Impact of Bone Harvesting Techniques on Cell Viability and the Release of Growth Factors of Autografts

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ABSTRACT

Background: Autogenous bone grafts obtained by different harvesting techniques behave differently during the process of graft consolidation; the underlying reasons are however not fully understood. One theory is that harvesting techniques have an impact on the number and activity of the transplanted cells which contribute to the process of graft consolidation.

Materials and Methods: To test this assumption, porcine bone grafts were harvested with four different surgical procedures: bone mill, piezosurgery, bone drilling (bone slurry), and bone scraper. After determining cell viability, the release of molecules affecting bone formation and resorption was assessed by reverse transcription polymerase chain reaction and immunoassay. The mitogenic and osteogenic activity of the conditioned media was evaluated in a bioassay with isolated bone cells.

Results: Cell viability and the release of molecules affecting bone formation were higher in samples harvested by bone mill and bone scraper when compared with samples prepared by bone drilling and piezosurgery. The harvesting procedure also affected gene expression, for example, bone mill and bone scraper samples revealed significantly higher expression of growth factors such as bone morphogenetic protein-2 and vascular endothelial growth factor compared with the two other modalities. Receptor activator of nuclear factor kappa B ligand expression was lowest in bone scraper samples.

Conclusion: These data can provide a scientific basis to better understand the impact of harvesting techniques on the number and activity of transplanted cells, which might contribute to the therapeutic outcome of the augmentation procedure.

KEY WORDS: autogenous bone, bone dust, bone graft, bone mill, bone particles, bone scraper, bone slurry, osteocyte, osteoinduction, piezosurgery

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INTRODUCTION

Over the past decades, autogenous bone grafts have consistently been described as the gold standard for bone grafting in implant dentistry as well as in oral and maxillofacial surgery.[1–3] Graft consolidation occurs faster when compared with allogenic and synthetic bone substitutes. However, the underlying reasons are not fully understood.[4] What clearly distinguishes autogenous bone grafts from any other grafts is the presence of viable cells. It is widely believed that the transplanted cells can

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turn into osteoblasts and directly contribute to bone formation.[5] However, the transplanted cells might also contribute to graft consolidation by the release of growth factors and other bioactive molecules. This assumption is supported by in vitro studies showing that culture expanded bone cells,[6] endothelial cells,[7] and osteocytes[8] release molecules in a paracrine mode of action.

Based on this experimental approach, the impact of the harvesting technique on the number and activity of bone cells has been studied. For example, more cells grow out from bone samples prepared by mill when compared with bone slurry.[9,10] However, other studies could not confirm this observation,[11] suggesting that the question has not been completely answered. Moreover, in vitro studies with culture expanded cells do not reflect the in vivo situation where autografts are harvested and immediately transplanted into the defect site. It thus requires freshly prepared bone samples, where the autocrine/paracrine function of the cells can be determined.

Overall these studies may help to make the decision about the selection of the most appropriate harvesting technique to obtain autografts with the best osteogenic potential. In a previous study, the behavior of culture expanded bone cells seeded onto freshly prepared bone samples was examined, considering the impact of the harvesting technique. Preparations from bone mill and bone scraper favored cell differentiation when compared with piezosurgery and bone slurry, clearly underlying the potential impact of the harvesting technique in the cellular response.[12] The impact of the harvesting technique on the viability and the biologic activity of the transplanted cells – which are responsible for the autocrine/paracrine effects – might provide a further clue to the reported findings.

In the present study, porcine bone samples were harvested by grinding of bone blocks with a bone mill, bone preparation with piezosurgery, collection of bone particles with a bone trap, following drilling of cortical bone with a 2.2-mm round bur, or using a bone scraper. Immediately thereafter, cell viability was determined as well as the expression of growth factor affecting bone formation and resorption including bone morphogenetic protein-2 (BMP2), transforming growth factor β1 (TGFβ1), vascular endothelial growth factor (VEGF), osteoprotegrin (OPG), and receptor activator of nuclear factor kappa B ligand (RANKL). Growth factors BMP2 and TGFβ1 are capable of stimulating rapid proliferation and/or differentiation of osteoblast progenitors,[13–19] while VEGF is responsible for the stimulation of angiogenesis.[20] RANKL induces osteoclast precursors to differentiate into osteoclasts by binding to its receptor RANK on the surface of osteoclast precursors. OPG is a decoy receptor that binds to RANKL and inhibits its ability to bind with RANK, thus preventing osteoclast precursors from committing to the osteoclast lineage.[21] To underlie the impact of the harvesting procedure on the autocrine/paracrine effects of the grafts, conditioned media (CMs) were collected and subjected to immunoassay and bioassay. Based on the overall hypothesis and the previous findings, we expected to find autograft preparations from bone mill, and bone scraper samples have higher viable cells and provoke a stronger autocrine/paracrine effect when compared with grafts obtained with piezosurgery and bone trap.

