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*Original Article*

# MAGNETIC CELL-HOMING STRATEGY FOR AUTOLOGOUS DENTAL PULP REGENERATION AS AN ALTERNATIVE IN NECROTIC TEETH: A PROOF-OF-CONCEPT STUDY

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**Abstract**

**Background**: This investigation aims to prove the concept of selective cell recruitment through the magnetic attraction of specific mesenchymal cells from the periodontal ligament for dental pulp regeneration. **Methods**: Human dental pulp cells (DPCs) and periodontal ligament cells (PDLCs) were investigated as enriched cluster of differentiation 90 positive ($CD90^+$). The behavior of the $CD90^+$ cell population was compared with that of the DPCs crude cell proliferation, alkaline phosphatase (ALP) activity, and osteogenic and odontogenic differentiation. Moreover, their ability to attract them to simulated root canals filled with a cell-free hyaluronic acid-based hydrogel was studied via magnetized endodontic files. **Results**: The immunophenotype differed across the study groups. Moreover, significant differences were observed among all the cell groups in terms of cell proliferation and osteogenic differentiation. PDLCs exhibited significantly greater values of cell proliferation than did $CD90^+$ PDLCs, whereas in terms of osteogenic differentiation, DPCs and $CD90^+$ PDLCs presented significantly lower values than did the other groups. In the magnetic recruitment assay, fluorescence microscopy, DNA, and ALP confirmed the attraction of a group of $CD90^+$ cells recruited to the hydrogel. **Conclusions**: Overall, the present work demonstrated the similarities between DPCs and $CD90^+$ PDLCs and the possibility of establishing a magnetic cell homing strategy, recruiting specific cells from neighboring tissues as an alternative for endodontic regeneration in cases of pulp necrosis.

**Keywords:** Dental pulp regeneration, magnetic cell homing, mesenchymal stem cells, hyaluronic acid, hydrogels.

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## Introduction

In dental pulp disease, conventional endodontic treatment provides pain relief and disinfection of root canals, removing vital or necrotic pulp tissue and preparing, enlarging, and disinfecting the root canal system to finally fill it with synthetic material [1]. Nevertheless, although this procedure allows the recovery of some tooth functions, such as aesthetics, chewing, phonation and swallowing, general homeostasis mechanisms, such as sensitivity, defense against recurrent infections, and the deposition of tertiary dentine, are compromised, impairing the long-term survival of endodontically treated teeth [2].

Several diverse biologically inspired procedures have been applied recently [3–5]. Some of those procedures have been designed with the objective of reinstating particular functionalities, encompassing aspects such as nutrition, innervation, and defense, as is the case in the revitalization process. Conversely, an alternative approach involves the use of blood-derived products, including platelet-rich plasma (PRP) or platelet-rich fibrin (PRF), along with other blood derivatives, to harness platelets as mediators for facilitating cell recruitment. These platelets are distinguished by the presence of three primary granule populations: (1) alpha granules, (2) dense granules, and (3) lysosomes. Alpha granules are the most prevalent and are responsible for releasing growth factors that influence angiogenesis, stem cell migration, and cellular proliferation [2,6]. Cell migration stimulated by alpha granules has demonstrated satisfactory outcomes comparable to those achieved through revascularization and allogeneic transplantation of mesenchymal

cells [3,4,7]. However, these processes lack odontoblasts, which might be attributed to the nonspecific nature of the recruited cells.

Consequently, alternative strategies have endeavoured to achieve full restoration of the pulp-dentin complex. Currently, the most widely adopted biologically inspired technique is regenerative endodontic procedures [3–5]. Regenerative endodontic procedures are biologically inspired procedures that seek the complete restoration of the dental pulp's form and function, including dentin and root structures and cells of the pulp-dentin complex [4].

In adult patients, regenerative endodontic procedures for infected or traumatized mature permanent teeth are explored via two distinctive approaches: (1) Cell transplantation of *ex vivo* cultivated stem/progenitor cells, and (2) cell homing by molecules that recruit the patient's endogenous cells [8]. Most current regenerative endodontic therapies focus on transplanting *ex vivo* autologous progenitor cells from teeth that are diagnosed with irreversible pulpitis [4,5]. These cells must be cultivated under good manufacturing practices (GMPs), which hinders their translation potential to daily clinical practice because of the high cost of autologous therapy and the need for a GMP laboratory for their development [4].

As a less expensive alternative and one that does not require GMP, we propose the use of a magnetic cell homing strategy, which is somewhat similar to the magnetic bifunctional cell engager performed by Cheng and colleagues [9]. In 2015, acute myocardial infarction was treated by targeting exogenous bone marrow-derived stem cells or endogenous CD34-positive cells to injured cardiomyocytes via magnetic attraction [9]. Previously, our group used magnetic nanoparticles to isolate selected adipose stem cells (hASCs) subpopulations that express different levels of stem cell markers and specific subpopulations that have been shown to be more prone to osteogenic, chondrogenic and endothelial differentiation potential [10–12]. This strategy has been further investigated for selecting subpopulations of hASCs that exhibit increased tenogenic commitment [10].

A variant of the magnetic bifunctional cell engager could have the potential to be applied in endodontics to increase the amount and cell specificity of existing biologically inspired procedures. It has already been demonstrated that stem cells can be recruited to the root canal by inducing bleeding from periodontal tissues [13,14]. This is particularly relevant considering that different tissues can be formed in the root canal after treatments such as revitalization, revascularization, or regenerative endodontics using blood-derived products [15,16].

Hence, this variant needs to recruit undifferentiated cells with the potential to deposit mineralized matrices. Cluster of differentiation 90 (CD90) is a cell surface marker typically found in the undifferentiated state of human mesenchymal stem cells (MSCs). These CD90-positive ($CD90^+$) MSCs highly express osteogenic genes such as runt-related transcription factor-2 (*RUNX2*), osteopontin (*OPN*), and osteocalcin (*OCN*), which are directly related to bone/dentin differentiation [17]. Dental pulp cells (DPCs) and periodontal ligament cells (PDLCs) express the CD90 marker, as confirmed by flow cytometry analysis [18–21]. These cells are derived from the same common embryologic source, the ectomesenchyme [22].

