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Research Article

Dental Pulp Cells Enhance Bone Healing in A Rat Osteotomy Model

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Abstract

Contemporary therapeutic approaches to treat unsuccessful union of long bone fractures include bone marrow stem cell-based enhancement of the healing process. This study examined whether dental pulp cells, known to contain stem cells of mesenchymal origin, could improve curing in a tibia fracture model. Osteotomies were performed in two groups of adult male rats and stabilized with intramedullary nailing. Dental pulp cells, harvested from human impacted third molars, were cultured and characterized using mesenchymal stem cell markers. Pulp cells or the vehicle were administered locally at the fracture site. Healing was evaluated radiologically from the 2nd to 8th week and histologically at the 8th week post surgery. Advanced and more efficient bone healing was observed in the cell-treated group compared to the control. This was deduced by the increased callus homogeneity and its earlier size decline as well as by the higher percentage of lamellar newly formed bone and the lower incidence of non-bridging and fibrous tissue detected in the experimental group. These findings confer indications that dental pulp cells, sharing mesenchymal stem cell properties, provide a healing advantage in tibia fractures and encourage further research towards their potential use in bone repair.

INTRODUCTION

Fracture healing in long bones is not always successful. The prevalence of non-unions can be considerably high, as for example in tibial fractures [1]. Therapeutic approaches to enhance bone healing involve improved biomechanical treatment and biological methods. The latter include autologous transplants, biomaterial scaffolds, growth factors and mesenchymal stem cells [2].

Bone marrow mesenchymal stem cells, naturally involved in bone remodelling and repair [3], have successfully been used to treat tibial fractures in animal models [4-6]. However, their use as autologous source of bone forming cells is restricted due to the invasive procedures involved in their harvest.

During the last decade, mesenchymal stem cells have been isolated and characterized from dental tissues [7]. Dental pulp stem cells in particular, produce dentin-like structures, while they are able to differentiate upon induction towards bone, muscle, neural, adipose and endothelial tissue [8]. Their osteogenic potential is analogous to that of bone marrow stromal stem cells [9]. Dental pulp stem cells can form bone tissue both *in vitro* and when ectopically transplanted *in vivo*. Moreover, dental pulp stem cells of human [10] or mouse [11] origin can induce bone formation upon transfer in a calvarial bone defect in the rat or mouse, respectively. In the only clinical study reported so far, autologous dental pulp stem/progenitor cells in a collagen scaffold could restore alveolar bone defects [12]. However, human pulp cells from adult dentition have not yet been examined in healing long bone defects.

The aim of the present study was to test whether locally applied human pulp cells of the permanent dentition can promote fracture healing in a rat model. For this purpose, tibial fractures were produced in adult male rats and stabilized with

intramedullary nailing. Cultured human dental pulp cells or the vehicle were administered locally at the fracture site. Healing was evaluated radiologically from the 2nd to 8th week and histologically at the 8th week post surgery.

MATERIALS AND METHODS

Animals and surgical procedure

In total, 20 male Wistar rats 11-13 weeks old at the beginning of the experiment (mean body weight 350g) were used, randomly assigned to either the control (11 animals) or the cell-treated group (9 animals). The experimental protocol was approved by the Ethics Committee of the School of Dentistry, University of Athens and all animal treatments were performed according to the guidelines of the European Communities Council Directive of 22 September 2010 (2010/63/EU) on the ethical use of animals.

