Journal of Cranio-Maxillofacial Surgery 43.7 (2016): 1161-1168

DOI: 10.1016/j.jcms.2015.026

Adipose-derived stem cells and platelet-rich plasma for preventive treatment of bisphosphonate-related osteonecrosis of the jaw in a murine model

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Keywords:
Adipose-derived stem cells
Platelet-rich plasma
Bisphosphonate-related osteonecrosis of the jaw
Zoledronic acid

a b s t r a c t

Objectives: The main challenge in treating bisphosphonate-related osteonecrosis of the jaw (BRONJ) is the absence of an effective established treatment. We aimed to compare different potentially preventive treatments for BRONJ after dental extractions in zoledronic acid (ZA)-treated animals. We studied the local application of different combinations of adipose-derived stem cells (ASCs) with or without previous stimulation with bone morphogenetic protein 2 (BMP-2) and platelet-rich plasma (PRP) in rats.

Material and methods: Fifty-six male Wistar rats were treated with ZA for 9 weeks. Dental extractions were performed in the eighth week, and the animals were divided into 4 groups. In group 1 (n ¼ 14), alveolar coverage with mucoperiosteal flap was performed. In group 2 (n ¼ 14), PRP was applied over the sockets and covered with the flap. In group 3 (n ¼ 15), allogeneic ASCs with PRP were applied and covered with the flap. In group 4 (n ¼ 13), animals were treated with ASCs cultured with BMP-2, PRP, and flap coverage. Histologic, fluorescence, and radiologic studies of the maxillae were performed.

Results: ASC-treated animals showed lower frequency of osteonecrosis (14% vs 50%, p ¼ 0.007) and greater bone turnover (p ¼ 0.024) and osteoclast count (p ¼ 0.045) than those not receiving the ASC treatment.

Conclusions: In this high-risk model, ASC-based treatments seem to prevent BRONJ more effectively than mucosal flap with or without PRP. The combination of ASCs and PRP appears to be synergistic, and the addition of BMP-2 could further improve the results.

1. Introduction

Osteonecrosis of the jaw is a well-known, severe side effect of bisphosphonate (BP) treatment, and was first reported as a rare but serious complication 3 decades ago (Marx, 2003; Wang et al., 2003). BPs are anti-bone resorptive drugs used routinely to decrease osteoclast-mediated bone loss in osteoporosis, multiple myeloma, Paget disease, and complications of metastatic disease. A high percentage of bisphosphonate-related osteonecrosis of the jaw (BRONJ) cases have been reported in patients treated with high doses of intravenous aminobisphosphonate (mainly zoledronic or pamidronic acid). An increased risk of BRONJ has been reported in intravenously treated cancer patients (odds ratio [OR] ¼ 4.27) compared to those treated orally (OR ¼ 1.18) (Lee et al., 2014). These patients often receive concomitant treatment with immunosuppressive and/or chemotherapy drugs, increasing the risk of osteonecrosis (Ruggiero et al., 2009; Anavi-Lev et al., 2013). Other risk factors include invasive dental procedures, oral infections, mechanical trauma to the jawbone, and long-term BP treatment (Dodson, 2009; Kuhl et al., 2012). In addition to its low incidence, the main challenges of this complication are the limited knowledge of its pathogenesis and the absence of effective established treatment. These difficulties have led several research teams to explore new therapeutic strategies for treatment and prevention of BRONJ disease. Mucosal coverage of alveolar sockets after dental extractions has been performed as treatment (Voss et al., 2012) and as a preventive measure (Abtahi et al., 2013) in order to avoid bacterial contamination and to increase blood supply to BP-affected bone during the healing process. Defective bone healing occurs in BRONJ because of suppressed bone turnover, likely favored by the antiangiogenic properties of potent BPs such as zoledronic acid (ZA)

