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SYSTEMATIC REVIEW

In-vitro Evaluation for Effects of Intracanal Medicaments on Viability, Proliferation, and Differentiation of Stem Cells From Apical Papilla – A Systematic Review

Morankar RAHUL, Nitesh TEWARI, Vijay MATHUR, Mohammad ATIF, Kalpana BANSAL, Sandeep AGRAWAL,* Riyaz MIR

ABSTRACT

This systematic review aimed to evaluate the cytotoxicity of intracanal medicaments used for root canal disinfection and assess their effect on the proliferation and differentiation potential of stem cells from apical papilla (SCAP). The PubMed/Medline, Cochrane, Scopus, EMBASE, CINAHL and Web of Science databases were searched. Studies evaluating the effect of intracanal medicament on human SCAP (*in-vitro* primary cell culture experiment) were included in this systematic review. The risk of bias analysis of included studies was carried out using the Toxicological data Reliability Assessment tool. The data was analysed for qualitative characteristics. A meta-analysis was not carried out considering the heterogeneity of selected studies in terms of cell culture experiments, methods of analysis and the interpretation of results. Four studies fulfilled the desired inclusion criteria. The different antibiotic pastes and their intracanal concentrations lead to reduction in the SCAP survival compared to calcium hydroxide medicament. The findings were insufficient to make a clear distinction between different antibiotic pastes regarding their cytotoxicity. Within the limitations of the present systematic review, it can be concluded that calcium hydroxide is a relatively better intracanal medicament than antibiotic paste mixtures in terms of their cytotoxicity and effect on proliferation of SCAP.

Keywords: Apical papilla cells, cytotoxicity, regenerative endodontics, root canal medicament, survival

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From the Department of pedodontics and preventive dentistry Centre for Dental Education and Research (M.R. ✉ captainrahul88@gmail.com, N.T., V.M., M.A., K.B.), AIIMS, New Delhi, India; Department of Biochemistry AIIMS Raebareli (S.A.), Uttar Pradesh, India; Department of Biochemistry and Molecular Biology (R.M.), AIIMS, New Delhi, India

**The current affiliation of the author: ICMR-National Institute of Pathology, Safdarjung Hospital Campus, New Delhi, India*

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HIGHLIGHTS

- *In-vitro* findings revealed that the calcium hydroxide is a preferred intracanal medicament over the antibiotic pastes prior to regenerative endodontic procedures in regards of toxicity.
- The quality of evidence is insufficient to give any recommendations regarding the use of concentration and duration of medicament.
- The heterogenicity among the included studies in this systematic review necessitates the need of standard guideline for *in-vitro* cell culture experiments.

INTRODUCTION

Regenerative endodontic procedures (REPs) are the biological alternatives to induce root development in non-vital immature permanent teeth (1-3). A microbe-free biological environment within the root canal system is considered prerequisite for any REP. This is achieved with the use of intracanal irrigants and medicaments. The American Association of Endodontists (AAE) (2016) recommend the use of calcium hydroxide or low concentration of triple antibiotic paste (TAP) which is a mixture of ciprofloxacin, metronidazole, and minocycline in a 1:1:1 ratio (4). Due to crown discolouration from TAP, the AAE also advocate the use of a modified triple antibiotic paste (mTAP) in which the minocycline component is replaced with clindamycin, amoxicillin, or cefaclor. They also recommend a double antibiotic paste (DAP) where the TAP has no minocycline component (5). The AAE (2010) recommends the placement of these pastes in the canal for 1 to 4 weeks. The rationale for the use of paste with multiple antibiotics is to cover the diverse microbial species present inside the root canal system (6). Calcium hydroxide [$Ca(OH)_2$] has been widely used as a root canal medicament. Its antimicrobial activity is mediated via the release of hydroxyl ions in the aqueous environment and alkaline pH (7). Calcium hydroxide-iodoform mixture, calcium hydroxide-chlorhexidine gel, and antibiotic-corticosteroid combinations have also been used for root canal disinfection and management of endodontic complications with variable

success rates (8-10). However, there is no evidence suggesting their use in regenerative endodontic procedures.

The different authors have elucidated the role of mesenchymal stem cells (MSCs) of dental origin in regenerative endodontics (11, 12). These include dental pulp stem cells, periodontal ligament stem cells, stem cells of human exfoliated dentition, stem cells of apical papilla, dental follicular cells, tooth germ progenitor cells, and induced pluripotent stem cells. The apical papilla is a dense reservoir of undifferentiated MSCs with great capacity of proliferation and odontogenic differentiation (13, 14). During the development of the root, Hertwigs epithelial root sheath regulates the SCAPs via epithelial-mesenchymal interactions (15). The apical papilla is present in continuity with the root canal space, in close appproximity to the tooth-apex. Thus, this rich source of stem cells should be preserved for performing REPs (16).

The antimicrobial effect of intracanal medicaments is well established; however, their cytotoxicity on the SCAP is still debatable. Several *in-vitro* studies have evaluated their effect on the survival and proliferation of apical papilla cells (17-24). The concept of regenerative endodontics revolves around the availability of SCAP and their ability to sustain in presence of pre-existing infection and proliferate further. The purpose of this systematic review was to determine the cytotoxicity of intracanal medicaments on the SCAP. An attempt was also made to perform the quality analysis of studies and identify the lacunae in the literature for future research.

MATERIALS AND METHODS

This systematic review was carried out according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (25). The protocol was registered with https://share.osf.io/registration/461CC-5FB-9CB.

Information sources

PubMed/Medline, Cochrane, Scopus, EMBASE, CINAHL, and Web of Science databases were searched till 15th December 2020 without any restriction of language and year of publication. Google Scholar and OpenGrey search was performed for the grey literature. The references of eligible studies were also checked to identify the additional papers. Hand searching was performed in the Journal of Dental Research, Journal of Oral Sciences, Acta Odontologica Scandinavica, Journal of Endodontics, International Endodontic Journal, and Pediatric Dentistry. Authors of the studies were contacted for additional details wherever required. Two authors (NT and MR) performed the literature search and selection of the studies independently as per the predefined search strategy. Any disagreement was resolved after consultation with a senior reviewer (VM).