METHODS

Harvesting of Bone Grafts

This study was performed in conjunction with an in vivo study to determine the role of each harvesting technique on graft consolidation. The four different harvesting techniques are routinely used and recommended in daily routine in implant dentistry. Miniature pigs were sedated with intramuscular injections of ketamine (20 mg/kg) and xylazine (2.0 mg/kg) and intravenous injections of atropine (0.05 mg/kg) and midazolam (0.5 mg/kg) at the Surgical Research Unit and Clinic for Large Animals, University of Bern, Switzerland. The protocol was approved by the committee for Animal Research, state of Bern, Switzerland (animal ethical approval #13/10). Through subangular incisions, the lateral portion of the mandible was exposed to harvest autogenous bone grafts prepared using four different techniques: (1) cortico-cancellous block grafts harvested with a 6-mm trephine and ground to particulate bone chips using a bone mill (R. Quétin, Leimen, Germany); (2) bone chips harvested with a sharp bone scraper (Hu-Friedy, Chicago, IL, USA); (3) bone particles collected from the suction tip with a bone trap filter after drilling of cortical bone with a 2.2-mm round bur under saline conditions (bone slurry; Schlumbohm GmbH & Co. KG, Brokstedt, Germany); and (4) bone particles harvested with a piezosurgery device under saline conditions (Mectron®, Carasco, Italy). In total, 12 animals were used to collect autogenous bone particles. All areas were subdivided into four sections and autogenous bone

was collected using clinical harvesting techniques and transported for in vitro experiments in α-Minimum Essential Medium (Gibco, Basel, Switzerland) supplemented with 1% antibiotics (10,000 units of penicillin, 10,000 µg of streptomycin, and 25 µg of amphotericin B/mL, Invitrogen, Basel, Switzerland).

Scanning Electron Microscopy

Autogenous bone particles were fixed in 1% glutaraldehyde and 1% formaldehyde for 2 days for scanning electron microscopy. Following serial dehydration with ethanol, samples were critical point dried (Type M.9202 Critical Point Dryer, Roth & Co., Hatfield, PA, USA) and allowed to dry overnight. The following day, samples were sputter coated using a Balzers Union sputtering device (DCM-010, Balzers, Liechtenstein) with 10 nm of gold and analyzed microscopically using a Philips XL30 field emission guns scanning electron microscope (Royal Philips Electronics, Amsterdam, Netherlands) to determine surface variations between samples.

Measurement of Particle Size

Autogenous bone particles were fixed in 1% glutaraldehyde and 1% formaldehyde for light microscopy. Images of 50 particles from each modality were captured using a ProgRes® C5 digital camera (Jenoptik, Optical Systems GmbH, Jena, Germany) connected to a Zeiss Axioplan microscope (Carl Zeiss, Göttingen, Germany). Projection area was determined as previously described[12] using the image surface area per autogenous bone particle (in square millimeter) and particle size was determined by measuring the maximal horizontal distance of each particle (in millimeter). The projection area and maximal horizontal distance of particles were measured using ImageJ software (NIH Image, Scion Corporation, Frederick, MD, USA). Data were averaged ± standard error (SE).

Quantification of Viable Cells in Autogenous Bone Particles

The cell viability in autogenous bone grafts was determined using the CellTiter 96® One Solution Cell Assay (MTS) (Promega, Madison, WI, USA). Briefly, 100 mg of harvested bone was incubated with 80 µL of CellTiter 96 aqueous solution dissolved in 400 µL of phosphate buffered solution. After 4 hours of culture, the cell viability was determined by measuring the absorbance at 490 nm on a 96 well plate reader. Experiments were performed in triplicate with three independent experiments for each condition. Data (± SE) were normalized to bone mill samples.

Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from 500 mg of autogenous bone particles using TRIZOL reagent and RNAeasy Mini Kit (QIAGEN, Basel, Switzerland) at time points 2 and 8 hours. Primer and probe sequences for genes encoding BMP2, TGFβ1, VEGF, OPG, and RANKL were purchased as predesigned gene expression assays (Applied Biosystems, Rotkreuz, Switzerland). Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed using 20-µL final reaction volume of TaqMan's One-Step Master Mix Kit (Applied Biosystems). RNA quantification was performed using a Nanodrop 2000c (Thermo Scientific, Waltham, MA, USA) and 50 ng of total RNA was used per sample well. All samples were assayed in triplicate and three independent experiments were performed. The ΔΔCt method was used to calculate gene expression levels normalized to glyceraldehyde 3-phosphate dehydrogenase values. Data (± SE) were log-transformed prior to analysis by two-way analysis of variance (ANOVA) with Bonferroni test, using Graphpad Software v. 4 (Graphpad Software, La Jolla, CA, USA).

Enzyme-Linked Immunosorbent Assay Protein Quantification

Bone grafts were incubated with serum-free medium at 500 mg/mL and CM was harvested after 2, 8, and 24 hours. BMP2, TGFβ1, VEGF, OPG, and RANKL were quantified using Quantikine® colorimetric sandwich enzyme-linked immunosorbent assays (RND Systems, Minneapolis, MN, USA) according to manufacturer's protocol. All samples were measured in duplicate and three independent experiments were performed. Statistical analysis was performed by two-way ANOVA with Bonferroni test (data ± SE).

Osteogenic Potential of CM from Bone Grafts

Bone grafts were incubated with serum-free medium at 100 mg/mL and CM was harvested after 24 hours. Serum-free medium previously not in contact with autogenous bone particles was used as a control. Primary porcine osteoblasts were isolated from spongy bone blocks by collagenase as previously described.[22]

TABLE 1 Characterization of Autogenous Bone Particle's Average Projection Area, Particle Size, and Range

	Projection Area ($mm^2 \pm SE$)	Range (mm^2)	Particle Size (mm ± SEM)	Range (mm)
Bone mill	1.734 ± 0.294	0.132–9.317	1.551 ± 0.137	0.525–4.467
Piezo	0.972 ± 0.109	0.256–3.743	1.352 ± 0.070	0.656–2.451
Bone slurry	0.026 ± 0.007	0.016–0.053	0.215 ± 0.010	0.115–0.361
Bone scraper	1.968 ± 0.295	0.069–7.118	1.805 ± 0.154	0.385–4.877

SE = standard error; SEM = scanning electron microscopy.

