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Autogenous bone chips: influence of a new piezoelectric device (Piezosurgery®) on chip morphology, cell viability and differentiation

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Abstract

Aim: The aim of the present study was to investigate the influence of a new piezoelectric device, designed for harvesting autogenous bone chips from intra-oral sites, on chip morphology, cell viability and differentiation.

Methods: A total of 69 samples of cortical bone chips were randomly gained by either (1) a piezoelectric device (PS), or (2) conventional rotating drills (RD). Shape and size of the bone chips were compared by means of morphometrical analysis. Outgrowing osteoblasts were identified by means of alkaline phosphatase activity (AP), immunhistochemical staining for osteocalcin (OC) synthesis and reverse transcriptase-polymerase chain reaction phenotyping.

Results: In 88.9% of the RD and 87.9% of the PS specimens, an outgrowth of adherent cells nearby the bone chips was observed after 6–19 days. Confluence of cells was reached after 4 weeks. Positive staining for AP and OC identified the cells as osteoblasts. The morphometrical analysis revealed a statistically significant more voluminous size of the particles collected with PS than RD.

Conclusion: Within the limits of the present study, it may be concluded that both the harvesting methods are not different from each other concerning their detrimental effect on viability and differentiation of cells growing out of autogenous bone chips derived from intra-oral cortical sites.

Key words: autogenous bone chips; differentiation; osteoblasts; Piezosurgery®; viability

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Autogenous bone grafting is a widely used augmentation procedure utilized within the frame of rehabilitation therapy. The mostly documented clinical applications refer to sinus floor elevations, periodontal surgery, implant surgery as well as horizontal and vertical alveolar ridge augmentations (Neukam 1990, Savant et al. 2001, Donos et al. 2002, Hallman et al. 2002, Blay et al. 2003, Capelli 2003, Clavero & Lundgren 2003, Schlegel et al. 2003). Nowadays, autogenous bone has been defined as a gold standard grafting material because of its osteoconductive, osteoinductive and osteogenetic properties (Nkenke et al. 2002, Schlegel et al. 2003, Springer et al. 2004). Furthermore, a major advantage of autogenous bone, especially in comparison with other augmentation materials such as xenogenic grafts, is the lack of possible immunological reactions (Schlegel et al. 2003). Autogenous bone chips can be obtained either by particulating previously harvested bone blocks from intra- or extra-oral sites with a bone mill, or by using a bone collector device during osteotomy procedures (Tinti et al. 1996, Chiapasco et al. 1999, Erpenstein et al. 2001, Young et al. 2002). Recent in vitro studies investigated the vitality of bone cells obtained in different reaming designs (Garcia et al. 2004, Hoegel et al. 2004, Springer et al. 2004). It was observed by a quanti-

tative and qualitative assay of the alkaline phosphatase activity that bone debris obtained after intra-medullary reaming contains vital osteoblasts, independent of the reamer sizes and reamer design (Hoegel et al. 2004). Furthermore, it has been shown that a higher percentage of viable cells were encountered in unmilled and spongy bone when compared with milled and cortical bone, respectively (Hoegel et al. 2004). However, cell viability seemed to be significantly influenced by the harvesting technique, as the least amount of vital cells was observed in the alloy ball reamer group, when compared with diamond ball or implant drill (Springer et al. 2004). Furthermore, conventional metallic drills may have some specific drawbacks such as overheating of bone when water cooling is insufficient or possible damages of the adjacent soft tissues (i.e. perforations of the Schneider membrane during sinus floor augmentation) (Danckwardt-Lilliestrom et al. 1970, Stürmer & Tammen 1986, Ercoli et al. 2004). Additionally, a metallic contamination of the bone has been observed, generating possible structural bone changes and having a toxic effect on living cells (Hobkirk & Rusiniak 1978). In order to overcome some of these problems, a newly developed piezoelectric device (Piezosurgery®) has been recently introduced for different bone augmentation procedures. The device is suggested to find clinical applications in periodontal surgery, sinus floor elevations and intra-oral gaining of bone chips (Vercellotti et al. 2001, Lambrecht 2004, Siervo et al. 2004, Vercellotti 2004). The main advantages of the piezoelectric device may be because of its modulated ultrasound microvibrations (29 kHz, ranging from 60 to 200 Hz) which should prevent damages to the adjacent soft tissues during osteotomy procedures. However, to the best of our knowledge, there are currently no data from in vitro studies examining the morphological characteristics and vitality of bone material obtained by means of this piezoelectric device when compared with conventional drills. Therefore, the aim of the present study was to investigate and compare the influence of both treatment approaches on (i) shape and size of intra-orally harvested bone particles obtained during standard surgical procedures, and (ii) morphology, viability and differentiation of cells growing out of the bone particles.

