Ozone therapy in dentistry: An overview of the biological mechanisms involved (Review)

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Abstract. At low medically-relevant concentrations, ozone serves as an oxidant with a wide spectrum of antimicrobial activity and the ability to promote healing and reduce inflammation. Despite providing therapeutic benefits in a range of diseases, certain adverse effects and contraindications of ozone treatment must be considered. These are primarily related to toxicity from inhalation and systemic types of administration and can be avoided by following relevant guidelines and recommendations. Ozone therapy has been implemented in a number of fields of dentistry and the most commonly used formulations for the oral cavity are gaseous ozone, ozonized water and ozonized oil. The biological mechanisms underlying the molecular effects of ozone have been increasingly reported, but currently remain largely unknown. The aim of the present review was to provide an overview of the mechanisms involved in ozone interaction with dental tissues. The present review focused on relevant evidence regarding the effect of ozone on dental tissues, including periodontal structures, dental cells, enamel and dentine, considering *in vitro* studies in addition to animal and human studies. A variety of biological mechanisms acting through multiple biochemical target pathways were reported to be responsible for the therapeutic effects of ozone. The main beneficial effects of ozone occurred in the following domains: antimicrobial activity, remineralization and microstructural changes of hard dental tissues, immunomodulation and biostimulation of dental and periodontal cells. Additional research could provide further insights into the use of ozone, increase its use for broader clinical applications and assist in the selection of targeted protocols.

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Contents

1. Introduction

Ozone (O_3) is a naturally occurring compound formed from three oxygen atoms as a result of conversion by ultraviolet radiation (1). At low medical concentrations, ozone acts as a powerful oxidant with wide-spectrum antimicrobial activity and the ability to promote healing processes and reduce inflammation through protective antioxidant pathways, thus providing therapeutic benefits in the treatment of a number of diseases (2-5).

The antimicrobial action is due to ozone-induced oxidation, which causes direct and indirect damage to microbial cell structures and metabolism (6-8). Ozone is also involved in pharmacological immunomodulation as it induces mild oxidative stress, which triggers antioxidant responses through the activation of specific molecular pathways, activating anti-inflammatory mechanisms (9,10). Additionally, topical

application of ozone has been reported to improve the rheological properties of blood by inducing functional and structural changes at the erythrocyte level, thereby enhancing peripheral oxygen perfusion and general metabolism (11-13).

Due to its strong oxidizing activity, ozone has been used as a disinfectant and germicidal agent for both industrial and medical purposes, with a variety of applications as a sterilizing agent for water treatment, medical equipment, dental settings and closed environments (14-18). Since 1930, ozone has also been studied for applications in dentistry (1,19). However, these studies were abandoned until the 1980s, due to the risk of inhalation toxicity and the difficulty of achieving optimal gas concentrations without dispersion (20). These issues have been addressed with modern technology and appropriate delivery and application techniques (19). A number of parenteral and topical routes are currently used to deliver ozone without toxic effects, with the exception of the inhalation route, which should be avoided due to broncho-pulmonary toxicity (11). The most widely used formulations for the oral cavity are gaseous ozone, ozonized water and ozonized oil (21).

The recommended concentration for a mixture of oxygen and ozone for medical use is between 5-50 μg ozone/1 ml oxygen (11). According to guidelines and good clinical practice recommendations in oxygen-ozone therapy published in the the World Federation of Ozone Therapy's Review on Evidence Based Ozone Therapy (20), an ozone concentration of 5-10 μg/ml is recommended for the application of ozonized products by intralesional injection or irrigation in order to obtain a therapeutic effect with no risk of toxicity, even in the event of ingestion (22,23).

Along with predominantly beneficial properties, the occurrence of some potential adverse effects must be considered, primarily in relation to toxicity upon inhalation. Excessive or prolonged exposure to gaseous ozone, which is more likely to occur in operators than in patients, may cause headaches, vomiting and irritation of the upper respiratory tract, which can manifest as a sore throat, cough, epiphora, rhinitis and bronchoconstriction (24,25). Additionally, certain forms of ozone therapy, namely those that induce systemic effects, are contraindicated in myocardial infarction, hyperthyroidism, acute alcohol intoxication, severe anemia, thrombocytopenia, active hemorrhage and pregnancy (21,26,27). However, ozone therapy is non-toxic to humans and is free of side effects when handled with care, using modern available technology in accordance with the manufacturer's instructions and current guidelines and recommendations (22).

