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# Chitosan-based endodontic irrigation solutions and TGF-β1 treatment: Creating the most favourable environment for the survival and proliferation of stem cells of the apical papilla *in vitro*

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## Abstract

**Background:** The dental pulp's environment is essential for the regulation of mesenchymal stem cells' homeostasis and thus, it is of great importance to evaluate the materials used in regenerative procedures.

**Aim:** To assess in vitro (i) the effect of chitosan nanoparticles, 0.2% chitosan irrigation solution, Dual Rinse®, 17% EDTA, 10% citric acid and 2.5% NaOCl on DSCS viability; (ii) the effect of different concentrations of TGF-β1 on DCSC proliferation; and (iii) whether treatment with TGF-β1 following exposure to the different irrigation solutions could compensate for their negative effects.

**Methodology:** (i) DSCS were treated with three dilutions (1:10, 1:100 and 1:1000) of the six irrigation solutions prepared in DMEM for 10 and 60 min to assess the effect on viability. (ii) The effect of different concentrations (0, 1, 5 and 10 ng/mL) of TGF-β1 on DCSC proliferation was assessed at 1, 3 and 7 days. (iii) The proliferative effect of TGF-β1 following 10-min exposure to 1:10 dilution of each irrigation solution was also tested. We used MTT assay to assess viability and proliferation. We performed statistical analysis using Prism software.

**Results:** (i) The different endodontic irrigation solutions tested showed a significant effect on cell viability ($p \leq .0001$). Significant interactions between the endodontic irrigation solutions and their dilutions were also found for all parameters ($p \leq .0001$). Chitosan nanoparticles and 0.2% chitosan irrigation solution were the least cytotoxic to DSCS whilst 2.5% NaOCl was the most cytotoxic followed by 17% EDTA. (ii) TGF-β1 at concentrations of 1 and 5 ng/mL resulted in significantly higher proliferation compared to the control group. (iii) Exposure to 17% EDTA or 2.5% NaOCl for 10 min was sufficient to make DSCS cells refractory to the proliferative effects of TGF-β1. DSCS groups treated with TGF-β1 following exposure to chitosan nanoparticles, 0.2% chitosan irrigation solution, Dual Rinse® and 10% CA demonstrated

significantly higher proliferation compared to non-TGF-β1-treated groups ($p \leq .0001$, $p \leq .0001$, $p \leq .0001$ and $p = .01$ respectively).

**Conclusions:** The current study offers data that can be implemented to improve the outcome of regenerative endodontic procedures by using less toxic irrigation solutions and adding TGF-β1 to the treatment protocol.

**KEYWORDS**

chitosan, citric acid, EDTA, nanoparticles, regenerative endodontic procedures, stem cells

## INTRODUCTION

Regenerative endodontic procedures (REPs) use the concept of tissue engineering to allow for continued increase in root length and dentine wall thickness in immature teeth (Hargreaves et al., 2013; Palma et al., 2019). To achieve this goal, a favourable environment for tissue regeneration must be created by disinfecting the root canal space and allowing for an interplay among (i) mesenchymal stem cells, (ii) a scaffold and (iii) growth factors (dos Reis-Prado, Oliveira, et al., 2022; Quijano-Guauque et al., 2023).

According to the American Association of Endodontists (AAE) and the European Society of Endodontology (ESE), regenerative procedures include careful disinfection of the root canal with sodium hypochlorite (NaOCl) whilst avoiding mechanical instrumentation and intracanal dressing with local antibiotics or calcium hydroxide pastes. This is followed by the removal of the dressing and rinsing with ethylenediaminetetraacetic acid (EDTA), stimulation of bleeding and coverage of the blood clot with collagen and finally, closure with calcium silicate cement followed by an adhesive restoration ('AAE Position Statement: Scope of endodontics: Regenerative endodontics', 2013; Galler et al., 2016).

Irrigating solutions in endodontics can be classified as antimicrobial, chelating or combinations of both (Basrani & Malkhassian, 2015). NaOCl is the most used irrigation solution in root canal treatment (Basrani & Malkhassian, 2015). This oxidizing solution has a high antimicrobial activity and causes the dissolution of organic matter as a function of its free available chlorine (OCl- and HOCl; Zehnder et al., 2005). It has been shown to be very effective in removing the organic part of the smear layer (Torabinejad et al., 2002). NaOCl, however, exerts no effect on the inorganic components of the smear layer; thus the need for another irrigation solution to be used in combination with NaOCl is essential (De-Deus et al., 2008; Giardino et al., 2019).

EDTA, an aminopolycarboxylic acid, is a chelator that is commonly used at a concentration of 17% and can remove the smear layer when in direct contact with the root canal wall for less than 1 min (Basrani & Malkhassian, 2015). Its prominence as a chelating agent arises from its ability to sequester di- and tricationic metal ions such as $Ca^{2+}$ and $Fe^{3+}$ (Basrani & Malkhassian, 2015). Citric acid, another chelator, is a weak organic acid that has been reported to remove the smear layer as efficiently as 17% EDTA at a concentration of only 10% (Dewi, 2020; Machado et al., 2017; Zehnder et al., 2005). When used in conjunction with NaOCl, however, both EDTA and citric acid strongly reduce the available chlorine, possibly rendering it ineffective (Zehnder et al., 2005).

Dual Rinse® HEDP (Medcem GmbH, Switzerland), an etidronate powder that forms etidronic acid (HEDP) in aqueous solution, is a chelating agent that has been recently approved for clinical use (Giardino et al., 2019). Arias-Moliz et al. (2015) reported that the free available chlorine remains stable over time when mixed with HEDP. Therefore, HEDP can be used in combination with NaOCl to remove the smear layer without any loss of its properties (Zehnder et al., 2005).