Search strategy

The research question posed for performing the search was: Which intracanal medicament have the least cytotoxic effect on the survival of SCAP? The PICOS for the eligibility criteria was: (P)- Cultured SCAP originally isolated from immature extracted human teeth (I)- Culture of SCAPs in the media containing intracanal medicament/s (C)- Cells cultured in different types of intracanal medicaments/without any medicament (positive control)/cytotoxic agents (negative control) (O)- SCAP cell viability and/or proliferation and/or differentiation (S)- *In-vitro* setting. Animal experiments and studies performed in clinical settings were excluded.

Keywords used for the literature search

The search strategy comprised of text words and MeSH terms. A broad-based search was implemented with individual keywords: "Intracanal medicaments", "Polyantibiotic paste", "TAP", "Triple antibiotic paste", "mTAP", "modified Triple antibiotic paste", "3-mix paste", "DAP", "Diantibiotic paste", "Antibiotic paste", "Calcium hydroxide", "Calcium hydroxide-Iodoform", "Iodoform paste", "Ledermix paste", "Intracanal steroids". Partial search with the Boolean tools AND and OR was done with above individual keywords and with "Stem cells from apical papilla", "SCAP" and "Apical papilla cells" in different possible combinations. The strategy for partial search was (1) Search ((Intracanal medicaments [Text Word]) OR Intracanal medicament [MeSH Terms]) (2) Search ((((Polyantibiotic paste [MeSH Terms]) OR Triple antibiotic paste [Text Word]) OR TAP [Text Word]) OR modified Triple antibiotic paste [Text Word]) OR mTAP [Text Word]) OR 3-mix paste [Text Word]) OR Diantibiotic paste [Text Word] OR DAP [Text Word] OR Antibiotic paste [Text Word] OR Calcium hydroxide [Text Word]) OR Calcium hydroxide-Iodoform [Text Word]) OR Iodoform [Text Word]) OR Ledermix paste [Text Word]) OR Intracanal steroids [Text Word]) (3) Search ((((((((((((SCAP [Text Word] OR Stem cells from apical papilla [Text Word] OR Apical papilla cells [Text Word]. The titles and abstracts were evaluated for suitability of inclusion in the systematic review, and duplicates were removed by the means of EndNote reference management software.

Study selection and eligibility criteria

Inclusion criteria- (a) studies evaluating the effect of intracanal medicament on human SCAP using *in-vitro* cell culture experiment, (b) studies should mention the details of culture media used for SCAPs culture, MSCs surface marker/s used for detection of SCAP, and the passage of cell lines used, (c) studies with results reported in the form of SCAP cell viability and/or proliferation and/or differentiation, (d) studies with complete details of its endpoint/s (results may be at one-time point or more than one-time point), (e) the type of assay used for cell viability/proliferation/differentiation analysis must be mentioned in the experiment. Exclusion criteria- (a) animal studies, (b) human trials carried out without culturing the cells derived from the apical papilla of extracted teeth, (c) studies evaluating the effects on any other type of dental stem cells, (d) studies evaluating the combinations of intracanal medicaments where a distinction cannot be made in the results of individual medicament, (e) systematic reviews or narrative reviews, (f) case reports or case series.

The authors of the full-text articles where the methodology was inadequately explained were contacted before exclusion (17, 18, 20). The list of excluded full-text articles and the reasons for their exclusion are given in Table 1.

Data collection process

A self-designed sheet, pilot tested on two of included studies was used for data extraction. Two calibrated reviewers (MR and