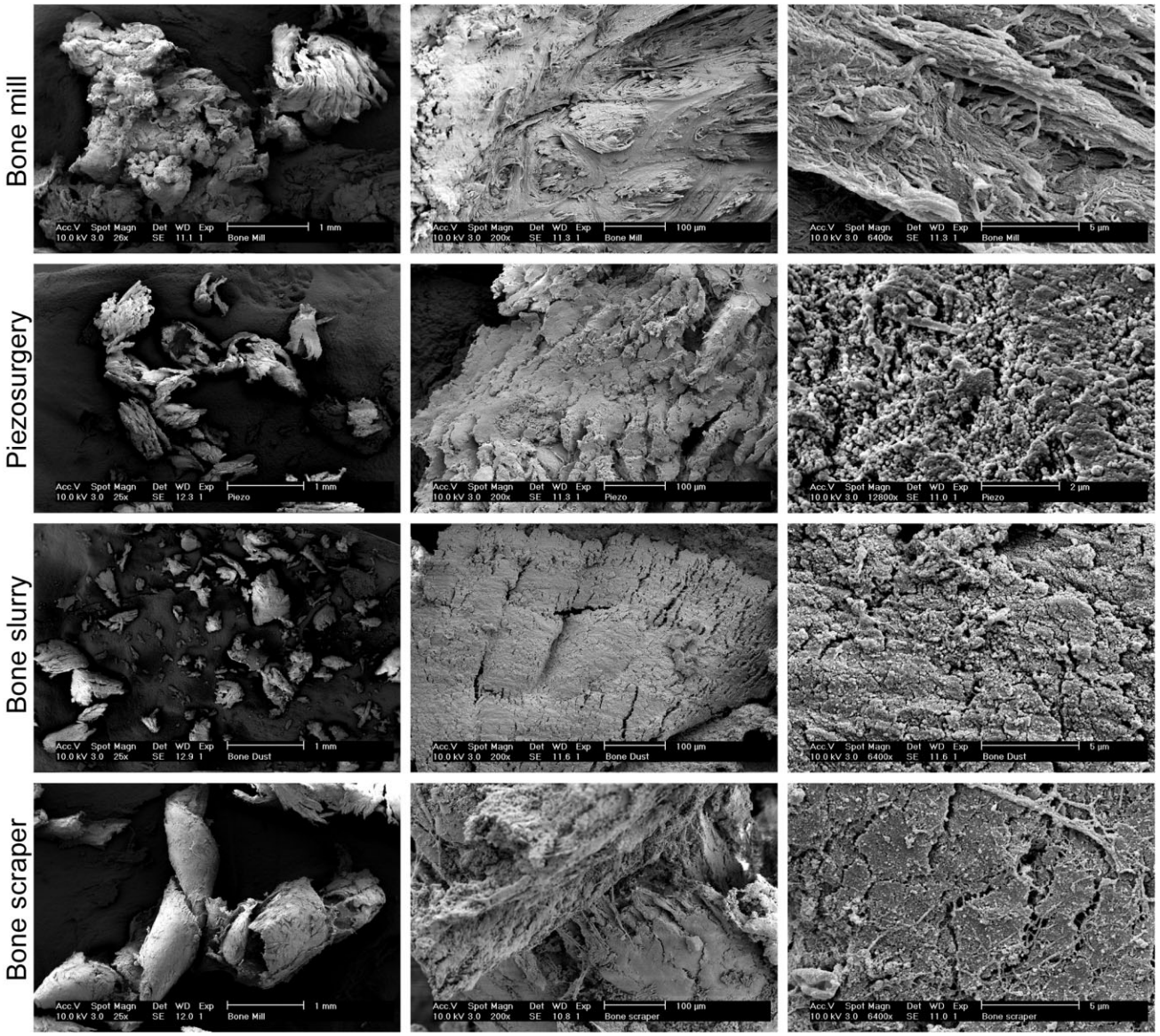

Figure 1 SEM analysis of autogenous bone harvested by four commonly employed techniques. SEM of bone mill samples displayed large particles with exposed collagen fibrils. Piezosurgery revealed dense cortical bone with many micro- and nanotopographies. SEM analysis of bone slurry samples revealed fine, powder-like particles. Bone scraper samples displayed large, swirly particles with a fibrin/collagen network still intact. SEM = scanning electron microscopy.

Primary osteoblasts from passage 3 were seeded at a density of 5,000/cm^2 (Becton Dickinson, Basel, Switzerland). After 8 hours, growth media were replaced by CM. After 5 days, cell proliferation was determined using CellTiter 96 One Solution Cell Assay. Cell differentiation was assessed at 5 days using real-time RT-PCR for Run×2, collagen1α1, and osteocalcin (OC) (Applied Biosystems) as previously described.[22]

Statistic

Date was analyzed for statistical significance using one-way ANOVA with Tukey's test.

RESULTS

Impact of the Harvesting Techniques on Particle Size and Cell Viability

Bone chips prepared by bone milling and bone scraper were larger compared with particle prepared by piezosurgery and bone drilling (Table 1). Micro- and nanotopographic images indicate that bone chips prepared by bone milling had collagen fibers on the surface, while the other modalities showed a smooth surface (Figure 1). Bone chips prepared by bone milling and bone scraper caused more formation of formazan, representing the number and/or activity of viable cells, than equivolumetric amounts of piezosurgery and bone slurry samples (Figure 2).

Expression of Genes That Are Related to Bone Formation and Resorption

To determine the impact of the harvesting technique on the expression of genes that are related to bone formation and resorption, BMP2, TGFβ1, VEGF, RANKL, and OPG were measured by RT-PCR and immunoassay. BMP2 and VEGF were significantly higher in samples of bone milling and bone scraper compared with samples from piezosurgery and bone slurry (Figure 3). TGFβ1 and OPG were higher in samples of bone milling only compared with all other samples (see Figure 3). The most significant finding with the expression of RANKL was that samples obtained with a bone scraper showed the least expression, while bone slurry the highest expression (Figure 4).

