

## Viability of maxillary bone harvesting by using different osteotomy techniques. A pilot study

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**Summary.** The use of autogenous grafts is still considered in bone regeneration surgeries. However, the bone cell viability of such grafts after being harvested from donor sites remains a matter of debate. The aim of the present study is to evaluate particulated and block bone cell viability, in terms of presence or absence of apoptosis and necrosis, obtained from different maxillary intra-oral harvesting methods: bone scraper, rotary carbide burs and piezoelectric device. Five healthy patients were enrolled in the study. The patients required sinus augmentation by lateral window approach. The bone was harvested by the bone scraper, piezoelectric device and rotary surgical instrument. The samples were processed with the Annexin V/FITC (fluorescein isothiocyanate stain) kit and were analyzed by means of Fluorescence-Activated Cell Sorted (FACS) technique. Within the limitations of this pilot study, the results indicated that autogenous bone chips collected from the three harvesting methods presented a large percentage of apoptotic cells, although large scale production of necrotic cells was not detected. In summary, although rotary surgical instrument and piezoelectric devices are frequently used instruments for oral osteotomy, fresh autogenous bone chips collected from them did not present a viable bone cell source.

**Key words:** Annexin, Piezoelectric device, Rotary surgical instrument, Cell viability

### Introduction

In a wide variety of clinical situations alveolar bone defects can hinder the insertion of dental implants or periodontal treatments. Therefore, their reconstruction is an important step in the patient's oral rehabilitation.

Autogenous bone grafts in blocks or particulated form have been used extensively in oral and maxillofacial surgeries. Thanks to their properties of osteoinduction, osteoconduction and the supposed ability to carry living bone cells. Autogenous grafts have been considered the gold standard biomaterial in bone regeneration (Wallace and Froum, 2003; Del Fabbro et al., 2004; Aghaloo and Moy, 2007).

Many techniques and devices are available to harvest intraoral autogenous bone grafts, such as: bone scraper, rotary instruments, bone chisels, rongeur pliers and piezoelectric devices (Horton et al., 1975; Khambay and Walmsley, 2000a,b; Wallace and Froum, 2003; Chiriac et al., 2005; Martos Diaz et al., 2007; Zaffe and D'Avenia, 2007; Johansson et al., 2010). However, few investigations have evaluated the quality and cell viability of the harvested bone (Bacci et al., 2011; Berengo et al., 2006; Zerbo et al., 2003). In these studies, viability of particulate, as well as block autogenous grafts obtained with different harvesting methods, were assessed using histological and histomorphometrical observations. Therefore, only percentages of vital and non-vital bone were obtained, and no other information regarding the status of bone cells at the time of harvesting could be studied. Other studies by Chiriac et al (2005) and Tetè et al (2009) compared the cell viability of particulated bone harvested with piezoelectric device and rotating drills. In these studies, however, cell survival was assessed by histomorphometric analysis and cell culture of bone cells

(Chiriac et al., 2005; Tetè et al., 2009). However, outgrowth, proliferation and differentiation of cells do not provide information on the viability of cells within the grafts (Chiriac et al., 2005).

Cells respond to stress in a variety of ways. If the stress stimuli goes beyond a certain threshold, it will activate the stress signal cascades that induce cell death pathway (Dragovich et al., 1998; Reed, 2000; Fulda et al., 2010). The most common cell death pathways are cell apoptosis and necrosis (Dragovich et al., 1998; Reed, 2000; Fulda et al., 2010).

Many techniques have now been developed to analyze apoptosis and necrosis. Flow cytometry has recently become the methodology of choice for the quantitative analysis of apoptosis. Consequently, flow cytometry with a combination of fluorescinated annexin V and propidium iodide allows the study of various aspects of cell death, including detection and quantification of apoptotic or necrotic cells. The earliest stage of apoptosis is the flipping of phosphatidylserine (PS) from the inner to outer layer of the plasma cell membrane. Annexin V is a  $Ca^{2+}$  dependent phospholipid-binding protein with high affinity for PS (Fadok et al., 1992). Therefore, this protein can be used as a marker of PS externalization. However, the translocation of PS to the cell membrane is not unique to apoptosis, and also occurs during necrosis. The additional staining with propidium iodide (PI), a nuclear dye for which living and apoptotic cells are impermeable, is necessary to distinguish between apoptotic and necrotic cells (Van Oostveldt et al., 1999; Willingham, 1999; Kikuyama et al., 2002). Consequently, staining cells simultaneously with Annexin V and propidium iodide allows the discrimination of intact cells (negative for both parameters), early apoptotic cells (annexin-positive, PI-negative) and late apoptotic or necrotic cells (double positive parameters) (Castedo et al., 1996; Levy et al., 1998; Lauber et al., 2004; Kim et al., 2006).

