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Abstract: The rat calvarial defect is an established model to evaluate craniofacial bone regeneration using cell-scaffold biocomplexes. Dental pulp harbors stem cells with significant osteogenic properties. Extracellular matrix (ECM)-like scaffolds simulate the environment that cells observe in vivo. In the present study, we evaluated the osteogenic effect of a biocomplex of human dental pulp cells and a hyaluronic-based hydrogel scaffold in calvarial defects of immunocompetent rats. Dental pulp cells at the 2nd passage were characterized by flow cytometry, osteodifferentiated ex vivo for 4 days and the whole population was encapsulated in the synthetic ECM matrix. Cell vitality was verified 24h upon encapsulation. 5 mm calvarial defects were created in 30 male rats and filled with the biocomplex, the scaffold alone, or nothing. Histological evaluation at 8 weeks showed incomplete bone regeneration in all groups. The scaffold was not fully degraded and entrapped cells were detected in it. Histomorphometry showed statistically significant superior new bone formation in the biocomplex-treated group and similarly lower regeneration in the two other groups. The present study provides evidence that the whole population of human dental pulp cells can advance bone healing when transplanted in immunocompetent animals in the absence of an osteoinductive or osteoconductive scaffold.

To the Editor-in-Chief of the **Journal of Cranio-Maxillo-Facial Surgery**

Prof. Dr. Jörg Wiltfang,

Klinik fur Mund, Kiefer-, und Gesichtschirurgie,Universitätsklinikum Schleswig-Holstein, Campus Kiel, Arnold-Hellerstr. 16, 24105, Kiel, Germany

Athens, July 30, 2014

Dear Professor Wiltfang,

Please consider for publication in the Journal of Cranio-Maxillo-Facial Surgery our manuscript entitled**: 'Bone regeneration in critical-size calvarial defects using human dental pulp cells in an extracellular matrix-based scaffold',** by Xenos Petridis, Evangelia Diamanti, George Ch. Trigas, Demos Kalyvas, and Efthymia Kitraki.

Submission declaration

We herein state that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint, that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder

Sincerely yours

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***Title Page**

Title:

Bone regeneration in critical-size calvarial defects using human dental pulp cells in an extracellular matrix-based scaffold

Short title: Dental pulp cells in healing calvarial defects

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Authors' contribution:

All authors had contribution to the conception and design of the study, acquisition of data, analysis and interpretation of data and preparation of the manuscript. They have all approved the submitted version.

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Authors' contribution:

All authors had contribution to the conception and design of the study, acquisition of data, analysis and interpretation of data and preparation of the manuscript. They have all approved the submitted version.

Summary

The rat calvarial defect is an established model to evaluate craniofacial bone regeneration using cell-scaffold biocomplexes. Dental pulp harbors stem cells with significant osteogenic properties. Extracellular matrix (ECM)-like scaffolds simulate the environment that cells observe *in vivo*. In the present study, we evaluated the osteogenic effect of a biocomplex of human dental pulp cells and a hyaluronic-based hydrogel scaffold in calvarial defects of immunocompetent rats. Dental pulp cells at the 2nd passage were characterized by flow cytometry, osteodifferentiated *ex vivo* for 4 days and the whole population was encapsulated in the synthetic ECM matrix. Cell vitality was verified 24h upon encapsulation. 5 mm calvarial defects were created in 30 male rats and filled with the biocomplex, the scaffold alone, or nothing. Histological evaluation at 8 weeks showed incomplete bone regeneration in all groups. The scaffold was not fully degraded and entrapped cells were detected in it. Histomorphometry showed statistically significant superior new bone formation in the biocomplex-treated group and similarly lower regeneration in the two other groups. The present study provides evidence that the whole population of human dental pulp cells can advance bone healing when transplanted in immunocompetent animals in the absence of an osteoinductive or osteoconductive scaffold.

Keywords: calvaria defects; dental pulp; stem cells; hydrogel scaffolds; bone healing; rat

1. Introduction

Bone tissue regeneration is one of the most challenging aspects of tissue engineering with a great impact on the lives of million people worldwide (*Greenwald et al.,* 2001). Although autogenous bone grafts constitute the 'gold standard' of bone defect reconstruction, their inherent donor-related limitations and resorption issues in large defects render their clinical use difficult and sometimes ineffective (*Dinopoulos et al.,* 2012). Bone tissue engineering aims at becoming the treatment of choice in bone tissue reconstitution by exploiting different combinations of the basic bioengineering principles: stem cells, scaffolds and growth factors (*Langer and Vacanti,* 1991).

