Dental pulp stem cell responses to novel antibiotic-containing scaffolds for regenerative endodontics

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Abstract

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Aim To evaluate both the drug-release profile and the effects on human dental pulp stem cells' (hDPSC) proliferation and viability of novel bi-mix antibiotic-containing scaffolds intended for use as a drug delivery system for root canal disinfection prior to regenerative endodontics.

Methodology Polydioxanone (PDS)-based fibrous scaffolds containing both metronidazole (MET) and ciprofloxacin (CIP) at selected ratios were synthesized via electrospinning. Fibre diameter was evaluated based on scanning electron microscopy (SEM) images. Pure PDS scaffolds and a saturated CIP/MET solution (*i.e.* 50 mg of each antibiotic in 1 mL) (hereafter referred to as DAP) served as both negative (nontoxic) and positive (toxic) controls, respectively. High-performance liquid chromatography (HPLC) was performed to investigate the amount of drug(s) released from the scaffolds. WST-1® proliferation assay was used to evaluate the effect of the scaffolds on cell proliferation. LIVE/DEAD® assay was used to qualitatively assess cell viability. Data obtained from drug release and proliferation assays were statistically analysed at the 5% significance level.

Results A burst release of CIP and MET was noted within the first 24 h, followed by a sustained maintenance of the drug(s) concentration for 14 days. A concentration-dependent trend was noticed upon hDPSCs' exposure to all CIP-containing scaffolds, where increasing the CIP concentration resulted in reduced cell proliferation ($P < 0.05$) and viability. In groups exposed to pure MET or pure PDS scaffolds, no changes in proliferation were observed.

Conclusions Synthesized antibiotic-containing scaffolds had significantly lower effects on hDPSCs proliferation when compared to the saturated CIP/MET solution (DAP).

Keywords: antibiotic pastes, drug delivery, nanofibres, regeneration, root canal, stem cells.

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Introduction

Treatment of pulpal necrosis of immature permanent teeth is challenging due to its uncertain prognosis

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(Andreasen *et al.* 2002, Ribeiro *et al.* 2008). Recently, regenerative endodontics has become a rational alternative to classical root apexification (Banchs & Trope 2004). It relies on copious chemical canal irrigation with minimal or no mechanical root canal preparation, followed by the application of a mixture of antibiotics for nearly 1 month (Banchs & Trope 2004, Geisler 2012). During the next visit, canal contents are removed via irrigation, and bleeding from periapical tissue is prompted (Murray *et al.* 2007). The evoked bleeding leads to the accumulation of

undifferentiated stem cells (Lovelace *et al.* 2011), which, according to the literature, can play an important role in the regeneration of pulpal tissues (Murray *et al.* 2007, Diogenes *et al.* 2014). Overall, this innovative endodontic procedure has led, according to clinical cases and case series, to increased root length, apical closure and continuing thickness of dentine walls, amongst other positive clinical findings (Banchs & Trope 2004, Kim *et al.* 2010, Petrino *et al.* 2010, Lin *et al.* 2013, Simon & Smith 2014).

One of the most critical steps in regenerative endodontics is the complete eradication of residual infection (Albuquerque *et al.* 2014). Typically, this is achieved by the application of calcium hydroxide. As an alternative, triple antibiotic paste (TAP) containing CIP, MET, and minocycline or double antibiotic paste (DAP), containing CIP and MET, are used (Hoshino *et al.* 1996, Sato *et al.* 1996). Notably, the use of both calcium hydroxide and antibiotic pastes has demonstrated several important side effects. The former has been associated with root weakening when used for both short (Sahebi *et al.* 2010, Yassen *et al.* 2013) and long (Andreasen *et al.* 1989, Yassen *et al.* 2013, Moazami *et al.* 2014) periods of time. On the other hand, the use of clinically recommended dosages of TAP and DAP pastes has led to unfavourable effects on dental pulp cells (Ruparel *et al.* 2012, Chuensombat *et al.* 2013), as well as periodontal ligament fibroblasts (Yadlapati *et al.* 2014), posing important questions towards the predictability of regenerative procedures. Moreover, current irrigation methods do not completely remove the remnants of antibiotic pastes from root canals (Berkhoff *et al.* 2014).