Material and Methods

Sample collection

A total of 69 patients (30 men and 39 women), aged between 18 and 68 years, undergoing standard surgical procedures (i.e. osteotomies, bone augmentation procedures) were included in the study based on signed informed consent. The study protocol was approved by the Ethics Committee of the Heinrich-Heine-University of Düsseldorf, Germany (reference number of the present project 2505/04). Criteria for patient selection were: (a) good health status (no systemic diseases), (b) non-smoker, (c) age >18 years, (d) no pregnancy in women. The patients were randomly assigned to the following treatment groups: (a) a piezoelectric device (Piezosurgery®, Mectron Medical Technology, Carasco, Italy) (piezoelectric device (PS), test group, $n = 33$), or (b) a conventional rotating drill (size: 4 mm, H141.104040, Komet, Lemgo, Germany) at a speed of 1000 r.p.m. (rotating drills (RD), control group, $n = 36$). Randomization was perfomed according to a computer-generated protocol (RandList®, DatInf GmbH, Tübingen, Germany). For the statistical analysis, samples were further divided into the following subgroups (i) age of patients (below 30 years, above 30 years), and (ii) donor site (upper jaw, lower jaw) (Table 1). Bone particles were pre-dominantly collected from cortical donor sites and collected in a bone filter KF-T2 (Schlumbohm, Brokstedt, Germany) connected to the suction device. The piezoelectric device was used according to the instructions given by the manufacturer (mode boosted, pump 5, burst C) utilizing a special application tip designed for bone chip harvesting. In both groups, bone harvesting was performed under sufficient water cooling with sterile saline. Precautions were taken in order to avoid contamination by saliva, represented by a mandatory utilization of two aspiration tips, one of them only for saliva aspiration and the other one attached to the collector. The bone collector was regularly rinsed with sterile saline in order to avoid a drying of the bone chips. Within 184 ± 165 s after collection, bone chips were transferred from the filter with a spatula into a sterile tube containing cell culture medium. The surgical procedures were perfomed by five previously calibrated surgeons.

Table 1. Number of patients per subgroup

	Piezoelectric device (PS)		Rotating drills (RD)	
Age (years)	<30	>30	<30	>30
Upper jaw	7	8	7	6
Lower jaw	9	9	11	12

Cell culture

Bone chips were cultivated in Dulbecco's modified Eagle's medium (DMEM, Gibco, Karlsruhe, Germany) with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin (Gibco) in 25 cm^2 cell culture flasks (Greiner, Flacht, Germany) and incubated in 5% CO_2 at 37°C. No osteogenic factors were added. Medium was changed every second day. After reaching confluency, the cells were harvested with EDTA-trypsin (Gibco) and transferred onto eight-well culture slides (Lab Tek Chamber Slide, Nunc, Wiesbaden, Germany) for immunhistochemical and histochemical observations. Subculturing produced vital cells for at least four passages.

Morphological investigations

For image acquisition a digital camera (Color View III, Olympus, Hamburg, Germany) was mounted on a binocular light microscope (Olympus BX50, Olympus). The shape and size of the bony particles were assessed morphometrically using a software program (Image J, Wayne Rasband, National Institutes of Health, USA). All measurements were performed by a calibrated examiner who was blinded to the design and purpose of the study.

