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Periodontal Regeneration using a Xenogeneic Bone Substitute seeded with Autologous Periodontal Ligament derived Mesenchymal Stem Cells: a 12-month quasi-randomized controlled pilot clinical trial.

Short running tittle "Periodontal regeneration with periodontal ligament stem cells"

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Conflict of Interest

Potential conflicts of interests of the authors are appended.

Abstract

Aim. To evaluate the safety and efficacy of autologous periodontal ligament-derived mesenchymal stem cells (PDL-MSCs) embedded in a xenogeneic bone substitute (XBS) for the regenerative treatment of intra-bony periodontal defects.

Material and Methods. This quasi-randomized controlled pilot phase II clinical trial included patients requiring a tooth extraction and presence of one intra-bony lesion (1-2 walls). Patients were allocated to either the experimental (XBS + $10x10^6$ PDL-MSCs/100mg) or the control group (XBS). Clinical and radiographical parameters were recorded at baseline, 6, 9 and 12 months. The presence of adverse events was also evaluated. Chi-square, Student`s t-test, U-Mann Whitney, repeated-measures ANOVA and regression models were used.

Results. Twenty patients were included. No serious adverse events were reported. Patients in the experimental group (n=9) showed greater clinical attachment level (CAL) gain [1.44, standard deviation (SD)=1.87] and probing pocket depth (PPD) reduction (2.33, SD=1.32) than the control group (n=10; CAL gain=0.88, SD=1.68, and PPD reduction=2.10, SD=2.46), without statistically significant differences.

Conclusion. The application of PDL-MSCs to XBS for the treatment of one-two wall intra-bony lesions was safe and resulted in low postoperative morbidity and appropriate healing, although its additional benefit, when compared with the XBS alone, was not demonstrated.

(ISRCTN 13093912)

Keywords: cell therapy, mesenchymal stem cells, periodontal ligament, periodontal regeneration, tissue engineering.

Clinical relevance

Scientific rational for the study: This phase II trial evaluated the added benefit of applying periodontal ligament-derived mesenchymal stem cells (PDL-MSCs) to xenogeneic scaffolds for the treatment of one-two wall intra-bony defects.

Principal findings: The application of autologous PDL-MSCs was safe and resulted in appropriate healing and low morbidity. However, the limited sample size prevented the evaluation of its clinical efficacy, although there was a trend towards better clinical attachment level gains when adding the cells.

Practical implications: The efficacy of therapeutic drugs containing PDL-MSCs needs further validation in studies with larger sample sizes, better-standardized periodontal lesions and longer follow-up.

INTRODUCTION

Advances in regenerative medicine and tissue engineering allowed to developed new strategies to improve the predictability and overcome some of the limitations of current approaches. One of these strategies has been the use of adult stem cells, since they have a nearly unlimited potential to replicate in an undifferentiated state as well as to differentiate into committed lineages. Among them, *ex vivo* expanded mesenchymal stem cells (MSCs) have proven self-renewing, high proliferation capacity and ability to differentiate into different stromal lineages (Garcia-Gomez et al., 2010; Santamaria, Sanchez, Sanz, & Garcia-Sanz, 2017). Furthermore, MSCs also exert paracrine or trophic effects, via secretion of soluble bioactive molecules that modulate immune cells to promote regeneration (Wang, Chen, Cao, & Shi, 2014).

The regenerative potential of MSCs in the reconstruction of orofacial tissues has been mainly evaluated in preclinical models, but also in some clinical studies. They have demonstrated their safety and applicability (Novello, Debouche, Philippe, Naudet, & Jeanne, 2019; Shanbhag et al., 2019). The clinical effectiveness of cell-based strategies for orofacial bone regeneration has been recently summarised in a systematic review, showing that cell therapy significantly enhanced regenerative outcomes in maxillary sinus augmentation procedures (Shanbhag et al., 2019).

In periodontal regeneration, the use of advanced therapies based on MSCs is derived from pre-clinical investigations, since there are very few controlled clinical trials (CCT) evaluating their efficacy in the treatment of human periodontal lesions (Novello et al., 2019). Pre-clinical studies have shown that MSCs, harvested from bone marrow, fat, periodontal ligament (PDL) and other oral sources, were capable of differentiating in cementum, functionally oriented PDL fibres and alveolar bone tissue, when implanted into different experimental periodontal defect models (Bright, Hynes, Gronthos, & Bartold, 2015; Nunez, Vignoletti, Caffesse, & Sanz, 2019). Our research group has evaluated the implantation of canine periodontal ligament-derived MSCs (PDL-MSCs) in three-wall intra-bony and critical-size supra-alveolar periodontal defects models (Nunez et al., 2018; Nunez et al., 2012), resulting in enhanced periodontal regenerative outcomes when compared with the carrier alone (Nunez et al., 2012).

The two published CCT, however, have reported conflicting results. Indeed, whereas the study by Dhote, Charde, Bhongade, and Rao (2015) reported significantly larger clinical attachment level (CAL) gains and radiographic defect fill, when applying a combination of allogeneic cord blood MSCs, platelet-derived growth factor-BB (PDGF-BB) and a β-tricalcium phosphate (β-TCP) scaffold, compared to open flap debridement (OFD), the study by Chen et al. (2016), comparing autologous PDL-MSCs seeded in scaffold of demineralized bovine bone mineral (DBBM) did not find significant differences in radiographical bone fill, when compared with the application of the scaffold alone.

This scarce and contradictory available clinical evidence justifies the attempt to perform a CCT to assess safety and efficacy of cell therapy in periodontal regeneration. It was, therefore, the aim of this 12-month pilot CCT, to evaluate the safety and clinical efficacy of using autologous PDL-MSCs embedded in a hydroxyapatite-collagen scaffold, for the regeneration of one- and two-wall intra-bony defects, when compared to the scaffold alone.