In the light of this, drug delivery-based strategies, to reduce the local drug concentration through the use of antibiotic-containing nanofibres, have been proposed recently (Bottino *et al.* 2011, 2013a, 2014, Albuquerque *et al.* 2014, Palasuk *et al.* 2014, Waeiss *et al.* 2014). Therefore, the hypotheses of the study were that the amount of drug(s) released from these novel antibiotic-containing scaffolds (i) would lead to reduced cell toxicity, and more importantly (ii), it would diminish the negative impact on hDPSC proliferation when compared to the double antibiotic (DAP) paste (*i.e.* ciprofloxacin and metronidazole mixture).

Materials and methods

Fabrication of the antibiotic-containing scaffolds

The synthesis of antibiotic-containing nanofibrous scaffolds has been described in detail (Bottino *et al.* 2011, 2013a, 2014, Palasuk *et al.* 2014, Waeiss *et al.* 2014). Briefly, MET (Sigma-Aldrich, St. Louis, MO, USA), and CIP (Sigma-Aldrich) bi-mix polydioxanone-based (PDS II®, Ethicon, Somerville, NJ, USA) solutions were prepared to obtain distinct MET/CIP-containing scaffolds. Pure MET and CIP scaffolds were also prepared. PDS pieces were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Sigma-Aldrich). MET and CIP were added alone and in different ratios (by weight, wt.%) into the PDS solutions and mixed overnight (Bottino *et al.* 2013a, Palasuk *et al.* 2014). The following groups of scaffolds were prepared: pure MET, pure CIP, 3:1MET/CIP, 1:1MET/CIP and 1:3MET/CIP (Palasuk *et al.* 2014). All antibiotic-containing PDS solutions were incorporated with 25 wt.% of antibiotic (s) in total relative to the PDS polymer weight (*i.e.* 150 mg of drug(s) in total) (Bottino *et al.* 2013a). Pure PDS scaffolds and a saturated CIP/MET solution (*i.e.* 50 mg of each antibiotic in 1 mL) (DAP) (Sabrah *et al.* 2013) served as negative (nontoxic) and positive (toxic) controls, respectively. Antibiotic-containing solutions and pure PDS were loaded into 5-mL syringes (Becton-Dickinson, Franklin Lakes, NJ, USA) fitted with 27G metallic blunt-tip needles. Electrospinning was conducted at 2 mL h^{-1}, from a distance of 18 cm and by applying electrical voltage between 15–18 kV. The scaffolds were processed at room temperature (RT) and then dried for at least 48 h (Bottino *et al.* 2013a). Scanning electron microscopy (SEM, JSM-5310LV, JEOL, Tokyo, Japan) was used to evaluate the morphological aspect of the synthesized scaffolds (Bottino *et al.* 2013a).

Drug release

Four scaffolds (15 × 15 mm^2) per group were initially weighed and incubated in 10 mL of phosphate-buffered saline (PBS, pH 7.4, Fisher Scientific, Pittsburgh, PA, USA) and kept in an incubator at 37 °C. Aliquots (1 mL) of each sample were collected at different time-points (1, 2, 5, 7 and 14 days). Equal amounts of fresh PBS were added back to the incubation media subsequent to each aliquot retrieval (Bottino *et al.* 2013a). HPLC–UV analysis was performed on an Agilent 1100 system (Palo Alto, CA, USA), using a Zorbax SB-phenyl chromatography column (5 μm, 150 × 4.6 mm i.d) with a 10-μL injection volume. A binary mobile phase consisting of solvent systems A and B were used in gradient elution where A was 0.1% formic acid (v/v) in ddH$_2$O and B was 0.1% formic acid (v/v) in acetonitrile. The

mobile phase flow rate was 1.0 mL min^{-1}. Initial conditions were set at 95 : 5 A:B, followed by a linear gradient to 40 : 60 from 0 to 15 min. Gradient conditions were re-equilibrated to 95 : 5 A:B from 15 to 16 min and held for 8 min at initial conditions until 23 min. Following separation, the column effluent was introduced into a UV–Vis photodiode array detector (Agilent). Quantitation wavelengths were 319 nm for MET and 278 nm for CIP. Retention times for MET and CIP were 5.4 min and 7.8 min, respectively. Standard curves for MET and CIP were generated using authentic standards, over a concentration range of 7–280 µg mL^{-1}, and then, the drug concentration in the aliquots was calculated. Drug-release data are shown as the mean value plus or minus the standard deviation ($\pm$SD) of the mean. The percentage of the released drugs was then calculated based on the initial weight of the drug incorporated into the electrospun scaffolds (Bottino *et al.* 2013a).