Response of Bone Cells Supernatants from Bone Grafts

To see how the findings on cell viability and gene expression translate into a paracrine function, CMs prepared from the various bone samples were harvested and incubated with primary bone cells. Consistent with these findings, CM of samples of bone milling and bone scraper significantly increased proliferation/viability and the expression of collagen type 1 and OC in bone cells. This was not the case when bone cells were exposed to CM obtained from samples from piezosurgery and bone slurry (Figure 5).

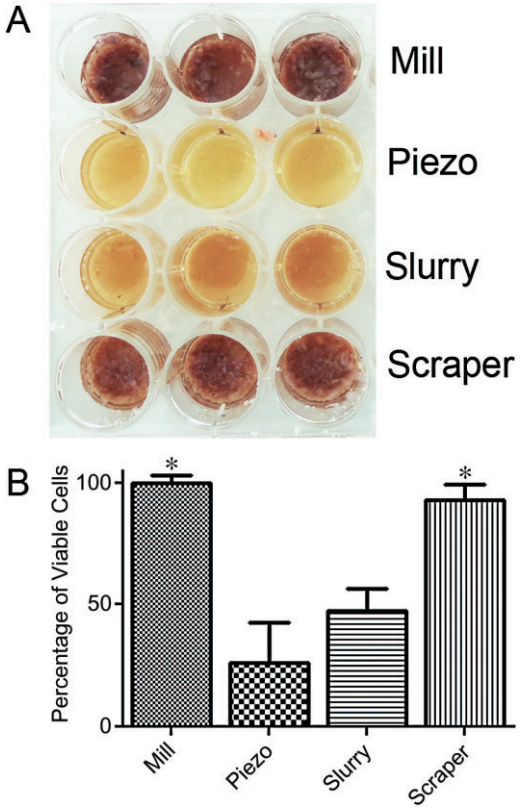

Figure 2 Cell viability of autogenous bone from four commonly employed harvesting techniques. *A*. Photographic image of autogenous bone particles incubated with MTS for 4 hours. *B*. Relative absorbances at 490 nm measured after transfer of incubation media into a fresh 96 well plate. Samples were normalized to bone mill, averages ± SE. (* denotes significant difference between bone mill and bone scraper when compared with piezosurgery and bone slurry). SE = standard error.

DISCUSSION

Over the past decades, characterization of isolated bone cells was performed.[23] However, these cells were extensively expanded and thus do not necessarily represent cells in bone grafts.[24] In the present study, the impact of four types of harvesting techniques was studied on the behavior of cells within autogenous bone grafts. The results demonstrated that cells in samples obtained by