The purpose of this pilot study is to evaluate the viability of particulated and block bone grafts, in terms of the presence or absence of apoptosis and necrosis, obtained from different maxillary intra-oral harvesting methods: bone scraper, rotary carbide burs and piezoelectric device.

## **Material and methods**

### *Patient Selection*

Four healthy patients with ASA type I and ASA type II classification system (Keats, 1978) were selected of different ages (32, 46, 52, 56 years old) and sexes. The patients were recruited to participate in this study from the university dental clinic of the International University of Catalonia (Barcelona, Spain). The inclusion criteria were as follows: the patients who required a sinus augmentation by lateral window approach under local anesthesia; presence of maxillary alveolar bone atrophy and presence of healthy systemic

conditions without any contraindicated surgery. Patients were not admitted to this study if any of the following criteria were presented: a) smoke more than 10 cigarettes per day, b) pregnancy, c) diabetes mellitus, d) suffer from disease that compromise the surgery, and e) medication with bisphosphonate. In case a patient needed bilateral sinus augmentation, it was decided to evaluate only the first unilateral site. If a patient was excluded from the investigation, the sinus elevation would be carried out to criterion of the surgeon. Medical history was recorded for each patient. Preoperatively, panoramic and computerized tomography (CT) were evaluated for maxillary sinus augmentation. One hour before surgery, antibiotic prophylaxis of two grams of amoxicillin was administered. The study was conducted in accordance with the standards of the Ethics Committee of the International University of Catalonia, Barcelona, Spain. All patients were carefully informed of the inherent risks of the operation, and patients accepted to participate in this study. A consent form was given and signed for each patient.

### *Surgical Procedure*

The surgical sites were anesthetized locally with 4% of articaine with epinephrine 1:100.000 (Ultracain, Norman, S.A., Madrid, Spain) via infiltration at the vestibule and palatal surgical sites. A full-thickness mucoperiosteal flap was elevated with a periosteum elevator until the anterolateral wall of the sinus cavity was fully exposed. A rectangular lateral window osteotomy approach was performed to access the Schneiderian membrane. The particulated bone samples (control group: Sc group, n=4) were harvested with the bone scraper (C.G.M.S.p.A. Divisione medicale meta, Reggio Emilia, Italy) from the central part of the exposed bone surface of the rectangular window (Fig. 1). Half of the lateral window osteotomy was performed using the piezoelectric device (Surgysonic II<sup>®</sup>, Esacrom, Imola-Bologna, Italy) with the diamond round tip of 1.8 mm in diameter (ESO8A) (P group, n=4) with copious sterile saline irrigation. The piezoelectric device has a maximum vibration frequency of 35 kHz, which was chosen in the present study. Gentle scratching movements and very little pressure was applied. Osteotomy was done until visualization of the Schneiderian membrane, then the bone samples were collected with a surgical curette. The other half of the lateral window osteotomy was accomplished by using a round carbide bur of number 8 size (Gebr. Brasseler GmbH&Co.KG, Lemgo, Germany) mounted in a 20:1 straight handpiece (S-11 W&H Dentalwerk, Bürmoos GmbH, Austria). A surgical implant motor unit was used as power source (Implantmed<sup>®</sup>, W&H Dentalwerk Bürmoos, Austria) (R group, n=4) at a speed of 600 rpm. Copious sterile saline irrigation was used to prevent overheating and using an intermittent pressure on bone in order to cause the least thermal changes. Osteotomy was done until exposure of the Schneiderian membrane;

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then, the bone samples were collected with a surgical curette. The rest of the sinus lift procedure continued normally for each of the four patients in the study. In one patient, the sinus lateral approach technique was performed bilaterally on different days. On one side, particulated bone samples were harvested for the study and the other side the bone lid resulting from the rectangular window osteotomy (performed only with rotary surgical instrumentation) was removed and analyzed as a block graft. All samples were collected with a sterile technique and contact with the patient's saliva was avoided by using aspiration tips.

Samples were then placed into microtubes that were filled with collagenase I solution (2 mg/ml). Samples were divided in 3 groups; bone from rotary surgical instrument (R), bone from piezoelectric device (P), bone from bone scraper (Sc), and bone block obtained from the rectangular window osteotomy was studied separately. After the operation, Amoxicillin-Clavulanic Acid (the dose depended on the patient's physical characteristics) was prescribed for one week. If patient presented allergy to penicillin, Clindamycin was