The *in vivo* surgical procedure and internal fixation of the fractured tibia, was performed under general anaesthesia using ketamine hydrochloride (100mg/kg b.w.) and xylazine hydrochloride (10mg/kg b.w.). An anteromedial 2cm incision was made just over the underlying tibial bone. The osteotomy site (mean distance from knee joint 1.47 ± 0.16cm) was exposed trying to preserve as much blood supply to the bone as possible, by minimizing soft tissue damage and detachment. The fracture site was prepared by drilling a 1mm hole through both cortices of the tibia, using a 1mm Kirschner nail, under continuous irrigation with normal saline to avoid overheating, tissue necrosis and a disturbed healing process. These predrilled holes produced a weak point in the bone, through which the tibia was fractured by the use of a pair of scissors, which caused no thermal damage, as opposed to the use of an electrical saw blade. The fibula was manually fractured to prevent interference with tibial union. A 1mm Kirschner nail was passed intramedullarly and in a retrograde direction through the proximal tibia bone fragment and exited at the level of the knee, by using intermittent drilling again to avoid overheating. After reducing the tibial fracture, the intramedullar nail was advanced distally until good gripping of the Kirschner on the distal bone was achieved. The stability of the osteosynthesis was checked by gentle manipulation of the limb and once confirmed, the proximally protruding Kirschner nail at the knee level, was cut flush to the underlying bone, so as not to obstruct knee movement. At this point, dental pulp cells or the sterile vehicle were placed in contact to the medulla at the fracture site using a micropipette. Then, fine absorbable sutures were used, for approximation of muscle fascia situated superficial to the tibial bone, as a means of keeping the cells in proximity of the fracture site. Finally the skin was closed with absorbable sutures. After surgery the animals were housed individually in stable temperature (24 +/- 2^0C) and on a 12-h light/12-h dark cycle. Immediate post-op care included analgesia with 0.1ml/kg b.w. Apotel Plus (600mg paracetamol + 20mg lidocaine/4ml) and hydration with subcutaneous administration of 20 ml dextrose solution (5%). The above medication was continued once daily for the next 3 days. Two animals per group did not recover from anesthesia or died within the first 24h from surgery. The rest of the animals (9 in the control and 7 in the experimental group) were sacrificed at 8 weeks post surgery.

Primary culture of dental pulp cells

Normal immature impacted third molars freshly extracted from three 16-18 year-old patients were used after informed consent was obtained in compliance with the Greek legislation. The teeth were cut with a diamond bur in two pieces and transferred into the culture hood where the pulps were removed and minced into tiny pieces under sterile conditions. The explants were cultured at 37°C, 5% CO_2 and 95% humidity in DMEM medium (Lonza) supplemented with 10% fetal bovine serum (FBS, Gibco), 100UI/ml penicillin, 100μg/ml (Gibco) streptomycin (Lonza), and 0.25μg/ml amphotericin B (Fungizone, Gibco). For the *in vivo* experiments, cells between the 3rd and 5th passage were used. Each animal received at the fracture site either $1x10^6$ cells diluted in 10μl of DMEM medium without FBS, or 10μl of sterile medium. The cells or vehicle were transferred directly into the bone fracture by a micropipette tip.

Characterization and in vitro osteogenic potential of dental pulp cells

Dental pulp cells at the 3rd passage were analyzed by flow cytometry (FACSCalibur, BD Biosciences) for the detection of surface stem cell markers. The cells ($1x10^6$) were incubated for 30min at 4^0C with the following mouse monoclonal anti-human antibodies: Fluorescein isothiocyanate (FITC)-conjugated CD44, FITC-CD90, phycoerythrin (PE)-conjugated CD166 and PE-CD34 (BD Biosciences). Pulp cells at the 3rd passage were cultured in the presence of osteo-inductive factors [50μg/ml ascorbic acid, 10^{-8} M dexamethasone and 10 mM β-glycerophosphate (Sigma)] for 28 days. Cultures were weekly tested for calcium depositions by Alizarin red staining and expression of dentinogenic and osteogenic markers by Western blot analysis using antibodies for dentin sialoprotein (DSP) (rabbit polyclonal, Santa Cruz, 1:200), runt-related transcription factor 2 (RUNX2, rabbit polyclonal, Santa Cruz, 1:200) and osteonectin (mouse monoclonal, Takara, 1: 2500), as previously described [13].

Radiological evaluation

Lateral radiographs of the fractured limbs were taken immediately after surgery and at 2, 4, 6 and 8 weeks post-op using a dental X-ray system (Villa) and occlusal dental film 42, size 4 (Kodak). For quantification of the callus size, the X-ray films were scanned and the callus area was measured using the program Image J v.1.41 software (NIH, Bethesda, USA) by two independent observers. The degree of agreement between observers was at least 90% and the mean values were used for statistical analysis.