The cellular attraction process holds substantial importance, but it is not the sole determinant. A vital aspect of cell recruitment involves replicating lost tissue structures effectively. Hyaluronic acid, found within the extracellular matrix of various tissues, plays a crucial role in this process. The use of hemoderivatives such as platelet lysate (PL) within hydrogels provides an autologous source of vital growth factors and fibrin matrices necessary for early-stage tissue regeneration, including pulp tissue. Our previous research demonstrated the efficacy of hyaluronic acid (HA) hydrogels supplemented with PL in facilitating the encapsulation of dental pulp cells (DPCs) [23].

Consequently, our proposal aims to demonstrate magnetic guidance and selective cell recruitment from periodontal-derived tissues into root canals filled with hyaluronic acid-based hydrogels as a potential treatment to regenerate dental pulp tissues in necrotic teeth.

## Materials and Methods

### *Isolation and Expansion of Human Dental Cells*

DPCs and PDLCs were derived from two healthy impacted third molars obtained from different donors at affiliated dental clinics. Upon receiving the teeth, the periodontal ligament was carefully separated from the middle portion of the root surface. The dental pulp was extracted from the pulp chamber and subjected to enzymatic digestion using a solution containing 3 mg/mL collagenase type I and 4 mg/mL dispase (both from Sigma, Steinheim, Germany) for one hour at 37 °C. The digested tissue was then centrifuged at 330 rpm for 5 min, and the pellet was resuspended in Dulbecco's modified Eagle medium (DMEM)-low glucose (Sigma, Steinheim, Germany) supplemented with 10 % fetal bovine serum (FBS, Thermo Fisher Scientific, Sioux, IA, USA) and 1 % antibiotic-antimycotic mixture (A/A, Thermo Fisher Scientific). The cell suspension was transferred to a 25 $cm^2$ tissue culture flask and incubated at 37 °C in a humidified atmosphere containing 5 % $CO_2$. The culture medium was changed every 2–3 days until the cells reached confluence.

### *Isolation of the $CD90^+$ Subpopulation via Immunomagnetic Bead Separation*

PDLCs were sorted via magnetic beads (Dynabeads™ Protein A, Invitrogen, Carlsbad, CA, USA) precoated with anti-CD90 (14090982). The procedure was based on the Dynabeads manufacturer's cell sorting protocol, as described elsewhere [10]. In summary, 50 $\mu$L of

**Table 1. Magnetic field charge values of the experimental instruments expressed in mili Tesla (mT).**

| Sample | Magnefect nano II | Brand-new endodontic file | Magnetized endodontic file |
|---|---|---|---|
| 1 | 346 | 0.330 | 4.065 |
| 2 | 350 | 0.315 | 3.765 |
| 3 | 325 | 0.435 | 4.165 |
| 4 | 364 | 0.457 | 4.295 |
| 5 | 362 | 0.420 | 3.585 |
| $\bar{x} \pm \sigma$ | 349.400 ± 15.646 | 0.391 ± 0.064 | 3.975 ± 0.293**** |

****$p < 0.0001$, indicating extremely significant differences compared to the control group.

Dynabeads$^{TM}$ protein A was mixed with a solution of 200 $\mu$L of CD90 antibody at a 50 $\mu$L/mL concentration in the washing and binding buffer provided in the antibody conjugation kit, forming the antibody-magnetic microparticle (Ab-MMP) complex. After that, PDLCs were suspended ($5 \times 10^6$ cells/mL) in DMEM supplemented with 10 % FBS/1 % A/A solution and incubated with Ab-MMPs for 45 min at 37 °C under gentle stirring. After this, the mixture of cells and particles was placed on a Dynal MPC magnet (MPC®-S, Dynal Biotech, Oslo, Norway) to remove cells that the Ab-MMPs had failed to recognize. The recruited cells ($CD90^+$ PDLCs) were cultured and observed via phase-contrast microscopy after 3 days of incubation.

*Subpopulation Culture and Characterization*

After cell sorting, the cells were divided into 6 groups: (a) DPCs in basal medium, (b) DPCs in osteogenic medium, (c) PDLCs in basal medium, (d) PDLCs in osteogenic medium, (e) $CD90^+$ PDLCs in basal medium, and (f) $CD90^+$ PDLCs in osteogenic medium. Each group was cultured in well plates for 7, 14, or 21 days in a humidified atmosphere at 37 °C with 5 % $CO_2$. Moreover, DPCs, PDLCs, and the $CD90^+$ PDLC subpopulation were screened for the stemness marker CD90 by flow cytometry. Cellular proliferation was evaluated via dsDNA quantification of a lysed cell suspension obtained after osmotic and thermal shock in the 6 groups via a PicoGreen dsDNA quantification kit (Invitrogen, Eugene, OR, USA) according to the manufacturer's specifications.

*Osteogenic Differentiation*

To determine which cell group has greater osteogenic potential, alkaline phosphatase (ALP) activity was assessed in a lysed cell suspension obtained after osmotic and thermal shocks from all the groups under study. A colorimetric endpoint assay was performed to measure ALP, which quantifies the conversion of p-nitrophenol phosphate to p-nitrophenol by the ALP enzyme. In summary, the samples and a substrate solution of 0.2 % (w/v) p-nitrophenyl phosphate (Sigma, St. Louis, MO, USA) in a substrate buffer of 1 M diethanolamine HCl (Merck, Darmstadt, Hesse, Germany) were added to a 96-well plate. A stop solution (2 M NaOH (Panreac, Castellar del Vallès, Spain) plus 0.2 mM EDTA (Sigma, St.Louis, MO, USA)) was added to each well after 1 hour of incubation, and the absorbance was read at 405 nm in a microplate reader (Synergy HT, Biotek, Winoosky, VT, USA). A standard curve was made from standards (0–0.3 $\mu$mol $mL^{-1}$) prepared with a p-nitrophenol (pNP; Sigma) solution. Samples and standard triplicates were analysed, and sample concentrations were read from the standard curve. Additionally, the ALP results were normalized to the DNA values to obtain the ALP expression per cell unit.

