Comparison of osteoblast activity and osteogenic potential of autogenous bone harvested with different methods: an in vitro study

Avideh Ramtin, Reza Amid, Fahimeh Sadat Tabatabaei, Parvin Torabzadeh, Mehdi Kadkhodazadeh

ABSTRACT

Aim: The purpose of this study was to compare three techniques of autogenous bone graft harvesting for intraoral surgeries namely the trephine bur, bone scraper and piezosurgery device. The viability of osteoblasts in the harvested bone was evaluated.

Materials and Methods: Bone grafts were harvested from 4 dogs. Tissue samples were obtained from the mandibles of dogs and stored in phosphate buffered saline (PBS) in sterile test tubes. Decellularization was done and the specimens were incubated under specific conditions for cell culture. Viability of osteoblasts was evaluated in the specimens harvested with the three techniques. Also, osteoinductive potential of bone graft, mineralized and demineralized bone allograft (MBA/DBM), and control group was evaluated using MTT assay, Alizarin Red staining and alkaline phosphatase (ALP) activity measurement.

Results: Two weeks after tissue culture, only the bone graft harvested by trephine bur contained viable cells; but number of isolated cells was small. At three days, the MTT assay revealed a significant reduction in cell proliferation in the trephine and the MBA/DBM groups. Alizarin Red staining demonstrated that number of calcified nodules was significantly higher in the MBA/DBM and the positive control groups at 3 days. These nodules were not observed in the other groups.

Conclusion: The results showed that only the bone graft harvested by the trephine bur contained viable cells. Allogenic bone showed higher osteoinductive potential than autogenous bone graft. Further in-vitro and in-vivo studies are required to find the most efficient technique for autogenous bone graft harvesting.

KEY WORDS: Bone graft, osteoblast, osteoinductivity, allogenous bone

INTRODUCTION

Bone graft substitutes play a critical role in regenerative dentistry (Sbordone, L., 2009). Many regenerative treatments have been recently suggested for tissue regeneration and restoring its ideal function. Considering the advances in cytology and related techniques and equipment, most clinicians and patients prefer regenerative and reconstructive treatments.

Bone grafting is widely performed in orthopedic and maxillofacial surgeries. Different bone graft materials are available namely the autogenous graft, allograft, xenograft and alloplasts or synthetic materials. Autogenous bone is considered as the gold standard of graft materials and has osteoconductive, osteoinductive and osteogenic properties. Cancellous bone has higher cellularity and consequently greater osteogenic potential. These grafts provide the most successful and reliable results; (Esposito, M., 2009) because the viable cells that provide growth factors and also the osteoblast progenitor cells may be traumatized or lost during graft harvesting.
Use of autogenous bone has the advantage of preserving the bone mineral and collagen. It also contains osteoblasts and bone morphogenetic proteins (BMPs) (Lezcano, F.J., 2007). Osteoblasts are the most important cells in the process of regeneration and formation of bone; however, only a small number of osteoblastic cells survive the transplantation process. There are two main types of avascular autogenous bone grafts: cortical bone and spongy bone (Kainulainen, V.T., 2006). Burchardt in his study described the three main differences of these two graft types (Burchardt, H., 1983). Cancellous bone grafts are re-vascularized more rapidly and more completely. Gradual revascularization occurs at an average speed of 1 mm/day. Spongy bone vascularization occurs within two weeks; while this process takes approximately two months in the cortical bone. (5) Cortical bone better tolerates the primary mechanical loads (O’Mahony, A., P. Spencer, 1999). A cortical graft is better adapted to the recipient site and therefore is recommended for use in porous areas with higher number of voids. These grafts are generally applicable in more areas and are also easier to use.

Autogenous bone grafts can be obtained via different techniques including the bone mill, trephine bur, bone scrapers and piezosurgery device. There is a gap of information about the possible differences of grafts harvested by different techniques. This study aimed to assess the viability of osteoblasts present in bone grafts harvested by a trephine bur, bone scraper and piezosurgery device.

**MATERIALS AND METHODS**

The principles of this study were based on the CONSORT guidelines. This in-vitro, experimental study was conducted on four Iranian hybrid (mixed breed) dogs fed with dog food. The animals were selected using non-randomized convenience sampling. The Ethics Committee of Dental Research Center, Shahid Beheshti University of Medical Sciences approved this study.

Fresh bone grafts were harvested from the mandibles of four dogs using a trephine bur (3 diameter, Medesy, Maniago, Italy), bone scraper and piezosurgery device. The study was performed in the Veterinary Hospital affiliated to Tehran University of Medical Sciences. The dogs were sedated by a veterinarian and lidocaine was injected into their left buccal vestibule. After ensuring a complete sedation, a flap was elevated at the left buccal vestibule using a surgical scalpel and samples were collected by a trephine bur, bone scraper and piezosurgery device, respectively. Specimens were transferred to vials and stored in ice. The surgical site was irrigated with saline solution and sutured with chromic gut absorbable suture. The same procedure was performed in the right buccal vestibule of all four dogs. Specimens were transferred to the laboratory. Finally, tramadol was injected for the dogs and the animals were fed soft food for one week. After a week, following the veterinarian’s approval, the animals resumed their regular diet.

