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WHAT THE FUTURE HOLDS FOR REGENERATIVE ENDODONTICS: NOVEL ANTIMICROBIALS AND REGENERATIVE STRATEGIES

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Abstract

Regenerative/revitalisation endodontic techniques are increasingly used as a treatment approach for the management of immature permanent teeth with necrotic pulps. Different chemical irrigants and medicaments are routinely used clinically for intra-canal disinfection. However, despite remarkable progress in this field, coronal discolouration, cell cytotoxicity, difficulty of removal of organic biofilm from the root canal, development of sensitisation and antimicrobial resistance are still challenges to this line of treatment. This review critically discusses and challenges the current status quo of antimicrobials used in regenerative endodontics and sheds the light on future alternative antimicrobial materials with regenerative potential.

Keywords: Antimicrobials, antibiotics, disinfection, biomaterials, regenerative endodontics, drug delivery, dental pulp stem cells, pulp regeneration.

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List of Abbreviations

A. naeslundii	*Actinomyces naeslundii*
A. radicidentis	*Actinomyces radicidentis*
Ag-GO	AgNPs synthesised on an aqueous graphene oxide matrix
AgNPs	silver nanoparticles
C. albicans	*Candida albicans*
C. longa	*Curcuma longa*
$Ca(OH)_2$	calcium hydroxide
CFUs	colony forming units
CHX	chlorhexidine gluconate
CLSM	confocal laser scanning microscope
DAP	double antibiotic paste
DMSO	dimethyl sulfphoxide
DPSCs	dental pulp stem cells
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
EDTA	ethylenediaminetetraacetic acid
EEP	ethanol extract of propolis
ERK	extracellular signal-regulated kinases
FtsZ	Filamenting temperature-sensitive mutant Z
L. monocytogenes	*Listeria monocytogenes*
LED	light emitting diode
MBC	minimum bactericidal concentration
MCJ	*Morinda citrifolia* juice
MIC	minimum inhibitory concentrations
MTA	mineral trioxide aggregate
NaOCl	sodium hypochlorite
NFkB	nuclear factor kappa B
P. acnes	*Propionibacterium acnes*
PBS	phosphate-buffered saline
pERK	protein R-like endoplasmic reticulum kinase
PDT	photodynamic therapy
POVI	povidone iodine
PPM	part per million
qPCR	quantitative real-time polymerase chain reaction
RET	regenerative/revitalisation endodontic techniques
rGO-Cur	reduced graphene oxide-curcumin
S. aureus	*Staphylococcus aureus*
S. enterica	*Salmonella enterica*
S. epidermidis	*Staphylococcus epidermidis*
S. mitis	*Streptococcus mitis*
S. mutans	*Streptococcus mutans*
SCAP	stem cells of the apical papilla
SEM	scanning electron microscope
TAMP	tailored amorphous multiporous
TAP	triple antibiotic paste
TGF β-1	transforming growth factor β1
TS	tryptone soy

Introduction

RET are becoming widely used in the management of immature permanent teeth with necrotic pulp tissues, in which other alternative treatment options offer less successful outcomes (Galler, 2016). RET are biologically based treatments that aim to replace damaged tissue structures, including the pulp-dentine complex (Murray *et al.*, 2007). This treatment strategy, if successful, could potentially allow the continuation of root maturation, therefore, improving the long-term prognosis of these compromised teeth (Conde *et al.*, 2016; Garcia-Godoy and Murray, 2012). Clinically this treatment involves no or very minimal root canal instrumentation, disinfection, followed by induction of apical bleeding into the root canal space (Banchs and Trope, 2004; Iwaya *et al.*, 2001). Due to the limited or no mechanical instrumentation, microbial elimination is mainly dependent on antimicrobial agents for sufficient canal disinfection. Despite remarkable progress in the field of RET over the past decade, several challenges remain (Tong *et al.*, 2017), such as the questionable efficacy of the currently available disinfection techniques in promoting continuation of root development (Fouad, 2020).

Furthermore, RET is a stem cell-based regeneration strategy. Hence the balance between root canal disinfection and keeping the microenvironment as hospitable as possible for the stem cells to regenerate the pulp-dentine complex is of prime importance (Kim *et al.*, 2018). Many of the currently used intra-canal irrigants such as NaOCl and CHX were proven to have a cytotoxic effect on stem cells (Martin *et al.*, 2014; Widbiller *et al.*, 2019). The use of topical antibiotic pastes are effective endodontic antimicrobials; however, in addition to tooth discoloration caused by some of the antibiotic combinations (Lenherr *et al.*, 2012), a level of cytotoxicity on stem cells in a concentration-dependent manner is of clinical concern (Ruparel *et al.*, 2012). Although using topical antibiotic pastes at lower concentrations provided adequate canal disinfection, such mixtures are difficult to prepare and obtain. The argument about their contribution to the global challenge of antimicrobial resistance at such low concentrations is yet to be settled (Ayoub *et al.*, 2020; Yadlapati *et al.*, 2017; Yadlapati *et al.*, 2014).

Therefore, the development of alternative disinfectant strategies with sufficient antimicrobial efficacy, biocompatibility, and regenerative potential (with the least harm to the surrounding conductive microenvironment) is of importance in this field. The achievement of a safer and more predictable regenerative outcome is clinically required (Diogenes *et al.*, 2014; Fouad, 2020). With the current advances in tissue engineering, this gap is being addressed through several innovative natural and synthetic strategies, such as alternative antimicrobial agents, nano-based delivery systems, and novel photosensitisers combined with PDT which initiate tissue regeneration and possess an innate antimicrobial activity (Chung and Park, 2017; Samiei *et al.*, 2016). A schematic illustration depicting limitations of currently used antimicrobial strategies and the potential benefits of alternative RET antimicrobial strategies is provided (Fig. 1).

Taking all of the above into consideration, it seems comprehensible to develop an alternative antimicrobial able to disinfect the root canal system without compromising the regenerative environment. The aim of this review is to critically discuss and challenge the current status quo of antimicrobials

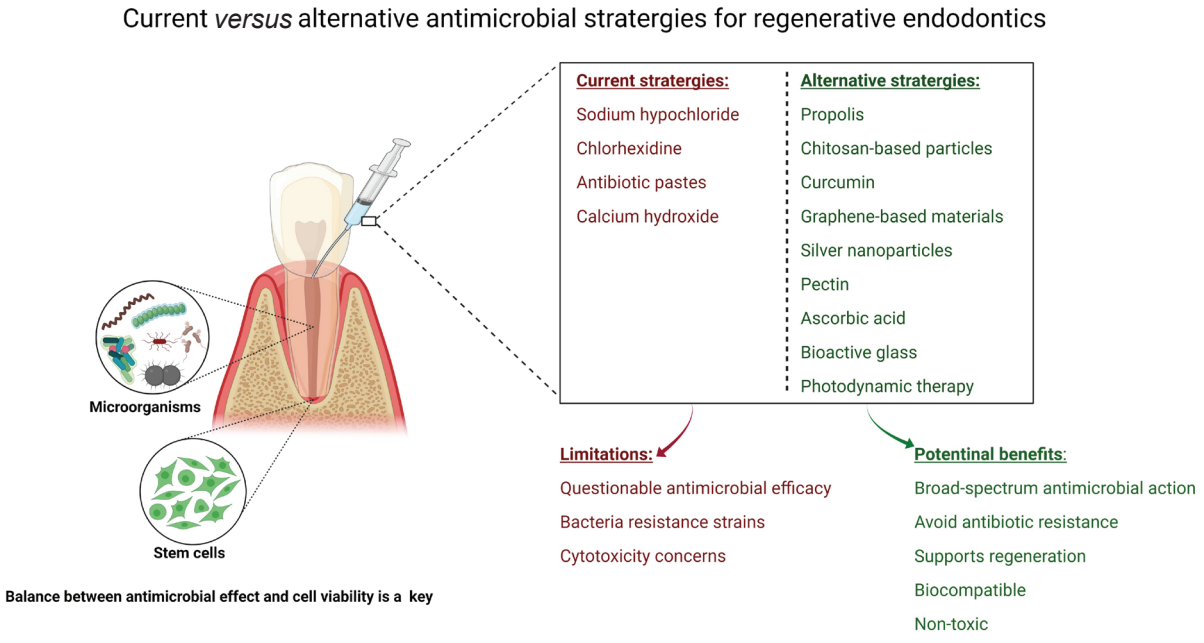

Fig. 1. Schematic illustration depicting limitations of currently used antimicrobial strategies and the potential benefits of alternative RET antimicrobial strategies. Created with BioRender.com.

used in regenerative endodontics, and to shed the light on future alternative antimicrobial materials with regenerative potential.