*Antibody-Magnetic Microparticle (Ab-MMP)-Loaded Hydrogels for Assisting in the Recruitment of Periodontal Odontogenic Subpopulations*

Elaboration of an Aldehyde-Hydrazide Hyaluronic Acid-Based Hydrogel Enriched with Platelet Lysate

The aldehyde-hydrazide hyaluronic acid-based hydrogel enriched with platelet lysate was produced via standard protocols established by our group and was described in previous publications [23]. Briefly, the injectable hydrogels were prepared at room temperature by mixing equal amounts of aldehyde and hydrazide derivatives of hyaluronic acid. A double-barrel syringe (A and B), fitted with a static mixer placed at the outlet and a gauge needle fitted to the mixer tip, was used to produce the injectable hydrogels. Barrel A was filled with hydrazide-modified hyaluronic acid previously dissolved in platelet lysate, and barrel B was filled with aldehyde-modified hyaluronic acid (2 % w/v). The solutions charged on the barrels were sterilized by ultraviolet (UV) irradiation (254 nm) for 30 min.

Preparation of Simulated Root Canals and Magnetization of Endodontic Files

To simulate a dental root canal space for cell recruitment, a 0.81 mm diameter transparent infusion tube with a length of 2 cm was utilized. Before use, these tubes were disinfected with 70 % ethanol and sterilized via UV irradiation at a wavelength of 254 nm for 30 min. For this assay, the magnetic charge of 32 brand-new sterile 25 mm 35-k endodontic files (Maillefer, Ballaigues, Switzerland, Batch No.: 1395750) was measured via a GM08 Gaussmeter (Hirst magnetic instruments, Falmouth, UK). After that, a stainless-steel endodontic file was placed in direct contact with the magnet array of a Magnefect nano II (nanotherics, Warrington, UK) system to induce magnetism. A Gaussmeter was used to determine the magnetism induction before

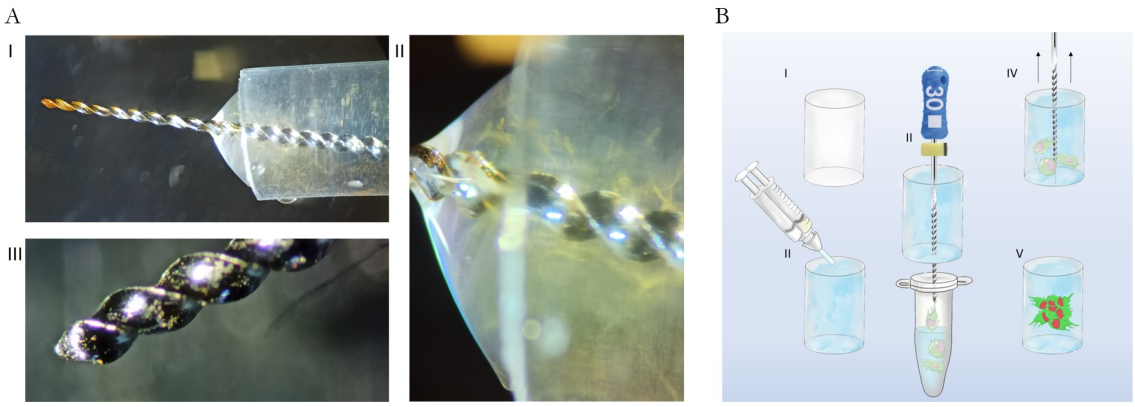


**Fig. 1. Magnetic attraction assay.** (**A**) Endodontic magnetic file assay. (I) The magnetized endodontic file was advanced through the hyaluronic acid-derived hydrogel, culminating in the tube containing the MMPs. (II) Utilizing a counterclockwise motion, the file was extracted, resulting in the retention of the MMPs within the hyaluronic acid-derived hydrogel. (III) Following the extraction of the file, MMPs adhering to the magnetized endodontic instrument remain observable. (**B**) Magnetic attraction scheme. (I) A 2 cm transparent infusion tube simulating a root canal. (II) The simulated root canal was filled with an injectable hyaluronic acid, which mimics the prospective implementation of a regenerative endodontic procedure. (III) The hydrogel was passed through a magnetized endodontic file directed towards the tube containing PDLCs conjugated with antibody-magnetic microparticles, emulating the magnetic attraction of grafted cells from the periodontal tissues. (IV) Upon removal of the magnetized file, the recruited cells were integrated into the hyaluronic acid-derived hydrogel. (V) Cell viability and proliferation were assessed in the recruited cells after predetermined time points. MMPs, magnetic microparticles; PDLCs, periodontal ligament cells.

and after placing the file in contact with the magnet. The Gaussmeter indicated that the six-well magnet array of the Magnefect nano II system had a magnetic charge of 349.400 (± 15.646) mili Tesla (mT) (herein used as a control). The new endodontic files presented a slight magnetic charge of 0.391 (± 0.064) mT. In comparison, after 12 hours of contact with the six-well magnet array of the Magnefect nano II system, the same endodontic files presented a magnetic charge of 3.975 (± 0.293) mT, which indicates a significant increase in the magnetic charge ($p < 0.0001$) (Table 1).

## Magnetic Attraction Assay

Each simulated root canal was filled with 30 $\mu$L of a completely cell-free hydrogel and incubated for 30 min at 37 °C. During this time, 80 $\mu$L of Dynabeads were washed three times in sterile phosphate-buffered saline (PBS). In an Eppendorf tube, 32 $\mu$L of CD90 antibody was suspended in 320 $\mu$L of binding and washing buffer (Dynabeads Protein A Kit, Invitrogen, Carlsbad, CA, USA). The Dynabeads were mixed with the previous solution for 10 min under rotation, forming the antibody-magnetic microparticles (Ab-MMPs).

The isolation of the $CD90^+$ subpopulation via the Ab-MMPs followed the same protocol as described above and according to the manufacturer's instructions.

A simulated root canal filled with a completely cell-free hydrogel (Fig. 1**A**) was fixed to a polydimethylsiloxane (PDMS) support previously disinfected and sterilized by UV radiation. The simulated root canals were placed inside each of the Eppendorf tubes through the PDMS support, preventing any part of them or their internal hydrogel from coming into contact with the solution in the Eppendorf tubes Ab-MMPs-PDLCs in the experimental group or Ab-MMPs in the control group (Fig. 1**B**).

