HUMAN MANDIBLE BONE DEFECT REPAIR BY THE GRAFTING OF DENTAL PULP STEM/PROGENITOR CELLS AND COLLAGEN SPONGE BIOCOMPLEXES

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Abstract

In this study we used a biocomplex constructed from dental pulp stem/progenitor cells (DPCs) and a collagen sponge scaffold for oro-maxillo-facial (OMF) bone tissue repair in patients requiring extraction of their third molars. The experiments were carried out according to our Internal Ethical Committee Guidelines and written informed consent was obtained from the patients. The patients presented with bilateral bone reabsorption of the alveolar ridge distal to the second molar secondary to impaction of the third molar on the cortical alveolar lamina, producing a defect without walls, of at least 1.5 cm in height. This clinical condition does not permit spontaneous bone repair after extraction of the third molar, and eventually leads to loss also of the adjacent second molar. Maxillary third molars were extracted first for DPC isolation and expansion. The cells were then seeded onto a collagen sponge scaffold and the obtained biocomplex was used to fill in the injury site left by extraction of the mandibular third molars. Three months after autologous DPC grafting, alveolar bone of patients had optimal vertical repair and complete restoration of periodontal tissue back to the second molars, as assessed by clinical probing and X-rays. Histological observations clearly demonstrated the complete regeneration of bone at the injury site. Optimal bone regeneration was evident one year after grafting. This clinical study demonstrates that a DPC/collagen sponge biocomplex can completely restore human mandible bone defects and indicates that this cell population could be used for the repair and/or regeneration of tissues and organs.

Keywords: Dental pulp stem/progenitor cells (DPCs), bone, human mandible, stem/progenitor cell graft, bioscaffold, regenerative medicine, clinical study.

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Introduction

The aim of tissue engineering (TE) is the regeneration of tissues through the combined use of biomaterials and biologic mediators in order to provide new tools for regenerative medicine (RM). Over the last years, TE has made significant progress, moving from being merely a biomaterial science towards being a genuinely multidisciplinary field, through the integration of biology, medicine and various engineering sciences. Importantly, future procedures will make increasing use of autologous transplants (i.e., material obtained from the same individual to whom they will be reimplanted); thus, the need for immunotherapy will be avoided. Ideally, these transplants will possess predictable patterns of vascularisation and nerve supply, which are both important aspects for a return to optimal functionality.

The need to develop tissue replacement and implementation strategies is particularly felt in the oro-maxillo-facial (OMF) field. Replacement of OMF structures is tricky and peculiar because orofacial functions – such as facial expression, articulation of speech, chewing and swallowing – are exquisitely delicate, being based on complex three-dimensional anatomical structures formed from soft (skin, mucosa and muscle) and hard (craniofacial skeleton and teeth) tissues (Bluteau *et al.*, 2008).

The repair and regeneration of bone is a major issue in the OMF field and for the whole human body in general. Bone loss is caused by many diseases (congenital or degenerative), traumas and surgical procedures; it is a problem for functionality and is having an ever-increasing social impact, especially in elderly subjects.

Bone is formed by extracellular matrix (ECM) rich in collagen and elastic fibres adherent to hydroxyapatite crystals. Adult bone is continuously remodelled through specific osteoblast/osteoclast interaction. Stem/progenitor cells residing in the periosteum and endosteum of bone possess a limited regenerative potential (Salgado *et al.*, 2006). For this reason, surgical intervention using biocompatible fillers or bone-grafting techniques is indispensable when significant bone loss occurs. To avoid side effects produced by the use of biocompatible materials and/or bone withdrawal, new biotechnological approaches for repair must be envisaged.

Although stem/progenitor cells have been isolated from different tissues and extensively studied *in vitro* and *in vivo* in the past years, there is no information yet on the application of human stem/progenitor cells for the repair of OMF structures at a clinical level. Unfortunately, there

are limits on the use of stem/progenitor cells in therapy, such as the low number of stem/progenitor cells that can be collected, morbidity at the site of collection and the difficulties in reaching the site of repair.

