



Original Article

Effect of dexamethasone on the growth and differentiation of osteoblast-like cells derived from the human alveolar bone

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والمخلص

أهداف الدراسة: هدفت هذه الدراسة إلى معرفة تأثير الديكساميثازون على نمو وتمايز الخلايا الشبيهة ببيانات العظم المشتقة من العظم السنخي البشري.

طرق البحث: تم جمع جزيئات العظام من المرضى أثناء تحضير موقع الزرع. زرعت العينات في وسط نمو، وزرعت الخلايا التي تكاثرت بعد أسبوعين إلى ثلاثة أسابيع في ثلاثة أنواع من وسط الاستنبات - المجموعة 1، وسط طبيعي؛ المجموعة 2، وسط منشأ للعظم بدون ديكساميثازون؛ والمجموعة 3، وسط منشأ للعظم مع ديكساميثازون - لمدد هي صفر، وأربعة وعشرين يوماً، وسبعة وعشرين يوماً. تم إجراء قياسات الحمض النووي والفسفاتاز القلوي وتطبيخ الإيزارين الأحمر/التولويدين الأزرق. تم تحليل البيانات باستخدام تحليل التباين مع اختبار بنفروني.

النتائج: كان قياس الحمض النووي أعلى بشكل ملحوظ للمجموعة 2 مقارنة بالمجموعة 1 في اليوم 7 والمجموعة 3 في الأيام 4 و 7 و 21. كان إجمالي قياس الفسفاتاز القلوي أعلى بكثير لعينات المجموعة 3 مقارنة مع عينات المجموعة 1 والمجموعة 2 في اليوم 21. لوحظ وجود قدر أكبر من تمعدن المصفوفة للمجموعة 3 مقارنة بالمجموعتين 1 و 2.

الاستنتاجات: أظهرت الخلايا العظمية السنخية البشرية فعالية عظيمة محسنة من حيث التمايز العظمي عند زراعتها في وجود ديكساميثازون. انخفض عدد الخلايا (إجمالي محتوى الحمض النووي) في وجود الديكساميثازون؛ ومع ذلك، لوحظ زيادة التمايز بين الخلايا الشبيهة ببيانات العظم.

الكلمات المفتاحية: نمو الخلايا؛ تمايز الخلايا؛ تكاثر الخلايا؛ ديكساميثازون؛ هندسة الأنسجة

Abstract

Objectives: This study aimed to investigate the effect of dexamethasone on the growth and differentiation of osteoblast-like cells derived from the human alveolar bone.

Methods: Bone particles were collected from patients during implant-site preparation. The samples were cultured in a growth medium, and the cells that propagated after two–three weeks were cultured in three types of culture media: group 1, normal medium; group 2, osteogenic medium without dexamethasone; and group 3, osteogenic medium with dexamethasone—for zero, four, seven, and 20 days. DNA and alkaline phosphatase (ALP) measurements and alizarin red/toluidine blue staining were performed.

Results: DNA levels were significantly higher in group 2 than in group 1 on day 7 ($p < 0.001$) and in group 3 on days 4, 7, and 20 ($p < 0.041$, $p < 0.006$, and $p < 0.001$, respectively). Further, total ALP levels were significantly higher in group 3 than in groups 1 on day 20 ($p < 0.023$). A greater amount of matrix mineralisation was observed in group 3 than in groups 1 and 2.

Conclusions: Human alveolar bone cells exhibit improved osteogenic efficacy in terms of osteogenic differentiation when cultured in the presence of dexamethasone. The cell number (total DNA content) decreased in the presence of dexamethasone; however, an increased differentiation of osteoblast-like cells was observed.

Keywords: Cell growth; Cell differentiation; Cell proliferation; Dexamethasone; Tissue engineering

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Introduction

Bone tissue engineering (BTE) mainly relies on the combination of osteogenic cells with an appropriate biomaterial scaffold and provides a source of cellularised bone substitutes that hold the potential to improve bone regeneration.^{1,2} A suitable cell source(s) for BTE is autologous cells, as compared to allogeneic cells, based on immunological compatibility and safety against the potential threats of pathogen transmission.³ Cells of different origins, such as bone marrow mesenchymal stem cells^{4–6} as well as periosteum^{7–10} and alveolar bone-derived osteoblast-like cells,^{11,12} have been studied for their potential clinical application in the fields of periodontology and oral reconstruction. The major advantage of alveolar bone cells compared with other cell sources is the availability of bone tissue without patient morbidity and a second surgical site.¹² The methods commonly employed for alveolar bone collections are the use of a bone trap, a bone scraper, or bone particles attached to the drill flukes during implant site preparations.^{13–15} However, by employing these strategies, only a small amount of bone could be collected, which could only be used either for minor grafting procedures or when mixed with other osteoconductive biomaterials.¹²

