

Response of intra canal medicaments on viability and survival of SHEDs

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Background: Regenerative endodontic procedures (REP) rely on the principles of tissue engineering and take advantage of the regenerative abilities of Stem Cells derived from human exfoliated deciduous teeth (SHEDs). Since REPs advise the minimal instrumentation of root canals, they are more dependent on intracanal medicaments with antimicrobial activity to provide a sterile environment for pulpal regeneration. Hence present trial was conducted to examine the influence of different intracanal medicaments on SHEDs proliferation and survival. **Study design:** SHEDs were cultured by using the long-term explant culture method and characterized using flow cytometry and exposed to different concentrations of calcium hydroxide, doxycycline, potassium iodide, triamcinolone, and glutaraldehyde. SHEDs were subjected to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, apoptosis using the Annexin V-binding assay and Alkaline Phosphatase (ALP) activity. **Results:** All medicaments significantly reduced cell viability at different concentrations over different exposure times. Highest number of live cells and ALP activity was observed in SHEDs cultured in calcium hydroxide. **Conclusion:** Potassium iodide and glutaraldehyde were the significantly less likely of all the medications examined to adversely affect the viability and survival of SHEDs.

Keywords: SHEDs, Intra canal medicaments, Regenerative endodontics

INTRODUCTION

SHEDs were discovered in the dental pulp of human exfoliated primary teeth and expressed the same characteristics as Mesenchymal Stem Cells (MSCs), such as fibroblastic traits, clonogenicity, cell surface antigen presentation, proliferative ability, and multidifferentiation capacity¹. SHEDs have recently been examined for their potential application in tissue engineering for bone tissue regeneration and cell-based therapies for a variety of refractory medical conditions, including hypoxic-ischemic brain damage, systemic lupus erythematosus, spinal cord injury, and diabetes. Additionally, exosomes generated from Dental Pulp Stem Cells (hDPSCs) are regarded as beneficial²⁻⁴.

In comparison to other human tissues, exfoliating primary teeth have the distinct advantages of being less ethically contentious, being a readily accessible source, being simple and minimally invasive to collect, and retaining high stem cell potential such as cell growth, multipotency, and immunoregulatory functions even after cryopreservation. Numerous associations have been developing a SHED bank for allogeneic and autologous cell treatment. As a result, they are regarded as a viable supply of cells for tissue regeneration. The following clinical approaches are used in regenerative endodontics: minimal instrumentation, root canal disinfection, intra-

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canal medicament application, canal bleeding induction and capping followed by a hermetic coronal seal⁶.

Microbes can have a detrimental effect on the growth and regeneration capacity of stem cells. Because Regenerative Endodontic Procedures (REP) advocate for little root canal instrumentation, they rely primarily on intracanal antimicrobial medicaments to maintain a sterile environment conducive to pulpal revival⁷. Calcium hydroxide is the disinfectant of choice for root canals⁸. Triple antibiotic paste and double antibiotic paste are more efficient disinfectants for root canals, according to studies^{9,10}. Intracanal medications affect mesenchymal stem cell survival and multiplication. In a study, it was discovered that triple and double antibiotic paste were detrimental to the viability of stem cells in the apical papilla¹¹.

However, a search of the literature reveals no research examining the response of intra canal medicaments to the growth and viability of SHEDs. As a result, the goal of this trial was to ascertain the influence of different intracanal medicaments on SHED proliferation and survival.

MATERIALS AND METHOD

The current *in vitro* experimental trial adhered to the Nagen-drababu V *et al.*¹², 2021 standards for research reporting. The sample size for this experiment was estimated using the “resource equation” method¹³. All analyses were carried out in three separate trials, with triplicates of each test specimen and reaction. Human exfoliating caries-free primary teeth indicated for orthodontic extraction in 7–14 year-old healthy children whose guardian/parents signed informed consent for participating in the present study were the source for the SHEDs. The institutional ethics committee gave its approval to this trial (IREB/2019/PHD/PEDO/01).