A magnetized endodontic file crossed all the cell-free hydrogels in the simulated root canals until the file tip was submerged entirely in the content of the Eppendorf tube. In this experiment, cells were attracted into the hydrogel through magnetically guided recruitment rather than direct seeding or pre-mixing cells with the hydrogel. For this purpose, following the Ab-MMP complex preparation, a magnetized endodontic file was positioned to draw $CD90^+$ PDLCs tagged with Ab-MMPs from a suspension directly into the cell-free hydrogel. This approach facilitated precise cell attraction and integration within the hydrogel structure, thereby simulating an *in vivo*-like recruitment environment. The magnetic attraction of the Ab-MMP-tagged cells to the hydrogel allowed us to avoid pre-mixing or surface seeding, supporting a controlled assessment of cell homing and viability within the hydrogel matrix. The endodontic file was subsequently removed from the Eppendorf tube and placed in a new Eppendorf tube containing 10 $\mu$L of PBS to wash the tip of the file. Finally, the endodontic file was withdrawn with a counterclockwise movement from the cell-free hydrogel, thus depositing everything that was magnetically attracted to it inside the hydrogel (Fig. 1). The simulated root canals were subsequently transferred to 24-well plates containing DMEM and cultured for 7 and 14 days in a humidified atmosphere at 37 °C with 5 % $CO_2$.

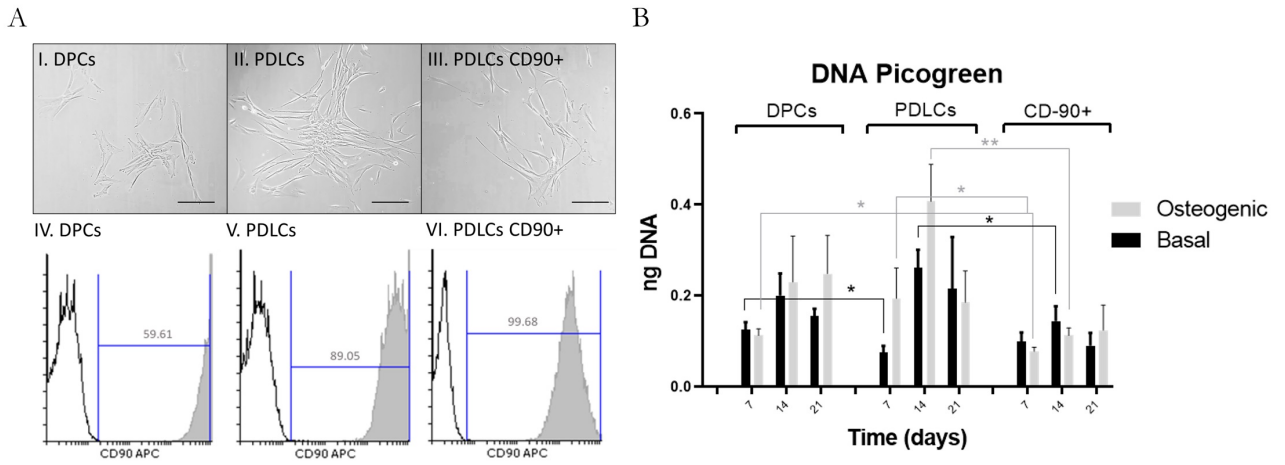


**Fig. 2. Cell characterization.** (**A**) Cell morphology and flow cytometry characterization. The first row of images corresponds to the cells observed under a phase-contrast microscope after 3 days in culture. (I) DPCs. (II) PDLCs. (III) $CD90^+$ PDLCs. Scale bar, 200 $\mu$m. The second row of flow cytometry plots indicates the percentage of CD90-positive cells. (IV) DPCs. (V) PDLCs. (VI) $CD90^+$ PDLCs, with their respective negative controls. (**B**) Cellular proliferation. dsDNA was quantified in basal and osteogenic media after 7, 14, and 21 days via a DNA PicoGreen assay. The symbols * and ** denote study groups with statistically significant differences: $p < 0.05$ and $p < 0.01$, respectively. DPCs, dental pulp cells; $CD90^+$, cluster of differentiation 90 positive; APC, allophycocyanin.

### Cell Viability and Proliferation Assays

For this assay, two groups were analysed: (A) an experimental group consisting of three samples of PDLCs grafted with Ab-MMPs carried by the magnetized endodontic files to the hydrogel and (B) a negative control group consisting of Ab-MMPs carried out by the magnetized file to the simulated root canals filled with hydrogel, both of which were cultured in basal medium for 7 and 14 days.

Cell viability was measured through the Alamar blue assay (AbtDseroTec, Hercules, CA, USA) following the manufacturer's instructions. Briefly, after 3, 7, 14, and 21 days, the medium was discarded, and the samples were washed twice with PBS and then incubated in a 10 % Alamar blue solution in DMEM at 37 °C and 5 % $CO_2$ for 3 hours. The fluorescence of the supernatant was read in triplicate in a microplate reader (Synergy HT, Biotek, Winoosky, VT, USA) at 560 nm excitation and 590 nm emission.

For the proliferation assay, lysed cell suspensions obtained after osmotic and thermal shocks from the experimental and negative control groups were measured via a PicoGreen dsDNA quantification kit (Invitrogen, Eugene, OR, USA).

### Alkaline Phosphatase Expression

ALP activity was quantified in the same cell lysate used for cell proliferation. ALP expression was evaluated in triplicate for each time point, using a protocol analogous to that outlined in the Osteogenic Differentiation section.

### Nuclei and Cytoskeleton Analysis

Immediately after the magnetic attraction assay, a simulated root canal was fixed in 10 % (v/v) neutral buffered formalin (Thermo Fisher) for 48 hours. Similarly, after each time point, the samples were immersed in formalin. After fixation, all the samples were washed in PBS and then permeabilized in 0.2 % (v/v) Triton X-100/PBS (Sigma–Aldrich) for 1 hour. The samples were subsequently incubated in 1 mL of 4′,6-diamidino-2-phenylindole (DAPI) dilactate (Biotium, Fremont, CA, USA) at a ratio of 1:1000 v/v in PBS for 1 hour and in phalloidin-tetramethylrhodamine B isothiocyanate (Sigma–Aldrich) at a ratio of 1:200 v/v in PBS for 1 hour. The samples were subsequently washed three times in PBS for 5 min to prevent background fluorescence. Finally, the samples were observed under a fluorescence inverted microscope (Zeiss, Jena, Thuringia, Germany, Axio Observer Z1), and images were acquired.