**Laboratory procedures:**

Harvested bone grafts were transferred to sterile tubes containing PBS (+1% antibiotic). After three times of washing, bone grafts were placed in sterile plates and chipped under sterile conditions. Specimens were then transferred to a 75 mL flask and stored in an incubator at 37°C under 5% CO2 and 95% humidity. Cultures remained intact for seven days and thereafter, the medium was refreshed twice weekly.

During the cell culture process, the isolated cells were evaluated under an inverted microscope (KYKY-EM 3200, China) to assess their proliferation and possible microbial contamination.

**Decellularization:**

Decellularization must be carried out before using the living tissue. For this purpose, the tissue was subjected to five thermal shock cycles and placed in a nitrogen tank for 5 minutes and then in a bain marie at 37°C for 10 minutes consecutively. The tissue was finally rinsed with PBS and was then ready for application on cells.

**Cell culture:**

The MG63 cell line procured from the Pasteur Institute cell bank was removed from the nitrogen tank and immediately transferred to a water bath at 37°C. After defrosting, culture medium was gradually added to the cells. After centrifuging and eliminating the previous culture medium, the cell suspension was transferred to a flask containing DMEM, 10% FBS and 1% antibiotic and stored in an incubator at 37°C under 5% CO2 and 95% humidity. The culture medium was refreshed every three days. After proliferation and reaching a confluence of 80%, the cells were passaged using trypsin.

**Cell preparation for testing:**

After reaching a confluence of 80%, the cells were detached from the bottom of the flask using Trypsin/ethylenediaminetetraacetic acid (EDTA) and counted using a Neubauer chamber.
Cell counting:
For cell counting, 20 μL of cell suspension was mixed with 20 μL of Trypan Blue; 10γ of this mixture was transferred to a Neubauer chamber and viable cells, light in color, were counted under a microscope.
Number of counted cells = (number of counted wells)/(total number of all counted cells) X dissolution coefficient X 10
A total of 40,000 cells in 2 mL of culture medium were transferred to each well of a six-well plate. Three plates were allocated for each test and a total of 18 six-well plates of cells were prepared and stored in an incubator. After four hours of incubation, the plates were removed from the incubator. The tissue harvested by the trephine bur was weighed and added to one well in each plate under sterile conditions. Commercial MBA and DBM (Iranian Tissue Bank Research & Preparation Center, Imam Khomeini Hospital Medical Complex, Tehran, Iran.) weighing equal to the tissue collected by the trephine bur, were added to the second well in each plate. One well was considered as the negative control and contained only the regular culture medium. Osteogenic medium (containing standard culture medium, 10 nm of dexamethasone, 10 nm of β-Glycerophosphate and 50 μg/ml of ascorbic acid) was added to one well in each plate as the positive control.
At day three, all plates were subjected to different tests. The ALP activity test was performed at three and seven days.

The MTT assay:
After three days of exposure to different materials, the cells were rinsed with PBS to wash off the overlaying culture medium. The MTT solution at a concentration of 5 mg/mL was prepared and diluted at a 1/10 ratio using PBS-free culture medium. One mL of this solution was added to each well. Plates were then incubated at 37°C under 5% CO2 and 98% humidity for three hours. During this time period, viable cells with active metabolism are capable of reducing yellow soluble MTT salt to insoluble, purple formazan crystals by the mitochondrial succinate dehydrogenase enzyme. These crystals are seen under a light microscope. After three hours of incubation, the overlaying medium was gently extracted and one mL of DMSO was added to each well to dissolve formazan crystals. The obtained solution was then transferred to a 96-well plate and the resultant color change (which has a direct correlation with the metabolic activity of the cells) was measured by ELISA Reader at 570nm wavelength with a 650nm filter and reported as optical density (OD). The percentage of cell viability was calculated by dividing the mean absorbance read for each group by the mean absorbance read for the negative control group multiplied by 100.

Alkaline phosphatase activity test:
Three days after exposure to different materials, cells were rinsed with PBS to wash off the overlaying culture medium. Then, 500 μL of the assay buffer solution available in the kit was added to each well according to the manufacturer’s instructions and after centrifugation (13,000 rpm for 10 minutes), the supernatant was transferred to a 96-well plate. Specific amounts of pNPP solution available in the kit were added to each well according to the manufacturer’s instructions. Specimens were kept in the dark for one hour at room temperature in the lab. Next, the test was stopped by adding the Stop Solution. The resultant color change, having a direct correlation with the ALP activity of the cells, was measured by ELISA Reader at 405 nm wavelength and reported as OD. All these procedures were repeated on day 7 as well.

