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STRUCTURAL AND ULTRASTRUCTURAL ANALYSES OF BONE REGENERATION IN RABBIT CRANIAL OSTEOTOMY: PIEZOSURGERY *VERSUS* TRADITIONAL OSTEOTOMES

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ABSTRACT

Clinical advantages of piezosurgery have been already proved. However, few investigations have focused on the dynamics of bone healing. The aim of this study was to evaluate, in adult rabbits, bone regeneration after cranial linear osteotomies with two piezoelectrical devices (Piezosurgery Medical – PM and Piezosurgery Plus – PP), comparing them with conventional rotary osteotomes (RO). PP was characterized by an output power three times higher than PM. Fifteen days after surgery, histomorphometric analyses showed that the osteotomy gap produced with PM and PP was about half the size of that produced by RO, and in a more advanced stage of recovery. Values of regenerated bone area with respect to the total osteotomy area were about double in PM and PP samples compared with RO ones, while the number of TRAP-positive (tartrate-resistant acid phosphatase positive) osteoclasts per linear surface showed a significant increase, suggesting greater bone remodelling. Under scanning electron microscopy, regenerated bone displayed higher cell density and less mineralized matrix compared with pre-existent bone for all devices used. Nanoindentation tests showed no changes in elastic modulus. In conclusion, PM/PP osteotomies can be considered equivalent to each other, and result in more rapid healing compared with those using RO.

Keywords: piezosurgery; cranial osteotomy; bone regeneration; nanoindentation; elastic modulus

1. Introduction

In maxillofacial, orthopedic, and hand surgery, as well as neurosurgery, and aesthetic and reconstructive surgery, there is an increasing demand for precise and safe bone cutting techniques (Chacon et al., 2006; Queiroz et al., 2008). Traditionally, rotating instruments, such as burs, have been used for bone surgery. However, the use of these traditional mechanical systems entails disadvantages, including bone overheating, bone fragmentation, formation of a 'smear layer' during osteotomy, and damage to adjacent tissues (Giraud et al., 1991; Tehemar et al., 1999; Chacon et al., 2006; Queiroz et al., 2008). Minimally invasive surgery is also important for providing fast healing after bone surgery (Yang et al., 2014). A precise and safe bone cutting technique is of the utmost importance for the preservation of delicate bony structures and protection of adjacent soft tissues (Avsar, 2012). Therefore, recent aims include the development of selective cutting instruments with improved surgical performance and manoeuvrability (Vercellotti, 1991; Schwieger et al., 2004).

The desire for minimally invasive surgical techniques in recent years has led to the introduction of ultrasonic bone (Yang et al., 2014). Ultrasonic bone surgery, also called 'piezoelectric bone surgery', or simply 'piezosurgery', is a micrometric selective technique that uses a defined ultrasonic frequency, in the range 24–32 kHz, that is capable of cutting bone (Schlee et al., 2006; Itro et al., 2012). Its mechanism of action is based on the ability of certain ceramics and crystals to deform when an electric current is passed across them, resulting in micro-vibration at ultrasonic frequency (Eggers et al, 2004; Leclercq et al., 2008). The tip cuts selectively mineralized tissues without cutting soft tissues, thereby limiting the risk of damage to blood vessels and nerves during bone surgery, which is especially important in the craniofacial skeleton. Intraoperative advantages of ultrasonic osteotomy lie in the better visibility of the surgical field, the precise control of cuts, and, above all, minimal damage to adjacent soft tissues (Labanca et al., 2008; Gonzales-Garcia et al., 2009; Farrel et al., 2011; Gabric et al., 2016). Additionally, the absence of macro-vibrations (typical of rotary or oscillating instruments) makes piezosurgery an advanced osteotomy technique, especially suited for oral and maxillofacial applications, where proximity to delicate structures, such as eyes, nerves, and teeth is a frequent concern.

Preclinical and clinical studies, combined with *in vitro* studies, have shown that piezosurgery produces clean and precise osteotomies with smooth walls and decreased bleeding (Sortino et al., 2008; Claire et al., 2013). However, up to now, most piezosurgical devices show poor capability of cutting dense bone and take longer compared with rotating burs or saws (Eggers at al., 2004; Gonzales-Garcia et al., 2009; Farrel et al., 2011).

Technological improvements have been recently introduced in some devices to provide safe, accurate, faster, and smaller osteotomy gaps, including in dense bone, such as the cranial vault, skull base, or vertebrae. These include new devices, such as Piezosurgery Plus® (Mectron Medical Technology, Carasco, Italy), Bone Scalpel® (Misonix, Farmingdale, New York, USA), and Sonopet® (Stryker, Kalamazoo, MI, USA), characterized by a greater output power (Table 1).

Scientific data in the current literature do not provide a conclusive answer on whether piezosurgery presents a clear advantage in terms of faster bone healing over the traditional osteotomy systems. Most publications on piezoelectric devices are clinical case reports; few of them are *in vivo* studies reporting on the use of piezoelectric surgical devices and their relation to healing speed compared with conventional rotary instruments (Table 2).