Literature search and scope of the review

An electronic search of PubMed and Elsevier's Scopus was undertaken with appropriate MeSH terms and various keyword combinations including "antimicrobial", "antibiotic", "disinfection", "dentistry", "pulp revascularisation", "pulp regeneration", "regenerative endodontic", "drug delivery", "biomaterials", and "dental pulp stem cells". The reference list of the relevant articles resulting from database searches was further hand-screened. No limits were applied to the year of publication and only English language literature was included. However, due to the scope and extent of this search, a wide-ranging comprehensive narrative review of antimicrobial strategies in regenerative endodontics rather than a systematic review was undertaken.

Current antimicrobial strategies used in RET

A critical summary of the most commonly used disinfectant agents (irrigants and medicaments) in terms of their antimicrobial efficacy and biocompatibility will be discussed below as these have been extensively reviewed in the literature (Diogenes *et al.*, 2014; Kim *et al.*, 2018; Martin *et al.*, 2014).

Intra-canal irrigation

NaOCl is one of the oldest endodontic irrigants and reported in most published RET studies, albeit in various concentrations ranging from 1 – 6 % (Tong *et al.*, 2020). NaOCl solution is regarded as the irrigant of choice mainly due to its bacteriostatic, bactericidal, and tissue dissolution properties (Bryce *et al.*, 2009; Zehnder, 2006). The ability of NaOCl to dissolve organic tissue is well-documented, as well as its negative effects on the mechanical properties of root dentine (Dotto *et al.*, 2020; Pascon *et al.*, 2009). These mechanical alterations are most likely due to the proteolytic action of concentrated hypochlorite solution on the collagen dentine matrix (Zehnder, 2006). Alterations in the dentine-matrix composition including the denaturation of growth factors and attachment proteins are likely to affect the fate of stem cells (Diogenes *et al.*, 2014). Furthermore, NaOCl at a concentration between 5-6 %, has shown detrimental effects on stem cell numbers and survival as well as loss of odontoblast-like phenotype differentiation both *in vitro* and *in vivo* (Casagrande *et al.*, 2010; Galler *et al.*, 2011).

From a biological perspective, a concentration-dependent effect of NaOCl on the survival of SCAP has been shown, with 6 % NaOCl showing the greatest reduction in stem cell survival. This resulted in the recommendation for using a low NaOCl concentration of 1.5 % (Web ref.1; Martin *et al.*, 2014; Trevino *et al.*, 2011). However, controversies remain in terms of the ability of low NaOCl concentrations to completely eradicate infected biofilms (Ma *et al.*, 2015; Tagelsir *et al.*, 2016). In an attempt to reduce such effect, 17 % EDTA has been recommended following the use of NaOCl irrigation. This step has been shown to reduce NaOCl detrimental side effects on stem cell survival, regardless of the NaOCl concentration used (Martin *et al.*, 2014; Trevino *et al.*, 2011). Furthermore, combining EDTA within a given irrigation protocol was also found to significantly increase the release of growth factors such as TGF β-1 into the root canal space, hence inducing DPSCs migration and differentiation to odontoblasts (Zeng *et al.*, 2016).

CHX is also one of the well known endodontic disinfectant agents used in RET and clinically available in the form of an aqueous solution or gel preparation with a dilution range of 0.12 % to 2 % (Okino *et al.*, 2004). However, the use of this irrigant is less prevalent within RET protocols as highlighted in a recent survey, in which only 11.4 % of respondents used CHX as the sole disinfectant, while 22.2 % reported a combined use of CHX and NaOCl (Tong *et al.*, 2020). CHX benefits from broad-spectrum antimicrobial and intra-canal substantivity (residual effect) properties (Martin *et al.*, 2014; Okino *et al.*, 2004; Trevino *et al.*, 2011). The use of 2 % CHX has also been shown to cause unfavourable effects on stem cell survival (Trevino *et al.*, 2011) and attachment (Ring *et al.*, 2008), with direct cytotoxicity effect in a concentration-dependent manner (Widbiller *et al.*, 2019). On the contrary, CHX lacks organic tissue dissolution ability (Okino *et al.*, 2004); therefore, its effect on biofilm disruption is questioned (Bryce *et al.*, 2009; Trevino *et al.*, 2011).

Intra-canal medicaments

Intra-canal medicaments are commonly used inter-appointment antimicrobial RET dressing (Tong *et al.*, 2020) and broadly divided into two groups; topical antibiotic pastes or $Ca(OH)_2$. Topical antibiotic pastes used within RET mainly include; TAP (ciprofloxacin, metronidazole, and minocycline), DAP (ciprofloxacin and metronidazole), and other modified formulations (Tong *et al.*, 2020). Although sufficient antimicrobial efficacy is one of the main advantages behind the use of topical antibiotic pastes, clinical limitations have been raised (Ribeiro *et al.*, 2020).

Complete removal of the applied antibiotic paste is crucially important for a successful regenerative outcome and to avoid unwanted, possibly long-term, side effects. Unfortunately, studies have demonstrated that a significant amount of antibiotic pastes (88 % residual) remains within the root canal system following current irrigation techniques (Berkhoff *et al.*, 2014). Indirect negative effects, such as reduction in dentinal strength and fracture resistance, are reported as early as 1 week post-

application. These effects were mainly linked to the strong demineralisation effect and the acidic nature of antibiotic pastes on the surrounding dentine matrix (Yassen *et al.*, 2013a; Yassen *et al.*, 2013b). These structural changes are thought to affect the fate of stem cells and hinder their regenerative potential, consequently resulting in inconsistent clinical results related to continuation of root maturation, thickening of root dentine, and apical closure (Tong *et al.*, 2017).

A concentration-dependent detrimental effect of various topical antibiotic pastes on human SCAP survival has been shown *in vitro* (Althumairy *et al.*, 2014; Ruparel *et al.*, 2012). Therefore, to achieve optimal results, various antibiotic mixtures and concentrations have been tested. A low TAP and DAP concentration of 1 mg/mL provided sufficient antimicrobial efficacy with no reported negative effects on the viability of SCAP when compared to higher concentrations (1,000 mg/mL) (Althumairy *et al.*, 2014). Furthermore, the potential development of bacterial resistance biofilms and/or sensitisation has been raised (Berkhoff *et al.*, 2014; Stewart and William Costerton, 2001). Coronal discolouration is also a commonly reported side effect of the TAP use, which is largely linked to minocycline, a semisynthetic tetracycline antibiotic (Kim *et al.*, 2010; Lenherr *et al.*, 2012; Sato *et al.*, 1996). However, despite omitting minocycline within RET protocols tooth discoloration continued to be reported (Tong *et al.*, 2017).

$Ca(OH)_2$ is another widely used intra-canal medicament advocated to overcome the undesirable effects of the topical antibiotic pastes and recommended by the European Society of Endodontology for short-term clinical application (Galler *et al.*, 2016). Material advantages such as availability and ease of removal from the root canal are documented (Berkhoff *et al.*, 2014; Nazzal *et al.*, 2018).

Nevertheless, conflicting antimicrobial efficacy of $Ca(OH)_2$, as an intra-canal dressing, has been reported (Ribeiro *et al.*, 2020). The ability of $Ca(OH)_2$ to eradicate specific bacteria, such as *E. faecalis*, and yeasts from the root canal systems has been questioned (Krithikadatta *et al.*, 2007; Zehnder *et al.*, 2004). More recently, a clinical molecular-based study showed comparable antimicrobial efficacy and regenerative outcome following the use of TAP and a combined $Ca(OH)_2$/CHX paste (de-Jesus-Soares *et al.*, 2020). From a biological perspective, $Ca(OH)_2$ provided an environment conducive to stem cell survival and proliferation (Althumairy *et al.*, 2014; Ruparel *et al.*, 2012). However, the possible side effects of $Ca(OH)_2$ on the biological property of dentine-matrix-derived growth factors have been highlighted and requires consideration (Kim *et al.*, 2018).

Possible alternative antimicrobial strategies with a regenerative potential for RET

The development and characterisation of the next generation novel materials that can enhance the regeneration of pulp-dentine complex as well as provide sufficient antimicrobial properties is a recent focus area for a safer and predictable regenerative outcome (Chung and Park, 2017). Indeed, a recent scoping review of the literature concluded that trends towards alternative antimicrobials are promising and deserve future consideration (Ribeiro *et al.*, 2020). Word cloud highlighting of both natural and synthetic alternative antimicrobial materials and strategies for potential use in RET was performed. The more a substance has been researched, within this specific field, the larger it appears in the word cloud (Fig. 2).