### *Statistical Analysis*

The data were processed via Microsoft Office Excel 2019 and analysed via GraphPad PRISM 9.5.1 (GraphPad Software Inc., La Jolla, CA, USA). The results are presented as the means ± standard errors of the means of three independent experiments, each performed in triplicate. Ordinary one-way analysis of variance (ANOVA) was used for multiple unpaired comparison tests, and the unpaired *t* test was used for individual comparison tests. For paired multiple comparisons, repeated measures one-way ANOVA was used, followed by paired *t* tests for the individuals.

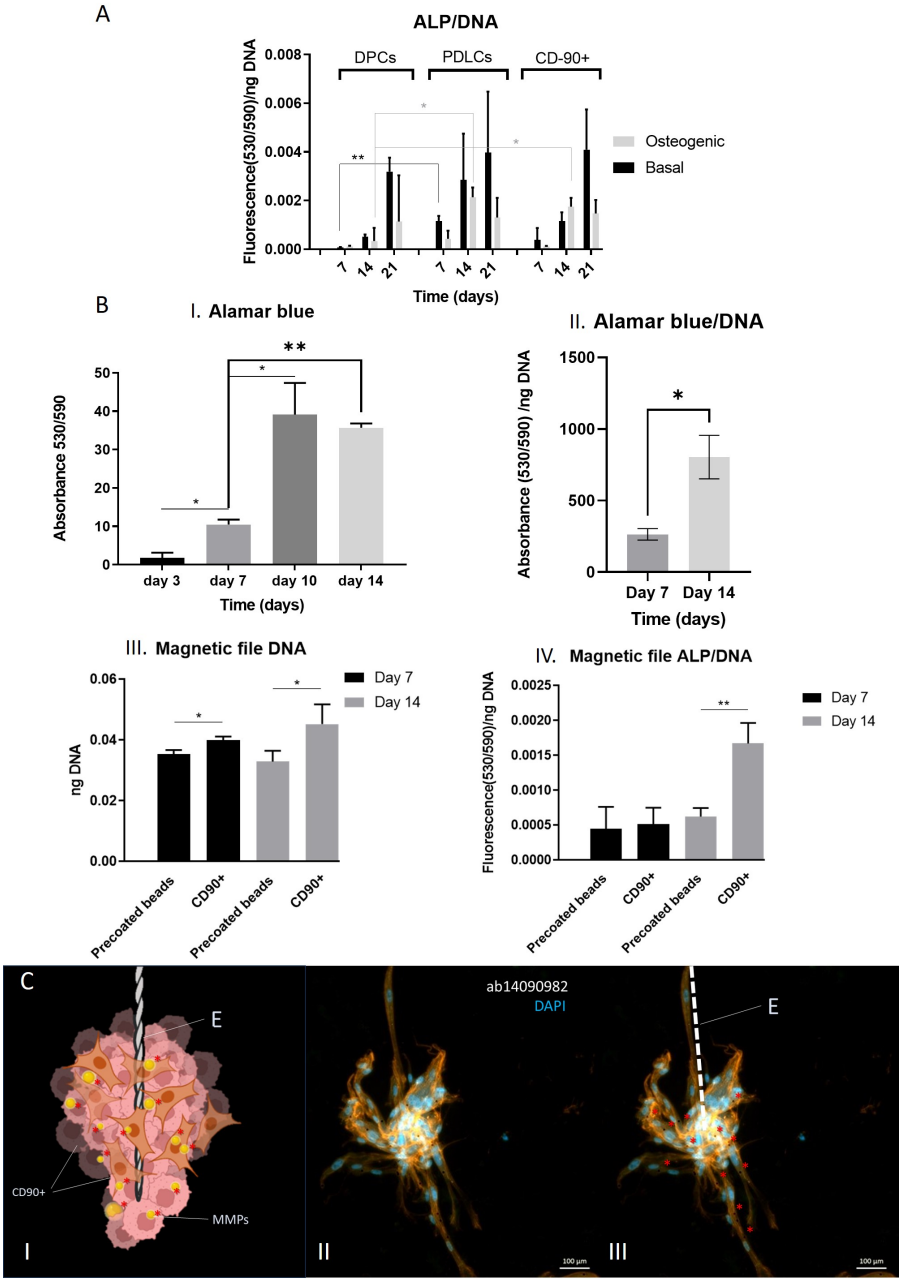


**Fig. 3. Cell differentiation and recruitment.** (**A**) Odontogenic differentiation. ALP expression per cell unit was analysed in basal and osteogenic media after 7, 14, and 21 days. The symbols * and ** denote study groups with statistically significant differences: $p < 0.05$ and $p < 0.01$, respectively. (**B**) Ab-MMPs assisted in recruitment. Using the endodontic cell engager, $CD90^{+}$ cells were selectively recruited from a pool of PDLCs and directed towards the simulated root canal, which had been filled with a hyaluronic acid hydrogel. These simulated root canals contained the recruited cells entrapped within the hyaluronic acid-based hydrogel and were subjected to culture in basal medium. The supernatant medium was collected for a comprehensive series of evaluations, including the following: (I) Cell viability test: This test was conducted via the Alamar blue assay at intervals of 3, 7, 10, and 14 days in basal medium. (II) Alamar blue per cell unit: Assessment of Alamar blue per cell unit after 7 and 14 days of culture in basal medium. (III) Cellular proliferation assay: Utilizing the DNA PicoGreen assay, cellular proliferation was quantified. (IV) ALP expression per cell unit: After 7 and 14 days in basal medium, the expression of alkaline phosphatase (ALP) per cell unit was evaluated for both the experimental and control groups. The symbols * and ** denote study groups with statistically significant differences: $p < 0.05$ and $p < 0.01$, respectively. (**C**) Immediate recruitment of Ab-MMPs. (I) Illustrative representation of the recruitment assay. (II) Fluorescence microscopy revealed IgG CD90 with an Alexa Fluor 598 secondary antibody for the cytoplasm (ab140909882) and DAPI to stain the nuclei. (III) A white line has been placed on the site of the endodontic file (E), which helps to identify its location within the image, and the red asterisks (*) were placed next to the MMPs to improve their identification. Scale bar, 100 $\mu$m. Ab-MMPs, antibody-magnetic microparticles; DAPI, 4′,6-diamidino-2-phenylindole; IgG, Immunoglobulin G.

