Impact of Harvesting Method and Donor Age on the Behavior of Human Osteoblast-Like Cells

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Autogenous particulate bone grafts are being utilized in oral implantology for minor grafting procedures. This study aimed to investigate the influence of the bone-harvesting technique, donor age, and donor site on proliferation and differentiation of human primary osteoblast-like cells in the cell culture. Autogenous bone particles (20 samples) were harvested from the maxilla and mandible during surgery using two different protocols, and two types of particulate bone grafts were collected: bone chips and bone sludge. Bone samples were cultured in growth medium and, after 2 to 3 weeks, the cells that grew from bone grafts were cultured in the normal and osteogenic medium for 0, 4, 7, and 20 days. DNA, alkaline-phosphatase (ALP), calcium-content measurements, and Alizarin red/toluidine blue staining were performed. Data were analyzed by repeated-measures analysis of variance with Bonferroni test. The level of statistical significance was set at 5% (P < .05). Total DNA, ALP, and calcium content were significantly higher for the bone chip samples compared to the bone sludge samples. Total DNA and ALP content were significantly higher for the patients in age group 1 (≤ 60 years) compared to age group 2 (> 60 years) and was significantly higher for mandibular samples than maxillary samples on day 20. However, the calcium measurement showed no significant difference concerning donor age and donor site. Data analysis revealed that harvesting technique (bone chips vs bone sludge), donor age (≤ 60 years vs > 60 years), and donor site (maxilla vs mandible) influenced the osteogenic potential of the collected particulate bone graft. The bone chips were superior in terms of osteogenic efficacy and should be considered a suitable option for particulate bone graft collection. Int J Periodontics Restorative Dent 2023;43:e35–e42. doi: 10.11607/prd.5711

Various grafting materials have been used in implant dentistry to regenerate bone; however, no bone substitute can completely replace autogenous bone, as it is the only material available containing vital osteoblasts, osteocytes, and growth factors.1,2 The autograft could be obtained from extraoral sites (the iliac crest or calvaria) or intraoral locations (tuberosity, chin, or ramus).2 The required quantity of autograft dictates the choice of a donor site.2 The major disadvantages of using an autograft are patient suffering (ie, pain or morbidity) and its limited availability.3 However, the need for a second surgical site can be circumvented by employing new methods of the bone collection, such as using a bone trap or bone scraper, or harvesting bone particles attached to the drill flutes during implant site preparations.2,4,5

The clinical success of autogenous bone graft is dependent on employing an accurate bone-harvesting technique that preserves the bone vitality and the ability of the osteoblast progenitor cells or osteocytes to stimulate an osteogenic response.6 The type (cortical or cancellous) and architecture (particulate or bone block) of the harvested bone can influence the regenerative ability of the grafts and, consequently, the de novo bone formation.7,8 Donor/patient age is
also considered a critical factor for the osteogenic efficacy of different autografts that must be taken into consideration. Therefore, this study aimed to investigate the influence of the bone harvesting technique, donor age, and donor site on proliferation and differentiation of human primary osteoblast-like cells in the cell culture environment.

Materials and Methods

Bone Samples

Twenty bone samples were collected from the maxilla or mandible of patients (1 sample per patient) during implant site preparation. Medical ethical committee approval was obtained from Vrije University Amsterdam (ethical approval no. 2013/151), and the study protocol was in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients, who were selected from the university’s dental hospital (ACTA; Amsterdam, The Netherlands).

The autogenous bone was collected using two different harvesting techniques: bone chips and bone sludge. For bone-chip harvesting, implant site preparation was performed at speeds of 150 to 200 rpm without saline irrigation, and the bone chips attached to the drill threads were collected (n = 10 samples). For bone-sludge harvesting, the implant osteotomy was prepared at the speed of 800 rpm with sterile saline irrigation, and the bone attached to the drill threads was obtained (n = 10 samples). All samples were sent immediately to the cell culture laboratory for further preparation and analysis (Fig 1).