Fig. 2. Word cloud representing alternative antimicrobial materials and strategies for potential use in RET. The more a substance was researched, within this specific field, the larger it appears in the word cloud. Created by Wordclouds.com.

Natural materials and strategies

Propolis

Propolis, also known as "bee glue", is a natural resinous substance crucial for both internal and external beehive protection (Ghisalberti, 1979; Grange and Davey, 1990). This resinous material is initially collected by bees (*Apis mellifera*) from exudates and plant buds, subsequently mixed with saliva secretions (bee enzymes) and wax (Ghisalberti, 1979; Grange and Davey, 1990). Historically, propolis has been used by on Egyptian, Greek and Roman traditional medicine since 300 BC (Khalil, 2006; Sforcin and Bankova, 2011).

Propolis comprises mainly resins, balsams, and wax in addition to amino acids, aromatic compounds, phenols, pollens, minerals, and vitamins (Ghisalberti, 1979; Grange and Davey, 1990; Uzel *et al.*, 2005). The resinous portion is mainly composed of flavonoid pigments (well-known plant compounds) and is regarded the main active component, linked to propolis broad-spectrum antimicrobial activity (Ghisalberti, 1979; Grange and Davey, 1990). Additional therapeutic features include anti-oxidant, anti-cariogenic, and anti-inflammatory properties (Khalil, 2006; Marcucci, 1995; Uzel *et al.*, 2005).

This resinous material has been highlighted as a promising natural additive to the chemical composition of toothpastes (Morawiec *et al.*, 2013) and mouthwashes (Dodwad and Kukreja, 2011; Halboub *et al.*, 2020). Possible avenues of propolis use in dentistry include prevention of dental caries and plaque formation (Koo *et al.*, 2000), a cell preservation medium for avulsed teeth (Martin and Pileggi, 2004; Ozan *et al.*, 2007), management of pulp exposures (Ahangari *et al.*, 2012), and as an antimicrobial agent for the root canal system (El-Tayeb *et al.*, 2019; Pagliarin *et al.*, 2016).

Although propolis possesses various therapeutic properties, its chemical composition varies according to the country of origin, botanical source, and time of collection (Marcucci, 1995; Uzel *et al.*, 2005). Clinically, the variation in chemical composition could ultimately result in a range of propolis therapeutic deficiencies and raises concerns in terms of quality control and batch-to-batch variability for standardised new drug development (Marcucci, 1995; Sforcin and Bankova, 2011). Despite such concerns, a standardised propolis extract, known as EPP-AF®, has been developed in Brazil (Berretta *et al.*, 2012). Due to propolis impurities, a series of various purification and extraction methods are required, such as maceration or Soxhlet extraction (Ghisalberti, 1979). The use of strong solvents, such as ethanol or DMSO, during propolis extraction are reported and will have a negative effect the on cell viability and their regeneration potential, even at concentrations as low as 0.1 % (Cunha *et al.*, 2004; Sut *et al.*, 2016). The lack of methodologically robust studies with detailed propolis chemical composition or its extraction method, lead to its limited clinical translation (Sforcin and Bankova, 2011). The use of nontoxic extraction solvents within well-controlled and designed comparative studies are required for further assessment of propolis as a promising material with potential clinical application in RET (Sut *et al.*, 2016).

Propolis biocompatibility and regenerative potential towards soft and mineralised tissues have been reported (Ahangari *et al.*, 2012; Al-Shaher *et al.*, 2004). Propolis, at concentrations of 4 mg/mL or lower, was found to be at least 10 times less cytotoxic to fibroblasts of the dental pulp and periodontal ligament when compared with $Ca(OH)_2$ (Al-Shaher *et al.*, 2004). The use of propolis as a vehicle for $Ca(OH)_2$ has also been suggested, with i*n vitro* studies concluding efficient diffusion throughout the dentinal tubules, and possibly extending to the external root surface (Baranwal *et al.*, 2017; Montero and Mori, 2012; de Rezende *et al.*, 2008). Furthermore, animal studies utilising propolis paste as an intra-canal medicament have shown promising results equal to or superior to TAP in-terms of soft and hard tissue deposition (El-Tayeb *et al.*, 2019; Pagliarin *et al.*, 2016). *In vitro* and *in vivo* studies utilising propolis as an intra-canal disinfectant agent are summarised in Table 1 and 2, respectively.

Chitosan-based particles

Chitosan (poly[1,4],-b-D-glucopyranosamine) is a cationic natural nontoxic biopolymer obtained by the alkaline deacetylation of chitin (Peter, 1995; Rabea *et al.*, 2003). Chitin is the second most abundant natural polymer found in the exoskeleton of marine crustaceans such as shrimps and crab shells (Peter, 1995; Rabea *et al.*, 2003).

Chitosan has a high nitrogen content with a strong chelating ability and great commercial interest (Rabea *et al.*, 2003). On a production scale, chitosan can be produced in several forms, such as a paste or powder, with particles at the macro- or nano-scale (Agnihotri *et al.*, 2004). Commercially, chitosan is available with an average molecular weight of 3,800 - 20,000 Daltons and 66 - 95 % deacetylation (Agnihotri *et al.*, 2004). Its versatile commercial applications include environmental, agricultural, food additive, and a hydrating agent in cosmetics (Peter, 1995; Rabea *et al.*, 2003). Chitosan-based particles are regarded as an effective drug delivery system (Li *et al.*, 2018), and tested as a vehicle for $Ca(OH)_2$ or TAP with promising results such as improved stability and promoting a sustained release of medicament (Ballal *et al.*, 2010; del Carpio-Perochena *et al.*, 2017; Shaik *et al.*, 2014). More recently, chitosan has been explored as an antimicrobial agent to disinfect the root canal system with proposed regenerative potential (Ducret *et al.*, 2019; Palma *et al.*, 2017).

This attractive research interest is linked to its unique biological properties such as biocompatibility, excellent bioadhesive, and broad-spectrum antimicrobial properties (Raafat and Sahl, 2009; Rabea *et al.*, 2003; Shrestha *et al.*, 2010). A postulated

mechanism of chitosan antimicrobial activity is linked to the interaction between positively charged chitosan (NH_3^+ groups of glucosamine) and negatively charged cell membrane causing a sequential of events, which alter the cell permeability and consequently cell death (Raafat and Sahl, 2009; Rabea *et al.*, 2003). Additional advantages for extended clinical applications include abundance in nature, ease of modification, nontoxic, and low production cost (Peter, 1995; Rabea *et al.*, 2003).

However, in spite of chitosan's proven biocompatibility and noncytotoxicity (Renard *et al.*, 2020), its regenerative potential in dentistry remains controversial. Preliminary *in vitro* work demonstrated stimulation of dental pulp tissue formation, in terms of mesenchymal stem cells viability and deposition of dental pulp-like collagen matrix following the use of a novel chitosan-enriched fibrin hydrogel (Ducret *et al.*, 2019). In-contrast, the clinical application of chitosan scaffolds in immature dog teeth with apical periodontitis resulted in no histologic evidence of pulp-dentine tissue regeneration nor newly formed mineralised tissue (Palma *et al.*, 2017). The degradation process of chitosan under inflammatory conditions requires careful assessment prior to clinical translation (Palma *et al.*, 2017; Yamada *et al.*, 2014). *In vitro* studies utilising chitosan-based particles as an intra-canal disinfectant agent are summarised in Table 1.

Table 1a. *In vitro* and *ex vivo* studies of alternative antimicrobial strategies highlighting antimicrobial type, origin, study design, usage, study and control groups, assessment method and duration, microorganisms tested and main findings.