BTE requires a large number of cells, which might be difficult to obtain by utilising primary cells because the total number of cells gained from the primary cell culture is limited.¹⁶ An adequate number of cells can be obtained by cell population expansion via the sequential passaging of cell cultures.¹⁶ To obtain cells with well-defined and reproducible osteoblast-like features, appropriate experimental procedures should be adopted.¹⁶ Various factors could affect the expression of the osteoblast-like cell phenotype in cell cultures (i.e. the cell culture medium, cell source, duration of culture, and composition of the cell culture medium, which might affect the proliferation and differentiation of cells).¹⁷ Different hormones, cytokines, and growth factors have been reported to influence the osteoblast differentiation process.¹⁸ To prevent the dedifferentiation of osteoblasts, cell culture media are often supplemented with various compounds such as dexamethasone,^{16,19,20} ascorbic acid,^{20–22} and β -glycerophosphate.^{20–22}

Dexamethasone (a synthetic glucocorticoid) has often been utilised to differentiate mesenchymal stem cells into osteogenic lineages.¹⁶ Particularly, dexamethasone decreases cell proliferation but increases ALP activity and collagen

synthesis in osteoblast-like cell cultures, which is indicative of cell differentiation.¹⁶ The effects of dexamethasone on collagen synthesis are debatable because both inhibition and stimulation of collagen expression have been observed depending on cell culture conditions and different cell sources.^{23,24} In addition, the effect of dexamethasone on the growth/proliferation and differentiation of osteoblast-like cells derived from human alveolar bone is not well documented in the literature. Therefore, this study aimed to evaluate the effects of dexamethasone on the growth and differentiation capacity of alveolar osteoblast-like cells. The null hypothesis of this study was that dexamethasone has no effect on the proliferation and differentiation of osteoblast-like cells derived from human alveolar bone.

Materials and Methods

Bone tissue harvesting

Bone samples were collected from the maxilla or mandible of five patients during implant site preparation. Alveolar bone chips attached to the drill flukes were collected. The bone that was collected would have otherwise been redundant. Informed consent was obtained from all patients. Bone samples were collected in sterile glass bottles with phosphate-buffered saline. The samples were sent to the laboratory for cell culture analysis.

Scanning electron microscopy

All samples were fixed in 2% glutaraldehyde for 5 min. Next, they were rinsed with 0.1 M of a Na-cacodylate buffer. Subsequently, they were dehydrated in a series of ethanol solutions before drying with tetramethylsilane. All the samples were kept in a dry atmosphere until scanning electron microscopy (SEM) was performed. The samples were attached to an aluminium base and a layer of gold was sputtered at the time of examination.¹²

Cell isolation and cell culture procedure

All samples for cell culture were rinsed twice with Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) and placed in tissue culture flasks (T25; Corning, NY, USA) in a normal culture medium containing DMEM, together with streptomycin, fungizone (Sigma–Aldrich, St. Louis, MO, USA), heparin (10 IU/mL; LEO Pharma A/S, Ballerup, Denmark), 1% penicillin, and 5% human platelet lysate (Sanquin, Amsterdam, the Netherlands). The cells were incubated at 37 °C in an atmosphere of 5% CO₂ and 95% air. The culture medium was replaced twice a week. After two–three weeks, the cells that propagated from the collected bone particles, called bone explants, were released using 0.25% trypsin (Invitrogen, Carlsbad, CA, USA) and 0.1%

ethylenediaminetetraacetic acid (Merck, Darmstadt, Germany) in phosphate-buffered saline and counted. The bone explants ($n = 15$, three samples from each patient) from passage 2 were counted, and the cells at a concentration of 20,000 cells/well/400 μL were placed in cell culture plates (24-well). The cells were cultured for four, seven, and 20 days after overnight attachment in three culture medium types¹²:

Group 1, normal medium; **Group 2**, osteogenic culture medium without dexamethasone; **Group 3**, osteogenic culture medium with 10^{-8} M dexamethasone. Osteogenic culture medium was supplemented with ascorbic acid (50 $\mu\text{g}/\text{mL}$) and sodium β -glycerophosphate (10 mM).