MATERIAL AND METHODS

Study design

This study was designed as a quasi-randomized, phase II, double-blinded, parallel-group pilot CCT, with a 12-month follow-up. All clinical procedures were performed in the Postgraduate Periodontal Clinic at the Faculty of Odontology, University Complutense of Madrid (Spain). Patient recruitment started on November 2014 and was completed on March 2019. Cell culture and provision of the experimental and control drugs were performed at the Cell Production Unit of the Institute of Sanitary Research Gregorio Marañón, Madrid (Spain). The trial was prospectively registered (www.isrctn.com ISRCTN13093912).

Ethics and approvals

The cell therapy regenerative application was approved by the Spanish Agency of Medicine and Medical Devices (EUDRACT 2013-00435-77), and the study protocol was approved by the Clinical Research Ethics Committee, Hospital Clínico San Carlos, Madrid. All procedures were performed according to the Good Clinical Practice and the Directives of the European Commission for Advanced Therapy Medicinal Products for human use.

Patients

Patients were consecutively screened (NS, LF) among those attending the Postgraduate Periodontal Clinic and were considered for inclusion if they fulfilled the following criteria: 1) from 25 to 70 years old; 2) absence of relevant medical conditions; 3) non-smokers; 4) moderate-severe chronic periodontitis (Armitage, 1999), or with stages III-IV periodontitis (Papapanou et al., 2018), 5) active periodontal therapy performed at least 4-6 weeks before recruitment; 5) presence of ≥1 tooth with CAL≥ 6 mm and an intra-bony defect of one or two walls (as predominant component) with a radiographic intra-bony component of ≥4 mm; 6) presence of ≥1 non-functional third molar or ≥1 tooth with a hopeless prognosis (extraction planned); 7) presence of ≥2 mm of keratinized gingiva at the tooth with the defect; 8) good oral hygiene (full-mouth plaque score, FMPS<25%); and 9) willingness to optimal compliance with the study procedures. Exclusion criteria are detailed in Appendix 1. Patients meeting the inclusion and exclusion criteria were invited to participate, informed about the objectives and risks of the study, and if willing to participate, signed the informed consent.

Investigational device and interventions

The extracted tooth was immediately transported to the Cell Production Unit, where the PDL was isolated by scraping the root, digested and cultured following the method previously described by our research group (Santamaria et al., 2017). In brief, PDL-MSCs were assessed for colony-forming unit fibroblasts (CFU-F) ability and characterized following the minimal criteria for MSCs (Dominici et al., 2006) (Figure 1). The expanded cells were subjected to sterility and mycoplasma contamination tests as well as to genomic stability tests to rule out chromosomal alterations by means of Microarray-based Comparative Genomic Hybridization (Nimgenetics S.L., Madrid, Spain).

The experimental treatment consisted of $1x10^7$ autologous PDL-MSCs (3^{th} passage) incubated for 1 hour (37ºC, 5%CO_2 and 95% humidity) in 100 mg of a xenogeneic bone substitute (XBS) (Bio-Oss Collagen, Geislich Pharma AG, Wolhusen, Switzerland). The control treatment comprised the use of the same XBS seeded in 200 μm of physiological saline solution.

The main surgical approach was the coronally advanced flap technique to minimize recessions (REC) (Zucchelli & De Sanctis, 2008) but other papilla preservation techniques could be performed if the surgeon deemed it advisable (Figure 2**)**. Teeth with mobility grade II were splinted before the surgery.

All subjects received systemic antibiotic therapy after surgery (amoxicillin, orally administered, 1 g, b.i.d., 7 days; Normon, Madrid, Spain) and anti-inflammatory medication only if necessary (ibuprofen 600 mg, t.i.d; Normon, Madrid, Spain). They were asked to abstain from mechanical oral hygiene in the surgical area and to use a 0.12% chlorhexidine (CHX) and 0.05% cetylpyridinium chloride mouth rinse, b.i.d. for 1 month (Perio-aid®, Dentaid, Cerdanyola del Vallés, Spain). Sutures were removed 14 days after surgery. A recall program, consisting of full-mouth supragingival prophylaxis, tooth polishing and oral hygiene reinforcement, was scheduled on a monthly basis.

Randomization, allocation concealment and blinding

Randomization followed a quasi-randomization approach, since patients were assigned to the treatment group according to whether the cell expansion process at the cleanroom was successful. Patients whose cells exhibited growth were assigned to the test group; in the opposite scenario, patients were assigned to the control group.

Allocation was concealed to the surgeons (JN and FV) until the end of the study, as both test and control drugs had identical appearance. Patients and examiners were blinded to the treatment assignment, since each patient received a code, whose identification was not provided by the cleanroom staff until the completion of the study.

Outcome variables

Clinical outcome variables

One trained and calibrated examiner collected the clinical data (NS). The calibration exercise consisted on duplicate measurements of PPD and CAL, 7 to 10 days apart, in two patients with ≥4

teeth with PPD ≥6 mm, and resulted in intra-class correlation coefficients for PPD and CAL of 0.801 and 0.856, respectively (Wang et al., 1995).

The primary outcome variable was CAL gain from baseline to 12-month visit, measured, at six sites per tooth, in mm, with an electronic pressure-sensitive periodontal probe (Florida Probe®, Florida Probe Corporation, FL, USA) (Gibbs et al., 1988), from the bottom of the pocket to the margin of a customized plastic splint made of thick polyethylene rigid sheets by using a thermoplastic vacuum forming machine (Technoflux, Barcelona, Spain), cut at the level of the interproximal cemento-enamel junctions (CEJs). Secondary outcome variables included CAL gain after 6 months, PPD and REC changes from baseline to 6 and 12 months. FMPS and full-mouth bleeding scores (FMBS) were calculated by dichotomously evaluating six sites per tooth at baseline,1, 3, 6, 9 and 12 months.

Intra-surgical outcomes

Once flaps were elevated and the intra-bony defects fully debrided, the number of walls was recorded, and the following intra-surgical measurements, using a periodontal probe to the nearest millimetre, were recorded; distance CEJ-bone defect (BD), distance CEJ-most coronal extension of interproximal bone crest (BC) (CEJ-BC). The intra-bony component of the defect (INFRA) was calculated by subtracting CEJ-BC from CEJ-BD (Tonetti, Pini Prato, Williams, & Cortellini, 1993).