Chitosan is a deacetylated derivative of chitin, the main component of the exoskeleton of crustaceans (Sashiwa & Aiba, 2004). It is the second most abundant natural biopolymer and it can be modified chemically (Raura et al., 2020). Chitosan was shown to effectively control the growth and reproduction of hazardous bacteria (del Carpio-Perochena et al., 2015; Kishen et al., 2008; Pascale et al., 2023). The most proposed mechanism of action of polycationic chitosan is by binding to the negatively charged residues of bacterial cell walls, altering permeability and causing cell death (del Carpio-Perochena et al., 2015; Kishen et al., 2008; Pascale et al., 2023). Modifications using sodium tripolyphosphate (TPP) have led to the production of chitosan nanoparticles, which are widely used in the biomedical field and drug delivery systems because of their distinctive physicochemical properties such as sensitivity, specificity and bioavailability (Aydın, Özyürek, et al., 2018). Chitosan nanoparticles have also been used in endodontics due to their antibacterial, antifungal, antiviral and chelating properties (Aydın, Özyürek, et al., 2018; Basrani & Malkhassian, 2015; Raura et al., 2020).

It is believed that growth factors have the potential to recruit endogenous mesenchymal stem cells to repair damaged tissue (Huang et al., 2021). In fact, several growth factors have been implanted into the root canal space to enhance cell recruitment, angiogenesis, re-innervation and dentinogenesis, with some success in the formation of pulp-like tissue (Chang et al., 2020). Transforming growth factor-β (TGF-β) is a multifunctional cytokine involved in the regulation of cell proliferation, migration, differentiation, apoptosis and extracellular matrix formation (Li et al., 2021). TGF-β1 is the most abundant and widely distributed of the TGF- β family (Li et al., 2021). It is a major growth factor involved in pulp tissue repair and dentinogenesis. It regulates collagen turnover, growth and differentiation of dental pulp cells (Chang et al., 2020; dos Reis-Prado, Abreu, et al., 2022).

The dental pulp's environment is essential for the regulation of mesenchymal stem cells' homeostasis (Huang et al., 2021). This warrants the evaluation of the various materials used in REPs since these materials would come into contact with apical papilla cells (APCs). APCs are cells isolated from teeth with incomplete rhizogenesis; they contain fibroblasts and stem cells of the apical papilla (SCAPs; Sonoyama et al., 2008). An ideal disinfection protocol for REPs would be one that combines antimicrobial effect and biocompatibility with mesenchymal stem cells (Sismanoglu & Ercal, 2021). To date, there is no standardization in the protocols applied in regenerative procedures (Sismanoglu & Ercal, 2021; Trevino et al., 2011).

The aim of the present study is to assess in vitro (i) the effect of chitosan nanoparticles, 0.2% chitosan irrigation solution, Dual Rinse®, 17% EDTA, 10% citric acid and 2.5% sodium hypochlorite on Dental Stem Cells SV40 or DSCS viability, (ii) the effect of different concentrations of TGF-β1 on DSCS proliferation and (iii) whether treatment with TGF-β1 following exposure to the different endodontic irrigation solutions could potentially compensate for their negative effect on proliferation.

## MATERIALS AND METHODS

The manuscript of this laboratory study has been written according to Preferred Reporting Items for Laboratory Studies in Endodontology (PRILE) 2021 guidelines (Nagendrababu et al., 2021). The study design is illustrated by a PRILE flowchart in Figure 1.

Ethical approval was attained from the Ethical Committee of the Hospital Odontologic of the University of Barcelona (Ceim HOUB; Approval code 42/2021). A previously characterized immortalized human SCAP cell line (Dental Stem Cells SV40 or DSCS; Sanz-Serrano et al., 2023) was used in all experiments.

All experiments were performed in triplicate and repeated at least twice. Three replicates are regarded as sufficient since cultured cells can be prepared in a uniform suspension in most cases and therefore the provision of large numbers of replicates is often unnecessary (Freshney, 2010).

### Preparation and characterization of chitosan nanoparticles

Chitosan nanoparticles were prepared based on the ionic gelation method following the protocol by Kouchak and Azarpanah (2015) to obtain nanoparticles with optimal properties. First, chitosan powder (≥75% deacetylated) obtained from *Pandalus borealitables* shrimp shells (Sigma-Aldrich, Spain) was added to 1% acetic acid (v/v) at a concentration of 4 mg/mL and the resulting solution was stirred at 400–600 rpm using a magnetic stirrer. Sodium tripolyphosphate (TPP; Sigma-Aldrich, Spain) powder was dissolved in Milli-Q water (Millipore Sigma, Spain) to obtain a concentration of 1 mg/mL, was then added drop by drop to the chitosan solution (CS) and was stirred at 400–600 rpm at room temperature to obtain a volume ratio of 4:1 CS/TPP. The solution was then centrifuged at 12 000 rpm and 25°C for 30 min (5415R Centrifuge; Eppendorf, Germany). The supernatant (containing CS/TPP nanoparticles) was sonicated at 40 kHz for 5 min in an ultrasonic bath (Ultrasons; J.P. SELECTA, Spain).

An aliquot of chitosan nanoparticles was dropped on a carbon grid and left to dry. The nanoparticle size and morphology were evaluated using a JEOL J2010F transmission electron microscope (JEOL Ltd, Japan) equipped with a Schottky field emission gun (FEG) and operated at an accelerating voltage of 200 kV. Images were recorded using an Orius CCD camera (Gatan Inc). Size determination was performed with Digital Micrograph software (Gatan Inc; version 3.7.4) in which the diameter of each particle in the various images taken was measured from four different starting points consisting of 250 total measurements.