Exposure of hDPSC to antibiotic-containing scaffolds

Low glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and 1% penicillin–streptomycin (Sigma-Aldrich) in a humidified incubator at 37 °C, with 5% CO$_2$, was used to culture hDPSC (catalogue number – DP003F, AllCells LLC, Alameda, CA, USA) obtained from permanent third molars. The medium was changed every 2–3 days, and cells at passages 4–6 were used.

Electrospun samples (15 × 15 mm^2, $n = 4$/group) cut from the distinct antibiotic-containing scaffolds and the control (pure PDS) were mounted in a plastic device (CellCrown™, Scaffdex, Tampere, Finland), disinfected in 70% ethanol for 30 min and rinsed twice in sterile phosphate buffer saline (PBS, Sigma-Aldrich). Meanwhile, hDPSCs were harvested by trypsinization, counted and seeded in 24-well plates at a density of 10^4/well (in 1500 µL of culture medium) on cell culture coverslips (Thermo Scientific Nunc, Rochester, NY, USA). After a 4-h incubation period to allow the cells to be attached, the plastic crowns with scaffolds were introduced into the wells. For standardization purposes, the crowns were separated from the bottom of the plate by a 2-mm distance achieved using plastic rings. Control (blank) columns were prepared with medium without cells and medium with cells but without any scaffolds (100% survival). In all the controls,

for quality purposes, the crowns without scaffolds were introduced. As previously explained, a saturated solution of MET and CIP in complete culture media was used as an equivalent of the DAP (toxic control). The antibiotics were mixed at a concentration of 50 mg mL^{-1} of each antibiotic (Sabrah *et al.* 2013). Then, the solution was stirred for 4 h at RT, centrifuged at 1425 *g* for 15 min, and supernatant was filter-sterilized with a 0.22-µm syringe filter.

Evaluation of hDPSCs proliferation and viability

To assess the effects of antibiotic-containing scaffolds on hDPSC proliferation, the WST-1 assay (Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer's protocol. Briefly, after 3, 5 and 7 days, the CellCrowns™ were removed and 500 µL of culture media was left in the wells, following the addition of WST-1 reagent at the ratio of 10 : 1. After 2 h of incubation in 5% CO$_2$ in a humidified atmosphere at 37 °C, the samples were transferred (100 µL) into 96-well plates. Full media with cells without exposure to scaffolds was assigned as the positive control, and full media without cells was used as the negative control. The optical density (OD) of the incorporated dye was measured by reading the absorbance at 450 nm in a microplate reader against a blank column (Allam *et al.* 2011, Bottino *et al.* 2013b). Cell proliferation was calculated as a ratio of OD from experimental value to positive control, both subtracted by negative control (Allam *et al.* 2011, Bottino *et al.* 2013b).

LIVE/DEAD® assay (Molecular Probes, Invitrogen, Eugene, OR, USA) was used to assess the effects of antibiotic-containing scaffolds on hDPSC viability (Kim *et al.* 2013). Five samples for each experimental group were analysed at day 3 of exposure according to the manufacturer's instructions. To obtain a negative control, cells were treated with 70% methanol for 30 min and cells with no treatment were prepared as a positive control. In brief, LIVE/DEAD® assay uses calcein and ethidium homodimer (EthD-1). Calcein is converted into green product by intracellular esterase, which is active only in viable cells. By contrast, EthD-1, a red staining, has an affinity to nuclei. Nonetheless, to enter the nucleus, the cell membrane must be disrupted. As a result, EthD-1 is detected only in dead cells. Excitation and emission values for calcein and EthD-1 are 494/517 nm and 528/617 nm, respectively. Briefly, after warming LIVE/DEAD® reagent stock solutions to RT, 20 µL of 2 m mol L^{-1} EthD-1 was mixed with 10 mL of sterile PBS to obtain

