Journal of Oral Biology and Craniofacial Research 14 (2024) 86–91

Contents lists available at ScienceDirect

Journal of Oral Biology and Craniofacial Research

journal homepage: www.elsevier.com/locate/jobcr

Evaluation of cytotoxicity of 3.8 % SDF and BioAKT solutions on the viability of dental pulp stem cells

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ARTICLE INFO

Keywords:
Regenerative endodontic treatment
5.25 % NaOCl
3.8 % SDF
BioAKT
DPSCs

ABSTRACT

Introduction: Nonsurgical endodontic therapies have evolved from classic endodontic therapies to regenerative endodontic treatments (RETs) in recent years. In context of the cytotoxic activity of the most commonly used endodontic irrigant, NaOCl, newer endodontic irrigating solutions should be tested for its effective use in RETs. The aim of this trial was to examine and assess the cytotoxic response of 3.8 % SDF and BioAKT irrigating solutions on the viability of DPSCs.

Methods: The viability of DPSCs cultivated in 5.25 % NaOCl, 3.8 % SDF & BioAKT at dilutions of 1:100,1:20 &1:10 were evaluated through MTT assay after 10 min, 60 min and 24 h incubation, detection of apoptosis and ALP activity after 7,14 & 21-days incubation. A two-way analysis of variance (ANOVA) with post hoc Turkey HSD was performed to determine significant differences between the specimens tested.

Results: When compared to the control at all time periods, all test specimens at varied dilutions (1:100, 1:20, and 1:10) caused no cytotoxic effects. The maximum number of live cells and ALP activity was observed with DPSCs cultivated in BioAKT followed by 3.8 % SDF and 5.25 % NaOCl at all time intervals.

Conclusion: Different doses of 3.8 % SDF and BioAKT solution revealed encouraging outcomes when compared to 5.25 % NaOCl in terms of viability, proliferation and long-term ALP functioning potential when cultivated in DPSCs.

Author contribution

Conceived & designed the analysis – MV, IM.
Collection of data – MV, IM, KB, LA.
Contributed data or analysis tool – MV, KB, HS.
Drafting of manuscript - MV, IM, LA, KB, HS.

Source of funding

Nil.

Data availability statement

Data would be made available on request to the corresponding author.

1. Introduction

Regenerative endodontic treatment (RET) is a therapeutic approach designed to restore and preserve the function of the pulp-dentin complex by substituting damaged pulp tissues with healthy ones. The success of RET relies on maintaining the viability of stem cells while ensuring proper disinfection.[1] Hence, the irrigant used in such procedures should not just facilitate antibacterial action, debridement, and necrotic tissue

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https://doi.org/10.1016/j.jobcr.2024.01.001
Received 27 September 2023; Received in revised form 31 December 2023; Accepted 2 January 2024