Alkaline phosphatase staining

Osseous alkaline phosphatase (AP), a membrane-bound tetrameric enzyme attached to phosphatidyl-inositol moieties located on the outer cell surface, was assayed using the release of *p*-nitrophenol from *p*-nitrophenolphosphate (Seibel 2000). After reaching confluency, the cells were fixed in 4% buffered paraformaldehyde, and incubated for 15 min. in an AP staining solution (Sigma, Deisenhofen, Germany). Counterstaining of the cell nuclei was carried out with 6% haematoxyline (DakoCytomation, Hamburg, Germany). As negative control, the endogenous AP

activity was blocked by 0.15 mg/ml levamisole (Sigma) (Van Belle 1972).

Immunhistochemical staining for osteocalcin (OC)

Cells were fixed in 4% buffered paraformaldehyde and incubated for 10 min. in 0.9% H_2O_2 in PBS in order to block the endogenous peroxidase. Non-specific sites were blocked with blocking solution (DakoCytomation, Hamburg, Germany) for 30 min. and the samples incubated for 60 min. at room temperature with the specific monoclonal anti-OC antibody 1:100 (Acris, Hiddenhausen, Germany). All antibodies used were diluted in antibody diluent (DakoCytomation). A non-specific mouse IgG1 antibody (DakoCytomation) in the same dilution served as negative control. Secondary antibody was an anti-mouse IgG biotin conjugate (1:100; DakoCytomation) for 60 min. Subsequently, samples were incubated with a steptavidin-HRP conjugate (1:300; DakoCytomation) for 30 min. and visualized using AEC (DakoCytomation) as substrate. After stopping the colour reaction within 5 min. the cells were counterstained with 6% haematoxyline.

Reverse transcriptase-polymerase chain reaction phenotyping (RT-PCR)

RT-PCR technique was used to analyse the expression of OC in the cells. Total RNA from 2×10^5 cells was isolated using the SV total RNA Isolation System® and c-DNA synthesized by using the Avian Myeloblastosis Virus Reverse Transcription System® (both from Promega, Mannheim, Germany) following the manufacturers' instructions. Specific primer pairs for the amplification of the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT) were used as described by Steinbrenner et al. (2003). For OC-PCR primers were newly designed using the primer select program DNA Star (DNAStar Inc., Madison, WI, USA) 5′CCA AGC AGG AGG GCA GCG AGG TAG TGA A (sense) and 5′CAG GGC AAG GGC AAG GGG AAG GGG AAG AGG AA (antisense). PCR for OC was performed for 3 min. at 95°C followed by 33 cycles of 30 s at 94°C, 30 s at 55°C and 1 min. at 72°C as well as a final elongation step for 10 min. at 72°C using the Taq DNA Polymerase (Promega). All primers were manufactured by MWG Biotech (Munich, Germany).

Statistical analysis

A software package (SPSS 11.0, SPSS Inc. Chicago, IL, USA) was used for the statistical analysis. Mean values and standard deviations were calculated for each group. As the data were normally distributed (Kolmogorow–Smirnow test), analysis of variance (ANOVA) was used for comparisons within and between the groups. Post hoc testing using Bonferroni's correction was used to confirm any significant values arising from multiple comparisons. The volume comparison of bone chips was carried out using Student's unpaired *t*-test. Results were considered to be statistically significant at $p < 0.05$.

Results

During the experimental period, there were no signs of any bacterial or fungal contamination of the culture flasks.

Cell proliferation

In 61 out of 69 samples, first adherent cells nearby the bone chips were observed after 6–19 days as evaluated by light microscopic observation (Fig. 1). In particular, outgrowing cells were detected in 32 samples (88.9%) of the RD group and in 29 samples (87.9%) of the PS group. In both groups, spreading of cells approached confluency on the bottom of the culture dish 20–25 days following incubation ($p > 0.05$, ANOVA). However, in both groups, four samples (total $n = 8$) revealed no signs of outgrowing cells up to 35 days following incubation.