TABLE 1. List of excluded full text articles and the reasons for their exclusion

Articles excluded	Reasons for exclusion
Eshaghali saberi, Narges farhad-mollashahi, Mersad saberi. Interaction of intracanal medicaments with apical papilla stem cells: quantitative cytotoxicity assessment by methyl thiazolyl tetrazolium, trypan blue and lactate dehydrogenase. Minerva stomatol. 2019;68(1):36-41.	Mesenchymal stem cell markers used for isolation of stem cells from apical papilla were not mentioned
Pattama Kitikuson and Tanida Srisuwan. Attachment Ability of Human Apical Papilla Cells to Root Dentin Surfaces Treated with Either 3Mix or Calcium Hydroxide. J Endod 2016;42(1):89-94.	
Panupat Phumpatrakom and Tanida Srisuwan. Regenerative Capacity of Human Dental Pulp and Apical Papilla Cells after Treatment with a 3-Antibiotic Mixture. J Endod 2014;40:399–405.	
Sorapong Chuensombat, Saengusa Khemaleelakul, Siriporn Chattipakorn, Tanida Srisuwan. Cytotoxic Effects and Antibacterial Efficacy of a 3-Antibiotic Combination: An *In Vitro* Study. Endod 2013;39:813–819.	
WE Kantz, PJ Ferrillo, ER. Zimmermanq. Cytotoxicity of three endodontic intracanal Medicaments. Oral surg.1974;38(4):600-604.	Gey strain of He La cells were studies Dental pulp stem cells were studied
Kamocki K, Nör JE, Bottino MC. Dental pulp stem cell responses to novel antibiotic-containing scaffolds for regenerative endodontics. Int Endod J. 2015;48(12):1147-1156.	
Labban N, Yassen GH, Windsor LJ, Platt JA. The direct cytotoxic effects of medicaments used in endodontic regeneration on human dental pulp cells. Dent Traumatol ,2014;30 (6):429-434.	
Palasuk J, Kamocki K, Hippenmeyer L, et al. Bimix antimicrobial scaffolds for regenerative endodontics. J Endod. 2014;40(11):1879-1884.	
Kamocki K, Nör JE, Bottino MC. Effects of ciprofloxacin-containing antimicrobial scaffolds on dental pulp stem cell viability-*In vitro* studies. Arch Oral Biol. 2015;60(8):1131-1137.	
Kim, K.W., G.H. Yassen, Y. Ehrlich, K. Spolnik, J.A. Platt, L.J. Windsor. The effects of radicular dentine treated with double antibiotic paste and ethylenediaminetetraacetic acid on the attachment and proliferation of dental pulp stem cells. Dent. Traumatol.2015; 31:374–379.	
Park M, Pang NS, Jung IY. Effect of dentin treatment on proliferation and differentiation of human dental pulp stem cells. Restor Dent Endod. 2015;40(4):290-298.	
Alghilan MA, Windsor LJ, Palasuk J, Yassen GH. Attachment and proliferation of dental pulp stem cells on dentine treated with different regenerative endodontic protocols. Int Endod J. 2017; 50:667–75.	
McIntyre PW, Wu JL, Kolte R, Zhang R, Gregory RL, Bruzzaniti A, et al. The antimicrobial properties, cytotoxicity, and differentiation potential of double antibiotic intracanal medicaments loaded into hydrogel system. Clin Oral Investig. 2019;23(3):1051-1059.	
Thesis, master of science in dentistry. Adam r. Everhart. The effects of nano-hydroxyapatite in a double antibiotic paste- loaded methylcellulose carrier on dental pulp stem cells. Indiana university school of dentistry,2019.	
Öncel Torun, Z, Torun D, Demirkaya K, Yavuz, S, Elçi, M, Sarper, M, et al. Effects of iRoot BP and white mineral trioxide aggregate on cell viability and the expression of genes associated with mineralization. Int. Endod. J. 2015, 48, 986–993.	
Chen, L, Zheng, L, Jiang, J, Gui, J, Zhang L, Huang, Y, et al. Calcium Hydroxide– induced Proliferation, Migration, Osteogenic Differentiation, and Mineralization via the Mitogen activated Protein Kinase Pathway in Human Dental Pulp Stem Cells. J Endod.2016;42(9):1355-1361.	
Saberi EA, Karkehabadi H, Mollashahi NF. Cytotoxicity of Various Endodontic Materials on Stem Cells of Human Apical Papilla. Iran Endod J. 2016;11(1):17-22.	Materials tested were not used as an intracanal medicament
Hosseini Matin M, Zare Jahromi M, Fesharaki M, Ostad Sharif M. Cytotoxicity of Triple Antibiotic Paste and Calcium Hydroxide against Cultured Human Dental Pulp Fibroblasts. J Dent Sch 2015; 33(3): 196-204.	Human dental pulp fibroblasts were studied
Ferreira MB, Myiagi S, Nogales CG, Campos MS, Lage-Marques JL. Time- and concentration-dependent cytotoxicity of antibiotics used in endodontic therapy. J. Appl. Oral Sci. 2010, 18, 259–263.	Human gingival fibroblasts were studied
Gougousis, K, Giannakoulas DG, Taraslia, V, Agrafioti A, Anastasiadou, E, Kontakiotis EG. Number of Dental Stem Cells on Root Canal Dentin after Application of Triple Antibiotic Paste or Calcium Hydroxide: An *In Vitro* Study. European journal of dentistry. 2019;13(2):161–165.	Stem cells from exfoliated and deciduous teeth (SHED) were studied
Gerald L. Vander Wall, John Dowson, Charles Shipman Jr. Antibacterial efficacy and cytotoxicity of three endodontic drugs. Oral Surgery, Oral Medicine, Oral Pathology.1972;33(2):230-241.	Baby hamster kidney cell line (BHK) and a diploid human embryonic lung cell line (HEL) were studied
Carla renata sipert, Aline pereira oliveira, Aelso luiz caldeira. Cytotoxicity of intracanal dressings on apical papilla cells differ upon activation with e. faecalis lta. j appl oral sci. .2019 27:e20180291.	
Juliana garuba rahhal, Emanuel da silva rovai,Marinella holzhausen,Celso luiz caldeira, Carlos ferreira dos santos,Carla renata sipert. Root canal dressings for revascularization influence *in vitro* mineralization of apical papilla cells. J appl oral sci. 2019;27:e20180396.	Characterization was done for mesenchymal origin using vimentin, however, surface markers used for SCAP identification were not mentioned