At present, ozone therapy is implemented in certain fields of dentistry, including implantology, oral surgery, periodontology, oral medicine and management of dental caries (28-31). Ozone applications have yielded promising results in the treatment of inflammatory and immune-mediated conditions of oral soft tissues, such as oral lichen planus and aphthous stomatitis (32,33). In addition, ozone has been applied in the context of post-operative wounds and complications such as alveolar osteitis, which alleviated painful symptoms and reduced healing times (34,35). Additionally, ozone has generally contributed to a reduction of possible side effects associated with traditional treatments of these oral ulcerative conditions and surgical wounds and complications (33,34,36). Due to its antimicrobial activity, ozone has been reported to be useful in the management of oral disease of infectious etiology, such as herpetic stomatitis and oral candidiasis (37). In addition, a beneficial remineralizing effect on hard dental tissues has been reported and used to treat dental demineralization and decay (38-41). In particular, ozone application has been suggested as a possible alternative or complementary strategy to manage dental caries in a less invasive and more comfortable procedure than traditional approaches, especially in young or poorly cooperative children (31,41,42). Since dental caries is considered a global public health challenge, especially in children and the socioeconomically disadvantaged population, the implementation of effective, safe and economic interventions to prevent and treat caries should be a priority in oral health care policies (43-45).

Overall, ozone therapy may indirectly help restore functional activities compromised by disease by targeting certain biological processes as a biological response modifier (10). Although ozone therapy is now used in a number of countries, it has mostly been adopted by private health care practitioners (46). Over the past four decades, practitioners in Europe have mainly used ozone therapy empirically, potentially due to a lack of adequate knowledge of the basic principles behind optimal use (46,47). This approach has sometimes led to the inappropriate or dangerous use of ozone, which has contributed to a general misconception of its intrinsic toxicity (46,47). These issues, combined with the difficulty and cost of conducting extensive clinical trials, lack of interest by pharmaceutical companies and health authorities, as well as a lack of sponsorship, have hindered progress in this field of research, so that ozone therapy remains a poorly known and controversial complementary medical practice (47). Nevertheless, a number of studies have been carried out with promising results (2-4,29). The general mechanisms of ozone interaction with human tissues have been increasingly studied and progress has previously been made in this area of research. However, the underlying mechanisms explaining the effects of ozone on dental tissues are complex and are not yet fully understood.

The aim of the present review was to provide an overview of the currently available evidence on the mechanisms involved in ozone interaction with dental tissues underlying the beneficial effects of ozone in dentistry.

2. Literature search methods

The present review focused on the current relevant evidence on the biological mechanisms underlying the interaction of ozone with dental tissues, including periodontal structures, dental cells, enamel and dentine.

A literature search was performed using the PubMed/MEDLINE database (https://pubmed.ncbi.nlm.nih.gov) until March 22, 2024. The online literature search was performed using combined terms related to ozone and possible biological effects [‘ozone’ AND ‘antimicrobial’, ‘remineralization’, ‘immunomodulation’, ‘biostimulation’, ‘regeneration’, ‘dental cells’, ‘periodontal cells’, ‘odontoblasts’, ‘cementoblasts’, ‘osteoblasts’ AND ‘(dental OR dentistry OR oral)’] (Fig. 1). Among all records retrieved through the literature search, reviews or papers with an experimental design specifically addressing ozone-related biological mechanisms