The use of progenitor / stem cells in tissue engineering approaches of osseous defects is associated with limitations such as locating easily accessible donor sites and isolating cell populations with adequate self-renewal capacity and specific differentiation potential; human dental pulp stem cells (DPSCs) seem to circumvent these problems. These ectomesenchymal-derived cells have exhibited notable differentiation capability *in vitro* (dentinogenic, osteogenic, chondrogenic, adipogenic, myogenic and neurogenic), depending on the culture conditions (*Gronthos et al.,* 2002; *Kawashima,* 2012), and significant osteogenic potential in particular (*Laino et al.,* 2005; *Riccio et al.,* 2010). In rodents, subcutaneous implantation of scaffolds seeded with human DPSCs has led to ectopic bone formation (*Laino et al.,* 2005; *d'Aquino et al.,* 2007; *Yang et al.,* 2009; *Chan et al.,* 2011), while their orthotopic use in calvarial bone defects has provided some very encouraging results (*Riccio et al.,* 2012; *Prisciotta et al.,* 2012; *Maraldi et al.,* 2013). Another remarkable feature of DPSCs is that they are immune-privileged and have immunosuppressive effects (*Pierdomenico et al.,* 2005; *Wada et al.,* 2009). These findings support the potential use of human dental pulp cells in the treatment of osseous defects.

A prerequisite for a successful bone tissue engineering approach is the use of biomaterials that will provide the osteogenic progenitor/ stem cells with the optimum conditions to function. Hydrogel delivery vehicles are commonly employed scaffolds (*Salinas and Anseth,* 2009). The hybrid synthetic hydrogels possess the inherent biological characteristics of the extracellular matrix (ECM), thus providing the incorporated cells with the necessary environmental cues, whereas the simultaneous addition of synthetic polymers allows for the optimal modification of their mechanical properties (*Salinas and Anseth,* 2009). A certain type of hydrogel scaffolds, called synthetic extracellular matrix (sECM), consists of chemically

modified hyaluronic acid, gelatin and a polymer solution that allows encapsulation of stem cells and the construction of three-dimensional cell cultures. It thus creates an environment that better mimics the *in vivo* conditions (*Shu et al.,* 2006). The performance of these scaffolds has not yet been evaluated adequately in bone tissue engineering.

Scaffolds with osteoinductive properties combined with a specific subpopulation of DPSCs have shown exceptional results in the calvarial defects of immunosuppressed rats (*Riccio et al.,* 2012; *Pisciotta et al.,* 2012; *Maraldi et al.,* 2013). In another report, scaffolds seeded with dental pulp cells did not significantly enhanced calvarial regeneration in nude mice (*Annibali et al.,* 2013). However, there is lack of reports on the effectiveness of orthotopically used sECM scaffolds seeded with the totality of dental pulp cells in immunocompetent rodents.

The aim of the present study was to evaluate the osteogenic effect of a biocomplex of human dental pulp cells encapsulated within a hyaluronic-based hydrogel scaffold in the calvarial bone defect model of non-immunosuppressed rats. The research hypothesis was that the defects treated with the above-mentioned biocomplex would exhibit enhanced bone regeneration, compared to those that received either no treatment or treatment with non-seeded scaffolds.

2. Materials and Methods

2.1. Isolation, culture and characterization of human dental pulp cells

Primary culture and osteoinduction of pulp cells

The pulp was collected from normal immature impacted third molars extracted for orthodontic reasons in the School of Dentistry, National and Kapodistrian University of Athens, from 16- to 18-year old patients after informed consent, in compliance with the Greek legislation. Immediately after extraction, the teeth were swabbed with sterile gauze, cut with a diamond bur in two pieces and then transferred into a culture hood where the pulps were removed with forceps and minced into tiny pieces with scalpels under sterile conditions. The explants were cultured at 37^{o}C, 5% CO_2 and 95% humidity in DMEM medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen, Life Technologies Co., Carlsbad, CA, USA), 100 UI/ml penicillin, 100 μg/ml streptomycin (Lonza), and 0.25

μg/ml amphotericin B (Fungizone®, Gibco, Invitrogen), as previously described (*Diamanti et al.,* 2013). The culture medium was changed every other day. Confluent cultures were collected by trypsination (trypsin / EDTA, Lonza) and subcultured. Cells at the 2nd passage were cultured in the presence of osteoinductive factors [50 μg/ml ascorbic acid, 10^{-8} M dexamethasone, 10 mM β-glycerophosphate (Sigma Aldrich, St. Louis, MO, USA)] for 4 days and subsequently used for the preparation of cell-scaffold biocomplexes.

Flow cytometry

Dental pulp cells before and after osteoinduction were analyzed by flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA) for the detection of surface stem cell markers. The cells (1×10^6) were incubated for 30min at 4^oC with the following mouse monoclonal anti-human antibodies: fluorescein isothiocyanate (FITC)-conjugated CD44, FITC-CD90, phycoerythrin (PE)-conjugated CD166 and PE-CD34 (BD Biosciences).

2.2. Preparation and analysis of biocomplexes

Preparation of scaffolds and cell encapsulation

Three-dimensional constructs were prepared by incorporating pulp cells within a hydrogel scaffold (HyStem™-HP Cell Scaffold Kit, Sigma Aldrich), composed by hyaluronic acid, heparin sulphate, gelatin and PEDGA solution. Initially, the scaffold components were mixed according to the manufacturer and placed in moulds (5 mm diameter, 1 mm thickness), prepared in the interior of 1 ml sterile insulin syringes. Subsequently, the osteoinduced cells were encapsulated (10^6 / scaffold) into the scaffold templates by gently mixing with a pipette. Following full polymerization of the material, the resulting biocomplexes were liberated from the syringes by moving the piston and placed into 12-well plates with osteoinductive medium for 1 more day. Scaffolds without cells were also prepared with the same procedure.