Cell Isolation and Culture Procedure

All samples for cell culture analysis were washed twice with Dulbecco’s Modified Eagle Medium (DMEM; Thermo Fisher Scientific) and placed into T25 tissue culture flasks (Corning) in a normal culture medium containing DMEM, with 1% penicillin, streptomycin, and fungizone (Sigma-Aldrich), 10 IU/mL of heparin (Leo Pharma), and 5% human platelet lysate (Sanquin). Incubation was performed in a humidified atmosphere of 95% air and 5% CO2 at 37°C. The culture mediums were replaced twice a week. After 2 to 3 weeks, the cells that grew out from the bone grafts (bone explants) were released by 0.25% trypsin (Invitrogen, Thermo Fisher Scientific), and 0.1% ethylenediaminetetraacetic acid (Millipore Sigma) in phosphate-buffered saline (PBS) and counted (passage 0). The cells were grown until passage 2. The number of outgrown cells from passage 2 was calculated per gram of wet bone tissue and cells (concentration: 20,000 cells/well/400 µL) and placed in 24-well plates. These cells from passage 2 were cultured for 0, 4, 7, and 20 days for further analysis. The cells were cultured in normal medium that had been converted to the osteogenic culture medium via the addition of 50 μg/mL of ascorbic acid, 10 mM of Naβ-glycerophosphate, and 108 M of dexamethasone.

The day after the overnight attachment of the cell cultures was considered day 0. At each time point (days 0, 4, 7, and 20), the culture medium was removed, and the cells were washed with PBS. The cells were submerged in 300 µL of Milli-Q water (Millipore Sigma) and frozen at −20°C until cell proliferation and differentiation assays. For calcium measurements,
the cells were stored dry in the freezer until analysis. For Alizarin red staining, cells were fixed in 4% formaldehyde.

**Cell Proliferation and Differentiation Measurements**

DNA content (a measure of cell number) was determined using the CyQuant Cell Proliferation Assay Kit (Molecular Probes). Alkaline phosphatase (ALP) activity was measured using the method described by Bessey et al. Calcium content in the samples was measured by the orthocresolphthalein complexone method.

Cells planned for alizarin red staining were fixed in 4% formaldehyde for 15 minutes and stored in PBS at 4°C until stained. Alizarin Red S mono sodium-salt solution (2%; C.I. 58005, Sigma Aldrich) was used alone or in combination with 0.2% Toluidine blue (C.I. 52040; Sigma Aldrich) in Milli-Q water.

**Statistical Analysis**

To ensure reproducibility, three separate runs of the experiments were performed, yielding near-identical results. Data analysis was performed using SPSS (version 20.0, IBM). Demographic variables were presented into frequencies and percentages, chi-square test was utilized for proportions of patient characteristics between bone chips and bone sludge groups. Numerical data were presented as mean ± standard deviation. The effects of donor age and donor site were analyzed, irrespective of the harvesting technique. Repeated-measures analysis of variance with post hoc Bonferroni test was performed for DNA and ALP analysis between groups. Wilcoxon signed-rank test was applied for pairwise comparison of data within groups. Wilcoxon Mann-Whitney U test was used to compare calcium levels. \( P < .05 \) was considered statistically significant.

**Results**

**Study Population**

The mean patient age was 57.6 years ± 13.1 years (range: 34 to 79 years). Demographic variables are presented in Table 1. Cell outgrowth was observed in all 10 bone chip samples (100% success rate). Osteoblast-like cell outgrowth was seen in 6 of 10 bone sludge samples (60% success rate). No

| Table 1 Demographic Characteristics Between Bone Sludge and Bone Chip Samples |
|---------------------------------|-----------------|-----------------|---|
|                                 | Bone sludge     | Bone chips      |  
| **Gender**                      |                 |                 |   |
| Male                            | 9 (90)          | 8 (80)          | > .05 |
| Female                          | 1 (10)          | 2 (20)          |   |
| **Donor age**                   |                 |                 |   |
| \( \leq 60 \text{ y} \)         | 6 (60)          | 6 (60)          | > .05 |
| \( > 60 \text{ y} \)           | 4 (40)          | 4 (40)          |   |
| **Donor site**                  |                 |                 |   |
| Maxilla                         | 4 (40)          | 6 (60)          | > .05 |
| Mandible                        | 6 (60)          | 4 (40)          |   |
| **Location**                    |                 |                 |   |
| Anterior                        | 1 (10)          | 2 (20)          | > .05 |
| Premolar                        | 4 (40)          | 3 (30)          |   |
| Molar                           | 5 (50)          | 5 (50)          |   |

Data are presented as n (%). Nonsignificant differences were observed for the proportions of patient characteristics between bone sludge and bone chip samples.
bacterial contamination was observed during the culture period for either group.