(Yamada et al., 2009; Anavi-Lev et al., 2013; Sharma et al., 2013). Therefore, it seems that stimulating angiogenesis locally could be an effective management strategy.
Mesenchymal stem cells (MSCs) have recently emerged as candidates for BRONJ treatment (Kikuiri et al., 2010; Cella et al., 2011; Gonzalvez-Garcia et al., 2013; Li et al., 2013) due to their capacity to differentiate to other mesenchymal tissues such as bone, cartilage, and adipocytes (Pittenger et al., 1999), and due to the secretion of numerous cytokines with immunomodulatory and proangiogenic activity. Although the mechanisms of the immunosuppressive effects of MSCs have not been clearly defined, it seems that MSCs modulate the function of different cells involved in immune response (Yoo et al., 2009; Kikuiri et al., 2010; Li et al., 2013) and tissue healing. MSCs have also been reported to be immunoprivileged cells, without human leukocyte antigen (HLA)e II expression and limited expression to HLA-I, a relevant feature since it would allow their allogeneic transplantation. Currently, allogeneic MSCs are used in different clinical trials (89 clinical trials registered on clinicaltrials.gov); no adverse events have been reported to date.
Adipose tissue is an abundant and easily accessible source of MSCs, known as adipose-derived stem cells (ASCs). ASCs have been widely studied since they were first described in 2001 (Zuk et al., 2001), and have shown great potential in enhancing wound healing (Toyserkani et al., 2014). Bone-inducing substances such as bone morphogenetic protein 2 (BMP-2) have been used to culture MSCs for treatment of skeletal defects and bone diseases by promoting their differentiation to bone tissue (Cowan et al., 2005).
Another emerging treatment that has been used in BRONJ is platelet-rich plasma (PRP). Although only a small number of observational studies have been performed to investigate their effects, the positive results seem to be related to growth-factor as well as cytokine secretion that stimulate healing (Martins et al., 2012; Longo et al., 2014; Mathias Duarte et al., 2014; Sarkarat et al., 2014). In addition, investigators in new experimental studies are searching for regenerative properties in the combination of MSCs with PRP, promoting MSC function because of the great amount of substances released by activated platelets (Van Pham et al., 2013). Research into bone regeneration is focused on osteoblastic potential differentiation (Pak, 2012; Tajima et al., 2014).

No well-established models have been published in which the effectiveness of both PRP and MSCs are tested in BRONJ treatment. Faced with the adverse situation of a lack of established preventive treatment for BRONJ after dental extractions together with suboptimal current treatments, we aimed to compare the effectiveness of different emerging therapies in preventing BRONJ development by using a rat model with dental extractions. To accomplish this, we studied the local application of different combinations of ASCs with and without previous stimulation with BMP-2 and PRP in rats.

2. Material and methods

2.1. Study design

A rat model was chosen to test four different treatments applied after dental extractions in bisphosphonate-treated animals; to do this, we followed our previously published BRONJ-like disease model (Barba-Recreo et al., 2014), which replicates the histologic and radiographic findings of the human disease. Following this BRONJ model, the bisphosphonate chosen was ZA because of its high relative potency.
The present study was approved by the Ethical Committee for Animal Care of the University Hospital La Paz, Madrid, Spain. It was conducted in accordance with the European Union guidelines for experimental animal use. Fifty-six adult male Wistar rats (Charles Rivers, France) with an average age of 8 weeks (mean weight 350 g) were used in this study. The animals were kept in an environment with a controlled temperature and 12-hour lightedark cycles, with food and water supplied ad libitum.
All animals received intraperitoneal ZA (Zometa 0.1 mg/kg, Novartis Pharma AG, Basel, Switzerland) three times a week for a period of 9 weeks. Extractions of the three right upper molars in each animal were performed in the eighth week of treatment (Barba-Recreo et al., 2014).
Oral surgery was carried out under general anesthesia (inhaled sevoflurane, 2 mL/min with oxygen 1 L/min) with the animal in supine position, with the assistance of an optical microscope. Tramadol 0.075 mg/kg was administrated subcutaneously each day for the first 3 postoperative days.
The animals were randomly assigned into four groups as follows.
In group 1 (n ¼ 14), animals were treated with a buccal mucoperiosteal flap to cover the alveolar bone postextractions. Light subperiosteal debridement and advancement of the buccal mucosa

were performed. Gingival borders were attached with a 6/0 silk continuous suture, covering the defect without tension.

In group 2 (n ¼ 14), animals were treated with allogeneic PRP applied over the alveolar sockets after dental extractions. The same mucoperiosteal flap as in group 1 was performed to retain and cover the PRP clot over the maxillary alveolar bone.

In group 3 (n ¼ 15), animals were treated with allogeneic ASCs.

One million ASCs included in a PRP clot were applied over the alveolar sockets. The aforementioned mucoperiosteal flap was used to retain the ASCs with PRP in the area of interest (Fig. 1).

In group 4 (n ¼ 13), animals were treated with 1 million ASCs cultured with BMP-2 applied over the alveolar sockets. A PRP clot and a mucoperiosteal flap affixed over the clot were used as covering layers.

One week after dental extractions, the animals were killed by decapitation while under general anesthesia (inhaled isoflurane with oxygen 3 L/min). The heads were harvested for further histologic, fluorescence, and radiologic studies.

