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REVIEW

The effect of ozone therapy in root canal disinfection: a systematic review

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Abstract

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Aim To answer the following focused question: 'As regards microorganism load reduction for patients undergoing root canal treatment, is the use of ozone therapy comparable to conventional chemomechanical techniques using sodium hypochlorite (NaOCl)?'

Data sources A systematic review was conducted using controlled vocabulary and free-text key words in the following databases: PubMed, Science Direct, Scopus, Web of Science and Open Grey until 2 November 2018. Additional studies were sought through hand searching of endodontic journals.

Study eligibility criteria, participants and interventions The inclusion criteria comprised studies that compared microbial reduction in root canals after treatments with ozone and NaOCl in extracted mature human teeth or randomized clinical trials.

Study appraisal and synthesis methods The quality assessment of included laboratory studies was performed with the following parameters: (i) sample size calculation, (ii) samples with similar dimensions, (iii) control group, (iv) standardization of procedures, (v) statistical analysis and (vi) other risk of bias. For randomized clinical trials, the qualitative analysis of the studies was performed from the bias risk assessment using the tool 'Bias Risk Assessment of Randomized Controlled Studies' Cochrane Handbook 5.0.2.

Results The search resulted in 180 published studies. After removal of duplicate studies and full-text analysis, eight studies were selected and seven were considered low risk of bias (seven *ex vivo* studies and one random clinical trial). Overall, the results demonstrated that ozone therapy provides significantly less microbial load reduction than NaOCl. As an adjunct in chemomechanical preparation, ozone was ineffective in increasing the antimicrobial effect of NaOCl. Ozone performance was strongly associated with the application protocol used: it is dose, time and bacterial strain dependent, besides the correlation with the use of complementary disinfection sources.

Limitations A restricted number of randomized clinical trial was found, and the difference amongst the methodology of the studies did not allow a meta-analysis to be performed.

Conclusions and implications of key findings Although the selected studies had limitations, this review reached a satisfactory methodological and moderate evidence quality contributing to important preliminary information regarding ozone therapy. As regards load reduction of microorganisms for patients undergoing root canal treatment, ozone is not indicated neither to replace nor to complement the antimicrobial action of NaOCl.

Keywords: disinfection, endodontics, ozone, sodium hypochlorite, systematic review.

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Introduction

Micro-organisms and their by-products are the main cause of pulp and periradicular diseases (Kakehashi *et al.* 1965, Siqueira Jr & Rocas 2007). Root canal preparation enlarges the main root canal promoting mechanical removal of infected dentine and simultaneously favours the penetration of irrigants through the canals, enhancing the decontamination process (Estrela *et al.* 2014). However, a significant percentage of the root canal surface remains untouched, regardless of the instruments used for mechanical preparation (Siqueira Jr *et al.* 2018). Such unreached areas may protect micro-organisms from root canal disinfecting protocols (Gomes & Herrera 2018).

Sodium hypochlorite (NaOCl) is the most commonly used root canal irrigant; it has an effective antimicrobial activity, a broad bacterial range, and creates a significant reduction in endotoxins levels (Zehnder 2006, Fidalgo *et al.* 2010, Neelakantan *et al.* 2019). However, several studies have demonstrated that complete bacterial elimination cannot be achieved consistently with any of the current disinfection protocols (Siqueira Jr *et al.* 2018, Silva *et al.* 2019). Therefore, efforts have been made to develop novel techniques to provide additional disinfection for the root canal system, such as passive ultrasonic irrigation (PUI) (Dioguardi *et al.* 2018), photodynamic therapy (PDT) (Eslami 2019), continuous irrigating techniques (Dioguardi *et al.* 2018, Silva *et al.* 2019) and apical positive and negative pressure irrigation methods (Dioguardi *et al.* 2018, Eslami 2019, Silva *et al.* 2019). Amongst these protocols, ozone therapy has been investigated aiming to increase microbial load reduction within the canal system and thus improve endodontic outcomes.

Ozone is a naturally occurring gas and a very strong and selective oxidant (Boch *et al.* 2016). Ozone therapy is based on the assumption that ozone (O_3) rapidly dissociates into water and releases a reactive form of oxygen that may oxidize cells, thus having antimicrobial efficacy without inducing drug resistance (Case *et al.* 2012). Firstly, ozone acts in glycolipids, glycoproteins or certain aminoacids, which are present in the cytoplasmic membrane of microorganisms (Rojas-Valencia 2011). The oxidation process of these unsaturated lipids and proteins generates a quantitative conversion of the present olefinic bonds to reactive species of lipid oxidation products (Junior & Lages 2012). These reactive species, named ozonide, signal and trigger metabolic changes that yield distant microbicide effects (Case *et al.* 2012, Junior & Lages 2012).

Following these principles, the use of ozone therapy has been tested both as an alternative agent to NaOCl and as a complementary disinfection source in chemomechanical canal preparation. However, conflicting results have been reported. Some authors demonstrated that ozone therapy has similar results compared to NaOCl in reducing various species of bacteria (Huth *et al.* 2009, Hubbezoglu *et al.* 2014, Kist *et al.* 2017), whilst others reported less effective disinfection (Case *et al.* 2012, Kaya *et al.* 2014, Boch *et al.* 2016). However, no effort has been made to evaluate the efficacy of ozone therapy by means of a systematic review of the literature. Relevant features such as the antimicrobial capacity of ozone when compared to NaOCl, ozone performance as an adjunct in chemomechanical preparation and its most indicated form of application are unknown. As ozone has gained attention in Endodontics, being currently suggested as an emerging and promising disinfection technique, these features are significant for clinicians in terms of supporting the use of ozone in clinical applications. Within this background, this systematic review aimed to answer the following focused question: 'As regards microorganism load reduction for patients undergoing root canal treatment, is the use of ozone therapy comparable to conventional chemomechanical techniques using NaOCl?'

