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ORIGINAL ARTICLE

Proliferation and osteogenic differentiation of osteoblast-like cells obtained from two techniques for harvesting intraoral bone grafts

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Abstract
Objectives The aims of our study were to verify the presence of viable osteoblasts in samples of bone tissue obtained by drilling or from cortico-cancellous bone blocks and to assess their growth and differentiation capacities.
Materials and methods Bone tissue samples were processed independently and cultured in Dulbecco's modified Eagle medium, in a CO_2 incubator at 37 °C. The proliferative capacity of osteoblasts was determined by spectrophotometry (MTT) at 24 and 48 h of culture. Cell cycle was analysed by flow cytometry. Cell differentiation was studied by red alizarin staining of nodules formed in mineralisation medium and by analysis of alkaline phosphatase activity.
Results In comparison to bone block-derived osteoblasts, the proliferative capacity was greater at 24 and 48 h of culture ($P<0.001$) in the drilling-derived osteoblasts, which showed significantly increased G2/M ($P=0.014$) and S ($P<0.001$) phases in the cell cycle study. The number of mineralised nodules was proportional to the incubation time, with no differences between the two types of sample, which also did not significantly differ in alkaline phosphatase activity.
Conclusion Superior autograft material is obtained by harvesting particulate bone from low-speed drilling fragments than from a cortico-cancellous bone block.
Clinical relevance These results suggest that bone obtained from low-speed drilling is a simple and effective alternative to the classic procedure for obtaining bone tissue.

Keywords Intraoral bone grafts · Biological drilling · Osteoblast · Proliferation · Differentiation · MTT

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Introduction

The number of dental implants used to replace missing teeth has increased exponentially over recent years [1, 2]. The predictability of implant procedures and the long-term stability of implants are directly related to the quality and quantity of bone tissue available for their placement [3]. Inadequate bone volume is the main limitation of this therapy, compromising implant survival and contraindicating this treatment in a large number of cases [4, 5].

Various procedures are used to overcome height and width deficiencies of the alveolar ridge [3]. Autologous bone remains the gold standard for bone augmentation because of the proteins (e.g. bone morphogenetic proteins), minerals and vital bone cells it contains, unlike allografts or xenografts [5]. Block [6, 7] or particulate [8, 9] autologous bone grafts or a combination of both [10, 11] is used, and multiple extra- and intraoral donor sites have been proposed. Numerous studies have demonstrated minimal resorption and superior revascularisation in bone grafts harvested from intraoral sites of membranous origin than in those from extraoral sites of endochondral origin [12–14]. It has been reported that osteogenic activity and post-insertion revascularisation are lower in block bone grafts than in particulate medullary bone graft [12] and that the internal structure of

cortical bone grafts contains areas that never revascularise and are therefore not replaced by viable bone [13, 14].

A technique using low-speed drilling has been proposed for implant bed preparation in order to avoid the biological changes to bone tissue induced by the heat generated at high drill speeds [15, 16]. Because the procedure does not involve additional irrigation, bone shavings trapped in drills can be harvested and used as particulate grafts for small bone defects in the implant bed [17].

It is important to establish the types of autologous graft that offer optimal predictability. The objective of this study was to compare the proliferative capacity and the capacity to form new bone tissue between osteoblasts harvested from fragments gathered during low-speed drilling and those harvested from a block bone graft.

Materials and methods

Tissues

Twenty bone biopsies were obtained from ten healthy volunteers aged 20–23 years in the course of mandibular surgery (two biopsies per volunteer), all students of the School of Dentistry (University of Granada, Spain) undergoing scheduled surgical extraction of impacted third molar at the school clinic. All participants signed their informed consent to participation in the study, which was approved by the Ethical Committee of the University of Granada (reference no. 598).

Surgical protocol

Two types of samples were obtained during the third molar extraction. For one type, an osteotomy was performed with a round drill and copious irrigation to obtain a small block of cortico-cancellous bone from the mandibular ramus. The second sample was taken from the area adjacent to the extraction site using the low-speed (20–80 rpm) drilling technique of Anitua et al. [17], with no irrigation.