2.2. PRP preparation Allogeneic PRP was obtained from the blood of 3 healthy donor Wistar rats by intracardiac puncture (2 mL blood collected perdonor animal) under general anesthesia (inhaled isoflurane with oxygen 3 L/min) and stored in sodium citrate tubes. The tubes were centrifuged for 10 min at 3500 rpm at room temperature. The first 500 mL of plasma (platelet-poor fraction) of each tube was discarded, and 450 mL of plasma from the closest region to the buffy coat was collected, avoiding cell collection. Aliquots (100-mL) of PRP were prepared and frozen at 20 ————————————C until use.

For clot formation, aliquots of PRP were defrosted, and 1.5 mL of CaCl2 dissolved in deionized water at a concentration of 2.3 mg/mL (10%) was added to each 25 mL of PRP. Aliquots of the final volume measuring 26.6 mL each were incubated for 3e5 min at 37 ————————————C until a manageable clot was obtained. These clots were later applied over the alveolar sockets (group 2 animals) within 20e30 min after activation.

For group 3 (PRP with ASCs), PRP was prepared as described above. After centrifugation of the ASCs at 1,500 rpm, 1 million cells were resuspended in a previously generated aliquot of PRP prior to clot formation by incubation at 37 ————————————C.

In group 4, ASCs with BMP-2 were not applied in a PRP clot due to BMP-2 interference with the CaCl2 during the clot activation.

ASCs with BMP-2 (1 million cells suspended in 25 mL of saline) were applied over the alveolar sockets and covered with a PRP clot in the same fashion as in group 2.

2.3. ASC isolation, cell culture, characterization, and labeling

Allogeneic ASCs were obtained from the subcutaneous fat tissue of three female donor Wistar rats (the same donor animals used for PRP preparation) according to a previously described protocol in humans, with minor modifications (Zuk et al., 2001). Fat tissue was ground into small pieces and digested with type I collagenase (0.075%; Gibco BRL, Paisley, Scotland, UK). The collagenase was then inactivated by the addition of an equal volume of Dulbecco's modified Eagle's medium (DMEM; Gibco BRL), which contained 10% of fetal bovine serum (FBS; Gibco BRL). The digested tissue was centrifuged at 250 g for 10 min, and the pellet was resuspended in 0.16 M NH4Cl for erythrocyte lysis. After filtering through a 70-mm nylon mesh, the cells were plated in culture dishes and cultured at 37 ————————————C in a humid atmosphere with 5% carbon dioxide in the DMEM (Gibco BRL) containing 10% FBS (Gibco BRL) and 1% penicillinestreptomycin (Gibco BRL). The medium was changed to remove nonadherent cells 24 h after seeding, and every 3e4 days thereafter. For subculturing, cells were detached with 0.05% (v/v) trypsineethylenediaminetetraacetic acid (EDTA; Gibco BRL) in phosphate-buffered saline when 70%e80% confluencewas reached.

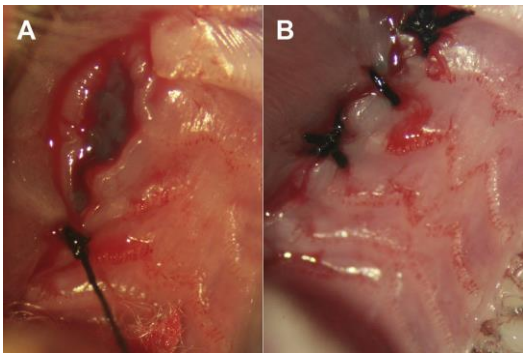

Fig. 1. (A) Adipose-derived stem cells in platelet-rich plasma clot covering alveolar sockets after molar extractions in a rat from group 3. (B) Coverage of alveolar sockets with the mucoperiosteal flap and 6/0 silk continuous suture.

In the third subculture, when 60%e70% confluence was reached, all cells were frozen until the week before their use.
For characterization of ASCs, cell cultures were analyzed by fourcolor flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) after staining with fluorochrome-conjugated monoclonal antibodies. The following conjugated antibodies were used at saturating quantities: Alexa Fluor 647econjugated CD90, CD29, CD45, and CD11b (Serotec, Spain). Briefly, 2

105 nucleated cells were incubated for 20 min at room temperature with the above-mentioned antibodies. Data acquisition and analysis were performed using the CELLQuest PRO software program (Becton Dickinson Biosciences, San Jose, CA, USA).
Once thawed, the cells were cultured for 1 week and, prior to implantation, ASCs were marked with PKH-26 (SigmaeAldrich, St Louis, MO, USA) following the protocol of the manufacturer, for groups 3 and 4.
For group 4, ASCs were cultured with 1 mg/mL BMP-2 (Noricum SL, Madrid, Spain) for 10 days, with the medium being renewed each 4 days under standard conditions.
One million allogeneic ASCs were applied in each maxilla in groups 3 and 4 according to the protocol described in the section on PRP preparation.