### Preparation of 0.2% chitosan irrigation solution, Dual Rinse®, 17% EDTA, 10% citric acid and 2.5% sodium hypochlorite

Chitosan irrigation solution was prepared by adding 0.2 g of chitosan powder to 100 mL of 1% acetic acid and stirring using a magnetic stirrer for 2 h at 600 rpm (Ozlek et al., 2019). Dual Rinse® was prepared following

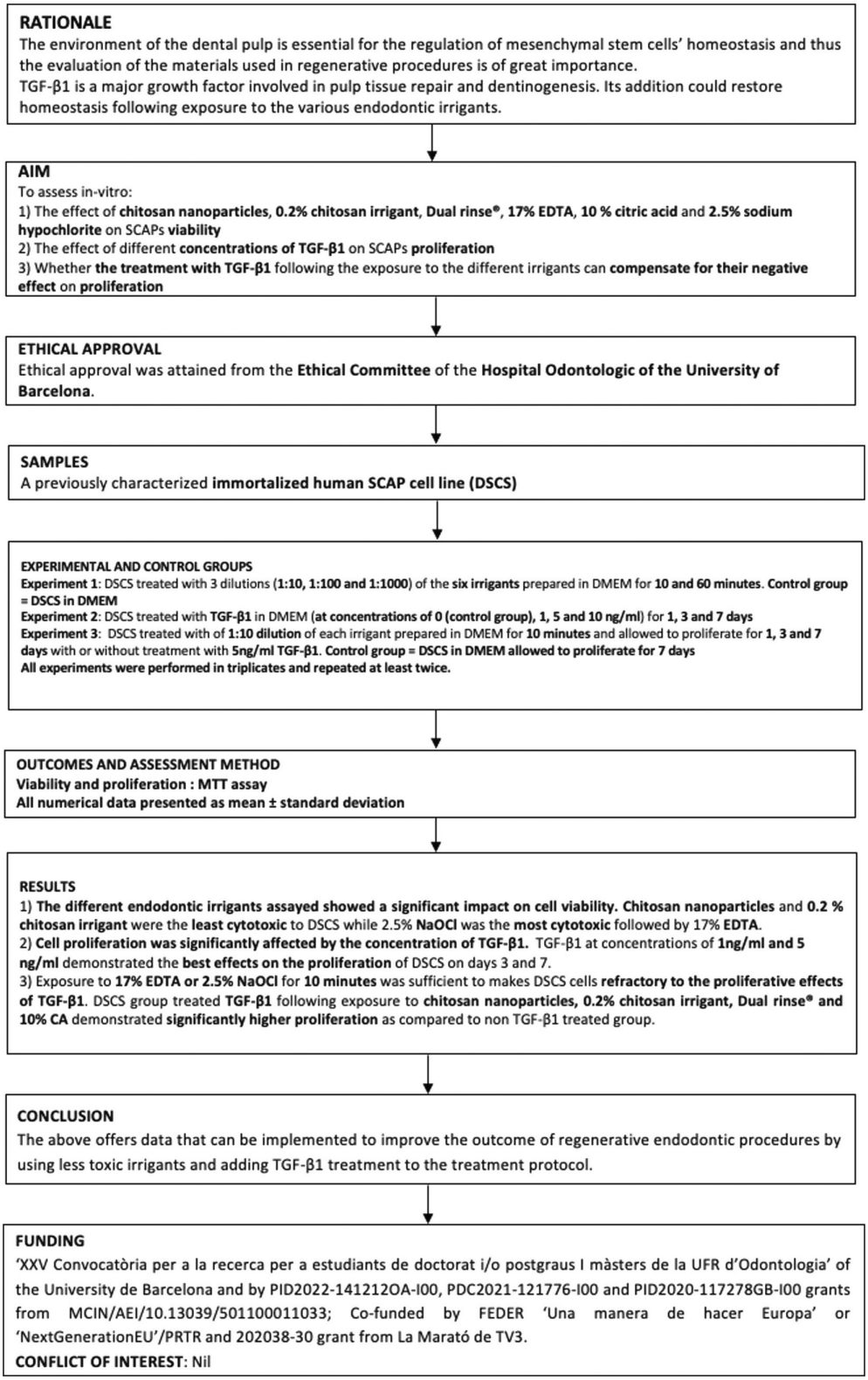


**FIGURE 1** Preferred reporting items for laboratory studies in endodontology (PRILE) 2021 Flowchart, as recommended by the *International Endodontic Journal*. Image software: Microsoft PowerPoint.

manufacturer's instructions where one capsule of Dual Rinse® containing approximately 0.9 g HEDP powder was added to 10 mL of sterile physiological saline. EDTA was prepared by adding 1.7 g of EDTA powder (PanReac AppliChem; ITW Reagents, Spain) to 10 mL of Milli-Q water. Citric acid was prepared by adding 1 g of citric acid

in powder (Merck, Germany) to 10 mL of Milli-Q water. NaOCl was diluted in Milli-Q water and adjusted to the desired concentration from a 6% solution (CanalPro; Coltene, USA). All solutions used in the experiment were filtered using a sterile syringe filter of 0.2 μL membrane pore size (Corning, USA).

## Preparation of TGF-β1

TGF-β1 powder (Sigma-Aldrich, Spain) was dissolved in Milli-Q water according to the manufacturer's instructions, and different concentrations of TGF-β1 were prepared and stored at −80°C for subsequent experiments.

## Cell culture

DSCS were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine and 100 U/mL penicillin–streptomycin (P/S), incubated at 37°C in 5% $CO_2$ and media were replenished every 2 days in all experiments.