Isolation and culture of osteoblasts

Bone biopsy samples were independently processed. Sections were thoroughly washed four times in phosphate-buffered saline (PBS, pH 7.4) to remove bone marrow and periosteum remains from the bone explants, which were then seeded into culture flasks (Falcon Labware, Oxford, UK) and cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen Gibco Cell Culture Products, Carlsbad, CA, USA) with 100 IU/ml penicillin (Lab Roger SA, Barcelona, Spain), 50 mg/ml gentamicin (Braum Medical SA, Jaen, Spain), 2.5 mg/ml amphotericin B (Sigma, St. Louis, MO, USA), 1 % glutamine (Sigma, St. Louis, MO, USA) and 2 % HEPES (Sigma, St. Louis, MO, USA), supplemented with 20 % foetal bovine serum (FBS, Gibco, Paisley, UK). Cultures were kept at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. Culture media was replaced twice a week. After 1 week, cells grew out from the mineral cortical bone. When the cells reached confluence, at 3 weeks of culture, they were detached from the culture flask with a solution of 0.05 % trypsin (Sigma, St. Louis, MO, USA) and 0.02 % ethylenediamine tetraacetic acid (EDTA, Sigma, St. Louis, MO, USA) and were then washed and suspended in complete culture medium with 20 % FBS. The morphological, biochemical [alkaline phosphatase (ALP) activity and osteocalcin secretion], and antigenic profiles of cells were characterised as described by Reyes-Botella et al. [18] and García-Martínez et al. [19] (see below). All trials were conducted in triplicate for each cell line.

Cell proliferation assay

Osteoblasts were seeded at 1×10^4 cells/ml per well into a 24-well plate (Falcon, Becton Dickinson Labware, NJ, USA) and cultured at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2 for 24 or 48 h in DMEM (Invitrogen Gibco Cell Culture Products, Carlsbad, CA, USA) FBS (Gibco, Paisley, UK). Culture cell proliferation was measured at 24 and 48 h of culture by MTT assay [20]. For this purpose, media were replaced with phenol red-free DMEM containing 0.5 mg/ml MTT (Sigma, Basel, Switzerland) followed by incubation for 4 h. Cellular reduction of the MTT tetrazolium ring resulted in the formation of a dark-purple water-insoluble deposit of formazan crystals. After incubation, the medium was aspirated and dimethyl sulphoxide (Merck Biosciences, Darmstadt, Germany) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with a spectrophotometer (Sunrise™, Tecan, Männedorf, Switzerland). Results were reported as mean absorbance (570 nm)±standard deviation (SD). At least three experiments were conducted for each treatment, using the mean value in the analysis.

Cell cycle assay

Human osteoblasts cultured from the blocks of cortico-cancellous bone or from the drilling-derived particulate bone were separated from the culture flask by treatment with a solution of 0.05 % trypsin (Sigma) and 0.02 % EDTA (Sigma); they were then washed and suspended in PBS and prepared for the cell cycle study using the procedure of Ormerod [21]. A suspension of single cells in 200 ml of PBS was prepared, and 2 ml of ice-cold 70 % ethanol and 30 % distilled H_2O were added and vigorously mixed. Cells

were left for at least 30 min in the cold and then harvested by centrifugation and resuspended in 800 ml PBS. Cells were checked microscopically; if clumped, they were passed through a 25-gauge syringe needle. Cells were then incubated at 37 °C for 30 min with 100 ml RNase (1 mg/ml) and 100 ml propidium iodine. Finally, samples were analysed using an argon-ion laser tuned to 488 nm (Fasc Vantage Becton Dickinson, Palo Alto, CA, USA) measuring forward and orthogonal light scatter and red fluorescence, measuring both the area and ether peak of the fluorescent signal when possible.