The purpose of this paper was to compare bone healing dynamics in experimental osteotomies, using two piezosurgical devices with different output power (Piezosurgery Medical® and Piezosurgery Plus®) and a conventional rotary osteotome, in an *in vivo* rabbit model.

2. Materials and Methods

2.1 Animal model

Sixteen healthy 6-month-old white New Zealand rabbits (Harlan Laboratories S.r.l., Correzzana, Monza e Brianza, Italy), with an average body weight of 5.00 kg, were used. The animals were maintained for acclimation to housing conditions, with food and water *ad libitum*, for at least for 1 week before surgery. The night before surgery, each animal was fasted in preparation for anaesthesia. All experiments were carried out according to the Bioethical Committee of the Italian National Institute of Health, and authorized with Decrees of the Italian Ministry of Health (protocol number 210/2013-B). Animal care, maintenance, and surgery were conducted in accordance with Italian law (D.L. no. 26/2014) and European legislation (EEC no. 63/2010).

2.2 Surgical Procedure

All animals were weighed and submitted to the same surgical procedure under general anaesthesia using a mixture of xylazine (4 mg/kg body weight) (Sedaxylan®, Dechra Veterinary Products S.r.l., Turin, Italy) and ketamine (30 mg/kg body weight) (Imalgene 1000®, Merial Italia S.p.A., Milan, Italy). If necessary, further sedation was carried out using propofol (7 mg/kg) (Propovet®, Ecuphar S.r.l., Piacenza, Italy) administrated in the marginal ear vein. After induction of anaesthesia, shaving and antisepsis of the areas to be operated (calvaria) was carried out. Four pairs of linear craniotomies (about 1 cm in length) were performed in each skull, using different surgical osteotomy techniques: i) conventional rotary osteotomy device (RO) (bur: H33L.316.016; Komet Italia S.r.l., Milan, Italy; motor system and drilling procedure: 1700 rpm, Physiodispenser 7000, Nouvag AG; Switzerland); ii) Piezosurgery® Medical (PM), and iii) Piezosurgery® Plus (PP), both provided by Piezosurgery® – Mectron Medical Technology (Carasco, Italy). The main difference between PM and PP is the higher output power of PP (75 W) compared with PM (23 W); automatic scansion is comparable in both instruments (24 KHz–36K Hz) (Figure 1). In each skull, all osteotomies (two by means of RO, two by PM, four by PP – see Figure 2) were performed by the same surgeon, using all aforementioned ultrasonic devices in automatic scansion mode (from 24 KHz to 36 KHz). The fascia-periosteal flaps were sutured with 4.0 glycolide/L-lactide copolymer (Vicryl®, Ethicon, Johnson & Johnson, Livingston, Scotland) and the skin with 3.0 silk (Perma-hand® Silk Suture, Ethicon, Johnson & Johnson, Livingston, Scotland). Postoperatively, single intramuscular injections of antibiotics (enrofloxacin, 10 mg/kg body weight) (Baytril 5%®, 50 mg/ml, Bayer S.p.A., Milan, Italy) and analgesic (buprenorphine, 0.05 mg/kg body weight) (Temgesic®, Indivior Italia S.r.l., Milan, Italy) were given.

2.3 Specimen collection

All animals were euthanized 15 days after surgical osteotomy with an intravenous injection of embutramide plus mebezonium iodide (0.3 ml/kg body weight) (Tanax® 50mg, MSD Animal Health S.r.l. Italia, Segrate, Milan, Italy), under general anesthesia with a mixture of xylazine (4 mg/kg body weight) (Sedaxylan®, Dechra Veterinary Products Srl, Turin, Italy) and ketamine (30 mg/kg body weight) (Imalgene 1000®, Merial Italia SpA, Milan, Italy). After euthanizing, the whole calvaria was removed from each animal, using a slow-speed saw, and the portion of bone

containing the four pairs of osteotomies was subdivided in four fragments, and then fixed for 12 h with 4% paraformaldehyde in 0.13 M phosphate buffer, pH 7.4, at 4°C. A total of 128 linear osteotomies were finally obtained (32 RO osteotomies, 32 PM osteotomies, 64 PP osteotomies), processed for light microscopy (LM), scanning electron microscopy (SEM)/nanoindentation, and transmission electron microscopy (TEM), according the following scheme:

	RO	PM	PP
LM	16	16	32
SEM/nanoindentation	8	8	16
TEM	8	8	16

2.4 Histology and enzymatic assay

Each fixed specimen was dehydrated in ascending ethanol series and embedded in polymethylmethacrylate (PMMA) at 4°C, without decalcification. For histological analysis, the embedded bone samples were cut along the perpendicular plane with respect to the skull surface, so that the entire calvaria thickness (external compact bone, diploë, internal compact bone) was visible adjacent to the osteotomy gap. Histological sections (5 μm thick) were obtained by means of a bone microtome (Autocut 1150-Reichert-Jung, Nussloch, Germany). Gomori trichrome stain and tartrate-resistant acid phosphatase (TRAP) reaction were performed on bone sections.