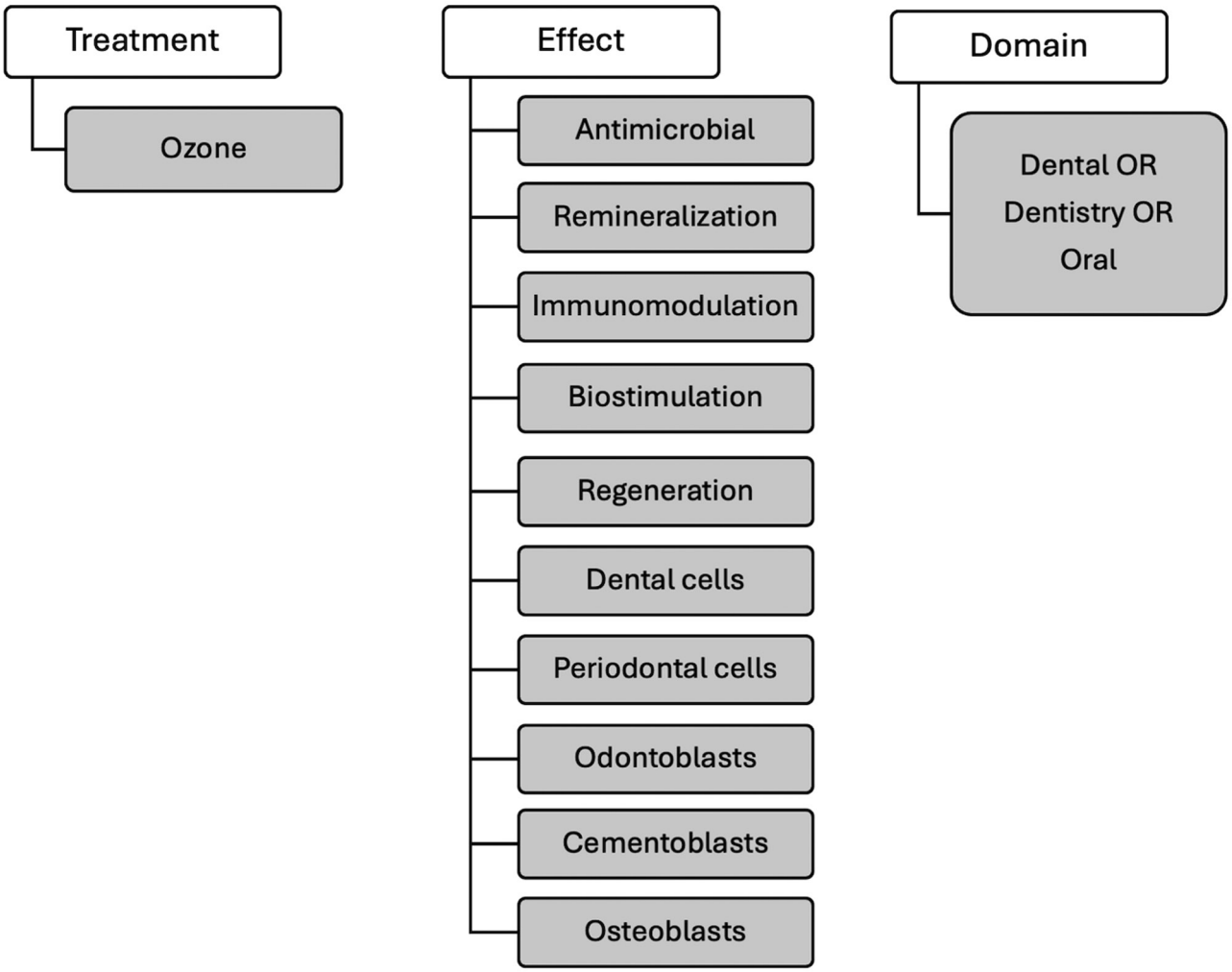

Figure 1. Schematic presentation of the search strategy used for literature screening.

in oral tissues were identified as key references. Relevant *in vitro* studies, in addition to animal and human studies, published in English were considered, without restrictions on the publication date.

3. General mechanisms involved in the interaction of ozone with dental tissues

A total of 487 potentially relevant records were retrieved as a result of the literature search. The number of studies decreased to 389 after duplicate removal, which was performed using the Rayyan software algorithm (48). Handsearching was conducted by manually screening the references of the considered studies to identify possible additional relevant papers.

The key references considered in the present review are listed in Table I. Of these studies, 16 were *in vitro* studies, seven were based on animal models including one with an additional human clinical phase, one was designed as a human *ex vivo* study and one was a review. In the field of dentistry, the main beneficial effects of ozone could be summarized in the following domains: Antimicrobial activity, remineralization through direct interaction with hard dental tissues, immunomodulation and biostimulation of dental and periodontal cells (Fig. 2).

4. Antimicrobial action

Ozone exhibits broad antimicrobial activity through oxidation, which causes dysfunction of the bacterial cell wall and cytoplasmic membrane, resulting in increased permeability to ozone molecules (7). Consequently, glycoproteins, glycolipids and amino acids are damaged and the enzymatic system is impaired, ultimately leading to cell lysis and death (6,8). Moreover, ozone reacts with unsaturated fatty acids in cell membranes, producing secondary reactive species such as aldehyde derivatives and lipid peroxides. These can reach the nuclei of intact cells, leading to nucleic acid breakdown that also results in the release of phosphate ions (49). The underlying mechanism by which this occurs remains largely unknown, but may explain the ozone buffering effect on biofilm fluids, which promotes a remineralizing environment (40). The bactericidal activity of ozone may be mediated by functional and structural disruption occurring at the level of the cytoplasmic membrane (50). Ozonized water (0.5-4.0 mg/l) has shown effective bactericidal properties against various strains of cariogenic bacteria and oral pathogens *in vitro*. Among these, however, gram-negative bacteria such as *Porphyromonas endodontalis* and *Porphyromonas gingivalis* were observed to be more sensitive to ozonized water

Table I. Key references concerning ozone mechanisms considered in the present review ordered by publication date.