Dental pulp is a niche housing neural-crest-derived stem cells. This niche is easily accessible and there is limited morbidity after collection (Jo *et al.*, 2007; Lensch *et al.*, 2006; Mitsiadis *et al.*, 2007). Previous studies have shown that dental pulp stem cells (DPSCs) are capable of differentiating into osteoblasts (Laino *et al.*, 2005; Laino *et al.*, 2006b) that secrete abundant extracellular matrix (ECM) and that can build a woven bone *in vitro* (Laino *et al.*, 2006a). Furthermore, DPSCs are capable of forming a complete and well-vascularised lamellar bone after grafting into immunosuppressed rats (d'Aquino *et al.*, 2007; Graziano *et al.*, 2008). The quality and quantity of regenerated bone formed by DPSCs was demonstrated in *in vitro* and *in vivo* experiments using stem cells and biomaterials (d'Aquino *et al.*, 2008; d'Aquino *et al.*, 2007; Graziano *et al.*, 2008; Laino *et al.*, 2005). Thus, dental pulp could be considered as an interesting and potentially important source of autologous stem/progenitor cells that are ready for use for therapeutic purposes, such as the repair/regeneration of craniofacial bones.

The aim of this study, therefore, was to demonstrate that DPCs could be used to repair bone defects in humans. Here we give evidence that DPCs seeded onto collagen sponge bioscaffolds repair alveolar defects of the mandible produced after extraction of impacted third molars. The autografts produced a fast regeneration of bone, which was of optimal quality and quantity when compared to standard techniques commonly used for guided bone regeneration and bone grafts of various origins (Jensen *et al.*, 2004).

Materials and Methods

Ethics
All procedures described here comply with Internal Ethical Committee guidelines, approved on June 12th, 2005 (Second University of Naples Internal Registry: Experimentation #914-Bone repair using stem cells).

Patients were invited, before being enrolled for the study, to carefully read and sign an informed consent form, drafted by us following instructions from our internal Ethical Committee.

Objectives
The objective of this clinical study was to repair an alveolar bone defect secondary to routine wisdom tooth extraction. Usually, after extraction of impacted unerupted or partially erupted wisdom (third molar) teeth, a proportion of patients risk reabsorption of the alveolar ridge distal to the second molar roots. In these patients, destruction of the tooth socket produces a pocket formed from 2 walls, which are represented by the root of the second molar and the distal ridge, with the lingual wall forming a third wall when present. This clinical condition (post-extractive alveolar bone loss), in which vertical loss has a probing depth of at least 7 mm, jeopardizes the second molar in an average of five years or less and does not allow bone repair with

normal techniques (Dodson, 2007). Thus, it represents a complication associated with the removal of the third molars that should not be underestimated. For this reason, we identified patients at risk of post-extractive alveolar bone loss as candidates that could benefit for a bone regeneration therapy.

Participants: patient selection and preparation
Eligibility criteria for participants and settings were the following: extraction needed for all wisdom teeth, with closely comparable conditions for the two lower impacted teeth; no systemic disease; no pregnancy (for females); no routine drug use. Patients with two similar lower molars were needed for the study so that we could use one as a test (T) site and the other one as a control (C) site. Seventeen out of the 100 patients initially contacted for this study consented to surgery. Of these 17 patients, 7 (6 females and 1 male) returned for the one-year follow-up. The template for the enrolment of patients was set within the limits of the approved clinical trial. All the procedures were performed at the Department of Odontostomatology of the Second University of Naples.

Patients were subjected to professional oral hygiene one week before surgery. They were then instructed to perform domiciliary hygiene of the oral cavity correctly, which consisted in washing the mouth with 0.2% Chlorhexidin (CHX) after tooth brushing, twice a day until surgery was performed.

Pre-surgery evaluation of dental pulp stem/progenitor cells
Before embarking on regeneration surgery, we needed to obtain stem/progenitor cells from the pulps of the patients. Patients were therefore subjected to the extraction of the upper (maxillary) molars and the pulps harvested as previously described (d'Aquino *et al.*, 2007). Briefly, teeth were washed in 0.2% CHX solution, the pulp chamber opened using a surgical drill and the pulp collected. Then, the pulp was rinsed in 1.5 ml saline solution and mechanically dissociated; using previously described procedures (Graziano *et al.*, 2008). After dissociation, cells were filtered through a 70μm strainer and cultured in α-minimal essential medium (MEM) (Cambrex, Charles City, IA, USA) with 20% FBS (Invitrogen, San Giuliano Milanese, Italy) and the medium changed twice a week. At day 21 cells were detached and analysed at the Fluorescence Activated Cell Sorter (FACS Vantage, Beckton Dickinson, Franklin Lakes, NJ, USA) for stem/progenitor antigen expression in good manufacturing practice (GMP) conditions. Cells were detached using 0.02% EDTA solution, centrifuged and incubated with 1μl of antibody in 100 μl of phosphate buffered saline (PBS) solution for 1h at 4°C. Antibodies were: anti-CD34 (clone AC136, Miltenyi Biotech, Calderara sul Reno, Bologna, Italy) and anti-flk-1 (c. sc-57135, Santa Cruz, CA, USA).