The statistical analysis of the characteristics of the first adherent cells revealed no significant difference in respect of age of the patients (<30 years against >30 years). Furthermore the donor site (upper jaw *versus* lower jaw) or harvesting method (RD against PS) did not seem to have any influence on the time needed for approaching cell confluency ($p > 0.05$, ANOVA, respectively).

Morphometrical analysis

Morphometrical analysis of the bone chips revealed that particles collected with PS were statistically significant and more voluminous than those collected by RD ($p < 0.01$, unpaired *t*-test) (Fig. 2a–c). However, all bone chips analysed showed a polygonal shape irrespective of the harvesting method.

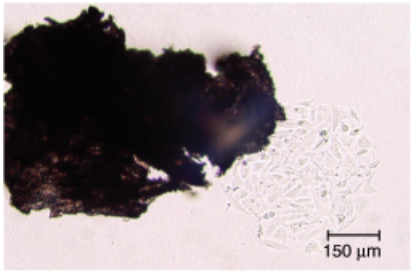

Fig. 1. Bone chip with outgrowing cells 9 days after incubation: rotating drill.

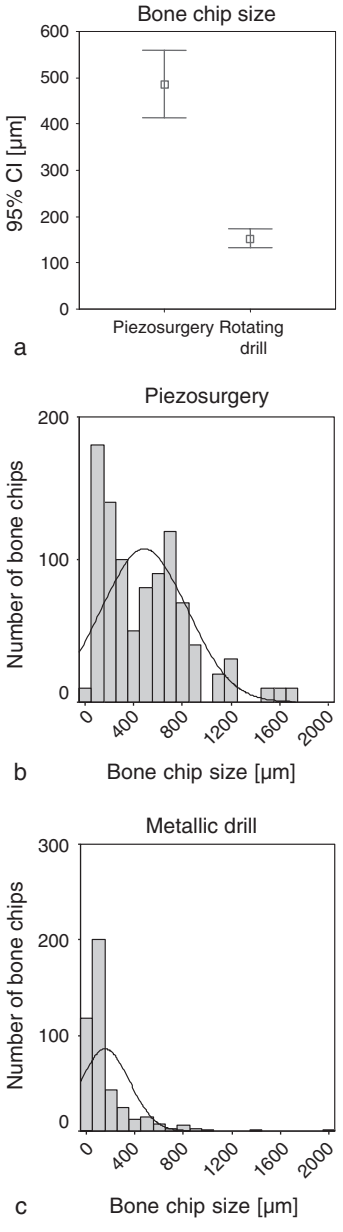

Fig. 2. (a) Errorbars of bone chip sizes (micrometres) in both groups (95% confidence interval). (b) Histogram of bone chip size (micrometresµ): Piezosurgery®. (c) Histogram of bone chip size (micrometres): rotating drill.

Cell characterization

In both groups, a positive staining for AP could be detected as an early maker for osteoblast differentiation. After subculturing the cells for 2 more weeks, an intense red staining of AP at the cytoplasma membrane was observed while the cell nuclei were counterstained blue (Fig. 3a). This positive AP reaction of all samples was proven to be intrinsic as its inhibition with levamisol as inhibitor of the endogenous AP was complete (Fig. 3b).