SCAP: Stem cells from apical papilla

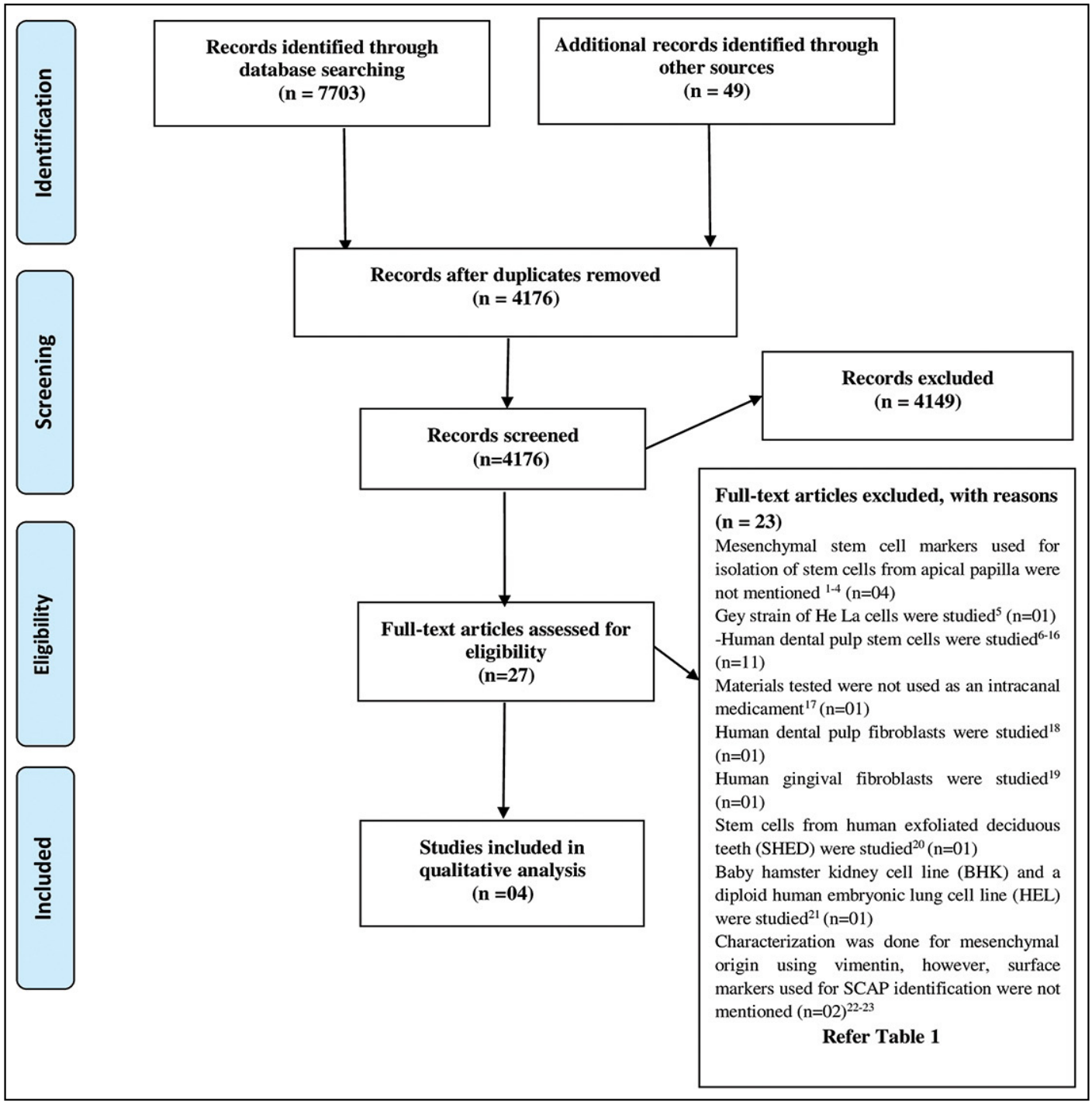

Figure 1. PRISMA flowchart

NT) collected the data from the included studies. Depending on the different variables collected, the Cohen's kappa values between examiners ranged from 0.70 to 0.80. In case of disagreement, the third reviewer (VM) was consulted and it was resolved by consensus.

Risk of Bias of individual studies

ToxRTool (Toxicological data Reliability Assessment tool) was used for the assessment of the quality of the included studies (26). This tool provides an exhaustive criteria and enlightenments for the evaluations of the quality of toxicological data. Each study was assessed for risk of bias using five criteria [test substance/drug identification (maximum points-4), test organism/cell characterization (maximum points-3), description of study design (maximum points-6), documentation of study results (maximum points-3), and plausibility of study design and results (maximum points-2)] by two reviewers (MR and MA) independently. In an event of disagreement, senior reviewer NT was consulted and his opinion was considered as final. ToxR tool comprised of an 18-point rating checklist for the assessment of methodological aspects of each study. Studies scoring less than 11 points were considered unreliable, those with scores 11-14 points were reliable with possible restrictions and studies with scores 15-18 points were reliable without restrictions.

TABLE 2. Demographic details and characteristics of studies included in the systematic review

Author	Year	Journal	Country	Type of study	Intracanal medicament	Concentration of intracanal medicament
Khoshkhounejad M et al. (19)	2019	European Journal of Dentistry	Iran	*In-vitro*	1. TAP (metronidazole, ciprofloxacin, and minocycline) 2. mTAP (metronidazole, ciprofloxacin, and clindamycin) 3. DAP (metronidazole, ciprofloxacin) 4. Ca $(OH)_2$ [Chemical Biodynamics e Pharmaceutica LTDA, Ibiporã, PR, Brazil] 5. Augmentin	Minimum inhibitory concentration, Minimum bactericidal concentration, Minimum biofilm inhibitory concentration
Bi J et al. (27)	2018	International Endodontic Journal	China	*In-vitro*	1. iRootFM (Innovative Bioceramix Inc., Vancouver, BC, Canada) composed of calcium silicates, zirconium oxide, calcium phosphate monobasic, calcium hydroxide and filler agents	
					2. Ca $(OH)_2$ [(Solarbio, Beijing, China) mixture of Calcium hydroxide powder and sterile water]	Ca $(OH)_2$ – Powder and sterile water at a ratio 0.17 g /0.1 mL
					3. TAP (Metronidazole, ciprofloxacin and minocycline in a 1: 1: 1 ratio)	TAP- 0.1mg/mL
Althumairy, RI et al. (23)	2014	Journal of Endodontics	USA	*In-vitro*	1. TAP (Metronidazole, ciprofloxacin, and minocycline in a 1:1:1 ratio) 2. DAP (Metronidazole and ciprofloxacin in a 1:1 ratio)	1 mg/mL (a watery consistency) Or
					3. Ca $(OH)_2$ [Ultracal (Ultradent, South Jordan, UT) composed of Calcium hydroxide and Barium sulphate]	1000 mg/mL (a paste consistency)
Ruparel NB et al. (28)	2012	Journal of Endodontics	USA	*In-vitro*	1. TAP (metronidazole, ciprofloxacin, and minocycline in a 1:1:1 ratio) 2. DAP (metronidazole and ciprofloxacin in a 1:1 ratio) 3. Modified TAP -mTAP (metronidazole, ciprofloxacin and cefaclor in a 1:1:1 ratio) 4. Augmentin 5. Ca $(OH)_2$ [Ultracal (Ultradent, South Jordan, UT) composed of Calcium hydroxide and Barium sulphate]	0.01, 0.1, 1, 10 and 100 mg/mL

TAP: Triple antibiotic paste, mTAP: Modified triple antibiotic paste, DAP: Double antibiotic paste

Statistical analysis

The included studies had variations in terms of medicaments used, their concentrations, assessment methods, intervals of assessment and interpretation of results. Considering the heterogeneity of the data, it was only analyzed for descriptive characteristics without meta-analysis. Cohen's kappa statistics was used to determine inter-reviewer agreement.