a 4 μ mol L^{-1} EthD-1 solution. Next, 5 μL of the component A (4 m mol L^{-1} calcein stock solution) was added to the 10 mL of 4 μ mol L^{-1} EthD-1 solution, yielding a 2 μ mol L^{-1} calcein AM solution. 100 μL of the LIVE/DEAD® working solution was added to each well, followed by incubation at RT for 30 min. Immediately after all staining procedures (in the dark room), the cells were evaluated directly in the 24-well plates using an inverted fluorescence microscope (Leica DMI 4000B, Wetzlar, Germany) at 25 × magnification with filters 480/527 and 546/600 for excitation/emission, respectively. At least six pictures per group were acquired and analysed using Image-J software (NIH, Bethesda, MD, USA).

Statistical analysis

Drug-release data were analysed by the Student's *t*-test. Data obtained from proliferation were analysed by one-way ANOVA. Tukey's *post hoc* test was used to compare differences amongst the groups at the same time-point. The level of significance was set at α = 0.05.

Results

Fibre morphology and drug release

Scanning electron microscopy (SEM) was used to examine the effect of drug(s) (MET, CIP and MET/CIP mixtures) incorporation on the fibre morphology of the various antibiotic-containing scaffolds synthesized via electrospinning. The results showed a submicron fibre diameter for all electrospun scaffolds. More importantly, fibres of the antibiotic-containing scaffolds had a visibly smaller mean fibre diameter than pure PDS (Fig. 1).

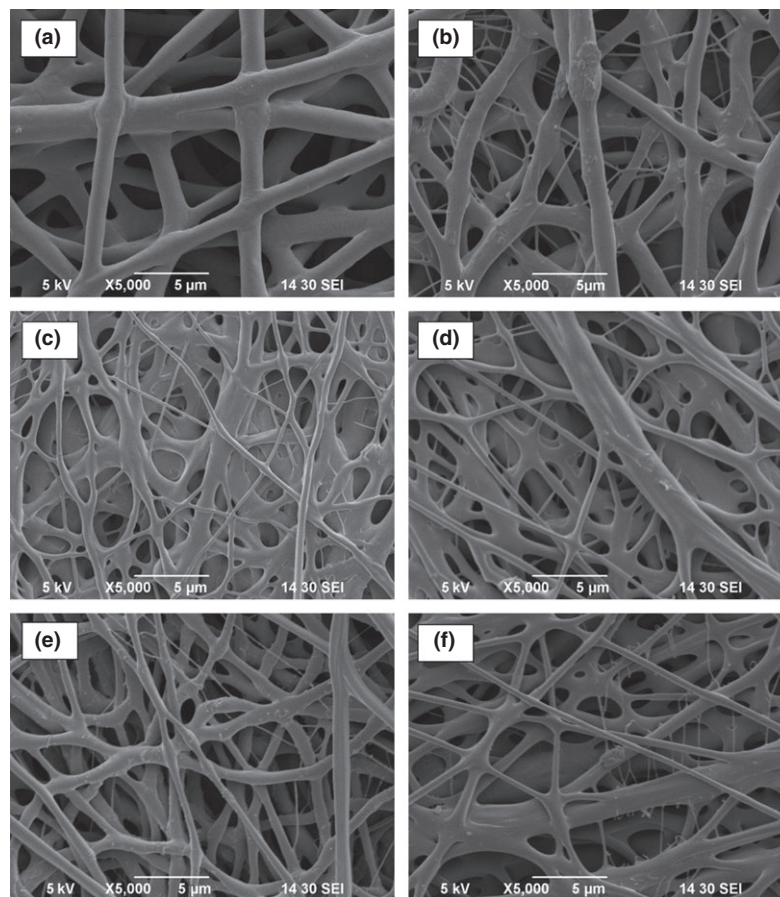Drug-release profiles from each group of antibiotic-containing scaffolds were examined using HPLC. The

Figure 1 (a–f) Representative SEM images of the electrospun antibiotic-containing scaffolds (5000 × magnification): (a) Pure PDS, (b) Pure MET, (c) 3 : 1MET/CIP, (d) 1 : 1MET/CIP, (e) 1 : 3MET/CIP and (f) Pure CIP.