Histological evaluation

Tibias were fixed in 4% paraformaldehyde solution for 48h at 4^0C. Decalcification was achieved by 10% EDTA (pH 7.4) replaced daily, under stirring, for 20 days at room temperature. Tibias were paraffin-embedded and 6μm transverse sections, serially numbered from the fracture site towards both the proximal and distal end of the tibia [14] were used for haematoxylin-eosin (H&E) and alcian blue staining, as well as for determining alkaline phosphatase (ALP) activity [15]. For histomorphometry, low magnification images (stereoscope M80 and camera DFC295, Leica Microsystems) including a region of 750μm proximally and distally from the fracture site (flanking all healing region) were

used to measure the areas of fibrous tissue, cartilage, ALP activity and bone marrow cavity as the % of total sectional area. Image J v.1.41 software was used to quantify the above measurements. For alcian blue and ALP measures, a thresholding procedure was used to subtract any non-specific background staining. Bridging of fractured sites and the percentage of mature or immature bone area over total cortical area was evaluated using a double-headed microscope (Leica Microsystems). All measurements were performed blindly by 2 independent observers. The degree of agreement between observers was at least 90 % and the mean values were used for statistical analysis. Eight slides were evaluated from each animal.

Statistical analysis

Statistical testing was performed using the SPSS 18.00 package. One-way analysis of variance (ANOVA) was used to test the effect of treatment between the two groups at 8 weeks. Repeated measures-ANOVA was applied to compare the callus size over time in radiographs, with the different time-points as the repeated measures and the experimental groups as the independent factor. All values are expressed as means +/- standard error of means. Significance was accepted for $P < 0.05$.

RESULTS AND DISCUSSION

Characterization and in vitro osteogenic potential of dental pulp cells

To evaluate the stem cell – like properties of the whole-pulp cell population used in the present study we determined the percentage of cells that were positive for established mesenchymal stem cell markers. Flow cytometry (Figure 1a) revealed that dental pulp cells at passage 3 were expressing at a high level the general mesencymal stem cell markers CD44 (88.6 ± 3.7%), CD90 (83.09 ± 8.4%), CD166 (88.0 ± 5.8%) and minimally expressing (4.5 ± 0.3%) the early hematopoietic and mesenchymal surface antigen CD34 [16,17], in accordance with previous studies [8,9,18]

To examine the osteogenic potential of the whole-pulp cell population we transdifferentiated these cells *in vitro* in the presence of osteo-inductive agents. At the 3rd week of osteo-induction, cultured cells showed positive alizarin staining, indicating their ability for mineral deposition (Figure 1b). In addition, the levels of the osteogenic markers Runx2 and osteonectin were increased in osteo-induced cells starting from