Furthermore, in both groups, OC could be identified in the cells as a late marker for osteoblast differentiation on the level of RNA expression and of protein synthesis. Phenotype analysis revealed an OC expression in all specimens. HPRT as housekeeping gene was used as an internal standard to verify the RT-PCR procedure and to make the amounts of osteoblast marker expression comparable within each experiment. The OC signal appeared at 277 bp, while the respective band for HPRT had a size of 439 bp (Fig. 4). Whereas HPRT was expressed in equal amounts in all samples, the various patient cultures differed significantly in the expression of OC. In immunhistochemical analysis OC was present in all samples (Fig. 5a), outlining that the cells are producing this extracellular matrix protein. In the negative control where no specific antibody was used, no reddish-brown signal appeared (Fig. 5b). Both marker enzymes AP and OC demonstrated the differentiation of the outgrowing cells into osteoblasts without any addition of osteogenic factors to the cell culture medium.

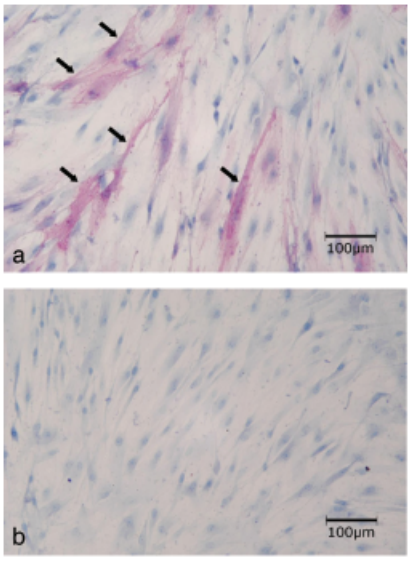

Fig. 3. (a) Histochemical staining for alkaline phosphatase (AP) (arrows): rotating drill (RD) (b) AP negative control; inhibition with Levamisol: RD.

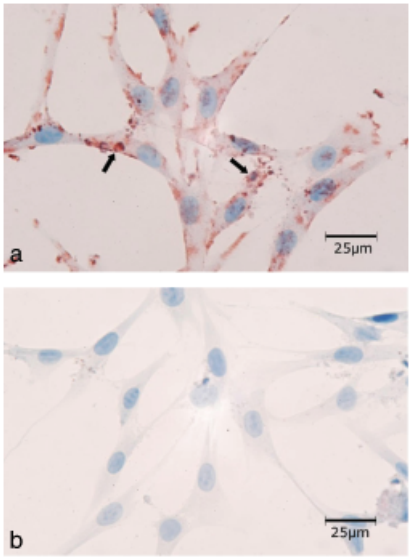

Fig. 5. (a) Immunhistochemical staining for Osteocalcin (arrows): Piezosurgery® (b) osteocalcin negative control: Piezosurgery®.

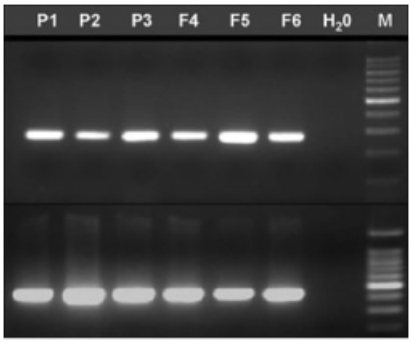

Fig. 4. Expression of Osteocalcin and the housekeeping gene hypoxanthine phosphoribosyl transferase in Piezosurgery® P1–P3 and rotating drill samples F4–F6.