## Effect of endodontic irrigation solutions on cell viability

DSCS were seeded at a density of $2\times10^4$ cells per well in 48-well plates (Corning, USA) and incubated at 37°C in 5% $CO_2$ for overnight attachment. The pH of the tested irrigation solutions was first adjusted (Sension+ pH 3; Hach, USA) to a pH of 7.4 with NaOH or HCl. Table 1 lists the initial pH of each solution. The cells were then treated with 400 μL of different dilutions (1:10, 1:100 and 1:1000) of the six irrigation solutions (as described above) and prepared in DMEM for 10 and 60 min. The control group consisted of DSCS in DMEM. After either 10 or 60 min, the media were then removed, wells washed with phosphate-buffered saline (PBS) and cells treated with 400 μL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] reagent (Sigma-Aldrich, Spain) per well prepared in DMEM (without serum and phenol red) at a concentration of 5 mg/mL and further incubated for 4 h at 37°C in 5% $CO_2$. The MTT-containing medium was then discarded, and DMSO (400 μL) was added to each well to dissolve the purple-coloured formazan crystals formed. Finally, 200 μL of each well was transferred to 96-well plates. DMEM alone was added to three wells in each plate to serve as blank wells and absorbance was read at 570 nm using a Tecan Sunrise Microplate Reader (Tecan Trading AG, Switzerland). The extent of formazan formed is directly proportional to cell viability, which was calculated using the following formula: %Viability = [(test OD - blank OD)/(control OD−blank OD)] × 100. According to the International Organization for Standardization (ISO) guideline 10993–5:2009(E), a reduction in cell viability greater than 30% means cytotoxicity (International Standard ISO 10993-5, 2009). Therefore, 30% was accepted as the threshold in the cytotoxicity assessment.

**TABLE 1** Initial pH of tested irrigation solutions.

| Irrigation solution | pH |
|---|---|
| Chitosan nanoparticles | 3.7 |
| 0.2% Chitosan irrigation solution | 3.4 |
| Dual Rinse® | 10.9 |
| 17% EDTA | 4.6 |
| 10% Citric acid | 1.8 |
| 2.5% Sodium hypochlorite | 12.0 |

## Effect of TGF-β1 on cell proliferation

DSCS were seeded at a density of $1\times10^3$ cells per well in 48-well plates and treated with 400 μL of TGF-β1 in DMEM (at concentrations of 0, 1, 5 and 10 ng/mL) for 1, 3 and 7 days. After each time-point, MTT was performed as described above. The absorbance was read at 570 nm and data were plotted and labelled as arbitrary absorbance units.

## Effect of endodontic irrigation solutions on cell proliferation with or without TGF-β1 treatment

DSCS were seeded in 48-well plates ($1\times10^3$ cells/well) and incubated at 37°C in 5% $CO_2$ for overnight attachment. The cells were then treated with 400 μL of a 10-fold dilution of each test solution prepared in DMEM for 10 min. After exposure to the different irrigation solutions, the wells were gently washed with PBS and allowed to proliferate for 1, 3 and 7 days with or without treatment with 5 ng/mL TGF-β1. The control group included DSCS in DMEM allowed to proliferate for 7 days without exposure to irrigation solutions or TGF-β1. MTT was performed at each time-point. The absorbance was read at 570 nm and data were plotted and labelled as arbitrary absorbance units.

## Statistical analysis

Statistical analysis was performed using Prism software (GraphPad; version 9.0). To determine the significant

differences between the tested groups, a two-way analysis of variance (ANOVA) with *post hoc* Tukey HSD was performed. The level of significance was fixed as 5% (a = 0.05) in all tests.

# RESULTS

## Chitosan nanoparticles characterization

TEM analysis showed dispersed spherical nanoparticles with an average diameter of 22.78 nm (Figure 2).

## Effect of endodontic irrigation solutions on cell viability

The different endodontic irrigation solutions tested showed a significant effect on cell viability ($p \leq .0001$). Significant interactions between the endodontic irrigation solutions and their dilution were also found for all parameters ($p \leq .0001$; Figure 3).

Chitosan nanoparticles and 0.2% chitosan irrigation solution were the only irrigation solutions that met the ISO 10993-5:2009(E) recommendations of a threshold of less than 30% reduction even with the lowest dilution (1:10) and the longest exposure time (60 min; Figure 3a,b). There was no significant difference in viability between the different dilutions or exposure periods within each of the two irrigation solution groups. There was also no significant difference between the two irrigation solutions under the different tested conditions (Figure S1a,b).

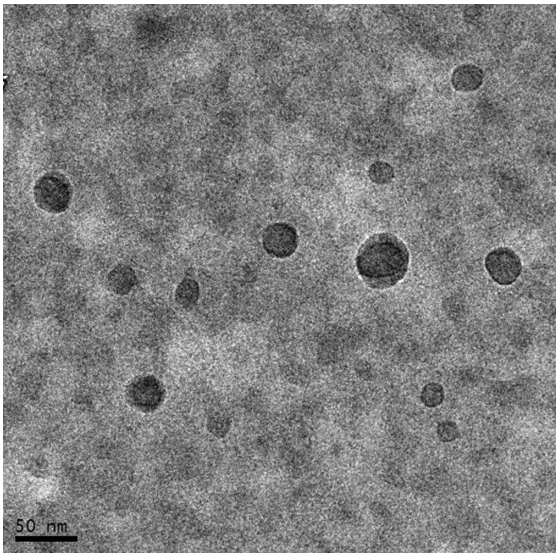


**FIGURE 2** Transmission electron microscopy showing spherical chitosan nanoparticles formation. Reference bar = 50 nm. Image software: Digital Micrograph (Gatan Inc; version 3.7.4).

The most cytotoxic endodontic irrigation solution was 2.5% NaOCl (Figure 3f). It caused an approximately 80% reduction in cell viability even at the highest dilution (1:1000) and the shortest exposure time (10 min) which is far beyond the 30% reduction threshold. 17% EDTA was the second most cytotoxic irrigation solution with a reduction of 87% and 66% at 1:10 and 1:100 dilutions, respectively, and with an exposure time of only 10 min (Figure 3d). The reduction in cell viability with the highest dilution (1:1000) for 10 min was the only condition falling under the 30% reduction threshold.

Dual Rinse® and 10% citric acid showed a similar pattern in their effect on cell viability, with no significant difference between them under the different tested conditions (Figure 3c,e and Figure S1). The lowest dilution (1:10) of each irrigation solution reduced viability far beyond the 30% reduction threshold with both a 10- and 60-min exposure time. Viability, however, was maintained fairly well at 1:100 and 1:1000 dilutions after a 10- and 60-min exposure time.