Cell viability and growth within the biocomplexes

Cell encapsulation and viability within the scaffold were microscopically evaluated 1 day after the biocomplex preparation. Specifically, biocomplexes were fixed with 4% neutral formalin solution, washed with blocking solution [BSA 3% and Tween 0.5% in phosphate buffered saline (PBS) pH 7.0] and incubated with 4,6-diamino-2-phenylindone (DAPI- Invitrogen, 1 / 2000 in PBS pH 7.0) for 20 min in order to stain the nuclei of viable cells. For the observation under a fluorescence microscope (Leica Microsystems, Wetzlar, Germany), biocomplexes were placed on slides and pressed with a coverslip. Formalin-fixed biocomplexes were also embedded in paraffin. Microtome sections (6 μm) were used to detect the cell proliferation marker Ki67 by immunohistochemistry using a primary antibody for human Ki67 (clone MIB1, Dako Corporation, Glostrup, Denmark). Slides were observed and photographed under an optical microscope (DMLB2, Leica).

2.3. Animals and surgical procedures

In total, thirty male Wistar rats 4 months old at the time of surgery were used for the *in vivo* experiments. The experimental protocol was approved by the Ethics Committee of the School of Dentistry, National and Kapodistrian 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. Animals were conditioned for 2 weeks prior to the experiments, housed at stable temperature (22 ± 2^{o}C) and a 12h light / dark cycle. The surgical procedure was performed under general anaesthesia, using ketamine hydrochloride (80 mg / kg b.w., Imalgene, Merial, Lyon, France) and xylazine hydrochloride (10 mg / kg b.w., Rompun, Bayer AG, Leverkusen, Germany). Calvaria defects (5 mm in diameter) were performed by the same researchers in two different time periods either bilaterally (14 animals) or unilaterally (16 animals). Defects were prepared using a trephine bur under constant saline irrigation with special care not to harm the underlying dura mater (Fig. 1). In bilaterally operated animals, the defects were randomly filled with a combination of scaffold and cell-scaffold (n = 6), scaffold and nothing (n = 3), or cell-scaffold and nothing (n = 5). In unilaterally operated animals the lesions were treated with scaffold (n = 5), cell-scaffold (n = 6) or left untreated (n = 5). The closure of the wound was done in layers (periosteum, skin) by absorbable sutures. Immediate post-operative care included

analgesia with 0.1 ml / kg b.w. Apotel Plus (600 mg paracetamol and 20 mg lidocaine / 4ml) and hydration with subcutaneous administration of dextrose solution (5%). After surgery, the animals were housed individually under constant conditions. No lethality was detected during the surgery or the post-operative period. Body weights were examined weekly to ensure proper feeding before and after surgery. The animals were sacrificed under anesthesia at 8 weeks post surgery. The calvaria were removed from the scalp, cleaned and placed in fixative.

2.4. Assessment of therapeutic effect

Histological evaluation

Cranial segments including the defects were immediately fixed in 4% paraformaldehyde solution for 48h at 4^{o}C. Decalcification was achieved by 10% EDTA (pH 7.4) replaced daily, under stirring, for 28 days at room temperature. Each cranial block was separated in two halves by a longitudinal cut through its center. Paraffin embedding was done in a way to achieve serial sectioning from the center towards the periphery of the defect. Coronal sections (6 μm) were stained with haematoxylin and eosin (H&E) for histological observations.

For histomorphometry, low magnification images (stereoscope SMZ800, Nikon and camera DFC500, Leica) of 4 central sections per lesion including the whole defect were used to measure areas of new bone as a percent of the total initial defect area (% new bone formation). Specifically, the area of new bone was defined as the sum of all the osseous areas in each section while the total defect area was calculated with the help of a reference defect that was created at a cranial segment following the exact experimental protocol. Image J v.1.41. software was used for quantification. 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.

Statistical analysis

Statistical analysis was performed using the SPSS 18.00 package. One-way analysis of variance (ANOVA) was applied using treatment as the independent variable and operation (one or two lesions) as a random factor, in order to check for a possible interaction of the operation design with the treatment.

Post hoc comparisons among different treatment groups (control, scaffold, cell-scaffold) were performed using Bonferroni test. Significance was accepted for $p < 0.05$.