## Results

*Cell Sorting and Flow Cytometry Characterization*

After 3 days of cell sorting, the morphology, amount, distribution, and disposition of the $CD90^+$ subpopulation were more similar to those of DPCs than to those of PDLCs, as shown by phase-contrast images (Axio Observer, Zeiss). Additionally, and as expected, it was possible to observe the presence of Ab-MMPs beside $CD90^+$ cells (Fig. 2**A**). The immunophenotype analysis of DPCs and PDLCs revealed high expression of the mesenchymal stem cell marker CD90. The percentage of $CD90^+$ cells positive for the CD90 marker was much greater than that in DPCs and moderately greater than that in PDLC samples (Fig. 2**A**).

*Cellular Proliferation*

The results pertaining to cell proliferation revealed that in the basal medium, DPCs exhibited significantly greater proliferation ($p < 0.05$) than PDLCs did on day 7. By day 14, PDLCs displayed notably greater proliferation ($p < 0.05$) than did $CD90^+$ PDLCs. In the osteogenic medium, both DPCs and PDLCs demonstrated significantly greater proliferation ($p < 0.05$) than did $CD90^+$ PDLCs at the 7-day mark in the basal medium. By the 14th day, PDLCs manifested significant differences in proliferation ($p < 0.01$) compared with $CD90^+$ PDLCs. On day 21, all the cell groups exhibited a reduction in proliferation across both basal and osteogenic media, with no discernible differences between them (Fig. 2**B**).

*Osteogenic Differentiation*

The osteogenic differentiation of DPCs, PDLCs, and $CD90^+$ PDLCs was assessed by quantifying ALP activity, which is considered an early marker of hard tissue formation. The activity of the ALP enzyme on day 7 of culture in basal medium was significantly greater ($p < 0.01$) in PDLCs than in DPCs. There were no other significant differences among the 3 groups of cells cultured in basal medium at the other time points. With respect to the group of cells cultured in osteogenic medium, on day 14, the DPCs demonstrated significantly lower ($p < 0.05$) ALP enzyme activity than the PDLCs and the $CD90^+$ PDLCs did. There were no significant differences in ALP expression on days 7 and 21 (Fig. 3**A**).

*Ab-MMPs Assisted in the Recruitment of Periodontal Odontogenic Subpopulations in Hydrogels*

Cell Viability and Cell Proliferation

The cells enclosed within the hydrogel matrices simulating root canals were cultured for a duration of up to 14 days. Subsequently, cell viability was determined through the Alamar blue assay, and cell proliferation was assessed via the PicoGreen assay. The Alamar blue assay revealed a noteworthy increase ($p < 0.05$) in the absorbance values from day 3 to day 10. No significant variations in viability were observed until day 14. However, between day 7 and day 14, a substantial difference ($p < 0.01$) became evident. To further investigate this trend, the Alamar blue values were normalized by dividing them by dsDNA, thereby providing an assessment of Alamar blue per cell unit. This analysis revealed a significant increase after 14 days of culture. Cellular proliferation was quantified by assessing the dsDNA content in the lysed cell suspensions obtained following osmotic and thermal shocks from both the experimental and negative control groups on days 7 and 14. Significant differences ($p < 0.05$) were detected between the groups on both day 7 and day 14 (Fig. 3**B**).

Alkaline Phosphatase Expression

The ALP activity per cell unit in the experimental and negative control groups after 7 days in culture was not significantly different, whereas after 14 days of culture in basal medium, significant differences ($p < 0.01$) were detected between the experimental group and the negative control group (Fig. 3**B**).

In the control group, the cells were not discernible after both 7 and 14 days. Conversely, following seven days of culture, the experimental group exhibited a cohort of cells orienting toward the magnetized file upon its extraction. A subset of these cells appeared to aggregate into diminutive colonies. Additionally, substantial cell conglomerates were observed within the interior region of the hydrogel, which subsequently proliferated throughout the hydrogel matrix by the 14th day (Fig. 3**B**).

Fluorescent Cell Stain Analysis

Immediately after Ab-MMP-assisted recruitment, a sample was observed via fluorescence microscopy, which revealed cell agglomeration around the magnetized endodontic file tip and a row of cells attached to the instrument (Fig. 3**C**). After seven days in culture, the experimental group exhibited an alignment of cells along the direction influenced by magnetization, which facilitated their removal. Some cells formed small colonies, and a large conglomerate of cells was observed within the inner region of the hydrogel. By day 14, the cells were evenly distributed throughout the hydrogel, forming both colonies and larger cell conglomerates (Fig. 4). In contrast, the negative control group contained no cells at either the 7-day or 14-day time points.