First author, year	Study type	Type of ozone and topic of research	(Refs.)
Ebensberger, 2002	*In vitro*	Ozonized water; proliferation of cementoblasts and fibroblasts	(83)
Celiberti, 2006	*In vitro*	Gaseous ozone; enamel properties	(6)
Grootveld, 2006	*In vitro*	Gaseous ozone; oxidation of salivary biomolecules	(56)
Huth, 2006	*In vitro*	Gaseous ozone, ozonized water; cytotoxicity on oral epithelial cells and gingival fibroblasts	(86)
Huth, 2007	*In vitro*	Ozonized water; NF-κB system	(10)
Cardoso, 2008	*In vitro*	Ozonized water; microbial endotoxins	(53)
Noguchi, 2009	*In vitro*	Ozonized water; odontoblast-like cells	(74)
Raafat Abdelaziz, 2011	*In vitro*	Gaseous ozone; dentinal tubules	(68)
Gürsoy, 2012	*In vitro*	Gaseous ozone; dentinal tubules	(70)
Alpan, 2016	Animal model	Gaseous ozone; bone regeneration	(89)
Estrela, 2017	*In vitro*	Gaseous ozone; dentine remineralization; biofilm	(40)
Amin, 2018	Animal model	Gaseous ozone injection; immunomodulation; candidiasis	(60)
Colombo, 2018	*In vitro*	Ozonized oil; biocompatibility; gingival fibroblasts	(85)
Wang, 2018	*In vitro*	Ozonized oil; gingival fibroblasts proliferation; immunomodulation	(84)
Krunić, 2019	*Ex vivo* human study	Gaseous ozone; dental pulp	(81)
Leewananthawet, 2019	*In vitro*	Ozonized water; oxidative stress; periodontal fibroblasts	(93)
Bayer Alinca, 2020	Animal model	Gaseous ozone; bone healing	(92)
Dikopova, 2021	Animal model and clinical human study	Gaseous ozone; dental pulp; dentine	(72)
Küçük, 2021	*In vitro*	Ozonized water; cytotoxicity; dental pulp	(73)
Cenci, 2022	Review	Ozone; antiviral immunomodulatory effects	(59)
Ferreira, 2022	*In vitro*	Ozonized water; dentine metalloproteinases; dental pulp cells	(71)
Floare, 2023	*In vitro*	Gaseous ozone; enamel microstructure	(24)
Vieira, 2023	Animal model	Gaseous ozone; bone inflammation and regeneration	(88)
De Souza, 2024	Animal model	Gaseous ozone; myofascial pain and inflammation related to temporomandibular disorders	(96)
Lima Neto, 2024	Animal model	Gaseous ozone; bone metabolism following biphosphate treatment for osteoporosis	(87)