Alternative antimicrobial/ origin	Study design/ usage	Study groups	Control groups	Microorganism	Assessment method/ duration	Main findings	Reference
Propolis/ Nature Home, Amman, Jordan	Human dentine block model Medicament	30 % propolis $Ca(OH)_2$	Saline Sterile uninoculated broth	*E. faecalis*	Microbiological samples were collected (paper points, headstrom files and disc immersion), incubated on agar plates and CFUs analysed 1 and 2 d	Propolis was significantly more effective than $Ca(OH)_2$ for short-term application	Awawdeh *et al.* 2009
Propolis	Human dentine block model Irrigant	Propolis MCJ 2 % POVI 2 % CHX gel $Ca(OH)_2$	Saline	*E. faecalis*	Dentine shavings were collected (200 and 400 µm depths), cultured on TS agar plates and CFUs analysed 21 d	CHX produced the highest antimicrobial efficacy followed by POVI, propolis and MCJ. $Ca(OH)_2$ was least effective	Kandaswamy *et al.*, 2010
Propolis/ Apis flora, Ribeirão Preto, Brazil	Human root model Irrigant	12 % propolis glycolic extract	Saline	*E. coli*	Microbiological samples were collected, incubated on agar plates and CFUs analysed	Propolis was effective to completely eliminate *E. coli* and reduce the amount of endotoxins	Valera *et al.*, 2010
Propolis/ Turkey (northeast and northwest areas)	Human dentine block model Irrigant	EEP 2 % CHX solution $Ca(OH)_2$	96 % ethanol PBS	*E. faecalis*	Dentine shavings were collected (300 µm depth), cultured on TS agar plates and CFUs analysed 7 d	Propolis antimicrobial efficacy was higher than $Ca(OH)_2$ and lower than CHX	Kayaoglu *et al.*, 2011
Propolis/ Calgary gold bee products, Canada	Human root model Medicament	$Ca(OH)_2$ TAP EEP ethanol	Saline	*E. faecalis*	Percentage reduction in colony counts 1, 2 and 7 d	Propolis was more effective than TAP at day 2 and equally effective at day 7	Madhubala *et al.*, 2011

Curcumin

Curcumin (diferuloylmethane), a dimeric derivative of ferulic acid, is the main bioactive substance of *C. longa* (turmeric), a wellknown oriental spice (Adamczak *et al.*, 2020). Curcumin pigment, a distinctive yellow-orange colouring matter of plant origin, was isolated back in 1842 by Vogel and Pelletier from the rhizomes of *C. longa*, originating from the ginger family tree native to South Asia (Adamczak *et al.*, 2020; Hewlings and Kalman, 2017).

Curcumin possesses a wide spectrum of bioactive and therapeutic properties such as antibacterial, antifungal, and antiviral properties (Praditya *et al.*, 2019; Rai *et al.*, 2008). Antioxidant and anti-inflammatory activities are also documented in the literature (Hewlings and Kalman, 2017; Rai *et al.*, 2020; Sinjari *et al.*, 2019). The antimicrobial action of curcumins is attributed to their ability to damage the bacterial cell membrane through the suppression of bacterial cytokinesis, the induction of filamentation, and the inhibition of the FtsZ assembly dynamics in the Z-ring (Rai *et al.*, 2008). Inhibition of cellular proliferation and alterations of gene expression are also reported to be behind the bactericidal mechanisms of curcumins (Rai *et al.*, 2008; Rai *et al.*, 2020; Tyagi *et al.*, 2015).

In the past, the clinical usage of curcumins was limited due to their poor water solubility, low oral bioavailability, and rapid metabolism (Chang *et al.*, 2018; Sinjari *et al.*, 2019). The use of liposomes, in order to solubilise curcumin phospholipidic bilayer, has been suggested to enhance curcumin delivery and improve its therapeutic efficiency (Chang *et al.*, 2018; Sinjari *et al.*, 2019). Sinjari *et al.* (2019) closely assessed the direct contact of human DPSCs with nanocarrier curcumin-loaded

Table 1b. *In vitro* and *ex vivo* studies of alternative antimicrobial strategies highlighting antimicrobial type, origin, study design, usage, study and control groups, assessment method and duration, microorganisms tested and main findings.

Alternative antimicrobial/ origin	Study design/ usage	Study groups	Control groups	Microorganism	Assessment method/ duration	Main findings	Reference
Propolis/ Beehives of Najaf Abad, Esfahan	Human root model Medicament	EEP $Ca(OH)_2$ ethanol	No medicament Sterile samples	*E. faecalis*	Microbiological samples were collected with a piezoreamer, plated and CFU analysed. MIC was also measured using dilution methods 7 d	CFUs and MIC of propolis were significantly less than $Ca(OH)_2$	Zare Jahromi *et al.*, 2012
Propolis/ RK's Aroma Products, Mumbai	Human root model Medicament	Propolis 2 % CHX gel $Ca(OH)_2$	Saline	*E. faecalis*	Dentine shavings were collected (depth of 400 µm), cultured on TS agar and CFUs analysed 1, 3 and 5 d	Propolis had greater antimicrobial efficacy than $Ca(OH)_2$ on day 1, with no significant difference in subsequent days	Bhandari *et al.*, 2014
Propolis/ Natural Bee Health Industry, Lima, Peru	Human root model Irrigant	$Ca(OH)_2$ Propolis 2 % CHX gel	Saline	*E. faecalis* and *C. albicans*	Dentine shavings were collected (100 and 200 µm depths), cultured on agar blood or agar Sabouraud plates and CFUs analysed 14 d	Propolis and CHX were the most effective against *E. faecalis*. However, CHX had the highest antifungal activity	Carbajal Mejía, 2014
Propolis/ Stakich, Royal Oak, Michigan, USA	Human root model Medicament	95 % propolis TAP 2 % CHX gel $Ca(OH)_2$	Saline	*C. albicans*	Dentine shavings were collected (200 and 400 µm depths) and CFUs analysed 1 and 7 d	Propolis was less effective on day 1 and equally effective to other medicaments on day 7	Chua *et al.*, 2014
Propolis/ Herbal Biosolutions, Delhi *C. longa*/ RYM exports, Mumbai, India	Human dentine block model Medicament	2 % CHX gel honey *Aloe vera* gel 20 % *C. longa* gel 11 % EEP $Ca(OH)_2$	Saline	*E. faecalis*	Dentine shavings were collected (200 and 400 µm depths) and CFUs were analysed. 1, 3 and 5 d	2 % CHX gel was most effective followed by propolis and *C. longa*	Vasudeva *et al.*, 2017

liposome in the presence of hydrophilic monomers (2-hydroxyethyl methacrylate). Quantitative cytokine release assessment showed a decreased secretion of tested pro-inflammatory cytokines (interleukin 6, interleukin 8, interferon-gamma, and monocyte chemoattractant protein-1), in response to curcumin liposome nanocarriers. The authors concluded that curcumin liposome nanocarriers had stimulated DPSCs proliferation and reduced inflammation *via* the NFkB/ERK/pERK pathways, but did not induce odontoblastic differentiation (Sinjari *et al.*, 2019). Various loading and encapsulation mechanisms for curcumin delivery such as nanoemulsion, nanosuspension, lipid nanoparticles, and hydrogel nanoparticles were further investigated in the literature (Dutta and Ikiki, 2013; Rai *et al.*, 2020).

Despite the above-mentioned therapeutic and bioactive properties, limited studies were conducted within the dental field, particularly in RET (Neelakantan *et al.*, 2013; Yadav *et al.*, 2018). Emerging *in vitro* studies, with promising results, expanding the use of curcumins as an intra-canal disinfectant agent are summarised in Table 1.

AgNPs

Inorganic metals, in their standard or ionic forms such as Ag or Ag^+, are regarded as antibiotic alternatives due to their broad-spectrum bactericidal effects coupled with the unlikely possibility of developing antibiotic-resistant bacterial strains (Oei *et al.*, 2012; Rai *et al.*, 2009). Specifically, AgNPs have gained recent popularity owing to their distinctive physical and biochemical properties (Bapat *et al.*, 2018; Rai *et al.*, 2009), synergistic antibiotic effect (Fayaz *et al.*, 2010), biocompatibility (Franková *et al.*, 2016; Gomes-Filho *et al.*, 2010), and antimicrobial properties (Lara *et al.*, 2010; Rai *et al.*, 2009). The ability of AgNPs to disrupt dental biofilms and prevent bacterial adhesion are also advantageous (Wu *et al.*, 2014).

The incorporation of silver particles within dental materials is not new and has been practiced for decades since the use of silver-containing dental amalgam (Noronha *et al.*, 2017). However, with advanced nanotechnology, AgNPs have gained considerable attention (Bapat *et al.*, 2018; Noronha *et al.*, 2017). The incorporation of AgNPs within a diverse range of dental materials have been proposed, such as composite resins (Cheng *et al.*, 2012; Durner *et al.*, 2011), dental implant coating (Cao *et al.*, 2011; Wang *et al.*, 2013), calcium silicates cements (Fan *et al.*, 2014; Samiei *et al.*, 2013), endodontic sealers (Vilela Teixeira *et al.*, 2017), and intra-canal disinfectant agents (Afkhami *et al.*, 2017; Afkhami *et al.*, 2015; Moazami *et al.*, 2018; Wu *et al.*, 2014).

The antimicrobial mode of action of AgNPs is largely associated with the release of Ag^+ ions, which consequently penetrate the cell membrane and interact with various cellular components, resulting in inhibition of cell replication and eventually cell death (Bapat *et al.*, 2018; Rai *et al.*, 2009). An immediate

Table 1c. *In vitro* and *ex vivo* studies of alternative antimicrobial strategies highlighting antimicrobial type, origin, study design, usage, study and control groups, assessment method and duration, microorganisms tested and main findings.