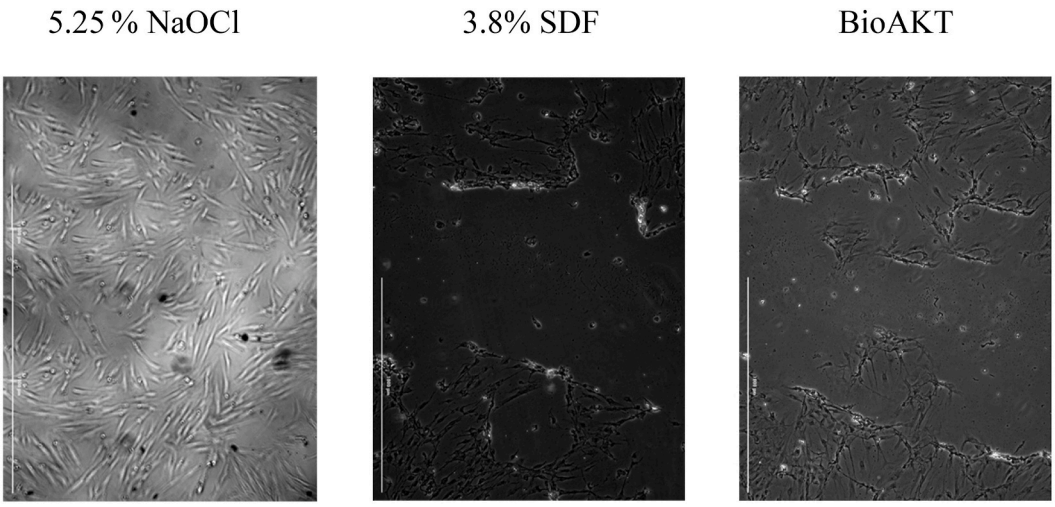

Fig. 1. Morphology of DPSCs cultured in various endodontics irrigants at dilution of 1:100 after 10 min of incubation.

dissolution but also be non-toxic to periradicular tissues and stem cells.[2] While 5.25 % NaOCl is a valuable and effectual irrigant, it hinders the propagation of dental stem cells and decreases protein expression levels by half compared to control when used at a concentration of 3 % in vivo.[3]

A solution of silver diamine fluoride (SDF) at a concentration of 3.8 % w/v has been employed for intracanal irrigation, representing a tenfold dilution of the SDF used in root canal therapy. Studies signify that this 3.8 % SDF solution possesses antimicrobial properties against *E. faecalis* biofilm and can effectively serve as an endodontic irrigant to impede bacterial growth.[4] Additionally, research has shown that 3.8 % SDF serves as a highly effective alternative to 1 % NaOCl as an endodontic irrigating solution.[5]

In a recent study,[6] a surface disinfectant consisting of electrolytically produced citric acid (4.846 %) and Ag^+ (0.003 %)—BioAKT (New Tech Solutions, Italy)—was assessed as a novel biomaterial for cleaning as well as disinfecting root canals. The bioavailability of Ag^+ in this solution aided swift efficacy against a broad spectrum of viruses, microorganisms, and fungi.[7]

Given the cytotoxic effects commonly associated with the widely used endodontic irrigant, NaOCl, it is crucial to explore alternative irrigating solutions for their suitability in RETs. It is imperative that endodontic irrigants do not impede the viability and propagation of dental pulp stem cells (DPSCs) during RET processes. This study was conducted to evaluate and compare the cytotoxic impact of three irrigating solutions—5.25 % NaOCl, 3.8 % SDF, and BioAKT—on DPSC viability. The null hypothesis posited that DPSC viability would be consistent across different irrigating solutions after exposure.

2. Material & methods

This study adhered to the protocols outlined by Nagendrababu V et al. (2021).[8] The sample size for this investigation was determined using the "resource equation" method.[9] All tests were conducted in triplicate, and every specimen and reaction was subject to analysis in triplicate. Approval for the current trial was obtained from the institutional ethical research committee (IERC-2019/45).

2.1. Cell culture preparation

Human dental pulp stem cells (DPSCs) were procured from HiMedia Laboratory, India. DPSCs, post 4th passage, were grown in 96-well plates at a density of 1×10^5 per well, following the manufacturer's guidelines. The cells were nurtured in Dulbecco Modified Eagle Medium (DMEM – Sigma Aldrich, USA) supplemented with 10 % fetal bovine serum (FBS -Gibco, USA) and 1 % penicillin (Gibco, USA) in an incubator with 5 % carbon dioxide, continually observed through an inverted phase microscope. Subculturing was performed twice per week upon reaching 80 % confluence.[10] Prior to the experiment, DPSC phenotypes were examined through immunofluorescence, utilising antibodies for CD73 (Santa Cruz, USA), CD90 (BD Biosciences), and CD105 (Abcam, UK). Following two phosphate buffer saline (PBS) washes, cells were incubated in the dark for 45 min with anti-mouse Alexa Fluor-conjugated secondary antibody (1:500 Molecular probes Invitrogen, USA). Microscopic slides exhibited over 95 % positive expression when observed under a fluorescence microscope.

