Effects of Ciprofloxacin-Containing Antimicrobial Scaffolds on Dental Pulp Stem Cell Viability — In Vitro Studies

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Abstract

Objective—A combination of antibiotics, including but not limited to metronidazole (MET) and ciprofloxacin (CIP), has been indicated to eradicate bacteria in necrotic immature permanent teeth prior to regenerative procedures. It has been shown clinically that antibiotic pastes may lead to substantial stem cell death. The aim of this study was to synthesize scaffolds containing various concentrations of CIP to enhance cell viability while preserving antimicrobial properties.

Design—Polydioxanone (PDS)-based electrospun scaffolds were processed with decreasing CIP concentrations (25 – 1 wt.%) and morphologically evaluated using scanning electron microscopy (SEM). Cytotoxicity assays were performed to determine whether the amount of CIP released from the scaffolds would lead to human dental pulp stem cell (hDPSC) toxicity. Similarly, WST-1 assays were performed to evaluate the impact of CIP release on hDPSC proliferation. Pure PDS scaffolds and saturated double antibiotic solution MET/CIP (DAP) served as both positive and negative controls, respectively. Antibacterial efficacy against E. faecalis (Ef) was tested.

Results—A significant decrease in hDPSC viability at concentrations 5–25 wt.% was observed. However, concentrations below 5 wt.% did not impair cell viability. Data from the WST-1 assays indicated no detrimental impact on cell proliferation for scaffolds containing 2.5 wt.% CIP or less. Significant antimicrobial properties were seen for CIP-scaffolds at lower concentrations (i.e., 1 and 2.5 wt.%).

Conclusion—The obtained data demonstrated that a reduced concentration of CIP incorporated into PDS-based scaffolds maintains its antimicrobial properties while enhancing viability and proliferation of dental pulp stem cells.

Keywords

antibiotics; scaffolds; cytocompatibility; stem cells; endodontics; regeneration

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1. Introduction

The dental pulp plays a vital role in tooth development as it harbors odontoblasts, highly specialized cells that synthesize dentin.\textsuperscript{1} Traumatic injuries or bacterial infection of the dental pulp lead to inflammation and, if not interrupted, to necrosis.\textsuperscript{2} In children, pulpal necrosis promotes the death of odontoblasts, resulting in the disruption of root development.\textsuperscript{3}

Traditionally, traumatic tooth injuries with incomplete apical development have undergone apexification therapy through placement of intracanal calcium hydroxide, or most recently, mineral trioxide aggregate.\textsuperscript{3,4} Nevertheless, while apexification therapy promotes merely apical closure, it does not allow for root development.\textsuperscript{4} Meanwhile, clinical evidence (e.g., root-end development with dentinal walls thickening) has been mounting following treatment of necrotic immature teeth with the revascularization technique.\textsuperscript{5–8}

In essence, in the currently advocated regenerative endodontics procedure, a combination of antibiotics, including but not limited to metronidazole (MET) and ciprofloxacin (CIP), has been indicated to obtain maximum bacteria eradication.\textsuperscript{3,5,9–11} Microbial elimination must be achieved with minimal root canal instrumentation to preserve undifferentiated cells.\textsuperscript{3,11} Following root canal decontamination, a tissue engineering-based approach, occurs through the process of provoking intentional bleeding in the periapical region, where a fibrin-based matrix is formed that ends up serving as a scaffold to support the attachment, proliferation and differentiation of stem cells from the apical papillae, thanks to the contribution of endogenously-produced growth factors.\textsuperscript{2,4} Most importantly, according to the literature, the demonstration of pulp-like tissue formation after revascularization has been observed only on a recent study.\textsuperscript{12} Meanwhile, the vast majority of studies, including both data from animal models and clinical cases, generally reports that the newly formed invaginated tissue to be composed of periapical tissue containing bone-like hard tissue and the root canal walls thickening is due to the formation of a cementum-like tissue.\textsuperscript{13,14}