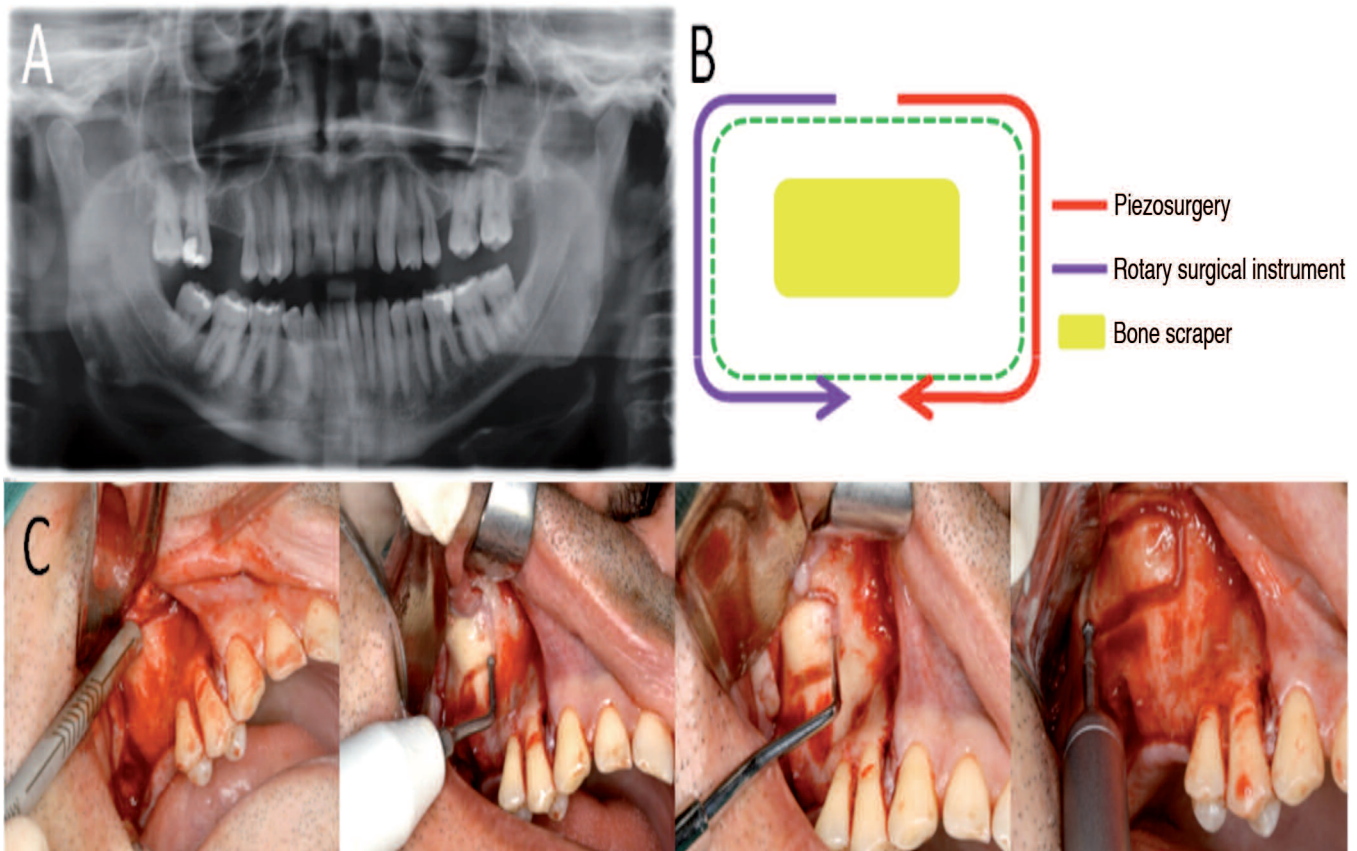
prescribed instead. For pain relief the patient was instructed to take Ibuprofen 600 mg/6 hours, unless the patient presented some contraindication. In case of stomach problems, Omeprazol 20 mg/day was prescribed.

### Laboratory Procedure

The samples were placed into microtubes and were fixed with 4% paraformaldehyde (Sigma) for 30 min at room temperature. Once placed in the laminar flow hood, they were gently washed twice with PBS 1% BSA (Sigma), and were incubated at 37°C for 45 minutes in a shaking water bath with collagenase I 3% solution for signal cell suspension. After that samples were centrifuged at 1800 rpm for 10 minutes at 4°C and were then washed with phosphate-buffered saline (PBS) 200 ml.