2.2. Test specimen preparation

All solutions for irrigation, encompassing 5.25 % NaOCl (Vishal Dentocare Private Limited, India), 38 % SDF (FAgamin; Argentina) diluted to 3.8 % using disinfected distilled water,[11] and BioAKT (New Tech Solution, Italy), were amalgamated with culture medium immediately preceding each analysis. The extract test method and biological assessment adhered to ISO 10993–12:2012 guidelines (E).[12] To scrutinise the dose-response correlation, the irrigating agents underwent dilution with growth media (DMEM) at volume ratios of 1:100, 1:20, and 1:10.[12] (Fig. 1).

2.3. Cell viability test by MTT assay

To facilitate cell adhesion, DPSCs were plated at a density of 1×10^4 cells/well in 200 μl of DMEM in 96-well plates and incubated overnight at 37 °C in a 5 % CO_2 incubator. Subsequently, DPSCs were exposed to 200 μl of test samples comprising 3.8 % SDF, 5.25 % NaOCl, and BioAKT at varying dilutions (1:20, 1:100, and 1:10) in growth medium. Exposure durations were set at 60 min, 10 min, and 24 h, respectively.

The viability of DPSCs was assessed using the EZ count MTT cell test kit (HiMedia, India). To halt the experiment, dimethyl sulfoxide was added after MTT incubation for 240 min in each well. By utilising a microplate reader (BioTek Instrument, USA) with a reference wavelength of AB_{630}, the AB_{570} value was computed. The absorbance of the control specimen, calibrated to 100 % in growth media, served as the baseline for calculating the percentage of cell viability for each test specimen relative to the control.[13] Each condition underwent analysis in triplicate.

2.4. Detection of apoptosis and necrosis

The Annexin V-FITC Apoptosis Detection Kit from BD Biosciences,

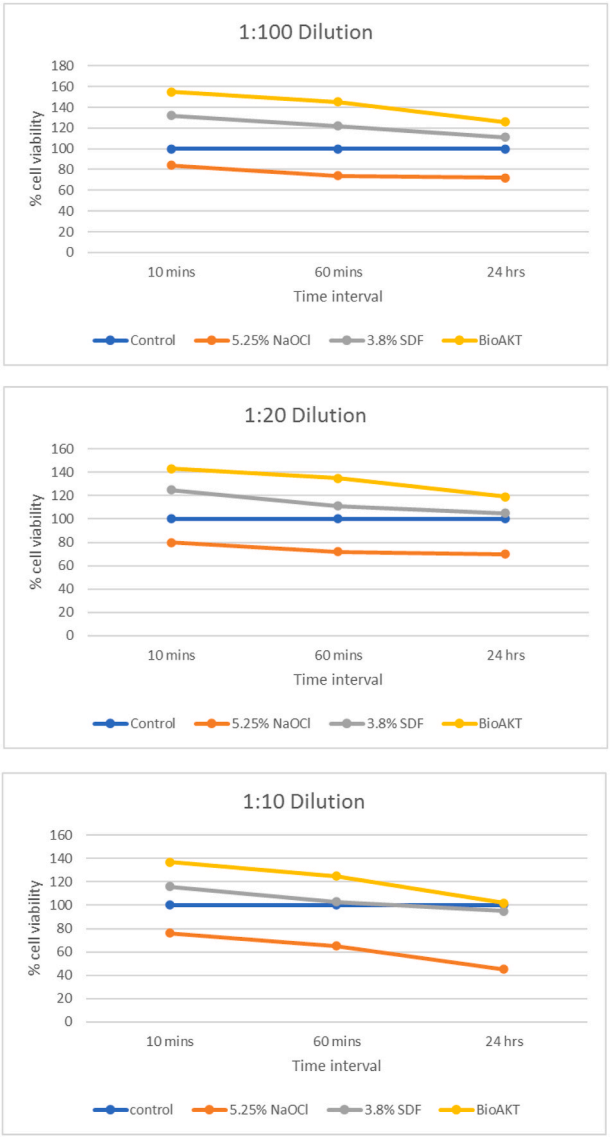

Fig. 2. Cell viability % of various endodontic irrigants at 1:100, 1:20 & 1:10 dilutions at different time intervals of incubation.