Osteoblast differentiation and matrix mineralization—alizarin red staining

Cells from the lines established from the two sample types were seeded (5×10^4 cells/ml/well) in a six-well plate (Falcon, Becton Dickinson Labware) and cultured in complete medium supplemented with 5 mM β-glycerophosphate and 0.05 mM ascorbic acid at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. The medium was replaced after 4 days and then every 3 days. We examined the matrix mineralisation of each cell line after 7, 15 and 22 days of culture. Red alizarin staining was used to visualise the precipitated calcium incorporated into the cellular matrix. Wells were washed with 150 mM sodium chloride, fixed in cold 70 % ethanol for 5 min and rinsed three times with distilled water. Wells were then incubated for 10 min with 1 ml of a 2 % red alizarin solution buffered at pH 4 with sodium hydroxide, then rinsed five times with distilled water and finally washed with PBS to reduce non-specific staining. Precipitate calcium present in the extracellular collagen matrix was coloured red, revealing the mineralisation nodules, which were counted under light microscopy. Images of stained cultures were captured with a digital camera. Results are expressed qualitatively based on the number of red nodules observed per well, considering (−)=no nodules, (+)= 5–20 nodules and (++)=>20 nodules per well.

Alkaline phosphatase activity

Cells from the lines established from the two sample types were seeded (1×10^4 cells/ml/well) in a six-well plate (Falcon, Becton Dickinson Labware) and cultured in complete medium supplemented with 5 mM β-glycerophosphate and 0.05 mM ascorbic acid at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. Cells were grown until day 15, when in vitro testing was performed. ALP activity was quantified by using a colorimetric endpoint assay (Diagnostic kit 104-LL, Sigma, St. Louis, MO, USA) to determine early osteoblastic differentiation. The assay measures the conversion of the colourless substrate *p*-nitrophenyl phosphate by the enzyme ALP to the yellow product *p*-nitrophenol, with the rate of colour change corresponding to the amount of enzyme present in solution. Standards of *p* nitrophenol (0–250 μM) were prepared from dilutions of a 1,000 μM stock solution and assayed in parallel. Cells were harvested by trypsinisation [0.05 % trypsin, 1 mM EDTA, (Invitrogen)] and lysed in 100 μl 1 M Tris pH 8.00 by ultrasonification for 4 min. Then, 10 μl of the suspension was mixed with 100 μl 7.6 mM *p*-nitrophenyl phosphate solution and incubated at 37 °C for 15 min. Substrate solution was prepared by mixing an aqueous solution of 4 mg/ml 4-nitrophenyl phosphate disodium salt (Sigma, St. Louis, MO, USA) with an equal volume of 1.5 M alkaline buffer (Sigma, St. Louis, MO, USA). The reaction was stopped by adding 1 ml 0.05 N NaOH, and the final absorbance was measured at 405 nm with a spectrophotometer (Fasc Vantage Becton Dickinson). All samples were run in triplicate, and specific ALP activity was expressed in U/mg cellular protein.

Statistical analysis

Data were expressed as means±standard deviation (SD) and compared using the Student's *t* test. $P<0.05$ was considered significant in all tests. At least three experiments were performed for each culture. SPSS version 17.0 (SPSS, Chicago, IL, USA) was used for all data analyses.

Results

The morphological and biochemical parameters of the cultured cells were characterised in a preliminary study. They formed a monolayer of spindle-shaped to roughly trapezoidal cells with round or elongated nuclei. ALP activity was present in both lines obtained and in 100 % of analysed cells, confirming the purity of the cultures. Overall, the cell lines under study had a mean concentration of 23.54±0.62 ng osteocalcin/2.5×10^4 cells. The cells showed positivity for CD10, CD13 and CD44 antigens.