Alternative antimicrobial/ origin	Study design/ usage	Study groups	Control groups	Microorganism	Assessment method/ duration	Main findings	Reference
Chitosan/ Sigma-Aldrich Inc.	Well plates irrigant	Chitosan-nanoparticle Zinc oxide-nanoparticle	-	2 strains of *E. faecalis* in planktonic and biofilm forms	LIVE/DEAD staining/confocal microscopy/ CFUs 90 d	The incorporation of nanoparticles enhanced antimicrobial efficacy with retained aging potential	Shrestha *et al.*, 2010
Chitosan/ Acros Organics	Human root model irrigant	15 % EDTA 0.2 % chitosan 10 % citric acid 1 % acetic acid	No final irrigation	-	SEM/ atomic absorption spectrophotometry	0.2 % chitosan was similar to 15 % EDTA in terms of smear layer and dentine demineralisation	Silva *et al.*, 2013
Chitosan/ Mahtani Chitosan Pvt. Ltd Veraval, India	Human root model irrigant	0.25 % chitosan 0.5 % chitosan 2 % CHX 3 % NaOCl	Saline	*E. faecalis* and *C. albicans* biofilms	Agar-well diffusion method/ MIC/ biofilm susceptibility assay/ SEM/ cytotoxicity assay/ CFUs	Similar antimicrobial efficacy was seen between study groups. However, chitosan showed significant less toxicity then NaOCl	Yadav *et al.*, 2017
Curcumin/ Biopurify Phytochemicals Ltd., Sichuan, China	Human root model irrigant	Curcumin 2 % CHX 3 % NaOCl	PBS	*E. faecalis* biofilms	MIC/ MBC/ CFUs 2nd d, 2nd and 8th weeks	Curcumin antimicrobial efficacy was similar to 3 % NaOCl at 2 d and 2nd week, and inferior to CHX at 8th week	Neelakantan *et al.*, 2013

bactericidal antimicrobial effect of AgNPs has been shown against several drug-resistant and drug-susceptible bacteria possibly through inhibiting cell wall, protein, and nucleic acid synthesis (Lara *et al.*, 2010).

However, there are some cytotoxicity concerns to the use of AgNPs, mainly related to adverse events of free Ag^+. Such concerns are controversial and at an early stage of research for sound conclusions to be drawn. Franková *et al.* (2016) assessed AgNPs against human dermal fibroblasts and epidermal keratinocytes. They concluded that AgNPs inhibit the production of pro-inflammatory cytokines and contributed positively towards the wound-healing process. Quantitative elution testing and qualitative on-growth of human osteoblasts also revealed no cytotoxicity following the use of 1 % nano-silver loaded bone cement *in vitro* (Alt *et al.*, 2004). Indeed, it was proven that AgNPs biocompatibility is concentration dependent with increased cytotoxicity at higher concentrations (Gomes-Filho *et al.*, 2010; Newby *et al.*, 2011). In addition to material concentration, material-specific characteristics (Cao *et al.*, 2011) and incorporation methods (Fan *et al.*, 2014) also determine the cytotoxicity of AgNPs. To investigate the latter, the cytotoxicity of nano-silver incorporated using two methods (template and absorption method) to mesoporous calcium silicate against human bone marrow mesenchymal cells was assessed (Fan *et al.*, 2014). Results indicated no obvious cytotoxicity following the adoption of the template method, in contrast to significant cytotoxicity associated with the absorption method (Fan *et al.*, 2014).

Despite the significant advantages of silver-based materials, potential adverse effects have been reported. Argyria, an irreversible skin pigmentation, is a well-known silver-related side effect (Drake and Hazelwood, 2005). Furthermore, potential tooth discoloration is also of concern and requires careful investigation prior to clinical usage (Afkhami *et al.*, 2015). *In vitro* spectrophotometric analysis showed no significant colour changes between AgNPs added to $Ca(OH)_2$ compared to $Ca(OH)_2$ alone when used as an intra-canal medicament (Afkhami *et al.*, 2017). In contrast, spectrophotometric analysis of AgNPs

Table 1d. *In vitro* and *ex vivo* studies of alternative antimicrobial strategies highlighting antimicrobial type, origin, study design, usage, study and control groups, assessment method and duration, microorganisms tested and main findings.

Alternative antimicrobial/ origin	Study design/ usage	Study groups	Control groups	Microorganism	Assessment method/ duration	Main findings	Reference
Photoactivated curcumin/ 20 µmol/L/ Sigma-Aldrich, St Louis, MO, USA	Human root model irrigant	Curcumin	Physiological solution	*E. faecalis* biofilms	Fibre optic LED of 100 mW/cm² Light duration of 5 and 10 min was investigated CFUs Before treatment, immediately after treatment, and after 7 d	Photoactivated curcumin reduced, however did not eliminate, bacterial count	da Frota *et al.*, 2015
Curcumin/ 2.5 mg/mL	Human root model irrigant	Curcumin 3 % NaOCl	Saline	*E. faecalis* biofilms	Ultrasonic activated for 30 s cycles for 4 min/ photoactivated with blue light of 1200 mW/cm² for 4 min/ SEM/ CFUs	Photoactivated curcumin produced the maximum elimination of biofilm bacteria	Neelakantan *et al.*, 2015
Photoactivated curcumin/ 2.5 mg/mL	Human root model	Photoactivated curcumin TAP DAP 2 % CHX $Ca(OH)_2$	No medicament	*E. faecalis* biofilm	LIVE/DEAD staining/ CLSM/ CFUs 14 d	Photoactivated curcumin produced superior antimicrobial and antibiofilm activity	Devaraj *et al.*, 2016
Curcumin/ 2.5 and 5.0 mg/ mL	Well plates irrigant	Curcumin-electrospun modified fibres Curcumin-free fibres 2 % CHX 1 % NaOCl TAP	Saline	*A. naeslundii* biofilms	CFUs	Photoactivated curcumin-based medicaments showed a high antibiofilm activity when used at low concentrations	Sotomil *et al.*, 2019

coated with imidazolium as a root canal irrigant resulted in statistically significant darker colour changes in comparison with the control groups (5.25 % NaOCl, 2 % CHX, and normal saline) (Moazami *et al.*, 2018). The colour change caused by AgNPs could be linked mostly to the direct contact of the irrigant material with the coronal dentine, which is practically difficult to control during irrigation. Taking the above results into consideration, well-controlled clinical studies are warranted for sound conclusions and clinical recommendations. Emerging studies utilising AgNPs within the endodontic field as an intra-canal disinfectant agent are summarised in Table 1.

Graphene-based materials

Graphene is a recently developed material made of densely packed carbon atoms arranged as a two-dimensional monolayer sheet forming a benzene hexagonal structure (Novoselov *et al.*, 2004). Examples of graphene derivatives include pristine graphene, graphene oxide, reduced graphene oxide, few-layered graphene, ultrathin graphite, graphene nanosheets, and graphene-based nanocomposites (Guazzo *et al.*, 2018; Tahriri *et al.*, 2019). These different derivatives mainly differ in the material size, number of layers, and specific surface characteristics (Guazzo *et al.*, 2018).

Graphene and its derivatives have a wide range of biomedical applications due to their unique mechanical, electrochemical, and physical properties such as mechanical strength, high surface area, thermal stability, and unique surface characteristics (Guazzo *et al.*, 2018; Tahriri *et al.*, 2019). Furthermore, their ability to combine and functionalise with various materials and bioactive molecules to enhance or alter specific properties for distinctive applications is of interest (Guazzo *et al.*, 2018).

Graphene-based materials also have broad-spectrum, yet variable, antimicrobial properties (Al-Jumaili *et al.*, 2017; Guazzo *et al.*, 2018). Its antimicrobial mode of action is explained through different mechanisms.

1. A sharp knife-edge cutting effect that consequently leads to cell membrane rupture, mechanical stress induction, and phospholipids extraction

Table 1e. *In vitro* and *ex vivo* studies of alternative antimicrobial strategies highlighting antimicrobial type, origin, study design, usage, study and control groups, assessment method and duration, microorganisms tested and main findings.