2.4. Macroscopic analysis

Mucosa healingwas assessed visually as either bone exposure or complete mucosa healing.

2.5. Sample preparation

The rat heads were fixed with 4% formaldehyde and sectioned sagittally with an electric metal saw. Plane radiographs (PHILPS Diagnostic 93) were taken of each half-head. Samples were then decalcified with nitric acid over 72 h. The half-jaws were harvested en bloc. Alveolar bone was sectioned sagittally with a scalpel in buccal and palatal/lingual fragments. Blocks of each half-jaw were placed in 70% ethanol and further dehydrated in graded alcohol solutions and embedded in paraffin. Histologic sections (5- to 6-mm thick) of each alveolar process were cut along the mesiodistal plane and stained with hematoxylineeosin and trichrome.

2.6. Histologic analysis and cell tracing

Histologic analysis was performed by a pathologist in blinded fashion (light microscopy,

40 magnification).

Several histologic parameters were evaluated, as follows. (1) Osteonecrosis or necrotic foci, defined as 8 to 10 adjacent empty lacunae in the alveolar bone, as previously described by Enlow and Yamashita (Basi et al., 2011; Yamashita et al., 2011); (2) osteoclast counting: arithmetic mean of their count in three different fields (

40), in two different random cuts in the alveolar process of upper jaw; (3) vascularization. evaluated at the level of the alveolar processes of the upper jaw and graded according to a visual scale from 1 to 5 (1 ¼ absence of vessels, 5 ¼ duplication of the vascular surface area compared to this area in rats with no ZA treatment (reference group from our previous study (Barba-Recreo et al., 2014))); and (4) degree of alveolar bone remodeling (after tooth extraction), using scale from 1 to 5 (1 ¼ absence of bone resorption, 5 ¼ 100% of alveolar bone area resorbed and substituted with fibrous tissue and new trabeculae).
Fluorescence analysis was performed for cell tracing. Sections were viewed under a fluorescent microscope (Leica DMI6000B Wetzalar, Germany). The sections obtained from groups 1 and 2 (no ASCs applied) were used as negative controls.

2.7. Radiographic analysis

Two maxillofacial surgeons evaluated the radiographs in a blinded fashion. Bone density was assessed by using the radiographic densitometric value of healthy post-extracted bone as a reference, as shown in our established BRONJ rat model (Barba-Recreo et al., 2014). Findings suggestive of osteonecrosis were defined as osteosclerosis, thickening and disorganization of the medullary trabeculation, and cortical disruption in the right upper jaw.

2.8. Statistical analysis

Qualitative variables were analyzed using the Pearson c2 test and Fisher exact test. Quantitative variables were analyzed with nonparametric KruskaleWallis test or ManneWhitney U test. The statistical significance level was set at 0.05. Analyses of the data were performed with the program SPSS v. 11.5 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Macroscopic results

None of the animals showed bone exposure at the extraction sites.

3.2. Histologic results

One specimen from group 1 and three specimens from group 2 were excluded from the final analysis due to technical problems with the histology, mainly with cutting samples. Osteonecrosis foci were found in 54% of animals in group 2 and in 46% of animals in group 1, contrasting with lower incidence in group 3 (20%) and group 4 (8%) treated with ASCs. The mean value of osteoclasts was higher in group 4 (9.9 osteoclasts per field at

40) and group 3 (8.4 osteoclasts per field at

40) than in the rest of groups (7.4 osteoclasts per field at

40 in group 2, and 7.2 osteoclasts per field at

40 in group 1). Vascularity analysis showed a high blood vessel count in group 4 (mean 2.5); this number decreased in group 3 (2.1), group 2 (2.3), and group 1 (2.2).
Nevertheless, alveolar bone remodeling analysis showed greater resorption in group 3 (mean 3.2) compared with the rest of groups (2.6 in group 4, 2.8 in group 2, and 1.9 in group 1).
Incomplete epithelial healing was more frequent in group 3 (60%) 1 week after the surgery. Small abscesses were found in close relation to impacted food and suture remains in the submucosa; these were more frequent in group 4 (54%). Histologic results are summarized in Table 1 and Fig. 2.
The original design of the four groups brought an analysis of limited potency due to the low number of animals in each group.
Therefore, we compared the treatments with and without ASCs at dental extraction sockets in ZA-treated rats (Table 2).
Osteonecrosis in alveolar bone was present in 50% of the animals without ASC treatment. This percentage decreased to 14% in animals treated with allogeneic ASCs. This difference was statistically significant (p ¼ 0.007). The difference in osteoclast count between groups treated with ASCs (9.0 osteoclasts per field

40) and animals without ASC treatment (7.3 osteoclasts per field

40) were also statistically significant (p ¼ 0.045). It was not possible to estimate significant differences during the vascularity analysis (2.2 in the group not treated with ASCs, and 2.3 in the ASC-treated group). Bone resorption was higher in the ASC-treated group (2.9). When compared to the animals not treated with ASCs, the difference in bone remodeling observed at healing alveolar sockets (2.3) was statistically significant (p ¼ 0.024). Disruption at the mucosal surface and inflammatory infiltrates were found more often in specimens from ASC-treated rats (43%), although no significant difference with non-ASC-treated group (32%) was found.