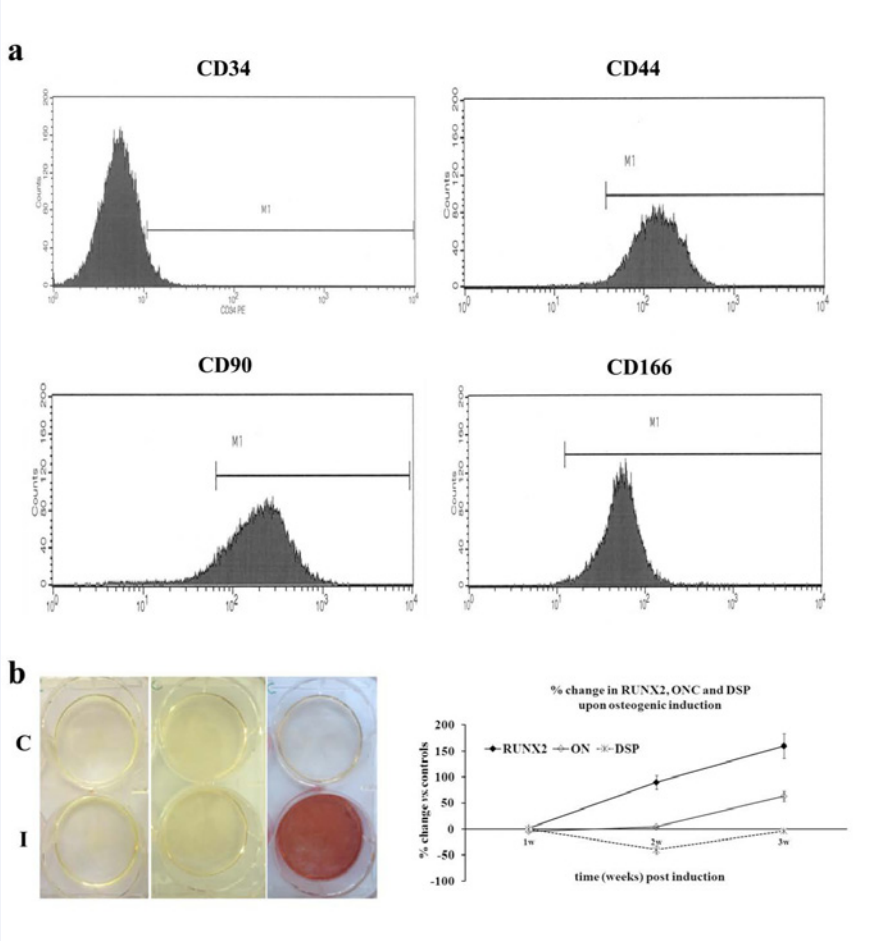

Figure 1 Characterization and osteogenic potential of dental pulp cells. (a) Detection of stem cell markers CD34, CD44, CD90 and CD166 in 3rd-passage dental pulp cells using FACS analysis. Representative results from one experiment. (b) Mineralization and osteogenic potential of dental pulp cells in culture. Detection of alizarin red staining in cells cultured on normal (C) or osteogenic (I) medium for 1, 2 and 3 weeks. Graph showing the % change vs. controls of RUNX2, osteonectin (ON) and dentin sialoprotein (DSP) protein levels in dental pulp cells during osteogenic induction. Data are expressed as mean ± standard error of the mean.

the 2nd and 3rd week, respectively, whereas the levels of dentin sialoprotein (DSP) did not change significantly (Figure 1b). These findings further support that the whole-pulp cell population has the potential to differentiate *in vitro* towards the osteogenic lineage and to express early osteogenic markers, implicated in osteoblast differentiation [19].

Characterization of bone healing in the *in vivo* model

A rat tibia osteotomy model was used to test whether dental pulp cells harvested from human impacted third molars and known to contain stem cells of mesenchymal origin, could improve bone repair. The healing process was monitored radiologically and histologically. The total callus area was measured from radiographs at 2, 4, 6, and 8 weeks post-fracture as an index of bone healing over time. Repeated measures one way ANOVA revealed significant effects of treatment on callus size over time ($F_{(3, 36)}$ = 6.28, p = 0.002) and a time x treatment interaction ($F_{(3, 36)}$ = 2.88, p = 0.049). Further -per week- analysis showed that at 2 and 4 weeks, callus size did not differ between the groups (p = 0.969 and p = 0.065, respectively). However, at 6 and 8 weeks post surgery the callus was significantly smaller in the group of cell-treated animals, compared to the controls (p = 0.029 and p = 0.05, respectively) (Figure 2). At 8 weeks post surgery, the remaining callus appeared more homogeneous in the cell-treated group (4 out of 7 rats, or 57%) compared to the control group (2 out of 9 rats, or 22%), denoting the existence of a lesser soft callus and advanced bone healing in the cell-treated animals.

Histological observations at 8 weeks post-operatively (Table 1 and Figure 3) support the radiologicall findings by demonstrating a lower incidence of non-bridging and fibrous tissue, accompanied by a higher percentage of lamellar new bone and the advanced reformation of the marrow cavity in the experimental group. More specifically, bone bridging was achieved in 6 of the 7 animals in the experimental group (85.8%) and in 6 of the 9 control animals (66.7%). The non-bridged gap, seen in only 1 experimental and 3 control animals (Figure 3a), was filled with well vascularized fibrous tissue. Foci of immature bone