Under sterile conditions, the pulp was collected and transferred to the laboratory (National Institute of Immunohematology, Parel, Mumbai) for further processing. Hank’s Balanced Salt Solution was used to disinfect the pulp tissues (Gibco, USA). Following that, collagenase-A (3 mg mL) was used for enzymatic action at 37 °C for 60 minutes. Cells (1.5×10^4 cells/cm²) were collected and cultured for 72 hours in 25-cm² plastic culture flasks in complete media (5% CO₂ at 37°C). The red blood cells were eliminated, as were any other non-adherent cells. To stimulate growth, fresh media was added. Passage zero (P₀) was defined as the density of adherent cells at 80% growth.

To enable future passaging, SHEDs were rinsed in Phosphate Buffer Saline (PBS) and isolated by culturing for 2-5 minutes at 37 °C with 0.25% trypsin. The trypsin action was deactivated by adding a culture medium. SHEDs were centrifuged at 500 g for 5 minutes before being seeded into 75-cm² flasks with 5×10^3 cells cm². Cells from passage 4 were used in this study. SHEDs phenotypes were assessed using a flow cytometer before the experiments, with specific antibodies for Cluster of Differentiation CD 90, CD 105, CD 73, CD 34, CD 45, and Human Leucocyte Antigen-DR Isotype (HLA-DR) (BD Biosciences, Pharmingen)¹⁴.

To examine the proliferation and survival of SHEDs, we performed MTT, Apoptosis assay and ALP assay. For the MTT assay, calcium hydroxide (Ca), doxycycline (Dy), potas-

sium iodide (PI), triamcinolone (Tr), and glutaraldehyde (Gt) (Sigma Aldrich, USA) were used. Various medicament was evaluated at concentrations of 0 (control), 10 g/mL, 25 g/mL, 50 g/mL and 100 g/mL. The SHEDs were exposed to the medicaments for 24 hrs, 48 hrs and 72 hrs.

Cell viability assay

The viability of SHEDs was assessed using an EZ count MTT cell assay kit (HiMedia, Ghatkopar, Mumbai, India). As per ISO 10993 standards¹⁵, the cell survival of less than 30% was considered cytotoxicity. MTT was introduced to each well and cultivated for 240 minutes before the experiment was aborted by the addition of dimethyl sulphoxide¹⁶. AB₅₇₀ nm was determined using a microplate reader (BioTek Instrument, Winooski, VT, USA) in conjunction with a reference wavelength of AB₆₃₀. The absorbance of the control group was set to 100% using growth media, and the % of viability in experimental groups was computed in relation to the control group.

Detection of apoptosis and necrosis

The Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, Pharmingen, USA) was used to determine the percentage distribution of live (7-AAD negative/Annexin-V negative), early apoptotic (7-AAD negative/Annexin-V positive), late apoptotic (7-AAD positive/Annexin-V positive), and necrotic (7-AAD positive/Annexin-V negative) cells using flow cytometry. The procedural steps recommended by the manufacturer were followed for performing this experiment. 24 well plates was used to seed 2×10^6 mL⁻¹ SHEDs¹⁷. SHEDs cultured in Dulbecco Modified Eagle’s Medium (DMEM) and H₂O₂ (1 mmol L⁻¹) were represented as negative control and positive control specimens respectively.

Alkaline phosphatase (ALP) assay

SHEDs were counted and grown overnight in 24-well plates in growth media at a density of 2×10^4 cells/well. SHEDs were cultured in a growth medium with test specimens (intra canal medicaments) the next day, while control groups were cultured in a growth medium only. ALP activity was examined after 7-days, 14-days, and 21-days of incubation. SigmaFast p-nitrophenyl phosphate tablets were used to measure ALP activity (Sigma-Aldrich, USA). SHEDs were cultured in an active dissolved solution for 60 minutes at 37 °C (in dark) after two PBS washes. 200 μL solution from every well was introduced to a 96-well plate, and AB₄₀₅ nm was assessed with an ELISA plate reader (BioTek Instrument, Winooski, VT, USA). The absorbance readings were transformed to ALP moles using a calibration curve built with known ALP moles¹⁸.