3. Results

Flow cytometry analysis of dental pulp cells at 2nd passage, prior to osteoinduction, showed two distinct populations based on size and granularity (areas R1 and R2, Fig. 2A). The minor population (R1) expressed the mesenchymal cell markers CD44, CD90 and CD166 at a percentage of 51.26 ± 4.06, 69.71 ± 11.76 and 65.60 ± 4.07, respectively and the CD34 antigen at a percentage of 3.27 ± 0.19. The R2 population was highly positive for all three mesenchymal markers (CD44: 84.53 ± 0.47 %, CD90: 89.22 ± 8.10 % and CD166: 88.07 ±1 4.42 %) and marginally positive for CD34 (3.85 ± 1.20 %). Upon 4 days of osteoinduction, flow cytometry revealed one more homogenous population of dental pulp cells that expressed CD44, CD90, CD166 and CD34 at a percentage of 95.03 ± 0.25, 89.65 ± 2.59, 87.54 ± 3.09 and 3.39 ± 1.25, respectively (Fig. 2B).

To check cellular distribution and viability into the scaffold, the biocomplexes were incubated with DAPI to label the nuclei of viable cells. Twenty four hours after cell encapsulation the cells were distributed in clusters and many nuclei were stained (Fig. 3A). Additionally, there were many cells dividing into the scaffold, as revealed by the Ki-67 immunopositive staining (Fig. 3B).

Qualitative histological observation of thin sections of the calvarial defects at 8 weeks post surgery showed that the bone lesions treated with the cell-scaffold biocomplex had superior healing compared to both untreated and scaffold-treated defects, though complete healing was not observed in any group of animals (Fig. 4). Specifically, the defects in the control group (n = 13) were mainly bridged with connective tissue whereas limited new bone formation was seen only at the defect edges (Fig. 4A). The connective tissue was loose towards periosteum and denser towards the dura mater. Within this tissue, leucocytes of chronic inflammation, blood vessels and red blood cells were also detected (Fig. 4E). In the scaffold-treated group (n = 12), the predominant observation was the presence of extended areas of non-degraded scaffold, surrounded by dense connective tissue (Fig. 4B, F, G). Similarly to the control group, areas of new bone formation were detected mainly at the defect edges and exhibited lamellar

organization. In the cell-scaffold treated group (n = 17), apart from new bone formation at the gap edges, isles of lamellar newly formed bone were seen dispersed into the dense connective tissue bridging the defect area (Fig. 4C, D, J, K, M, N). The newly formed bone exhibited lamellar organization with lacunae and osteocytes in it. Several areas of scaffold remnants were also evident at the defect area, which were less extended compared to the scaffold-treated group (Fig. 4C, D, H, K, L, M, N). Scaffold remnants in the cell-scaffold group contained in several occasions dead cells and cell debris and were surrounded by dense leukocyte infiltration. Neovascularization was equally detected in all groups.

To quantitate the healing process in terms of bone formation, the areas of new bone synthesis were calculated as a percentage of the initial osseous gap area. Histomorphometry showed that new bone formation was 21.98 ± 8.30 % in the control group, 24.40 ± 8.29 % in the scaffold group and 32.78 ± 9.24 % in the cell-scaffold treated group (Fig. 5). No significant interaction of the operation scheme (one or two cranial defects per animal) with the treatment was detected ($p = 0.906$). Statistical evaluation of the results revealed that there was a significant effect of treatment (i.e. control, scaffold, cell-scaffold) on the percent of new bone formation [$F_{(2,41)} = 75.7$, $p = 0.013$]. Subsequent post-hoc analysis for group comparisons showed that the percent of new bone formation in the cell-scaffold treated group was significantly higher compared to both control ($p = 0.003$) and scaffold treated groups ($p = 0.029$). No significant differences were detected between untreated and scaffold treated animals ($p = 1.0$).

4. Discussion

The objective of this study was to evaluate the healing response of cranial defects in rats treated with a biocomplex comprised of human dental pulp cells and a hyaluronic-based scaffold. The histological/ histomorphometric observations showed superior bone regeneration within the biocomplex-treated defects, compared to the untreated or scaffold-treated ones, confirming our hypothesis.

In this study, we used the heterogeneous population of cultured dental pulp cells as a whole to treat bone defects, instead of isolating specific stem cell-like subpopulations with angiogenic properties (*Riccio et al.,* 2012; *Pisciotta et al.,* 2012; *Maraldi et al.,* 2013), in order to examine whether the totality of isolated dental pulp cells can be used therapeutically. Our choice was based on the previous knowledge that

dental pulp harbors a high percentage of stem/ progenitor cells (*Alge et al.,* 2010) that can differentiate to osteoblast-like, bone-forming cells (*d'Aquino et al.,* 2008; *Mori et al.,* 2010). Although the bone forming capacity of dental pulp cells has been related in part to the existence of the specific subpopulation of c-kit^{+}/CD34^{+}/CD45^{-}/STRO-1^{+} DPSCs that also promotes neovascularization (*Laino et al.,* 2005; *d'Aquino et al.,* 2007), we hypothesized that including all subpopulations existing in primary culture (such as endothelial and osteoprogenitor cells) could act synergistically on new bone formation (*Moioli et al.,* 2008; *Dissanayaka et al.,* 2012; *Guerrero et al.,* 2013). This study used early passage pulp cells based on previous studies reporting decreased proliferative and differentiation capacity during extended culture (*Patel et al.,* 2009; *Min et al.,* 2011). We also adopted a short-term osteoinduction period with the rationale of providing the pulp cells with the osteoinductive environmental cues without strictly committing them to the osteoblastic phenotype, in order to maintain substantial part of their stemness (*van der Dolden et al.,* 2002; *Sikavitsas et al.,* 2003; *Castano-Izquierdo et al.,* 2007). Flow cytometry results of our study verified the initial existence and retention of mesencymal stem cell properties of the dental pulp cells used in the *in vivo* experiment.