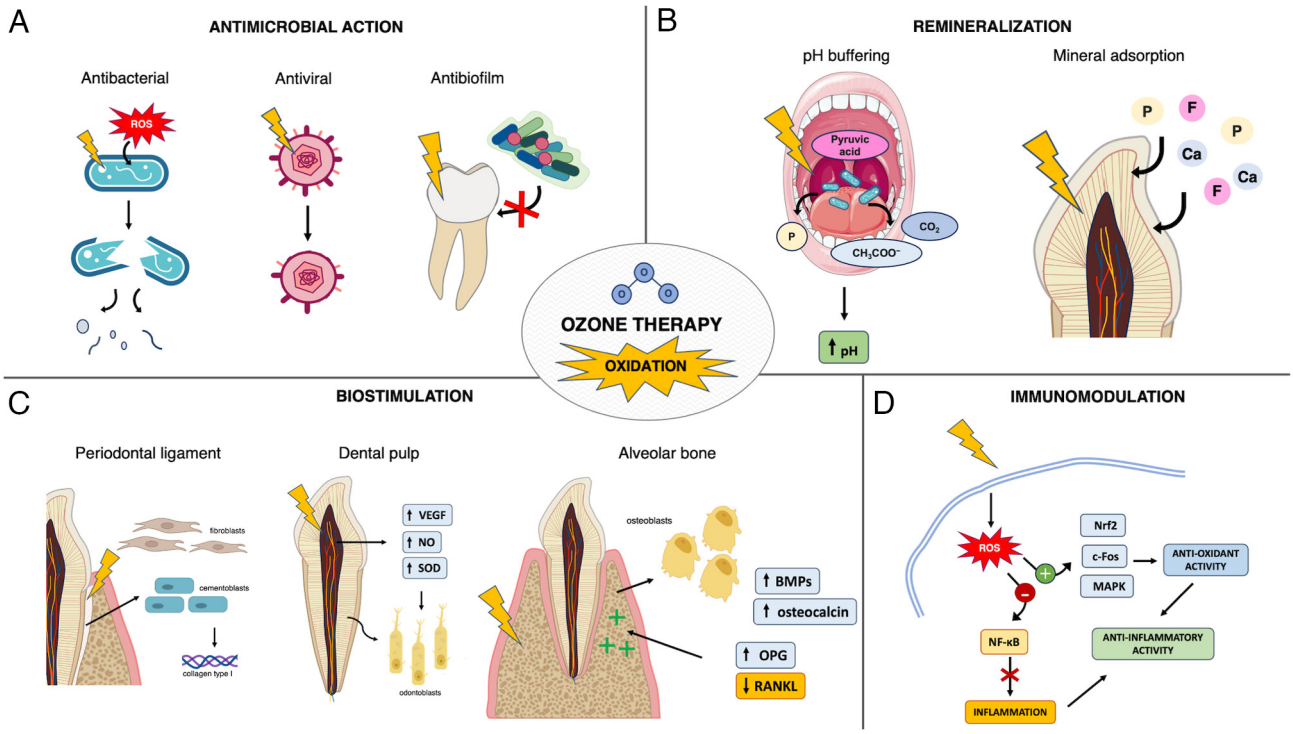

Figure 2. Mechanisms involved in ozone interaction with dental tissues. (A) Antibacterial effect: Ozone causes damage to cytoplasmatic membrane and cell walls of bacteria and fungi, allowing ROS to enter cells and cause functional damage, cell lysis and death. Antiviral effect: Ozone induces conformational changes to viral structures such as the envelope and spike proteins, which leads to virus inactivation. Antibiofilm effect: Ozone induces alteration of salivary biomolecules and related binding sites, thus hampering biofilm formation and adhesion on dental surfaces. (B) Oxidation of the nucleic acids of microorganisms and of pyruvic acid in the oral cavity results in a pH buffering effect, facilitating a remineralizing environment. Oxidation causes microstructural surface changes, removal of surface organic components and proteins from enamel and dentine, which leads to enhanced deposition and diffusion of minerals within enamel and dentin, thus promoting remineralization and reduction of dentine hypersensitivity. (C) Effects on dental pulp: Mild oxidation promotes dental pulp homeostasis, vascularization and regeneration associated with increased expression of VEGF, NO and SOD, which leads to odontodifferentiation of dental pulp cells, odontoblast proliferation and the production of mineralized matrix. Effects on periodontal ligament: Ozone induces proliferation of periodontal cells and enhanced collagen type I synthesis, which results in periodontal tissue regeneration. Effects on alveolar bone: Ozone induces increased osteoblast proliferation and activity, increased expression of osteocalcin and BMPs, reduced RANKL levels and increased OPG levels, which results in the promotion of bone healing and formation. (D) Ozone byproducts and oxidation cause mild ROS signaling and mitochondrial stress, which lead to activation of antioxidant pathways and inhibition of pro-inflammatory pathways, resulting in an anti-inflammatory effects. Ozone also stimulates T-cell mediated immunity. Arrows indicate a causal effect, lightning bolts indicate ozone-induced oxidation and red crosses indicate prevention. ROS, reactive oxygen species; NO, nitrous oxide; SOD, superoxide dismutase; BMP, bone morphogenetic protein; RANKL, receptor activator of NF-κB ligand; OPG, osteoprotegerin; Nrf2, nuclear factor erythroid 2-related factor.

compared with gram-positive streptococci and *Candida albicans* (50). Such discrepancies have been linked to differences in the structure of microorganism cell walls (51). The effect of ozone was also investigated on *C. albicans* and *Enterococcus faecalis* in infected root dentinal tubules, alone or in association with other antimicrobial agents (52). Ozonized water significantly reduced the number of *C. albicans* and *E. faecalis*, but showed no residual effect and activity against lipopolysaccharide (LPS) endotoxin (53). The authors hypothesized that these findings were related to the poor penetration of irrigants into the inner regions of dentinal tubules, allowing the remaining microorganisms to multiply over the course of the experiment (53).

The aforementioned findings were supported by a study that investigated the effects of ozone on an oral biofilm of *Streptococcus mutans* and *Lactobacillus acidophilus*, which reported no evidence of bacterial colonization on ozone-treated dentin surfaces *in vitro* (54). Due to the short half-life of ozone, however, it is unlikely that ozone could have interfered with the activity of the bacterial glycosyltransferase enzyme (54). This enzyme is responsible for the synthesis of polysaccharides that serve an essential role in biofilm formation and adhesion of the microorganism to tooth surfaces (55). It is more likely for ozone treatment to alter the dentin surface, making it more difficult for organisms to colonize the surface (6,54). Ozone oxidation is capable of altering organic surface components, such as collagen, and inducing the release of specific low molecular weight salivary biomolecules from macromolecular binding sites (56). This may result in 'smoothing' of the protein molecules, thereby affecting the wettability of the dentin surface.