### Flow Cytometry (Annexin V-FITC/ PI Assay)

Flow Cytometry Analysis (FAC) was carried out the



**Fig. 1.** Surgical procedure. **A.** Maxillary alveolar bone atrophy at first quadrant. **B.** Diagram showing the areas of bone sample harvest in the lateral window design for the sinus augmentation procedure. **C.** Bone was harvested with bone scraper and then half of the lateral window osteotomy was performed with piezoelectric device. The other half of the lateral window osteotomy was then performed with rotary surgical instrument.

same day of the intervention. For the analysis of control samples different IgG isotypes coupled to FITC fluorochromes (BD Pharmingen, San Diego, CA) were used. The cell suspension was incubated for 45 minutes at 4°C in darkness. Later, the cells were washed twice with PBS and centrifuged for 6 minutes at 1,800 rpm. In this way, the fluorochrome residues were removed, since they would give a false fluorescence percentage. All flow cytometry measurements were made using a FACScan™ cytometer (FACSCalibur system) and analyzed with the CellQuest Software. More than 500,000 cells were used from each sample in order to detect unspecific unions or auto fluorescence. Following the protocol of Annexin V-FITC Kit (Miltenyi Biotec), cells were washed with resuspended cells in 1X Binding Buffer at a concentration of  $1 \times 10^6$  cells per ml, then the samples were filtered with 0.22  $\mu\text{m}$  single use filter (Millipore). This step was followed by centrifuge at 300xg for 10 minutes. Afterwards the cell pellets were resuspended in 100  $\mu\text{L}$  of 1x Binding Buffer and 10  $\mu\text{L}$  of Annexin V-FITC was added ( $10^6$  cells in the microtubes R, P and Sc group). Afterwards, samples were incubated for 45 minutes in the dark at 4°C and then centrifuged at 300xg for 10 minutes. Finally, samples were resuspended cell pellet in 500  $\mu\text{L}$  of 1x Binding Buffer per  $10^6$  cells and add 2  $\mu\text{L}$  of

propidium iodide. Then, the samples were analyzed by FACS. To verify our technique, osteoblast cells cultured were tested with Annexin kit according to the manufacturer's protocol (data not shown).

#### Immunofluorescence analysis

Anterolateral maxillary sinus bone lid, which served as a cortical block graft study model, was fixed with 4% paraformaldehyde (Sigma) for 30 min at room temperature after washing twice with PBS 1% BSA (Sigma). Later, samples were sectioned at a thickness of 4  $\mu\text{m}$  for Immunofluorescence staining. Slides were incubated at 4°C overnight with fluorescein as a negative control and with Annexin V-FITC (fluorescein isothiocyanate stain) (1:400, Miltenyi Biotec) and propidium iodide (1:400, Miltenyi Biotec). After that, the slides were washed twice with PBS plus 1% BSA (Sigma). Slides were examined by confocal fluorescence microscopy (Confocal 1024 microscope, Olympus AX70, Olympus Optical, Tokyo).

#### Statistical analyses

Mean values and standard deviation were calculated. Apoptosis, necrosis, living cells and apoptosis and necrosis among the three instruments were examined by multifactorial variance analysis (ANOVA). In all analyses, a P-value of 0.05 was considered to represent statistical significance.

**Table 1.** Data of rotary surgical instrument group.

	Apoptosis	Necrosis	Living cells	Apoptosis+Necrosis
Patient 1	97.4	0.2	2.4	97.6
Patient 2	96.2	0.18	3.62	96.38
Patient 3	94.8	0.5	4.7	95.3
Patient 4	95.2	0.3	4.5	95.5
MEAN	95.9	0.295	3.805	96.195

**Table 2.** Data of piezoelectric device group.

	Apoptosis	Necrosis	Living cells	Apoptosis+Necrosis
Patient 1	96.8	0.1	3.1	96.9
Patient 2	95.2	0.16	4.64	95.36
Patient 3	97.9	0.28	1.82	98.18
Patient 4	96.3	0.9	2.8	97.2
MEAN	96.55	0.36	3.09	96.91

**Table 3.** Data of Bone scraper group.

	Apoptosis	Necrosis	Living cells	Apoptosis+Necrosis
Patient 1	98.1	0.16	1.74	98.26
Patient 2	97.5	0.16	2.34	97.66
Patient 3	96.9	0.41	2.69	97.31
Patient 4	97.5	0.081	2.419	97.581
MEAN	97.5	0.20275	2.29725	97.70275

## Results

#### Annexin V-FITC/ PI Assay

The analysis was performed on the same day of intervention. Specific antibodies to a particular cell were used to label the cells in flow cytometry; they are directly conjugated to a fluorochrome. In this study, specific antibodies were used to detect cell apoptosis and necrosis (AnnexinV FITC). The control of fluorescence used FITC- conjugated IgGI antibody (BD Pharmingen) to detect non-specific binding or autofluorescence. Non-specific bindings were not detected, as shown in Fig. 2.

The percentage of cells undergoing apoptosis or necrosis was measured with annexin V and propidium iodide staining, respectively, followed by FACS. Compared with control group (Fig. 2), cells exhibited apoptosis. As shown in table 1, data of R group, mean percentage of apoptosis, necrosis, living cells and apoptosis/necrosis is 95.9, 0.29, 3.80, and 96.19, respectively. Data of P group, mean percentage of apoptosis, necrosis, living cells and apoptosis/necrosis is 96.55, 0.36, 3.09, and 96.91, respectively (Table 2). Data of Sc group, mean percentage of apoptosis, necrosis, living cells and apoptosis/necrosis is 97.5, 0.20, 2.23, and 97.70, respectively (Table 3).