Despite encouraging evidence, intrinsic patient-to-patient variability in cell populations and the quantity and variety of growth factors,\textsuperscript{3} combined with the extreme stem cell toxicity linked with the use of irrigants and antibiotics,\textsuperscript{15,16} have suggested the opportunity of using antibiotic-containing fibrous scaffolds capable of functioning as a drug delivery system to eliminate root canal infection while preserving stem cell viability.\textsuperscript{17,18} Based on the aforementioned considerations, the aim of this study was to synthesize, via electrospinning, fibrous-based scaffolds containing distinct CIP concentrations to enhance dental pulp stem cell viability while preserving antimicrobial properties.

2. Materials and Methods

2.1. Synthesis of ciprofloxacin-containing fibrous scaffolds

Ciprofloxacin-containing (CIP, Sigma-Aldrich, St. Louis, MO, USA) polydioxanone-based (PDSII\textsuperscript{®}, Ethicon Inc, Somerville, NJ, USA) polymer solutions were prepared to synthesize scaffolds with selected CIP concentrations, following standard procedures as recently reported.\textsuperscript{17–20} First, violet-colored PDS suture filaments were cut into pieces of similar
length (i.e., 3 cm) and de-stained by immersion in dichloromethane (Sigma-Aldrich) for 48 h. Second, de-stained PDS was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Sigma-Aldrich) at a concentration of 10 wt.% overnight by using vigorous stirring. Lastly, CIP powder was added to the prepared PDS solutions at increasing concentrations (1, 2.5, 5, 10, 15, 20, and 25 wt.%, in respect to the polymer weight – i.e., 6, 15, 30, 60, 90, 120, and 150 mg, respectively), and then mixed overnight. The distinct CIP-containing PDS solutions and pure PDS (control) were spun into fibers (hereafter referred to as scaffolds) at room temperature (RT) onto an aluminum foil covered rotating (120 rpm) stainless steel mandrel by loading the solutions into plastic syringes (Becton-Dickinson & Company, Franklin Lakes, NJ, USA) fitted with a 27G metallic blunt-tip needle using previously optimized parameters (i.e., flow rate 2 mL/h, distance tip-collector 18 cm, and 15–18 kV). The scaffolds were vacuum dried at RT for at least 48 h prior to testing.

Scanning electron microscopy (SEM, JSM-5310LV, JEOL, Tokyo, Japan) was used to evaluate fiber morphology of the synthesized scaffolds. The fiber diameter was evaluated using Image-J software (NIH, Bethesda, MD, USA).

2.2. Cell culture for viability and proliferation assays

Low glucose Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% FBS (HyClone Laboratories Inc, Logan, UT), and 1% penicillin–streptomycin (Sigma-Aldrich) placed in a humidified incubator at 37°C, with 5% CO$_2$, was used to culture human dental pulp stem cells (hDPSC, 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.

2.3. Cytotoxicity assays

To evaluate the potentially toxic effects of CIP-containing scaffolds on human dental pulp stem cells, cytotoxicity assays according to the guidelines provided by the International Standards Organization (ISO, 1993) were performed. All scaffolds (15×15 mm$^2$) were first sterilized by UV radiation (30 min each side), followed by 30 min immersion in 70% ethanol. After rinsing with sterile PBS (5 min, 2×), extracts from the distinct scaffolds were prepared. The assay evaluates the toxic effects of any leachable compound(s) (e.g., released ciprofloxacin) when in contact with the cells. Sterile scaffolds (surface ratio = 1 cm$^2$/mL of medium) were incubated in DMEM at 37°C for 2 days under a 5% CO$_2$ humidified atmosphere. Extracts of sterile ultra-high molecular weight polyethylene (UHMWPE) were prepared as a negative control. Supernatants were filtered through a Millipore$^\circledR$ membrane, and serial dilutions were obtained from the extracts. The dilutions were also prepared for a 0.3 vol % phenol solution to serve as the positive control. Cytotoxicity tests at each dilution were carried out in quadruplicate. Briefly, hDPSCs at a density of 3×10$^3$/well were seeded and allowed to adhere in the wells of 96-well tissue culture plates. After 4 h, the media was replaced by distinct extract concentrations. 100 µL of each extract was pipetted into each well. Control columns of four wells were prepared with medium without cells (blank), and medium with cells but without the extract (100% survival). The plates were incubated under a 5% CO$_2$ humidified atmosphere. Next, 20 µL of CellTiter 96 AQueous One Solution Reagent (Promega Corporation, Madison, WI, USA) was added after 3 days. The reagent was allowed to react for 2 h at the same conditions.
Incorporated dye was measured by reading the absorbance at 490 nm in a microplate reader against a blank column.\textsuperscript{17,18}