Statistical Analysis

The mean and standard deviation of three independent experimental values were calculated. For pair-wise evaluations, a two-way analysis of variance (ANOVA) with post hoc Tukey Honestly Significant Difference (HSD) was employed to evaluate whether there were significant differences among the

specimens tested. Statistical tools were used to examine the data (Prism; GraphPad Software, USA). All tests were conducted with a significance threshold of 5% ($\alpha = 0.05$).

RESULTS

Characterization of SHEDs

Flowcytometric examination of SHEDs revealed the strong manifestation of the positive marker CD 73 (93.90%), CD 90 (94.60%), and CD 105 (35.30%). The progeny of SHEDs did not manifest negative markers CD 34 (2.60%), CD 45 (2.75%), or HLA-DR (1.65%).

Cell viability assay

The medicaments reported variable cytotoxicity at different concentrations and time intervals. Calcium hydroxide showed a significant decrease in cell viability at 50 $\mu\text{g}/\text{mL}$ or higher after all time intervals of incubation. Calcium hydroxide reported cytotoxicity at a concentration of 500 $\mu\text{g}/\text{mL}$ and 1000 $\mu\text{g}/\text{mL}$ after 24 hrs, 48 hrs and 72 hrs interval (Fig. 1). Doxycycline reported a statistically significant reduction in cell survival at a concentration of 50 $\mu\text{g}/\text{mL}$ or more after 24-hrs, 48-hrs and 72-hrs incubation. Cytotoxicity was seen at a concentration of 250 $\mu\text{g}/\text{mL}$ or more at all time intervals (Fig. 2).

Potassium iodide (Fig. 3), triamcinolone (Fig. 4) and glutaraldehyde (Fig. 5) showed a statistically substantial decrease in cell viability at concentration level of 50 $\mu\text{g}/\text{mL}$ or more at all time intervals. Triamcinolone reported cytotoxicity at a concentration of 100 $\mu\text{g}/\text{mL}$ or more after 24-hrs, 48-hrs and 72-hrs incubation. In the present study, SHEDs reported the highest cell survivability when cultured in calcium hydroxide followed by potassium iodide, doxycycline, triamcinolone and glutaraldehyde at a concentration of 25 $\mu\text{g}/\text{mL}$, 10 $\mu\text{g}/\text{mL}$, 10 $\mu\text{g}/\text{mL}$, 25 $\mu\text{g}/\text{mL}$ and 25 $\mu\text{g}/\text{mL}$ respectively after a time interval of 72 hours of treatment. Hence the subsequent experiment involving these test specimens was performed with these dilutions only.

Detection of apoptosis and necrosis by flow cytometry

The mean \pm SD variation among various medicaments, were statistically significant for % of live cells ($p = 0.045$), early ($p = 0.001$) and late ($p = 0.036$) apoptotic cells when compared to control. The highest % of live cells were found in calcium hydroxide (97.50 ± 1.46), followed by triamcinolone (95.00 ± 2.01), negative control (94.50 ± 1.79), glutaraldehyde (93.7 ± 1.55), potassium iodide (93.20 ± 0.36), doxycycline (92.00 ± 1.70) and positive control (15.40 ± 10.36) (Fig. 6).

Alkaline phosphatase assay

The difference in ALP activity between all test specimens was statistically significant at all the time intervals—7 days, 14 days and 21 days ($p = 0.001$). The highest amount of ALP activity was observed in SHEDs incubated in calcium hydroxide (1375.4 ± 321.89), followed by control (1150.7 ± 222.32), potassium iodide (1050.2 ± 155.12), triamcinolone (1030.7 ± 122.98), glutaraldehyde (1025.3 ± 164.26)

& doxycycline (900.3 ± 64.21) after 72 hours of incubation. Pair-wise comparison of ALP activity among all test specimens was statistically significant than the control specimen at all time intervals (Fig. 7).