USA, was employed to assess the percentage distribution of live (7-AAD negative/Annexin-V negative), late apoptotic (7-AAD positive/Annexin-V positive), early apoptotic (7-AAD negative/Annexin-V positive), and necrotic (7-AAD positive/Annexin-V negative) cells using flow cytometry. The experiment strictly adhered to the manufacturer's recommended procedural steps.[14] A 24-well plate was utilized to seed 2×10^6 mL^{-1} DPSCs.[14] Following two washes with PBS, the cells were re-suspended in 1X binding buffer, and 5 μl of Annexin V and 7-AAD were added. After a 15-min incubation in the dark, the cells were re-suspended in binding buffer and subjected to flow cytometry. Cells cultured in DMEM served as the control. Each condition underwent analysis in triplicate.

2.5. Alkaline phosphatase assay (ALP)

DPSCs were enumerated and cultured overnight at a density of 2×10^4 cells/well in 24-well plates with growth media. Subsequently, DPSCs were cultured the next day in a growth medium with test specimens, whereas the control group underwent cultivation in a growth medium alone. The activity of ALP was assessed after 7, 14, and 21 days of incubation. SigmaFast *p*-nitrophenyl phosphate tablets (Sigma-Aldrich, USA) were utilized to determine ALP function. After two PBS washes, DPSCs were exposed to an actively dissolved solution for 60 min at 37° Celsius (in the dark). Each well of a 96-well plate was filled with 200 μl of the solution, and AB_{405} was measured using an ELISA plate reader (BioTek Instrument, USA). A calibration curve with known ALP moles was employed for converting absorbance values to ALP moles.[14] Each condition underwent analysis in triplicate.

Table 1
Table 1: Comparison of cell viability in percentage between various test specimens and different time intervals at three different dilutions.

Dilution 1:100			
Viability in %			
Incubation time -	10 min	60 min	24 h
Control	100	100	100
5.25 % NaOCl	84	74	72
3.8 % SDF	132	122	111
BioAKT	155	145	126
Dilution 1:20			
Viability in %			
Incubation time -	10 min	60 min	24 h
Control	100	100	100
5.25 % NaOCl	80	72	70
3.8 % SDF	125	111	105
BioAKT	143	135	119
Dilution 1:10			
Viability in %			
Incubation time -	10 min	60 min	24 h
Control	100	100	100
5.25 % NaOCl	76	65	45
3.8 % SDF	116	103	95
BioAKT	137	125	102

2.6. Statistical analysis

The experiments were conducted in three independent trials with triplicates for every experimental group and outcome. The collected data was utilized to compute the mean and standard deviation. Statistical significance between test samples was evaluated using Two-way ANOVA followed by Post-hoc Tukey HSD. Statistical analysis was performed (Prism 9.5.0; GraphPad Software, USA). A significance level of 5 % was applied to each test.

3. Results

3.1. Cell viability test by MTT assay

In comparison to the control across all time intervals—10 min, 60 min, and 24 h—none of the test specimens at different dilutions (1:100, 1:20, and 1:10) exhibited cytotoxic effects. BioAKT (155 ± 12.9 %) demonstrated the highest cell viability percentage, followed by 3.8 % SDF (132 ± 9.4 %) and 5.25 % NaOCl (84 ± 4.7 %) at a 1:100 dilution following a 10-min incubation. Consequently, subsequent experiments with the test specimens were specifically carried out using a 1:100 dilution. Statistically significant differences in cell viability percentage were observed at all time points compared to the control ($p = 0.01$). Pairwise comparisons of cell viability percentage (5.25 % NaOCl vs 3.8 % SDF, 3.8 % SDF vs BioAKT, and BioAKT vs 5.25 % NaOCl) revealed statistically significant differences across all time periods for all dilutions ($p = 0.01$) in comparison to the control (Fig. 2 and Table 1).