2.5 Histomorphometric analysis

Stained sections were observed under a light microscope (Leica DM 2500, Leica Microsystems, Wetzlar Hessen, Germany) connected to a digital camera (DSC295m, Leica Microsystems, Wetzlar Hessen, Germany), and digital pictures were recorded. The osteotomy gap width was evaluated using the Image-J software ‘straight line tool’, measuring the distance between the two native bone surfaces surrounding the osteotomy (Figure 3).
Bone regeneration was evaluated by measuring, on digitalized pictures, the amount of bone present inside the gap, and expressed as BV/TV%. The area of bone present inside the osteotomy (BV) and the total area of the osteotomy (TV) were measured using the of Image-J ‘polygon tool’, in order to calculate the BV/TV% value (Figure 4). In addition, as an index of remodelling activity, the number of TRAP-positive cells (stained in red) was counted within the osteotomy gap and expressed as number of osteoclasts per surface of bone (N OCL/mm); the bone surface was measured manually by outlining the perimeter of the bony trabeculae within the osteotomy gap, using Image-J software (see Figure 5).

To evaluate the minimal distance (md) from the osteotomy edge (OE) at which osteocytes (OC) were recognized as viable cells (OC-OE_md) in all osteotomized native bones, Gomori-trichrome-stained histological sections were observed under LM using an ocular containing a micrometric scale.

2.6 Transmission electron microscopy (TEM)

Each sample to be processed for TEM observation (see ‘*Specimen collection*’ above) was post-fixed for 1 h with 1% osmium tetroxide in 0.13 M phosphate buffer, pH 7.4, dehydrated in graded

ethanol, and embedded in epoxy resin (Durcupan ACM). Samples were then sectioned with a diamond knife mounted in an Ultracut-Reichert microtome (Reichert-Jung GmbH, Nussloch, Germany). Semi-thin sections (1 µm) were stained with toluidine blue and examined with an Axiophot-Zeiss light microscope (Zeiss Axiophot; Jena, West Germany); ultrathin sections (70–80 nm) were mounted on Formvar- and carbon-coated copper grids stained with 1% uranyl acetate and lead citrate, and examined using Zeiss EM109-TEM (Carl Zeiss AG, Jena, Germany).

2.7 Scanning electron microscopy (SEM)

For SEM analysis, some PMMA-embedded samples were polished with a series of increasing grit emery papers, and finally with liquid alumina powder, before being sputter-coated with a 10 nm gold-palladium layer (Emitech K550, Emitech Ltd, Ashford, Kent, UK). Samples were observed with an ESEM (SEM-QUANTA 200, FEI Company, the Netherlands) under low vacuum, using the backscatter mode in order to gain information on the degree of bone mineralization.

2.8 Nano-mechanical analysis

Nanoindentation tests were carried out in order to evaluate the maturity of newly formed bone for the different osteotomy methods used. In particular, the reduced elastic modulus (Er) of novel (within the osteotomy gap) and pre-existent native (far from the osteotomy edge) bone tissue was investigated. Reduced elastic modulus (Er) has been reported to be directly related to the degree of mineralization of bone tissue (Bala et al., 2011; Gupta et al., 2005). Thus, by evaluating the mechanical properties of regenerating bone and comparing these with those of pre-existent bone, additional insights into bone growth dynamics can be obtained (Bianchi et al., 2015). Indentation tests were performed on polished PMMA-embedded histological sections from five calvaria, using a nanoindentation tester, NHT2 (CSM Instruments SA, Peseux, Switzerland) equipped with a diamond Berkovich tip, after calibration with a fused quartz (indentation modulus ~ 72 GPa). Matrix variable-sized 2D indents were drawn; the distances between subsequent indents were set at 25 µm in both *x* and *y* directions to avoid any influence of residual stresses due to adjacent indentations. Fifty to 130 indents were performed on each sample. The protocol applied included a trapezoidal load control profile with maximum load of 8 mN and holding time of 30 s; loading and unloading time was 10 s. Modulus data were extracted from the load-depth curve using the Oliver-Pharr method (Oliver and Pharr, 2004).

2.9 Statistical analysis

For histomorphometric evaluations, one-way analysis of variance (ANOVA), followed by Bonferroni post hoc tests between groups, were performed using the software STATA 11.0 (StataCorp, Texas, USA). Values of $p < 0.05$ indicated significant differences between groups.

For nano-mechanical data, statistical analysis was performed using MATLAB software (version 7.13.0.564, Mathworks, Natick, MA, USA). After verifying the normal distribution of data with a Lillie test, the Kruskal-Wallis non-parametric test was used to compare elastic modulus values for pre-existent bone in all animals, whereas the Wilcoxon-Mann-Whitney non-parametric test was applied to compare the modulus values for newly formed and pre-existent bone for each osteotomy

method (RO, PM, PP). All data have been reported as mean values ± standard deviation, at a significance level of $p < 0.05$.

3. Results

3.1 Histology and histomorphometric analysis

Histological analysis showed that osteotomies performed using different devices produce different sizes of osteotomy gap: PM and PP devices result in bone gaps around half the thickness of those from RO devices ($p < 0.001$ ANOVA, with Bonferroni post hoc) (Table 3 and Figure 3).