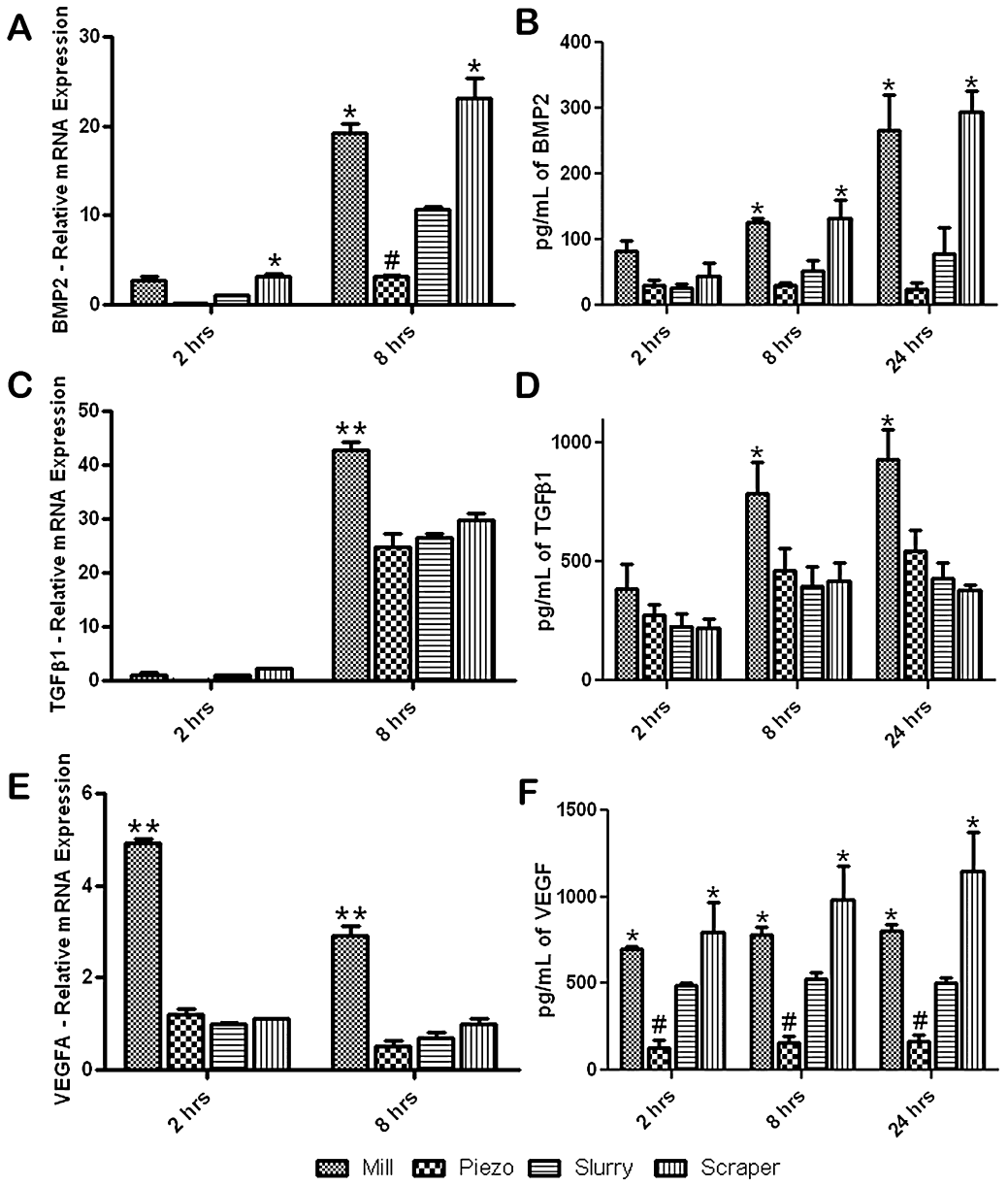

Figure 3 Relative mRNA levels and protein contents for BMP2 (A, B), TGFβ1 (C, D), and VEGF (E, F) in autogenous bone chips harvested using different modalities. Bone mill and bone scraper brought the highest mRNA and protein expression of BMP2 (A, B). Elevated expression of TGFβ1 was seen in all modalities with bone mill having significantly higher expression over all other modalities (C, D). VEGF protein content was highest in bone mill and bone scraper samples with significantly less expression in piezosurgery samples (E, F). Samples were normalized to piezosurgery at 2 hours, averages ± SE (* denotes significant difference between bone mill and bone scraper when compared with piezosurgery and bone slurry, ** denotes significant increases over all other modalities, and # denotes significant decreases over all other modalities). BMP2 = bone morphogenetic protein-2; SE = standard error; TGFβ1 = transforming growth factor β1; VEGF = vascular endothelial growth factor.

bone mill and bone scraper showed higher viability and a stronger paracrine potential compared with piezosurgery and bone slurry samples. When comparing the observed data with those of similar studies, the basic findings correspond well: for example, more cells grow out from samples prepared by bone mill when compared with bone drilling.[9] Our findings are also in line with observations that cell outgrowth occurred more rapidly in bone chips than in bone sludge.[10] However, the present findings represent the cellular situation directly within the autografts.

Harvesting techniques such as bone milling and bone scraper had a positive impact on gene expression favoring bone formation when compared with