2.4. Proliferation assays (WST-1)

hDPSCs were harvested by trypsinization, counted, and seeded on 13-mm treated cell culture coverslips (Thermanox, Thermo Fisher Scientific Inc, Rochester, NY, USA) in 24-well plates at a density of $10^4$/well (1500 μL of culture medium). Meanwhile, scaffolds (15×15 mm$^2$, n=4–6/group) were mounted in plastic CellCrown\textsuperscript{TM} (Scaffdex, Tampere, Finland), disinfected in 70% ethanol for 30 min, and rinsed twice in sterile PBS (Sigma). After a 4 h incubation period to allow cell attachment, the CellCrowns with scaffolds were introduced into the wells. A 2 mm distance between the cells and scaffold was achieved using plastic rings for standardization purposes. Control (blank) columns were prepared in medium without cells and in medium with cells but without scaffold (100% survival). A saturated solution (50 mg/mL) of CIP in DMEM was prepared by stirring for 4 h, at RT, followed by centrifugation (3000 rpm) for 15 min. Clear supernatant was filter sterilized using a 0.22 μm syringe filter.

WST-1 (Roche Diagnostics, Mannheim, Germany) assay was used to evaluate the effects of CIP-containing scaffolds on hDPSC proliferation up to 7 days of cell/scaffold exposure.\textsuperscript{20} After 3, 5, and 7 days, the CellCrowns with scaffolds were removed. A 500-μL quantity of DMEM was left in each well. Next, WST-1 reagent was added at the ratio of 10:1 (i.e., 50 μL) into each well. After 2 h of incubation in 5%CO$_2$ in a humidified atmosphere at 37°C, 100 μL of the total volume present in each well was transferred into 96-well plates.\textsuperscript{20} Full medium with cells without scaffold exposure and full medium without cells were assigned as positive and negative controls, respectively. Optical density (OD) of the incorporated dye was determined by reading the absorbance at 450 nm in a microplate reader against a blank column.\textsuperscript{20} Proliferation was calculated as a ratio of OD from experimental value to positive control; both were subtracted by the negative control.\textsuperscript{20}

2.5. Antimicrobial testing

Agar diffusion assays were used to determine the antimicrobial efficacy of CIP-containing scaffolds against \textit{Enterococcus faecalis} (Ef) (ATCC 29212).\textsuperscript{17,18} According to our recent findings, similarly prepared CIP-containing scaffolds at concentrations of 5 and 25 wt.% have demonstrated significant antimicrobial activity against \textit{Ef}.\textsuperscript{17} Thus, here we proposed to investigate whether or not scaffolds containing lower CIP concentrations (e.g., 1 and 2.5 wt %) would still preserve the antimicrobial properties. \textit{Ef} was aerobically cultured for 24 h in 5%CO$_2$ at 37°C in brain heart infusion broth (BHI).\textsuperscript{17,18} Next, UV sterilized disk-shaped (6 mm in diameter) samples (n=4) from 3 experimental groups (i.e., 1, 2.5, and 5 wt.%CIP) were placed on blood agar plates containing \textit{Ef} bacterial lawns.\textsuperscript{17,18} Chlorhexidine (0.12%) and 0.9% sterile saline solution were used as positive and negative controls, respectively. After 3 days of incubation, the inhibition zones (in mm) were recorded.\textsuperscript{17,18}

2.6. Statistical analysis

One-way analyses of variance (ANOVAs) followed by Tukey’s multiple comparisons were used to evaluate differences in fiber diameter. Meanwhile, cell viability (cytotoxicity) and
proliferation data were analyzed using two-way ANOVA. The comparisons for inhibition zones were performed using mixed-model ANOVA. The significance level was set at p<0.05.