RESULTS

Study selection

The search conducted in six electronic databases identified the 7703 articles. Forty-nine additional records were found via other sources. After elimination of duplicates and evaluation of titles and abstracts, 27 full-text articles were short-listed. They were further assessed for eligibility and finally four articles were included for qualitative data analysis, and reliability assessment. The details of the search have been presented as PRISMA chart in Figure 1.

Study characteristics

Table 2 describes the demographic details of the included studies-author name, year, journal name, country, study design, type of intracanal medicament used, and their concentration. Table 3 describes the characteristics of SCAP cells used in individual studies such as their source, MSC surface markers used for identification, the passage of cell line, and the cell culture media used. The assessment of the viability, proliferation, and differentiation of SCAP with details of their methods, intervals, and the results have been described in Table 4 and Table 5.

Quality assessment of individual studies according to the ToxR Tool

The agreement scores between the two reviewers (MR and MA) were 75% which shows the substantial agreement. The four eligible studies were assessed for the inherent quality of toxicological data. As shown in Figure 2, three studies were considered reliable without restrictions (score 15 or more) and one study with a score of 14 was reliable with restrictions.

TABLE 3. Source of stem cells from apical papilla (SCAP) and their characteristics

Author	Year	Source of SCAP cells used	Passage cell line used	Surface marker/s for SCAP cell identification	Culture media used
Khoshkhounejad M et al. (19)	2019	Immature extracted human mandibular third molar	Third to fourth	STRO-1	DMEM with 10% FBS, L-glutamine and antibiotics
Bi J et al. (27)	2018	Human impacted third molars with immature roots	Third	Positive for CD73, CD90, CD105, CD146 Negative for CD31 and CD34	α-MEM with 15% FBS, L-ascorbic acid and antibiotics
Althumairy, RI et al. (23)	2014	Previously characterized SCAP cell line (RP-89) obtained from immature human extracted third molars	Fifth to seventh	Previously characterized SCAP cell line (RP-89) with molecular markers CD73, CD90, and CD105	α-MEM with 10% FBS, L-glutamine and antibiotics
Ruparel NB et al. (28)	2012	Apical papillae of extracted immature human mandibular third molars	Fifth to eighth	STRO-1	α-MEM with 10% FBS, L-glutamine and antibiotics

DMEM: Dulbecco's Modified Eagle Medium, α-MEM: Alpha minimum essential medium, FBS: Fetal bovine serum

Calcium hydroxide vs. Triple antibiotic paste (TAP)- effect on SCAP survival

All the included studies compared calcium hydroxide and triple antibiotic paste in multiple concentrations in terms of SCAPs survival (19, 23, 27, 28). A study (19) had reported nearly similar cytotoxicity with TAP and calcium hydroxide at minimum inhibitory concentration (0.2 mg/mL) whereas it was significantly high for the calcium hydroxide at a minimum bactericidal concentration (16 mg/mL). Two studies (23, 28) found increased viability of SCAPs with calcium hydroxide as compared to TAP, whereas no significant change was observed in the loss of viability and cell membrane integrity with various doses of calcium hydroxide and low doses (0.01 and 0.05 mg/mL) of TAP in one study (27). Although, the changes were significant when SCAPs were cultured in more than 0.1 mg/mL of TAP.

Calcium hydroxide vs. modified Triple antibiotic paste (mTAP)- effect on SCAP survival

A study (19) had reported higher cytotoxicity to SCAPs with calcium hydroxide compared to mTAP at minimum bactericidal concentration (MBC) whereas it was less for mTAP at minimum inhibitory concentration (MIC). Another study (28) had compared calcium hydroxide and modified Triple antibiotic paste at concentrations of 0.01, 0.10, 1.00, 10.0, and 100 mg/ml and found that calcium hydroxide has no effect on SCAP cells' viability at any concentration. However, there was a significant dose-dependent cytotoxic effect on cell viability with mTAP.

Calcium hydroxide vs. Diantibiotic paste (DAP)- effect on SCAP survival

One study (28) found the detrimental effects of DAP on the SCAPs with less than 20% viability when exposed to 10 mg/ml and 100 mg/ml. The survival rate was observed to be 33% to 56% when exposed to 1 mg/ml of medicament. No detrimental effect was found at any of the concentrations of calcium hydroxide. Another study (23) had found no effect of DAP on the viability of SCAP at low concentration (1 mg/ml) whereas the concentration of 1000 mg/ml was highly cytotoxic. They also found the increased SCAP survival with calcium hydroxide compared to DAP whereas more cytotoxicity was observed with calcium hydroxide compared to DAP at MBC in one study (19).

TAP vs. mTAP vs. DAP- effect on SCAP survival

Three studies have compared the cytotoxicity of different antibiotic combinations on SCAP (19, 23, 28). One study (19) found that at MIC, MBC, and MIBC, the cytotoxicity of TAP, DAP, and mTAP was nearly same with TAP being least cytotoxic. No significant difference was observed between TAP and DAP in terms of SCAP survival both at 1mg/ml and 1000 mg/ml concentrations as per the results of another study (23). One of the studies (28) also showed TAP, DAP, and mTAP to have detrimental effects on the survival of SCAP with less than 20% viability when exposed to each of the three combinations at concentration 100 mg/ml and 10 mg/ml, while the cell survival rate ranged from 33% to 56% at 1 mg/ml.

Assessment of proliferation of SCAP

Two studies have evaluated the proliferation of SCAP after exposure to intracanal medicaments (19, 27). One study (19) had used the WST-1 assay at MIC, MBC, and MBIC that demonstrated the highest proliferation rate with TAP followed by DAP and mTAP. The proliferation was lowest for Augmentin at MIC and calcium hydroxide at MBC. Another study (27) with CCK-8 assay found that the SCAP cultured in a medium with iRoot FM was associated with greater proliferation compared to those cultured in calcium hydroxide or TAP.