## Discussion

This study aimed to assess a magnetically mediated cell homing strategy for the targeted migration of specific mesenchymal cells from periodontal tissues to a root canal filled with a hyaluronic acid-based hydrogel. The evaluation was conducted to introduce an alternative therapeutic approach in regenerative endodontics for treating teeth affected by pulp necrosis. We first analysed a specific population of PDLCs as a potential source of odontogenic cells and then investigated a magnetically mediated selective recruit-

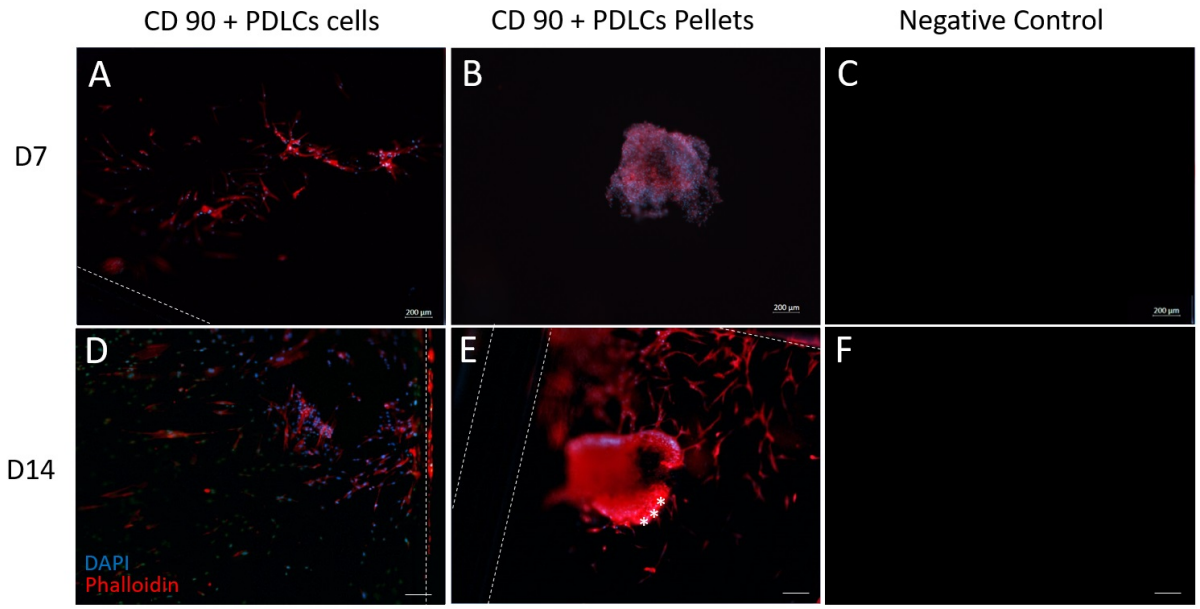


**Fig. 4. Ab-MMPs assisted in recruitment. DAPI-phalloidin staining after the magnetic recruitment assay.** (**A**) Micrography showing dispersed cells forming small colonies organized along the path defined by the removal of the magnetized file from the simulated root canal after 7 days of culture in basal medium. (**B**) A larger conglomerate of cells incorporated and formed pellets in the hydrogel after 7 days of culture in basal medium. (**C**) Negative control. (**D**–**F**) Micrographs **D**, **E**, and **F** represent the same regions as **A**, **B**, and **C**, respectively, following a culture period of 14 days in basal medium (scale bar 200 $\mu$m). White asterisks screen the MMPs. The limits of the simulated root canal are delineated by white dashed lines.

ment process, emphasizing its potential utility in augmenting therapeutic approaches. Magnetically mediated cell recruitment enhances the specificity of cell homing for endodontics. Therefore, it has the potential to reduce the procedural constraints inherent to autologous therapies, facilitating a smoother transition to clinical application.

Current clinical approaches for endodontic regeneration predominantly target teeth with vital pulp tissue [4,5]. Teeth diagnosed with dental pulp necrosis undergo a loss of all the cellular components within the dental pulp, including those exhibiting stem cell-like properties [24]. The only feasible method for dental pulp regeneration under these conditions is to culture dental pulp stem cells from an alternative healthy tooth [5], other autologous sources [25], or even allogeneic sources [3]. The primary challenges associated with this method arise from donor site morbidity and the requirement for a GMP-compliant laboratory for processing. The implementation of GMP protocols significantly increases the expenses associated with regenerative endodontic therapy, rendering it economically unviable. This finding was corroborated by Meza *et al*. in 2019 [4], who cultivated autologous pulp cells under GMP conditions to administer regenerative autologous cellular therapy utilizing mesenchymal stem cells derived from inflamed dental pulp in conjunction with leukocyte platelet-rich fibrin in a mature tooth.

As a result, we postulate that leveraging periodontal tissues as a viable cell source for cell homing may present a more efficacious and promising clinical approach, avoiding increased surgical expenses and morbidity [8,26]. However, while dental pulp cells (DPCs) and PDLCs exhibit notable distinctions, they both express certain mesenchymal markers, such as CD90 [19], which is extensively distributed across the cell membrane [27].

In this context, one of the objectives of this research was to identify appropriate mesenchymal subpopulations that are proficient in generating mineralized matrices that can enhance current endodontic revitalization treatments, such as revascularization. While revascularization has successfully fostered the ingrowth of vascularized and innervated connective tissues into devitalized root canals, it has not achieved the restoration of an odontoblast-like layer. Consequently, our strategy was to pinpoint a suitable cellular population within tissues proximal to the root canal and substantiate the concept of magnetically recruiting and guiding these cells into the dental root canal via magnetized files. As a result, this research evaluated the osteogenic differentiation potential of three populations derived from the above-mentioned tissues: DPCs, PDLCs, and $CD90^{+}$ PDLCs. This study evaluated the osteogenic phenotype of cell populations using standard protocols and markers commonly documented in the scientific literature on osteogenic differentiation [28–31]. Specifically, cells were

cultured under standardised osteogenic conditions, and alkaline phosphatase (ALP) activity, a key protein involved in bone matrix mineralisation, was used as a primary bone marker. The quantitative results for ALP expression are presented in Fig. 3. The 14-day osteogenic culture revealed a pronounced increase in ALP activity in both PDLCs and $CD90^+$ PDLCs, exceeding the activity observed in DPCs. No statistically significant disparities were discerned between the basal and osteogenic cultures across any of the cell groups, including DPCs, PDLCs, and $CD90^+$ PDLCs, at various timepoints. This observation can be attributed to the composition of the basal medium, which was formulated with low-glucose DMEM. Notably, low-glucose DMEM has been previously shown to induce alkaline phosphatase activity and exert a robust stimulatory effect on matrix mineralization within cell cultures [32,33].