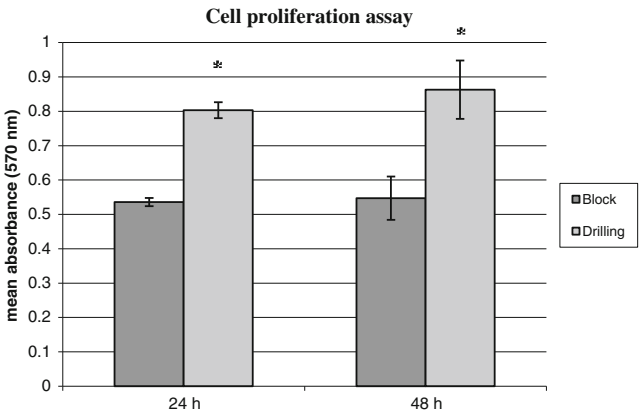

Fig. 1 Measurement of proliferation of cultured human osteoblasts by MTT assay at 24 and 48 h after culture establishment. Data are reported as mean absorbance (570 nm)±standard deviation (SD). *$P<0.001$, osteoblasts isolated from drilling vs. osteoblasts isolated from bone blocks

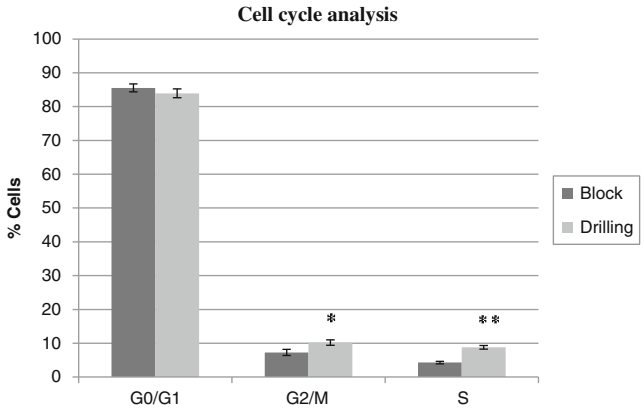

Fig. 2 Flow cytometry results showing the percentage of cells in G0/G1, G2/M and S phases. Data are reported as means±standard deviation (SD). *$P<0.05$, **$P<0.001$, osteoblasts isolated from drilling vs. osteoblasts isolated from bone blocks

Cell proliferation assay

Figure 1 depicts the MTT proliferation for each osteoblast cell line at 24 and 48 h after culture. At both time points, the mean spectrophotometrically determined absorbance was significantly greater ($P<0.001$) in the line derived from samples obtained by drilling than in the line isolated from the blocks of cortico-cancellous bone. The absorbance was directly proportional to the number of viable cells and, therefore, to the cellular proliferation.

Cell cycle assay

In the cell cycle study, no significant difference in stage G0/G1 was observed between sample types, but significantly increased G2/M ($P=0.014$) and S ($P<0.001$) stages were found in cells derived from drilled bone samples in comparison to those isolated from cortico-cancellous blocks (Fig. 2). The cell cycle profile was normal in all cases, and no DNA aneuploid cells or signs of neo-plastic transformation were observed (Fig. 3a, b).

Osteoblast differentiation and matrix mineralisation—alizarin red staining

Table 1 exhibits data on the number of red alizarin-stained mineralisation nodules counted under light microscopy after 7, 15 and 22 days of culture in mineralisation medium. At 7 days of culture, no nodules were observed in any well (Fig. 4, A, A′); at 15 days of culture, small nodules started to appear in both cell lines (Fig. 4, B, B′); and at 22 days of culture, the number and size of nodules had increased in both cell lines (Fig. 4, C, C′); no difference were observed between the cell lines at any time point.

Alkaline phosphatase activity

ALP activity was present in both lines obtained and in 100 % of analysed cells, confirming the purity of the cultures. Mean ALP activity did not differ between cells derived from bone blocks (2.43±0.15 U) and those in cells obtained via the drilling technique (2.37±0.11 U; Fig. 5).