Assessment of differentiation of SCAP

Only one study (27) evaluated the effects of intracanal medicament on the differentiation potential of SCAP. They found an increase in mineralization with the iRoot FM compared to calcium hydroxide, TAP, and control groups. They also observed a decrease in mineralization after exposure to TAP.

TABLE 4. Intracanal medicaments used, methods of viability assessment and results

Author	Intracanal medicament	Concentration of intracanal medicament	Viability assessment method	Intervals of viability assessment	Observations
Khoshkhounejad M et al. (19)	1. TAP (metronidazole, ciprofloxacin, and minocycline) 2. mTAP (metronidazole, ciprofloxacin, and clindamycin) 3. DAP (metronidazole, ciprofloxacin) 4. $Ca(OH)_2$ [Chemical Biodynamicse, Pharmaceutica LTDA, Ibiporã, PR, Brazil] 5. Augmentin	Minimum inhibitory concentration, Minimum bactericidal concentration Minimum biofilm inhibitory concentration	LDH Assay-cytotoxicity WST-1 Assay - viability	Day 3	At minimum inhibitory concentration, the cell cytotoxicity for TAP, DAP, mTAP, Augmentin and calcium hydroxide were 12.19%, 15.82%,16.14%,23.89 and 13.18% respectively At minimum bactericidal concentration, the cell cytotoxicity for TAP, DAP, mTAP, Augmentin and calcium hydroxide were 17.79%,23.56%,21.75%,24.22 and 94.73 % respectively At minimum biofilm inhibitory concentration, the cell cytotoxicity for TAP, DAP, mTAP and Augmentin was 17.79%,18.62%,22.73%, and 24.22% respectively
Bi J et al. (27)	1. iRoot FM 2. $Ca(OH)_2$ [(Solarbio, Beijing, China) mixture of Calcium hydroxide powder and sterile water 3. TAP (Metronidazole, ciprofloxacin and minocycline in a 1: 1: 1 ratio)	$Ca(OH)_2$ – mixing powder and sterile water at a ratio 0.17 gm /0.1 mL TAP-0.1mg/ml	CCK-8 assay	1, 3, 5 days	CCK-8 assay showed iRoot FM (0.5 mg/mL) had a higher cell viability than $Ca(OH)_2$ (0.5 mg/mL) and TAP (0.01 mg/ml at both 3 and 5 days
Althumairy, RI et al. (23)	1. TAP (Metronidazole, ciprofloxacin, and minocycline in a 1:1:1 ratio) 2. DAP (Metronidazole and ciprofloxacin in a 1:1 ratio) 3. $Ca(OH)_2$ [Ultracal (Ultradent, South Jordan, UT) composed of Calcium hydroxide and Barium sulphate]	1 mg/mL (a watery consistency) Or 1000 mg/mL (a paste) consistency	Cell Titer-Glo luminescence assay	7 and 28 days	At 1000 mg/mL concentration of either TAP or DAP was completely lethal to SCAP after 7 days, they were not tested further for 28 days whereas same concentration of $Ca(OH)_2$ increases the cell survival and proliferation At 1 mg/mL concentration of either TAP or DAP, no significant difference in SCAP survival compared with the control group whereas $Ca(OH)_2$ produced significantly greater SCAP survival and proliferation when compared with TAP, DAP, or the control group.
Ruparel NB et al. (28)	1. TAP (metronidazole, ciprofloxacin, and minocycline in a 1:1:1 ratio) 2. DAP (metronidazole and ciprofloxacin in a 1:1 ratio) 3. Modified TAP -mTAP (metronidazole, ciproflo-xacin and cefaclor in a 1:1:1 ratio) 4. Augmentin 5. $Ca(OH)_2$ [Ultracal (Ultradent, South Jordan, UT) composed of Calcium hydroxide and Barium sulphate]	0.01,0.1, 1, 10 and 100 mg/mL	TC10 automated cell counter after incubation with TC10 trypan blue	Day 3	TAP evoked a concentration-dependent decrease in viable SCAPs. TAP at the concentrations 1, 10, and 100 mg/mL resulted in 58.0%±12.4%, 8.0%±1.8%, and 1.3%±0.5% SCAP survival, respectively. TAP, DAP, mTAP, and Augmentin have detrimental effects on the survival of stem cells with less than 20% viability observed when exposed to each of the 4 drugs at 100 mg/mL and 10 mg/mL whereas, a survival rate from 33% to 56% was observed when cells were exposed to 1 mg/mL either medicament. A 100% survival rate was observed when greater dilutions were used. Calcium hydroxide (Ultracal) had no detrimental effect on the SCAP survival at any of the concentrations tested, it significantly increased the proliferation/ survival of SCAPs at the concentration of 1 mg/mL, resulting in an increase of 68.3% ±15% in the viable SCAP numbers.

TAP: Triple antibiotic paste, mTAP: Modified triple antibiotic paste, DAP: Double antibiotic paste, CCK-8: Cell counting kit 8

TABLE 5. Assessment of proliferation and differentiation of stem cells from apical papilla