The day the cells (bone explants) were cultured was considered day 0. At each time point (days 4, 7, and 20) after removal of the culture medium, the cells were rinsed with phosphate-buffered saline. The cells were immersed in MilliQ water (300 μL ; Merck KGaA, Darmstadt, Germany) and frozen at -20°C until further evaluation. The cells were fixed in 4% formaldehyde for alizarin red/toluidine blue staining.¹² a schematic representation of the cell culture protocol is depicted in Figure 1.

Measurements of cell proliferation and differentiation

DNA measurements, that is, representative of cell proliferation or cell number, were evaluated using the CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Leiden, the Netherlands). Next, 485 nm excitation and 528 nm emission wavelengths in a microplate reader were used to read the absorbance (Synergy HT; BioTek®, Germany). The DNA content was analysed using a standard curve. Alkaline phosphatase (ALP) activity was estimated using the method reported by Bessey et al.²³ For the ALP assay, 100 mL of a 5 mM paranitrophenylphosphate solution, 20 mL of an alkaline buffer (Sigma), and 80 mL of the sample were added. Serial dilutions of 4-nitrophenol (0–25 nmol) were prepared for the standard curve. The plates were then incubated at 37°C for 60 min. The reaction was stopped by adding 0.3 M NaOH. An ELISA plate reader (BioTek, Santa Clara, CA, USA) was used to read the sample plates. Subsequently, the measurements were normalised to the protein concentrations.

Alizarin red and toluidine blue staining

First, a 2% alizarin red S (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany; C.I.58005) mono sodium salt solution was utilized alone or was used together with 0.2% toluidine blue (Sigma–Aldrich-Chemie GmbH, Taufkirchen, Germany, C.I.52040) in MilliQ water. Alizarin red and toluidine blue staining were conducted as a sign of cell differentiation. The mineralised nodules that contained calcium were stained red by Alizarin red staining. Toluidine blue staining was used after alizarin red staining. Toluidine blue stained the nucleic acids of the cells. This increased the acuity of the images and improved their distinguishability.

Statistical analysis

SPSS 20.0 (IBM product, Chicago, USA) was used for data analysis. The numerical data, including the DNA and ALP measurements taken on days 4, 7, and 20 in the normal and osteogenic media with and without dexamethasone, are presented as mean \pm standard deviation. The DNA and ALP levels between normal and osteogenic media were compared using the Wilcoxon Mann–Whitney U-test. The DNA and ALP measurements at various time intervals within each medium were compared using the Wilcoxon signed-rank test. The confidence level to assess associations was set at 95%.

Results

Scanning electron microscopy

An SEM image of the collected bone particles is shown in Figure 2B (magnification 1000 \times ; Figure 2B). SEM revealed intact collagen fibrils on the outer surface of autogenous bone particles. Conavities (represented by *), micropores (see the arrow), and irregular topography were observed. The image shows a dense microstructure, which might be due to the presence of organic components attached to inorganic minerals.

Explant cell morphology

After two–three weeks, cells propagated from the bone grafts, called bone explants, as shown in Figure 2B. The cells exhibited an elongated fibroblast-like morphology.

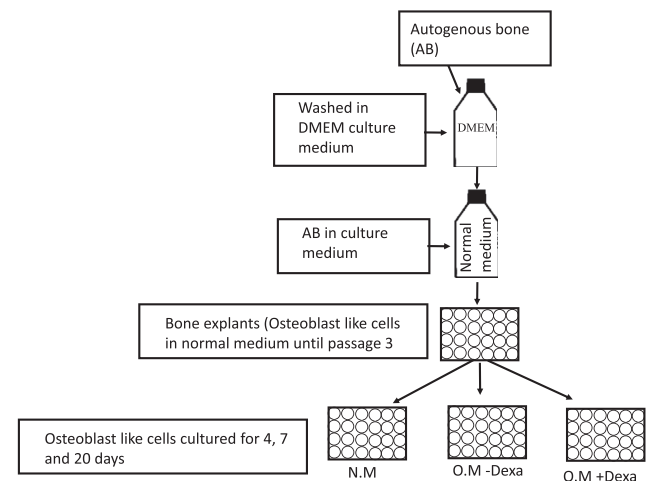


Figure 1: Schematic presentation of cell culture protocol. The bone particles were placed into a T25 flask. After two to three weeks, the cells that grew out from the bone grafts were placed in 24-well plates. The cells were then cultured in three culture medium types: 1: N.M (normal medium); 2: O.M – Dexa (osteogenic culture medium without dexamethasone); 3: O.M + Dexa (osteogenic culture medium with dexamethasone).