Extraction of wisdom teeth
Patients were prepared for surgery by decontamination of the oral cavity with CHX. Then lower (mandible) impacted third molars were extracted following a standard procedure: after making a horizontal incision in the gum,
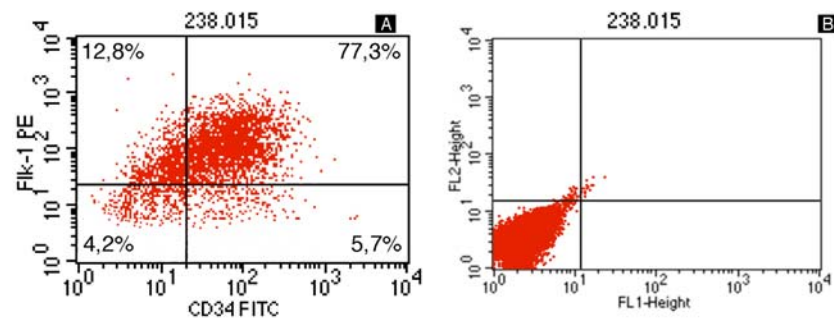
Fig. 1. Fluorescence activated cell sorting before surgery. (A) Cells collected from maxillary (upper) third molars were challenged for CD34 and flk-1. Representative 2D plot image showing high positivity levels for both flk-1 and CD34 antigens (77,3%). (B) Isotype control.

the muco-periosteal flap is reflected and the bone covering the tooth is removed using a round bur. The area is irrigated with a steady stream of saline solution until the crown is entirely exposed.

When the oral surgeon is not able to extract the whole tooth in one go, a groove is created vertically (along the long axis of the tooth) at the cervical line of the tooth, using a fissure bur, in order to separate the crown from the root. The groove created by the bur must not be deep, since the mandibular canal is often found in close proximity to the tooth and the risk of injuring or severing the inferior alveolar nerve must be avoided. After being placed in the groove, a straight elevator is used to separate the crown from the root, with a rotary movement. The crown is removed separately, using the same elevator, with a rotary movement upwards, and the root is then easily removed, using a straight or angled elevator, the blade end of which is placed in a purchase point created on the buccal side of the root.

In the case of a tooth with multiple roots, the crown must be sectioned and removed, as above described. Afterwards, if the roots of the impacted tooth are separated during crown sectioning, they can be easily removed one in succession, starting with the distal root and then the mesial root.

After smoothing the bone, the area is irrigated with saline solution and the distal root of the second molar is planned with a Gracey curette, and all the necrotic tissue is taken away.

Stem/progenitor cells, obtained as above described, were gently endorsed with a syringe onto a collagen sponge scaffold (Gingistat, Vebas, San Giuliano Milanese, Italy). The sponge-cell implant was used to fill the space left by the extraction procedure (test (T) site). A sponge without cells was used to fill the control (C) site.

A flap of gum was then sutured as a tendon in order to avoid any contact with the oral cavity. A suture was then placed at the distal portion of the second molar and the others were placed at the interdental papillae and at the posterior end of the incision. For both sites, a replacement jig was placed to ensure correct localization for sample withdrawal.

Post-surgery patient evaluations
Clinical and radiological controls were performed. The first control was scheduled at day 7 after surgery, when X-ray

(for each patient the Ethical Committee permitted to perform a maximum of 4 X-ray Orthopantomographies (OPTs) per year and a maximum of 8 endo-oral X-rays per year), clinical observation and suture removal were performed. Oedema, presence of inflammation and functionality were clinically evaluated.

Patients were controlled once a month thereafter, up to the third month. During these controls, clinical observations and X-rays were performed. During the fourth control, at three months after surgery and before bone sampling, probing depth was performed to evaluate the retrieved clinical attachment.

A sample was then collected from the T and C sites of each patient for histological and immunofluorescence (IF) analyses. Each bone sample was collected using a drill with a replacement jig. Bone specimens were used for histological observations. For this purpose, each sample was decalcified in 10% EDTA in distilled water for 2 months. Then, each specimen was sectioned and stained. Other than haematoxylin-eosin staining, samples were used for IF analyses, using the following antibodies: anti-osteocalcin (OC), anti-osteonectin (ON), anti-bone alkaline phosphatase (BAP), anti-bone morphogenetic protein (BMP)-2 and anti-vascular endothelial growth factor (VEGF) (all purchased from Beckton Dickinson). One year from surgery, further analyses were performed.