In all samples, we observed different amounts of both fibrous tissue and newly forming bone, depending on the device used; in particular, larger amounts of fibrous tissue compared with bone tissue were present in RO samples (Figure 6). Histological differences were observed in the perivascular stromal spaces between bone-forming trabeculae, which appeared wider in RO osteotomies compared with PM and PP ones (Figure 3). Moreover, close to the forming bony trabeculae, numerous osteoblasts, arranged in cords and involved in preliminary bone regeneration, were observed in RO samples, whereas, in PM and PP samples, bony trabeculae were mostly covered with typical prismatic osteoblasts, arranged in monostratified laminae and involved in bone compaction (Figure 7).

Concerning BV/TV, differences were found between RO and PM/PP samples: the values are about double in PM and PP osteotomies with respect to RO ones, although this was not statistically significant (Table 3). TRAP reaction, on the other hand, showed a significant increase in N OCL/mm in PM and PP devices with respect to RO ones; the significance was higher in PP vs RO than in PM vs RO (Table 3 and Figure 8).

The values for minimal distance from osteotomy edge (OE) at which osteocytes (OC) were recognized as viable cells in osteotomized native bones by means of RO, PM, and PP devices (OC-OE_md) are shown in Table 3: in PP osteotomies, viable osteocytes were observed closer to the osteotomy edge (mean value of minimal distance 38.2 µm) compared with RO (83.4 µm) and PM (65.7 µm) – with statistical significance.

3.2 TEM

Ultrastructural analysis confirmed evidence from histological observations: inside the repairing bone gap in RO osteotomy, osteoblasts arranged in cords involved in preliminary bone regeneration were frequently recorded; whereas, in PM and PP osteotomies, prismatic osteoblasts arranged in monostratified laminae were present close to the formed preliminary trabeculae (Figure 9). Observations performed on the native bone close to the edge of the osteotomies showed the presence of abundant, non-viable osteocytes in RO, and confirmed the presence of viable osteocytes in PP and PM (Figure 10).

3.3 SEM

For all osteotomies, independently from the device used, SEM analysis showed, as expected, that the newly formed bony trabeculae inside the osteotomy gap were less mineralized compared with the adjacent pre-existent bone; moreover, the regenerated bone was characterized by higher cell density compared with the pre-existent bone. When comparing bone gap filling, it was possible

to observe, only in RO osteotomies, the presence of some fragments/remnants of osteotomized bone, which were absent in PM and PP osteotomies (Figure 11).

3.4 Nano-mechanical analysis

Figure 12 shows the results of the nano-mechanical tests. Indents were performed on pre-existent and newly formed regenerated bone tissue after osteotomy using RO, PM, and PP. Significant differences were found between modulus values for pre-existent bone, so mechanical data from the five investigated rabbits were not pooled to avoid animal-dependent effects. PP and RO methods applied alternately resulted in newly formed bone with similar mechanical properties to pre-existent bone (PP in R1 and R3; RO in R2 and R4).

4. Discussion

Safe bone cutting techniques are required in maxillofacial, orthopedic, and hand surgery, as well as neurosurgery, and aesthetic and reconstructive surgery (Chacon et al., 2006; Queiroz et al., 2008). Several studies report the extensive clinical use of these techniques and/or provide an evaluation of the bone cutting qualities of the surgical instruments used, above all when bone needs to be cut adjacent to important soft tissues, such as the nerves, vessels, Schneiderian membrane, or the dura mater, and where mechanical or thermal injury should be avoided (Pavlíková et al., 2011).

In piezosurgery, most technological advances have arisen in maxillofacial surgery and neurosurgery (Table 2). The versatility of these devices has then allowed their application to hand, orthopedic, aesthetic, and reconstructive surgery.

The use of piezosurgical instruments for osteotomy of dense bone (e.g. the cranial vault, skull base, or vertebrae) has been considered by some neurosurgeons and spine surgeons to be unnecessarily time-consuming compared with traditional instruments, above all when bone osteotomy is a step in approaching deeper and complex structures requiring long surgical procedures. However, new, higher-power piezoelectric devices have recently been developed that can cut dense bone more effectively. These have a power output of ≥75 watts, which is at least three times the value for most piezosurgical devices available on the market (Table 1). The effect on bone tissue of the higher output power generated by these new ultrasonic generators is not known. Furthermore, few *in vivo* studies on bone healing after osteotomy performed with piezosurgical instruments are to be found in the English-language scientific literature.

The first histological description was carried out by Horton et al. (1975) in an *in vivo* study on dog alveolar bone; these investigators described accelerated bone formation in alveolar defects generated by chisels and ultrasonic instruments in comparison with traditional drills. The authors suggested that piezoelectric devices were much less effective as osteotomy systems; however, the ones they used were quite different from modern piezoelectrical instruments, Therefore, in our opinion, the observations by Horton et al. (1975) are not comparable to more recent studies. Vercellotti et al. (2005) evaluated the level of alveolar bone crest after osteotomy with piezosurgery and burs using an *in vivo* model of dog alveolar ridges: histological analysis showed a bone level gain in the group treated with piezosurgery, and bone loss in the diamond and carbide bur groups. The viability of cells after piezosurgery was evaluated histologically by several authors in bone grafting procedures that used a dental piezoelectrical device (Happe et al., 2007; Sohn et al., 2007).