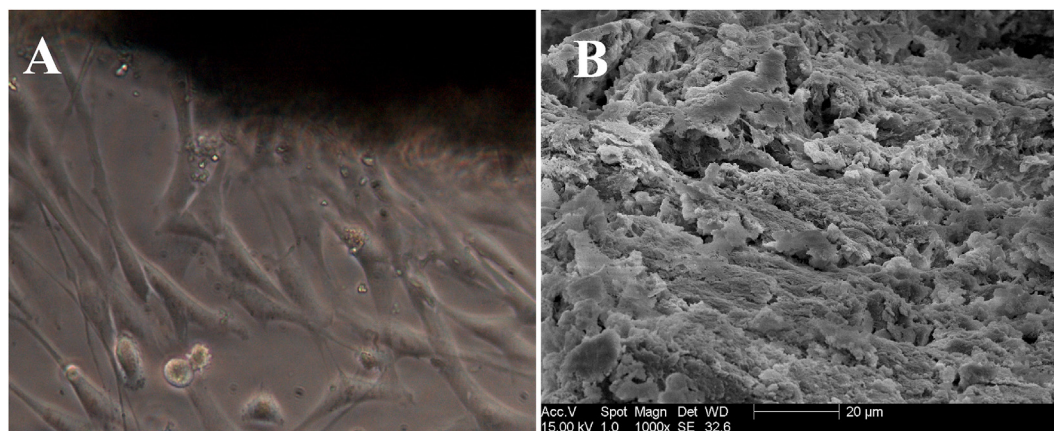


Figure 2: **A:** Light microscope image of osteoblast-like cell outgrowth from the autogenous bone. **B:** Scanning electron microscope image of harvested autogenous bone (Magnifications: 1000 \times , bar = 20 μ m).

Table 1: Comparison of DNA of normal medium with osteogenic medium with and without dexamethasone.

Duration	Medium (mean \pm SD)			Sig. (<i>p</i> -value)		
	Normal (N)	OST + Dexa (M1)	OST – Dexa (M2)	N vs. M1	N vs. M2	M1 vs. M2
Day 4	2423.0 \pm 736.3	1507.1 \pm 954.3	2308.0 \pm 823.9	0.023	0.999	0.041
Day 7	3039.5 \pm 862.4 ^a	1980.1 \pm 1344.7 ^a	4132.5 \pm 1290.8 ^a	0.233	0.001	0.006
Day 20	3968.9 \pm 839.5 ^{a,b}	1830.6 \pm 1198.5	3980.3 \pm 2141.5 ^a	0.001	0.733	0.001

A significant difference of mean DNA normal medium.

^a Significant difference at the 5% level of significance compared with day 4.

^b Significant difference at the 5% level of significance compared with day 7.

Cell proliferation assays

DNA levels were significantly higher in Group 2 than in Group 3 on days 4, 7, and 20 (Table 1). It was also significantly higher for the Group 1 samples than for the Group 3 samples on days 4 and 7; no significant difference was observed on day 20 (Table 1). Additionally, it was significantly higher in Group 2 than in Group 1 on day 7; no statistically significant difference was observed on days 4 and 20 (Table 1).

Regarding the proliferation rate within the groups, for Group 1, a statistically significant difference was observed on days 7 and 20 compared with day 4 ($p = 0.001$), as well as on

day 7 compared with day 20 ($p = 0.003$). A significantly higher amount of DNA was present on days 7 ($p = 0.001$) and 20 ($p = 0.027$) than on day 4 for Group 2 samples. However, in the case of Group 3 samples, a significant difference was detected only between days 4 and 7 ($p = 0.015$). No statistically significant differences were noted at the other time points.

Cell differentiation assays

The total ALP measurement was significantly higher for Group 3 samples than for Group 1 samples on day 20. No statistically significant differences were observed

Table 2: Comparison of ALP of normal medium with osteogenic medium with and without dexamethasone.