Materials and methods

Protocol and registration

This systematic review followed the recommendations of PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analysis) guideline (http://www.prisma-statement.org) and registered in PROSPERO (CRD 42019134748).

Search strategy

The search process was performed independently by two examiners (E.J.L.N.S. and T.K.S.F.). The electronic databases PubMed, Science Direct, Scopus, Web of Science and Open Grey were searched for articles published until 2 November 2018, without language, year restrictions or limits. The electronic search strategy was developed using most cited descriptors in the previous publication on this theme combining Medical Subject Heading (MeSH) terms and text word (tw).

For each database, the following terms were combined: 'Periapical abscess', 'Periapical lesion', 'Root Canal Obturation', 'Dental Pulp Necrosis', 'Dental Pulp Devitalization', 'Endodontic', 'Ozone', 'Microbial Consortia', 'Disinfection', 'Bacteria', 'Polymerase chain reaction', 'Culture', 'Microb*' and 'Microorganism*'. The Boolean operators 'AND' and 'OR' were applied to combine the terms and create the search strategy. The search strategies defined for each databases are detailed in Table 1. A complementary screening on the references of the selected studies was performed, and a hand search in *Journal of Endodontics* and *International Endodontic Journal* was performed to find any additional study that did not appear in the primary database search. Articles from different sources were imported into the EndNote Web reference manager

Table 1 Search strategy in the databases

Database	Search strategy	Findings
Pubmed	#1 ((((((((((periapical abscess[MeSH Terms]) OR Periapical Abscess[Title/Abstract]) OR periapical lesion[Title/Abstract]) OR Root Canal Obturation[MeSH Terms]) OR Root Canal Obturation[Title/Abstract]) OR Dental Pulp Necrosis[MeSH Terms]) OR Dental Pulp Necrosis[Title/Abstract]) OR Dental Pulp Necrosis[MeSH Terms]) OR Dental Pulp Necrosis[Title/Abstract]) OR Dental Pulp Devitalization[MeSH Terms]) OR Dental Pulp Devitalization[Title/Abstract]) OR Endodontic*[Title/Abstract]	24 310
	#2 (Ozone[MeSH Terms]) OR Ozone[Title/Abstract]	21 115
	#3 ((((((((((Microbial Consortia[MeSH Terms]) OR Microbial Consortia[Title/Abstract]) OR Disinfection [MeSH Terms]) OR Disinfection[Title/Abstract]) OR bacteria[MeSH Terms]) OR bacteria[Title/Abstract]) OR polymerase chain reaction[MeSH Terms]) OR polymerase chain reaction[Title/Abstract]) OR culture[MeSH Terms]) OR culture[Title/Abstract]) OR Microb*[Title/Abstract]) OR Microorganism*[Title/Abstract]	2 578 854
	# 1 AND # 2 AND #3	22
Scopus	# 1 TITLE-ABS-KEY (periapical abscess) OR TITLE-ABS-KEY (periapical lesion) OR TITLE-ABS-KEY (Root Canal Obturation) OR TITLE-ABS-KEY (Dental Pulp Necrosis) OR TITLE-ABS-KEY (Dental Pulp Devitalization) OR TITLE-ABS-KEY (Endodontic*)	44 516
	#2 TITLE-ABS-KEY (Ozone)	98 334
	#3 TITLE-ABS-KEY (Microbial Consortia) OR TITLE-ABS-KEY (Disinfection) OR TITLE-ABS-KEY (bacteria) OR TITLE-ABS-KEY (polymerase chain reaction) OR TITLE-ABS-KEY (culture) OR TITLE-ABS-KEY (Microb*) OR TITLE-ABS-KEY (Microorganism*)	4 369 630
	# 1 AND # 2 AND #3	37
Web of science	#1 TS=('Periapical abscess' OR 'Periapical lesion' OR 'Root Canal Obturation' OR 'Dental Pulp Necrosis' OR 'Dental Pulp Devitalization' OR 'Endodontic')	15 641
	#2 TS=('Ozone')	84 558
	#3 TS=('Microbial Consortia' OR 'Disinfection' OR 'bacteria' OR 'polymerase chain reaction' OR 'culture' OR 'Microb*' OR 'Microorganism*')	1 962 295
	# 1 AND # 2 AND #3	28
Science direct	#1 Periapical abcess OR periapical lesion OR Root Canal Obturation OR Dental Pulp Necrosis OR Dental Pulp Devitalization OR E Endodontic*	28 566
	#2 Ozone	139 890
	#3 Microbial Consortia OR Disinfection OR bacteria OR polymerase chain reaction OR culture OR Microb* OR Microorganism*	2 723 950
	# 1 AND # 2 AND #3	88
Cochrane	#1 Periapical abscess OR periapical lesion OR Root Canal Obturation OR Dental Pulp Necrosis OR Dental Pulp Devitalization OR E Endodontic*	1036
	#2 Ozone	672
	#3 Microbial Consortia OR Disinfection OR bacteria OR polymerase chain reaction OR culture OR Microb* OR Microorganism*	48214
	# 1 AND # 2 AND #3	4
Open Grey—SIGLE	#1 Periapical abscess OR periapical lesion OR Root Canal Obturation OR Dental Pulp Necrosis OR Dental Pulp Devitalization OR E Endodontic*	17
	#2 Ozone	1488
	#3 Microbial Consortia OR Disinfection OR bacteria OR polymerase chain reaction OR culture OR Microb* OR Microorganism*	33 927
	# 1 AND # 2 AND #3	1

(EndNote™), to catalogue the references and automatically remove duplicate records.