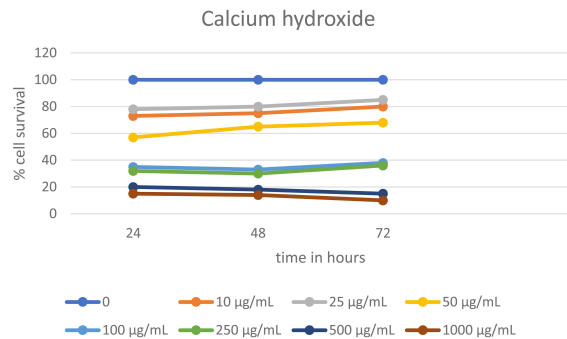


Figure 1: MTT analysis for SHEDs cultured in calcium hydroxide at various dilutions after 24 hrs, 48 hrs and 72 hrs incubation.

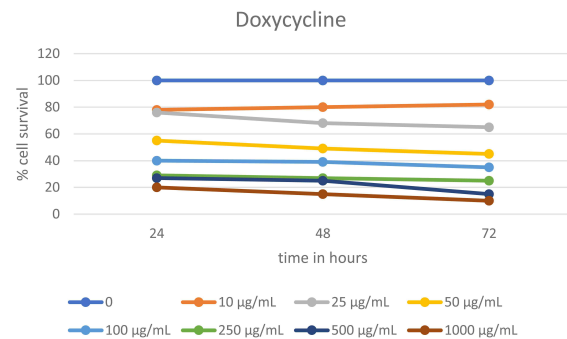


Figure 2: MTT analysis for SHEDs cultured in doxycycline at various dilutions after 24hrs, 48 hrs and 72 hrs incubation.

DISCUSSION

The goal of this trial was to examine the effect of several antibacterial intracanal medicaments at varying concentrations on the viability and survival of SHEDs over time. The results suggested that SHEDs were more viable when exposed to all medicaments at lower concentrations for 48 or 72 hours, but were tolerated at elevated doses when exposed for 24 hours. Both the concentration and duration of intracanal medication exposure affected the survivability of SHEDs.

MSCs population is expected to be positive for CD 105, CD 73, and CD 90 as per the phenotype requirements of International Society for Cell Therapy (ISCT). Furthermore, such cells should not express CD 14, CD 45, CD 34, CD 19, HLA class II, CD 79a or CD11b¹⁹. The present study used CD 45, HLA-DR, and CD 45 as negative markers, while CD 105, CD 73, and CD 90 were used as positive markers. Phenotypical analysis of SHED in the present study showed high positivity for positive markers CD 73 (93.9 %), CD 90 (94.60 %)

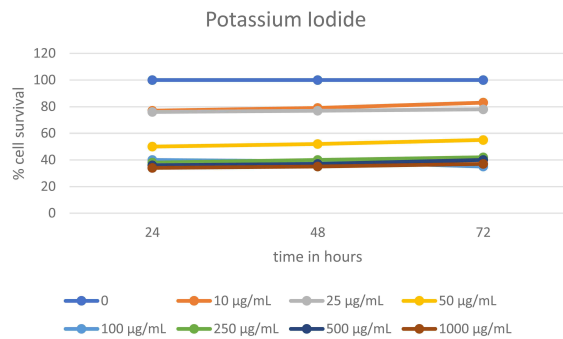


Figure 3: MTT analysis for SHEDs cultured in potassium iodide at various dilutions after 24hrs, 48 hrs and 72 hrs incubation.