Comparison among three groups, (Table 1-3) mean percentage of apoptosis in R group (95.9) and P group

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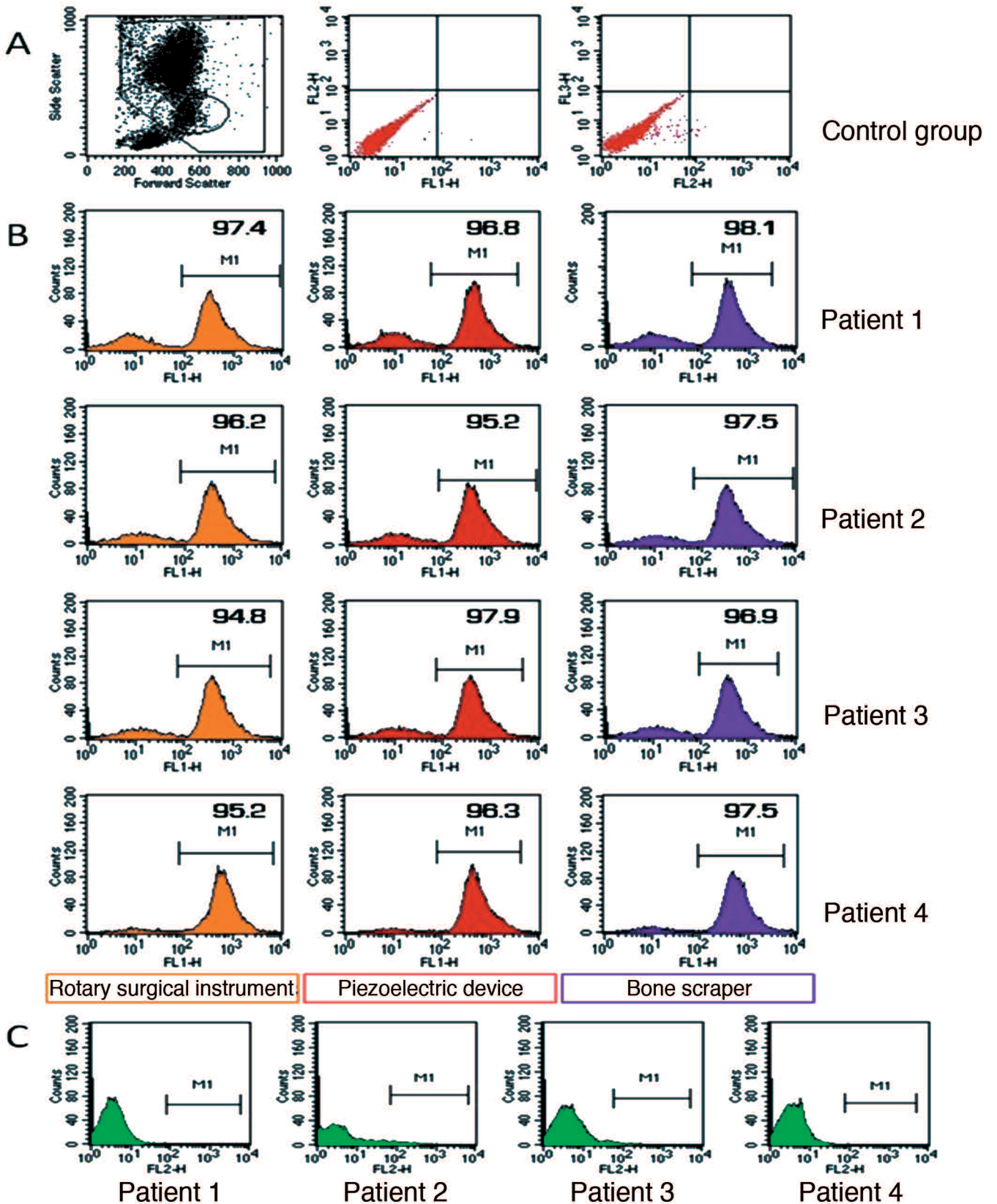


Fig. 2. Annexin V-FITC assay of apoptotic effect by FACS analysis.

(96.55) are similar to Sc group (97.5). Likewise, mean percentage of necrosis in R group (0.29) and P group (0.36) are similar to Sc group (0.20). Among the three groups a low percentage of living cells were detected; R group (3.80), P group (3.09) and Sc group (2.23). Finally, total percentage of apoptosis/necrosis are 96.19 in R group, 96.91 in P group and 97.70 in Sc group.