3.3. Fluorescence results

ASCs were observed fully packed in the PRP clot remains 1 week after dental extractions in group 3. Furthermore, the cells were found throughout the whole alveolar socket in groups 3 and 4, from the submucosal area down to the bottom of the alveolar socket and the new trabecular bone (Fig. 3). No ASCs were located in the mucosa or in the alveolar bone.

3.4. Radiographic results

Plane radiograph analysis showed BRONJ features, including
osteosclerosis, bone thickening, and cortical disruption of alveolar bone (Fig. 4 and Table 3). Bone density analysis showed osteosclerosis in more than 85% of the rats in each group, a common consequence of BP treatment.
Bone thickening was more frequent in group 2 (54%) and group 3 (47%). Nevertheless, cortical disruption of alveolar bone in the upper jaw was higher in group 1 (38%). It was not possible to estimate significant differences among the radiologic findings, either for the original four-group comparison or for the ASC/non-ASC group analysis.

4. Discussion

In order to lower the risk of BRONJ after traumatic injuries including dental extractions, several treatments are gaining popularity among oral surgeons, ranging from simple procedures such as mucosal flaps, to more sophisticated ones such as PRP and stemcell applications. In the present work, we showed that adding ASCs could further decrease BRONJ incidence in a BRONJ high-risk model in rats, compared to non-ASC treatments (PRP and mucosal flap), with statistical significance. In order to not interfere with the immunological properties of the studied treatments (PRP and ASCs) and to allow us to better analyze the results without confounding factors, no other drugs were used (e.g., corticoids) to increase BRONJ incidence.
Some authors defend the use of local flaps, especially for resistant cases, in advanced stages of disease (Williamson, 2010; Voss
et al., 2012), or for preventive reasons (Abtahi et al., 2013). For our experimental study, we chose a mucoperiosteal flap as a standard local preventive treatment after dental extractions. When the flap was the only measure applied, BRONJ incidence decreased from 80% (Barba-Recreo et al., 2014) to 46% in the present model.

However, the percentage of BRONJ in animals treated with high doses of ZA remains too high to recommend the flap alone as a sufficient preventive treatment following dental procedures. PRP is also an emerging BRONJ-preventive (Scoletta et al., 2011) and treatment method (Bocanegra-Perez et al., 2012; Mozzati et al., 2012), and has been used in clinical practice in a more empirical fashion than based on higher scientific evidence. Platelets secrete several growth factors and cytokines that could decrease and shorten initial local inflammation, stimulate neoangiogenesis, and therefore promote tissue healing (Marx, 2004). PRP clot also seems to be less favorable to bacterial growth due to its pH values (Marx, 2004). Platelets release almost 95% of their presynthesized growth factors within the first hour of their activation and continue producing these proteins until their senescence (5e10 days).

Therefore, the influence of PRP over bone healing, which is a slow process, seems to be concentrated exclusively at the beginning of the process, and other long-lasting treatments are thought to be more beneficial. This observation could explain why, in our model, PRP does not seem to add benefits (no significance) over the flap alone. ASCs are MSCs obtained from adipose tissue (Zuk et al., 2001) and are capable of secreting a great variety of growth factors (Salgado et al., 2010) with a significant impact on tissue regeneration (Tsuji et al., 2014). Endothelial lineage cell recruitment by MSCs could potentially play an interesting role in improving the vascular supply (Chen et al., 2008). A significant number of MSCs grafted into the wound can result in the augmentation of the local blood supply and improvement of regeneration capacity (Cella et al., 2011), and subsequently could allow for better osteogenesis of the damaged bone (Gonzalvez-Garcia et al., 2013). We found that animals treated with stem cells showed a lower rate of osteonecrosis (14%; Table 2) and higher osteoclast density (Table 2) (p ¼ 0.007 and p ¼ 0.045, respectively). Consequently, bone remodeling was increased in stem celletreated specimens (p ¼ 0.024). Nevertheless, no significance was observed during vascularity analysis. MSCs also have unique immunomodulatory properties (Nauta and Fibbe, 2007; Wolf and Wolf, 2008).

Table 1
Histologic findings of each group of treatment.