Discussion

In this study of two methods of bone harvesting for grafts, the cells survived the procedures and proved able to grow

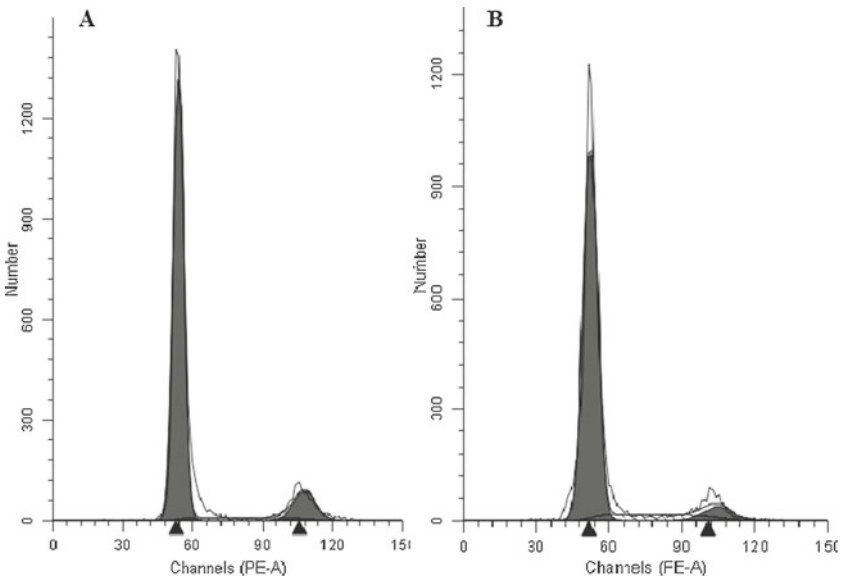

Fig. 3 Fluorescence profile of cell cycle of cultured human osteoblasts obtained by flow cytometry. **a** Cells from cortico-cancellous blocks. **b** Cells from bone samples obtained by drilling

Table 1 Number and size of mineralisation nodules after 7, 15 and 22 days of culture, in both cell lines

	7 days		15 days		22 days	
	Number of nodules[c]	Size of nodules	Number of nodules	Size of nodules	Number of nodules	Size of nodules
Drilling[a]	–	–	+	40–80 μm	++	130–350 μm
Block[b]	–	–	+	50–90 μm	++	133–360 μm

[a] Osteoblast line isolated from drilling

[b] Osteoblast line isolated from bone blocks

[c] (−) absence of nodules, (+) 5–20 nodules per well, (++) more than 20 nodules per well

out from bone grafts into tissue culture dishes. It was previously reported in clinical studies that bone obtained by drilling the implant bed is useful as a graft in small bone defects [22, 23]. In vitro studies were also able to isolate viable osteoblasts from drill-derived bone samples for grafting in human and porcine bone [24, 25].

In MTT proliferation assays, the osteoblasts isolated from drilled bone samples showed a greater proliferative capacity