Safety assessment and patient-reported outcome measures

Postoperative morbidity was evaluated by accounting the anti-inflammatory drugs taken and by assessing the patient well-being and quality of life through the fill of the Oral Health Impact Profile (OHIP-14) questionnaire at 15 days and 12 months after the procedure (Montero-Martin, Bravo-Perez, Albaladejo-Martinez, Hernandez-Martin, & Rosel-Gallardo, 2009). In the last visit, participants were asked to rate their perceived aesthetic outcome in the treated area using a 0-10 visual analogue scale (VAS). The occurrence of adverse events was monitored throughout the 12 postoperative months, with careful interviews and by observation of the affected tissues. Postoperative wound healing was assessed at one, two and four weeks using a qualitative index (complete healing, thin or thick fibrin line, incomplete healing or necrosis). Presence of oedema was evaluated as present or absent.

Data analysis

As this was a pilot, phase II, CCT, primarily aiming to assess the safety of this cell-based regenerative therapy, the sample size was estimated adequate with 20 patients (10 per group).

The data were analysed on a patient-based basis (per protocol), using as primary outcome variable CAL gains from baseline to 12-months. Descriptive statistics, including percentiles, means and standard deviations (SDs) for quantitative data, and percentages for categorical data were determined.

In order to compare groups (at each visit and in the changes between visits), Chi Square (or Fisher`s exact) tests, unpaired Student`s t-test (or Mann-Whitney U-test) and repeated-measures ANOVA were applied. The significance of the treatment effect on the primary outcome was assessed by constructing multiple linear regression models, considering CAL change as the dependent outcome, group as the exposure, and age, Rec, INFRA, defect angle, number of walls, smoking and operators as potential confounding and interaction factors. Final models were selected using the backward elimination method that allowed factors to remain in the model whenever their significance was $p<0.1$.

RESULTS

Study population and enrolment

Twenty patients, from a total of 46 subjects evaluated in the screening process (Figure 3), were selected. Ten patients [mean age=48.8 (SD=10.6), range 38-60 years, three females) received the test intervention (XBS + PDL-MSCs) and 10 patients [mean age=57.5 (SD=7.9), range 49-65 years, 3 females] the control intervention (XBS + saline). All treated patients completed the 12-month follow-up with the exception of one patient from the test group, who left the study after 6 months for personal reasons, not related to the study. The treatment groups were homogeneous at baseline in terms of patient and defect characteristics ($p>0.05$), although defects in the control group exhibited significantly more bony walls than the test group (2.0 *vs* 1.7; $p=0.08$), especially in the coronal aspect of the defects (2.0 *vs* 1.7; $p=0.04$) (Table 1). The most frequently treated teeth were incisors and canines, followed by premolars and molars.

Tooth extraction and cell isolation

Twenty teeth were extracted (Appendix 2) and taken to the cleanroom, where cell isolation was performed. The most frequently extracted tooth was the third molar (11/20), followed by other molars (3/20), premolars (3/20) and canines/incisors (3/20). Half of the teeth provided cells with the capability of developing CFU-F under culture, and were consequently allocated to the test group. The most suitable tooth for cell isolation was the third molar (7/10). All cells were positive for CD73, CD90, CD105, CD44 and CD29 and negative for CD34, CD45, CD14, CD19 and HLA-DR by flow cytometry analysis. They were also successfully differentiated to osteoblasts, chondroblasts and adipocytes (Figure 1). All PDL-MSCs showed lack of mycoplasma contamination and exhibited genomic stability after array-based comparative genomic hybridization (CGH) test.

Safety evaluation

No patient reported any adverse event other than the common side effects of periodontal regenerative surgeries. Mild-moderate pain and swelling, during the first week, and mild dentine hypersensitivity, during the following weeks, were the most frequently reported side effects in both groups. Even though primary wound closure was attained in all sites at the end of the surgery,

different levels of healing were recorded at 1, 2, 4 and 12 postoperatively (Table 2), but differences between groups were not statistically significant ($p>0.05$).

Patient-reported outcome measures

The number of anti-inflammatory drugs taken for the first week after surgery was greater in the participants from the test (4.6, SD=4.2) than from the control group (2.4, SD=3.2), although no significant differences were found ($p>0.05$). The VAS mean score of satisfaction with the aesthetic appearance at the surgical area was lower in the test group (7.38, SD=2.9) than in the control group (8.80, SD=2.82), with no statistically significant differences ($p>0.05$). Similarly, no differences could be found, between groups, in terms of patient well-being and quality of life after the intervention ($p>0.05$), although control subjects reported better oral health-related quality of life indicators, 15 days (4.2, SD=2.9) and 12 months (2.7, SD=3.4) after the intervention than the test group (5.7, SD=3.1; and 7.0, SD=10.73; respectively).

Efficacy evaluation

Although mean FMPI levels increased from baseline, all patients presented FMPS percentages below 25% throughout the entire follow-up. Regarding the intragroup comparisons, both groups showed CAL and PPD improvements over time; nevertheless, the analysis did not reveal statistically significant differences for any of the measured clinical variables from baseline to 12 months ($p>0.05$). (Appendix 3).

When analysing the intergroup comparisons, the experimental group attained greater mean CAL gain (1.44, SD=1.87) and PPD reduction (2.33, SD=1.32) than the control group (0.8, SD=1.68 and 2.10, SD=2.46), respectively), although these differences were not statistically significant ($p>0.05$ for both). An average REC increase of 1 mm was observed in both the test (1.11, SD=1.16), and control groups (1.2, SD=1.54), ($p\geq0.05$) (Table 3). Percentiles for changes between baseline and 12 months are shown in Appendix 4. The frequency distribution analysis revealed a larger proportion of sites showing CAL gain ≥2 mm and ≥3 mm in the test group (56% and 33%) than in the control group (40% and 20%) ($p>0.05$) (Table 4, Appendix 5).

At the final 12-month visit, 50% and 60% of the defect sites in the test and control group, respectively, exhibited PPD <4 mm ($p>0.05$). Nevertheless, the mean residual PPD was higher in the control than in the test group ($p>0.05$) (Table 4).