Group	Osteonecrosis	Osteoclasts per ×40 field	Vascularity	Bone resorption	Mucosal disruption	Inflammatory infiltrate (abscess)
1	46%	7.2 (3.0)	2.2 (0.8)	1.9 (0.8)	15%	15%
2	54%	7.4 (3.5)	2.3 (0.6)	2.8 (0.9)	18%	27%
3	20%	8.4 (3.7)	2.1 (0.8)	3.2 (0.7)	60%	13%
4	8%	9.9 (2.7)	2.5 (0.7)	2.6 (0.8)	23%	54%

Percentage of animals in each group showing osteonecrosis, mucosal disruption, and abscess are presented. Number of osteoclasts at ×40 magnification field, vascularity (scale 1–5; see text) and bone resorption (scale 1–5; see "Histologic results" section of "Material and methods") of each group are presented as mean (standard deviation).

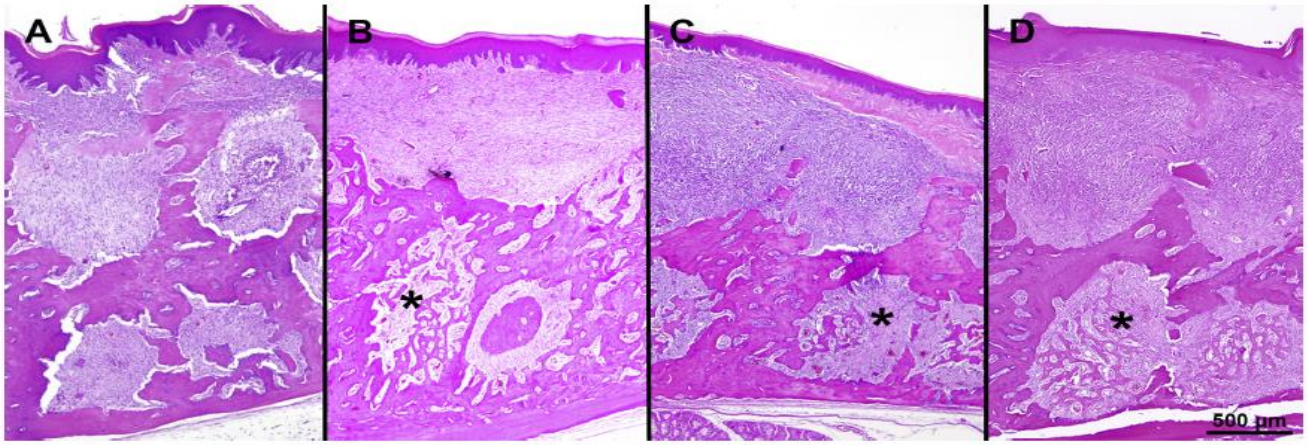

Fig. 2. Histologic images of dental sockets 1 week after extractions. Hematoxylin–eosin staining. (A) Group 1 animal: limited bone turnover with high alveolar crest. (B) Group 2 animal: partial crestal bone resorption and new trabecular bone (*) at apical half of the socket. (C) Group 3 animal: high bone turnover. (D) Group 4 animal: high bone turnover with multiple new trabeculae forming (*).

Table 2
Histologic findings after regrouping in adipose-derived stem cells (ASC) or non-ASC treatment groups.

Group	Osteonecrosis	Osteoclasts per ×40 field	Vascularity	Bone resorption	Mucosal disruption	Inflammatory infiltrate (abscess)
No ASC treatment	50%	7.3 (3.2)	2.2 (0.8)	2.3 (0.9)	17%	21%
ASC treatment	14%	9.0 (3.3)	2.3 (0.8)	2.9 (0.8)	43%	32%
Significance	$p = 0.007$	$p = 0.045$	NS	$p = 0.024$	NS	NS

Percentage of animals of each group showing osteonecrosis, mucosal disruption, and abscess are presented. Number of osteoclasts at ×40 magnification field, vascularity (scale 1–5; see "Histologic results" section of "Material and methods"), and bone resorption (scale 1–5; see text) of each group are presented as mean (standard deviation). NS, not significant.