Eligibility criteria

Studies that evaluated the microbial reduction in root canals after treatment with both ozone and NaOCl were included. The eligibility criteria were based on the PICOS strategy (PRISMA-P 2016), as follows:

- *Population (P):* mature human teeth;
- *Intervention (I):* ozone;
- *Comparison (C):* sodium hypochlorite (NaOCl);
- *Outcome (O):* micro-organism reduction counting;
- *Study design (S):* laboratory or clinical trials.

The following were excluded: reviews, letters, opinion articles, conference abstracts, studies performed in animals, studies that included artificial teeth and studies in which it was not possible to recover the reduction rates of microorganism counting.

Selection of the studies

Two authors (E.J.N.L.S. and T.K.S.F.) independently selected the retrieved studies by examining the titles and abstracts. The full text was accessed when it was not possible to judge the studies by title and abstract. A second stage consisted of reading the full texts and judging the potential studies to be included based on the eligibility criteria through the PICOS strategy. Disagreements on study inclusion were solved by consensus with a third author (M.C.P.). Duplicated studies in the databases search were considered only once.

Data extraction

Two authors (E.J.N.L.S. and T.K.S.F.) collected the data independently from the included studies. Disagreements were solved by a third author (M.C.P.). Information regarding publication (author and publication year), tooth type, micro-organisms, sample size, irrigant, micro-organism reduction values and statistical analysis was extracted. In cases of missing data, the authors were contacted three times by electronic message.

Quality assessment

Each selected study was evaluated for inner methodological risk of bias independently by two authors (E.J.N.L.S. and T.K.S.F.).

For laboratory studies, a quality assessment was adopted with adaptations used in previous systematic reviews (Sarkis-Onofre *et al.* 2014, Rosa *et al.* 2015, Silva *et al.* 2018). For the quality assessment of the included studies, the following parameters were considered: (i) sample size calculation, (ii) samples with similar dimensions, (iii) control group, (iv) standardization of procedures, (v) statistical analysis and (vi) other risk of bias. Each parameter for all included studies was judged as ‘low’, ‘high’ or ‘unclear’ risk of bias. During the quality assessment, disagreements between authors were resolved through discussion with a third author (M.C.P.). When any parameter was judged as ‘unclear’, the author was contacted by electronic message in order to obtain more information and to enable the judgement of ‘low’ or ‘high’ risk of bias.

For randomized clinical trials, the qualitative analysis of the studies was performed from the bias risk assessment using the Cochrane risk of bias tool: ‘Bias Risk Assessment of Randomized Controlled Studies’—Cochrane Handbook 5.0.2 (Higgins & Green 2011). Due to the methodological characteristics of the studies, the following domains were considered: (i) random sequence generation; (ii) allocation concealment; (iii) blinding of participants; (iv) blinding of outcome assessment; (v) incomplete outcome data; (vi) selective reporting; and (vii) other source of bias. The blinding of operators was not considered since it is impossible to perform in these types of interventions.

To make the general judgment of the risk of individual bias, each included study was judged as ‘high’ risk of bias for negative domain response (red), ‘low’ risk of bias for positive domain response (green) and risk of ‘uncertain’ bias (yellow) when response was not clear. When the study was judged as ‘uncertain’, the authors were contacted via e-mail at least three times for more information and allowed to be classified as ‘low’ (green) or ‘high’ (red) risk of bias. Once this contact was not possible, the articles remained with some ‘uncertain’ bias risks.

Results

Selection of studies

Figure 1 shows the flow diagram of the search strategy. Initially, the search resulted in 180 published studies the searched databases, but 92 were excluded as they were duplicates. Then, from 88 eligible papers, the analysis of titles and abstracts resulted in the inclusion of 11 studies. The main reason for rejection of the articles was the tested groups that did not match the