Due to the heterogeneity of DPSCs and the lack of a 'universal' stem cell marker, general mesenchymal stem cell markers were used to characterize the osteogenic potential of pulp cells in this study. The surface epitopes CD44, CD90 and CD166 are commonly used for DPSCs characterization (*Kawashima,* 2012). These markers are highly expressed in progenitor populations derived from the dental pulp (*Min et al.,* 2011; *Kitraki et al.,* 2014), which is in compliance with our findings. In addition, CD34, a typical marker of hematopoietic progenitor and endothelial cells (*Burn et al.,* 1992), is also detected at low levels in cells of mesenchymal origin (*Lin et al.,* 2012), which comes in agreement with the present observations as well. Pulp cells' heterogeneity was also reflected at the two distinct subpopulations detected by flow cytometry prior to osteoinduction and has been reported in previous studies as well (*Laino et al.,* 2005; *d'Aquino et al.,* 2007; *Min et al.,* 2011; *Kitraki et al.,* 2014). These two populations may be considered as subgroups of dental pulp cells at different developmental stages in culture (*Neuhuber et al.,* 2008).

To evaluate bone regeneration, the 5 mm critical-size defect was used (*Bosch et al.,* 1998). Histological observations at 8 weeks post implantation in the control group showed limited bone healing with a thin connective tissue bridging. This verified the impotence of the endogenous mechanisms to fully regenerate

defects of this size, without exogenous treatment approaches. To characterize the osteogenic potential of the cell population, an inert scaffold was chosen. In scaffold-treated defects, bone healing was similar to that of the control group, confirming the absence of any osteoconductive or osteoinductive properties of the sECM scaffold. An important observation in this study was that the scaffold was not degraded properly. Possible causes of the poor degradation could be found on the chemical composition of the sECM and/or intrinsic enzyme mechanisms; for example glycosaminoglycans (heparin sulphate) have been reported to block the inflammatory events leading to degradation (*Sterling et al.,* 1998; *Wrenshall et al.,* 1999). Since scaffold degradation and tissue deposition are closely interrelated (*Drury and Mooney,* 2003; *Rice & Anseth,* 2004), the observed incomplete healing might be possibly attributed to this event. The specific characteristics and biobehavior of this sECM could explain the different outcomes reported in other studies using different types of scaffold (*de Mendonça Costa et al.,* 2008; *Riccio et al.,* 2012; *Zanchetta et al.,* 2012; *Pisciotta et al.,* 2012; *Maraldi et al.,* 2013).

Bone regeneration was clearly superior in the cell-treated group compared to the control and scaffold-treated groups, with isles of bony tissue scattered along the whole defect area, apart from the bone deposition in the periphery. However, no complete healing was detected as reported in previous studies using a discrete subpopulation of DPSCs (*Riccio et al.,* 2012; *Pisciotta et al.,* 2012; *Maraldi et al.,* 2013). Apart from using different cell populations, possible reasons for incomplete healing could be related to cell distribution and viability into the scaffold. While DAPI and Ki67 staining confirmed viability and division *in vitro,* the encapsulation process did not yield a homogenous cell distribution that could explain non-optimal regeneration capacity (*Zhou et al.,* 2006; *Hasegawa et al.,* 2010). Furthermore, the entrapped dead cells detected within the scaffold remnants suggest that spreading of the encapsulated pulp cells into the defect was problematic; mainly the cells closer to the scaffold periphery were able to interact with the host tissue and trigger healing, a process evidenced by the presence of the sparsely distributed bony isles. These histological findings in the cell-treated group could explain the incomplete bone regeneration (approximating 30%) and at the same time the increased leukocyte recruitment due to the dead cells.

The use of immunocompetent rats in the present study could have an impact on the inflammation-related incomplete healing. However, given the immunosuppressive properties of DPSCs (*Pierdomenico et al.,*

2005; *Wada et al.,* 2009), this is not very likely. In addition, complete bone healing was reported after the treatment of cranial defects of non-immunosuppressed rats with human DPSCs (*de Mendonça Costa et al.,* 2008). Moreover, the same population as in the present study has recently been used to treat tibia fractures in immunocompetent rats without eliciting a different inflammatory response compared to the untreated group (*Kitraki et al.,* 2014). Inevitably, additional studies are needed to investigate the fate of the transferred cells and the early cellular interactions and immunological responses at the recipient tissue, in order to elucidate the underlying healing mechanism.

5. Conclusion

The present study provides some evidence that early passage dental pulp cells from impacted third molars, without cell sorting for isolating stem cell-like subpopulations, possess osteogenic potential and can advance bone healing in the rat calvarial model, when transplanted in immunocompetent animals in the absence of an osteoconductive or osteoinductive scaffold. Further research is required to improve cell viability at the transplantation site.

