 nm (Biotech, USA) based on formazan, the intensity of light absorption).

For alginate samples, the granules were first washed twice with PBS solution and the remaining steps were similar to those of monolayer culture.

2.4.3 Cell counting with Neubar:

In monolayer culture, petri dishes after medium evacuation, were washed by PBS, and the cells were removed from the dish by adding enzymes and the centrifugal solution and supernatant were evacuated. At this stage, cells were counted by homocytometry. In the alginate scaffold, the granules were first dissolved in sodium citrate, then the PBS was added and supernatant were evacuated then, the cells were counted by the homocytometry.

2.5. Statistical analysis:

One type of analysis was applied for compression control and alginate group. Data was analysed using the t-test. It must be emphasized that these ratios are all significant based on $P < 0.001$ for both groups. Analysis was carried out using the SPSS software with version 16.

3. Result

3.1. Culture of osteoblast cells:

In early culture, cells with spherical morphology were observed. In the first passage, these cells began to grow

Table 1: Count the number of cells after two weeks. The amount of Mean ± SD is presented. Counts done per 10,000 cells transmitted in alginate droplet or monolayer culture

Group	Mean	Standard deviation	P-value
Monolayer	17574	2834	<0.001
Alginate	3996	3063	<0.001

Table 2: Cell survival after two weeks. Cell survival read per 10,000 cells transmitted in alginate droplet or monolayer culture by reader ELISA

Group	Mean	Standard deviation	P-value
Monolayer	10507	0.190	<0.001
Alginate	18928	0.296	<0.001

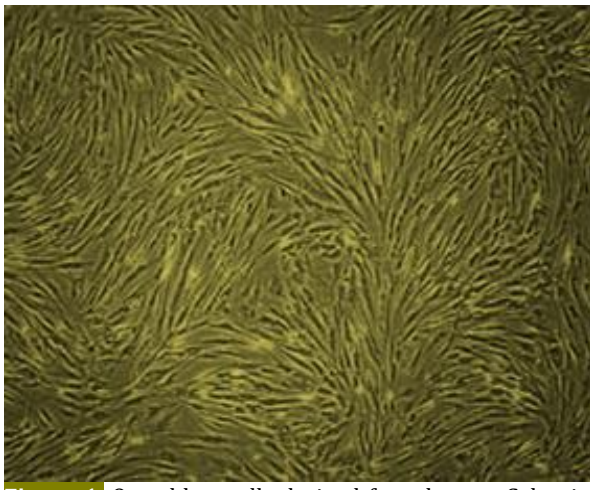

Figure 1. Osteoblast cells derived from human Calvaria bone after second passage: These cells can be seen with spindle morphology (Magnification x40).

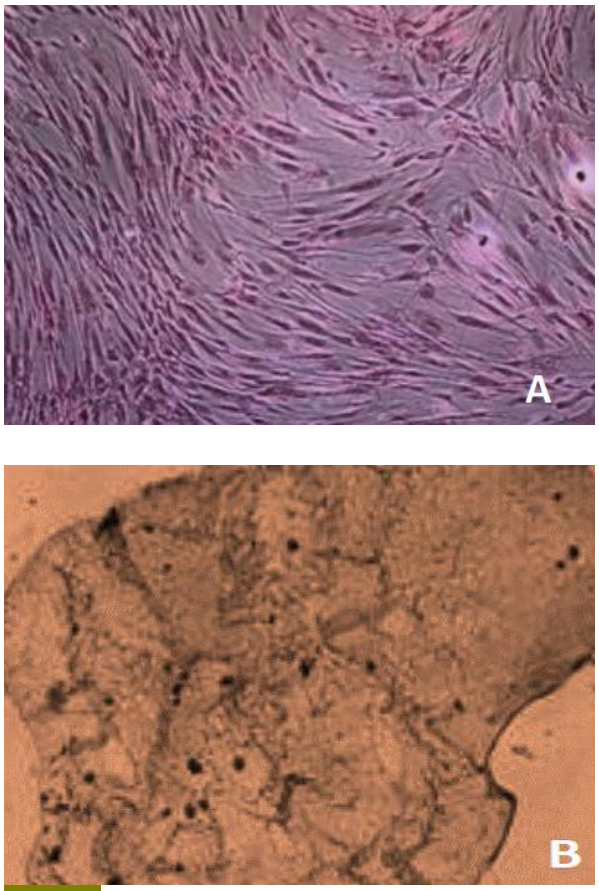

Figure 2. Van Kossa staining A (Monolayer culture) B (Alginate culture). The presence of a dark-coloured deposit indicates the matrix of mineral formed by osteoblast cells (Magnification x300).

with the formation of colonies. Finally, the number of spindle cells increased by performing the cell passage, so that in the second passage, cells occupied the entire surface of the dish.