The only *in vivo* study that combined histomorphometric and molecular analysis was conducted by Preti et al. (2007). These researchers evaluated the level of osseointegration of titanium implants placed in surgical beds prepared with piezosurgery versus conventional drilling in mini-pig tibia. They observed lower numbers of inflammatory cells, higher numbers of osteoblasts, increased expression of bone morphogenetic proteins, and lower expression of pro-inflammatory cytokines; the authors concluded that piezosurgery may accelerate the earlier phases of the implant osseointegration when compared with rotating drilling. Maurer at al. (2008) evaluated the microscopic differences in *ex vivo* bony samples of rabbit skull. After using three different osteotomy techniques, they observed that ultrasonic piezoelectric osteotomy preserved the original structure of bone, in contrast to rotatory drilling and sawing. A histomorphometric analysis in *ex vivo* models on rabbit skull was provided by Hollstein et al. (2012), who used five different piezoelectrical devices to perform osteotomies: the bone microstructure appeared to be substantially preserved. Heinemann et al. (2012) evaluated histomorphometric differences in *ex vivo* bony samples of porcine lower jaw segments, comparing two different piezoelectrical devices and a rotating bur: the bone matrix adjacent to the defect showed viable osteocytes with all three instruments. A recent histomorphometric study conducted by Ma et al. (2013) compared bone healing after osteotomy performed with piezosurgery versus oscillatory saws: no statistically significant differences were found in terms of histomorphometry; however, the authors observed a higher degree of formation of vascularized tissues and connective preliminary matrix, as well as increased bone remodelling activity, at 7 and 14 days after piezoelectric surgery.

A large study on rat tibia was conducted by Esteves et al. (2013). Bony samples were subjected to histological, histomorphometrical, immunohistochemical, and molecular analysis. Comparative evaluation of bone healing after osteotomic lines accomplished by rotating drill or piezosurgery did not demonstrate significant differences between the two animal groups. Yang and Girod (2014) performed subcritical-size calvarial defects with piezosurgical devices in the parietal bones of adult mice. Bone healing was evaluated with non-invasive micro-CT for 8 weeks. These authors reported that bone remodelling and regeneration were faster in the piezosurgical group of mice compared with the rotating instrument group. Different animal models for evaluating bone healing after piezosurgical osteotomy have been described in the scientific literature (Table 2). Dense bone and a wide bone surface are needed to perform a reproducible osteotomic framework in which several osteotomic lines can be executed (Figure 2). Bone density is highest in the skull and is related to intra-operative drilling resistance (Norton et Gamble, 2001; Smolka et al., 2006). Dog alveolar bone is not particularly dense, though it has been employed by numerous authors in oral surgery studies using piezosurgery (Table 2). Rabbit skull has also been used by many authors to evaluate bone healing after piezosurgery (Maurer et al., 2008; Hollstein et al., 2012; Ma et al., 2013). Mouse, rat, and mini-pig are small animal models in which the skull and vertebrae do not have not enough surface for a number of osteotomic lines. Rabbit skull allowed us to carry out several parallel osteotomic lines in a quite flat bone surface, which simplified the osteotomic framework and harvesting of samples for histological/histomorphometrical evaluation. Large animal models (sheep, pork, dog) would also have been suitable for our study design, but not the first choice according to the ethical use of animals in research directive (European Communities Council Directive, 23 September 2010 [2010/63/EU]).

In crucial finding of our study is that PM and PP produce thinner bone gaps, which in turn lead to easier and faster recovery; for this reason, it is not surprising to observe that in RO osteotomies all healing processes require longer times.

It is also important to discuss the form/quality of osteogenesis processes involved in the recovery of bone gaps produced by the different types of osteotomy. Over the last decade, we have demonstrated for the first time that two different mechanisms of bone formation exist, occurring in sequence during intramembranous ossification in both physiological and pathological conditions (Ferretti et al., 2002, 2006; Palumbo et al., 2003, 2004; Marotti, 2004; Marotti et al., 2010). We named these two processes of bone formation static osteogenesis (SO) and dynamic osteogenesis (DO): SO is characterized by pluristratified cords of 'stationary' osteoblasts, which differentiate through inductive stimuli (Streeten and Brandi, 1990; Villanueva and Nimni, 1990; Kasperk et al., 1997; Inoue et al., 2000) at a fairly constant distance from the network of blood capillaries, without moving from the differentiation site during their transformation into osteocytes; DO occurs, instead, through the familiar mono-stratified laminae of 'movable' osteoblasts. The temporal sequence of events is as follows: firstly, variously polarized stationary osteoblasts are irregularly arranged inside cords and give rise (in the same place where they differentiate) to osteocytes clustered within confluent lacunae, thus allowing the formation of a preliminary woven bone made up of thin trabeculae – this is not valid from the mechanical viewpoint due to its highly random cellularity. Subsequently, dynamic osteogenesis occurs on the surfaces of the woven-textured SO-trabecular framework, mainly involved in bone compaction, i.e. in filling primary Haversian spaces with primary osteons.