Conflict of interest statement:

None of the authors has any conflict of interest regarding this research.

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Figure captions:

Figure 1. Surgical procedure. (A) Preparation of the surgical defect. (B) Creation of the 5-mm critical size defect in the rat calvarium. (C) Placement of the cell-scaffold biocomplex. (D) Closure of the periosteum. (E) Closure of the overlaying skin.

Figure 2. Flow cytometry findings. Analysis of dental pulp cells before (A) and following osteoinduction (B) for the detection of CD44 (a), CD90 (b), CD166 (c) and CD34 (d). Two distinct cell populations (R1 and R2) were evidenced initially (A) that were homogenized to a single population after osteoinduction (B). The percentage of positive cells in each case is provided as means ± standard deviation.

Figure 3. Dental pulp cells' viability and growth within the scaffold 24h post-encapsulation. (A) DAPI staining. Nuclei of the viable cells are stained blue. (B) Ki-67 immunostaining. Nuclei of the proliferating cells are stained dark brown. Counterstaining with haematoxylin.

Figure 4. Histological findings in H&E stained sections. Representative low magnification (x1.25) photomicrographs of the defect site in the control (A), scaffold (B) and cell-scaffold (C, D) groups. Arrowheads indicate defect margins. Areas of new bone formation are indicated by asterisks. Rectangles indicate sites within defect for which higher magnification (x20) images (E-N) are provided. (E): Connective tissue (ct) in the framed area of the control group; (F, G): Large areas of scaffold (s) remnants surrounded by dense connective tissue in the framed area of the scaffold group; (H-N): Non-degraded scaffold with entrapped cells (sc), and sites of new bone (nb) formation within the defect in the framed areas of the cell-scaffold group in C (H-K) and D (L-N). Note the inflammation (in) around areas of cell-scaffold remnants and dead cells into the scaffold (arrows in L).

Figure 5. New bone formation within the calvaria defect. Bars represent means ± standard deviation of the percent of new bone in the untreated (control), scaffold treated and cell-scaffold treated lesions. The number of tested defects is given in parentheses. * Significantly increased, compared to the control ($p = 0.003$) and to the scaffold treated ($p = 0.029$) groups.