The treatment effect, in terms of CAL gain, was assessed by multiple regression models, incorporating several factors as potential sources of variability (Appendix 6). The model containing treatment group, baseline REC, INFRA, defect angle and smoking as covariates, explained 52.8% of the variability (p-value=0.056). Although there were differences between groups in favour of the test group (1.23 mm), these were not statistically significant (p=0.116). However, the intra-bony component (p=0.003) and the defect angle (p=0.027) demonstrated a significant effect on the primary outcome.

Study power calculation

Post hoc power analysis, using an effect size of 0.645 mm in CAL gain (baseline-12 months), a variability of 1.76, a sample size of 10 and 9 patients (control and test group) and an α error of 5%, detected a study power of 12.2%.

DISCUSSION

The aim of this pilot CCT was to evaluate the safety and efficacy of PDL-MSCs seeded in hydroxyapatite-collagen scaffolds for the regeneration of periodontal intra-bony defects. The results demonstrated that: (1) this cell therapy was safe and that the adverse events associated with the utilization of PDL-MSCs were not different from the common side effects of a standard regenerative periodontal surgery (Cortellini & Tonetti, 2015); and that, (2) although a statistically significant added beneficial effect could not be detected in terms of CAL gain and PPD reduction between groups, positive trends, favouring the cell-based protocol, were evident (25% of subjects in the control group suffered a CAL loss of 0.25 mm while 25% of the test subjects showed no changes in CAL).

The CAL gains and PPD reductions reported in the present investigation in both groups were modest in comparison with other studies evaluating different regenerative technologies (Matarasso et al., 2015; Nibali et al., 2019). These limited clinical improvements may be explained by the patient and defect selection, since only one- and two-wall non-contained intraosseous lesions were selected, with the objective of assessing the added value of MSCs in the treatment of those defects lacking a predictable regenerative outcome. The tested material was not able to increase the predictability of results of the proposed therapy, even though the test group resulted in higher

CAL gains and PPD reductions at 12 months than the control group (mean differences 0.64 mm and 0.23 mm) (Table 2). The evaluation by multiple regression analysis (Appendix 6) demonstrated that changes in the primary outcome variable after therapy could not be explained by the group only, but rather by the depth of the intra-bony component and the angle of the defect. These results agree with other studies where both covariates were significantly associated with the treatment outcomes (Tonetti, Pini-Prato, & Cortellini, 1993; Tsitoura et al., 2004).

Furthermore, the treatment effect within each group was neither statistically significant. Although conservative surgical techniques were used to minimize the apical migration of the gingival margin, REC increased in both groups during healing. This effect was probably due to the relevant baseline REC (table 1), what may have prevented a stable coronally position of the flaps during healing. Despite these findings, the test group showed a constant improvement pattern, in terms of CAL gain and PPD reduction over time, whilst the control group exhibited improvements in the first 6 months, followed by a subsequent deterioration of the clinical parameters (Appendix 2), suggesting a beneficial role of the cells at this late stage.

When compared with other clinical studies using expanded MSCs applied to periodontal regeneration, the outcomes of the present investigation were inferior to those described in prospective case series (Baba et al., 2016; Yamada et al., 2013) and in a CCT in which allogeneic cord blood cells plus PDGF-BB/β-TCP scaffold were compared to an OFD (Dhote et al., 2015). However, in the other published CCT with expanded MSCs, using a similar cell therapy construct to the one used in the present investigation (PDL-MSCs/DBBM versus DBBM) (Chen et al., 2016), differences between groups were not statistically significant, as in the present study.

A recent randomized CCT using cell populations other than pure expanded MSCs, has described a significant added clinical effect for the regeneration of deep intrabony defects. Ferrarotti et al., 2018 reported that a heterogeneous cell population containing MSCs (i.e micrografts containing pulp-derived MSCs) resulted in statistically significant better CAL gain and PPD reduction than the control group (without cells) not only for the treatment of 1, 2-wall defects but also for treating well-contained 3-wall intrabony defects.

This was a phase II pilot study, aiming to evaluate the safety and performance of a cell therapy based on combining PDL-MSCs with a commercially available xenogeneic bone substitute,

resulting in no adverse effects, no significant morbidity and good acceptance by the patients. In terms of performance, although clinical differences were noticed in favour of the cell group, no statistically significant differences were detected between groups, mainly due the small sample size of this pilot study, which resulted in a low statistical power (12.2%). Another limitation was the lack of a proper randomization. The original protocol included block randomization by a computer-generated list, but the lack of cell growth in some cases, forced to assign the test and control therapy in accordance with the cell expansion results. This issue deeply limits the applicability of autologous expanded PDL-MSCs from patients with periodontitis in the clinical practice, as the predictability of isolating sufficient counts of viable cells with proper proliferation capability has been shown to be low. At the present time, the cost-effectiveness of this cell therapy protocol is very limited, as the added value of the application of PDL-MSCs from subjects with a reduced periodontium is scarce and the current costs of ex-vivo expanded MSCs make them barely affordable. Therefore, the use of other autologous cell sources or even allogeneic MSCs should be strongly addressed in future trials. Besides, well-design randomized CCT, with a greater number of patients and longer follow ups should be carried out.

Within the limitations of the present pilot study, it can be concluded that the use of autologous PDL-MSCs for periodontal regeneration purposes is safe. The results have not demonstrated a significant added clinical benefit, after 12 months, of this cell therapy protocol, when compared with the control.

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Table 1. Baseline patient and defect characteristics.

