Insights into Functional Erythromycin/Antioxidant Containing Chitosan Hydrogels as Potential Bio-active Restorative Materials: Structure, Function and Antimicrobial Activity

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Abstract

Substantial data are available in the literature on the role of Reactive Oxygen Species (ROS) and antioxidants in disorders such as the inflammatory and in chronic immune mediated conditions such as rheumatoid arthritis. However, remarkably little information is available on the periodontal diseases, which show many of the pathological features of other chronic inflammatory diseases. The periodontal tissues also provide an ideal medium within which to study mechanisms of ROS mediated tissue damage and of antioxidant defense in response to bacterial colonization, through the non-invasive collection of GCF. Bio-adhesive polymers appear to be particularly attractive for the development of alternative etch free dentin bonding systems with an added advantage of additional therapeutic delivery systems to improve intradental administration of therapeutic and prophylactic agents if necessary. Chitosan, which is a biologically safe biopolymer, has been proposed as a bio-adhesive polymer and are of continuous interest to us due to their unique properties and flexibility in broad range of oral applications reported by others and us recently. The objectives of this study is to evaluate the novel chitosan based functional drug delivery systems which can be successfully incorporated into “dual action bioactive restorative materials” containing common antibiotic such as erythromycin, krill oil, aloe and aspirin as commonly used antioxidant species.

Methods: The novel hydrogels will be investigated with respect to the antioxidant capacity and drug release capacity of the tetracycline as well as erythromycin from the designer drug delivery system, the use of SEM imaging for the characterization of the surfaces and reactive features of novel materials with antimicrobial potential as well as use of the newly designed materials as an effective adhesive restorative materials.

Results: A continuous release of erythromycin, while maintaining antibiotic effects against the tested bacteria, for at least 24 hours was shown from designer chitosan-antioxidant hydrogels. The increase antioxidant capacity of the designer material, significant antimicrobial capacity as well as adhesive dentine bond strength make the chitosan-containing restorative materials suitable and potentially advantageous materials for restorative and periodontal applications in-vitro. The addition of antioxidants to the functionalized restorative material acts as an alternative free radical defense mechanism and therefor increases the shear bond strength teeth by managing the excess of free radicals produced during common restorative procedure. Future investigations are necessary to validate this hypothesis.

Keywords: Erythromycin; Free radicals; Chitosan hydrogels; Functional restorative materials; In-vitro

Introduction

Substantial data are available in the literature on the role of reactive oxygen species (ROS) and antioxidants in disorders such as the inflammatory lung diseases and in chronic immune mediated conditions such as rheumatoid arthritis [1,2]. However, remarkably little information is available on the periodontal diseases, which show many of the pathological features of other chronic inflammatory diseases [3]. The periodontal tissues also provide an ideal medium within which to study mechanisms of ROS mediated tissue damage and of antioxidant defense in response to bacterial colonization, through the non-invasive collection of GCF [4].

Bio-adhesive polymers appear to be particularly attractive for the development of alternative etch free dentin bonding system with an added advantage of additional therapeutic delivery systems to improve intradental administration of therapeutic and prophylactic agents if necessary [5-10]. Chitosan, which is a biologically safe biopolymer, has been proposed as a bio-adhesive polymer and are of continuous interest to us due to their unique properties and flexibility in broad range of oral applications reported by others and us recently [11,12]. The objectives of this study is to evaluate the novel chitosan based functional drug delivery systems which can be successfully incorporated into “dual action bioactive restorative materials” containing common antibiotics such as erythromycin, krill oil, aloe and aspirin as commonly used antioxidant species. The novel hydrogels will be investigated with respect to the antioxidant capacity and drug release capacity of the tetracycline as well as erythromycin from the designer drug delivery system, the use of SEM imaging for the characterization of the surfaces and reactive features of novel materials with antimicrobial potential.

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as well as use of the newly designed materials as an effective adhesive restorative materials.