Duration	Medium (mean \pm SD)			Sig. (<i>p</i> -value)		
	Normal (N)	OST + Dexa (M1)	OST – Dexa (M2)	N vs. M1	N vs. M2	M1 vs. M2
Day 4	0.176 \pm 0.254	0.153 \pm 0.112	0.252 \pm 0.414	0.363	0.084	0.307
Day 7	0.273 \pm 0.386 ^a	0.471 \pm 0.411 ^a	0.312 \pm 0.381 ^a	0.156	0.011	0.307
Day 20 ^b	0.203 \pm 0.181	0.386 \pm 0.302 ^a	0.288 \pm 0.265	0.023	0.099	0.053

A significant difference of mean DNA normal medium.

^a Significant difference at the 5% level of significance compared with day 4.

^b Significant difference at the 5% level of significance compared with day 7.

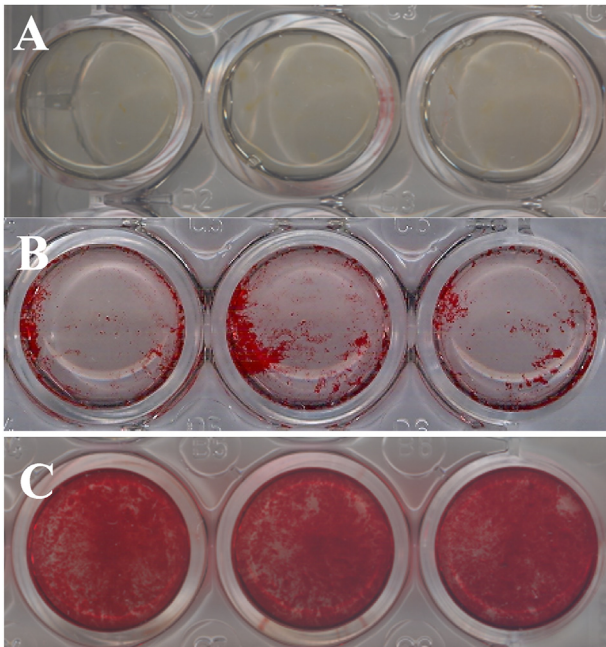


Figure 3: Alizarin red staining results on Day 20: No mineralisation in the normal medium (A) and negligible mineralization in the osteogenic medium without dexamethasone (B). A higher amount of red mineralised matrix could be observed in the osteogenic medium with dexamethasone (C).

at any other time points (Table 2). It was also significantly higher in Group 2 than in Group 1 on day 7. No significant differences were observed at any other time points (Table 2). The total ALP content was higher in Group 3 than in Group 2 on day 20. No statistically significant difference was observed in ALP activity between Groups 2 and 3 (Table 2).

Regarding ALP activity within the groups, for the Group 1 and Group 3 samples, significantly higher ALP content was present on day 7 than on day 4 ($p = 0.001$ and $p = 0.016$, respectively). No significant differences were observed at other time points (Table 2). However, in Group 2, a significantly higher mean ALP level was observed on days 7 and 20 than on day 4 ($p = 0.002$ and $p = 0.001$, respectively).

Alizarin red and toluidine blue staining

Microscopic images of alizarin red staining for all three groups on day 20 are shown in Figures 3 and 4. Visual examination of the cell cultures showed no mineralisation of osteoblast-like cells in the normal medium. Almost negligible mineralisation was observed on day 20 for osteoblast-like cells in osteogenic medium without dexamethasone (Figures 3B and 4B). A greater quantity of red mineralised nodules was observed in samples treated with dexamethasone (Figures 3C and 4C).