Variable	Control group	Test group	*p-value* *
Subject number	10	10	-
Age, mean (SD)	57.5 (7.9)	48.8 (10.6)	0.053†
Gender (% females)	30	30	1.000‡
%FMPS, mean (SD)	8.4 (4.7)	6.2 (7.1)	0.432†
%FMBS, mean (SD)	12.9 (9.2)	12.8 (12.1)	0.978†
CAL in mm, mean (SD)	9.9 (1.6)	10.6 (2.2)	0.435†
PPD in mm, mean (SD)	6.8 (1.03)	6.6 (1.07)	0.676†
REC in mm, mean (SD)	3.2 (1.8)	4.0 (1.7)	0.494†
CEJ-BD in mm, mean (SD)	10.8 (2.1)	10.2 (2.4)	0.524†
Intra-bony component in mm, mean (SD)	5.95 (1.7)	5.2 (1.3)	0.302†
Defect angle in degrees, mean (SD)	25.3 (6.9)	27.8 (5.1)	0.374†
Predominantly one wall (%)	0	30	0.211‡
Predominantly two walls (%)	100	70	0.211‡
Defects in maxilla (%)	50	50	1.000‡
Defects in mesial aspect (%)	38.5	61.5	0.350‡
Strictly interproximal defects	8	6	0.628‡
Interproximal & lingual/palatal defects	2	4	0.628‡
Defects accessed with CAF	5	7	0.650‡
Defects accessed with other PPT	5	3	0.650‡

SD, standard deviation; FMBS, full-mouth bleeding score; FMPS, full-mouth plaque score; CAL, clinical attachment level at the deepest defect site; PPD, probing pocket depth at the deepest defect site; REC, Recession of the gingival margin; CEJ-BD, distance from the

cemento-enamel junction to the base of the defect; CAF: coronally advanced flap; PPT: papilla preservation techniques.

† *Student´s t-test was used for continuous variables.*

‡*Fisher's exact text was used for categorical variables.*

Statistically significant differences were considered for p≤0.05.

Table 2. Primary closure of the Defect-Associated Interdental Papilla at follow-up visits.

VISIT	Control, N=10 (Scaffold)	Test, N=9 (Scaffold/PDL-MSCs)	p-value
1 Week	4*	4†	0.721
2 Weeks	6‡	5¶	0.384
4 Weeks	7Ω	9∫	0.549
12 Weeks	10	10	NA

* One defect showed necrosis, one fibrin line and four thin fibrin line.

† Two defect showed necrosis, two fibrin line and two thin fibrin line.

‡ One defect showed incomplete healing and three thin fibrin line.

¶ One defect showed incomplete healing, three fibrin line and one thin fibrin line.

Ω One defect showed incomplete healing, one thin fibrin line

∫ One defect showed incomplete healing.

NA, not applicable; PDL-MSCs, Periodontal ligament- derived mesenchymal stem cells

Pearson Chi- Square test was used for the comparisons.

Table 3. Clinical outcomes, presented as mean changes and standard deviation (mean [SD]), between baseline and 12 months.

Outcome variable	Control, N=10 (Scaffold)	Test, N=9 (Scaffold/PDL-MSCs)	Mean difference (95% CI)	*p*-value
CAL gain in mm	0.80 (1.68)	1.44 (1.87)	0.64 (-2.36; 1.48)	0.441
PPD reduction in mm	2.10 (2,46)	2.33 (1.32)	0.23 (-2.18; 1.71)	0.804
REC increase in mm	1.2 (1.54)	1.11 (1.16)	0.08 (-1.42; 1.25)	0.890

CAL, clinical attachment level; PPD, probing pocket depth; REC, Recession of the gingival margin; PDL-MSCs, Periodontal ligament- derived mesenchymal stem cells; CI, confidence interval.

Repeated-measures ANOVA was used for evaluating intergroup changes between baseline and 12 months. Statistically significant differences were considered for $p \leq 0.05$.

Table 4. **Frequency distribution (%) of clinical attachment level gain and probing pocket depth reduction, residual probing pocket depth (mean [SD]) and locations with PPD<4mm (%) at the 12 month-visit.**

	CAL gain		*PPD reduction*		*Residual PPD*	*Sites with PPD<4mm at the defect site (%)*
	<2 mm	≥2 mm	<2 mm	≥2 mm		
Test (n=9)	44	56	22	78	4.33 (1.00)	5 (50)
Control (n=10)	60	40	40	60	4.70 (2.11)	6 (60)
p-value*	0.65		0.62		0.64	1.00

SD, standard deviation; CAL, clinical attachment level; PPD, probing pocket depth; CI, confidence interval;

**Unpaired Student´s t-test and Fisher`s exact test were used for the analysis. Statistically significant differences were considered for* p≤0.05.

Figure 1. Cell isolation, characterization and surgery in a patient from the experimental group. ***a****. Periodontal ligament scraping, periodontal ligament-derived mesenchymal stem cells (PDL-MSCs) in culture and test device ready for transplantation.* ***b****. Characterization of PDL-MSCs by flow cytometry and differentiation (dif.) to osteoblasts, chondroblasts and adipocytes after staining with Alkaline Phosphatase, Alcian Blue and Oil Red Staining.* ***c.*** *Upper left canine with a preoperative probing pocket depth of 14 mm.* ***d.*** *Preoperative periapical x-ray showing a deep intra-bony defect in the mesial aspect of the tooth.* ***e.*** *After flap elevation and debridement, visualization of a two-wall bony defect with an intra-bony component of 10 mm.* ***f.*** *Occlusal view of the defect.* ***g****. Adaptation of the test device into the defect.* ***h.*** *Primary wound closure.*

Figure 2. Surgical procedure and wound healing in a control subject and in a test subject. CONTROL GROUP: a. Incisions; b. Two-wall intra-bony defect (lingual and distal wall); c. Graft placement; d. Suture; e. Baseline x-ray; f. 1-week healing; g. 2-weeks healing; h. 1-month follow-up; i. 12-month follow up; 12-month x-ray. TEST GROUP: a. Incisions; b. Flap elevation; c. Two-wall intra-bony defect (10 mm intra-bony component); d. Graft adaptation; e. Baseline x-ray; f. Suture; g. 2-weeks healing; h. 1-month follow-up; i. 12-month follow up; 12-month x-ray.

Figure 3*.* ***Study flowchart for the 12-month trial.*** *PDL-MSCs, periodontal ligament- derived mesenchymal stem cells; CAL, clinical attachment level; PPD, probing pocket depth; REC, recession.*

APPENDICES

Appendix 1. Diseases, conditions and characteristics that represent exclusion criteria for the study.