Materials and Methods

While Chitosan (Aldrich, Australia), glycerol (Sigma, USA), glacial acetic acid (E. Merck, Germany) were used as received. The degree of de-acetylation of typical commercial chitosan used in this study is 87%. Chitosan with molecular weight 2.5 x 10^6 KD was used in the study. The isoelectric point is 4.0-5.0.

Preparation of the gels of the various antibiotic induced hydrogels

Chitosan hydrogels have been prepared using the methodology previously described [13]. Briefly, the corresponding antibiotic and antioxidant mixtures, were incorporated by dispersion of corresponding antioxidant powder 0.02 grams in glycerol (5% w/w) using a mortar and a pestle and 1 milliliter of glacial acetic acid (3% w/w). The corresponding antioxidant mixtures were incorporated into the mixture and the summary of the newly prepared materials was highlighted in Table 1.

Determination of gel pH

One gram of the prepared gels was accurately weighed and dispersed in 10 ml of purified water [13]. The pH of the dispersions was measured using pH meter (HANNA instruments, HI8417, Portugal).

In-vitro erythromycin release

The release study was carried out with USP dissolution apparatus type 1, Copley U.K., slightly modified in order to overcome the small volume of the dissolution medium, by using 100 mlbeckers instead of the jars. The basket of the dissolution apparatus (2.5 cm in diameter) was filled with 1 gm of the gel on a filter paper. The basket was immersed to about 1 cm of its surface in 50 ml of phosphate buffer pH 6.8, at 37ºC ± 0.5 and 100 rpm [14]. Samples (2 ml) were collected at 0.25, 1, 2, 3, 4, 5, 6, 7, 8, 10, and 20 hours [15] and were analyzed spectrophotometrically by U.V. Spectrophotometer (Cintra 5, GBC Scientific equipment, Australia). The in-vitro erythromycin (E) release kinetics from chitosan gels were evaluated using a method identical to the investigations previously described for release were spectrophotometrically assayed at 286 nm, evaluated in PB, pH 6.8. Three replicate measurements were performed for each designed formulation.

Microbiological investigations

A type strain of Staphylococcus aureus (ATCC 12600), obtained from the American Type Culture Collection (Manassas, USA) was used as test bacterium for estimating the antibacterial activity of the hydrogels. The selection of these species was made based on the previously reported work on the antibiotic: nanomaterials [16]. The antibacterial activity of the prepared hydrogels was compared to antibiotic sensitvity discs (Mast Laboratories, Merseyside UL) containing 5 µg of erythromycin per disc respectively.

Morphology of the gels

The samples were prepared by freezing in liquid nitrogen for 10 min, and then were freeze-dried for 24 h. The prepared samples were fractured in liquid nitrogen using a razor blade. The fractured samples were dried under vacuum, attached to metal stubs, and sputter coated with gold under vacuum for the SEM study. The interior and the surface morphology were observed under scanning electron microscope (SEM, Hitachi S4800, Japan).

Gel stability

Stability of the gel formulations was also investigated. The organoleptic properties (color, odor), pH, drug content, and release profiles of the gels store at 20ºC were examined on days (0, 15, 30 and 178). The performance of the hydrogels was not affected by the storage conditions, suggesting remarkable stability of the novel biomaterials under investigations.

Studies of equilibrium swelling in the alternative drug delivery systems

The known weight tea infused-containing dry gels were immersed in pH 4.0, pH 9.0 buffer solutions, respectively, and kept at 25ºC for 48 h until equilibrium of swelling had been reached.

The swollen gels were taken out and immediately weighed with microbalance after the excess of water lying on the surfaces was absorbed with a filter paper. The equilibrium swelling ratio (SR) was calculated using the following equation:

\[ SR = \frac{W_e - W_d}{W_d} \times 100\% \]

Where Ws and Wd are the weights of the gels at the equilibrium swelling state and at the dry state, respectively [18]. Experiments were repeated in triplicate for each gel specimen and the mean value calculated.