Lastly, ozone has also exhibited promising virucidal effects. A recent study on severe acute respiratory syndrome coronavirus 2 reported that ozonized water rapidly caused conformational changes in the S1 subunit of the S protein (57). Based on previous studies, the authors also hypothesized that ozone could oxidize the glycoprotein on viral particles such as the viral envelope, which may contribute to virus inactivation (57,58). These mechanisms may be similarly involved in viruses commonly affecting the oral cavity, such as herpes simplex virus type 1 (59). Additionally, ozone activated the immune system in immunosuppressed rats by stimulating

T-cell mediated immunity and enhancing the immune response against pathogens (59,60).

5. Remineralization and interaction with dental hard tissues

With regard to the direct interaction of ozone with hard dental tissues, studies were retrieved that investigated the effects of ozone applications on physical and microstructural properties of enamel and dentin.

Celiberti *et al* (6) reported that gaseous ozone applications on sound enamel caused no significant changes to enamel physical properties, such as microhardness and acid resistance, without compromising etching and adhesive procedures for the placement of resinous restorative materials. The authors also reported that after initial reversible slight dehydration, ozone did not significantly change the free surface energy of the enamel. Consequently, the enamel-acid contact angle and interfacial area remained unaltered, which could explain the unchanged acid resistance. Similarly, Floare *et al* (24) measured an increase in enamel microhardness by dehydration, which was based on an early assessment after gaseous ozone application on demineralized enamel. At a microstructural level, the aforementioned study observed a progressive leveling of the enamel surface, opening of interprismatic canals and enamel prism pattern homogenization resulting from oxidation-induced disruption of the proteins embedded in the enamel matrix. Ozone oxidative proteolytic action can break down the organic contents of demineralized enamel and enhance diffusion of remineralizing agents. Additionally, ozone-induced oxidation of microbial pyruvic acid into acetate and CO_2 has a buffering effect, which counteracts the acid environment sustaining the carious process, thus promoting surface remineralization (24,38). Furthermore, a randomized controlled clinical trial reported that gaseous ozone treatment of non-cavitated carious lesions in primary teeth produced results comparable with fluoride varnish application in terms of fluorescence values and visual inspection index, with little or no caries progression reported in both treatment groups (61). These findings suggested that in young or uncooperative patients where accidental ingestion of topical products may occur, ozone may be a valid alternative to professionally applied topical fluorides to achieve remineralization (62). Parents or caregivers may also object to the use of fluorides, as early excessive exposure to fluoride has been implicated, albeit controversially, in a number of potential adverse health effects (63-66).

Although the effect of ozone on dentin is less well studied, it has been reported that, in addition to improving diffusion of salivary ions to the surface of degraded dentin, ozone can neutralize acidic proteins produced by cariogenic bacteria. These constitute the osmotic stimuli responsible for the movement of fluids in dentin tubules that causes hypersensitivity (67,68). Moreover, promising results have been reported in preliminary studies investigating ozone management of dentine hypersensitivity (67,68). Ozone oxidation of organic matter occurs through a selective reaction with molecules containing double bonds, amines or activated organic groups (68). Additionally, the resulting ozone byproducts, such as free hydroxyl radicals, undergo a stronger oxidizing action that selectively involves the organic components of dentin (69). These direct and indirect reactions may be responsible for the preferential degradation of demineralized peritubular dentin, which results in an increase in dentinal tubule diameter (68). This, associated with the local presence of remineralizing agents, enhances their deposition in open dentinal tubules, which can lead to increased tubular occlusion rates (68). Furthermore, *in vitro* evidence suggested that gaseous ozone autonomously induces occlusion of dentinal tubules, similarly to erbium-doped yttrium-aluminum-garnet laser treatment. This resulted in a significant reduction in the diameter and number of open dentinal tubules, which may be due to the immediate precipitation of minerals from oxidation induced-disruption of the surface matrix (70). Such ozone-induced effects could be affected by specific experimental setup and protocols, which have not been compared in the currently available literature.

Lastly, it has been reported that ozone interferes, albeit partially, with the activation of embedded dentin metalloproteases (MMPs) more than other common antiseptic agents (71). Dentin MMPs serve an important role in the progression of dental caries, as they can degrade the collagen matrix and negatively affect the adhesive interface of restorations (71).