Statistical analysis
Student t-test (two-tailed) was used for statistical evaluation. The level of significance was set at $p<0.05$.

Results

The dental pulp cells of the third maxillary molars collected for pre-surgery evaluation were strongly positive to both CD34 and Flk-1 (Fig. 1A, B) and clearly comprised stem/progenitor cells in sufficient quantity to perform the *in vivo* experiments. All the selected patients were therefore subjected to surgery to extract third mandible molars and regenerate bone defects at the level of the lower, impacted third molars (Fig. 2 A, B, C, D, E, F, G, H, I).

Seven days after implantation of the biocomplex, clinical and radiological controls revealed that T and C sites did not display differences (Fig. 3A, B, C). There was slight oedema and inflammation at the sites of surgery,
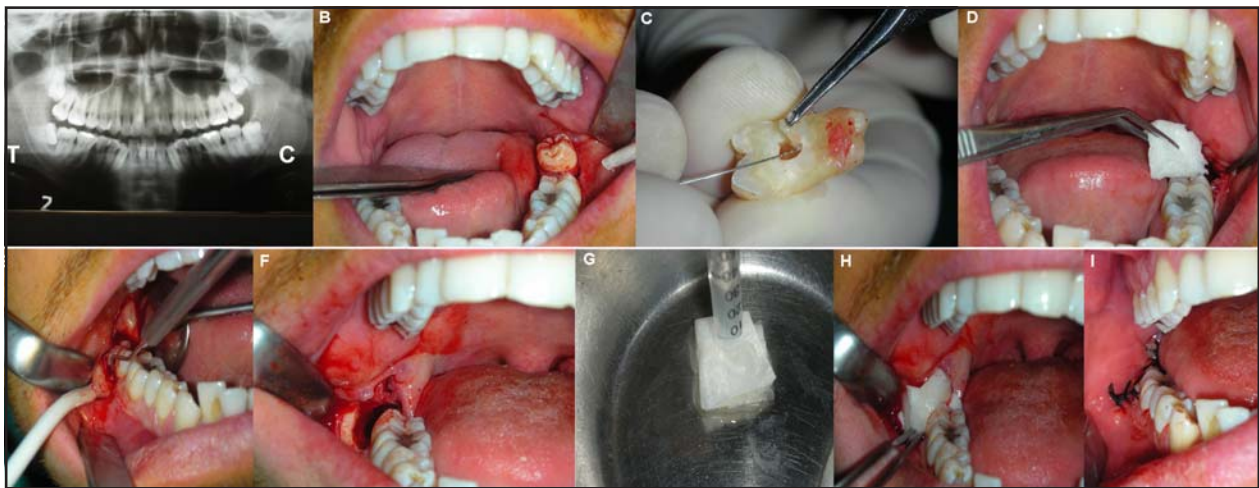
Fig. 2. Surgical procedure. (A) Pre operatory X-ray (T: test site; C: control site). In this representative X-ray it is possible to see how the lower right third molar is in close contact with the second molar root. (B) Control site tooth extraction. (C) Pulp withdrawal from the extracted tooth. (D) In the control site, a collagen sponge without DPCs is put in the gap left by the surgery and is then sutured. (E) Evaluation of the depth of the defect produced by tooth extraction carried out with a parodontal probe at the test site. (F) Test surgical site. After extraction of the third molar, a gap is left in the mandible. (G) Construction of the collagen-cell biocomplex. Stem/progenitor cells obtained from pulp being seeded onto the collagen sponge. (H) Grafting of the biocomplex at the test site. (I) Surgery ends with the placing of sutures.

Fig. 3. X-ray and clinical control 7 days after surgical intervention. (A) Control X-ray (T: test site; C: control site) of patient N. 3. At the T site it is possible to observe the wide gap behind the distal roots of the right second molar. The yellow double-headed arrow evidences the vertical gap and the white double-headed arrow indicates the horizontal gap. (B) Test site. (C) Control site.

but patients did not present with morbidity or infections after intervention. In all cases, the post-surgery time for recovery was normal. Patients did not complain about particular post-operative pain, so no analgesic medication was given. Functionality was normal in all cases except for one patient, who suffered a little distortion in mouth opening and an increased level of oedema at both sites. Post-operative clinical observation revealed normal healing without scar tissue formation or functional disturbances; no bleeding, no swelling or other side effects were observed.