## Effect of TGF-β1 on cell proliferation

Cell proliferation was significantly affected by the concentration of TGF-β1 ($p \leq .0001$). Significant interactions between the concentration of TGF-β1 and time-point were also found for all parameters ($p \leq .0001$).

TGF-β1 at concentrations of 1 and 5 ng/mL demonstrated the best effects on the proliferation of DSCS on days 3 and 7, with significant difference between each group and both the control and the 10 ng/mL groups (Figure 4). There was no significant difference between the 1 ng/mL group and the 5 ng/mL groups, however, at any time-point.

## Effect of endodontic irrigation solutions on cell proliferation with or without TGF-β1 treatment

Differential effects of the distinct treatments were first visualized after 3 days and reached significant differences after 7 days (Figure 5). DSCS groups treated with 5 ng/mL TGF-β1 following 10-minute exposure to chitosan nanoparticles, 0.2% chitosan irrigation solution, Dual Rinse® and 10% citric acid (Figure 5a–c,e) demonstrated significantly higher proliferation compared to the non-TGF-β1-treated groups ($p \leq .0001$, $p \leq .0001$, $p \leq .0001$ and $p = .01$ respectively). There was no significant difference in proliferation between the TGF-β1-treated groups and the nontreated groups following a 10-min exposure to 17% EDTA or 2.5% NaOCl (Figure 5d,f).

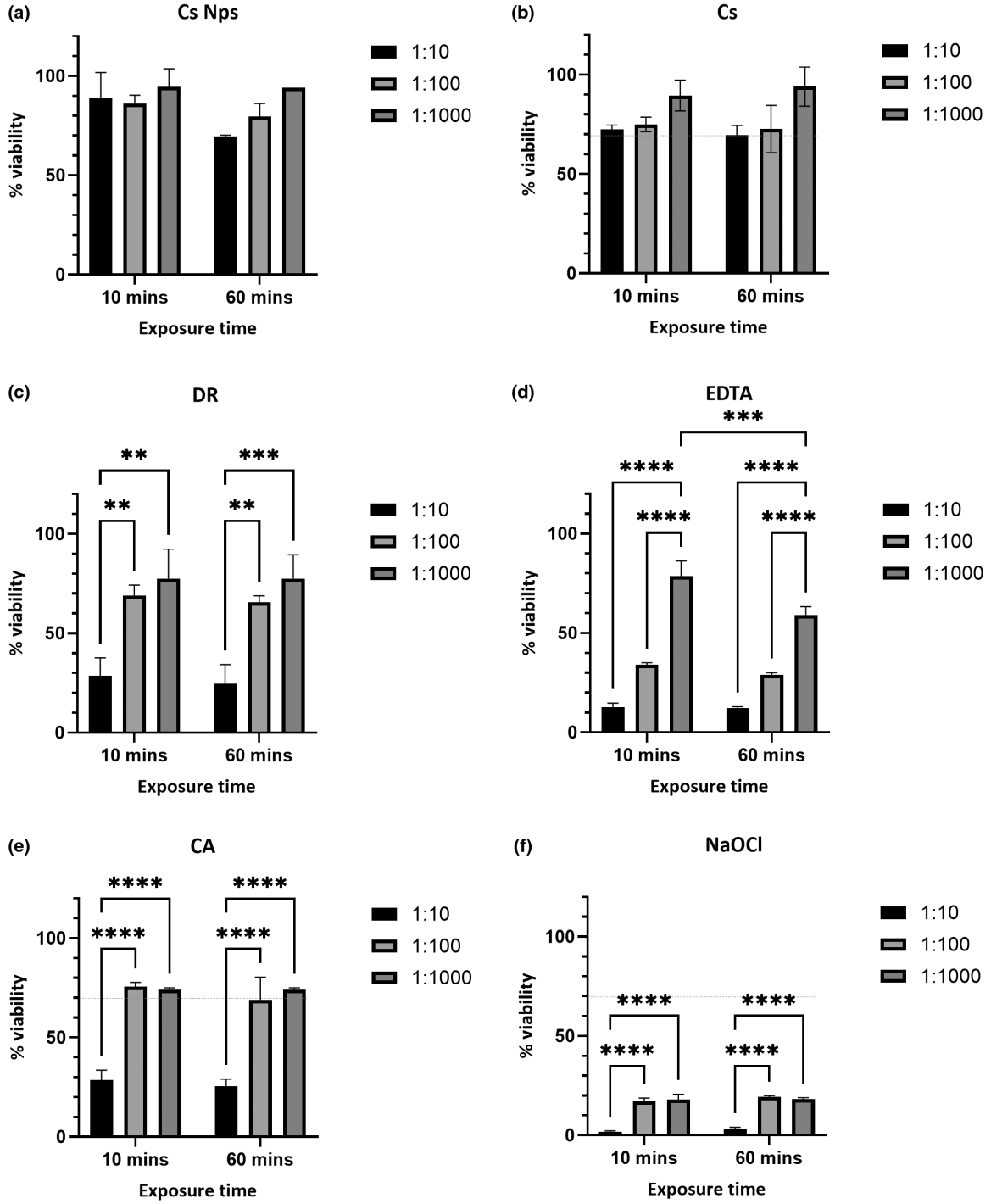


**FIGURE 3** In vitro assessment of DSCS viability after exposure to three dilutions (1:10, 1:100 and 1:1000) of six endodontic irrigation solutions (a–f) for 10 or 60 min determined by MTT assay. (a) Cs Nps group, (b) Cs group, (c) DR group, (d) EDTA group, (e) CA group, (f) NaOCl group. Bar graph presenting the mean of three replicates. The percentage of viability in the experimental groups is calculated according to the absorbance of the control group which is set to 100%. The horizontal line (in red) at 70% represents the 30% reduction threshold in accordance with the ISO 10993-5:2009(E) recommendation considering values below this line to be cytotoxic. Cs Nps, Chitosan nanoparticles; Cs, 0.2% chitosan irrigation solution; DR, Dual Rinse® HEDP; CA, 10% citric acid; EDTA, 17% EDTA; NaOCl, 2.5% sodium hypochlorite. The asterisk symbol (*) indicates a significant difference between groups: *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$. Image software: Prism (GraphPad; version 9.0).