Generally, in comparison with SO-trabecular bone, DO-bone is a lamellar bone that is mechanically more resistant, because it is less microporous and arranged in a more orderly fashion with respect to both cells and the fibrillar components of its intercellular matrix; moreover, it occurs in response to mechanical stimuli, instead of the vascular-derived inductive factors associated with SO. This aspect acquires particular importance during osteotomy healing when, as normally occurs in bone histogenesis, static and dynamic osteogenesis are temporally correlated and thus follow each other (Marotti, 2004). Our observations on bone gap healing, concerning (i) osteoblast cords involved in preliminary bone regeneration in RO osteotomies (SO-I step) and (ii) osteoblast laminae covering preliminary bony trabeculae in PM/PP osteotomies (DO-II step), are in line with our previous observations on the steps occurring in sequence during bone histogenesis. In this context, it should be emphasised that in the thinner PM/PP bone gaps, the osteoblasts are in more favourable conditions (i.e. a suitable distance from the network of blood capillaries), increasing the supply of vascular-derived inductive factors, which, in turn, allow the progression from the first to second step of osteogenesis. This is also supported by BV/TV values, which are about double in PM and PP osteotomies compared with RO ones; these data are also in line with the presence of smaller amounts of bone tissue compared with fibrous tissue in RO.

The improved performance of these modern devices in terms of bone regeneration is also supported by evidence on bone remodelling. A significant increase in osteoclast number (N OCL/mm) was observed in PM and PP osteotomies compared with RO ones, in particular concerning PP vs RO. It is indeed well known that bone remodelling requires osteoclast differentiation and activation, in turn triggering the cells of the reversal phase (probably of stromal-fibroblast origin) that differentiate into osteoblasts, so that bone formation can occur in a way that improves bone quality (Palumbo et al., 2001).

Besides the influence of the osteotomy device on bone regeneration, it impact in terms of potential injury to the osteotomized bone (i.e. on bone cells close to the edge of osteotomy) must also be taken into consideration. As described, our results clearly show more viable osteocytes

closer to the osteotomy edge where the modern devices have been used, in particular concerning PP with respect to RO.

Some limitations of our study can be identified: i) the higher osteotomy thickness in RO than in PM; ii) the unknown relevance to humans of results for a rabbit model. The authors are aware of the greater osteotomy thickness in the drilling group (RO) due to the larger diameter of the bur compared with both piezosurgical tips. This discrepancy could have been avoided if a thin surgical saw had been used; however, lack of fine control of a saw in the inner calvarial cortex makes it difficult to preserve the dura mater and is so this is not suitable for small surgical fields. Large animal models (pork, sheep, bovine, non-human primate) could provide more valid data in terms of human bone healing. However, if a small animal model is possible, a larger animal model is deemed unjustifiable according to ethics in animal research guidelines (European Communities Council Directive of 23 September 2010 [2010/63/EU]). Large mammals have not been used for piezosurgical evaluation in the scientific literature; moreover, there have been no previous studies on an animal model using piezosurgical devices with output power of ≥75 watts. For this reason, a small animal model was our first choice.

5. Conclusion

In conclusion, using the animal model described, we have been able to demonstrate the validity of new-generation devices in performing surgical osteotomy in the cranial vault. Our results indicate that osteotomies performed using piezosurgery – by means of PM and PP – show more advanced stages of bone healing compared with RO, in part due to the lower osteotomy thickness in PM and PP. Moreover, PP, despite being more powerful than PM, does not alter the process of bone healing; thus it can be considered at least equivalent to PM. Overall, piezosurgery is shown to be more effective, compared with the traditional techniques, in improving the progression of skeletal repair.

The authors are aware that additional studies are required to better characterize the progression of, and related changes occurring during, bone regeneration after piezosurgery, especially in terms of the expression of specific markers by bone cells engaged at the onset of bone healing.

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Captions

Figure 1. Osteotomy devices. RO: conventional rotary osteotomic instrument; PM: Piezosurgery Medical®; PP: Piezosurgery Plus® (Mectron Medical Technology, Carasco, Italy).

Figure 2. Photographs showing the osteotomies performed in each rabbit skull – two by means of RO, two by PM, and four by PP.

Figure 3. LM micrographs showing the thickness (red dotted line) of the osteotomy (between the two black dotted lines) obtained using the three different devices (RO, PM, PP), producing different bone gaps. Note that the PM and PP devices produce a gap about half the width of that produced by RO. Note also that perivascular stromal spaces among the bone-forming trabeculae appear wider in RO osteotomy.

Figure 4. Representative digitized picture used for histomorphometric evaluations of BV/TV %. The total area of osteotomy (TV) is marked by the yellow polygon; the regenerated bone areas (BV) are outlined in blue.

Figure 5. Representative digitized picture used for histomorphometric evaluations of the number of TRAP-positive cells developing red color (indicated by arrows) counted within the osteotomy gap; the surface of bone was manually measured by outlining the perimeter of bony trabeculae (yellow profiles).

Figure 6. LM micrographs showing the newly formed bone during gap recovery for the three different osteotomies.