This activity is thought to be largely based on inhibition of T-cell and B-cell proliferation and dendritic cell maturation (Nauta and Fibbe, 2007) and the secretion of a large number of cytokines and growth factors (Chen et al., 2008). MSCs also are able to restore the immunologic imbalance produced by zoledronic treatment (between T helper cells and regulatory T-cells), which may alter the inflammatory response of alveolar socket healing in ZA-treated animals (Kikuiri et al., 2010; Li et al., 2013). However, in our study, inflammatory infiltrates were detected in animals of all groups, with no close relation with osteonecrosis foci. No adverse reaction against allogeneic ASCs or PRP was observed. Although autologous PRP use is the most

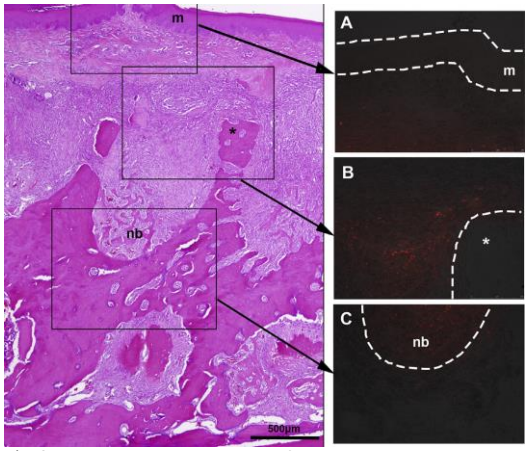

Fig. 3. Fluorescence study in an animal from group 3. (A) Adipose-derived stem cells (ASCs) labeled with PKH-26 (red fluorescence) located at submucosa. (m, mucosa). (B) ASCs located within the whole alveolar socket. (*crestal bone). (C) ASCs located within new bone trabeculae (nb).

common form of PRP used in humans, allogeneic PRP use in small animals makes it possible to obtain a large amount of this product from few donor animals (Rajabi et al., 2015). ASCs have a great advantage over bone marrow mesenchymal stem cells (BM-MSCs) in terms of cell yield. Both types of MSCs seem to have similar properties. However, ASCs are characterized by faster cell proliferation, stable population doubling, and lower levels of senescence than BM-MSCs. Some papers describe inferior osteogenic but superior angiogenic capacity of ASCs over that of BM-MSCs (Liao and Chen, 2014). ASCs have been shown to survive in ischemic environments and to increase secretion of growth factors (VEGF, HGF, and bFGF) that are necessary for angiogenesis under hypoxic conditions (Rehman et al., 2004; Lee et al., 2009).

This fact is interesting for treatment of bone osteonecrosis and ischemic wounds as in BRONJ. Basic studies have found important and synergic effects between PRP and MSCs (Van Pham et al., 2013). Both PRP and MSCs stimulate macrophage recruitment and shorten the acute inflammatory response, which leads to early progress to chronic inflammation and tissue regeneration (Georgiev-Hristov et al., 2012). In our study, we used PRP clot for packing and retaining the ASCs transplanted in group 3, as PRP is an osteoconductive agent that provides stability and adhesion to ASCs. This combination decreased BRONJ incidence compared to mucosal flap and increased bone turnover. The paradoxically high percentage of mucosal disruption observed in the histologic analysis in group 3 could be explained by mucosal slight dehiscence favored by the large volume of PRP-ASC clot, although PRP and submucosa continued covering the bone on the seventh day after the oral surgery. It was found that PRP seems to decrease VEGF

Table 3
Radiologic findings of each treatment group.

Group	Osteosclerosis	Bone thickening	Cortical disruption
1	85%	23%	38%
2	100%	54%	18%
3	87%	47%	20%
4	92%	31%	8%

Percentage of osteonecrosis, bone thickening, and cortical disruption of each group of animals.

secretion of ASCs in PRP-cultured cells (Van Pham et al., 2013). This fact could explain the difference in bone vascularization between groups 3 and 4. In group 4, ASCs cultured with BMP-2 were directly spread over alveolar sockets and covered with a PRP clot, which might have less direct influence over the ASCs. In order to enhance the osteogenic capacities of the ASCs, we introduced BMP-2, as this protein has been reported to be an osteogenic enhancer (Cowan et al., 2005) and PRP has been described as an enhancer of osteoblastic differentiation in the presence of BMPs (Tomoyasu et al., 2007). With this combination, we achieved the lowest incidence of BRONJ. This group also had the highest osteoclast count and vascularity 1 week after dental extractions, but bone turnover or bone resorption was higher in the group with ASCs without BMP-2, although these findings were not statistically significant. Further preclinical and clinical studies are needed to elucidate all of the implications of PRP and BMP-2 in ASC bone regeneration.