3. Results

Representative SEM micrographs of the synthesized micro/nano fibrous scaffolds are shown in Figure 1. Mean fiber diameter ranged from 1139±348 nm (PDS, control) to 748±231 nm (25 wt.% CIP). Generally, CIP concentrations above 5 wt.% led to the formation of fibers with significantly (p<.001) smaller diameters than PDS.

Based on the intended clinical use of the current antibiotic-containing scaffolds as a disinfection strategy prior to regenerative endodontics procedures, the cytotoxic effects of all scaffolds were assessed to determine whether the amount of CIP released would indeed lead to cell toxicity. Cytotoxicity assays revealed a significant decrease in cell viability at concentrations 5–25 wt.% (Fig. 2). Then, we processed CIP-containing scaffolds at lower concentrations (i.e., 1 and 2.5 wt.%) to determine the optimal, non-toxic CIP concentration that can be incorporated without impairing cell viability. The assay was run again using similar controls but with CIP-containing scaffolds with concentrations from 1–5 wt.%. Fig. 2B shows the cell viability data, where concentrations lower than 5 wt.% can be seen not to impair hDPSCs viability. Similarly, we evaluated the indirect effects of CIP release from the scaffolds on the proliferation ability of hDPSC by incubating the cells in the presence of scaffolds up to 7 days. Data revealed that scaffolds containing 5 to 25 wt. % of CIP inhibited cell proliferation at all-time points (Fig. 3). No inhibitory effects on hDPSC proliferation were seen after exposure to 1 and 2.5 wt.% CIP-containing scaffolds (Fig 4). The cytocompatibility data presented in this study agree with our previous work. However, since decreasing CIP concentrations to 1 and 2.5 wt% revealed no significant impact on cell viability/proliferation, we proposed to examine whether these scaffolds would indeed present antimicrobial properties. Agar diffusion data demonstrated significant growth inhibition of *E. faecalis* associated with the use of CIP-containing scaffolds at concentrations lower than 5 wt.% (Fig. 5).

4. Discussion

Eradication of infection from necrotic teeth is a vital step prior to regenerative endodontics procedures. It is now well-established that antibiotic mixtures, such as the so-called double (MET and CIP) and triple (MET, CIP, and minocycline) antibiotic pastes are excellent in providing a bacteria-free niche; however, they decrease viability of dental pulp stem cells involved in tissue regeneration. Lately, our group has proposed a drug delivery strategy based on the use of antibiotic-containing electrospun scaffolds, with significant clinical potential in endodontics for the disinfection step involved in the current clinically advocated regenerative strategy. Taken together, our recent data has proven that a variety of antibiotic drugs (i.e., MET, CIP and MET/CIP bi-mix formulations) can be successfully spun into fibrous scaffolds, with significant antimicrobial efficacy against sensitive bacteria. Nevertheless, despite markedly lower antibiotic concentrations being released from these scaffolds when compared with actual antibiotic concentrations used
Clinical studies have also reported a considerably negative impact on hDPSCs survival after exposure to CIP-containing scaffolds, not to MET-containing scaffolds.\(^{17}\)