Shear bond strength tests for dentine bonding

Extracted non-carious, intact, human molars stored in water containing a few crystals of thymol at 4ºC were used within two months. Samples were checked before use for any damage caused by their removal. All teeth were thoroughly washed with water to remove residual thymol and transferred into an artificial solution of saliva for 48 hours prior subjecting the teeth to the experimental solution.

The roots of the teeth were removed with a separating disc and the occlusal enamel removed by grounding wet on 60-grit silicon carbide (SiC) paper. The teeth were embedded in PVC (Consjit Tubing, SA PVC, JHB, RSA) pipe containers with cold cure acrylic resin so that

### Table 1: Gel formulation prepared in the study.

<table>
<thead>
<tr>
<th>Gel formulation</th>
<th>Chitosan (w/w%)</th>
<th>Erythromycin (w/w%)</th>
<th>Aspirin (w/w%)</th>
<th>Aloe (w/w%)</th>
<th>Krill Oil (w/w%)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan-H-E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Gel-1</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5.30</td>
</tr>
<tr>
<td>Chitosan-H-EA1</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Gel-2</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>6.74</td>
</tr>
<tr>
<td>Chitosan-H-EAK2</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Gel-3</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>6.14</td>
</tr>
<tr>
<td>Chitosan-HAsE1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Gel-4</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>6.24</td>
</tr>
<tr>
<td>Chitosan-HAsEK1</td>
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<tr>
<td>Gel-5</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>6.36</td>
</tr>
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</table>
the ground occlusal surfaces projected well above the resin. The 10 mm length pipes were put on a glass surface with one end blocked by the glass and the embedding done through the open end. Immediately after embedding the occlusal surfaces were ground wet with 180-grit followed by 600-grit SiC on a polishing machine to expose the superficial dentin. The samples were washed under a stream of tap water. Two of these studs (next to each other) were then bonded to the polished dentine surface of each tooth via the bonding agent XP bond (Dentsply, New York, USA), as suggested by the manufacturer. A standardized jig (Ultradent ISO A2-70) with an internal diameter of 2.5 mm and height of 3 mm was used to shape the composite resin stud (SDR, Dentsply, CA, USA, Batch number 1105000609, Exp 2013-04), which was cured, as suggested by the manufacturer. The bonding agent XP Bond contained: carboxylic acid modified dimethacrylate (TCB resin), phosphoric acid modified acrylate resin (PENTA), urethane dimethacrylate (UDMA), triethylene glycol dimethacrylate (TEGDMA), 2-hydroxyethylmethacrylate (HEMA), butylated benzenediyl (stabilizer), ethyl-4-dimethylaminobenzoate), camphorquinone, functionalized amorphous silica, t-butanol.

In this way were 64 teeth samples (each containing 1 stud) prepared and divided into 8 groups of 8 each, A, B, C, D, E, F and K (Table 2) and stored in a solution of artificial saliva in order to replicate the oral cavity conditions. These groups were then treated as outlined in Table 2. After 24 hours one stud of each tooth was tested for shear bond strength. An Instron Universal Testing Machine (Griffith University, G12, QLD) at a crosshead speed of 0.5 mm/minute was used to test the de-bonding strength. All data were analysed using the non-parametric ANOVA test.

Free radical damage in the test tube, in-vitro model

We reported earlier that protein cross-linking as a model for detection of free radical activity and activation of "molecular defense forces". Bovine serum albumin (BSA), a completely water-soluble protein, was polymerized by hydroxyl radicals generated by the Fenton reaction system of Fe²⁺/EDTA/ H₂O₂/ascorbate [19]. As a result, the protein loses its water-solubility and the polymerized product precipitates. The decrease in the concentration of the water-soluble protein can easily be detected. The in-vitro incubation mixtures contained reagents, added in the sequence as follows, at the final concentrations: bovine serum albumin (0.8 mg/ml), phosphate buffer, pH 7.4 (10 mM), water to reach 2.5 ml total volume, antioxidant tested to reach required concentration as shown in results, EDTA (0-4.8mM), Fe(NH₄)₂(SO₄)₂ (0-4mM), ascorbate (4 mM) and H₂O₂ (0.2%).