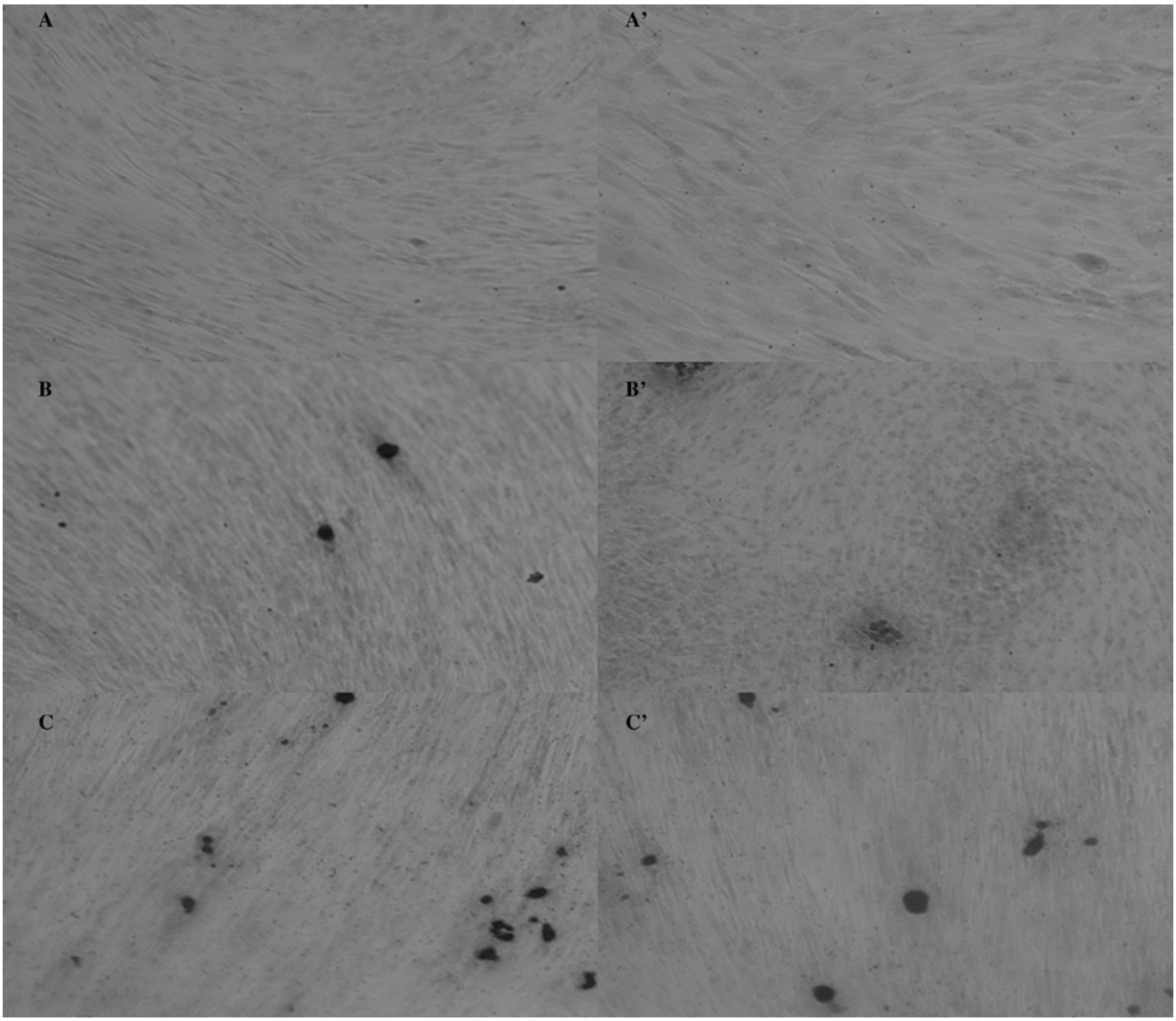

Fig. 4 Calcium deposits evidenced with alizarin red staining in osteoblasts cultured in mineralisation medium at 7 (*A*, *A′*), 15 (*B*, *B′*) and 22 (*C*, *C′*) days of culture. *A–C* Osteoblasts from bone blocks; *A′–C′* osteoblasts from low-speed drilling (scale, 100 μm)

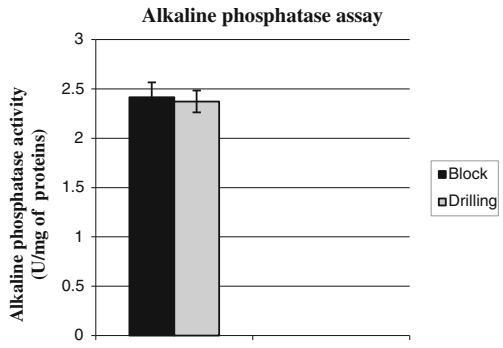

Fig. 5 Alkaline phosphatase activity of osteoblasts isolated from bone blocks (block) and osteoblasts isolated from drilling (drilling). The activity was determined in cell lysates and normalised to total cellular protein. Data are reported as means±standard deviation (SD). There were no statistically significant differences between groups