## DISCUSSION

Recent advances in regenerative endodontics have used revascularization procedures to treat immature permanent teeth with open apices (Limoeiro et al., 2015; Palma et al., 2019). There is sparse and low-quality evidence, however, to support their effectiveness in treating apical periodontitis in those teeth (Meschi et al., 2022).

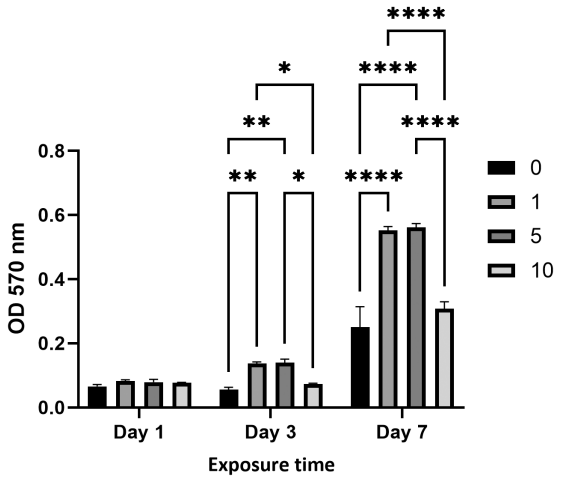


**FIGURE 4** In vitro assessment of the effect of different concentrations of TGF-β1 on the proliferation of DSCS for 1, 3 and 7 days determined by MTT. Absorbance presented as a bar graph exhibiting the strongest proliferative activity when stimulated with 1 and 5 ng/mL TGF-β1 on days 3 and 7. OD, optical density. The asterisk symbol (*) indicates a significant difference between groups: *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$. Image software: Prism (GraphPad; version 9.0).

The search for ways to improve the outcome of REPs is ongoing in both materials used and techniques applied.

In this study, the cytotoxicity of six endodontic irrigation solutions with different dilutions and exposure periods was evaluated. The direct exposure of tissue culture cells to irrigation solutions in this laboratory study necessitated the dilution and pH adjustment of these solutions. The AAE recommends using lower concentrations of NaOCl for 5 min followed by saline or EDTA for another 5 minutes in regenerative procedures (AAE Clinical Considerations for a Regenerative Procedure, 2021). We tested the cytotoxicity of diluted irrigation solutions after 10- and 60-minute exposure periods. We also investigated whether treatment with TGF-β1 following the exposure to the different irrigation solutions would compensate for their cytotoxic effect and therefore help improve the outcome of REPs.

Chitosan nanoparticles and 0.2% chitosan irrigation solution were found to be the least cytotoxic irrigation solutions meeting the ISO 10993-5:2009(E) recommendations regardless of dilution or exposure time. A study assessing toxicity and oxidative DNA damage to fibroblast cells found 0.2% chitosan-treated groups to have statistically higher viability and lower oxidative DNA damage compared to the 5.25% sodium hypochlorite group (Aydın, Akpinar, et al., 2018). Chitosan gained interest for use in endodontics due to its tissue regeneration qualities as well as its antimicrobial and chelating properties (Pascale et al., 2023). It is derived from natural renewable sources and has been shown to be biodegradable and biocompatible whilst displaying negligible toxicity (Kong et al., 2010). Chitosan treatment has also been shown to improve the resistance of dentine to degradation by bacterial collagenase whilst reinforcing dentine structure (Pascale et al., 2023). A recent study found 0.2% chitosan irrigation solution to be as effective as 5.25% NaOCl in reducing the number of CFUs of *Enterococcus faecalis* (Pascale et al., 2023). Whilst 0.2% chitosan irrigation solution and chitosan nanoparticles showed similar results in their effect on DSCS being the least toxic irrigation solutions tested in our study, the development of nanoscale systems from natural polymers, such as chitosan, for use as biological carriers might have the added advantage of the ability of biopolymer nanoparticles to diffuse across the biofilm structures and exert their antimicrobial effects (Gondim et al., 2018).

The results of our study showed that 2.5% NaOCl was the most cytotoxic irrigation solution tested. It did not meet the 30% reduction threshold in cell viability even when diluted 1000 folds and used for only 10 min. Other studies also found NaOCl to adversely affect mesenchymal stem cell viability (Sismanoglu & Ercal, 2021), proliferation capacity and differentiation potential (Liu et al., 2018). Furthermore, NaOCl was found to cause damage to dentine structural integrity and affect its microhardness (Pascon et al., 2009), which can be considered another drawback to using it in REPs, especially due to the reduced dentine wall thickness in immature necrotic permanent teeth.

The use of EDTA for 10 min has been recommended as a final irrigation solution during regenerative endodontic procedures due to its chelating properties since it removes the smear layer and may expose growth factors entrapped in the dentine matrix (Deniz Sungur et al., 2019). Our results showed that 17% EDTA was the second most cytotoxic irrigation solution. It met the 30% reduction threshold only when used for 10 min and diluted 1000-fold. After a 10-min exposure time, there was no significant difference in viability between the 10- and 100-fold dilutions; there was, however, a significant difference in viability between each of the later dilutions and the 1000-fold dilution. A similar pattern was seen after a 60-min exposure time; the 1000-fold dilution, however, did not meet the 30% reduction threshold. In a systematic review on the influence of EDTA on regenerative endodontics, 10 studies showed no influence on cell viability from the use of 17% EDTA, 6 studies showed a reduction in cell viability, whilst another 6 reported higher cell viability after using EDTA (dos Reis-Prado, Abreu, et al., 2022). The absence of the dentine factor in some of the studies, as well as concentration and exposure time differences, might have caused variations between studies.