Figure 1-web

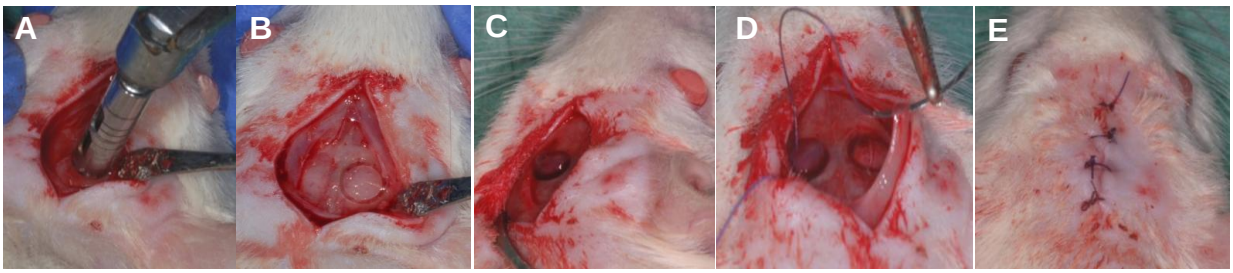


Figure 2-web

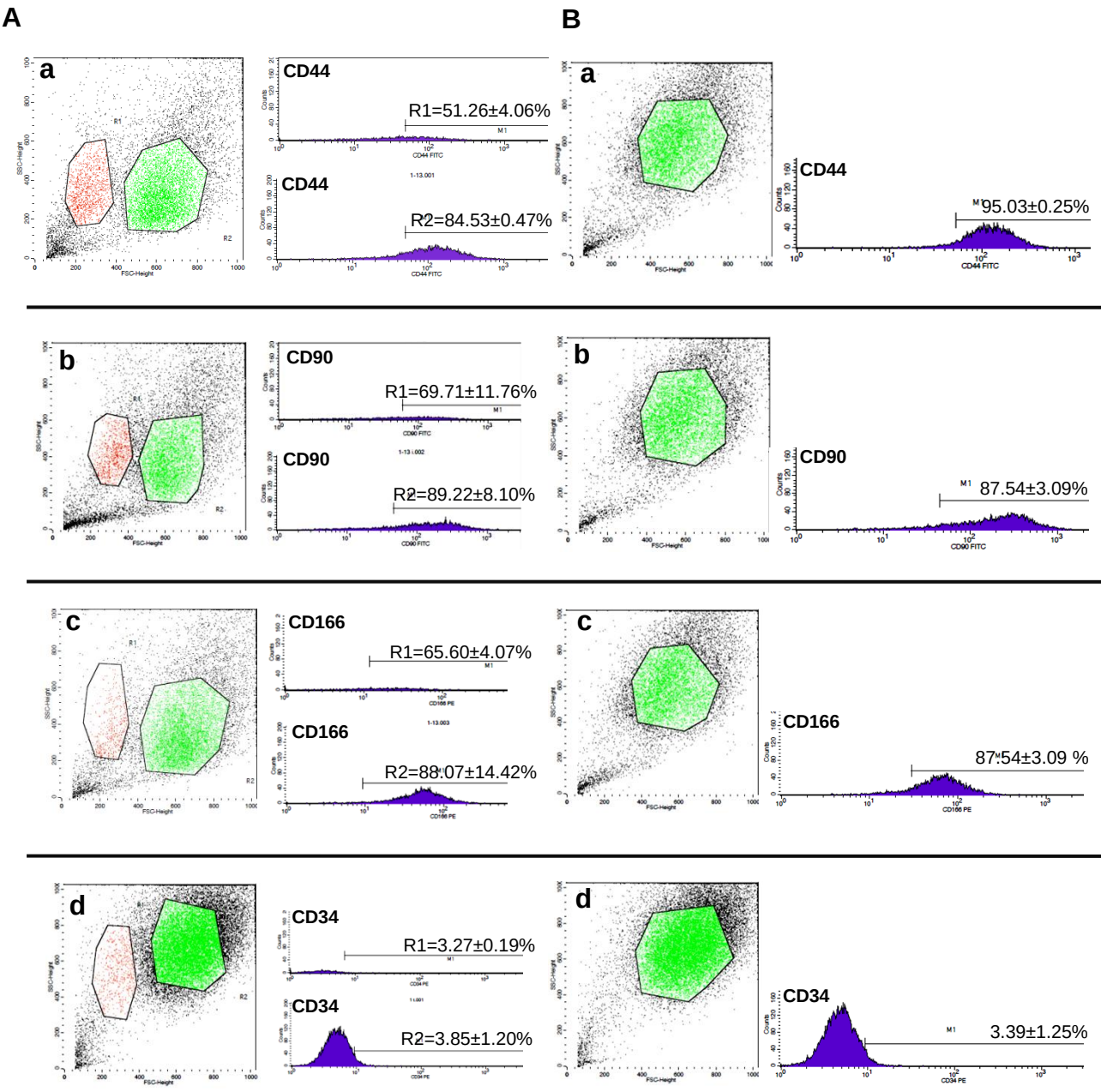
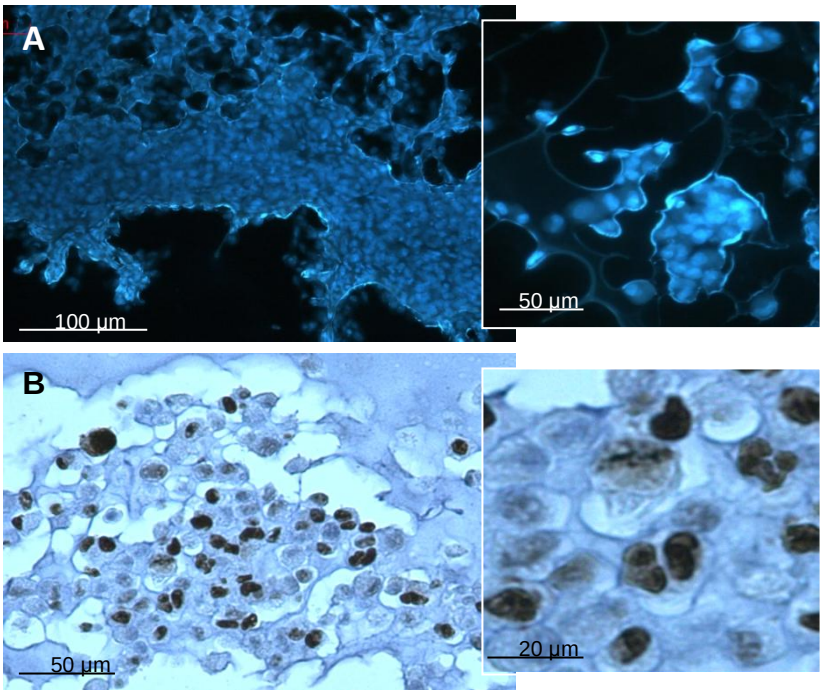

A
B
a
CD44
R1=51.26±4.06%
CD44
R2=84.53±0.47%
a
CD44
M1 95.03±0.25%
b
CD90
R1=69.71±11.76%
CD90
R2=89.22±8.10%
b
CD90
M1 87.54±3.09%
c
CD166
R1=65.60±4.07%
CD166
R2=88.07±14.42%
c
CD166
87.54±3.09 %
d
CD34
R1=3.27±0.19%
CD34
R2=3.85±1.20%
d
CD34
M1 3.39±1.25%