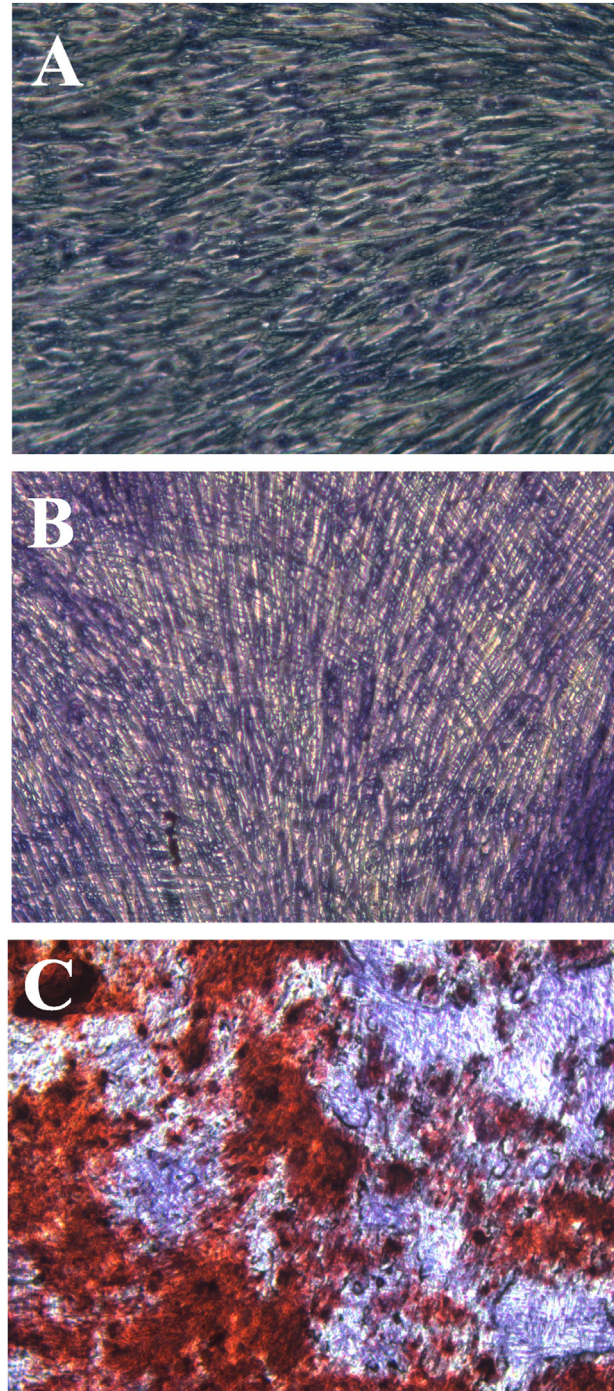


Figure 4: Alizarin red and toluidine blue staining of bone explants on Day 20 (magnification 40 \times): No mineralisation in normal medium (A) and negligible mineralization in the osteogenic medium without dexamethasone (B). A higher amount of red mineralised nodule formation was seen in the samples with dexamethasone (C).

Discussion

This study aimed to examine the effect of dexamethasone on human alveolar bone-derived osteoblast-like

cell differentiation. The null hypothesis that dexamethasone has no effect on the proliferation and differentiation of osteoblast-like cells derived from the human alveolar bone was rejected. In this study, we observed that dexamethasone in the osteogenic medium reduced the proliferation and increased the differentiation of osteoblast-like cells. In addition, the findings of this study confirmed that cells isolated from human alveolar bone are a good source of cells that can differentiate into the osteoblast lineage.

A series of events, such as cell proliferation, the expression of osteoblast markers, the synthesis and maturation of an extracellular matrix, and matrix mineralisation, have been reported for the development of an osteoblast phenotype from osteoprogenitor cells.²⁵ In our study, ALP biochemical assays were conducted to measure osteoblast differentiation. ALP is a well-known biochemical marker of osteoblasts because the ALP enzymes are released during osteoblast differentiation.¹² Our results showed that the total ALP content was higher for Group 3 (osteogenic culture medium with dexamethasone) compared with Groups 1 and 2 (normal medium and osteogenic culture medium without dexamethasone, respectively) on Day 20. Our results are in accordance with those of previous studies that demonstrated that when human bone marrow stem cells were treated with dexamethasone, either uninterruptedly or after the first cell passage, they differentiated into the osteoblast phenotype.²⁶ This was confirmed by the expression of biological markers that are distinctive for the osteoblast phenotype, such as ALP measurement and matrix mineralisation.²⁶