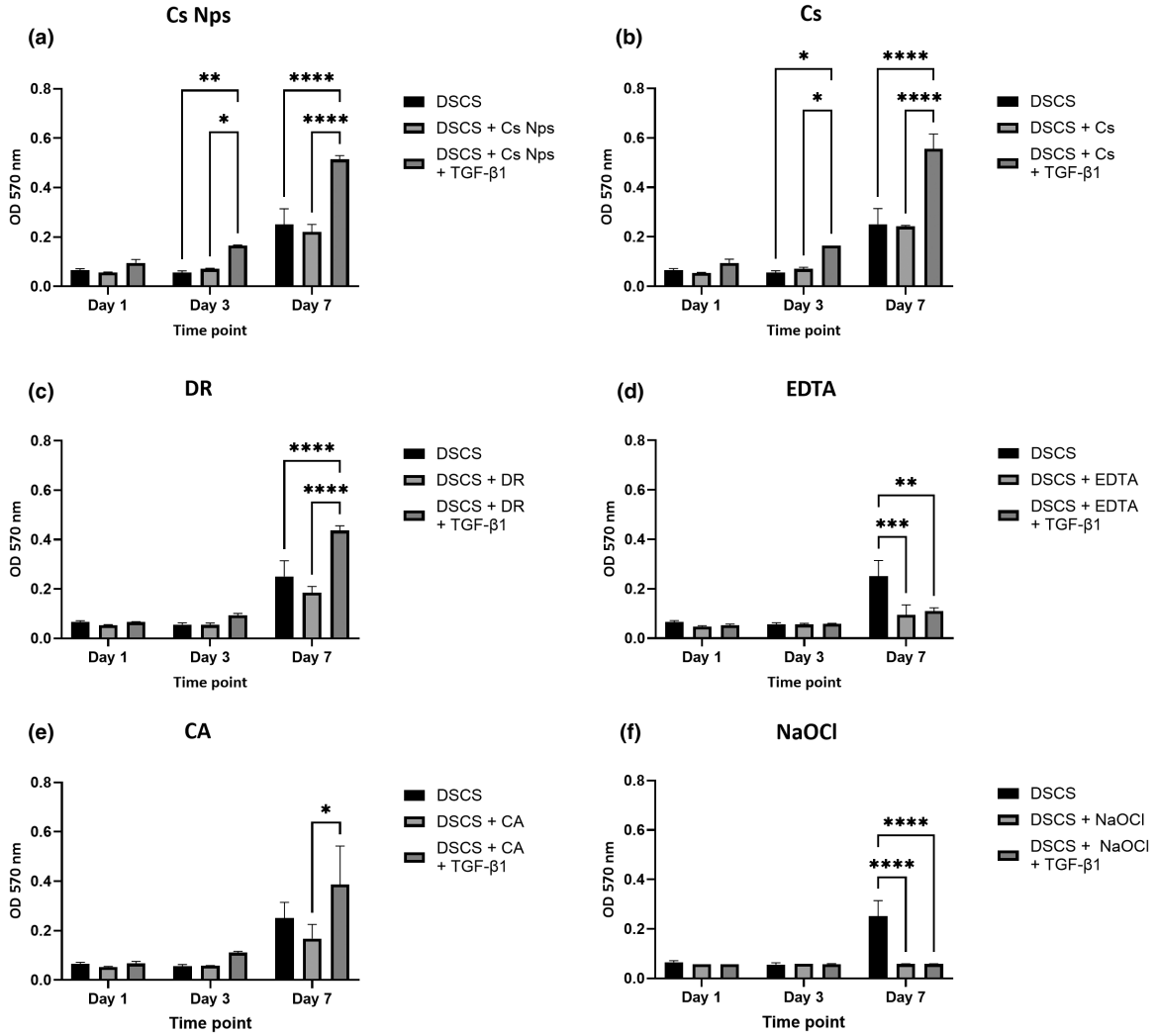


**FIGURE 5** In vitro assessment of the effect of 5 ng/mL TGF-β1 treatment on the proliferation of DSCS for 1, 3 and 7 days after 10-min exposure time to the 1:10 dilution of the six endodontic irrigation solutions. Absorbance presented as a bar graph showing DSCS proliferation with or without TGF-β1 treatment and a control group, where DSCS were allowed to proliferate without exposure to irrigation solutions or TGF-β1. Exposure to 17% EDTA or 2.5% NaOCl was sufficient to make DSCS cells refractory to the proliferative effects of TGF-β1 (d, f). DSCS group treated with 5 ng/mL TGF-β1 following exposure to chitosan nanoparticles, 0.2% chitosan irrigation solution, Dual Rinse® and 10% citric acid (a–c, e) demonstrated significantly higher proliferation compared to non-TGF-β1-treated group. OD, optical density; DSCS, dental stem cells SV40; Cs Nps, chitosan nanoparticles; Cs, chitosan irrigation solution; DR, Dual Rinse® HEDP; CA, 10% citric acid; EDTA, 17% EDTA; NaOCl, 2.5% sodium hypochlorite. The asterisk symbol (*) indicates a significant difference between groups: $*p < .05$, $**p < .01$, $***p < .001$, $****p < .0001$. Image software: Prism (GraphPad; version 9.0).