To chelate iron completely, 1.2 molar excess of EDTA was always used [20]. The reaction mixture was incubated for 20 min at ambient temperature. The supernatant was precipitated with an equal volume of trichloroacetic acid (10%) at 0°C. The precipitate thus obtained was re-dissolved in 1 ml of Na₂CO₃ (10%) in NaOH (0.5 M) and the final volume made up to 2.5 ml by water. An aliquot of the solution was used for protein determination [21]. The yield of OH. radicals generated in the incubations were determined on the basis of degradation of deoxyribose [22]. Bityroosine formation was monitored by measuring fluorescence at 325 nm (excitation) and 415 nm (emission) according spectrophotometer [23].

Results

The characterization of prepared chitosans gels (Gel-1-5)

The SEM images were obtained to characterize the microstructure of the freeze-dried naproxen composite gels and are presented in Figure 1. It could be seen that the gels displayed a homogeneously pore structure. It was thought that the micro-porous structure of the gels could lead to high internal surface areas with low diffusional resistance in the gels. The surfaces of the gels were also presented (Figure 1). The 'skin' of the gels can be seen, and the collapse of the surface pores may be due to freeze-drying process.

Studies of equilibrium swelling in chitosan gels (Gel-1-5)

The hydrogels remain in the cylindrical form after swelling. Compared with dry state hydrogels, the swollen state hydrogel volume displays significant increases and is summarized in Figure 2.

Equilibrium swelling ratio (SR) of hydrogels exerts an influence on their release rates. The reduction in equilibrium swelling capacity is due to the formation of a tight network structure in high content. Environmental pH value has a large effect on the swelling behavior of these gels. From Figure 2, it is clear that the SR value increases with the increase of pH. Such pH dependent properties of the hydrogels come from the polyelectrolyte nature of chitosan segments in the hydrogel network. Namely, when the pH value of the buffer solution (pH 9.0)...
was far higher than the isoelectric point (PI) of GEL (PI 4.0–5.0), the carboxyl groups were de-protonized to carry negative charges, which made molecular chains repulsed to each other. The network became looser and it was easy for the water molecules to diffuse into the cross-linked network. According to above results, we believed that the erythromycin results release mechanism could result from the superposition of various effects, such as swelling property of hydrogels, the solubility of the drug and erosion property of matrix; it is not necessarily based on a single factor. Importantly, the solubility of the otherwise poorly soluble erythromycin has significantly improved due to the positive host-guest interactions between the chitosan and the therapeutic agent.

**In-vitro release of antibiotics from chitosan gels (Gels 1-5)**

The in vitro release of erythromycin expressed as the cumulative percent of drug released as function of time from the designed formulations of gels, is presented in Figure 3.

**In-vitro antibacterial activity of erythromycin/antioxidant containing chitosan hydrogels**

All the test samples give inhibition zones larger than the clinical breakpoint inhibition zone diameters (European Committee on Antimicrobial Susceptibility Testing, Basel, Switzerland) for *S. aureus* sensitivity for erythromycin. Using the students’ T test, no statistically significant difference (p<0.05) between the averages of the inhibition zone diameters for all the samples except for the Gel 3 containing aloe, erythromycin and krill oil were found (Table 3). Gel 4 was excluded from investigation due to the well documented aspirin/erythromycin direct drug interactions and therefore leading to further optimization of the material prior to microbiology to be conducted [24].

Clinical breakpoints for erythromycin: for 15 µg/disc >21 mm is sensitive and <18 mm is resistant.