Cell Growth in Culture Medium

On average, osteoblast outgrowth (bone explants) for bone sludge and bone chips was observed after 23.2 ± 6.1 and 23.6 ± 4.1 days, respectively (Table 2). Donor age showed a statistically significant difference (P < .002) in the mean number of days needed for cell outgrowth: 20 days for group 1 (patients ≤ 60 years old) and 27 days for group 2 (patients > 60 years old). However, no significant difference was observed in the duration of cell outgrowth between maxilla and mandible. All data are presented in Table 2.

Cell Proliferation Assays

On day 20 in a normal medium, total DNA content was significantly higher for the bone chip samples than for the bone sludge samples. No significant difference was observed on days 0, 4, or 7 (Fig 2a). Additionally, total DNA content was significantly higher for bone chip samples than for bone sludge samples on day 7 in the osteogenic medium. No significant difference was observed on days 0, 4, or 20 (Fig 2a). Total DNA content was significantly higher for the patient age group 1 as compared to age group 2 on day 20 in an osteogenic medium; no significant difference could be observed at other time points in regards to donor age (Fig 2b). Concerning the donor site, DNA content in normal medium was significantly higher for mandibular samples than maxillary samples on day 20 (P = .010). However, no significant difference could be observed on days 0, 4, and 7. DNA measurements in osteogenic medium on day 0 were only marginally higher in mandibular samples than in maxillary samples (F = 2.43, P = .079; Fig 2c).

Table 2 Time Interval for Osteoblast Outgrowth in Cell Cultures According to the Study Variables

<table>
<thead>
<tr>
<th>Variables</th>
<th>Duration passage 0–1, d</th>
<th>Duration passage 1–2, d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harvesting technique</td>
<td></td>
<td></td>
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<tr>
<td>Bone sludge</td>
<td>23.2 ± 6.1</td>
<td>8.8 ± 6.0</td>
</tr>
<tr>
<td>Bone chips</td>
<td>23.6 ± 4.1</td>
<td>5.9 ± 1.8</td>
</tr>
<tr>
<td>Donor age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 (≤ 60 y)</td>
<td>20.6 ± 3.0</td>
<td>5.89 ± 3.0</td>
</tr>
<tr>
<td>Group 2 (&gt; 60 y)</td>
<td>27.1 ± 4.2*</td>
<td>8.43 ± 4.9</td>
</tr>
<tr>
<td>Donor site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maxilla</td>
<td>21.9 ± 3.2</td>
<td>6.7 ± 3.3</td>
</tr>
<tr>
<td>Mandible</td>
<td>24.7 ± 5.6</td>
<td>7.2 ± 4.7</td>
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*Statistically significant difference (P < .05).
Data reported here represent the cell outgrowth until passage 2.

Cell Differentiation Assays

Regarding ALP activity in the osteogenic medium, no significant difference was observed between the bone chip samples and bone sludge samples at any time point. In the bone chip samples, ALP activity was significantly higher (P < .05) on day 20 (0.401 ± 0.287) than on day 0 (0.132 ± 0.107) and day 4 (0.139 ± 0.149). Regarding donor age, significantly higher ALP activity was observed in age group 2 (0.595 ± 0.467) than in age group 1 (0.225 ± 0.141) on day 20 in the osteogenic medium.

Calcium Content Measurements

The calcium content of five randomly selected samples was measured as an indication of matrix mineralization (Fig 3). No significant difference could be observed regarding calcium content between
bone chip samples vs bone sludge samples at any time point in the normal medium. However, in the osteogenic medium, bone chip samples had a significantly higher amount of calcium than bone sludge samples on day 20 (P < .05; Fig 3a). Regarding donor age and donor site, no significant difference could be observed between the age groups nor between the maxilla and mandible in both normal and osteogenic mediums (Figs 3b and 3c).

**Alizarin Red and Toluidine Blue Staining**

The microscopic images of samples stained with toluidine blue and Alizarin red at days 7 and 20 are depicted in Fig 4. A larger amount of the red mineralized nodule formation was observed in bone chip samples than in bone sludge samples in the osteogenic medium at day 20. No mineralized red nodules were observed in the normal medium for either type of bone sample.