The interval plot in multifactorial ANOVA shows the mean and 95% confidence intervals in order to detect differences between apoptosis, necrosis, living cells and apoptosis/necrosis among the three different instruments. As shown in Fig. 3, there were no significant differences of presence of apoptosis processes among instruments or patients (P-value=0.15 and 0.51, respectively). The analysis of necrosis also had no statistical significance among instruments or patients (P-value=0.66 and 0.41, respectively). Significant differences in the presence of living cells could not be found among instruments or patients (P-value=0.15 and 0.65, respectively). There were no significant differences of apoptosis/necrosis processes among instruments or patients (P-value=0.15 and 0.65, respectively) (Fig. 3).

*Immunofluorescence analysis*

To confirm our result, we performed a study of immunofluorescence analysis in order to see the viability of the cortical bone block obtained. The results showed a high positive percentage of Annexin V-FITC (green) and

low positive percentage of propidium iodide (red) as shown in Fig. 4.

**Discussion**

The reason why sinus lift procedure with the lateral approach was used in the present study is because while performing the osteotomy with such a technique great amounts of particulated bone samples can be obtained, as well as the lateral cortical bone lid. Often these grafts are discarded after the procedure. Therefore, the study is in compliance with the ethical guidelines on human research. Also, another advantage is that the area where these bone grafts are obtained (the facial surface of the maxillary bone and part of the malar process) is away from the resorbed and atrophic alveolar process and presents a healthy bone source.

Only four patients participated in the study, although statistical results are strong since bone samples for control and test groups were obtained from the same patient and same bone area.

The bone lid resulting from the lateral window osteotomy was analyzed as a block graft and compared as a frequently used intraoral donor site. Frequently used autogenous donor sites are mandibular bone grafts from either the ramus or symphysis area. However, grafts from the mandible (ramus and symphysis) and the maxillary sinus lateral wall have the same cortical composition, but different embryologic origin. A study

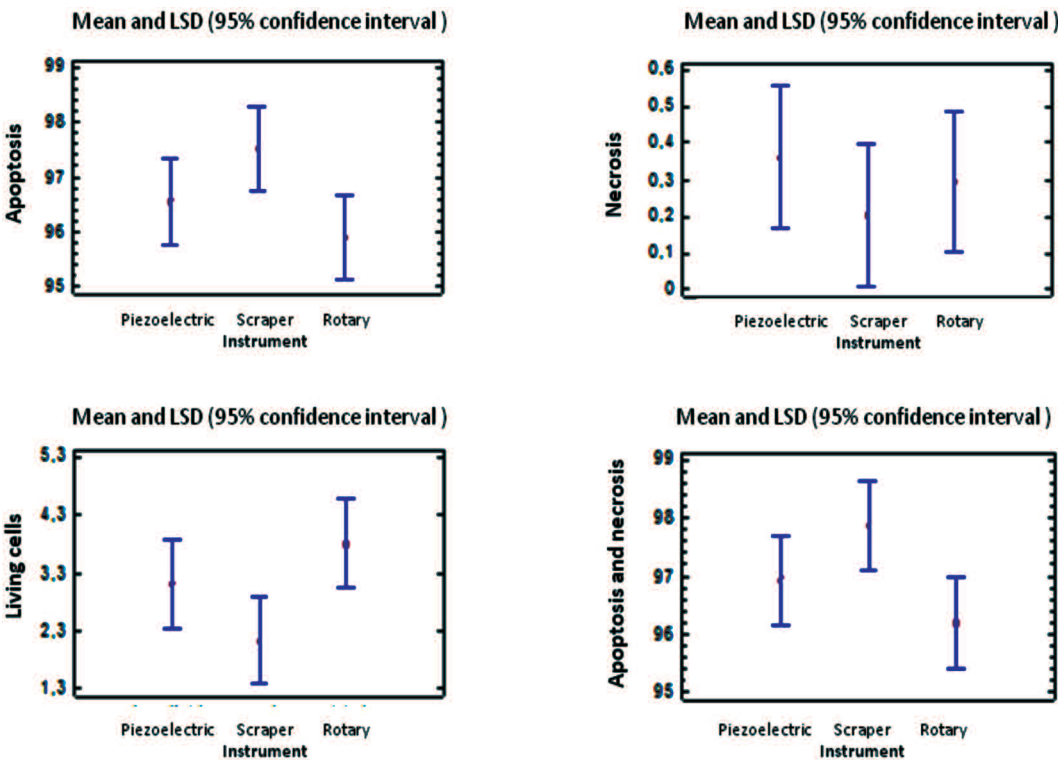


Fig. 3. Mean and LSD of Apoptosis, Necrosis, Living cells and Apoptosis/Necrosis among three instruments.