Thirty days after surgery, clinical parameters were well balanced in all the patients. X-ray controls showed significant differences between the C and T sites: the latter presented with a high rate of mineralisation (Fig. 4A,B).

Clinical control performed two months after surgery did not reveal the presence of any alteration. X-ray analyses clearly revealed different levels of cortical bone at the T (Fig. 4C) and C sites (Fig. 4D): whereas at the T site the cortical margin reached the cementum-enamel junction

(CEJ) level of the second molar, demonstrating vertical regeneration, this was not seen at the C site in any of the patients.

Three months after the surgery, X-ray analyses confirmed that the T sites were completely regenerated and that the cortical level was much higher than at the C sites (Fig. 5A,B). From day 7 up to the third month, patients did not show signs of local or general infections or diseases. All the parameters (oral and general) were within normal ranges. The functionality (dental functions, chewing in particular) and quality of life were optimal in all cases. Samples of bone were collected for histology and IF analyses (Fig. 5C,D).

The probing depth analyses revealed an increase of clinical attachment that was quantitatively higher at the T site than at the C site: whereas the C sites presented with a gain of 4.4±1.2 mm, the gain at the T sites was 6.2±2.3 mm. In addition, we collected a bone sample for each site, using mini-invasive surgery. The bur was positioned in the right place using a replacement jig placed before the
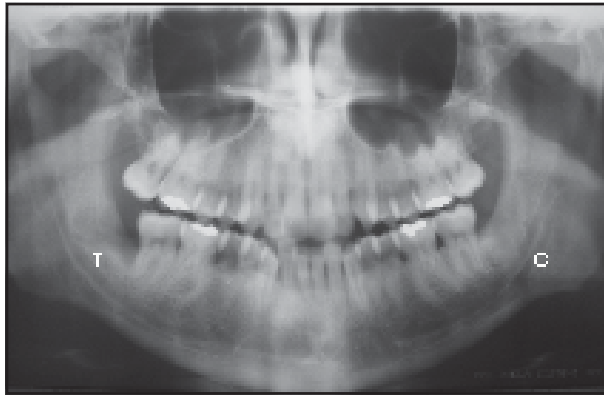
Fig. 4. X-ray control 30 days after graft (T: test site; C: control site). The image shows that significant bone regeneration has started at the T site of patient N. 3 (compare with Fig. 3A).
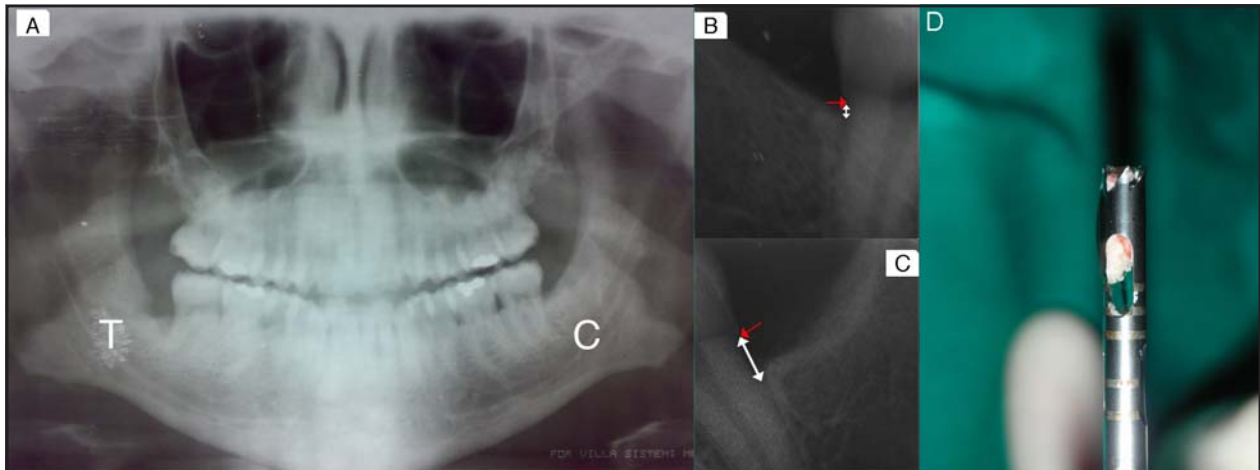