mean drug release, as well as the percentage (%) of drug release, was assessed up to 2 weeks as an initial screening of the potential ability of these scaffolds to function as drug delivery systems. A burst drug(s) release was noted within the first 24 h in all samples. The initial burst release was followed by an overall linear and sustained maintenance of the drug(s) concentration in the incubation media throughout the 14 days (Fig. 2). The mean MET drug released after 14 days for pure MET, 3 : 1 MET/CIP, 1 : 1 MET/CIP and 1 : 3 MET/CIP were 1870 ± 663.1 µg, 1260 ± 229 µg, 1068 ± 305.9 µg and 536 ± 71.6 µg, respectively (Fig. 2a). Meanwhile, the percentage (%) of MET drug released after 14 days was 63% (3 : 1 MET/CIP), 69% (pure MET) and 93% for the 1 : 1 MET/CIP scaffolds (Fig. 2b). 1 : 1 MET/CIP scaffolds released all MET content by day 5 (Fig. 2b). The mean CIP drug released after 14 days for pure CIP, 3 : 1 MET/CIP, 1 : 1 MET/CIP and 1 : 3 MET/CIP were 966 ± 166.6 µg, 418 ± 96.8 µg, 674 ± 105 µg and 900 ± 96.8 µg, respectively (Fig. 2c). After 14 days, the percentage (%) of CIP drug released ranged from 32% for pure CIP to 63% for 3 : 1 MET/CIP scaffolds (Fig. 2d). No statistical differences in mean CIP release between pure CIP and 1 : 3 MET/CIP scaffolds (Fig. 2b) were observed. The percentage of CIP drug release was significantly lower ($P < 0.05$) in pure CIP when compared
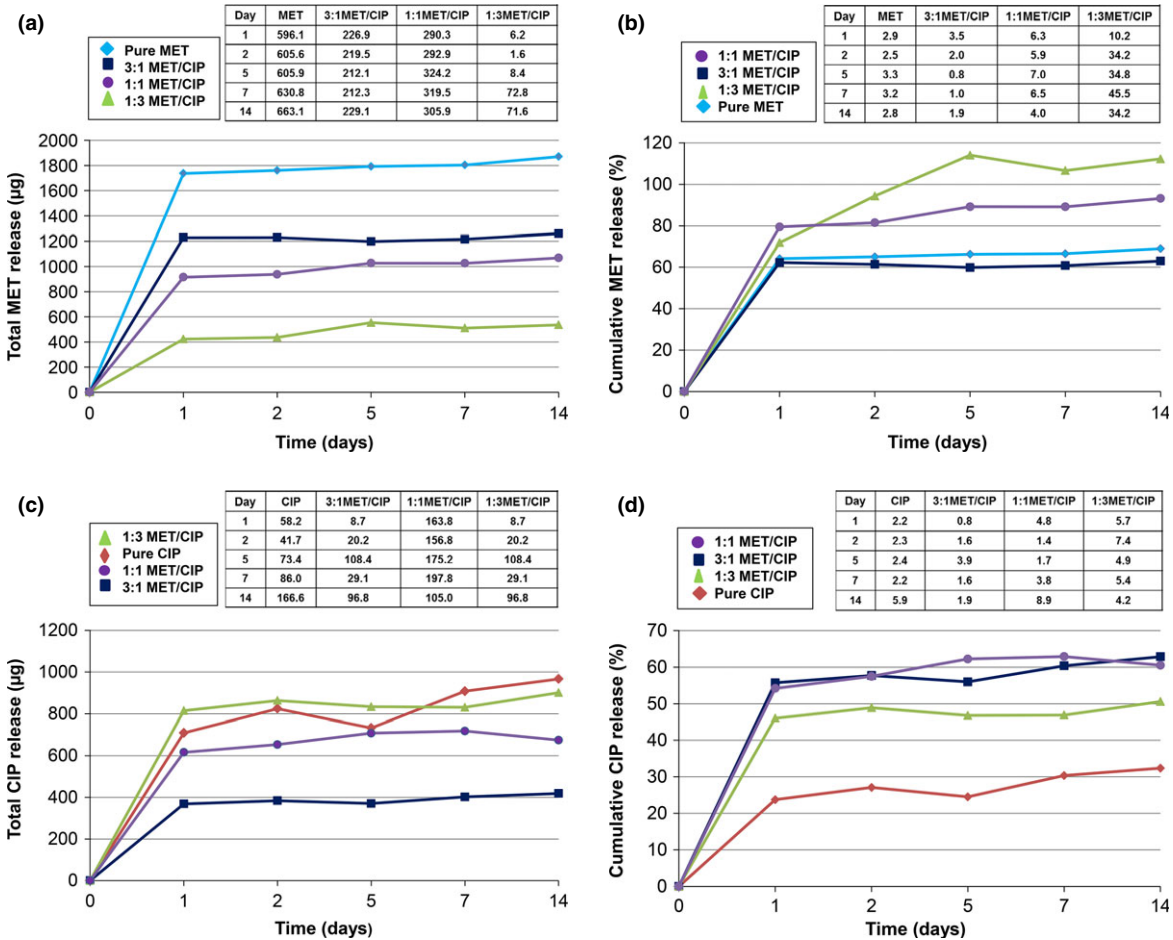