6. Biostimulation of dental and periodontal structures

With regard to the effect on dental cells, ozone was reported to stimulate the odontodifferentiation potential of dental pulp cells and the production of mineral nodules *in vitro*, possibly acting as an indirect biostimulator of tertiary dentin formation *in vivo* (71). Evidence of ozone enhancing dental pulp regenerative potential has been reported by additional previous studies (72-74). An *in vitro* study by Noguchi *et al* (74) reported that, while bacterial LPS toxin inhibits the formation of mineralized nodules by odontoblast cells, ozonized water exposure can restore this property by reducing inflammatory response in odontoblast cells, exhibiting a direct toxic effect against bacterial LPS *in vitro*. Similarly, gaseous ozone application followed by the placement of mineral trioxide aggregate (MTA) was reported to improve pulp regenerative potential by increasing odontoblast proliferation and dentin bridge formation in an *in vivo* animal study, when compared with the placement of MTA alone (72). The increased ability to normalize pulp microcirculation and reduce inflammation was also observed in a concurrent human clinical study in cases of accidental pulpal exposure (72). The interaction of ozone with protein-lipid complexes of cell membranes and blood plasma promotes the synthesis of biologically active compounds, enhances the activity of immunocompetent cells and improves rheology and oxygen-carrying capacity of the blood (75). Similar findings were reported by Küçük *et al* (73) who observed that ozonized water at different concentrations demonstrated a good biocompatibility according to cell viability and enhanced the proliferation of primary dental pulp cells *in vitro*. This finding may also support the potential application of ozone as an adjuvant irrigant in the field of regenerative endodontics.

Ozone has also been reported to stimulate tissue regeneration of dental pulp. Similar to healing and regeneration processes occurring in other types of tissues, the mechanisms involved in the aforementioned process include increased

expression of VEGF, increased levels of nitric oxide (NO) and reduced oxidative stress due to increased activity of antioxidant enzymes such as superoxide dismutase (SOD) (76-79). NO is involved in pulp healing as a regulator of vascular homeostasis and pulp afferent sensitivity, as an indicator of cell differentiation and as a mediator of inflammatory activity (80). In particular, one of the three isoenzymes of NO synthase (NOS), neuronal NOS (nNOS), is typically present in healthy human pulp tissue, which demonstrates its involvement in pulp homeostasis (81). VEGF is responsible for the angiogenesis, activity and differentiation of odontoblasts and a slight increase in VEGF levels may be related to an increase in pulp vascularization inducing a beneficial response (81,82). A previous study reported increased VEGF and nNOS levels and decreased SOD levels in pulp tissue following a single application of gaseous ozone in the deep cavities of healthy teeth, where increased SOD activity and subsequent depletion may have occurred in response to mild oxidative stress induced by ozone (81). These findings also indicated that ozone could effectively diffuse through dentin to pulp tissue after a single application (81).

Additionally, promising effects of ozone on periodontal cells and structures have been previously reported, which may support the notion that ozone has biostimulating effects. Cementoblasts and fibroblasts in the residual periodontal ligament (PDL) adhering to the root surface of healthy avulsed teeth showed a higher proliferation rate when irrigated with ozonized water compared with saline (83). This suggested that ozone, in addition to effectively decontaminating the root surface, may also promote periodontal regeneration after the reimplantation of avulsed teeth. These findings have been supported by several studies investigating the biostimulatory potential of ozone on the periodontal structures and bone remodeling. Ozonized oil has been reported to significantly promote the production of type I collagen by human gingival fibroblasts, which was associated with the regenerative capacity of periodontal tissues (84). Furthermore, ozone, particularly ozonized water, has shown optimal biocompatibility with gingival fibroblasts, outperforming other antiseptics commonly used in oral care such as chlorhexidine digluconate (85,86).

Beneficial effects of ozone on bone metabolism have also been reported. The application of gaseous ozone was reported to improve bone metabolism and homeostasis in a zoledronate-treated rat model of osteoporosis, which showed a synergistic effect between zoledronate and ozone associated with increased bone regeneration (87). This ozone-induced biostimulatory effect was further confirmed by other previous studies that reported increased bone regeneration of calvarial defects of both healthy rats and rats with diabetes mellitus (88,89). Diabetes mellitus and hyperglycemia are associated with impaired healing processes due to activation of the NF-κB pathway, as well as increased alkaline phosphatase expression and suppressed osteocalcin, MMP-13, VEGF and glycolytic enzyme levels in osteoblasts, resulting in osteoporosis (90,91). Conversely, an increased number of osteoblastic cells and increased expression levels of osteocalcin and bone morphogenetic protein-2 (BMP-2) were observed in diabetic rat calvarial defects treated with xenografts and ozone compared with a xenograft-only group, which suggested ozone-induced improved bone formation and remodeling (89).

Similar results have been reported for periodontal disease-induced bone destruction (92,93). Untreated periodontal pockets caused by the presence of anaerobic pathogens, vascular endothelial damage, edema and increased inflammatory cell infiltration, exhibit low oxygen levels (94). HIF-1α (hypoxia-inducible factor 1α) is activated by pro-inflammatory signals in periodontal cells and gingival tissue and is the major regulatory protein that responds to hypoxia (94). Hypoxia also causes an increase in the release of receptor activator of NF-κB ligand (RANKL), which causes bone resorption and a decrease in the level of osteoprotegerin (OPG), which is responsible for bone formation (95). Furthermore, a study investigating the effects of ozone therapy on periodontal disease-induced bone destruction in rats reported a lower number of HIF-1-α positive cells in the ozone-treated group compared with both positive and negative controls, as well as significantly fewer RANKL-stained cells. Conversely, OPG levels were higher in the ozone-treated group, which suggests that ozone therapy may potentially be effective in promoting bone healing (92).