Fig. 5. X-ray, withdrawal and bone sampling 3 months after graft. (A) X-ray control 3 months after grafting (patient N. 3) (T: test site; C: control site). (B) Enlargement of the T site X-ray. The red arrow indicates the cementum-enamel-junction; the white double-headed arrow indicates the minimal exposure of the second molar root due to significant bone regeneration. (C) Enlargement of the C site X-ray. The red arrow indicates the cementum-enamel-junction; the white double-headed arrow indicates the considerable exposure of the second molar root due to a lack of bone regeneration. (D) Bone sample collected 3 months after grafting.

surgery. In this way we were able to collect bone from the regenerated sites, avoiding any potential loss of periodontal tissue. The samples belonging to the T sites were very different when compared with those withdrawn from the C sites: T site samples were made up of well organized and well vascularised bone with a lamellar architecture surrounding the Haversian channels (Fig. 6A); bone from C sites was immature, with fibrous bone entrapped among new lamellae, incomplete and large Haversian channels and evidence of bone reabsorption (Fig. 6B). In all cases the collagen sponge was completely reabsorbed.

IF analyses were performed to assess the expression of bone proteins (ON, OC and BAP), as well as BMP-2 and VEGF, which are both important molecular signals during bone regeneration. ON, OC and BAP expression was slightly different between T and C sites: they were expressed at both sites, but with a different distribution (Fig. 6 C,D,E,F,G,H). Significant differences were observed for BMP-2 and VEGF expression: these two molecular signals were expressed at much higher levels ($p<0.001$) in the T samples with respect to the C ones (Fig. 6 I, J, K, L, M).

One year after surgery, patients presented with a normal oral cavity without signs of alterations. The mucosae were normal at both sites. X-ray analysis confirmed that the bone regeneration at the T site was complete and stable (Fig. 7). Final scores for the bone regeneration per patient are given in Table 1. Quality of life, chewing, oral cavity and relative functions remained optimal in all the cases.

Discussion

Approximately 1,500,000 subjects undergo craniofacial reconstruction each year in Europe, and about 20% of these patients experience a loss of function despite reconstruction. 30,000 of these subjects suffer from donor site morbidity relating to flap reconstructions after oral and maxillofacial surgery. The missing parts that are involved in the defect of a given tissue or organ have specific functions, and replacement is often quite difficult. For example, the closure of a bone defect is commonly associated with the transfer of tissue (e.g., a flap), which may not fully restore the unique function of the lost part. Furthermore, tissue transfer is associated with donor site morbidity, accompanied by scarring, infection and loss of function. Craniofacial reconstruction aims mainly to repair gross aesthetic and functional disfigurement in cases of

Table 1. Patient Characteristics and Final Clinical Score

Patient N.	Age (YEARS)	SEX	FINAL SCORES	
1	27	F	0	2
2	25	F	1	2
3	29	M	2	3
4	31	F	1	2
5	40	F	2	3
6	36	F	1	2
7	24	F	2	2

Score Legend: 0, NO regeneration; 1, =30% regeneration; 2, =70% regeneration; 3, complete regeneration (80-100%). F, female; M, male. $p < 0.01$, T *vs* C for all patients except N. 7.

congenital malformations, tumour resections and post-traumatic deformities.

Recent TE products are based on novel biomaterials integrating stem/progenitor cells that are capable either of self-renewal or of differentiating into several specific cell types. The use of adult stem/progenitor cells can be extensive, since stem/progenitor cells can be harvested from various tissues such as adipose tissue, bone marrow, dental pulp and periodontal ligament. Dental stem/progenitor cells collected from dental pulp can be differentiated *in vitro* and then transplanted with biomaterial scaffolds into the host without immunologic rejection (d'Aquino *et al.*, 2007; Graziano *et al.*, 2008). The use of appropriate biomaterial scaffolds combined with selected growth factors can significantly improve the survival and differentiation of the transplanted stem/progenitor cells.

To date, only few cases of translation from biological studies and TE to patients have been reported (Gurtner *et al.*, 2008). The difficulties linked to cell manipulation and the quantity and quality of stem/progenitor cells are the main reasons for the slow progress. In addition, cell differentiation is more a limitation than an implementation for results. Stem cells are more capable of regeneration than their differentiated daughter cells. For example, mesenchymal stem cells exhibit properties that allow their use in regenerative medicine, including their immunosuppressive activity (Dazzi and Horwood, 2007; Guo *et al.*, 2006; Le Blanc and Ringden, 2007; Locatelli *et al.*, 2008; Samuelsson *et al.*, 2009). It has been shown that this property is also displayed by DPSCs (Pierdomenico *et al.*, 2005; Wada *et al.*, 2009). Moreover, it has been demonstrated that infusion of mesenchymal stem cells (MSCs) expanded *in vitro* exert a therapeutic effect in patients with steroid-resistant severe graft-versus-host disease (Le Blanc *et al.*, 2008), paving the way to new clinical use of MSCs, even though they derive from an allogenic source.