1) Diseases affecting connective tissue metabolism and/or conditions that represent specific exclusion criteria for cell therapies according to the Spanish regulations: i) History of rapidly progressive dementia/ neurological illness of unknown aetiology, ii) Previous treatment with pituitary-derived hormone, iii) Uncontrolled systemic or local infection in the tissue of interest, iv) History, risk factors, clinical evidence or positive tests for human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV) and human T-lymphotropic virus I/II (HTLV I/II), v) History of chronic autoimmune disease that could caused injury of the tissues of interest, vi) Disease-transmission risk factors related to travelling history and local prevalence of local infections, vii) Diseases that affect connective tissue metabolism, viii) Risk of invalidation of diagnostic tests due to hemodilution or immunosuppressive-treatment, ix) Presence of physical signs that may entail a risk for disease transmission, x) History of recent vaccination with attenuated virus that may entail a source of infection. Source: https://www.boe.es/buscar/act.php?id=BOE-A-2006-19625&tn=0&p=20140704

2) Pregnant or lactating women.
3) Participation in an intervention trial in the same quadrant in the previous two months. 4) Type III tooth mobility, furcation involvement, periapical pathology or vertical root fracture in the selected tooth.

Appendix 2. Reasons for tooth extraction in patients from control and test groups.

	Control	**Test**
	Number of patients	*Number of patients*
Third Molar without occlusion	3	7
Hopeless periodontal prognosis	7	3
Total number of patients	10	10

Appendix 3. Clinical outcomes at baseline and 12 months and changes in clinical outcomes between baseline, 6 and 12 months.

Outcome variable		N (C/T)	Control, (Scaffold)	Test (Scaffold/PDL-MSC)	*p-value*
FMPI in %, mean (SD)	Baseline	(10/10)	8.44 (4.70)	6.22 (7.11)	0.432
	12mo.	(10/9)	21.04 (24.44)	18.80 (15.82)	0.818
CAL in mm, mean (SD)	Baseline	(10/10)	9.90 (1.66)	10.60 (2.22)	0.435
	12mo.	(10/9)	9.10 (2.18)	9,44 (2.35)	0.745
PPD in mm, mean (SD)	Baseline	(10/10)	6.80 (1.03)	6.60 (1.07)	0.676
	12 mo.	(10/9)	4.70 (2.11)	4.33 (1.00)	0.641
REC in mm, mean (SD)	Baseline	(10/10)	3.20 (1.81)	4.00 (1.76)	0.890
	12 mo.	(10/9)	4.40 (1.35)	5.33 (2.29)	0.288
CAL change in mm, mean (SD)	0-6mo.	(10/10)	1.80 (1.93)	0.80 (1.98)	0.269
	6-12mo.	(10/9)	-1.00 (2.00)	0.33 (1.41)	0.222
	0-12mo.	(10/9)	0.80 (1.68)	1.44 (1.87)	0.441
PPD change in mm, mean (SD)	0-6mo.	(10/10)	2.30 (2.16)	1.50 (1.50)	0.350
	6-12mo.	(10/9)	-0.20 (1.75)	0.66 (0.86)	0.197
	0-12mo.	(10/9)	2.10 (2.46)	2.33 (1.32)	0.804
REC change in mm, mean (SD)	0-6mo.	(10/10)	-0.40 (1.83)	-0.70 (1.25)	0.675
	6-12mo.	(10/9)	-0.80 (1.31)	-0.55 (1.01)	0.659
	0-12mo.	(10/9)	-1.20 (1.54)	-1.11 (1.16)	0.890

PDL-MSCs, Periodontal ligament- derived mesenchymal stem cells; CI, confidence interval. FMPI, full-mouth plaque index; 12mo., 12 months; CAL, clinical attachment level; PPD, probing pocket depth; REC, Recession of the gingival margin; 0-6mo., between 0 and 6 months; 6-12mo., between 6 and 12months; 0126mo., between 0 and 12 months.

Unpaired Student´s t-test and Mann Whitney U test were used for the analysis. Statistically significant differences were considered for $p \leq 0.05$.

Appendix 4. Percentiles for changes between baseline and 12 months for the main quantitative variables.

		Percentiles					
Variable	**Group**	**5**	**10**	**25**	**50**	**75**	**90**
CAL change	Control	-2.00	-1.90	-0.25	0.50	2.25	3.00
(mm)	Test	-2.00	-2.00	0.00	2.00	3.00	
PPD reduction	Control	-2.00	-1.90	-0.25	3.00	4.25	5.00
(mm)	Test	0.00	0.00	1.50	2.00	3.50	
REC change	Control	-4.00	-3.90	-2.25	-1.00	0.00	0.90
(mm)	Test	-3.00	-3.00	-2.00	-1.00	0.00	

CAL, clinical attachment level; PPD, probing pocket depth; REC, Recession of the gingival margin.

Appendix 5. **Frequency distribution (%) of clinical attachment level gain and probing pocket depth reduction at 12 months.**