Figure 2 (a–d) Graphs represent both MET and CIP drug release for all antibiotic-containing scaffolds. (a) Mean MET drug release (in µg, ±SD, refer to inset Table). (b) Percentage of MET drug release (±SD, refer to inset Table) from the scaffolds based on initial weight. (c) Mean CIP drug release (in µg, ±SD, refer to inset Table). (d) Percentage of CIP drug release (±SD, refer to inset Table) from the scaffolds based on initial weight.
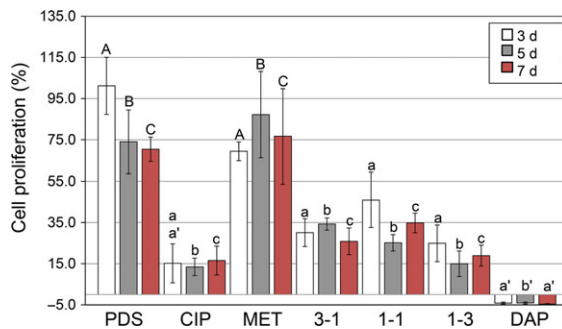
Figure 3 Effects of pure PDS, antibiotic-containing scaffolds and DAP on hDPSCs proliferation. Proliferation was assessed via WST-1 assay after 3, 5 and 7 days of hDPSC exposure to the scaffolds and DAP solution. Data were plotted with mean (±SD). Statistical analyses were compared within the results from the same day. Correlation of upper case letter versus lower case letter versus lower case letter with apostrophe denotes statistical significance at the level of $P < 0.05$.

to all other groups at all time-points investigated (Fig. 2d).

Human DPSCs proliferation and viability

The WST-1 proliferation assay was used to determine the indirect effects (i.e. drug(s) release) of antibiotic-containing scaffolds on hDPSCs proliferation. In brief, the colorimetric-based WST-1 assay makes use of a tetrazolium salt that generates a very water-soluble formazan upon cellular reduction (Tominaga *et al.* 1999). The results of the assay revealed a significant ($P < 0.05$) decrease in proliferation for all groups exposed to CIP-containing scaffolds (Fig. 3) at all time-points, in a concentration-dependent manner. In contrast, groups exposed to MET and pure PDS showed no change in proliferation. In this study, LIVE/DEAD® assay was performed specifically to qualitatively evaluate cell morphology and viability after 3 days of scaffold exposure. Analysis of hDPSC viability by fluorescence microscopy revealed important differences amongst the groups. Cells exposed to either pure PDS or MET displayed a spindle-shaped fibroblastic cell body with mainly long cellular processes, with strong substrate attachment and associated multiple proliferating cells (Fig. 4a–b). A visual decrease in cell number with a higher % of dead cells, indicated by the colour red, was observed after exposure to CIP-containing scaffolds. In addition, these cells showed a rounded form without cytoplasmic branched projections (Fig. 4c–f). No cells were seen after 3 days of exposure to the DAP-mimic solution (data not shown).

Discussion

Selection of the best method for eradication of root canal infection prior to regenerative endodontic procedures should be dictated by two major outcomes, that is the maximization of the antibacterial efficiency whilst preserving the proliferation/differentiation potential of pulp stem cells (Trevino *et al.* 2011, Martin *et al.* 2014). Over the past few years, a myriad of studies has been performed (Bottino *et al.* 2013a, 2014, Palasuk *et al.* 2014, Waeiss *et al.* 2014) focusing on the synthesis of antibiotic-containing scaffolds based on the central hypothesis that controlling the release rate of significantly lower (compared to the double and triple antibiotic pastes), yet effective, antibiotic doses might lead to enhanced human dental pulp stem cell viability (hDPSC), whilst preserving antimicrobial activity.