Our study found that viability can be maintained fairly well when diluting Dual Rinse® 100- or 1000-fold as opposed to 10-fold dilution. Viability was significantly increased with the 100-fold and 1000-fold dilutions compared to the 10-fold dilution regardless of the exposure time. Dual Rinse® was developed as a capsule containing approximately 0.9 g HEDP powder to be mixed with NaOCl immediately before treatment for an all-in-one root canal irrigation solution with combined proteolytic, antimicrobial and chelating properties and a minimum loss of available chlorine (Ballal et al., 2019). In our study, Dual Rinse® powder was added to 10 mL of saline instead solely to evaluate its cytotoxicity. Deniz Sungur et al. (2019) also evaluated the single use of 9% HEDP without mixing it with NaOCl and found dental pulp stem cell proliferation to be significantly lower in the 9% HEDP group compared to the control group (distilled water) at day 1, day 3 and day 5. In our study, 10% citric acid was less cytotoxic than 17% EDTA under all tested conditions. Ivica et al. (2019) also found 10% citric acid to have a significantly better effect on cell survival than 17% EDTA. Many *in vitro* studies have shown citric acid to have greater biocompatibility and less tissue irritation than EDTA (Dewi, 2020). Our results show that

viability can be maintained fairly well when 10% citric acid is diluted 100- or 1000-fold but not when it is diluted 10-fold only. Viability was significantly increased with 100-fold and 1000-fold dilutions compared to the 10-fold dilution regardless of the exposure time.

Another factor that can aid in creating the ideal environment for regenerative procedures by enhancing mesenchymal stem cell recruitment, proliferation and differentiation is the presence of growth factors, such as TGF-β1 (Chang et al., 2020; dos Reis-Prado, Abreu, et al., 2022). Our study found TGF-β1 at concentrations of 1 and 5 ng/mL to demonstrate significantly higher proliferation of DSCS compared to the other tested concentrations. Li et al. (2021) evaluated the effect of different concentrations of TGF-β1 on the proliferation of human dental pulp stem cells (hDPSCs) for 7 days using MTT assay and found TGF-β1 at a concentration of 1 ng/mL to have the best promoting effects on proliferation.

Given that TGF-β1 promoted the proliferation of DSCS, we wondered whether these effects could compensate for the reduction in viability induced by the irrigation solutions. Therefore, we analysed DSCS proliferation at days 1, 3 and 7 after 10-min exposure to the 10-fold dilution of each of the six endodontic irrigation solutions with or without the addition of TGF-β1. We chose a concentration of 5 ng/mL since it had a slightly higher effect on proliferation than the concentration of 1 ng/mL on both days 3 and 7. The results showed that exposure to 17% EDTA or 2.5% NaOCl for 10 min was sufficient to make DSCS cells refractory to the proliferative effects of TGF-β1. A 10-min exposure to chitosan nanoparticles, 0.2% chitosan irrigation solution, Dual Rinse® and 10% citric acid, however, did not interfere with the proliferative properties of TGF-β1, so therapeutic co-treatments could be envisaged. Multiple studies have found chelating agents, such as EDTA and citric acid, to aid in the release of TGF-β1 (Atesci et al., 2020; Chae et al., 2018; Ferreira et al., 2020; Galler et al., 2015; Hancerliogullari et al., 2021; Sadaghiani et al., 2022; Zeng et al., 2016). A recent study also found chitosan and chitosan nanoparticles to release TGF-β1 (Quijano-Guauque et al., 2023). In our study, however, we evaluated the effect of TGF-β1 on DSCS as an adjunctive treatment following the use of different irrigation solutions.

It is critical to point out that the treatment rationale in REPs differs from that in the classic use of irrigation solutions in root canal treatment. The results of this study suggest that the most used irrigation solutions, NaOCl and EDTA, can be quite toxic to mesenchymal stem cells and might alter the outcome of regenerative procedures. It also suggests that chitosan-based irrigation solutions could be a promising irrigation solution to be used in these procedures. The study also offers data that can be implemented to improve the outcome of regenerative procedures such as adding TGF-β1 to the treatment protocol. All the experiments were conducted using an immortalized human multipotent stromal cell line developed in vitro from SCAPs with higher proliferative capacity and retention of the expression of mesenchymal surface markers and multipotency (Sanz-Serrano et al., 2023).

A possible limitation of the present study is the neglect of the effect of dentine tissue and the positive and negative pressures in the periapical area, which in turn affect the movement of the irrigation solution inside the root canal. The direct exposure of the cells to the irrigation solutions necessitating the dilution and pH adjustments is also a limitation. The MTT cytotoxicity test, one of the most widely used assays for measuring the cytotoxic effects of materials, also has some limitations (Ghasemi et al., 2021). Future studies using an organotype root canal system with standardized irrigation delivery rates could better represent clinical settings. Additional studies using different cytotoxicity assays could also be valuable.

## CONCLUSION

We conclude that chitosan-based irrigation solutions are the endodontic irrigation solutions least cytotoxic to DSCS, whilst 2.5% NaOCl and 17% EDTA are the most cytotoxic. We also conclude that TGF-β1 has significant proliferative effects on DSCS, which were seen despite a 10-min exposure period to chitosan nanoparticles, 0.2% chitosan irrigation solution, Dual Rinse® and 10% citric acid. Exposure to 17% EDTA or 2.5% NaOCl for 10 min, however, made DSCS refractory to the proliferative effects of TGF-β1.

### AUTHOR CONTRIBUTIONS

**Roumaissa Belkadi** was involved in conceptualization, methodology, investigation, data curation, data analysis and writing of original draft. **Diana Sanz-Serrano** was involved in conceptualizing, methodology and article review. **Francesc Ventura** was involved in supervision, conceptualization, methodology, funding acquisition and article review. **Montse Mercade** was involved in supervision, conceptualization, methodology, funding acquisition and article review.

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## CONFLICT OF INTEREST STATEMENT

The authors certify that they have no conflict of interest of any kind.

## DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available within the article or its supplementary materials. Raw data are available upon request.

## ETHICS STATEMENT

Ethical approval was attained from the Ethical Committee of the Hospital Odontologic of the University of Barcelona (Ceim HOUB; Approval code 42/2021).

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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