In this study, the total cell number (DNA measurement) was significantly higher in Groups 2 and 1 than in Group 3. Our results indicated low proliferation and high differentiation capacity of human alveolar bone cells in the presence of dexamethasone. Bella et al. conducted a study investigating the role of dexamethasone in the osteogenic differentiation of human mesenchymal stromal cells. Their study data illustrated that dexamethasone stimulates osteogenic differentiation by constraining the expression of SOX9.²⁷ Our results are in accordance with those of Beloti and Rosa, who found that cell numbers decreased in the presence of dexamethasone.²⁶ However, the increased differentiation of bone marrow stem cells into mature osteoblasts was also observed. Owen et al. demonstrated a reciprocal correlation between the induction of cell differentiation and a decline in cell proliferation in cell cultures of rat calvarial cells.²⁸ In addition, Kira et al. reported that the osteogenic differentiation ability of bone marrow mesenchymal stromal/stem cells was enhanced by the addition of ascorbic acid phosphate and dexamethasone. Higher ALP activity and osteocalcin levels were observed in the presence of dexamethasone.²⁹ However, our results contrast those of Atmani et al., who utilised rat BMSCs and showed that the presence of dexamethasone increased cell proliferation as well as differentiation.³⁰ Should this be 'the cause of these differences is not known'? Because these variable results indicate that cells derived from different sources or origins exhibit different behaviours or diversity in the isolation process and cell culture settings, such as the type of culture medium, foetal serum, or the concentration of dexamethasone.²⁶ The methods utilised for the evaluation of

the cell number (cell proliferation) might have also influenced the final results. While Atmani et al. performed an indirect method (i.e. 6-h [methyl-3H] thymidine incorporation), in this study, the CyQUANT cell proliferation assay kit (Molecular Probes, Leiden, Netherlands) was used.³⁰

In this study, alizarin red and toluidine blue staining revealed a lack of mineralised matrix formation in Groups 1 and 2. Our results are in line with those of Beloti and Rosa and Ter Brugge and Jansen, who also found no matrix mineralisation in rat BMSCs in the absence of dexamethasone.^{16,26} Rat BMSCs were differentiated into the osteogenic lineage only in the presence of dexamethasone.¹⁶ Gasson et al. evaluated the effect of BMP-2 and dexamethasone on the osteogenesis of autogenous and induced pluripotent stem cells derived from canine mesenchymal cells. Their results also confirmed that dexamethasone and BMP-2 could improve the osteogenic capacity of canine mesenchymal stromal cells.³¹

Bone samples collected from alveolar bone can be utilised for the construction of cell-based bone scaffolds.^{17,32} The primary advantage of this approach is minimal patient morbidity and reduced treatment costs for the patient, as no additional surgical procedures are required for bone tissue harvesting.^{12,32} Several studies have been published wherein different cell sources, such as alveolar bone collected during routine periodontal surgeries and periosteal cells,^{8,9,33,34} were utilised as sources of osteogenic cells for tissue-engineered bone substitute preparations. In addition, the clinical application of tissue-engineered bone substitutes derived from alveolar bone cells has been reported.^{33,35–37} Pradel et al. utilised second-passage alveolar osteoblast-like cells harvested from the mandible and maxilla to construct collagen scaffolds, which were used for bone regeneration after mandibular cyst enucleation and for osteoplasty of the alveolar cleft defect.^{35,36} The clinical relevance of this study arises from the fact that the harvested autologous bone during implant site preparation might be particularly advantageous for minor bone augmentation procedures in dental implant cases that require such augmentation. In addition, the outgrown cells obtained from harvested bone via cell-culturing techniques can be utilised as tissue-engineered bone substitutes.

A limitation of this study was that the evaluation of the effect of dexamethasone was only performed on passage 2 of the cell culture. Therefore, additional *in vitro* studies should be conducted to evaluate the role of DEX in cell subcultures. There is a wide variation in cell culture protocols, including cell isolation procedures, cell passages, and bone substitutes used in tissue engineering approaches. Therefore, it is difficult to draw general conclusions. Further research is required to compare the efficiency of different cell sources from different origins for the preparation of tissue-engineered bone substitutes.

Conclusions

Based on our results, we conclude that human alveolar bone-derived osteoblast-like cells exhibit improved osteogenic efficacy in terms of higher ALP activity and greater matrix mineralisation when cultured in the presence of dexamethasone. The cell number (total DNA content) decreased in the presence of dexamethasone; however, an

increased differentiation of osteoblast-like cells was observed. Therefore, the addition of dexamethasone to osteogenic medium is recommended for osteoblast-like cells derived from the human alveolar bone.

Source of funding

This research received a grant from funding agencies, that is, the Oral Reconstruction (OR) Foundation (Camlog Foundation; Project CF41206) in not-for-profit sectors.

Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

Medical ethics committee approval (ethical approval no. 2013/151) was obtained from Vrije University Amsterdam, and the study protocol was in accordance with the Declaration of Helsinki: October 2013.

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