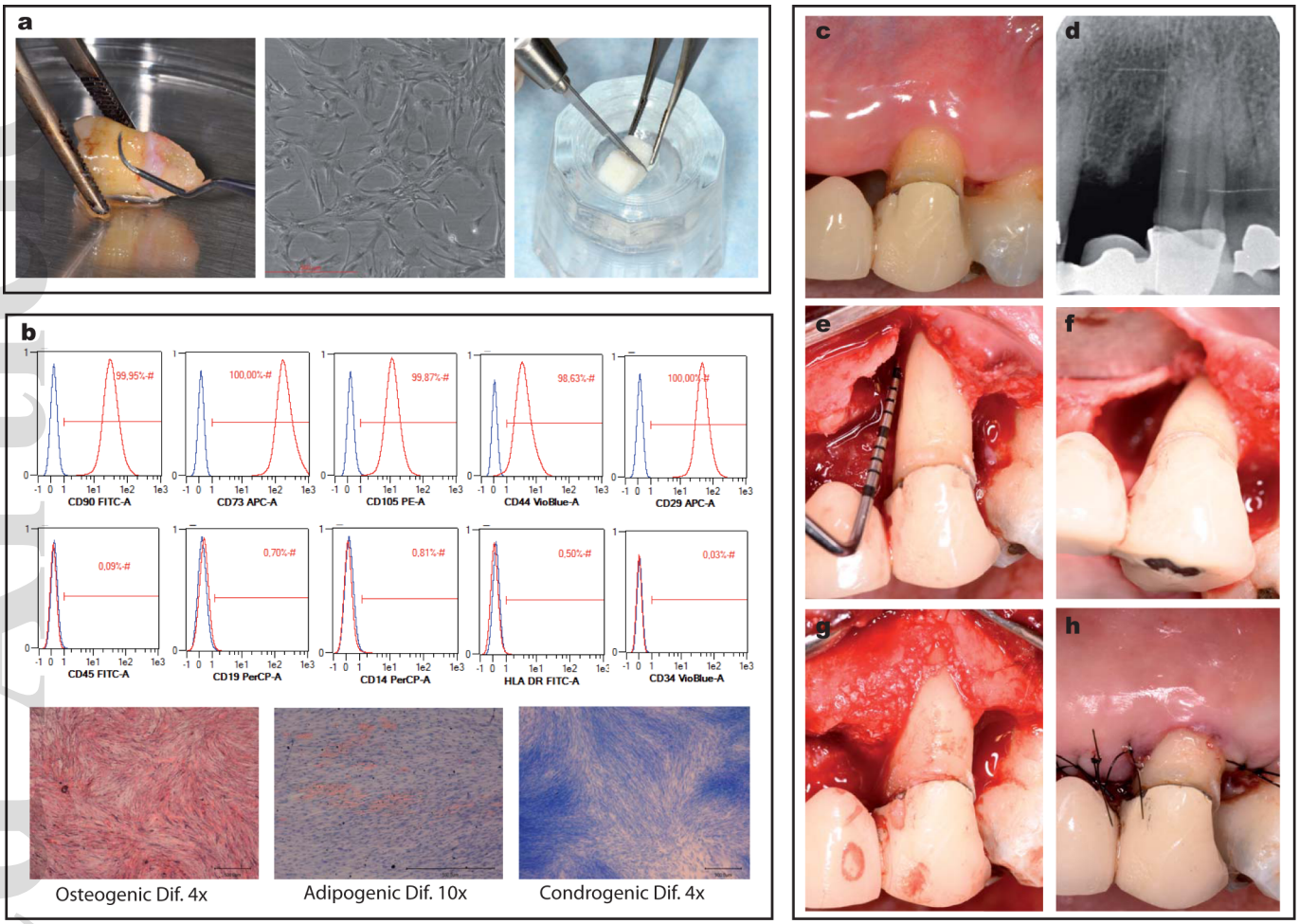
	CAL gain		***PPD reduction***		p-value*
	<3 mm	≥3 mm	<3 mm	≥3 mm	
Test (n=9)	67	33	56	44	p≤0.05
Control (n=10)	80	20	40	60	

**Fisher`s exact test was used for the comparisons between groups.*

Appendix 6. Multiple Lineal regression models using clinical attachment level change (baseline-12 months) as primary outcome.

MODEL	Parameter	Estimate	Std. Error	Beta	t	p-value	95% CI	
							Lower Bound	Upper Bound
MODEL 1	*Constant*	*0.8*	*0.56*		*1.42*	*0.17*	*-0.387*	*1.98*
p-value=0.441								
Adjusted	**Treatment group**	0.64	0.81	0.18	0.78	0.44	-1.08	2.36
R^2*=0.035*								
MODEL 2	*Constant*	*11.95*	*7.05*		*1.69*	*0.125*	*-4.01*	*27.91*
p-value=0.307	**Treatment group**	1.42	0.98	0.41	1.44	0.18	-0.80	3.64
Adjusted	**Age**	-0.04	0.06	-0.24	-0.60	0.55	-0.19	0.11
R^2*=0.172*	**Baseline Recession**	0.41	0.28	0.42	1.45	0.17	-0.22	1,05
	INFRA	-1.33	0.49	-0.92	-2.71	0.02	-2.45	-0.22
	Defect angle	-0.21	0.11	-0.74	-1.91	0.08	-0.46	-0.03
	Defect Morphology (apical)	0.82	0.95	0.23	0.86	0.40	-1.32	2.98
	Smoking	-1.63	1.13	-0.46	-1.44	0.18	-4.19	0.91
	Operators	-0.86	1.80	-0.15	-0.48	0.64	-4.95	3.21
MODEL 3	Constant	12.50	3.76		3.32	0.006	4.37	20.64
p-value=0.135	**Treatment group**	1.23	0.73	0.36	1.68	0.11	-0.35	2.82
Adjusted	**Baseline REC**	0.43	0.22	0.44	1.95	0.07	-0.04	0.92
R^2*=0.294*	**INFRA**	-1.43	0.40	-0.99	-3.55	0.003	-2.30	-0.56
	Defect angle	-0.20	0.08	-0.73	-2.48	0.027	-0.38	-0.02
	Smoking	-1.55	0.80	-0.43	-1.93	0.074	-3.27	0.17

Std. Error, standard error; CI, confidence interval; INFRA, infra-bony component of the defect; Smoking (categorical: never smoker, former smoker<12 months, former smoker>12 months), REC, Recession of the gingival margin.