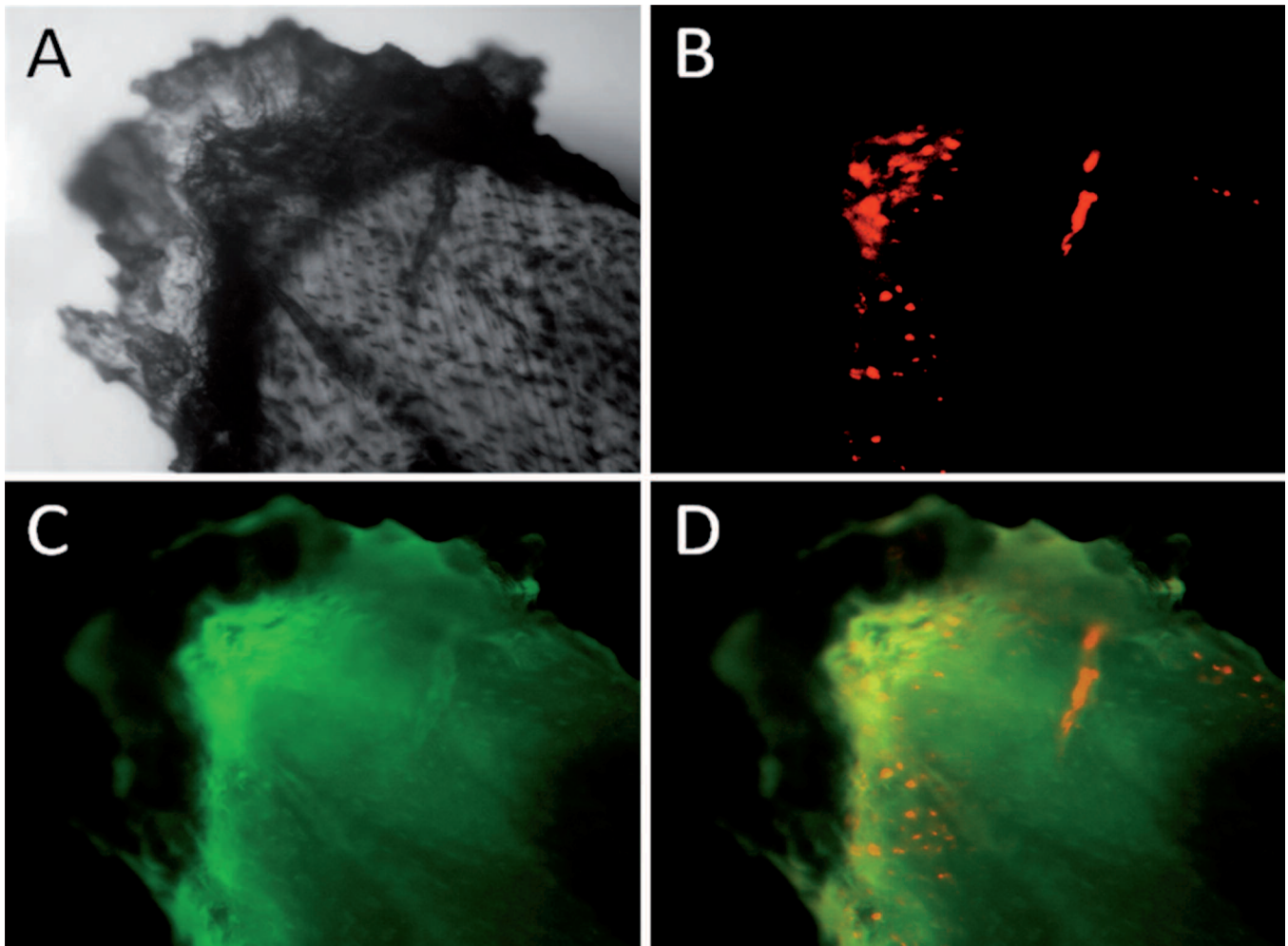
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by Rosenthal and Buchman (2003), states that volume stabilization and integration of the intraoral bone graft is directly related to the bone micro-architecture rather than its embryologic origin. Therefore, comparisons between mandibular and maxillary bone blocks can be made.

In this study, we evaluate the viability of particulated and block bone grafts measured with annexin V and propidium iodide staining. There is no other study found in the literature that used such analysis to evaluate the viability of human intra-oral maxillary bone grafts. Therefore, exact comparisons between similar studies cannot be done due to different instruments used and different analytical approaches.

Past studies regarding the time period which bone cells can survive showed the following results: Ellegaard et al. (1975) showed that 1 week after bone grafting, most of the osteocyte lacunae were empty, Chugh et al.