Alizarin Red staining:
After three days of exposure to different materials, in order to assess the formation of calcified nodules, cells were washed with PBS for three times and fixed in absolute alcohol for 30 minutes. The cells were washed again with PBS and stained with 2% Alizarin Red at a pH of 4.2-4.4 for 10 minutes at 37°C. Photographs were taken by an inverted microscope.

Cell culture on bone tissue:
To assess the adhesion of cells to bone tissue and the possibility of using the harvested bone grafts as scaffolds for tissue engineering, the bone grafts harvested with the trephine bur were weighed; the same amounts of DBM and MBA were also added and tissues were covered with 1052 MG63 cells in 200 μL of culture medium. For primary adhesion of cells, plates were incubated at 37°C for one hour. Next, 500 μL of the regular culture medium (containing DMEM, 10% FBS, 1% antibiotic-antimycotic and 2 mmol/L of glutamine) was added to half the specimens. The other half received the same amount of osteogenic culture medium (containing standard culture medium, 10 nm of dexamethasone, 10 nm of Beta-Glycerophosphate and 50 μg/ml of ascorbic acid). All specimens were then incubated at 37°C under 5% CO2.
One day after cell culture, specimens were evaluated under a scanning electron microscope (SEM). For this purpose, scaffolds were rinsed with PBS twice and fixed with 2.5% glutaraldehyde for 2 hours followed by 1% osmium for one hour. Specimens were then dehydrated using different concentrations of ethanol (30, 50, 70, 90,
Specimens were placed under a sterile hood overnight to ensure drying. They were then gold-coated and evaluated under an electron microscope (KYKY-EM3200, China).

Statistical analysis:
Data obtained via the MTT assay and ALP activity test were analyzed using GraphPad Prism V.5 software (GraphPad, San Diego, USA). Data analysis was carried out using one-way ANOVA. Differences between groups were analyzed using post-hoc Tukey’s test (multiple comparison). P<0.05 was considered statistically significant.

Results:
Results of cell isolation from the tissues:
Two weeks after tissue culturing in plates, tissues harvested by bone scraper and piezosurgery device showed no activity and no cell was isolated from them. Only the tissue harvested by the trephine bur contained cells; however, number of cells isolated from this tissue was scarce and insignificant.

Results of the MTT assay:
After three days of culture, cell proliferation in the positive control group was not significantly different from that in the negative control group; however, cell proliferation in the trephine group had significantly decreased (P<0.05). In the MBA & DBM group, this reduction was strongly significant (P<0.05) (Diagram 1).

Diagram 1: The results of MTT assay at three days.
*Indicates statistically significant values (P<0.05)
Negative control group: Regular culture medium
Positive control group: Osteogenic medium
Bone tissue: Bone tissue harvested by the trephine bur

Results of ALP activity test:
The ALP activity of different groups was compared with that of the control groups at three and seven days. In MBA & DBM, trephine and positive control groups, ALP activity significantly decreased at three days. This reduction in activity in the trephine group was more significant than in other groups (P<0.05) (Diagram 2).

Diagram 2: The results of ALP activity test after three days.
The ALP activity of MBA & DBM and the positive control groups did not change significantly after seven days; but, in the trephine group, ALP activity significantly increased on day seven compared to day three (P<0.05) (Diagram 3).

**Diagram 3:** The results of ALP activity test after seven days.

**Results of Alizarin Red staining:**
At three days, mineralized nodules were clearly seen following Alizarin Red staining in the MBA & DBM and the positive control groups. The amount of calcified nodules was significantly higher in the MBA & DBM group; while these nodules were not observed in the negative control or the trephine group (Figure 1).

**Fig. 1:** Alizarin Red staining of groups containing MBA & DBM (a), osteogenic medium (b), tissues harvested by the trephine bur (c) and the negative control (d).

**SEM analysis:**
Figure two shows the SEM image of cell-containing scaffolds. Numerous cell stacks and collagen-like fibers were observed on both scaffolds. But the size of porosities in the bone scaffold harvested by the trephine bur seemed to be larger. Small calcified nodules were also observed on the surface of MBA & DBM scaffold.
**Fig 2:** SEM image of cells adhered to the surface of bone tissue harvested by the trephine bur and the MBA & DBM scaffold.

**Discussion:**

Assays used in the current study are among the standard tests for the measurement of osteogenic activity of cells. First, we briefly describe these tests:

The MTT assay is a colorimetric assay for evaluation of cell viability. By measuring oxidative-reductive mitochondrial enzymes in this test under specific conditions, the number of viable cells is counted. This test is conducted in the dark because the test materials are photosensitive.