Alternative antimicrobial/ origin	Study design/ usage	Study groups	Control groups	Microorganism	Assessment method/ duration	Main findings	Reference
C. longa extract/ Curenext, Abbott, India	Human root model Medicament	*C. longa* extract 1 % CHX gel $Ca(OH)_2$	-	*E. faecalis* biofilms	CFUs 24 h	CHX gel showed highest antimicrobial efficacy followed by *C. longa* extract, while $Ca(OH)_2$ was least effective	Yadav *et al.*, 2018
Nano-silver liquid form/ Nanocid Company, Tehran, Iran	Test tubes and inoculated plates Irrigant	Nano-silver 2 % CHX 5.25 % NaOCl	Vancomycin bacterial susceptibility papers Saline	*E. faecalis*	MIC/ agar diffusion/ zones of inhibition 6, 18, 24 and 48 h of incubation	Nano-silver showed equal bactericidal action as 5.25 % NaOCI	Lotfi *et al.*, 2011
Silver nitrate/ Factory of Shanghai Chemical Reagent Ltd, China	Longitudinal split root human teeth model	Nano-silver incorporated mesoporous calcium silicate	$Ca(OH)_2$ deionised water	*E. faecalis*	LIVE/DEAD staining/ cell counting kit-8 Variable	The incorporation of Ag particles resulted in enhanced antibacterial efficacy and reduced bacterial colonisation	Fan *et al.*, 2014
AgNPs/ Huzheng Nano Technology Ltd, Shanghai, China	Longitudinal split root human teeth model Irrigant/ medicament	Stage 1; 0.1 % AgNPs solution 2 % NaOCl Stage 2; 0.01 % AgNPs gel 0.02 % AgNPs gel $Ca(OH)_2$	Sterile saline	*E. faecalis*	SEM/ LIVE/DEAD staining / CLSM Stage 1; for 2 min Stage 2; for 7 d	0.02 % AgNPs gel as a medicament resulted in significant reduction in residual biofilms	Wu *et al.*, 2014
Ag-GO	Human root model Irrigant	1 % NaOCl 2.5 % NaOCl 2 % CHX 17 % EDTA 0.25 % Ag-GO	Saline	*P. acnes*, *A. radicidentis*, *S. epidermidis*, and *S. mitis*	Paper point sampling/ microbial counts/ CLSM	Ag-GO resulted in significant bacterial reduction, however, 2.5 % NaOCl showed highest antimicrobial efficacy	Ioannidis *et al.*, 2019

from the cell membrane (Al-Jumaili *et al.*, 2017; Zhou and Gao, 2014)
2. Cell entrapment. Aggregated graphene sheets can trap and separate microorganisms from their microenvironment. This environmental disconnection results in preventing nutrient consumption, reducing proliferative activity, and eventually inactivation of the microorganism (Akhavan *et al.*, 2011)
3. Oxidative stress induction can also act as an electron pump transferring electrons out of microorganisms (Liu *et al.*, 2011).

However, the exact antimicrobial effectiveness is difficult to predict and is thought to be dependent on several factors such as type of microorganisms, duration of exposure, and most importantly specific structural properties – such as number of layers, porosity, size, and shape of the individual graphene sheets (Li *et al.*, 2014; Liu *et al.*, 2011; Zhu *et al.*, 2017).

In general, graphene-based materials are biocompatible, promoting cell adhesion, proliferation, and differentiation (Menaa *et al.*, 2015; Tahriri *et al.*, 2019). However, the exact degree of biocompatibility and potential cytotoxicity is highly dependent on their oxygen functional groups (Guazzo *et al.*, 2018). Despite the limited *in vivo* studies, *in vitro* studies of variable methodologies concluded that most graphene-based hydrophilic forms are less toxic than the hydrophobic forms (Guazzo *et al.*, 2018; Tahriri *et al.*, 2019).

To further investigate graphenes' biocompatibility and and dental tissue regeneration, Rosa *et al.* (2016) investigated the proliferation and differentiation potential of DPSCs on graphene oxide-based substrates. Their results indicated an upregulation of odontogenic gene expression with graphene oxide-based substrates, in comparison to the control (glass-based substrates). The same group later observed higher osteogenic rather than odontoblastic differentiation (Xie *et al.*, 2017). However, *in vitro* studies do not provide robust data distinction between osteogenic and odontogenic differentiation, and further *in vivo* studies are required to make this distinction.

More recently, graphene-based materials have gained significant attention within the dental field, including graphene coated implants (Jung *et al.*, 2015; Ren *et al.*, 2017), incorporation into commercial dental materials such as adhesives and resins (Bregnocchi *et al.*, 2017; Sun *et al.*, 2018), and as

Table 1f. *In vitro* and *ex vivo* studies of alternative antimicrobial strategies highlighting antimicrobial type, origin, study design, usage, study and control groups, assessment method and duration, microorganisms tested and main findings.

Alternative antimicrobial/ origin	Study design/ usage	Study groups	Control groups	Microorganism	Assessment method/ duration	Main findings	Reference
AgNPs in an aqueous vehicle/ Khemia, São Paulo, Brazil	Bovine dentine blocks Irrigant	94 ppm AgNPs solution 2.5 % NaOCl 2 % CHX	Saline without inoculum	*E. faecalis* biofilm and infected dentinal tubules	LIVE/ DEAD staining Variable contact time of 5, 15 and 30 min	AgNPs showed variable antimicrobial efficacy; however, this was inferior to NaOCl	Rodrigues *et al.*, 2018
Bioactive glass S53P4	Contra-lateral human premolar teeth Medicament	Bioactive glass $Ca(OH)_2$	No medicament	*E. faecalis*	Sampling of dentine chips/ SEM 10 d	Bioactive glass, in its current format, was less efficient than standard $Ca(OH)_2$	Zehnder *et al.*, 2006
Bioactive glass S53P4	Human dentine block model Medicament	2 % CHX gel 2 % metronidazole gel Bioactive glass $Ca(OH)_2$	Saline	*E. faecalis*	CFUs/ inhibition of growth 1, 3 and 5 d	Bioactive glass S53P4 antimicrobial efficacy was superior to $Ca(OH)_2$, but inferior to metronidazole and CHX gel	Krithikadatta *et al.*, 2007
Bioactive glass S53P4	Human root model Medicament	$Ca(OH)_2$ 1 % CHX gel bioactive glass $Ca(OH)_2$ plus point Activ point	Saline	*E. faecalis* and *S. mutans*	CFUs 7 d	CHX-impregnated medicaments were more efficient than alkaline-pH-acting medicaments	Atila-Pektaş *et al.*, 2013
TAMP bioactive glass	Human dentine block model Medicament	TAMP-bioactive glass 100 mg/mL DAP 1 mg/mL 0.02 % AgNPs gel	Without bacterial contamination	*E. faecalis* biofilms and infected dentinal tubules	Biofilm disruption assay/ CFUs/SEM 1 and 7 d	All tested medicaments resulted in significant biofilms reduction	Sadek *et al.*, 2019

bioactive cements (Dubey *et al.*, 2017). To enhance root canal disinfection, the synthesis of AgNPs on an aqueous graphene oxide matrix has shown promising preliminary results in terms of antimicrobial activity and biofilm disruption (Ioannidis *et al.*, 2019).

Their versatile and promising properties position graphenes as suitable candidates for investigation within RET. Emerging studies on them as intra-canal disinfectant agents are summarised in Table 1.

Pectin

Pectin is a natural plant-specific carboxylated polysaccharide extracted from fruits or vegetables. Pectin provides mechanical strength for the cell walls of plants and are important for various cellular processes, such as water absorption, morphological development, and ripening of fruits (Redondo-Nevado *et al.*, 2001; Vincken *et al.*, 2003). Pectins are known for their gelling, thickening and emulsifying properties, hence their wide use in the food industry. Its emulsification property is affected by the chemical characteristics of the raw material as well as extraction methods used and can be modified using chemical and enzymatic treatments.

The gelling properties of pectin became of interest due to its possible use as an *in situ* biocompatible gelling system for bone tissue engineering (Munarin *et al.*, 2010a; Munarin *et al.*, 2011; Munarin *et al.*, 2012), an injectable cell delivery system (Mishra *et al.*, 2008), and a drug delivery system (Ishii and Matsunaga, 2001; Munarin *et al.*, 2010b). The structure and features of pectins depend on the plant species and tissues. However, some characteristics are common to all of these polysaccharides. Two main structural categories have been recognised, the smooth and hairy (or ramified) regions (Varoni *et al.*, 2012).

Plant-derived pectins have been investigated as candidates for surface nano-coating of orthopaedic and dental titanium implants, due to their ability to enhance osteogenic differentiation of osteoblasts. Folkert *et al.* (2016) investigated the effect of coating titanium implants, with unmodified pectin and enzymatically modified pectin extracted from potato pulps, on *in vitro* cell proliferation, mineralisation, and osteogenic differentiation of osteoblast-like cell lines and primary mice osteoblasts. The study confirmed that both types of pectin coating enhanced cell proliferation, mineralisation, and osteoblastic differentiation – particularly modified pectins with a high content of galactose (Folkert *et al.*, 2016).