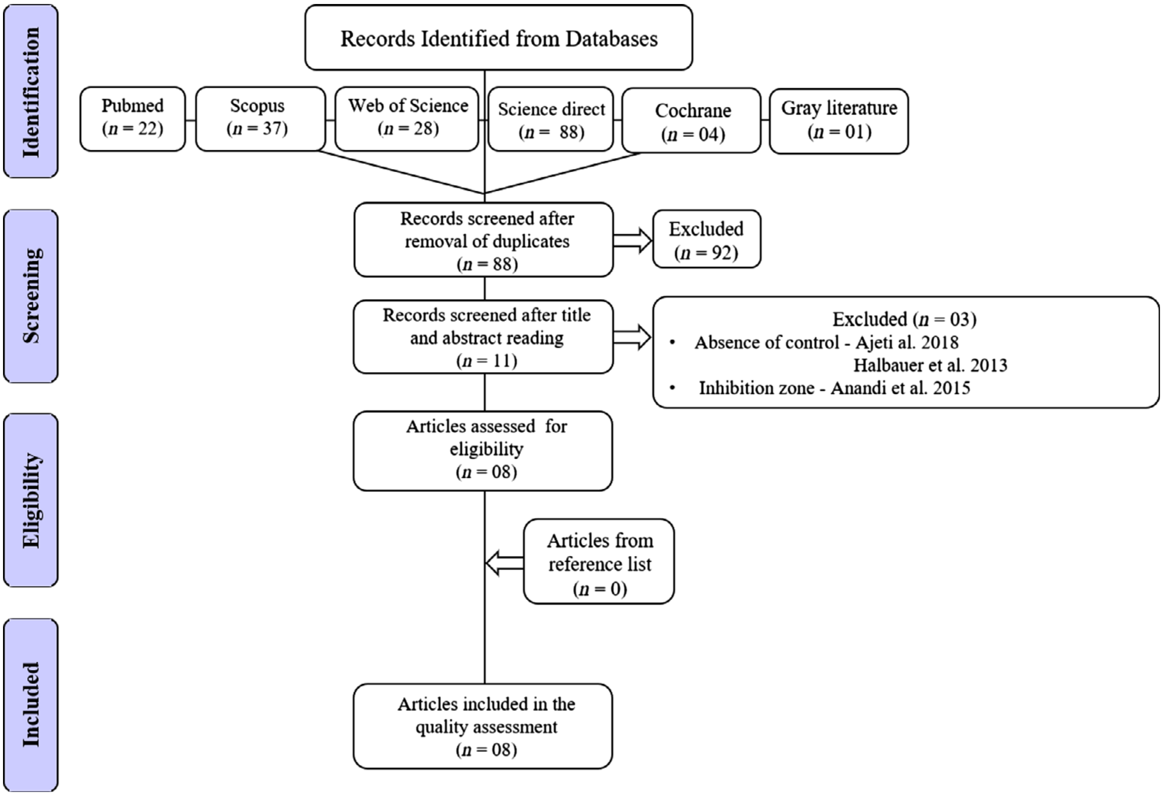

Figure 1 A flow chart of the literature search and the selection process according to the PRISMA statement.

inclusion criteria. After a comprehensive reading, three studies were excluded due to the absence of control groups (Halbauer *et al.* 2013, Ajeti *et al.* 2018) and performance of inhibition zones (Anand *et al.* 2015). Therefore, a total of eight studies were selected for the systematic review. Amongst these eight selected papers, seven are laboratory studies and only one was a randomized clinical trial. After the electronic search, the references of the selected studies were hand searched, but no further articles were found.

Data collection

The data collected from the eight included studies (Nagayoshi *et al.* 2004, Huth *et al.* 2009, Case *et al.* 2012, Hubbezoglu *et al.* 2014, Kaya *et al.* 2014, Noites *et al.* 2014, Boch *et al.* 2016, Kist *et al.* 2017) are summarized in Table 2.

Risk of bias

Regarding the evaluation of inner methodological risk of bias, seven studies were considered 'low' risk of bias (Nagayoshi *et al.* 2004, Huth *et al.* 2009, Hubbezoglu *et al.* 2014, Kaya *et al.* 2014, Noites *et al.* 2014, Boch *et al.* 2016, Kist *et al.* 2017) and one study was considered as having 'moderate' risk of bias (Case *et al.* 2012). The risk of bias results of laboratory studies is summarized in Fig. 2, and the risk of bias of the randomized clinical trial study is detailed in Fig. 3.

Disinfection effect of ozone therapy

Ozone therapy as an alternative to NaOCl

Overall, the results demonstrated that ozone therapy reduces the microbial load significantly less than NaOCl. Ozone used alone was not able to match the outcomes of NaOCl in any of the studies evaluated. From eight included studies, five reported that although ozone reduced microbial counts significantly, this reduction was lower than that achieved by NaOCl (Nagayoshi *et al.* 2004, Huth *et al.* 2009, Case *et al.* 2012, Kaya *et al.* 2014, Boch *et al.* 2016).

Some studies demonstrated that ozone was capable of reaching a similar performance to NaOCl; but with

Table 2 Characteristics of the included studies

Authors, year country (type)	Microorganisms	Tooth type	Groups/sample size	NaOCl protocol	Ozone protocol	Outcome
Boch *et al.* (2016) Germany (*in vitro*)	*Enterococcus faecalis*	Anterior teeth and premolars with a single patent root canal	*Control* • 20% EDTA (*n* = 25) • 3% NaOCl (*n* = 25) *Experimental* • Ozone (*n* = 25) • 20% EDTA–ozone (*n* = 25) • 3% NaOCl–ozone (*n* = 25)	*Concentration*: 3% *Volume*: 5 mL *Time application*: – *Delivered system*: 30-gauge irrigation needle	*Physical state/Concentration*: gaseous/2100 ppm *Time application*: 60 s (exchange of × 100/s) *Application form*: via the ozone-generating equipment HealOzone® 2130 C (Kavo, Biberach, Germany), according to the manufacturer's instructions.	*Reduction (CFU) in groups*: • 20% EDTA: 80.6% • 3% NaOCl: 99.9% • Ozone: 85.4% • 20% EDTA–ozone: 98.3% • 3% NaOCl–ozone: 99.9 % *Conclusion*: ozone reduced *E. faecalis*, even organized in a biofilm. However, this reduction was lower than NaOCl. NaOCl and NaOCl–ozone, which presented similar results, were the most effective disinfecting agents, followed by EDTA–ozone, ozone alone and EDTA alone.
Case *et al.* (2012) Australia (*in vitro*)	*Enterococcus faecalis*	Single-rooted anterior teeth	*Control* • 1% NaOCl (*n* = 14) • Saline (*n* = 14) • Saline–PUI (*n* = 14) *Experimental* • Ozone (*n* = 14) • Ozone–sPUI (*n* = 14)	*Concentration*: 1% *Volume:* 5 mL *Time application:* 2 min *Delivered system*: 35-gauge irrigation needle.	*Physical state/Concentration*: gaseous/140 ppm *Time application*: 24 s (2 L min^{-1}), repeated times times giving a total ozonation time of 2 min. *Application form*: via the ozone-generating equipment dental ozone system Prozone (W&H Dental Werk Burmoos GmbH, Burmoos, Austria), which was introduced into 100 mL sterile saline in the root canal until 2 mm short of the working length.	*Reduction (CFU) in groups*: • 1% NaOCl: 93.5 % • Saline–PUI: 50.2% • Ozone: 71.6% • Ozone–PUI: 83.8% *Conclusion*: ozone reduced bacteria significantly. However, this reduction was lower than NaOCl. NaOCl was the most effective disinfecting agent, followed by ozone–PUI, ozone alone, and finally PUI alone.