In the present study, we prioritised qualitative assessments of cell viability and morphology to establish an initial proof of concept for the magnetic recruitment system within the hydrogel matrix. Although this approach has yielded significant preliminary insights, we acknowledge that the absence of quantitative analyses, such as flow cytometry, may limit a comprehensive evaluation of cell homing efficiency and recruitment outcomes. Moving forward, we intend to expand our analytical framework to incorporate quantitative metrics that will complement our qualitative findings, thereby enhancing the translational relevance and rigor of this magnetic guidance strategy.

The magnetic attraction process ensures the targeted recruitment of specific cells, facilitating the attraction of cell aggregates that manifest osteogenic and odontogenic traits. Given that this is the first time that a magnetized endodontic instrument has recruited cells grafted with antibodies conjugated to magnetic microparticles (MMPs), our research endeavoured to characterize the recruited cell group incorporated within the hyaluronic acid-based hydrogel, aiming to evaluate its potential for future applications. In the same way, while our study was guided by previous findings indicating that MMPs have minimal negative effects on cell viability and function, we recognise that specific MMP types and concentrations may vary and could impact cell behaviour differently. Although our viability assays yielded positive results, further investigation into MMP concentration-specific effects on viability, proliferation, and odontogenic differentiation potential will enhance understanding. Future studies will thus incorporate additional assays to explore these effects more comprehensively. Additionally, while this study primarily aimed to establish a proof-of-concept for the feasibility of magnetic recruitment, we acknowledge that the lack of inflammatory response evaluation is a limitation in the current approach. Understanding the immunological implications of biomaterials is indeed crucial, especially in regenerative therapies, where a favourable immune environment supports tissue healing and integration. Future investigations will include thorough assessments of inflammatory responses, using *in vitro* and *in vivo* models, to provide a comprehensive view of the therapeutic potential and safety profile of our system. This approach will allow us to evaluate pro-inflammatory cytokine levels, macrophage polarisation, and tissue compatibility, thereby enhancing the translational relevance of our findings.

Following the execution of the recruitment assay, metabolic activity was assessed via the Alamar blue assay. Observations from this assay highlighted a peak in activity around the tenth day, which persisted until the fourteenth day. This trend may be attributable to an increase in cellular proliferation, which appears to correlate directly with hydrogel degradation. This implies that a substantial cell population competes for diminishing nutrient resources. The observed outcomes align with findings reported by Jooybar *et al*. in 2020 [34], who attributed the activity peaks on the tenth and fourteenth days to the incorporation of platelet lysate within the hyaluronic acid-based microgel.

Similarly, DNA analysis confirmed the viability of the cells encapsulated within the hydrogel, underscoring an efficacious recruitment process and a conducive environment for dental pulp regeneration. The efficacy of the recruitment procedure employed in this research was further validated by DAPI-phalloidin staining conducted immediately post assay. Upon examination via fluorescence microscopy, one sample revealed that a cohort of $CD90^+$ cells were successfully recruited into the hydrogel. Moreover, the presence of Ab-MMPs within the cell aggregate was discernible (Fig. 3**C**). After 7 days, the cells assumed a fusiform morphology and initiated the formation of spherical colonies within the hydrogel, a phenomenon that was amplified by the 14th day. Notably, no cells were detected within the hydrogel samples of the control group, which contained only Ab-MMPs (Fig. 4).

## Conclusions

Compared with DPCs, the $CD90^+$ PDLCs population exhibited superior osteogenic differentiation potential, albeit inferior to that of PDLCs. Endodontic files can be magnetized, facilitating the recruitment of $CD90^+$ cells from a heterogeneous cohort when labelled with magnetic microparticles. The magnetic recruitment of specific cellular populations has been identified as a potential alternative in scenarios of autologous pulp regeneration for teeth exhibiting pulp necrosis. This approach is pertinent for revitalization cases, the utilization of blood products, or the use of tissue engineering biomaterials, including hyaluronic acid.

## List of Abbreviations

Ab-MMPs, antibody-magnetic microparticles; ALP, alkaline phosphatase; $CD90^+$, cluster of differentiation 90 positive; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle medium; DPCs, dental pulp cells; FBS, fetal bovine serum; MMPs, magnetic micropar-

ticles; PBS, phosphate-buffered saline; PDLCs, periodontal ligament cells; PDMS, polydimethylsiloxane; UV, ultraviolet; GMP, good manufacturing practice; MSCs, mesenchymal stem cells; PL, platelet lysate; A/A, antibiotic-antimycotic mixture; mT, mili Tesla; hASCs, adipose stem cells; ANOVA, analysis of variance.

## Availability of Data and Materials

The datasets generated and analysed during the current study are available from the corresponding author upon reasonable request. All data underlying the findings, including raw data, processed data, and statistical analysis files, have been archived and can be provided to researchers interested in verifying the results of this study. The materials and protocols used are detailed in the manuscript, and additional information regarding reagents and experimental conditions can be shared upon request.

## Author Contributions

EAO contributed to the conceptualization, investigation, methodology, formal analysis, validation, and drafting of the original manuscript. PSB contributed to the conceptualization, methodology, investigation, validation, supervision, and revision of the manuscript. AIG contributed to the investigation, validation, supervision, and critical revision of the manuscript for important intellectual content. MEG contributed to supervision, funding acquisition, and manuscript revision. All authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work, ensuring that any questions related to accuracy or integrity were appropriately investigated and resolved.

## Ethics Approval and Consent to Participate

All collected human samples, including teeth, cells, and blood derivatives, were handled in compliance with national and international guidelines. Human teeth were extracted for orthodontic purposes at the Malo Clinic Dental Care (Portugal) in accordance with quality and safety standards (as outlined in 2004/23/EC, 2006/17/EC, and 2006/86/EC). These procedures were conducted under an institutional protocol approved by the health ethical committee on December 23, 2014.

Platelet components from whole blood or apheresis for the production of the PL batches were collected from healthy adult volunteers and regular blood donors. These donors met the requirements set forth in annexes II and III of directive 2004/33/EC and annex IV of directive 2002/98/EC. The collections took place at Serviço de Imunohemoterapia-CHSJ (Porto, Portugal) under an approved institutional protocol (ethical commission approval no. 363/18, December 2012). All donations were anonymous, adhering to the European Data Protection Directive 95/46/EC.

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## Conflict of Interest

The author(s) declare no conflicts of interest.

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