Quinolones, including CIP, are broad spectrum antibiotics, commonly used against a wide range of both gram-positive and gram-negative bacteria.\(^{22}\) The mechanism of action\(^{23}\) involves the inhibition of bacterial DNA topoisomerase II (gyrase) and topoisomerase IV, which are enzymes involved in untwining bacterial DNA during the replication process. Regrettably, CIP has also been shown to decrease the viability of eukaryotic cells, leading to multiple adverse effects\(^{24,25}\), such as tendinopathies\(^{26}\) and peripheral neuropathy\(^{27,28}\), due to a decline in the synthesis of proteins encoded in mitochondrial deoxyribonucleic acid (mtDNA). Analysis of mtDNA from CIP-treated cells revealed the presence of site-specific, double-stranded DNA breaks\(^{29}\), which were attributed to inhibition of mitochondrial topoisomerase II activity, functionally and structurally similar to that found in bacteria.\(^{30}\)

Taken together, mammalian cells are therefore susceptible to CIP toxic effects, particularly cells with high proliferation rates, \(i.e.,\) human hematopoietic stem cells\(^{31,32}\), embryonic stem cells (hESCs)\(^{33}\), and dental-related cells.\(^{15,16}\) Worth mentioning, one should note that the cytotoxic effects associated with CIP also depend on the dosage and time of exposure. In a recent \textit{in vitro} study, embryonic stem cells were able to regain baseline metabolic activity after CIP withdrawal\(^{33}\); thus, decreasing the drug concentration or/and time of exposure may significantly improve stem cells viability. Based on the aforementioned, here we investigated whether synthesis, via electrospinning, of the CIP-containing scaffolds with reduced CIP concentrations would enhance dental pulp stem cell viability, while preserving antimicrobial properties. In our previous study\(^{17}\), we demonstrated that CIP at 5 wt.% had comparable antimicrobial efficacy to 25 wt.%\(^{.}\) The data obtained herein revealed that scaffolds containing lower CIP concentrations (\(e.g.,\) 1 and 2.5 wt.%) not only sustained the antimicrobial properties but more importantly they significantly reduced the negative impact on hDPSC viability/proliferation.

5. Conclusions

In conclusion, data from this study demonstrated that reducing the amount of CIP incorporated into PDS-based scaffolds enhances hDPSCs viability and proliferation while preserving its antimicrobial properties.

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References


Fig. 1.
Representative SEM micrographs of the scaffolds (5000×): (A) pure PDS, (B) 1 wt.% CIP, (C) 2.5 wt.% CIP, (D) 5 wt.% CIP, (E) 10 wt.% CIP, (F) 15 wt.% CIP, (G) 20 wt.% CIP, and (H) 25 wt.% CIP.
Fig. 2.
Cytotoxic effects of extracts prepared from scaffolds with selected CIP concentrations. hDPSCs were exposed to increasing extracts concentration, namely 6.25%, 12.5%, 25%, 50%, and 100%. Cell viability (%) was determined after 72 hours. *Significant differences from the control were set at p < 0.05.
Fig. 3.
Proliferation (%) of hDPSCs after exposure to CIP-containing scaffolds (5 – 25 wt.%).
Proliferation was determined at days 3, 5 and 7. Data were analyzed using one-way ANOVA and Tukey’s multiple comparison tests. *Significant differences between experimental groups and the control, at the same time point, were set at $p < 0.05$. 

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Fig. 4.
Proliferation (%) of hDPSCs after exposure to CIP-containing scaffolds (1 – 5 wt.%). Proliferation was determined at days 3, 5 and 7. Significant differences from the control (PDS) and other groups were set at $p < 0.05$ and represented using lower case letters.
Fig. 5.
Effects of CIP-containing scaffolds on the growth inhibition of *Ef*. (A-B) Representative macrophotographs of agar diffusion assays on *Ef*, with 1 wt.%, 2.5 wt.%, and 5 wt.% CIP-containing scaffolds. Sterile saline (Sal) and 0.12% chlorhexidine (CHX) solutions were used as negative and positive controls, respectively. (C) Data from the agar diffusion assays at day 3 (n=4). Significant difference between groups is denoted with an asterisk (p < 0.05).