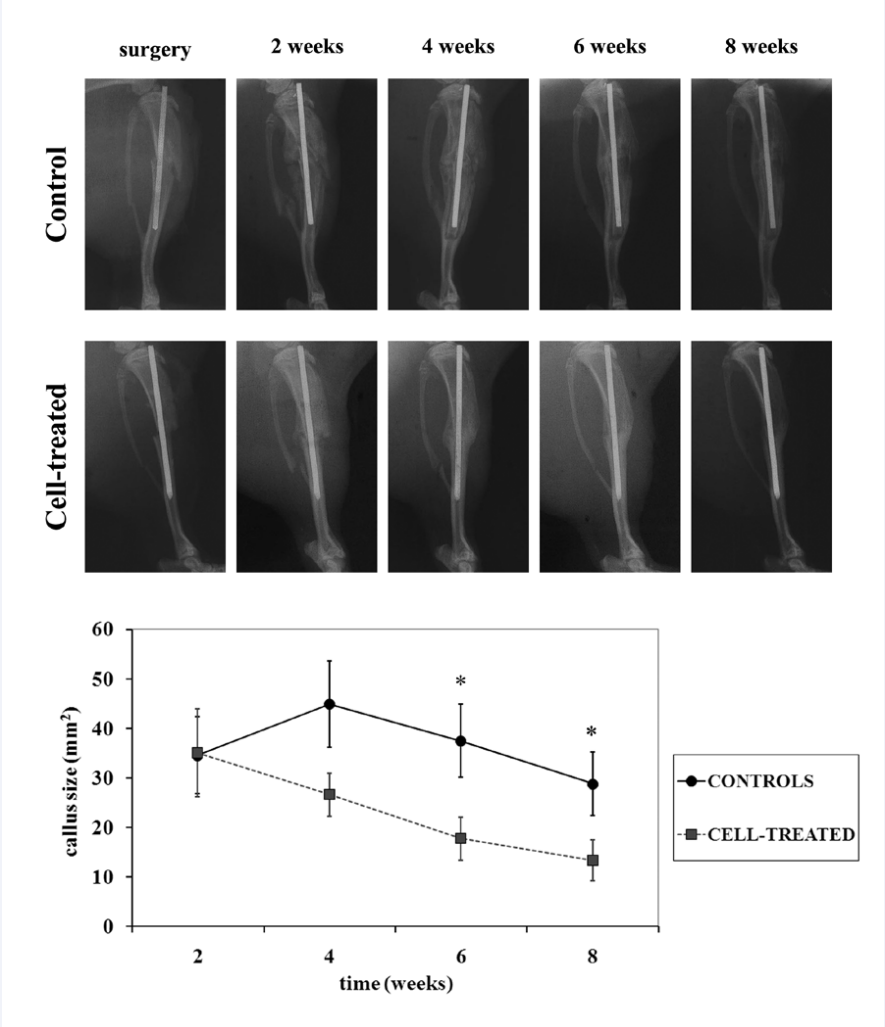

Figure 2 Representative radiographs during tibia healing of a control (upper panel) and a cell-treated (lower panel) rat immediately after surgery and at 2, 4, 6 and 8 weeks post operation. For scale reference, the intramedullar nail is 1mm in diameter. Data are expressed as mean ± standard error of the mean. * Statistically significant differences between the groups at the 6th and 8th week.

Table 1: Histomorphometric data at the 8th week post surgery.

Aminal group	**Medullar cavity** (% of total area)	**Cartilage** (% of total area)	**Fibrous tissue** (% of total area)	**Cortical area** (% of total area)	**Mature bone** (% of cortical area)	**Immature bone** (% of cortical area)	**ALP activity** (% of total area)
Control	36.6±4	1.2±0.4	8.6±2	57.6±5.7	15.5±3.4	84.5±3.4	2.7±1.9
Cell-treated	51.0 ±4*	1.3±0.4	0.8±0.4*	54.2±8.2	32.5±6.6*	67.5±6.6*	1.1±0.2

Data represent mean ± standard error of mean and are expressed as percentage of the total sectional area, or the cortical area. Cortical area was determined by subtracting medullar cavity, fibrous area and cartilage from the total sectional area. Asterisk (*) denotes statistical difference, compared to the control.