3.2. Van Kossa staining:

This staining was used to prove the existence of a matrix of mineral, in two experimental groups (monolayer and alginate). During the second week, the formation of dark sediment proved the existence of a matrix of mineral. As a result, the calcium deposits turned black and the cells core became red.

3.3. Mean Activity of Live Cells: MTT

The average activity of live cells in alginate scaffolds was significantly higher than that of live cells in monolayer culture. In living cells, purple crystalline formazan is formed. The formation of these crystals represents the activation of the respiratory chain enzymes and a criterion for the survival of the cells.

3.4. Cell counting:

By cell count, it was determined that the mean of cell proliferation in alginate scaffold was significantly higher than that of monolayer scaffold ($P < 0.001$).

4. Discussion

So far, many studies have been carried out on the culture of bone marrow cells and researchers have used different materials as cellular scaffolds (16). However, this study aimed to deal with the ability of cultures of osteoblasts on alginate scaffolds in order to analyse the behaviour of these cells in alginate in comparison with monolayer culture.

Calvaria bone is a sponge bone which is used in most exploratory studies on explanation culture (17); since cells grow earlier on the bone, Pape et al. reported the time needed to remove cells from the tissues by studying the samples from the head bone (18).

Our study lasted 10 days on average, which is consistent with their study. Past studies reported a period of 4 to 5 weeks in which the cells fully covered the petri dish floor (19). The duration of this study was an average of 20 days, which may be due to the difference in the addition of various compounds such as ascorbic acid and beta glycerol phosphate with different densities in the culture in our study, since none of the above materials were used in their study.

On the other hand, it should be noted that the culture medium also has a significant effect on cell growth and proliferation, since different culture media contain different nutrients and can affect the growth and proliferation of cells (20). In the studies listed above, αMEM medium was used, while in our study, DMEM medium was used as the basic culture medium (20).

Most studies have revealed that scaffolds are essential for the progression of the process of repairing and rebuilding tissues in the living organism and laboratory conditions, and act as a suitable substrate for migration - sticking - to determine the form of a new tissue and its

transplantation to the living body (21, 22). In addition, the structure of the scaffold must facilitate the transfer of regulating and nutrient molecules to cells and metabolites from the cell to the environment. Also, the used scaffold should not cause inflammatory reactions or the production of toxins in the body, and must have mechanical properties that are appropriate to the tissue (23, 24).

Abbah et al., with the culture of stem cells of fatty tissue in alginate and its differentiation into osteoblast, showed that the environment of this gel facilitates the proliferation and survival of osteoblast. In 2008, the researcher and colleagues cultured stem cells derived from bone marrow in alginate gel, differentiated it into osteoblasts and reaffirmed the results of previous studies on alginate gel (25). Liao et al. showed that osteoblast cells were grown on alginate gel and used to repair bone defect in the skull. It was observed that after 12 weeks, the repair was complete (26). In addition to studies carried out in vitro, Alsberg et al. reported that rat osteoblasts with alginate scaffold were grafted to the bone defect and bone repair was reported within 10 weeks (27).

In the present study, alginate scaffold has been used which increases growth and proliferation of cells due to the presence of hydrogel alginate and the high amounts of water, since in this case, the exchange of nutrients and metabolites is better performed. On the other hand, alginate becomes easily gelatinized with no organic solvents at the room temperature and does not require a toxic activator (14). This feature has led to more scaffold alginate in the setting of the tissue.

In different studies, Van Kossa staining was used to prove the existence of a matrix. In this study, the results of Van Kossa staining on the two groups showed the presence of minerals in the matrix in the second week. The Von Kossa stain is used to mineralization in cell culture and the basics is a precipitation reaction in which silver ions react with phosphate in the presence of acidic material. This result was consistent with that of Abbah et al., who used the Van Kossa stain to prove the existence of a matrix (25).

The results of this study showed that the osteoblast cells derived from Calvaria bone has significantly more proliferation and also has significantly more live cells on the alginate scaffold, compared with monolayer. Perhaps, because the gelatine scaffold is one of the three-dimensional scaffolds in the field of cell culture.

5. Conclusion:

Our research shows that alginate gel better can support proliferation and survival of osteoblasts rather than monolayer culture. Most likely the cause of this difference can be found in the biological properties of this gel; alginate gel porosity provides conditions where cellular and metabolic activities are accelerated.

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7. Conflict of interest:

The authors have no conflicts of interest.

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9. Author's contributions:

Fereshteh Aliakbari project development; Data collection and data analysis, Faezeh Azizi and Sahar Omidpanah; Manuscript writing, Afshin Moradi; Manuscript editing, Mohamad Ali Hossini.

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