Table 2 Continued

Authors, year country (type)	Microorganisms	Tooth type	Groups/sample size	NaOCl protocol	Ozone protocol	Outcome
Hubbezoglu *et al.* (2014) Turkey (*in vitro*)	*Enterococcus faecalis*	Single-rooted mandibular premolar teeth	*Control* • 5.25% NaOCl (*n* = 10) • 5.25% NaOCl–PUI (*n* = 10) • Ozone 8 ppm (*n* = 10) • Ozone 8 ppm–PUI (*n* = 10) • Ozone 12 ppm (*n* = 10) • Ozone 12 ppm–PUI (*n* = 10) • Ozone 16 ppm (*n* = 10) • Ozone 16 ppm–PUI (*n* = 10)	*Concentration*: 5.25% *Volume*: – *Time application*: 3 min *Delivered system*: –	*Physical state/Concentration*: gaseous/8, 12 or 16 ppm *Time application:* 180 s *Application form*: via a custom-made ozone-generating equipment (TeknO3zone Company, Izmir, Turkey). The aqueous ozone concentration of the distilled water was measured with a probe in the reactor tank and shown by a digital indicator on the generator. Infected root canals were irrigated by manual or ultrasonic technique.	*Reduction (CFU) in groups*: • 5.25% NaOCl: 100% • 5.25% NaOCl–PUI: 100% • Ozone 8 ppm: 62.6% • Ozone 8 ppm–PUI: 74.9% • Ozone 12 ppm: 73.3% • Ozone 12 ppm–PUI: 87.2% • Ozone 16 ppm: 91.4% • Ozone 16 ppm–PUI: 100% *Conclusion*: ozone reduced bacteria significantly. However, this reduction was lower than NaOCl. NaOCl, NaOCl–sPUI and ozone 16 ppm–sPUI, which presented similar results, were the most effective disinfecting agents. Higher concentrations and the use of PUI yielded better results of ozone. The bactericidal activity of high concentration of aqueous ozone combined with ultrasonic application technique showed efficacy similar to that of 5.25% NaOCl in root canals.

Table 2 Continued

Authors, year country (type)	Microorganisms	Tooth type	Groups/sample size	NaOCl protocol	Ozone protocol	Outcome
Huth *et al.* (2009) Germany (*in vitro*)	*Enterococcus faecalis, Candida albicans, Peptostreptococcus micros, Pseudomonas aeruginosa*	Single-rooted teeth	*Control* • 5.25% NaOCl (*n* = 14) • 2.25% NaOCl (*n* = 14) • 2% CHX (*n* = 14) • 3% H_2O_2 (*n* = 14) • PBS (*n* = 14) *Experimental* • Ozone gas (*n* = 14) • Ozone water (*n* = 14)	*Concentration*: 5.25% and 2.25% *Volume*: – *Time application*: 1 min *Delivered system:* –	*Physical state/concentration*: Gaseous/1–53 g m^{-3} or Aqueous/1.25–20 μ g^{-1} *Time application:* 1 min *Application form:* gaseous: via the ozone-generating equipment Ozonosan photonic (Dr Hänsler, Iffezheim, Germany) in a self-constructed glass chamber with simultaneous concentration measurement (GM-6000-NZL; Anseros, Tübingen, Germany). Aqueous: bidistilled water was treated with ozone gas using the ozone generator, which resulted in a final photometrically confirmed concentration water.	*Reduction (CFU) in groups:* *General mean reduction for all tested bacteria: • 5.25% NaOCl: 100% • 2.25% NaOCl: 100% • 2% CHX: 100% • 3% H_2O_2: reduced, but did not eliminated bacteria totally • Ozone gas: O Minimum tested concentration (1 g m^{-3}): >99% • Ozone water: O Concentrations between 5 and 20 μg^{-1}: 100% O 1.25 and 2.5 μg^{-1}: Reduced substantially, but did not eliminate bacteria totally. *Conclusion*: ozone gas presented similar results to NaOCl in reducing different species of bacteria. However, as regards ozone water, in order to achieve reduction levels similar to NaOCl and ozone gas, higher concentrations were necessary. 5.25% and 2.25% NaOCl, 2% CHX and ozone gas, which presented similar results, were the most effective disinfecting agents; followed by 3% H_2O_2 and ozone water. Aqueous and gaseous ozone were dose- and strain dependently effective against the biofilm microorganisms.

Table 2 Continued

Authors, year country (type)	Microorganisms	Tooth type	Groups/sample size	NaOCl protocol	Ozone protocol	Outcome
Kaya *et al.* (2014) Turkey (*in vitro*)	*Enterococcus faecalis*	Mandibular premolars with straight root canals	*Control* • 2.5% NaOCl (n = 12) • Saline (n = 6) • Low-temperature atmospheric pressure plasma (n = 12) *Experimental* • Ozone (n = 12)	Concentration: 2.25% Volume: 5 mL Time application: 2 min Delivered system: 27-gauge dental injector placed 1 mm far from the working length.	*Physical state/Concentration:* gaseous/140 ppm *Time application:* 24 s (2 L min^{-1}), repeated four times giving a total ozonation time of 2 min. *Application form:* via the ozone-generating equipment Prozone (W&H Dental Werk Burmoos GmbH, Burmoos, Austria), which was introduced into 100 mL sterile saline in the root canal until 2 mm short of the working length.	*Reduction (CFU) in groups:* • 2.5% NaOCl O Coronal: 2.34 (2.46) O Middle: 2.03 (2.52) O Apical: 3.19 (2.82) • Saline O Coronal: 6.54 (0.27) O Middle: 6.51 (0.61) O Apical: 6.38 (0.61) • Plasma O Coronal: 3.34 (2.47) O Middle: 0.00 (0.00) O Apical: 2.97 (2.63) • Ozone O Coronal: 4.69 (2.28) O Middle: 4.72 (2.29) O Apical: 4.69 (2.26) *Conclusion:* ozone reduced bacteria significantly. However, this reduction was lower than NaOCl. NaOCl and Plasma were the most effective disinfecting agents, followed by ozone and saline in descending order.