3.2. Detection of apoptosis and necrosis

In comparison to the control group, the mean ± SD (%) difference for

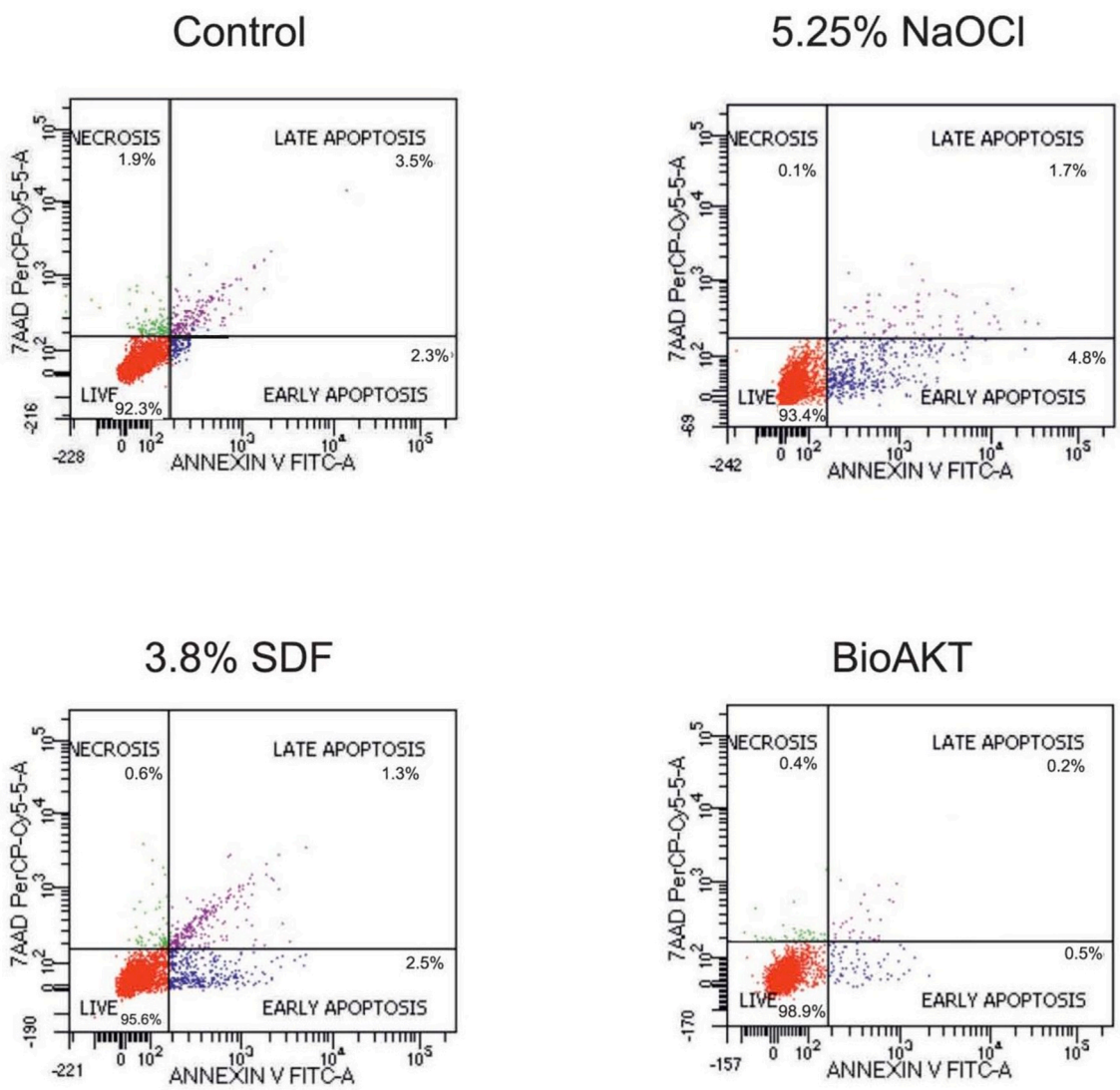

Fig. 3. Representative 2D flow cytometry dot plot of data derived from FITC – AnV and7-AAD stained DPSCs cultured in various intra canal medicaments.