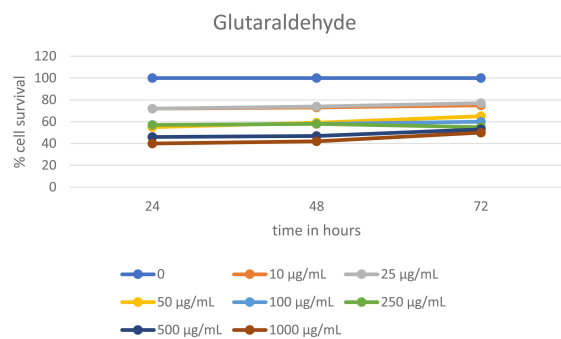


Figure 5: MTT analysis for SHEDs cultured in glutaraldehyde at various dilutions after 24hrs, 48 hrs and 72 hrs incubation.

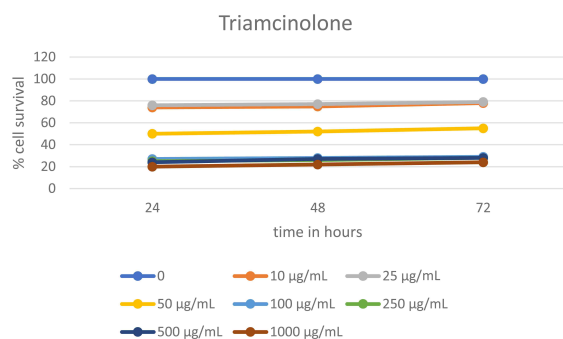


Figure 4: MTT analysis for SHEDs cultured in triamcinolone at various dilutions after 24hrs, 48 hrs and 72 hrs incubation.

and low positivity was observed for CD 105 (35.30 %) which is commonly expressed by endothelial progenitors. These results were consistent with other studies²⁰⁻²².

Tetrazolium compounds are employed to produce a quantifiable colorimetric assay to evaluate human cell proliferation and survival. Hence, the MTT assay has been used extensively to the extent that it is now the typical technique for assessing cell viability¹⁶. Therefore, the present study used the same technique for determining cell viability. Cells that have been constantly exposed to medications for up to 72 hours are analyzed to assess the medication’s optimum efficiency and efficacy²³. As a result, the MTT assay was assessed at intervals of 24, 48, and 72 hours.

Calcium hydroxide is a disinfectant that is approved for intracanal use during regenerative endodontic operations²⁴. Calcium hydroxide increases the proliferation, osteogenic differentiation, and mineralization of Dental Pulp Stem Cells (DPSCs) via the mitogen-activated protein kinase pathway²⁵. This could be the probable reason for calcium hydroxide exposure up to 25 g/mL, not significantly affecting the viability (after 72 hrs incubation), ALP activity (after 21-days) and apoptosis of SHEDs in the present trial. This finding corroborated with a study showing calcium hydroxide as non-toxic to apical papilla stem cells (SCAPs) and promotes cell survival and proliferation²⁶. However, cytotoxicity testing of

calcium hydroxide against SCAPs at its minimal bactericidal concentration revealed that it was more harmful than antibacterial combinations²⁷. This fact was supported by a study that highlighted that both dosage and exposure time contribute to calcium hydroxide cytotoxic response²⁸. However according to Neelakantan *et al.*²⁹ calcium hydroxide inhibits microbial growth for 48 hrs; after which, it declines.

Doxycycline has previously been shown to be an effective intracanal antibacterial irrigant and medicament^{30,31}. However, the amounts of doxycycline utilized in these trials were far higher than those identified in our research to be cytotoxic. The present trial reported cytotoxicity with doxycycline at a concentration of 250 µg/mL after 24 hrs incubation. This result contradicted the findings of Bhandi and colleagues²⁸, who reported that doxycycline induced considerable cytotoxicity after 24 hours and 48 hours at a concentration level of 25 g/mL and 10 g/mL respectively. These were the lowest concentrations observed for any of the medicaments examined. Due to its increased tolerance for brief exposure, it is well suited for use as an intracanal irrigant. However, the concentration must be kept low due to the substance’s substantivity, which lasts up to three weeks following 10 minutes of exposure³⁰. The present trial found doxycycline with the least number of live cells which was in accordance with the study conducted by Bhandi and *et al.*²⁸ who reported the highest apoptosis of DPSCs with doxycycline. The likely explanation is that prior research has established that the second-generation tetracycline derivative doxycycline disrupts mitochondrial proteostasis and physiology, decreases proliferation of a variety of cell types, and promotes apoptosis³⁰.