Author	Proliferation assessment method and intervals	Differentiation assessment method and intervals	Observations
Khoshkhounejad M et al. (19)	WST-1 assay 1, 3, 5 days	-	At minimum inhibitory concentration, the cell proliferation for TAP, DAP, mTAP, Augmentin and calcium hydroxide were 219.17%, 151.25%,164.63%,84.66% and 159.41% respectively At minimum bactericidal concentration, the cell proliferation for TAP, DAP, mTAP, Augmentin and calcium hydroxide were 220.87%,180.53%,130.53%,66.93% and 17.94 % respectively At minimum biofilm inhibitory concentration, the cell proliferation for TAP, DAP, mTAP and Augmentin was 220.87%,206.87%,99.69%, and 66.93% respectively
Bi J et al. (27)	CCK-8 assay 1, 3, 5 day	Osteogenic-Alizarin red S after 4 weeks	Low doses of iRoot FM (0.1 and 0.5 mg/mL) significantly elevated the proliferation rate of SCAP (P<0.001), especially at the concentration of 0.5 mg /mL. High doses of iRoot FM (1.0 and 2.5 mg/mL) had no significant effects on the proliferation rate of SCAP. Low doses of $Ca(OH)_2$ (0.1 and 0.5 mg/mL) also improved the proliferation rate of SCAP (0.1 mg/mL, P=0.043; 0.5 mg/mL, P<0.001), whilst high doses (1.0 and 2.5 mg/mL) decreased the proliferation rate (1.0 mg/mL, P=0.009; 2.5 mg/mL, P<0.001) However, TAP significantly suppressed SCAP proliferation at all doses except at the dose of 0.01 mg/mL (P=0.082). iRoot FM had a greater capacity to elevate SCAP proliferation in 3 and 5 days than the $Ca(OH)_2$ and TAP groups. After 4 weeks of differentiation iRoot FM increased mineralized nodules formation compared with the $Ca(OH)_2$, TAP and control groups, whilst TAP decreased the formation of mineralized nodules of SCAP.
Althumairy, RI et al. (23)	-	-	Proliferation and survival data presented as a single entity
Ruparel NB et al. (28)	-	-	Proliferation and survival data presented as a single entity

WST-1: Assay for Cell Proliferation and Viability, iRoot FM: A Novel intracanal medicament, CCK-8: Cell counting kit 8, TAP: Triple antibiotic paste, DAP: Double antibiotic paste, mTAP: Modified triple antibiotic paste, SCAP: Stem cells from apical papilla

DISCUSSION

The objective of this systematic review was to analyze the cytotoxicity of various intracanal medicaments used for root canal disinfection prior to regenerative endodontic procedures. The results revealed that calcium hydroxide was relatively lesser cytotoxic to SCAP cells compared to the different antibiotic paste combinations. The regeneration of pulp-dentin complex with REPs requires disinfection of root canal space which can be achieved with the use of suitable intracanal medicaments. AAE guidelines (2016) recommend the use of calcium hydroxide or antibiotic paste for 1-4 weeks for REPs. Similar to all regenerative medicine approaches, these procedures also require an interplay of stem cells, scaffolds, and growth factors. As a result, it is important that the SCAP available after disinfection are in adequate number and quality. This is a difficult proposition due to the known cytotoxic properties of popular intracanal medicaments, although, the findings of this systematic review revealed that calcium hydroxide and low concentration antibiotic pastes are less cytotoxic.

Due to the accessibility to the collateral circulation, the apical papilla tends to wall off the necrosis of pulp and survive even though both the tissues are in continuity to one another (13, 29). This can be also due to the infection-resistant nature of SCAP (30, 31). It is also important to understand that the presence of SCAP is essential for root development/maturation (13, 32-34). Therefore, maintaining the survival of SCAPs is essential for the success of REPs. Considering the crucial role of SCAP, their survival was assessed by several authors after exposure to intracanal medicaments. Furthermore, the proliferation and differentiation of SCAP were also investigated by researchers (19, 27). To evaluate the evidence exclusively for SCAP, this systematic review included only the studies mentioning MSC markers for SCAP identification. The proliferation, differentiation, and expression of MSC markers demonstrate the stemness of cultured cells derived from the apical papilla. SCAPs are located in the perivascular region and show positive expression of MSC markers such as STRO-1 and CD146 with both fading with each passage (35-37). Moreover, SCAPs also show positive expression of CD73, CD90, and CD105 (38). The low passage cell lines are generally preferred for studying the differentiation of cells, however, using the cell lines with consistent growth properties should be pursued as a rule (39, 40).

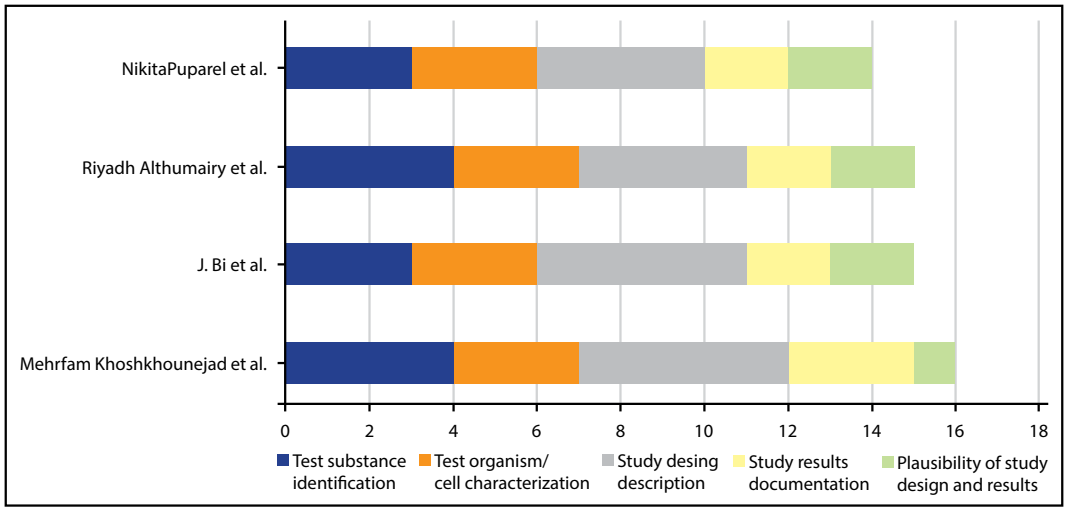

Figure 2. Quality assessment of the selected articles according to the Toxicological data Reliability Assessment Tool (ToxR Tool) *in-vitro* criteria

This systematic review involved the qualitative synthesis of four *in-vitro* studies fulfilling the inclusion criteria. We do not come across any substantial evidence supporting the AAE recommendation for the use of calcium hydroxide or low concentration of triple antibiotic paste (0.1-1.0 mg/ml) as an intracanal medicament prior to REP. In actual clinical scenario, the standard formulations of triple antibiotic paste are not readily available and it is formulated by mixing the powders of different antibiotics in a desired proportions with the normal saline. In order to obtain a paste-like consistency more antibiotic powder is required that further increases the concentration to as high as 1000 mg/ml (23). Concentrations in this range can be highly detrimental to SCAP survival. The modifications of TAP (mTAP, DAP) have been considered for its use as an intracanal medicament pertaining to its tooth discoloration potential. Similarly, Nashaat et al. (2021) (41) in their study compared the cytotoxicity of TAP in combination with an anti-inflammatory drug Catafast (TAPC) in its Nano and regular forms with calcium hydroxide and found that the cytotoxicity of Nano TAPC was lower than calcium hydroxide whereas it was higher compared to regular TAPC.