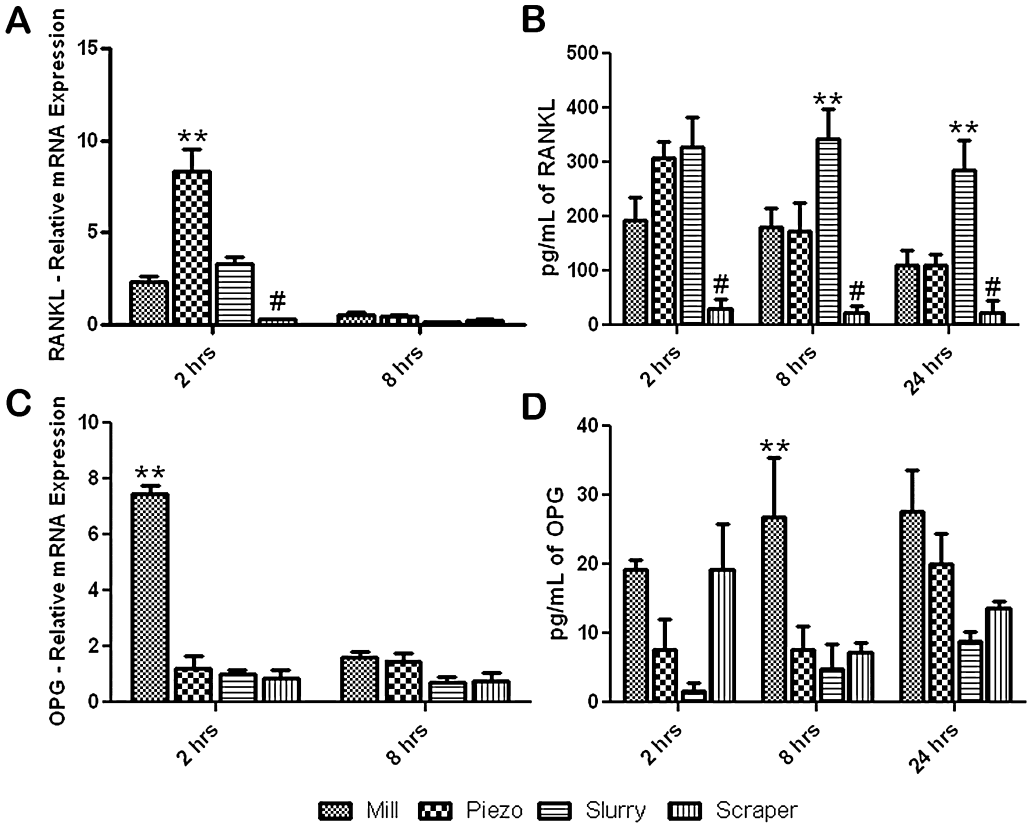

Figure 4 Relative mRNA levels and protein contents for RANKL (A, B) and OPG (C, D) of autogenous bone chips harvested using different modalities. Mechanical fragmentation by bone mill, piezo-electric, and bone slurry significantly increased relative to mRNA and protein expression of RANKL, whereas bone scraper samples showed significantly lower expression of RANKL (A, B). Elevated mRNA expression of OPG was observed in bone mill samples (C) but low protein release was observed in all samples with significant increases only observed on bone mill at 8 hours (D). Samples were normalized to piezosurgery at 2 hours, averages ± SE. (** denotes significant increases over all other modalities and # denotes significant decreases over all other modalities). OPG = osteoprotegrin; RANKL = receptor activator of nuclear factor kappa B ligand; SE = standard error.

piezosurgery and bone slurry samples. These changes in gene expression cannot be explained by changes in cell number because RT-PCR was normalized to housekeeping genes. Hence, the harvesting technique might have not only affected gene expression but also caused changes in the remaining cell population. Changes in gene expression can be a cellular response to the biomechanical stress induced by the different harvesting techniques[25] or a shift in the remaining cell population including osteoblast, lining cells, and/or osteocytes. In particular, osteocytes have gained substantial recognition in recent years, as they appear to be central regulators of bone turnover.[26,27] A detailed analysis, prior to any cultivation steps, would help to understand the cellular composition of our sample grafts.

A strong relationship between mechanically induced harvesting techniques via electrically powered instruments and RANKL expression was observed. Clinical evidence has speculated that bone slurry (which undergoes high mechanical destruction during harvesting) has a fast rate of bone resorption.[28,29] It is likely overheating during mechanical harvesting by bone slurry preparations and/or by vibrations generated during piezosurgery samples affecting cell viability. Interestingly, it has previously been shown that osteocytes undergoing cell death release apoptotic bodies expressing RANKL, which is capable of inducing de novo osteoclastogenesis.[30] The effects of electrically powered instruments on osteoclastogenesis require further investigation.