(1998) also reported that 50% of osteocytes did not survive after 1 week post grafting, and Berggren et al (Berggren et al., 1982) reported that osteocytes and osteoblasts can survive up to 25 hours after grafting if they are stored in a cold culture solution. It is important to notice that the samples used in this study were analyzed as soon as they were extracted from the patient, therefore, the status of bone cell activity at the time of bone harvesting was revealed. High percentages of apoptosis and necrosis were found: 96.1% in R group, 96.9% in P group and 97.7% in Sc group. Interestingly, according to our results, particulated and block graft presented programmed cell death and necrosis as soon as they were extracted from the donor site. However, in the present study, cell death activity could not be assigned to a particular bone cell type, either from osteoclasts, osteoblasts or osteocytes, since histological and histomorphometrical analysis were not performed.



**Fig. 4.** Immunofluorescence analysis of anterolateral sinus bone wall. **A.** Slide of the bone block. **B.** Small red areas indicating presence of necrosis. **C.** Green stain, indicating presence of apoptosis. Notice that almost all the block slide underwent apoptosis. **D.** Apoptosis and necrosis stain overlapped. x 10

More recent studies regarding cell viability with different harvesting instruments showed different results. Chiriac et al. (2005) reported higher percentages of cultured osteoblast cells for both rotary drill and piezoelectric device, 88.9 % and 87.9% respectively. They concluded that the harvesting methods are not different concerning the detrimental effect on viability. Another study by Springer et al. (2004) showed higher amounts of cultured osteoblasts in samples with large particle size spongy bone chips, indicating that large particle size bone marrow grafts contain more bone cells and they have more chance to survive. These findings are in agreement with an histological study by Berengo et al. (2006) where they reported higher vital bone cells when bone grafts had large particle sizes. Another study by Bacci et al. (2011) showed contradictory results, their histological findings revealed that smaller particle size bone chips, obtained with a piezoelectric device, had less vital bone (64.83%) compared with the small particle size bone chips harvested with a bone scraper (75.34%).

In the present study, although no statistical differences were obtained between groups, the worst results in terms of cell viability were for the control group of the bone scraper, 97.7% of apoptosis and necrosis and the least amount of living cells: 2.2%. However, this group showed the least amount of necrosis percentage of 0.2%. These findings are in agreement with the study of Berengo et al. (2006) where they found 100% of non vital bone cells in bone grafts harvested with the bone scraper. But at the same time these findings differ from another histological study of Zaffe and D'Avenia (2007) where the bone scraper was used and obtained a mean bone viability between 45-72%. The R group showed the least amount of apoptosis percentage (95.9%) but showed the greatest amount of living cells (3.8%) among the three groups. The P group showed very similar results compared with the rotary group, although it showed an increased percentage of apoptosis and necrosis and a decreased percentage of living cells (96.55, 0.3 and 3.09 respectively). Therefore, it remains unclear to what extent particle size and device used can influence the viability of bone cells after harvesting. However, the bone quality of the patient seems to be an important factor, as some studies reported higher percentages of bone cells when trabecular bone was harvested (Zerbo et al., 2003; Springer et al., 2004).

The bone block that was harvested from the lateral window osteotomy was analyzed using immunofluorescence analysis. A high positive percentage of Annexin V-FITC (green) and low percentage of propidium iodide (red) areas were found (Fig. 4), indicating that most of the bone block underwent apoptosis and small areas of necrosis appeared. These results differ from the histomorphometrical results obtained by Berengo et al. (2006) and Guillaume et al. (2009) where they found high percentages of vital bone when block grafts were harvested. However, another study by Zerbo et al. (2003) evaluated the survival of osteocytes and graft viability after ramus bone block

regeneration. They reported that after a healing time of 7 months 11.1% of non-vital bone was present and that the majority of the osteocytes did not survive the grafting procedure.

Recent investigations have reported that osteocytes are involved in sensing mechanical stimuli inside the lacunar-canalicular system (Weinbaum et al., 1994; Klein-Nulend et al., 1995; Guillaum et al., 2009). Therefore, certain forces or vibrations, like the ones provoked by certain devices or instruments during harvesting, can induce osteocytes to undergo apoptosis or necrosis. However, this observation remains unanswered by scientific studies.

### Conclusions

The analytical methods used in this pilot study allowed the gathering information regarding bone cell status at the time of bone harvesting. Within the limits of the present study, the results showed that none of the intra-oral harvesting methods used (piezoelectric device, rotary instrumentation and bone scraper) could get viable bone cells after the extraction of bone particles, and higher percentages of apoptosis were found in all samples. Moreover, when cortical bone block was harvested bone cells also underwent apoptosis and necrosis processes. More well-designed prospective clinical studies are needed to better understand bone healing process in grafting procedures.

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*Ethical regulations.* Bone tissues used for these experiments were obtained with informed consent from donors. All experiments were performed in accordance with the guidelines on human research issued by the Committee on Bioethics of the International University of Catalonia.

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