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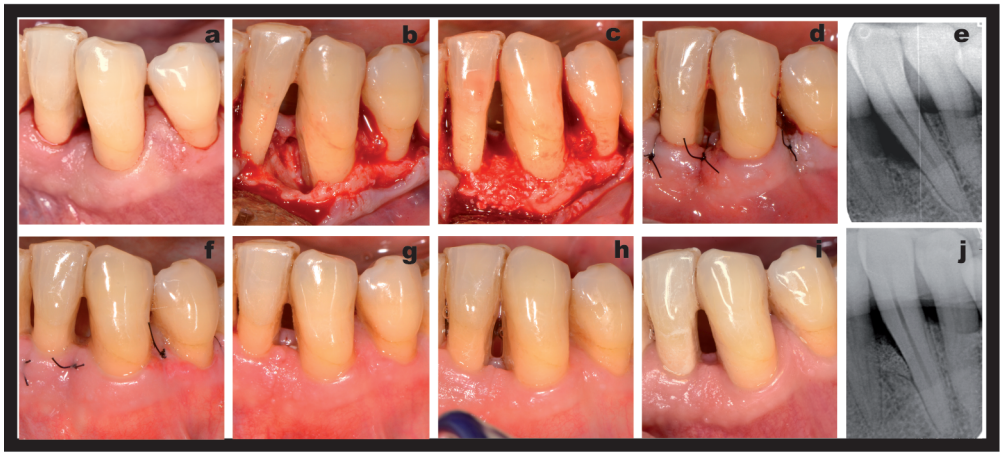
CONTROL GROUP



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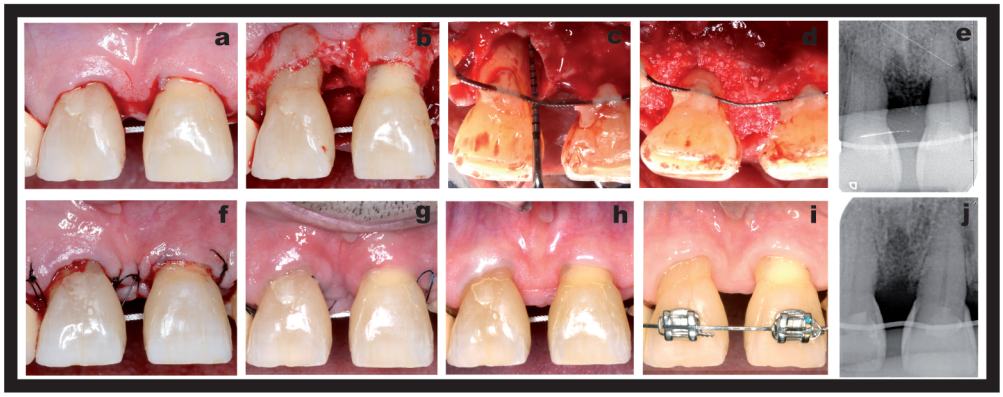

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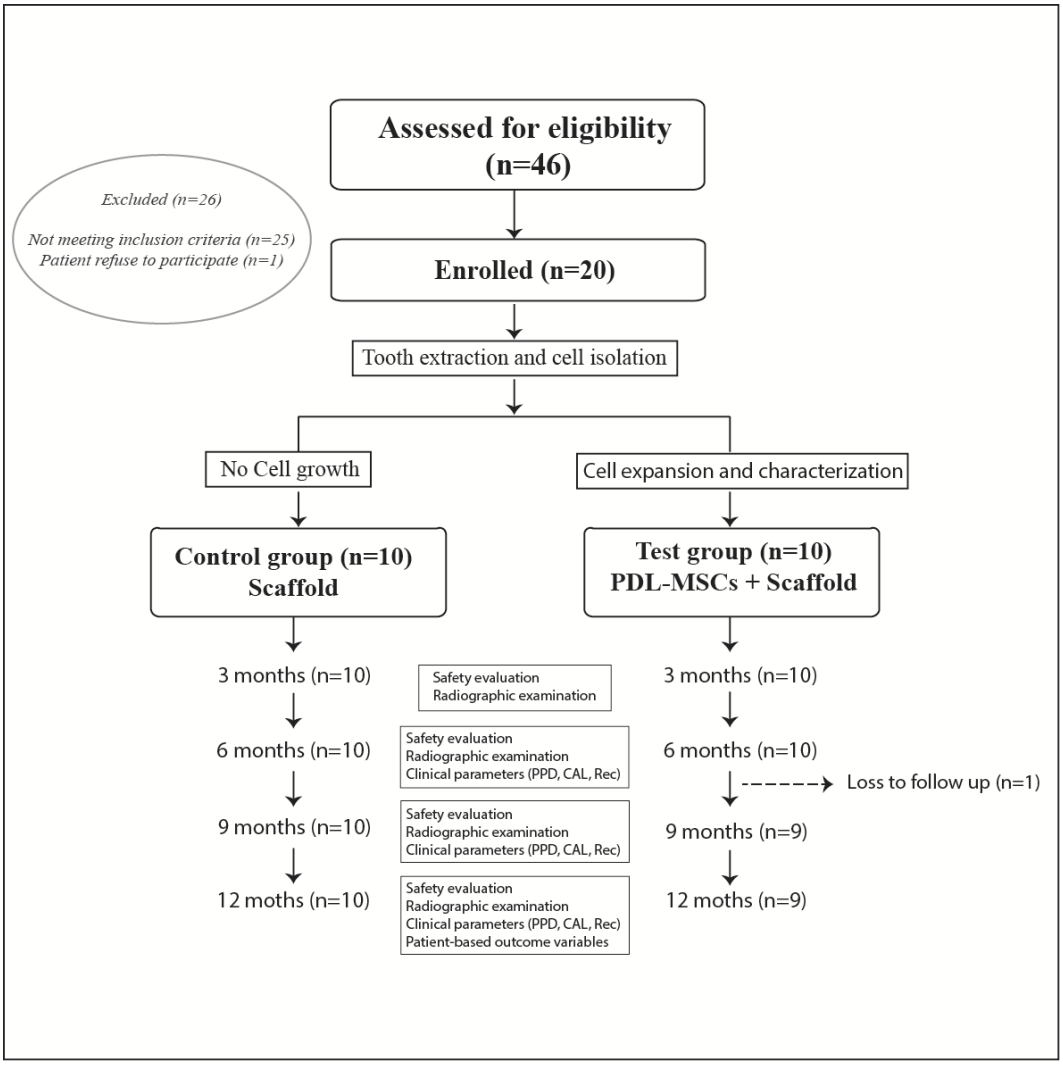

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