Figure 7. LM micrographs showing RO samples with osteoblasts arranged in cords (dashed ovals) close to the forming bony trabeculae, and PP samples with bony trabeculae covered by typical prismatic osteoblasts arranged in monostratified laminae (arrows).

Fig. 8. LM micrographs showing TRAP reaction in the three osteotomies. Note the higher level of positivity (red color) in PM and PP osteotomies compared with RO.

Figure 9. TEM micrographs of repairing bone gaps showing osteoblasts arranged in cords (dashed oval) involved in preliminary bone regeneration in RO, and prismatic osteoblasts (arrows) arranged in monostratified laminae present close the preliminary formed trabeculae in PP.

Figure 10. TEM micrographs of native bone close to the osteotomy border, showing the presence of abundant non-viable osteocytes in RO, and the presence of viable osteocytes in PM and PP.

Figure 11. SEM micrographs showing that the newly formed bony trabeculae inside the osteotomy gaps are less mineralized and display higher cell density compared with adjacent, pre-existent native bone in RO, PM, and PP samples. Note the presence of some fragments/remnants of osteotomized bone only in RO osteotomies (arrows).

Figure 12. Histograms showing the elastic modulus (GPa) of pre-existent bone (PB) and newly formed bone after osteotomy with PM, PP, and RO devices. For clarity, only $p > 0.05$ values are indicated in the graphs.

Table 1 Technical features of the main piezosurgical devices on the market. The data were obtained from the online user manuals published by each producer (see *web references*).

DEVICE	TIP WORKING FREQUENCY (kHz)	MAXIMUM OUTPUT POWER (W)	TIP RESONANCE AUTOMATIC SCANSION	INTENDED USE	PRODUCER
Bone Scalpel	22.5 kHz	130 W	Absent	Indicated for use in the fragmentation and aspiration of both soft and hard (e.g. bone) tissue, as performed in the following surgical specialties: orthopedic surgery, plastic and reconstructive surgery, thoracic surgery, neurosurgery, wound care, general surgery	Misonix Inc. – Farmingdale, NY, USA
Cusa NXT Ultrasonic Surgical Aspirator	24.35 kHz	Data not available	Absent	Indicated for use in the fragmentation, emulsification, and aspiration of both soft and hard (e.g. bone) tissue, as performed in the following surgical specialties: neurosurgery, gastrointestinal and affiliated organ surgery, urological surgery, plastic and reconstructive surgery, general surgery, orthopedic surgery, gynecological surgery, thoracic surgery, laparoscopic surgery, and thoracoscopic surgery	Integra LifeSciences Corporation – Plainsboro, NJ, USA
Piezoelectric System	28–36 kHz	Data not available	Present	Indicated for use in the osteotomy, osteoplasty, decorticating, drilling, shaping and smoothing of bone, bone substitutes, and teeth, as used in the following surgical specialties: general surgery, orthopedic surgery, otolaryngological surgery, maxillofacial and oral surgery, hand and foot surgery, neurosurgery, spinal surgery, and plastic/reconstructive surgery	Synthes Inc. – West Chester, PA, USA
Piezomed	22–35 kHZ	24 W	Present	Indicated for treatment of organic hard and soft tissue in dental surgery, implantology, maxillofacial surgery, and periodontics	W&H Dentalwerk Bürmoos GmbH – Bürmoos, AUSTRIA
Piezon Master Surgery	24–32 kHz	25 W	Present	Indicated for use in osteotomy and osteoplasty, as used in periodontal surgery, implantology, oral surgery, and maxillary surgery	EMS – Nyon, SWITZERLAND
Piezosurgery Medical	24–36 kHz	23 W	Present	Indicated for use in osteotomy, osteoplasty, and hole drilling, as used in the following surgical specialties: otorhinolaryngology, oral-maxillofacial surgery, hand and foot surgery, neurosurgery, spinal surgery, plastic/reconstructive surgery, orthopedics, and chest surgery	Piezosurgery® – Mectron Medical Technology – Carasco, ITALY
Piezosurgery Plus	24–36 kHz	75 W	Present	Indicated for use in osteotomy, osteoplasty, and hole drilling, as used in the following surgical specialties: otorhinolaryngology, oral-maxillofacial surgery, hand and foot surgery, neurosurgery, spinal surgery, plastic/reconstructive surgery, orthopedics, and chest surgery	Piezosurgery® – Mectron Medical Technology – Carasco, ITALY
Piezotome M+	28–36 kHz	Data not available	Present	Indicated for use in the osteotomy, osteoplasty, decorticating, drilling, shaping, and smoothing of bone, bone substitutes, and teeth, as used in, but not limited to, the following surgical specialties: general orthopedic surgery, otolaryngological surgery, maxillofacial and oral surgery, hand and foot surgery, neurosurgery, spinal surgery, and plastic/reconstructive surgery	Satelec – Acteon Group – Merignac Cedex, FRANCE
Sonoca	25 kHz, 35 kHz, 55 kHz	Data not available	Absent	This ultrasonic generator is solely intended for use in ultrasonic surgery and wound treatment on humans; this results in applications for the selective dissection of tissue, the cutting of tissue, and the coagulation of tissue.	Söring GmbH – Quickborn GERMANY
Sonopet	25 kHz	100 W	Absent	Indicated for use in the fragmentation, emulsification, and aspiration of both soft and hard (e.g. bone) tissue, as performed in the following surgical specialties: neurosurgery, gastrointestinal and affiliated organ surgery, urological surgery, plastic and reconstructive surgery, general surgery, orthopedic surgery, gynecological surgery, thoracic surgery, laparoscopic surgery, and thoracoscopic surgery	Stryker – Kalamazoo, MI, USA
Surgysonic II	22–35 kHz	Data not available	Present	Indicated for use in the osteotomy, osteoplasty, decorticating, drilling, shaping, and smoothing of bone, bone substitutes, and teeth, as used in the following surgical specialties: dental surgery, oral surgery, implantology, endodontics, periodontics, maxillofacial surgery, ENT, neurosurgery, and hand and foot surgery	Esacrom – Imola (BO), ITALY
VarioSurg 3	28–32 kHz	25 W	Present	It is indicated for use in the osteotomy, osteoplasty, decorticating, drilling, shaping, and smoothing of bone, bone substitutes, and teeth, as used in the following surgical specialties: dental surgery, oral surgery, implantology, endodontics, and periodontics	NSK, Nakanishi Inc – Tochigi, JAPAN