**Discussion**

In the present study, cell outgrowth was observed in 6 out of 10 samples for the bone sludge group; this is comparable to Gruber et al, who noticed cell growth in 5 out of the 10 bone samples collected using a bone trap. Analysis of the cell culture data in the present study demonstrated an inferior cell activity and osteogenic potential of the bone sludge compared to the bone chips. These results are in line with Liang et al, who confirmed the presence of a significantly higher number of osteoblast-like cells in particulate bone grafts collected via low-speed drilling without saline irrigation than in bone particles collected with bone traps. The inferior osteogenic response of bone harvested from the maxilla and mandible.

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sludge is most likely due to the decomposition and washing off of living cells and various growth factors/biologic proteins caused by saline irrigation during implant osteotomy preparation. Further, particulate bone collected with electrical equipment presents a reduced number of viable cells; the vibrations from drilling equipment can injure the cell membranes of autograft bone cells. Bone sludge was collected at a speed of 800 rpm, and the bone chips were collected using drilling speeds of 100 to 150 rpm. It can be speculated that using a high drilling speed damaged the cell membranes of the cells in the bone sludge.

There is a scarcity of the studies that compare the influence of donor sites and/or donor age on the osteogenic potential of autografts harvested from maxilla or mandible. From a clinical point of view, these factors both hold great interest for periodontics and for oral and maxillofacial surgeons. Most clinicians are acquainted with different intraoral harvesting techniques and often utilize bone sludge and bone chips for minor augmentation procedures in oral implantology. In the present study, significantly higher cell counts and ALP activity were observed for mandibular osteoblast-like cells at day 20 in a normal medium. However, no significant difference was observed at any other time point. The present results are in accordance with Pradel et al, who reported no significant difference in terms of cell count and differentiation markers between the maxilla and mandible; however, they suggested that trabecular maxillary bone chips possessed better potential for growth of osteoblast-like cells in vitro. Kasperk et al suggested that bone cells from different origins might have different intrinsic programs, which could influence the pattern of their proliferation and differentiation, and that osteoblast phenotypes can differ by the origin of the cells. These findings are further confirmed by Kelder et al, who reported that different donor sites present site-specific variations between the cells, and that the cells derived from alveolar bone vary from long bone in their expression of osteogenic markers and osteoclastic activity.

Tissue engineering approaches are becoming a reality and entering clinical practices. The autogenous bone particles collected during implant site preparation could be used as a cell source for tissue engineering. Pradel et al have reported using bone chips and bone sludge collected from the maxilla and
mandible as a cell source to fill dental cystic caries lesions.\textsuperscript{16}

In the present study, a significantly higher cell count was observed among patients aged 60 and younger compared to the patients over age 60. In stem cell therapy, cell dysfunction is seen with natural aging,\textsuperscript{19,20} and donor/patient age is considered a critical factor.\textsuperscript{21} However, no studies have been performed to see the effect of donor age on the behavior of autografts and bone explants from these bone grafts. There are many conflicting works of literature concerning the influence of donor age on cell growth and function of adult mesenchymal stem cells. Some studies have observed no significant age-associated differences in the differentiation of human bone marrow–derived stem cells (BMSCs)\textsuperscript{22–25}; however, several studies have observed a difference in cell proliferation, cell attachment, and cell renewal in mouse,\textsuperscript{26} rat,\textsuperscript{27} and human\textsuperscript{28} BMSCs.

The limitation of the present study is that the evaluation of osteogenic potential was only performed in an in vitro environment. Therefore, additional in vivo studies are warranted to evaluate the role of collected bone particles in new bone formation.

**Conclusions**

The analysis of all collected data revealed that harvesting technique (bone chips vs bone sludge), donor age (≤ 60 years or > 60 years), and donor site (maxilla vs mandible) influenced the osteogenic potential of the collected particulate bone graft. No significant difference was observed between samples harvested from the maxilla and mandible. The bone chips were superior in terms of osteogenic efficacy and should be considered a suitable option for particulate bone graft collection. Animal studies should also be conducted to evaluate the regenerative (osteogenic, osteoinductive, and osteoconductive) ability of these particulate bone grafts.

**Acknowledgments**

This manuscript was supported by a grant from the Oral Reconstruction Foundation in Basel, Switzerland (previously Camlog Foundation; Project CF41206). The authors have no commercial relationship with the funding organization. The authors declare no conflicts of interest.

**References**