Conversely, Gurzawska *et al.* (2017) evaluated the effect of nano-coating titanium implants with plant-cell-wall-derived rhamnogalacturonan-I (pectins), on the bone healing and osseointegration of implants in the tibia of a rabbit model. The results showed no significant difference between coated and non-coated implants.

In dentistry, Nguyen *et al.* (2013) found that different pectins (LM-, HM- and AM-pectin) coated liposomes, adsorbed the hydroxyapatite to the tooth surfaces *in vitro* which suggested their possible usage as a protective coating on tooth enamel. Furthermore,

Table 1g. *In vitro* and *ex vivo* studies of alternative antimicrobial strategies highlighting antimicrobial type, origin, study design, usage, study and control groups, assessment method and duration, microorganisms tested and main findings.

Alternative antimicrobial/ origin	Study design/ usage	Study groups	Control groups	Microorganism	Assessment method/ duration	Main findings	Reference
Poly (lactic- *co* -glycolic acid) nanoparticles with methylene blue as a photosensitiser	Human root model Irrigant	Methylene blue loaded nanoparticles with/without light	No light/ no methylene blue nanoparticles	*E. faecalis* in planktonic and biofilm forms	CFUs/ SEM Light application for 5 min with a wavelength of 665 nm.	Methylene blue loaded nanoparticles reduced *E. faecalis* counts in both planktonic phase and within root canals	Pagonis *et al.*, 2010
Curcumin/ indocyanine green as photosensitiser	Well plates Irrigant	5.25 % NaOCl, 0.2 % CHX, 2 % CHX, 40 mmol/L curcumin with LED 1 mg/mL indocyanine green with diode laser	No exposure to irrigation solutions or photosensitisers	*E. faecalis* in planktonic and biofilm forms	CFUs/ crystal violet assay LED wavelength of 450 nm Diode laser wavelength of 810 nm	Photoactivated curcumin antimicrobial efficacy was very close to standard irrigating solutions tested	Pourhajibagher *et al.*, 2018
rGO-Cur photosensitiser	Human root model Irrigant	rGO-Cur LED group rGO-Cur with LED 2.5 % NaOCl	Only bacterial suspension	*E. faecalis* biofilms	MIC/ SEM/ real-time qPCR Wavelength of 450 ± 30 nm with 300 s	The combined usage of rGO-Cur with LED showed promising results in terms of antimicrobial efficacy and biofilm inhibition	Ghorbanzadeh *et al.*, 2020

in vivo usage of pectin in combination with a chitosan scaffold within RET in dog endodontic lesions was assessed (Palma *et al.*, 2017). However, the mineral content and root closure were better using a blood clot alone, in comparison to a pectin-chitosan scaffold (Palma *et al.*, 2017).

Pectin is reported to have bactericidal effects against the most widely distributed pathogenic and opportunistic microorganisms. It was also found that higher concentrations (> 2 %) had an inactivating effect on a therapeutic bacteriophage and decreased the antimicrobial activity of penicillin (Men'shikov *et al.*, 1997). Cinnamaldehyde pectin extracted from the papaya puree were found to have antimicrobial effects against *E. coli*, *L. monocytogenes*, *S. aureus*, and *S. enterica* (Otoni *et al.*, 2014).

Although it is still early days for pectin usage in the dental field, its gelling properties, unique structural variations, and antimicrobial properties, makes injectable pectin gels an excellent candidate to investigate for regenerative endodontics.

Ascorbic acid

Ascorbic acid, commonly known as vitamin C, is an essential vitamin for the health of body tissues and especially for natural collagen synthesis. This white to light-yellow water-soluble ketolactone with two ionisable hydroxyl groups (Du *et al.*, 2012), is the most abundant antioxidant vitamin within plant cells (Smirnoff, 2000).

As an electron-donor, ascorbic acid is known to have several physiological and biochemical properties (Arrigoni and De Tullio, 2002; Du *et al.*, 2012). Ascorbic acid has antimicrobial properties against several bacteria and viruses, ranging from bacteriostatic to bactericidal that was reported to increase by time (Goldschmidt, 1991).

Ascorbic acid also has potent anti-inflammatory properties (Diomede *et al.*, 2020), therefore; positively influencing host-defence mechanism towards tissues repair during infection (Goldschmidt, 1991). Its antiscorbutic properties play a pivotal role in the synthesis of collagen through its ability to act as an electron-donor maintaining the ferrous in collagen hydroxylases in an active state (Du *et al.*, 2012). Ascorbic acid's potential influence on the formation of dentine collagen matrix is of particular interest to researchers in pulp-dentine complex regeneration (Balic *et al.*, 2009).

Within the dental field, ascorbic acid has been advocated as a biocompatible reducing agent integrated into commercial monomers and composite systems. Indeed, 10 % ascorbic acid was found to restore the dentine bonding strength to adhesive resins after deproteinisation with 5 % NaOCl irrigation solution (da Cunha *et al.*, 2010; Furuse *et al.*, 2014; Morris *et al.*, 2001). Additionally, the integration of ascorbic acid within dental monomer increased DPSCs proliferation rate and decreased unwanted cellular morphology alterations due to the monomer treatment (Diomede *et al.*, 2020).

Furthermore, its potential role in promoting regeneration of the dental pulp, as a novel endodontic compound, has been suggested (Diomede *et al.*, 2020). Its relative cheap cost, ease of use, antioxidant properties, and tissue repair properties makes it an

Table 2. *In vivo* human and animal studies of alternatives antimicrobial strategies highlighting antimicrobial type, origin, study design, usage, study and control groups, assessment method and duration and main findings.

Material/ origin/ preparation	Study design/ usage	Study groups	Control groups	Assessment method/ duration	Main findings	Reference
EEP/ Commercially available	Chronically exposed primary teeth Irrigant	3 % NaOCl 12.5 % alcoholic extract of miswak 11 % EEP	0.9 % saline	Paper points sampling/ CFUs Pre- and post-irrigation samples	11 % EEP showed disappointing results with no significance difference from the control	Shingare and Chaugule, 2011
Egyptian propolis/ El Monofia province	Immature non-vital dogs' teeth with induced periapical infection Medicament for RET	Propolis paste with propolis plug Propolis paste with MTA plug TAP with propolis plug TAP with MTA plug	No medicament No intervention	Antimicrobial efficacy: CFUs/reduction in colony counts 3 weeks Regenerative outcome: radiographic/ histopathologic evaluation 2 weeks, 1 and 2 months	Propolis showed promising results, comparable to TAP, in terms of antimicrobial efficacy and hard and soft tissue deposition	El-Tayeb *et al.*, 2019
Propolis/ Farmácia de Manipulação Nova Derme	Immature dog teeth with induced periapical infection Medicament for RET	TAP 1 % propolis paste	No medicament No intervention	Histological analysis 7 months	Propolis showed promising results, superior to TAP, in terms of hard and soft tissue deposition	Pagliarin *et al.*, 2016

excellent candidate to investigate for regenerative endodontics.

Synthetic materials and strategies

Bioactive glass

In the early 90s, Hench and colleagues developed a novel Class A bioactive glass-ceramic material composed of silica and other components such as calcium and phosphate (Hench and Paschall, 1973; Hench *et al.*, 1971). Following this discovery, bioactive glass (calcium sodium phosphosilicate) received considerable clinical interest within the dental field as a versatile material –as a bone substitute for tissue regeneration (Norton and Wilson, 2002; Pereira *et al.*, 2018), an implant coating material (Xuereb *et al.*, 2015), a drug delivery material (Wu and Chang, 2014), and an additional component within various restorative materials (Sauro *et al.*, 2012; Tirapelli *et al.*, 2011). This attracted research attention was largely related to its inherent material advantages such as antimicrobial activity (Munukka *et al.*, 2008; Waltimo *et al.*, 2007; Zhang *et al.*, 2010), biocompatibility, and hard tissue regenerative potential (El-Gendy *et al.*, 2015; El-Gendy *et al.*, 2013; El Shazley *et al.*, 2016).
The mechanism of antimicrobial action of bioactive glass is attributed to different factors – its alkaline pH, the sustained release of silica and/or calcium phosphate ions, and specific glass composition (Gubler *et al.*, 2008; Zhang *et al.*, 2010). However, because conventional micron-sized bioactive glass demonstrated mild to moderate antimicrobial activity, specifically against *E. faecalis*, material improvements were consistently ongoing (Krithikadatta *et al.*, 2007; Waltimo *et al.*, 2007; Zehnder *et al.*, 2006).