The additional advantage of utilizing a biocompatible and bio-adhesive chitosan functionalized materials lie in the potential to extend the site-specific antibiotic delivery, avoid the release of products of breakdown as well as add additional benefits of antioxidants in the novel restorative materials. The work is currently on the way in our laboratory to investigate the host guest interaction between the antibiotic and bio-polymeric core as well as extended therapeutic uses of nontraditional antibiotic agents in overcoming resistance that is developed by several pathogenic microorganisms against.

**Shear bond strengths**

Figure 4 give the shear bond strength values after 24 hours respectively (MPa).

Mean shear bond strength values and difference between the groups are summarized in Figure 4 for bonding to dentin after 24 hours. In general there was an increase in bond strength of the dentin treated with the antioxidant containing hydrogels compared to the bond strength of the conventionally bonded teeth. An increase in the shear bond strength was also previously reported [25]. Interestingly the increase in bond strength was also observed in the groups of hydrogen peroxide exposed samples suggesting that there additional benefits associated with chitosan: antioxidant system are in need of further investigations [26].

The results of this study suggest that the optimum results for the strengthening of dentin can be achieved throughout the immediate treatment with antioxidant: chitosan with the increase of dentin bond strength. Also, impressively an almost immediately after the corresponding gel treatment and proceeding with bonding procedures is recommended with the significant increase in bond strength. The results of this study suggest that the optimum results for the increased enamel dentin bond strength can be achieved throughout the immediate treatment with gels. The additional advantage of the system may suggest that, antioxidant release from chitosan gel depends on the physical network structure (open cell like structure) as well as pH properties and flexibilities of the material. Antioxidant release is directed and controlled through the pores of the low polymer concentration; consequently slower antioxidant release from the gel base was achieved and therefore weaker adhesive properties of the materials such as Gel-1 in case of groups [26].

It was shown by others and us earlier, that the swelling properties and antioxidant release from gels were increased under acidic conditions due to the protonation of the primary amino group on chitosan [27]. Chain relaxation due to protonation of amino groups leads to a faster hydrogen bond dissociation and efficient solvent diffusion. Thus, the appreciable increase in water uptake at lower pH values can be attributed to the high porosity of the gels, which seems

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**Table 3: Gels 1,2,3 and 5 with erythromycin as an active antibacterial ingredient tested for antibacterial activity against Staphylococcus aureus NCTC 12600.**

<table>
<thead>
<tr>
<th>Sample no. (n=10)</th>
<th>Erythromycin inhibition zone diameters (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Erythromycin disc (5 µg)</td>
</tr>
<tr>
<td>Average</td>
<td>30.7</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.5</td>
</tr>
</tbody>
</table>

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**Figure 2:** Water uptake degree of the gels Gel-1–Gel-5 (n=6, p<0.05).

**Figure 3:** Cumulative release of erythromycin from chitosan gels (Gels 1-5) over the period of time (hours).
Figure 4: Shear bond strength of hydrogels after 24 hours of bonding to dentin. Y axis is in MPa should be inserted

Figure 5: Antioxidant capacity measured at 450 nm using the previously described spectrophotometric assay to assess the hydrogels and corresponding ingredients antioxidant capacity after 24 hours under storage under ambient temperature condition. Antioxidant capacity was measured during the first 2 hours of exposure.

Figure 6: Antioxidant capacity measured at 450 nm using the previously described spectrophotometric assay to assess the hydrogels and corresponding ingredients antioxidant capacity after 6 month under storage under ambient temperature condition. Antioxidant capacity was measured during the first 2 hours of exposure.

to govern the diffusion of the solvent in the gel matrix, and thus, the release of the antioxidant from the gel [26]. The additional benefit of using chitosan: antioxidant system as a bonding/pre-bonding to enamel and dentin system lies in its ability to show favorable immediate results in terms of bonding effectiveness. It is well documented that the hydrostatic pulpal pressure, the dentinal fluid flow and the increased dentinal wetness in vital dentin can affect the intimate interaction of certain enamel and dentin adhesives with dentinal tissue. Therefore the newly developed chitosan: antioxidant systems might at least be able to address the shortfalls in the current perspectives for improving bond durability through understanding factors affecting the long-term bonding performance of modern adhesives and addresses the current perspectives for improving bond durability.