Potassium iodide has been utilized as a disinfectant and antibacterial agent. It is a disinfecting irrigant solution that is highly effective³¹. The SHEDs survival significantly decreased at a dose of 50 µg/mL post 24 hrs incubation. The number of live SHEDs cultured in potassium iodide was less when compared to control. The ALP activity of SHEDs cultured in potassium iodide was significantly low when compared to control. However, the present trial found potassium iodide better than doxycycline. This finding is in accordance with a trial conducted by Bhandi and co-workers²⁸.

Triamcinolone, a corticosteroid, has been used in combi-

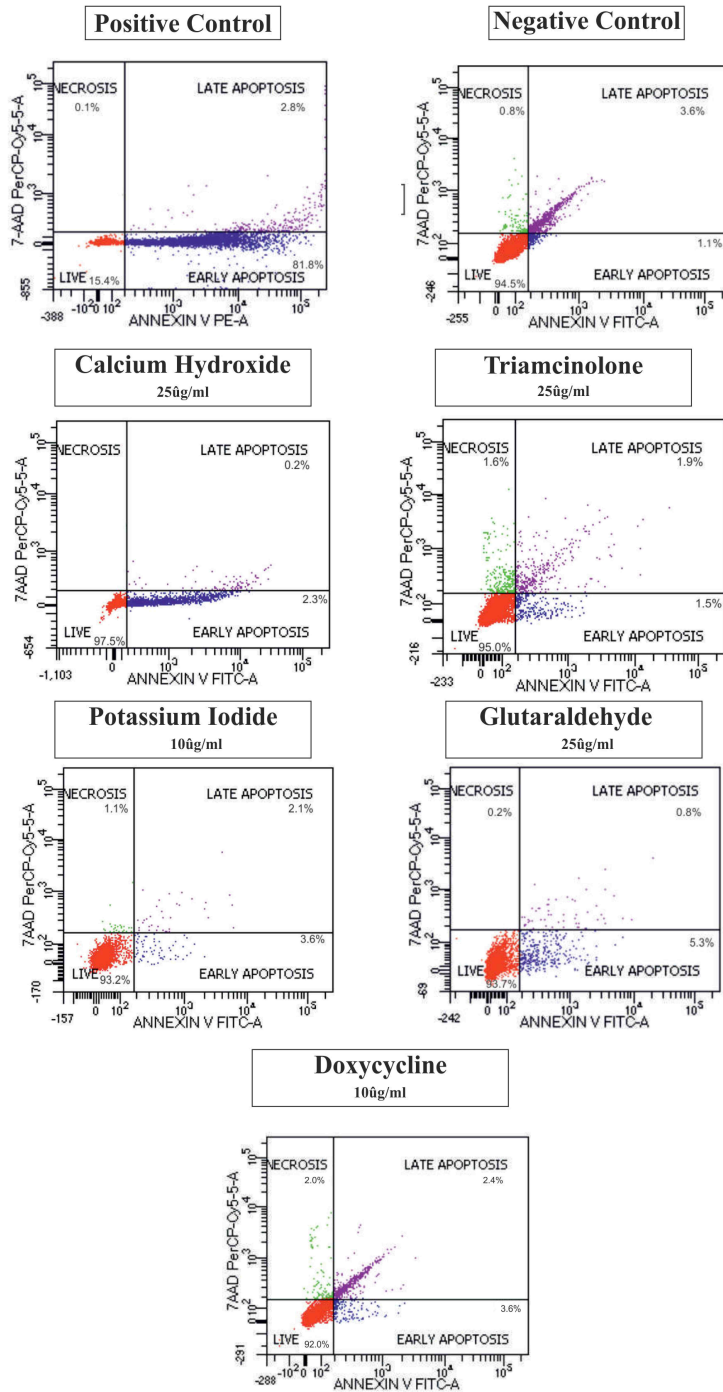


Figure 6: Representative 2D flow cytometry dot plot of data derived from FITC-AnV and 7-AAD stained SHEDs cultured in various intra canal medicaments.

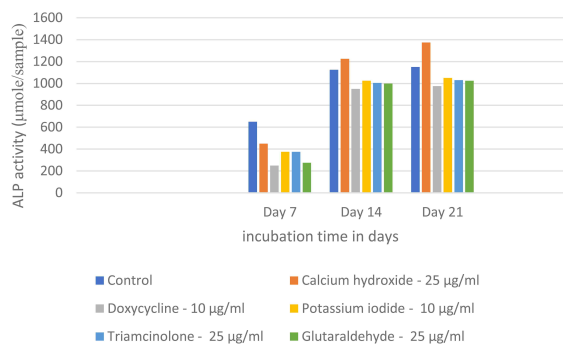


Figure 7: ALP activity of SHEDs cultured in various intra canal medicaments.

nation with tetracycline as an initial dressing for individuals with endodontic discomfort undergoing pulp capping procedures³². The present trial reports triamcinolone with cytotoxicity at concentration of 100 µg/mL or more after 24-hrs incubation. This finding is further confirmed by the results of the study conducted by Bhandi *et al.*²⁸ The number of live cells was suggestively greater than the control. However, the ALP activity was significantly lower than the control. Triamcinolone's role in the hepatogenic development of stem cells, such as the differentiation and proliferation of macrophages, adipocytes, and osteocytes, may explain these data³³. These findings were in accordance with the study conducted by Wemes and co-workers³⁴ that evaluated the response of corticosteroids and found a dose-dependent association on the viability of MSCs.