Table 2 Main animal studies on piezosurgical devices available in the scientific literature

Authors	Animal model	In vivo/ex vivo/bone healing	Material and methods	Ultrasonic device	Conclusions
Horton JE et al., 1975	Dog alveolar bone	In vivo	Light microscope	No data	The bur produced the smoothest surface. Histological evaluation in later periods showed the best healing with the use of the chisel.
Vercellotti T et al., 2004	Dog alveolar bone	In vivo	Histomorphometric analysis	Piezosurgery – Mectron Dental Technology	By day 56, the surgical sites treated by burs showed a loss of bone, versus bone gain in the piezo-treated sites.
Preti G et al., 2007	Mini pigs	In vivo	Histomorphology and evaluation of levels of bone morphogenetic protein (BMP)-4, transforming growth factor (TGF)-β2, tumor necrosis factor-alpha, and interleukin-1β and -10 in the peri-implant osseous samples	Piezosurgery – Mectron Dental Technology	Piezoelectric bone surgery appeared to be more efficient in the first phases of bone healing; it induced an earlier increase in BMPs, controlled the inflammatory process better, and stimulated bone remodeling as early as 56 days post-treatment.
Maurer P et al., 2008	Rabbit skull	Ex vivo	Light microscopy, environmental surface electron microscopy (ESEM), and confocal laser scanning microscopy (CLSM)	Rotating instrument, micro-saw, Piezosurgery – Mectron Dental Technology	Bony structure integrity was observed after the ultrasonic technique
Hollestein S et. al, 2012	Rabbit skull	Ex vivo	Light microscopy, (ESEM), and confocal laser scanning, microscopy (CLSM).	Piezosurgery 3, Piezon Master Surgery, Piezosurgery – Mectron Medical Technology, VarioSurg, Piezotome 2	The osseous microstructure was preserved. Five different piezosurgical devices were evaluated.
Heinemann D et al., 2012	Porcine fresh lower jaw segments	Ex vivo	Light microscope	Piezosurgery – Mectron Medical Technology, SONICflex, and the conventional bur method	The bone matrix adjacent to the defect radius showed intact osteocytes.
Ma L et al., 2013	Rabbit skull	Bone healing	Light microscopy and histomorphometric analysis	Piezosurgery – Mectron Medical Technology, and two types of oscillating steel saw blade	More advanced bone healing was observed compared with the use of a traditional saw.
Esteves JC et al., 2013	Rat tibia	Bone healing	Histomorphometric analysis, immunohistochemical staining, and RT-PCR (reverse transcriptase-polymerase chain reaction)	Piezo Master Surgery, and conventional drilling with a 2 mm round diamond-coated tip	Bone healing dynamics after piezosurgery were comparable to those observed with conventional drilling.
Yang BE et al., 2014	Mice skull	Bone healing	Micro-CT (micro–computed tomography)	Surgystar diamond round tip and Piezoelectric System (Synthes)	Piezosurgery resulted in faster bone healing compared with mechanical instrumentation.

Table 3 Histomorphometric parameters (mean values) evaluated in regenerated bone (osteotomy gap, BV/TV, N OCL/mm), and native bone (OC-OE_md) for the three different osteotomies

Device	Osteotomy gap (µm)	BV/TV %	N OCL/mm	OC-OE_md (µm)
RO	1415 ± 206[a]	22.7 ± 18.7	0.88 ± 0.54[b,c]	83.4 ± 33.5
PM	626 ± 54	39.8 ± 21.9	1.51 ± 0.71	65.7 ± 23
PP	637 ± 62	38 ± 21.1	1.77 ± 0.64	38.2 ± 21.9[b,d]

[a]$p < 0.001$ vs PM, PP; [b]$p < 0.05$ vs PM; [c]$p < 0.01$ vs PP; [d]$p < 0.001$ vs RO. (ANOVA followed by Bonferroni post hoc test)