compared to osteoblasts isolated from blocks of cortico-cancellous bone, both at 24 and 48 h of culture. This finding may be explained by the energy transmitted by the drill to the bone of the implant bed, given that in vitro stimulation with various types of low-intensity energy (e.g. laser or even shock wave) have been found to exert a biostimulatory effect on osteoblasts, increasing their proliferation and viability [26, 27]. Thus, low-energy Nd:YAG laser treatment increased the viability and proliferation of SaOS-2 osteoblasts [28]. Low-energy laser therapy, which has no thermal effect, has become widely used in implant and periodontal treatments and is reported to favour tissue healing and regeneration through the stimulation of cells of different origins [29]. Other authors have reported the stimulatory effects of laser phototherapy on the growth and differentiation of human osteoblasts in vitro [30, 31]. Histological studies in animals showed that laser phototherapy can promote and increase collagen fibre deposition by osteoblasts, which can accelerate the bone repair process [32–34]. However, other authors found that laser therapy had no significant influence on the proliferation and viability of osteoblasts [35].

Shock waves have also shown osteogenic effects at low energy levels, including stimulation of the growth of bone marrow stromal cells, differentiation towards osteoprogenitors and an increase in the proliferation and activity of osteoblasts. This activity is associated with the production of TGFβ1, which plays an important role in regulation and stimulating the differentiation of osteoprogenitor cells during fracture repairs [36–38]. However, high-intensity energy can generate the production of heat-induced free radicals and disturb the homeostasis of cellular calcium, resulting in cell and tissue damage [39, 40]. High-energy shock wave treatment produced aseptic necrosis and damaged osteocytes in rat bone marrow [41], whereas lower energy treatment enhanced cell growth without producing cell damage [26], as found in the present study with a low-speed drilling technique.

In an in vitro study, Gruber et al. [24] found no statistically significant differences in proliferative capacity between drilling-derived porcine osteoblasts harvested during implant bed preparation and cortical bone cylinders obtained using a trephine. This contrasts with our observation of higher proliferative capacity in drilled samples than in blocks of cortico-cancellous bone. However, in common with our study, they found no significant difference between the two types of bone sample in the differentiation capacity of the osteoblasts as measured by mineralisation study or ALP activity analysis. When cultured in mineralisation medium, both cell lines in our study produced calcium salt deposits in the form of nodules that increased in size and number over time. Our observation of no significant differences in the size or number of the nodules between the cell lines indicates that there was no difference in cell differentiation as a function of the harvesting technique. Similar findings were obtained when the cells were cultivated in a medium that favoured differentiation and ALP activity analysis was performed. Our results are in agreement with a previous report that the application of low-intensity energy (via laser) to osteoblast cultures had no significant effect on their differentiation [42]. However, this issue is controversial because another study found that irradiation with low-intensity laser energy significantly increased the formation of mineralised nodules and number of cells after 21 days of culture [43]. Our cell cycle study results were consistent with the MTT assay finding of greater proliferation in the cell line from drilled fragments vs. cortico-cancellous blocks. In comparison to the latter, the fluorescence profile of the drilling-derived osteoblasts revealed a significantly higher proportion of cells in the G2-M phase and especially in the S phase, which indicates that the cells are synthesising the genetic material required for cell division [21].

In conclusion, the harvesting of bone from the low-speed drilling of the implant bed is a widely utilised technique. It may be the method of choice for filling small bone defects, fenestrations and dehiscences associated with implantation. There is no need for a second surgical field, and it is simpler and faster in comparison to the classic technique of harvesting block bone grafts. The utilisation of osteoblasts obtained by this method is supported by our findings that they have a greater proliferative capacity and that their differentiation capacity is not impaired. Hence, this particulate bone may also be useful in combination with other types of bone graft (allografts or xenografts) for filling larger defects.

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Conflicts of interest statement The authors declare that they have no conflict of interest.

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