The drug-release data indicated that after an initial burst release (i.e. within the first 24 h), a continued maintenance of the drug(s) concentration for all time-points was observed, suggesting a sustained level of drugs that might be effective in the eradication of existing infection. In fact, a recent study (Palasuk *et al.* 2014) revealed significant antimicrobial activity of these novel antibiotic-containing electrospun scaffolds over a 14-day period for different bacteria, comparable to activity provided by 0.12% chlorhexidine. Notably, the percentage of CIP was higher for 1 : 3 MET/CIP scaffolds when compared to those containing CIP only. This observation could be explained by pharmacokinetic interactions of both drugs. Solubility of MET is inversely proportional to pH, with the lowest solubility at about pH 8.0 (Rediguieri *et al.* 2011), whereas the solubility of CIP, a zwitterionic molecule, is optimal at neutral pH (Breda *et al.* 2009). Thus, MET presence in 1 : 3 MET/CIP could facilitate the release of CIP from the scaffolds due to physicochemical interactions. The important differences (i.e. a sizeable standard deviation) in mean fibre diameter amongst the antibiotic-containing groups might also play an important role in drug-release kinetics. Full optimization of the various electrospinning conditions, such as voltage and distance tip-collector, amongst others, will be explored in a future investigation to reduce the fibre diameter variability normally associated with electrospinning.

The long-term goal of this research is to translate into clinical practice a two-step tissue engineering-based strategy for regenerative endodontics by first
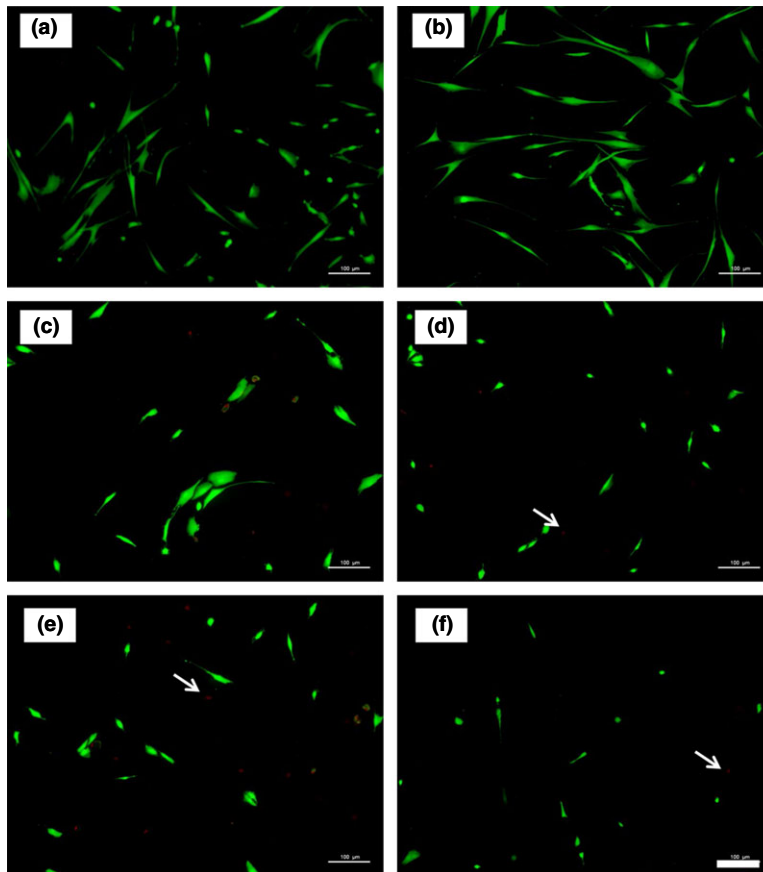
Figure 4 (a–f) Representative fluorescence microscopy images (25 × magnification, scale bar = 100 μm) show hDPSCs viability assessed with LIVE/DEAD® assay after exposure to scaffolds for 3 days: (a) Pure PDS; (b) MET; (c) 3 : 1MET/CIP; (d) 1 : 1MET/CIP; (e) 1 : 3MET/CIP and (f) CIP. Cellular bodies stained green show viable cells. Arrows indicate red stained ethidium bromide-positive nuclei (dead cells).