The clinical implications of the present findings have to be based on the assumption that the paracrine factors released from the transplanted cells may contribute to graft consolidation. If this is true, autografts harvested by bone mill and bone scraper might be more favorable compared with autografts harvested by piezosurgery and bone drilling. Still, measures such as cell viability and gene expression remain surrogate parameters to predict the process of graft consolidation and

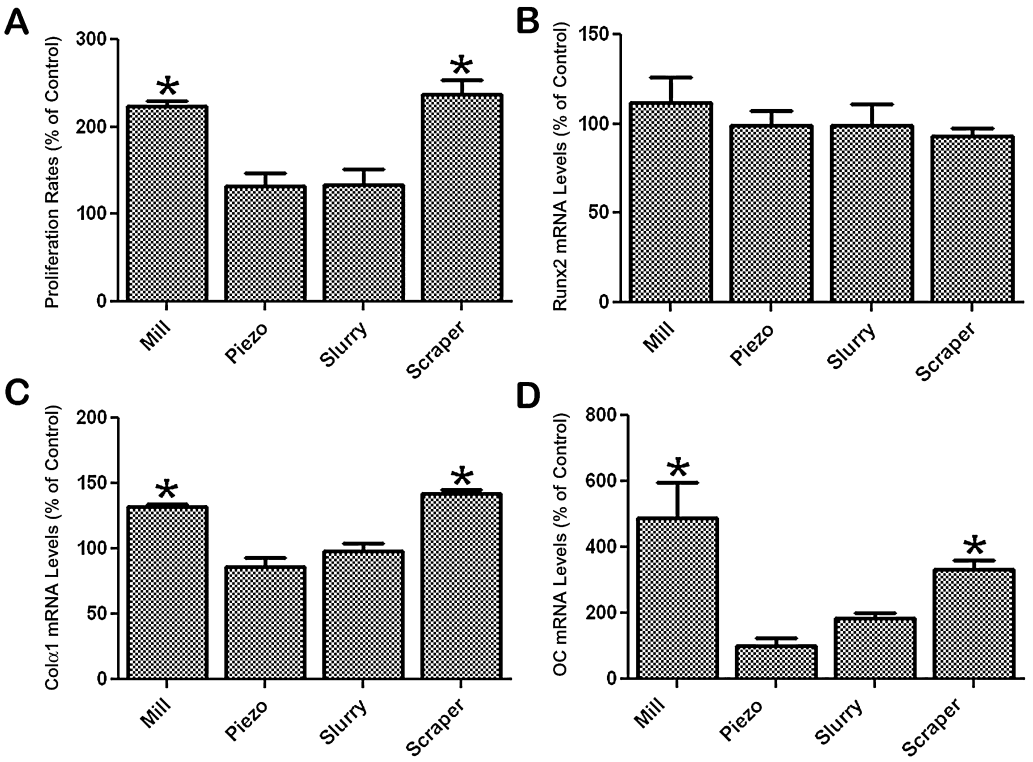

Figure 5 Relative proliferation rates of autogenous bone chips harvested using different modalities (A). Bone mill and bone scraper samples displayed significantly elevated values when compared with piezosurgery and bone slurry (A). Relative mRNA levels of (B) Run×2, (C) Col1α1, and (D) OC of primary minipig osteoblasts seeded on autogenous bone as assessed by real-time PCR. Significantly elevated levels of Col1α1 and OC were observed in bone mill and bone scraper samples when compared with piezosurgery and bone slurry. Conditioned media from bone mill samples improved OC mRNA values by over 400% when compared with control, unconditioned media (D). Samples were normalized to culture media without media, averages ± SE. (* denotes significant difference between bone mill and bone scraper when compared with piezosurgery and bone slurry). OC = osteocalcin; PCR = polymerase chain reaction; SE = standard error.

thus the therapeutic success of the augmentation surgery. Cell cultures cannot fully simulate the in vivo situation where transplanted cells are exposed to hypoxia and to a myriad of molecules released at the injury site, some of which might alter the cell viability and activity.[31,32] Nevertheless, the present study shows that the harvesting technique affects cell viability and activity in a potential graft. The present study can serve as a primer for preclinical models to understand the impact of the harvesting technique on the process of graft consolidation.

In conclusion, autografts harvested by bone mill and bone scraper better maintain cell viability and allow the cells to provide a paracrine environment when compared with autografts obtained with piezosurgery and bone drilling. At least part of the clinical response of the organism to grafts obtained with different harvesting techniques may result from the ability of the viable cells to provide a favorable paracrine microenvironment.

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