Differentiation of stem cells into osteoblasts *in vitro* was assessed by osteocalcin expression, a cell membrane protein that identifies osteoblasts. In addition, the extracellular matrix that is secreted by these cells was measured by the expression of ON, a marker of bone matrix, and BAP, a tetrameric glycoprotein found on the surface of osteoblast and an indicator of bone turnover. Morphologically, bone biopsies collected from the treated sites and used for histological analysis revealed the presence of well differentiated lamellar bone, with typical Haversian channels and lamellae and spider-like osteocytes. Interestingly, the significantly higher amount of BMP-2 and VEGF observed at the treated sites, when compared to the control sites, explains and confirms the different levels of bone maturation. In fact, these two molecules are responsible for fine-tuning the balance of secreted bone ECM and neo-angiogenesis during bone formation. For bone development and healing, vigorous vessel sprouting is extremely important to sustain mineralisation and maturation.

Regarding the most appropriate scaffold for bone regeneration, we have previously demonstrated that complete bone regeneration can be obtained *in vivo* with DPSCs seeded on reabsorbable collagen sponges (Gingistat). These gave optimal results, allowing cells to proliferate and differentiate into osteoblasts (Graziano *et al.*, 2008). Therefore, we used the same scaffold in this clinical study. The results that we have obtained and shown above allow us to assume that this scaffold is an optimal support for stem/progenitor cells in cell-guided regeneration.