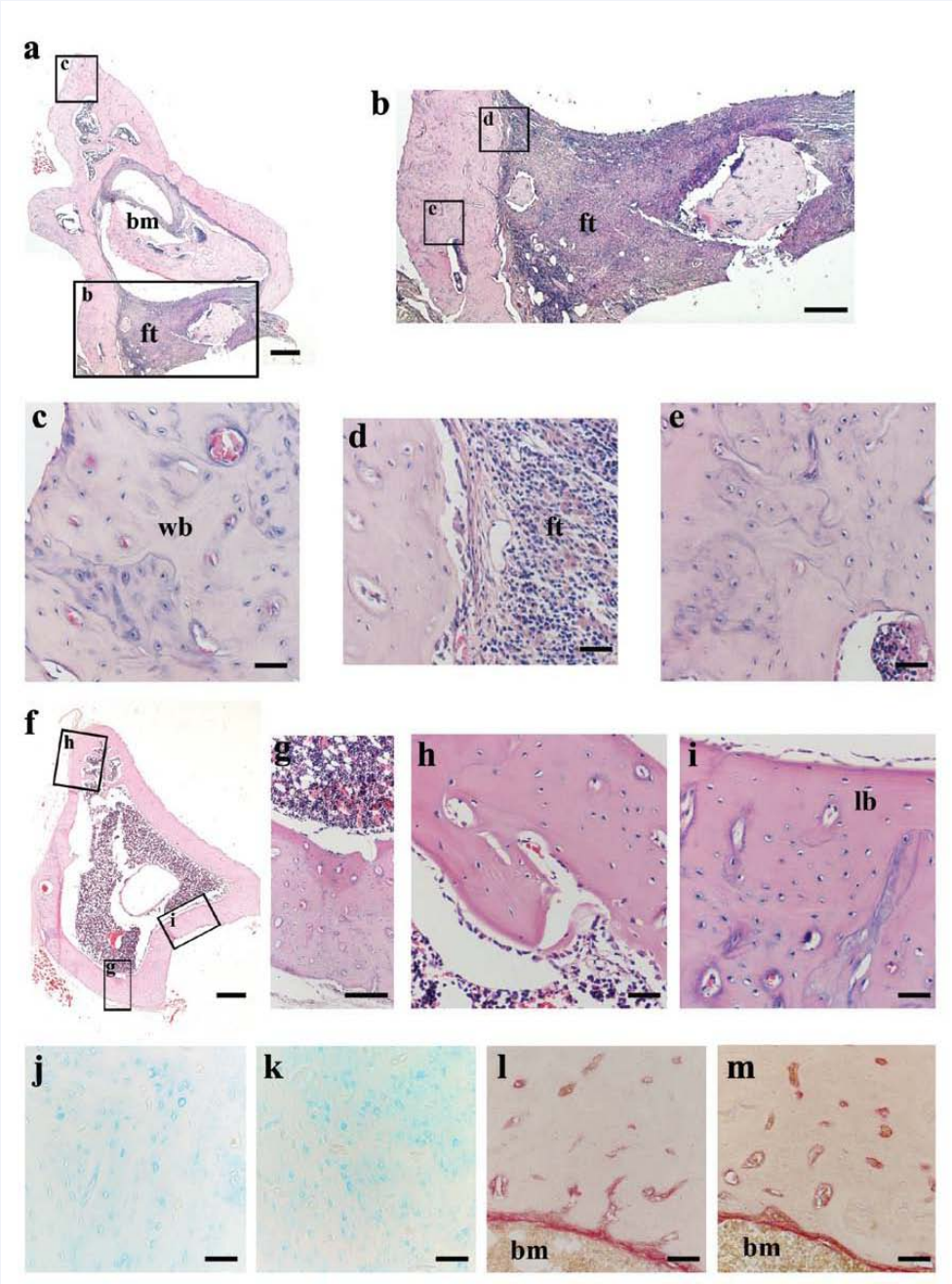

Figure 3 Examples of histological sections at 8 weeks post-fracture in tibias of control (a, b, c, d, e, j, l) and cell-treated (f, g, h, i, k, m) animals. H&E stained sections showing smaller bone marrow cavity (a), non-bridging (a, b), extended fibrous tissue (a, b, d) and mostly woven bone (c, e) in the controls, compared to cell-treated (f - i). Framed areas in a, b and f refer to higher magnification images, according to the letter inside the upper left side of the frame. Alcian blue staining of cartilage (j, k) and alkaline phosphatase (ALP) activity (l, m) in control (j, l) and cell-treated (k, m) animals. bm: bone marrow; ft: fibrous tissue; lb: lamellar bone; wb: woven bone. Scale bars in a, b, f and g correspond to 200μm and in c-e and h-m to 50μm.