Table 2 Continued

Authors, year country (type)	Microorganisms	Tooth type	Groups/sample size	NaOCl protocol	Ozone protocol	Outcome
Kist *et al.* (2017) Germany	(Randomclinical trial)	Overall bacterial load	All groups of teeth except for maxillary molars	*Control* • 3% NaOCl (n = 20) *Experimental* • Ozone (n = 21)	*Concentration*: 3% *Volume*: 5 mL *Time application*: 15 min *Delivered system*: –	*Physical state/concentration*: gaseous/32 g m^{-3} *Time application*: 120 s *Application form*: via a specific endo-cannula of the healOzone Compact X4 device (Curozone GmbH, Wiesbaden, Germany). In both groups, the root canal was mechanically cleaned and irrigated with NaOCl and EDTA. Then, ozone or NaOCl was applied followed by a 1-week inter-appointment dressing (Ca (OH)2). As final disinfection, ozone (ozone group) or CHX 2 % (NaOCl group) was applied.
Reduction (CFU) in groups: *After*	*chemomechanical preparation:* • NaOCl: 99.87 ± 0.33 • Ozone: 99.19 ± 3.98 *After inter-appointment dressing and final irrigation:* • NaOCl: 99.40 ± 1.92 • Ozone: 98.52 ± 4.92 *Conclusion:* ozone presented similar bacterial reduction to NaOCl after chemomechanical treatment and after inter-appointment dressing.					

Table 2 Continued

Authors, year country (type)	Microorganisms	Tooth type	Groups/sample size	NaOCl protocol	Ozone protocol	Outcome
Nagayoshi *et al.* (2004) Japan (*in vitro*)	*Enterococcus faecalis* and *Streptococcus mutans*	Bovine incisors	*Control* • 2.5% NaOCl (n = 20) • Distilled water (n = 20) • Distilled water–PUI (n = 20) *Experimental* • Ozone (n = 20) • Ozone–PUI (n = 20)	*Concentration*: 2.5% *Volume*: 5 mL *Time application*: 2 min (30 mL min^{-1}) *Delivered system:* –	*Physical state/concentration*: aqueous/4 mg L^{-1} *Time application:* 10 min (30 mL min^{-1}) *Application form*: via flushing specimens with ozone water.	Conclusion: ozone reduced bacteria significantly. However, this reduction was lower than NaOCl. NaOCl was the most effective disinfecting agent, followed by ozone–PUI and ozone alone.
Noites *et al.* (2014) Portugal (*in vitro*)	*Enterococcus faecalis* and *Candida albicans*	Single-rooted teeth	*Control* • 1% NaOCl (n = 20) • 3% NaOCl (n = 20) • Saline (n = 20) • 5% NaOCl (n = 20) • 0.2% CHX (n = 20) • 2% CHX (n = 20) *Experimental* • Ozone 24 s (n = 20) • Ozone 60 s (n = 20) • Ozone 120 s (n = 20) • Ozone 180 s (n = 20) • Ozone 180 s- 5% NaOCl (n = 20) • Ozone 24 s- 2% CHX (n = 20) *In each group, 10 specimens were contaminated with Enterococcus faecalis, whilst the other half of sample, 10 teeth, were contaminated with Candida albicans	*Concentration:* 1%, 3%, 5% *Volume*: 10 mL *Time application:* – *Delivered system:* –	*Physical state/concentration*: gaseous/4 mg L^{-1} *Time application:* 24 s, 60 s, 120 s, and 180 s *Application form*: –	*Conclusion*: as regards *E. faecalis*, ozone presented similar bacterial reduction to NaOCl groups. As regards *C. albicans*, ozone reduced bacteria significantly; however, this reduction was lower than NaOCl. Ozone in association with CHX was the most effective disinfecting agent, followed by ozone in association with NaOCl. The application of gaseous ozone during short periods (24 and 60 s) was not sufficient to eliminate neither *C. albicans* nor *E. faecalis*. Higher periods (120 s and 180 s), although not completely efficient, were significantly better than lower doses.

	Sample size calculation	Samples with similar dimensions	Control group	Standardization of procedures	Statistical analysis carried out	Other risk of bias
Boch *et al.* (2016)	−	−	+	+	+	+
Case *et al.* (2012)	−	+	+	+	+	+
Hubbezoglu *et al.* (2014)	−	+	+	+	+	+
Huth *et al.* (2009)	−	+	+	+	+	+
Kaya *et al.* (2013)	−	+	+	+	+	+
Nagayoshi *et al.* (2004)	−	+	+	+	+	+
Noites *et al.* (2014)	−	+	+	+	+	+

Figure 2 Risk of bias results of *in vitro* studies. (+) indicates low risk of bias whilst (−) negative indicates high risk of bias.

	Random sequence generation	Allocation concealment	Binding of participants and personnel	Binding of ooutcome assessment	Uncomplete outcome data	Selective reporting	Other sources of bias
Kist *et al.* (2017)	+	+	+	+	+	+	+

Figure 3 Risk of bias of the randomized clinical trial study. (+) indicates low risk of bias whilst (−) negative indicates high risk of bias.