In Alizarin Red staining test, the calcified materials in the specimens absorb the dye and appear red under a microscope. By observing the calcified materials produced by the transplanted cells, it can be concluded that these cells are viable and functional.

Sila-Asna et al. (2007) assessed the viability of osteoblasts using ALP activity and Alizarin Red staining tests. Both these tests were well capable of depicting cell function. The results of these tests were eventually compared with those of a test for assessment of a gene containing information regarding the protein products of osteoblasts. This test accurately shows the function of osteoblasts. All three tests were successful in assessing the viability of osteoblasts.

Favorable efficacy of the aforementioned tests for the assessment of bone cell viability has also been reported in similar studies by Ciapetti et al. (2003), Hicok et al. (2004) and Evangelista et al. (2007).

The current study aimed to assess and compare the viability of osteogenic cells harvested by bone scraper, piezoelectric device and the trephine bur. The results showed that only the bone harvested by the trephine bur contained viable cells. Thus, only the specimens obtained by the trephine bur underwent further testing. In the current study, three methods were used for culturing viable bone cells isolated from the bone tissue harvested by the trephine bur. Three 6-well plates were allocated for each of the three methods. Specimens were subjected to three tests on day three. Alkaline phosphatase activity test was performed at three and seven days. The tests and the obtained results were as follows:

**The MTT assay:**

This assay showed that after three days of culture, cell proliferation in the positive control group was not significantly different from that in the negative control group; however, cell proliferation in the trephine and MBA & DBM groups had significantly decreased; which indicates cell differentiation in the MBA & DBM and lack of cell differentiation in the trephine group.

**The ALP activity test:**

The results revealed that in the study groups, ALP activity significantly decreased at three days compared to that in the negative control group. This reduced activity in the trephine group was greater than in other groups. On day seven, no significant change occurred in the positive control and MBA & DBM groups; but in the trephine group, ALP activity significantly increased compared to that on day three. The results of this test also indicated cell differentiation in the MBA & DBM and lack of differentiation in the trephine group.

**Alizarin Red staining test:**

This test demonstrated the formation of calcified nodules in the MBA & DBM and the positive control groups at day three. No such nodules were seen in the negative control or the trephine group.

Atari et al. (2011) evaluated the viability of maxillary bone harvesting by three methods of bone scraper, rotary carbide burs and piezoelectric device. They reported that bone chips obtained by the three methods showed significant amounts of apoptotic cells and did not provide a suitable source of viable bone cells. Similarly in our study, no viable cell could be isolated from the bone grafts harvested by the bone scraper and piezoelectric device.

Papadimitrou et al. (2013) studied the morphology of bone particles harvested by four different instruments namely back action chisels I and II, a safescraper and a sonic flex handpiece. All specimens contained viable
cells; but the shape of bone chips was distinctly different among groups. In their study, only the results of light and electron microscopy were reported.

Mouraret et al. (2014) compared piezosurgery device and a conventional bur used in oral and maxillofacial surgeries. They assessed bone function by measuring ALP activity and reported that the bone graft harvested by piezosurgery contained more viable cells compared to the bone tissue collected by the bur. In the recent years, piezosurgery has yielded successful results.

In the current study, we evaluated the viability of bone cells in bone grafts harvested by the three instruments namely bone scraper, trephine bur and piezoelectric device. The results showed that the graft materials collected by the bone scraper and piezoelectric device did not contain viable bone cells; while the bone chips obtained by the trephine bur contained viable cells. Thus, it appears that procuring bone graft with the aforesaid techniques yields different results from what is expected from the autogenous bone block graft as the gold standard. The more traumatizing the bone graft harvesting technique, the lower the chance of survival of osteoblasts. Consequently, the process of osteogenesis will be completely impaired. The surgical technique, the elapsed time, method of storage, temperature, etc. may be responsible for not obtaining viable cells by the bone scraper and piezosurgery. However, this issue needs further investigations.

Moreover, taking samples from the maxilla of dogs may yield different results because the composition, quality and quantity of the maxillary bone are different from those of the mandible. However, a final conclusion can be drawn after sampling in human studies.

Conclusion:
Two weeks after tissue culture, only the bone graft harvested by the trephine bur contained viable cells; but the number of isolated cells was small. The MTT assay on day three showed a significant reduction in cell proliferation in the trephine and MBA & DBM groups. Alizarin Red staining on day three indicated that number of calcified nodules was significantly higher in MBA & DBM and the positive control groups and these nodules were not observed in the other groups. The results showed that only the bone tissue collected by the trephine bur contained viable cells. All three tests were well capable of showing cell viability. Further in-vitro and in-vivo studies are required to find the most efficient technique for autogenous bone graft harvesting.

ACKNOWLEDGMENTS

This study was supported by Dental Research Center, Shahid Beheshti University of Medical Sciences.

Notes:
No potential conflict of interest relevant to this article was reported.

REFERENCES