Glutaraldehyde has been utilized as an irrigant and as an intracanal medicament in the past³⁵. The present study reported a significant reduction in SHEDs survival at a concentration of 50 µg/mL or more after 24 hrs incubation. The number of live cells and ALP activity of SHEDs cultured in glutaraldehyde was significantly less when compared to control. These facts were further confirmed by the study conducted by Bhandi and co-workers²⁸. Shi J observed that the presence of glutaraldehyde in mouse lymphoma cells could cause considerable cytotoxic and mutagenic consequences³⁶.

The study's shortcoming was that it examined the impact of the medications up to 72 hours after administration for survival and up to 21 days for ALP activity. Clinical recommendations currently recommend that the medication be left in place for 1–4 weeks³⁷. It is critical to understand which medications can be safely and efficiently employed in regeneration therapies without compromising SHEDs viability or regenerative capacity. Hence the present trial assessed MTT assay, apoptosis and necrosis and ALP activity of intra canal medicaments. ALP plays a role in cell growth modulation and serves as an early marker of osteogenic proliferation and differentiation¹⁸.

CONCLUSION

The current research reveals that potassium iodide and glutaraldehyde were significantly less likely of all the medications examined to adversely affect the viability and survival of

SHEDs. Calcium hydroxide, doxycycline and triamcinolone does affect the SHEDs viability and survival at a concentration of 250 µg/mL or higher. After 14 and 21 days of incubation, calcium hydroxide exhibited the maximum ALP activity of all the medicaments examined.

There is also a need for us to concentrate on the long-term impact of these medicaments on SHEDs due to their presence in the dentinal tubules after the medicament is withdrawn from the root canal. Additional research is necessary to determine the antibacterial effect of potassium iodide and glutaraldehyde alone or in combination with other antimicrobial agents.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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