Advances with nano-technology fabrication led to the development of nano-scale bioactive glass (Lei *et al.*, 2010). The nano-scale bioactive glass 45S5 was found to increase the pH in a solution and increase silica release by a factor of 10 in comparison to μm-sized bioactive glass, resulting in improved antimicrobial effectiveness (Waltimo *et al.*, 2007). Increased ion release of nano-scale bioactive glass also enhanced cytocompatibility and tissue regeneration properties (Lei *et al.*, 2010; Wang *et al.*, 2020); nano-scale bioactive glass also promoted dentine mineralisation of higher stability and acid resistance compared to micron-scale glass particles (Sheng *et al.*, 2016).

Various types of bioactive glass have been specifically developed and investigated such as TAMP bioactive glass (Sadek *et al.*, 2019) and mesoporous bioactive glass (Wu *et al.*, 2011; Yan *et al.*, 2004). Furthermore, with improved material science, the ability to customise bioactive glass functionalised structures with the addition of antimicrobial and regenerative agents became possible (Ribeiro *et al.*, 2020). The combined addition of silver ions to mesoporous bioactive glass has shown promising results in terms of improved antimicrobial effectiveness (Gargiulo *et al.*, 2013). More sophisticated hybrid systems have also been developed, such as the incorporation of silver-doped bioactive glass within hydrogels with promising antimicrobial, anti-inflammatory, and DPSCs differentiation potential (Wang *et al.*, 2015; Zhu *et al.*, 2019).

Although at an early stage of research, studies expanding its use as a disinfectant agent for endodontics (Atila-Pektaş *et al.*, 2013; Krithikadatta *et al.*, 2007; Zehnder *et al.*, 2006) and RET (Sadek *et al.*, 2019) have been performed and summarised in Table 1.

PDT

PDT, also known as photodynamic inactivation or photoactivated disinfection, is advocated as an adjunct antimicrobial approach for clinical disinfection of the complex root canal system (Gursoy *et al.*, 2013; Plotino *et al.*, 2019). This specialised technique involves the vibrant interaction between a photosensitising agent (photosensitiser) and a light source (Hamblin and Hasan, 2004; Konopka and Goslinski, 2007). This interaction leads to the production of reactive oxygen species, such as free radicals and singlet oxygen, resulting in oxidative and cytotoxic damage to the target cells (Hamblin and Hasan, 2004; Konopka and Goslinski, 2007).

The antimicrobial mechanism of PDT can be explained based on the direct effect on extracellular molecules mediated by singlet oxygen of high chemical reactivity and the indirect photodamage to the polysaccharide bacterial biofilm (Konopka and Goslinski, 2007; Wainwright and Crossley, 2004). This dual activity is reported as a significant advantage over currently used antibiotics (Konopka and Goslinski, 2007; Plotino *et al.*, 2019), with effectiveness against antibiotic-sensitive and antibiotic-resistant microorganisms (Wainwright and Crossley, 2004). Furthermore, there is no evidence of bacterial resistance to the various metabolic pathways associated with the action of singlet oxygen or free radicals (Dias *et al.*, 2020; Konopka and Goslinski, 2007).

The effect of PDT on the surrounding stem cells has been investigated. Li *et al.* (2020) found that the application of PDT provided an inductive microenvironment for SCAP growth. Quantitative reverse transcriptase-polymerase chain reaction also resulted in a positive expression of platelet-derived growth factor and vascular endothelial growth factor. In-line with the above, PDT resulted in greater viability of apical papilla cells (Deluca *et al.*, 2021) and significantly less cytotoxicity compared to NaOCl irrigation (George and Kishen, 2007; Gomes-Filho *et al.*, 2016).

Within dentistry, the application of PDT is expanding into different areas, such as treatment of head and neck cancer (Grant *et al.*, 1993; Hopper, 2000), oral plaque biofilms (Tahmassebi *et al.*, 2015; Wood *et al.*, 2006), treatment of peri-implantitis

(Bassetti *et al.*, 2014; Bombeccari *et al.*, 2013), and root canal disinfection (Asnaashari *et al.*, 2017; Bonsor *et al.*, 2006; de Miranda and Colombo, 2018; Soukos *et al.*, 2006). More recently, PDT has been recommended as a positive adjunct in RET protocols (Deluca *et al.*, 2021; Devaraj *et al.*, 2016). However, to date, this is scarcely documented *in vivo*. Successful clinical and radiographic outcomes, in terms of thickening of dentinal walls and apical closure, following the adjunct use of tolonium chloride photosensitiser followed by platelet-rich fibrin (Johns *et al.*, 2014) or collagen resorbable matrix (Abdel Hafiz Abdel Rahim *et al.*, 2019) are reported.

Although most reported studies utilised PDT with aid of an intra-canal optic fibre, Nunes *et al.* showed no significant difference in bacterial reduction when an intra-canal optic fibre was not used. However, in this *in vitro* study, all teeth were decoronated and only standard 15 mm root segments were utilised (Nunes *et al.*, 2011). The reduced oxygen concentration within the root canals, especially in deep irregularities, can directly affect the formation of cytotoxic oxygen derivatives and reduce the antimicrobial efficacy (Nunes *et al.*, 2011). Modifying the optical fibre tip to improve the clinical performance has been recommended (George and Walsh, 2011).

Chemical phenothiazine dyes such as methylene blue and toluidine blue (tolonium chloride) are often reported within endodontic protocols (Gursoy *et al.*, 2013; Siddiqui *et al.*, 2013). However, potential adverse effects, such as staining and discoloration are documented (Plotino *et al.*, 2019; Ramalho *et al.*, 2017). To overcome this clinical limitation, attempts such as evaluating a therapeutic efficacy window of the chemical dyes have been tested (Gursoy *et al.*, 2013; Plotino *et al.*, 2019). Obliteration of dentinal tubules as a result of viscous photosensitiser substances impregnating the dentine surface is also reported (Plotino *et al.*, 2019). Clinically, this could reduce the bond strength between the root filling material and dentinal walls (Shahravan *et al.*, 2007).

Therefore, to overcome the above limitations and enhance clinical outcomes, research has recently focused on the development of novel formulations, such as polymer-based nanoparticle photosensitiser (Gil-Tomás *et al.*, 2007; Koo *et al.*, 2007; Shrestha and Kishen, 2014). Nanoparticle-based photosensitisers have several advantages over standard photosensitising molecules such as:

1. increasing production of reactive oxygen species;
2. reducing the possibility of multiple-drug resistance;
3. providing selective treatment by localised delivery agents;
4. having a nonimmunogenic nature of nanoparticle matrix (Koo *et al.*, 2007; Pagonis *et al.*, 2010).

Examples of novel photosensitiser suggested for intra-canal disinfection include poly(lactic*co*glycolic) acid nanoparticles loaded with methylene blue (Pagonis *et al.*, 2010). The use of curcumin solution as a photosensitiser has gained significant scientific interest (da Frota *et al.*, 2015; Ghorbanzadeh *et al.*, 2020; Neelakantan *et al.*, 2015; Pourhajibagher *et al.*, 2018; Sotomil *et al.*, 2019). Chitosan nanoparticles functionalised with rose-bengal photosensitiser were also found to stabilise the structural integrity of root dentine *in vitro* by photo-crosslinking the collagen, resulting in sufficient elimination of biofilms, the stabilisation of the dentinal matrix (Shrestha and Kishen, 2014), and significant inactivation of bacterial endotoxin lipopolysaccharides (Shrestha *et al.*, 2015). Emerging studies are currently being published of a novel photosensitiser as an adjunct for intra-canal disinfection, with promising results, as summarised in Table 1.

Concluding remarks and future perspectives

Adequate disinfection of the root canal system during RET is a prerequisite for successful regeneration of the pulp-dentinal complex. However, it should be achieved while maintaining a conducive environment for stem cell survival and proliferation. Although currently adopted antimicrobial protocols provide acceptable disinfection, the clinical outcomes are still unpredictable and far from ideal. Key limitations, such as coronal discolouration, cell cytotoxicity, difficulty of removal from the root canal, development of sensitisation and resistant bacterial strains are widely documented within the literature.

There is a growing interest in the exploration of alternative antimicrobial strategies within RET for a predictable biological outcome. Despite the above-mentioned promising results of various new strategies, it is noteworthy that currently available data are mostly drawn from *in vitro* and limited animal studies using single bacterial species, mainly *E. faecalis*. Further investigations into the effect of the proposed antimicrobials against complex polymicrobial biofilms involved in endodontic infections is of extreme importance to finalise the conclusion concerning the use of these materials in RET. Therefore, the development and testing of the proposed alternative antimicrobial materials within a well-controlled *in vitro*, followed by *in vivo*, studies are required.

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