We advocate local application of the MSCs (Cella et al., 2011) and not intravenous infusion (Kikuiri et al., 2010; Li et al., 2013). This application allows the benefits of ASCs to be

concentrated in the area of interest. The fluorescence study revealed ASCs within the fibrous tissue and remodeled bone at the alveolar socket 7 days after their application, suggesting that ASCs are able to survive and disperse within the healing tissue in ZA-treated rats 1 week after dental extractions. Murine models have the disadvantage of a rapid metabolism and better healing than in humans, providing results that may be difficult to extrapolate to humans. Small animal models are indicated for the initial study of new therapies because of their costeffectiveness, easy handling, and fewer ethical and logistic issues, especially for long-lasting treatments, as in the case of BRONJ. No bone exposurewas archived in our study; this situationwas favored

Despite the limitations of this experimental study performed in a limited number of animals, the study exemplifies preventive treatment of BRONJ after dental extractions in rats. As no other drugs were applied, the BRONJ-like lesions in our study are totally bisphosphonate dependent, which allows us to reach conclusions on the role of the studied treatments. We found that ASC-based treatments seem to prevent BRONJ in this high-risk model, providing greater efficiency than mucosal flap with or without PRP.
The combination of ASC and PRP appears to be synergistic, and the addition of BMP-2 could improve results even further. As all of these treatments are already in clinical use and as their safety profile has been widely studied, we believe that the combinations that we propose will soon be studied in clinical settings to determine their value in high-risk patients.

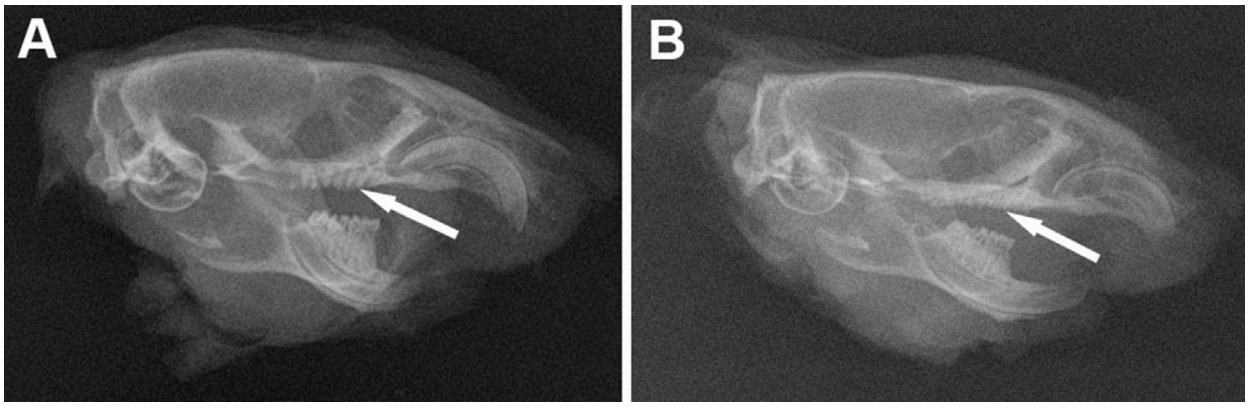

Fig. 4. Plane radiographs of hemiheads 1 week after extractions. (A) Cortical disruption at alveolar ridge in an animal from group 1 (arrow). (B) Alveolar bone thickening in an animal from group 4 (arrow).

by the different treatments applied for BRONJ prevention and by the fact that no other drugs that may enhance osteonecrosis (e.g., corticoids or other immunosuppressive drugs) were administered, with the exception of ZA. Even with the limitations of this study, histologic osteonecrosis should be considered a relevant finding in animals with high metabolism and high bone turnover. Histologic osteonecrosis with no clinical bone exposure seems to be an initial phase of the disease, although histologic features are not included in the American Association of Oral and Maxillofacial Surgeons (AAOMS) definition of BRONJ in humans. The preventive effect of ASCs shown in this study provides new data for further advancing research on potential treatments for this severe disease in humans.

5. Conclusion

Simultaneously, preclinical studies are needed to better describe the long-term results of these novel treatments and to obtain further details on the exact molecular mechanisms involved in the pathogenesis of BRONJ that could be prone to influence.

Conflict of interest statement

All authors declare that they have no competing interests, with the exception of MGA. MGA is a co-author of 2 patents entitled "Identification and isolation of multipotent cells from nonosteochondral mesenchymal tissue" (10157355957US) and "Use of adipose tissue-derived stromal stem cells in treating fistula" (US11/167061). The Universidad Autonoma de Madrid (UAM) and Cellerix share the patent rights.

Acknowledgements

We thank Carlota Largo, veterinarian in the Experimental Surgery Department, for logistical support and experimental animal care, and Luz Vega, technician in the Cell Therapy Laboratory of Instituto de Investigacion Sanitaria Fundacion Jimenez Díaz, for technical assistance. The authors also thank Oliver Shaw (IIS-FJD) for revising the text for aspects related to the English language.
This study was supported by a grant from the Spanish Ministry of Health and Consumer Affairs (FIS PI10/01991) and a RETICS grant from ISCIII (RD12/0019/0035) and the Education Council of Madrid (P2010/BMD-2420).

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