Antioxidants, chitosan macro-encapsulation and stability

Chitosan, a linear abundant polysaccharide, is selected as the wall material of the delivery system [26]. Due to its biodegradable, biocompatible, muco-adhesive and non-toxic nature, it has been widely used in numerous drug delivery systems. Compared to other delivery systems, chitosan nanoparticles have a special feature. They can adhere to the mucosal surface and transiently open the tight junction between epithelial cells. Some reports have indicated that chitosan can increase membrane permeability; both in-vitro [28] and in-vivo [29]. Microencapsulation of antioxidants has been important area of research for several years in order to preserve the beneficial effects of antioxidants [30]. In this work, the gelation method was used to prepare the chitosan: aspirin, chitosan: krill oil and chitosan: aspirin: krill oil microencapsulation. The stability of antioxidant-loaded chitosan complexes has been measured during storage and results suggest there is no significant decomposition observed after 6 months storage at room temperature (24°C) as antioxidant capacity of the materials stored for 6 month have showed no diminished capacity compare to the freshly prepared hydrogels as indicated in Figures 5 and 6.

Investigations into stability of antioxidants in the chitosan hydrogels during storage

Stability of various conventional antioxidants in the newly designed drug delivery system during storage is an important factor to determine whether chitosan-coated nano-size delivery vehicle can protect various conventional antioxidants. So the stability of the microencapsulated antioxidants has been measured by UV absorbance. Stabilities of microencapsulated antioxidants have been compared and after 6 months of storage at 24°C, the stability of antioxidant-
molecular carrier vehicle was not significantly diminished as indicated in Figures 5 and 6. The increased stability and the antioxidant capacity demonstrate that the antioxidant had been protected by the molecular carrier.

Insights into performance of erythromycin-chitosan gels as an effective free radical defense functional material

We considered worthwhile to study the antioxidant properties of krill oil, aspirin, and chitosan as a “build in defense mechanism” for the in-vitro generated free radical production and counter reaction of the antioxidant defense of the chitosan, krill oil and aspirin. Therefore we adopted the method for recording changes in water solubility of the model protein bovine serum albumin (BSA) exposed to free radicals generated by an inorganic chemical system. In the present study we used the Fenton reaction system of Fe3+/EDTA/H2O2/ascorbate as a source of free radicals to prove the ability of NSAIDs as well as complexed form
of chitosan: antioxidants to protect BSA against free radical mediated cross-linking, in comparison with an efficient hydroxyl radical scavenger such as trolox. As clearly demonstrated by the Figure 7, upon exposure to standard H$_2$O$_2$ in the form of Fe$^{2+}$/EDTA/ H$_2$O$_2$/ascorbate solution as a base line determine free radical generation under "prototype in-vitro free radical damage", upon incorporation of the chitosan substituted hydrogels, the build in antioxidant capacity and therefore free radical defense of the in-vitro model has been activated and are of significant value to take notice. This model represents the practical approach of in-situ monitoring and test the amount of free radical production and synergistic antioxidant defense of the system. Further investigations and fine-tuning of the system are currently on the way in our laboratory.

Conclusion

A controlled release of erythromycin, while maintaining antibiotic effects against the tested bacteria, for at least 24 hours was shown from designer chitosan-antioxidant hydrogels. The increase antioxidant capacity of the designer material, significant antimicrobial capacity, as well as adhesive dentine bond strength make the chitosan-containing restorative materials suitable and potentially advantageous materials for restorative and periodontal applications in-vitro.

The addition of antioxidants to the functionalized restorative material acts as an alternative free radical defense mechanism and therefor increases the shear bond strength teeth by managing the excess of free radicals produced during common restorative procedure. Future investigations are necessary to validate this hypothesis in-vivo.

References