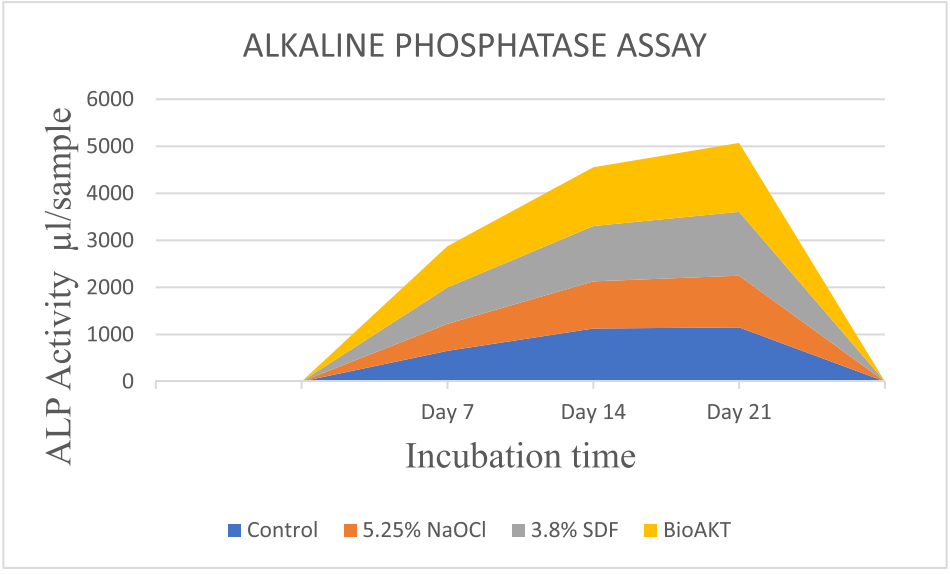

Fig. 4. ALP activity of various endodontic irrigants after 7, 14- & 21-days incubation.

the number of live, early, and late apoptotic cells was statistically significant across various test specimens ($p = 0.001$). The highest percentage of live cells was recorded in BioAKT (98.90 ± 1.66), followed by 3.8 % SDF (95.68 ± 3.01), 5.25 % NaOCl (93.47 ± 2.79), and the control (92.30 ± 4.36) (Fig. 3).

3.3. Alkaline phosphatase assay (ALP)

At each observed time point, a substantial disparity in ALP activity among the test specimens was noted (p 0.01). The highest ALP activity was recorded in DPSCs incubated with BioAKT (1465 ± 221.9), followed by 3.8 % SDF (1357.7 ± 122.2), control (1150.2 ± 145.2), and 5.25 % NaOCl (1100.5 ± 114.1) following a 21-day incubation period. Throughout all time intervals, the ALP activity of 5.25 % NaOCl was significantly decreased compared to the control. Statistically significant variations in ALP activity were observed between test and control samples for all time points (Fig. 4).

4. Discussion

Nonsurgical endodontic treatments have progressed from traditional endodontic approaches to regenerative endodontic treatments (RETs), aiming to overcome the drawbacks of conventional methods by preserving teeth's physiological attributes, including dental proprioception, tertiary dentinogenesis, and innate immunity. The goal is to maintain natural dentition throughout a person's life. In RETs, the selection of an appropriate endodontic irrigant is crucial due to its role in antibacterial efficacy and the ability to dissolve organic waste without extensive instrumentation.[1] In RET, the irrigant is needed to warrant the survival of DPSCs for the success of RETs. Therefore, this study was undertaken.

The study revealed that 5.25 % NaOCl, at different concentrations, did not induce cytotoxicity during a short incubation period. This finding contradicts the research by Sismanoglu and team, who demonstrated the negative impact of NaOCl on the viability of mesenchymal stem cells (MSCs), with cytotoxicity reliant on concentration and time.[1] Notably, the same research indicated that 6 % NaOCl (1:100), as against the control, did not significantly decrease MSC viability following 10 and 60 min of exposure, aligning with the current investigation's results.

Alkahtani and team proposed that rising NaOCl levels and treatment duration might lead to reduced cell viability.[16] A separate in vitro study showed that NaOCl use decreases cell viability, proliferative capacity, and odontogenic differentiation, with responses dependent on dilution and incubation time.[17] These findings align with the present trial, where maximum cell viability across all test samples was observed after a 10-min incubation, contrasting with the 60-min and 24-h periods.