establishing a bacteria-free environment conducive to tissue regeneration (Step 1) through the use of antibiotic-containing scaffolds, followed by placement of a scaffold encapsulated with growth factors and/or stem cells (Step 2) to drive regeneration of the pulp–dentine complex (Albuquerque *et al.* 2014). Data from previous studies demonstrated convincing evidence of the antibacterial efficacy of PDS-based antibiotic-containing scaffolds (e.g. vancomycin, rifampicin, metronidazole, ciprofloxacin) against osteomyelitis-, periodontitis-, and root canal infection-associated pathogens (Bottino *et al.* 2013a, 2014, Palasuk *et al.* 2014, Waeiss *et al.* 2014). Here, novel antibiotic-containing scaffolds were evaluated in terms of their potential toxic effects to hDPSC using both proliferation and viability assays. Based on the data presented, the hypothesis was accepted, as the antibiotic-containing

scaffolds promoted significantly lower cytotoxic effects when compared to the clinically advocated double antibiotic paste (DAP).

Calcium hydroxide [$Ca(OH)_2$] is considered to be an excellent antimicrobial medication in endodontics, but its effectiveness can be limited to the main root canal with inadequate dentine penetration (Haapasalo & Ørstavik 1987, Ercan *et al.* 2006), mostly due to low solubility and buffering dentine characteristics (Haapasalo *et al.* 2000, Portenier *et al.* 2001). Recently, antibiotic-containing pastes (i.e. TAP and DAP), at clinically advocated concentrations, have been shown to significantly affect the proliferation and survival of dental pulp cells (Chuensombat *et al.* 2013) as well as stem cells from the apical papilla (Ruparel *et al.* 2012). The present study confirms the highly toxic effects of DAP through the proliferation and viability

assays after hDPSC exposure to the DAP-mimic, MET/CIP saturated solution.

From a clinical perspective, antimicrobials may act unfavourably on dental stem cells via two main mechanisms, that is diffusion to apical papilla during time of introduction into the root canal system, and/or by interference of residual drugs with migrating stem cells into the preformed blood clot. Of the two drug components in DAP, no change in either proliferation or viability of MET-only containing scaffolds was noticed. Even though there are studies indicating MET as being responsible for dose-dependent cytotoxic effects both *in vitro* (Kapoor *et al.* 1999) and *in vivo* (Chatzkel & Vossough 2010, Kuriyama *et al.* 2011), it has demonstrated favouring eukaryotic cell viability. MET has been shown to increase lymphocyte proliferation (Elizondo *et al.* 1994); however, the mechanism is not well understood. One plausible explanation is that MET can modulate proliferation through the release of inflammatory cytokines as observed in human periodontal ligament cells (Rizzo *et al.* 2010). Thus, MET, despite its proven antimicrobial characteristics, could also be considered to be a potential stem cell proliferation inducer when delivered locally using scaffolds to eliminate root canal infection. Regarding CIP, an inhibitory effect of all CIP-containing polymer scaffolds on both proliferation and viability of hDPSCs was observed. Dose-dependent cytotoxic effects of antibiotics from the fluoroquinolones group, including CIP, were described elsewhere (Sobolewska *et al.* 2013). Lastly, an important decrease in hDPSC viability for all samples exposed to CIP with a dose-dependent trend, where the least toxicity was seen in cells exposed to scaffolds with the lowest CIP content (3 : 1 MET/CIP). Future studies will focus on the determination of an optimal concentration of CIP with minimal or no toxic effects to stem cells, whilst still maintaining antimicrobial efficacy. In a clinical setting, the lowest possible CIP concentration within scaffolds with modulatory effects of MET might lead to improvement in the survival, proliferation and regenerative capacity of dental pulp stem cells.

Conclusion

Synthesized antibiotic-containing scaffolds had significantly lower effects on hDPSCs' proliferation and viability when compared to the saturated CIP/MET solution (DAP).

Conflict of interests

The authors declare no conflict of interests.

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