The present study is the first that accomplishes autologous bone regeneration in humans. This was obtained with the use of a biocomplex constructed from dental pulp stem/progenitor cells seeded onto a collagen-based scaffold. We have given evidence here that the procedure described results in optimal bone repair, with the restoration of complete periodontal tissue behind the
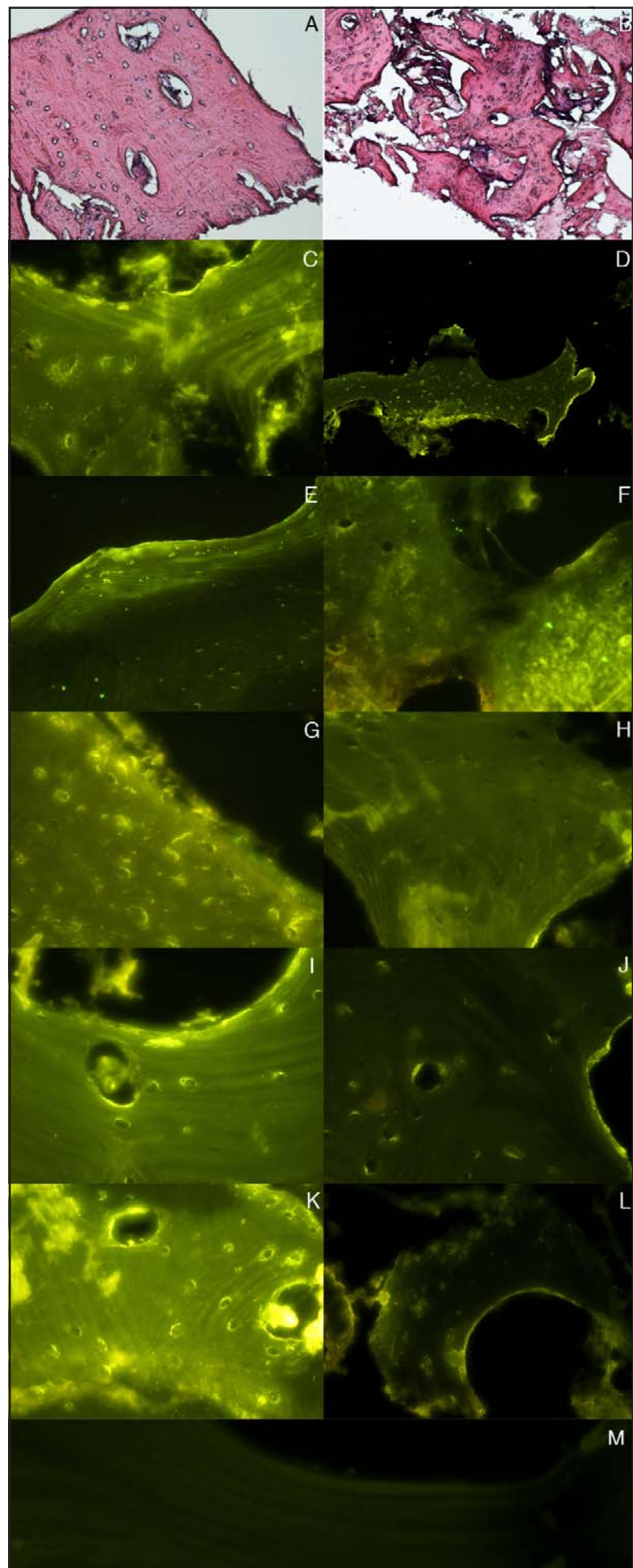
Fig. 6. Histological and Immunofluorescence analyses on samples collected after 3 months from surgery. Haematoxylin-eosin staining reveals better bone formation at the T site (A) than that at the C site (B). IF analyses were performed on bone samples to assess the expression of bone proteins including Osteonectin (C - Test site; D - Control site), Osteocalcin (E - Test site; F - Control site), BAP (Bone Alkaline Phosphatase) (G- Test site; H- Control site) and growth factors such as BMP-2 (I- Test site; J- Control site) and VEGF (K- Test site; L- Control site). M: isotype negative control for fluorescein isothiocyanate (FITC).
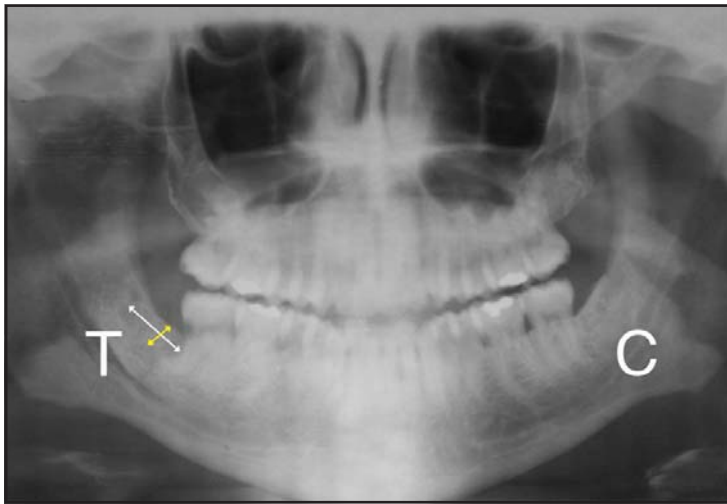
Fig. 7. X-ray control 1 year after graft. X-ray performed on patient N. 3 one year after surgery, evidencing the optimal regeneration at the Test site (T: test site; C: control site). The yellow double-headed arrow indicates the gain in vertical bone height and the white double-headed arrow indicates the antero-posterior gain.

second molars, as assessed by clinical and X-ray evaluation.

The technique that we developed for this clinical study can be easily applied to any other area of reconstructive and orthopaedic surgery. Stem cells represent an easy and natural alternative to repair/regenerate damaged tissues. This is essential especially when bone loss subsequent to degenerative or traumatic diseases cannot be amended through conventional therapies.

Within the craniofacial region, maxillary and mandible bones often undergo reabsorption following degenerative diseases, including periodontal disease (the first cause of tooth loss in the elderly), mandible necrosis or tumour resections. Autologous DPCs are a new tool for bone tissue engineering. The procedure is efficient, exhibits low morbidity of the collection site, and is free from diseases incurred by transmission of pathogens. The regeneration process is fast and efficient.

Conclusions

This clinical study has demonstrated the following: (i) dental pulp stem/progenitor cells can be used for OMF bone repair; (ii) the use of DPCs on appropriate reabsorbable scaffolds produces an efficient biocomplex; (iii) collagen sponges can be considered an optimal support for the stem/progenitor cells in cell-guided regeneration.

We have given evidence here that autologous DPCs can be used in a low-risk and effective therapeutic strategy for the repair of bone defects. The result we have obtained is encouraging and prompts further clinical trials on a larger scale of bone loss.

Limitations

Despite the optimal results, the flaws of this study reside mainly in the small number of patients enrolled. Longer patient follow-up would ascertain the lifespan of the regenerated bone. In further studies, regeneration will be ascertained, other than in bone, in other tissues of the OMF area.

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