$Ca(OH)_2$ is one of the commonly used intracanal medicaments for root canal disinfection during the REPs. However, the conventional CH paste may not be optimal as paste carriers may not reach the apical area, thus affecting the efficiency of the disinfection procedure (42). Different vehicles, such as propylene glycol, distilled water, physiological saline, anaesthetics, and glycerin have been used to improve their canal insertion (43). They determine the speed of ionic dissociation, thus allowing the absorption of the paste by the root canal and the periapical tissues (9). Barium sulphate has also been included in commercial products to improve their radiopacity whereas camphorated para-monochlorophenol, and chlorhexidine, have also been incorporated to enhance the antimicrobial effect (7, 44). Thus, the composition of different $Ca(OH)_2$ pastes varies greatly which influences their ability of canal disinfection as well as their cytotoxic potential to the stem cells.

AAE guidelines (2016) recommend the intracanal medicaments for 1-4 weeks for adequate disinfection of the root canal. However, there were considerable variations in the endpoints of SCAP viability assessment among the included studies. It has been observed that the concentration of intracanal medicaments used has more impact on the survival of stem cells compared to the duration of contact (23, 27). It was found that when the concentration of antibiotic paste was 1000 mg/ml, it leads to total loss of viability of stem cells in the first 7 days. However, when the concentration used was 0.01 mg/ml, no significant effect was seen on the viability (23). Another study revealed that low concentration of iRoot FM (0.5 mg/ml), calcium hydroxide (0.5 mg/ml), and TAP (0.01 mg/ml) results in significantly more number of viable stem cells compared to higher concentration when analyzed at day 1, 3 and 5 days (27). Among the included studies, one study (19) had used LDH Assay and WST-1 Assay for viability assessment, whereas others had used CCK-8 assay, Cell Titer-Glo luminescence assay, and TC10 automated cell counter. Therefore, even though the outcome was similar, the method of outcome assessment was variable among the different studies.

The risk of bias assessment is an integral component of any systematic review. It helps to identify the quality of evidence available in the literature and whether it is reliable to draw any conclusion from their results. There is a number of tools to assess the quality of animal studies and human trials. However, a standardized quality assessment tool for *in-vitro* cell culture studies is not available. The Toxicological Data Reliability Assessment tool was the most appropriate tool available for the reliability of *in-vitro* cell culture studies included in the present review. It comprised of 18 items; however, many items were subjective thus can lead to an assessment bias. Although, the risk of bias assessment was done by two reviewers (MR and MA) with 75 percent agreement scores, still we emphasize the need for a better risk of bias assessment tool for *in-vitro* cell culture studies.

The heterogenicity of the included studies in terms of designs, methods, and interpretation of results make it difficult to pool the results and perform a quantitative synthesis. Although, iRootFM a novel bio-ceramic material was used in only one study, it was found better compared to TAP and calcium hy-

droxide (27). It has significantly elevated the proliferation rate of SCAP at low doses (0.1 and 0.5 mg/ml) whereas no significant effects were observed at high doses (1.0 and 2.5 mg/ml). The majority of included studies revealed calcium hydroxide better in terms of viability and proliferation of SCAP compared to other medicaments. However, a contradictory observation was made in one study (19) that found calcium hydroxide being more cytotoxic than antibiotic pastes (TAP, mTAP, DAP and Augmentin) at their minimum bactericidal concentration (16 mg/ml). This concentration was found to be much higher than the concentrations used in other studies. When the results were compared for TAP, DAP, and mTAP, a distinction could not be made in this review. Only one study (27) had assessed the differentiation potential of SCAP in presence of intracanal medicament using Alizarin red staining (Osteogenic). They found that iRoot FM increases the formation of mineralized nodules as compared to calcium hydroxide, TAP and control groups after cultured in an induction medium for 4 weeks. Thus, in order to draw a conclusion pertaining to differentiation, future studies should include differentiation potential as an outcome variable.

Strenghts

This systematic review addressed an important research question. According to the authors an appropriate methodology, precise inclusion criteria, and lower risk of bias among included studies make the observations of this systematic review more reliable.

Limitations

The present systematic review observed a high degree of heterogenicity among the studies and hence the meta-analysis could not be carried out. This could be attributed to the absence of clear guidelines for *in-vitro* cell culture experiments regarding the passage of cell lines to be used, time points for assessment of viability and proliferation. The subjectivity of risk of bias analysis can also be a limitation of the present systematic review.

CONCLUSION

Within the limitations of the present systematic review, it can be concluded that calcium hydroxide is a relatively better intracanal medicament than antibiotic paste mixtures in terms of their cytotoxicity and effect on the proliferation of SCAP. The evidence is inadequate to evaluate their effect on the differentiation potential of SCAP. Commonly used medicaments like triantibiotic paste, modified triantibiotic paste and diantibiotic paste are comparable and less cytotoxic when they are used in appropriate concentrations.

Clinical significance

The findings of this systematic review suggested that the calcium hydroxide is a relatively less cytotoxic intracanal medicament compared to antibiotic paste mixtures. However, comparable clinical and radiographic success outcomes have been observed in the literature for their use in regenerative endodontic procedures (45). This necessitates need for further clinical research to prove the superiority of one over other in terms of clinical success, radiographic outcomes, tendency for tooth discoloration and ability to regain the pulp sensibility.

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