Discussion

The results of the present study have indicated that both PS and RD are not different from each other concerning their detrimental effect on viability and differentiation of cells growing out of autogenous bone chips harvested during standard surgical procedures. In this context, it must be emphasized that differentiation of cells was assessed using OC, a hydroxyapatite-binding protein which is exclusively synthesized by osteoblasts, odontoblasts and hypertrophic chondrozytes (Gallop et al. 1980, Hauschka et al. 1989, Hopyan et al. 1999, Raymond et al. 1999, Seibel 2000). When interpreting the present results, it has also to be noted, that patients age, gender and harvesting region did not seem to have any influence on the investigated parameters. However, it was observed that bone samples collected by PS (mean size: $486 \pm 355\,\mu$m) were statistically significant and more voluminous than RD (mean size: $153 \pm 201\,\mu$m). Yet, particle size did not seem to have any influence on outgrowth, proliferation, viability and differentiation of cells. There might be several explanations for the lack of a difference between both groups. First of all, it is important to point to the results of an experimental study in monkeys which have shown that particles in the range of 125–1000 μm possess a higher osteogenic potential than do particles below 125 μm, as smaller particles may be more easily absorbed by macrophages with little or no new bone formation (Shapoff et al. 1980, AAP 2001). Furthermore, the optimal particle size has been reported to range between 100 and 300μm (Shapoff et al. 1980). All these data taken together with the results of the present study indicate that bone samples in both groups seem to be within the range of the optimal particle size. In this context, however, it must also be emphasized that outgrowth, proliferation and differentiation of cells do not provide information on the viability of cells within the graft. The finding that RD may represent a suitable device for bone harvesting corroborates to a certain extend previous in vitro studies (Hoegel et al. 2004, Springer et al. 2004). In particular, Hoegel et al. (2004) analysed the bone debris obtained after reaming sheep tibiae with two different reamer designs regarding cell viability by measuring AP activity. A positive AP activity in most of the samples indicated that osteoblasts may survive the reaming process irrespective of the reamer sizes and reamer design (Hoegel et al. 2004). However, Springer et al. (2004) reported that trabecular or cortical bone particles obtained by a ball reamer revealed smaller amounts of vital cells when compared with a diamond ball or an implant drill. There might be several explanations for this discrepancy. First of all, it is important to realize, that in the present study, bone particles were harvested predominantly from cortical

donor sites and collected in a bone filter for 184 ± 165 s. In this context, it is impossible to estimate the influence of the passing of time in the bone filter on cell viability. However, the results of the present study have demonstrated a survival of osteogenic cells as determined by AP activity and OC synthesis. On the other hand, it is important to point to the results of experimental animal studies analysing the effects of increased temperature during cortical drilling (Danckwardt-Lilliestrom et al. 1970, Stürmer & Tammen 1986, Hoegel et al. 2004). According to the authors, thermal side effects may result in bone damages because of changes in protein structure and protoplasma lipids, modified metabolism and enzyme activity (Danckwardt-Lilliestrom et al. 1970, Hoegel et al. 2004). In this context, it is important to point to the results of the present study which have shown that in both groups, a small number of bone samples revealed no signs of outgrowing cells following 35 days of incubation, which might be attributed to critical temperature increases during cortical preparation. To the best of our knowledge, these are the first data evaluating the influence of PS on viability and differentiation of primary osteoblasts grown out of bone chips harvested during standard surgical procedures, and therefore a comparison with other studies with a similar design is not possible. As described above, this system has been recently introduced for gentle cutting and collecting of bone particles because of its modulated vibrations (Eggers et al. 2004). Our working hypothesis was, that the generated microvibrations of the PS might have an influence on the vitality of the collected bone chips. However, the results of the present study have indicated that both harvesting methods led to comparable cell proliferation times and cell differentiation into osteoblasts. Nevertheless, it must be pointed out that results obtained by using an in vitro experimental model cannot recreate the complex interactions of cells in vivo. Further studies using controlled experimental in vivo models are needed in order to verify the present results.

Within the limits of the present study, it may be concluded that autogenous bone chips harvested with both PS and RD contained vital cells which differentiated into osteoblasts in vitro. In both groups, patient's age and donor site did not seem to have any influence on the investigated parameters.

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Clinical Relevance

Scientific rationale for the study: The working hypothesis of the present study was, that the generated microvibrations of a newly developed ultrasonic device (Piezosurgery®) might have an influence on the vitality of collected autogenous bone chips when compared with a conventional RD.

Principal findings: The present results have indicated that both harvesting methods are not different from each other concerning their detrimental effect on viability and differentiation of osteoblasts grown out of bone chips harvested during standard surgical procedures.

Practical implications: Autogenous bone chips collected by both harvesting methods seem to possess an osteogenic potential.