7. Immunomodulation

Knowledge of the underlying immunomodulatory mechanisms of ozone is developing and is important in a number of medical fields, including oral medicine. The pharmacological activity of medical ozone mainly depends on the ability of ozone byproducts to induce mild reactive oxygen species (ROS) signaling or mitochondrial stress that triggers an antioxidant response (9). This occurs through the activation of the Nrf2 (nuclear factor erythroid 2-related factor)-mediated system and inhibition of NF-κB pathway, which modulates immunity toward anti-inflammatory mechanisms (9). Ozone has also been used to treat myofascial pain and inflammation associated with temporomandibular disorders in rats (96). Despite a lack of improvement in nociception and inflammation that could have been due to the local infiltrative route of ozone administration and mechanically damaged muscle tissue, there was significant evidence of ozone stimulating collagen deposition and tissue repair (96). This finding was attributed to ozone-induced stimulation of fibroblast migration and increased immunohistochemical expression of platelet-derived growth factor, TGF-β and VEGF, which serve essential roles in tissue repair (96).

With specific reference to the dental field, Huth *et al* (10) investigated the effect of aqueous ozone on NF-κB-associated signaling both in periodontal ligament tissue from root surfaces of periodontally damaged teeth and in oral cells stimulated with TNF. It was reported that the NF-κB pro-inflammatory pathway was inhibited following incubation with ozonized medium, through ozone-mediated prevention of NF-κB inhibitor IκBα proteolysis, cytokine expression and κB-dependent transcription (10,59). Furthermore, the inhibitory effect was not directly caused by ozone, but was mediated, to varying degrees, by the formation of specific ozonized amino acids (10). Similarly, Leewananthawet *et al* (93) reported that ozone ultrafine bubble water induced oxidative stress through ROS production in human primary periodontal ligament fibroblasts. Consequently, certain genes and pathways involved in oxidative stress responses, such as *c-Fos*, *Nrf2* and *p38*-MAPK signaling were upregulated after ozone treatment.

Furthermore, the MAPK pathways have been reported to be associated with BMP-9-mediated differentiation of PDL fibroblasts to osteoblastic-like cells, possibly serving a pivotal role in regenerating mineralized dental tissues (93,97).

8. Limitations

There are certain limitations to the present review, mainly related to the available evidence. A number of the studies considered were published >10 years ago. However, a number of these studies were informative and were important sources for the purposes of the present review. Furthermore, most of the evidence reviewed was based on *in vitro* studies which, unlike animal models, may not be able to replicate real clinical conditions and may therefore only provide partial insights into the *in vivo* mechanisms of action of ozone. Finally, the limited availability and heterogeneity of the relevant literature prevented the assessment of the specific effects of different ozone formulations and protocols of application.

9. Clinical relevance and future perspectives

Future studies should focus on investigating different formulations, concentrations and application protocols of ozone in order to induce the specific desired effects. In addition, further research is warranted in the less-studied fields, such as dental cell biostimulation and pulp regeneration. These are topics of increasing interest to clinicians, therefore, in-depth research could expand clinical applications of ozone in a number of areas of dentistry, including cariology and regenerative endodontics. The development of *in vitro* experimental models through modern technologies could help overcome the limitations of animal studies, such as cost and ethical issues and traditional *in vitro* settings, which fail to replicate the biological environment. Selected optimal protocols should then be tested in well-designed randomized clinical trials. This would allow comparisons between established traditional treatments for specific oral health conditions, both in terms of clinical efficacy and patient acceptance. For example, the use of ozone for oral ulcerative lesions of various etiologies, as well as for the treatment of dental caries in young children, has shown promising results (31,32). This has provided an alternative treatment option that is less invasive compared with traditional approaches, free of side effects and with good patient compliance.

10. Conclusions

A variety of biological mechanisms acting through multiple biochemical target pathways are responsible for the therapeutic effects of ozone. These mechanisms are complex and at present not fully understood, especially with respect to specific effects on the oral and dental environment. Overall, they include antimicrobial action, immunomodulatory and biostimulatory effects and the direct modifications of hard dental tissues. Additional research in this area could provide further insights, enhance the use of ozone for broader medical purposes and dentistry applications and assist in the selection of targeted and more effective ozone treatment protocols.

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Authors' contributions

FV, TF and LG conceptualized the topic of the review and methodology. FV retrieved the relevant literature. FV and TF prepared the manuscript draft. UC, MV and LG reviewed and edited the manuscript. All authors read and approved of the final version of the manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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