The current research indicates that 3.8 % SDF promotes DPSCs survival across all concentrations during short-term incubation. This contradicts a study by Kim and team, who reported an 8 % decrease in DPSC viability when exposed to SDF. The potential explanation for this discrepancy could be that SDF diminishes pulpal-like cell viability by depleting glutathione.[18] Apoptosis, a crucial aspect for tissue homeostasis, is tightly regulated, and it can be triggered by extracellular or intracellular stimuli, activating extrinsic or intrinsic pathways.[19] ALP, an early marker of osteogenic proliferation and differentiation, plays a role in controlling cell growth.[15] Therefore, ALP assay tests and apoptosis detection were conducted in the current trial.

The cytotoxic activity of SDF has been attributed to both F^- and Ag^+ contents, creating a synergistic connection which induces oxidative stress and cellular damage. The heightened toxicity of Ag^+ and F^- is believed to result from a rise in reactive oxygen species (ROS) or lipid peroxidation, coupled with a decline in overall antioxidant properties, leading to apoptosis and inflammatory processes.[20] SDF is associated with the "zombie effect," where non-vital cells act as a reservoir of Ag^+, affecting the proliferative and differentiation capacity of neighboring healthy cells.[21] This could explain why 3.8 % SDF demonstrated more live cells and ALP activity than NaOCl but fewer compared to BioAKT. As per Nakade et al., F^- can enhance the propagation and ALP activity of human dental pulp cells at micromolar doses.[22]

BioAKT, a patented antibacterial irrigant, is defined by an Ag^+ complex created through an electrochemical process involving silver and citric acid. In this process, Ag^+ gently binds to a citrate ion, forming the compound $AgC_6H_7O_7$. Consequently, this disinfectant generates a stable form of Ag^+ in an organic acid context.[23] BioAKT exhibited the highest cell viability in this research across all doses and time points tested. These results contradict the study conducted by Generali and team, which confirmed cytotoxicity of BioAKT at concentrations >0.5 % when incubated with L-929 mouse fibroblast cell lines. The potential toxic effect of these irrigants could be associated with the citric acid in their composition, leading to a pH reduction to 1.7.[24]

BioAKT, when incubated with DPSCs, exhibited the highest number of live cells in the current experiment. Interestingly, this observation contradicted the observations of Lan and team, who asserted that BioAKT's detrimental impact on live cells was attributed to citric acid's capacity to reduce the pH level of the growth medium, leading to extracellular acidosis.[25] Citrate concentrations in bones and teeth range from 20 to 80 mol/g, surpassing plasma levels by 100–400 times. Recent trials highlight its crucial role in bone structure and biology as a basic constituent of bone nanocomposites.[26] Approximately one-sixth of the free surface of apatite nanocrystals contains citrate compound, fostering linkages between mineral layers and modifying the crystalline structure of bone, primarily associated with bone formation. Carrying more carboxylate receptors than all other proteins combined, citrate aids in binding calcium ions. In both in vivo and in vitro scenarios, the addition of citrate to the culture media or its release from biomaterials during resorption improves ALP expression.[27] This may explain why BioAKT, being a citrate-based irrigant, demonstrated the highest ALP activity at all time points during incubation in the present trial.

The current study faces several drawbacks, including its reliance on a two-dimensional cell culture model. Other constraints include a restricted 24-h timeframe for the MTT assay and a 21-day limit for evaluating ALP activity in DPSC-cultured endodontic irrigants. The time-sensitive nature of the MTT assay, apoptosis detection test, and ALP activity assessment underscores the necessity for a more extensive investigation over an extended duration. Additionally, the available information on the dosage, material safety data, therapeutic recommendations, and antibacterial properties of the various components comprising the BioAKT root canal irrigant is inadequate.

5. Conclusion

In conclusion, the study's findings indicate promise with regards to proliferation, survivability, and long-term ALP functional capacity when cultivating DPSCs in various concentrations of 3.8 % SDF and BioAKT solution. To determine the optimal concentration of such solutions for endodontic irrigation in regenerative therapies utilising DPSCs, further clinical research works are necessary.

Declaration of competing interest

Nil.

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