Figure 3-web



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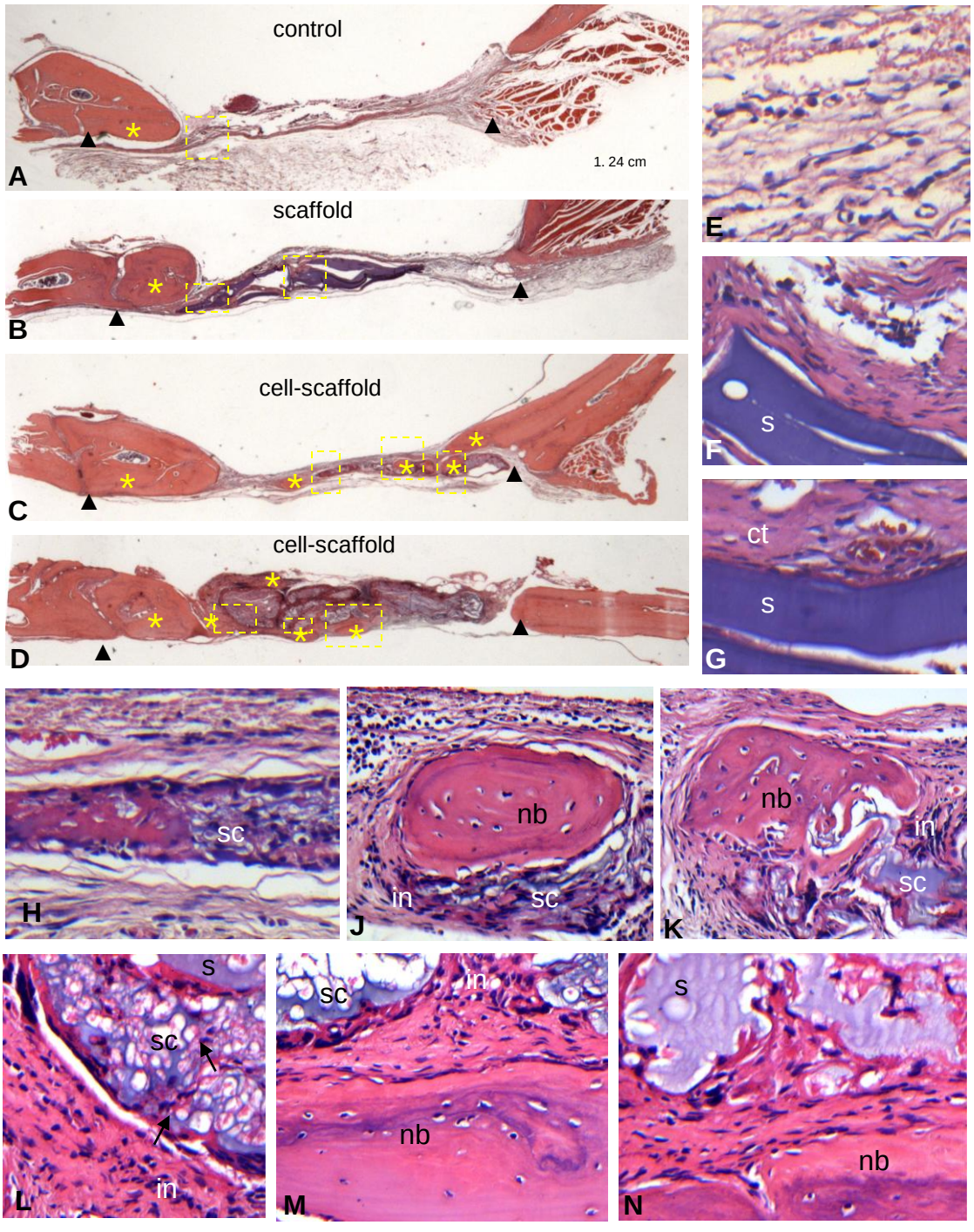
control
1. 24 cm
A
scaffold
B
cell-scaffold
C
cell-scaffold
D
E
s
F
ct
s
G
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H
nb
in
sc
J
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in
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K
s
sc
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L
sc
in
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M
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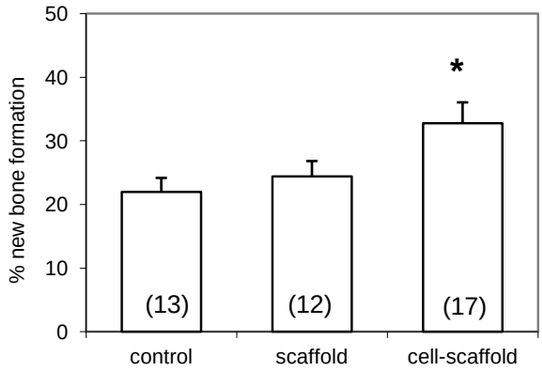
nb
N

Figure 5-web


50
40
30
20
10
0
% new bone formation
*
(13)
(12)
(17)
control
scaffold
cell-scaffold

Highlights:

- Use of human dental pulp cells (DPCs)/ ECM biocomplex in calvarial defects of immunocompetent rats
- Enhanced bone regeneration was detected in biocomplex-treated defects vs. controls
- The whole population of DPCs exhibits osteogenic potential in models of craniofacial defects