higher concentrations or periods of use, mainly if associated with complementary treatments such as ultrasound, NaOCl or chlorhexidine (CHX) during chemomechanical preparation. Hubbezoglu *et al.* (2014) observed that higher concentrations of gaseous ozone associated with the use of PUI yielded better antibacterial results, which solely in these conditions was associated with comparable results to NaOCl and NaOCl–PUI groups. In accordance, Noites *et al.* (2014) concluded that the antibacterial effect of ozone was significantly increased when associated with CHX or NaOCl and more effective with *C. albicans* than *E. faecalis* strains. Also, the authors demonstrated that the application of gaseous ozone during longer periods of use (120 s and 180 s), although not completely effective, was significantly better than shorter periods of use. In the clinical trial included, ozone had similar bacterial reduction to NaOCl (Kist *et al.* 2017). Nevertheless, ozone/NaOCl application and antimicrobial evaluation were performed after chemomechanical preparation using the Mtwo-System rotary files (VDW, Munich, Germany), NaOCl and EDTA and after inter-appointment dressing with calcium hydroxide. Huth *et al.* (2009) proposed a methodology for the analysis of bacterial elimination using a self-constructed glass chamber with simultaneous measurement of concentration, showing similar results between gaseous ozone and NaOCl. However, as regards aqueous ozone, higher concentrations were necessary to achieve reduction levels similar to NaOCl. In this context, the antimicrobial effect of ozone was strongly associated with the application protocol used: it is dose, time and bacterial strain dependent, as well as the correlation with the use of complementary disinfection techniques.

There were considerable variations regarding the material and methods used amongst the selected studies, such as the ozone application protocol and NaOCl concentrations (1–5.25%). As regards microorganisms evaluated, all studies selected the bacteria *E. faecalis* in the analysis. Besides this, three studies also included other bacteria: *Candida albicans* (Huth *et al.* 2009, Noites *et al.* 2014), *Peptostreptococcus micros* (Huth *et al.* 2009), *Pseudomonas aeruginosa* (Huth *et al.* 2009) or *Streptococcus mutans* (Nagayoshi *et al.* 2004).

Ozone therapy as an adjunct to NaOCl

Considering the pool of selected studies, ozone therapy, as an adjunct in chemomechanical preparation, was ineffective in increasing the antimicrobial effect of NaOCl. In the study of Noites *et al.* (2014), ozone increased the reduction in bacterial counts when using CHX and NaOCl. However, according to Hubbezoglu *et al.* (2014) and Boch *et al.* (2016), the association of NaOCl with ozone did not demonstrate greater bacterial load reduction in comparison with the use of NaOCl alone.

Additionally, ozone significantly increased the antibacterial action of EDTA when used in association with this solution (Boch *et al.* 2016).

Discussion

Ozone was first suggested for root canal treatment because of its reported high antimicrobial action (Nagayoshi *et al.* 2004, Huth *et al.* 2009). In addition to that, a significant decrease in the cytotoxicity to oral cells was observed for ozone gas in comparison with established endodontic irrigants such as 2.25% NaOCl and 2% CHX (Hyslop *et al.* 1988, Filippi 2001). In fact, aqueous ozone (up to 20 mg mL^{-1}) was not toxic to oral cells (Filippi 2001, Ebensberger *et al.* 2002, Nagayoshi *et al.* 2004, Huth *et al.* 2009). Therefore, ozone is currently being discussed as a possible alternative or complementary antimicrobial agent during root canal treatment.

Although reducing bacterial levels significantly, ozone, when used alone, is not able to yield similar results to NaOCl (Nagayoshi *et al.* 2004, Case *et al.* 2012, Hubbezoglu *et al.* 2014, Kaya *et al.* 2014, Boch *et al.* 2016). In this systematic review, ozone on its own achieved comparable results to NaOCl solution in laboratory studies with higher concentrations (Huth *et al.* 2009) or periods of use (Noites *et al.* 2014), mainly when associated with PUI (Hubbezoglu *et al.* 2014), NaOCl (Boch *et al.* 2016) or CHX (Noites *et al.* 2014). In the only randomized clinical trial included, previous relevant antimicrobial steps such as root canal preparation, irrigation with EDTA and use of calcium hydroxide for 1 week were accomplished before using ozone or NaOCl. Therefore, it could be stated that in the studies where ozone and NaOCl had comparable results, which were the minority of studies detected, irregular comparison between experimental and control groups was detected. Consistently, most reports demonstrated that ozone is associated with bacterial load reductions significantly lower than NaOCl (Nagayoshi *et al.* 2004, Case *et al.* 2012, Hubbezoglu *et al.* 2014, Kaya *et al.* 2014; Boch *et al.* 2016). In this sense, ozone therapy should not replace conventional chemomechanical techniques using the most commonly used root canal irrigant, NaOCl.

Beyond its antimicrobial effect, the use of ozone intervention replacing NaOCl has also been suggested in other clinical conditions: resorbed apex and/or wide open foramen, due to its lower cytotoxicity, and in cases of resistant bacteria and persistent infections when NaOCl was previously used (Boch *et al.* 2016). However, these indications should be evaluated with caution. Firstly, if an adequate irrigation technique is used, the toxicity of NaOCl is controlled, even in adverse clinical conditions with higher chances of contact with periapical tissues (Zehnder 2006, Slaughter *et al.* 2019). Furthermore, other auxiliary chemical substances with lower cytotoxicity could be used for this reason, such as CHX, which has more evidence supporting its use than ozone (Ferraz *et al.* 2001, Gomes & Herrera 2018, Neelakantan *et al.* 2019). As regards bacterial resistance, this undesirable effect does not apply to NaOCl, since its antimicrobial action relies on chemical reactions that lead to unspecific destruction of microbial cells (Zehnder 2006, Dioguardi *et al.* 2018). Considering the resistance of oral bacteria towards CHX, some concerns have been raised regarding multidrug efflux pumps and cell membrane changes since this agent acts in bacterial cytoplasmic membranes (Cieplik *et al.* 2019).

Ozone has also been recommended as an adjunct in chemomechanical preparation, since it may increase the disinfection effect of root canal irrigants. Nevertheless, the association of NaOCl and ozone has been shown to be ineffective, with similar antimicrobial effects in comparison with the use of NaOCl alone (Hubbezoglu *et al.* 2014, Boch *et al.* 2016). Thus, the actual need of ozone as a secondary disinfection source for conventional root canal treatment using NaOCl is not supported.