were also present. In the control group there was approximately 10 times more fibrous tissue (8.6±2% of total area, Figure 3a, b, d) compared to that in the cell-treated group (0.8±0.4%), (one-way ANOVA, $F_{(1, 14)} = 8.69$, $p = 0.011$). The cell-treated group showed advanced bone healing with less fibrous tissue and significantly more mature bone ($F_{(1, 14)} = 6.34$, $p = 0.026$) at the fracture site (Figure 3c, e *vs*. h, i). The presence of mature bone in the cortical area of the cell-treated animals (32.5±6.6%), was twofold higher compared to the control group *(*15.5±3.4%). The bone marrow cavity area was larger ($F_{(1, 13)} = 4.85$, $p = 0.046$) in the cell-treated group (51.0 ±4%) compared to the control (36.6±4%), denoting a faster bone remodeling at 8 weeks post-fracture. In the experimental group, the cavity appeared histologically normal, containing all three haemopoietic cell lineages and mature adipose tissue (Figure 3f, g). In contrast, in the control group, the cavity was still partially filled with immature bone and fibrous tissue (Figure 3a). The cartilage tissue, as measured in alcian blue stained sections, comprised the 1.3±0.4% of the total tissue area in the experimental group and the 1.2±0.4% in the control group (Figure 3j and k, respectively). ALP activity was low (approximately 1.5% of total area) and localized in osteoblasts and cells lining the surface of trabecular bone, as well as around blood vessels (Figure 3l, m). No differences in cartilage tissue or ALP activity were detected between the two groups.

To our knowledge, this is the first application of human dental pulp cells from permanent dentition in the treatment of long bone fractures. Bone marrow mesenchymal stem cells, having a comparable osteogenic potential with dental pulp cells [9], have been used to successfully treat segmental tibia fractures in the rat [4].In that study, calcification at the fracture site could be radiologically detected at 6 weeks post operatively. Scaffold-seeded human dental pulp stem/progenitor cells (preselected to be CD34+/flk+) have been used to heal cranial bone defects in immunocompromised rats [10] and as autologous grafts for alveolar bone repair in humans [12]. In line with our findings, better bone repair was reported. Stem cells isolated from the pulp of deciduous human teeth, sharing osteogenic properties with cells from the adult pulp, have also been used to treat cranial bone defects in rats [20] and mandibular defects in minipigs [21], with promising results. Compared to the above-mentioned studies, our work has provided evidence that placement of the whole population of cultured pulp cells from adult human dentition at the fracture site, without prior stem cell sorting or osteo-induction, is sufficient to promote bone healing.

Characterization of stem and progenitor cells within the pulp is based on the detection of several surface antigens, used to identify mesenchymal stem cells from other sources [9]. Previous studies have revealed the existence of discrete subpopulations of pulp stem/progenitor cells with varying frequency and potential. For example, CD34+ cells, a small population within the human pulp, can induce the formation of bone modules upon subcutaneous transplantation in rats [22]. Subpopulations of human dental pulp cells characterized as CD34+/c-kit+/CD45- or CD34+/flk+ have also been reported to possess significant osteogenic potential [10,23]. Different clones of dental pulp cells from mouse teeth exhibit either low or high osteogenic potential [11], further supporting the co-existence of discrete subpopulations of stem-like cells within the dental pulp. More studies are required before concluding which subpopulation(s) is/(are) the best in diverse tissue repair models, since relevant orthotopic assays in animal models are limited. Notably, extended culturing of pulp cells prior to an *in vivo* application, necessary for expansion of an isolated population or *in vitro* trans-differentiation, can possibly reduce their potential [24]. Based on the above, we conducted this study by using the whole population of cells obtained from the pulp, without prior osteo-induction or scaffolding that could modify cell responses.

The present study aimed to investigate if dental pulp cells would have a beneficial effect on the biological outcome of fracture treatment. Thus, tibias were harvested for histological examination two months post surgery, at the phase of bone remodeling [14]. Although the healing progress was followed radiographically from the 2nd post operative week onwards, the present experimental design poses the limitation that we cannot examine the fate and the role of the added dental pulp cells. According to previous studies, mesenchymal stem cells at the injured site can act by trans-differentiating into bone forming cells and /or by inducing an effective host response [6]. Furthermore, dental pulp cells, like many other mesenchymal stem cells, exhibit anti-inflammatory effects in allogenic tissues [25], suggesting that they could have induced a beneficial immune response during the initial phase of healing, important for adequate repair. Future studies at earlier stages of bone healing in our model, will help define the underlying mechanisms.

CONCLUSION

This study showed that human dental pulp cells can enhance the healing of tibial fractures in an animal model and suggests that these cells represent an easily accessible, low morbidity at the collection site, source of mesenchymal multipotent cells for tissue repair of long bones. In the future, stem/progenitor cells of dental origin could serve as a convenient therapeutic approach towards fracture healing, as well as for the filling of pelvic or femoral defects after hip arthroplasty. The enhancing effect of dental pulp cells in bone repair could reduce the long immobilization time required for healing and improve patients' quality of life.

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