Study design can be highlighted as a limitation of the present systematic review. Most of the available information on ozone therapy was from laboratory studies, and only one randomized clinical trial study was retrieved. Aiming to provide a comprehensive answer to the PICOS question and highlight possible conflicts between different types of studies, no effort has been made to select only one type of study (Caputa *et al.* 2019). Although representing different levels of evidence, both laboratory and clinical papers were selected for this systematic review. The type of each study was clearly identified, and it was also taken into account during the synthesis of the evidence.

Another limitation that can be pointed out was the variability amongst the studies regarding their methodology, such as ozone application protocol (e.g. different ozone-generating equipment, ozone concentration, ozone physical state and time and technique of application) and NaOCl concentrations (1–5.25%). Considerable failing in equivalence of parameters between control and experimental groups (Hubbezoglu *et al.* 2014, Kist *et al.* 2017), limited sample size with the absence of sample size calculation (Nagayoshi *et al.* 2004, Huth *et al.* 2009, Case *et al.* 2012, Hubbezoglu *et al.* 2014, Kaya *et al.* 2014, Noites *et al.* 2014, Boch *et al.* 2016), missing relevant information regarding distributions of groups (Nagayoshi *et al.* 2004, Huth *et al.* 2009) and presentation of results (Nagayoshi *et al.* 2004) were detected. Regarding the similarities in sample dimensions, the study conducted by Boch *et al.* (2016) was classified as high risk since the authors used human extracted anterior and also premolar teeth. Only Kist *et al.* (2017) had a low risk as in all domains, the authors reported that they followed the CONSORT statement to conduct the clinical trial, performed the sample size calculation, the randomization and the dblindness.

These fails and divergences are reflected in the results and may lead to erroneous interpretations within selected papers as regards the performance of disinfection methods. Moreover, it is important to emphasize that, from eight included studies, seven were considered 'low' risk of bias (Nagayoshi *et al.* 2004, Huth *et al.* 2009, Case *et al.* 2012, Hubbezoglu *et al.* 2014, Kaya *et al.* 2014, Noites *et al.* 2014, Kist *et al.* 2017) and only one study was considered as having 'moderate' risk of bias (Boch *et al.* 2016) and the outcomes were consistent amongst the studies. However, seven of the eight included studies were laboratory based; therefore, the overall evidence is considered moderate. The present study revealed that ozone antimicrobial effect is strongly associated with the application protocol used: it is dose, time and strain dependent, besides the correlation with the use of complementary disinfection sources. In respect of ozone strain-dependent action, the ozone effect is based on the interaction with the lipid layers of microorganisms (Rojas-Valencia 2011, Junior & Lages 2012). Within this rationale, it could be inferred that ozone has different antimicrobial effects according to different groups of bacteria (Gram positive and Gram negative). Once Gram-negative bacteria structure contains lipopolysaccharides (LPS) and phospholipids in the membrane, this group seems more susceptible to ozone since its interaction occurs directly on these structures. All laboratory studies selected for this review evaluated the antimicrobial action of ozone against *Enterococcus faecalis*, a Gram-negative bacteria. Only three studies also selected other micro-organisms for analysis, including fungus, Gram-positive bacteria and other Gram-negative bacteria (Nagayoshi *et al.* 2004, Huth *et al.* 2009, Noites *et al.* 2014). The findings of the above-mentioned studies suggest that the antimicrobial results of ozone against the tested microorganisms were not associated with the gram classification into Gram-negative or Grams-positive bacteria.

As a result of this substantial variance in methodology between studies, it remains unclear what the best application protocol for ozone therapies is. Further better quality studies are certainly needed, but some assumptions can already be made so far by this systematic review. As previously stated, higher concentrations and longer periods of application of ozone permits improved disinfection results. Moreover, better outcomes are also found when using ultrasound, NaOCl or chlorhexidine associated with ozone therapy (Hubbezoglu *et al.* 2014, Noites *et al.* 2014, Boch *et al.* 2016). However, important features such as the specific ozone concentration or time application to use according to variables are still unknown.

In this context, due to the heterogeneous methodologies and impossible comparison between the various treatments applied and the various study designs of included studies, a meta-analysis is not recommended; therefore, only restricted clinical recommendations could be formulated. Consequently, it should also be pointed that the level of evidence and thus the strength of this review are not considered high. Nevertheless, the overall completeness of the present study reached satisfactory standards and, as it represents the best effort to collect the highest available information regarding ozone performance, its applicability remains significant.

As future research implications, the importance of yielding a fair comparison amongst experimental and control groups should be highlighted. Moreover, the accomplishment of studies evaluating the antimicrobial potential against overall bacterial load in biofilms attached in dentinal walls is suggested. Finally, the present results do not encourage the clinical use of ozone therapy as it demonstrated no benefit for patients undergoing root canal treatment. One concern is that clinical studies present considerable workforce and costs and, for this reason, the cost–benefit should be considered before conducting these trials. It is indicated that, prior to conducting clinical trials on ozone approach, further high powdered laboratory studies assessing the antimicrobial action of ozone and elucidating other relevant questions, such as the best protocol of use, are performed.

Conclusion

Although the selected studies have limitations, this review reached a satisfactory methodological quality and moderate evidence to provide important preliminary information regarding ozone therapy. As regards microbial load reduction for patients undergoing root canal treatment, ozone therapy has inferior results when compared with conventional chemomechanical techniques using NaOCl. As an adjunct during chemomechanical preparation, ozone intervention was ineffective in increasing the antimicrobial effect of NaOCl. Therefore, ozone is not indicated either to replace nor to complement the antimicrobial action of NaOCl.

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Conflict of interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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