Copazan Oral Gel: Functional Biomaterial and Periodontal Disease in Veterinary Medicine from Concept to Application in Vitro

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Abstract
Periodontal disease is considered an inflammatory disorder that damages tissue through the complex interactions between periodonto-pathic bacteria and host defense systems. It is likely that the role of reactive oxygen species (ROS) is common to both bacterial- and host-mediated pathways of tissue damage. In recent years, there has been a tremendous expansion in the veterinary, medical and dental research concerned with free radicals, ROS and antioxidant defense mechanisms. This review is intended to summarize and evaluate the recent advances in bio-material application in veterinary dental medicine as well as highlight performance of Copazan Oral Gel® as designer bio-materials capable for various applications in periodontal treatment of animals and humans in the future.

Introduction
Periodontics is a science that aims to study the periodontium and the diagnosis, prevention and treatment of periodontal diseases, in order to promote and restore the periodontal health [1].

The periodontium is the set of adjacent structures to the teeth that provides them with support and protection. These structures are: gingiva, cementum, alveolar bone and periodontal ligament [1].

Periodontal disease is the most common oral disease in dogs with up to 80% of animals affected [2]. This disease is progressive and involves two stages: gingivitis (reversible) and periodontitis (irreversible, but often controllable). It is caused by plaque buildup on teeth. The plaque is a smooth membrane, adhesive, contaminated with saliva bacteria and debris. Bacteria and bacterial products cause inflammation of soft tissue. The plaque becomes mineralised to form calculus, which migrates into the gingival sulcus, causing additional inflammation, loss of periodontal ligament, bone loss and ultimately tooth loss [3].

Medical problems that affect the oral cavity should be identified in its early stages, so that the animals can be treated before showing serious secondary systemic disorders related to malnutrition and/or infections [4]. One should also be aware of ways to prevent the disease, as animal tooth brushing and the use of antimicrobials as an adjunct in periodontal therapy [5].

Dental Anatomy of Dogs
As in most domestic mammals and in humans, dogs have diphyodont dentition, featuring two sets of teeth, a deciduous or primary and a permanent, although edentulous at birth [1]. The oral anatomy of dogs has subdivisions and similar structures to those of humans (Figure 1), differing in the shape of the cavity, which also varies between breeds, anatomy and quantity of teeth and in the teeth apex [1]. Dogs have, like humans, incisors, canines, premolars and molars, differing among themselves in functions and numbers of roots [5].

Domestic dogs have in their primary teeth, 28 teeth (12 incisors, four canines, 8 premolars and 4 molars), and in the permanent, 42 teeth (12 incisors, four canines, 16 premolars and 10 molars) [5]. Regardless of the number of roots, function, size and shape, the teeth have subdivisions that are common to all types, and form the dental organ together with some adjacent structures [5].

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periodontal tissue, named false pocket, although when there is a loss of the support tissue and protection of the tooth, the sulcus is called periodontal pockets, which can be of two types: suprabone, when the bottom of the sulcus is coronal to the support alveolar bone, and intrabone, when the bottom is located apically in relation to the adjacent alveolar bone. The pocket depth can vary between regions of the mouth and even between neighbouring teeth [13]. According to the location, the plaque can be classified as supra or sub-gingival. The supragingival plaque corresponds mainly to microbial aggregates found on tooth surfaces (mostly in the gingival third of the crown), however may extend into the gingival sulcus, where they are in immediate contact with the marginal gingiva. The subgingival plaque corresponds to bacterial aggregates found entirely within the gingival sulcus or periodontal pockets [1]. The bacterial constituents present in dental plaque are modified according to the disease evolution. In healthy gingiva, the cocci represent nearly two-thirds of the bacteria, followed by non-motile small rods. The bacteria present are mostly gram-positive and there is no significant representation of more virulent bacterial types. At the stage of gingivitis, the gram- positive rods (non-motile) gradually increase, surpassing the cocci, and the number of gram- negative bacteria also grows. This change continues to go on until the periodontium involvement phase (periodontitis), when the more pathogenic gram-negative microorganisms become the majority, so that the spirochetes represent almost half of the bacteria, while gram-positives are underrepresented [14]. Without interference in the plaque formation an inflammatory process can occur, which marks the beginning of a periodontal disease and that provides a favourable environment for change in the microbial composition of the plaque, that become a biofilm with more pathogenic characteristics, continuing with later stages of the disease [15].

In dogs, the progression of periodontal disease can be divided into stages based on the clinical appearance of the

For a better understanding of periodontal disease it is important to have further information about a set of structures that constitute the alveolar-dental joint, the periodontium [6]. There is a division of these structures according to their functions, so there is a periodontal support formed by the cementum, the periodontal ligament, alveolar bone, and gingiva that besides participating in the support also comprises the protection periodontium [5].

The gingiva (Figure 1) is the part of the masticatory mucosa that surrounds the cervical portion of the tooth and covers the alveolar process [6]. Its main function is to protect structures adjacent to the tooth, being the first line of defence against periodontal disease [7]. Two parts can be distinguished: the free and attached gingiva [8].

The free gingiva can be pink or pigmented in some breeds, with firm consistency and an opaque surface [9]. The margin of the free gingiva is the edge of it. Between the free gingiva and the tooth, a groove is formed known as the gingival sulcus, which, in normal conditions in the dogs, varies in depth from one to three millimeters [10]. The sulcus is surrounded by an adhered epithelium that secretes a fluid with inflammation mediatory cells, immunoglobulins and antibacterial substances important in the physical and immunological protection of the junctional epithelium and deeper tissues [11].

The junctional epithelium (Figure 2) is located at the bottom of the sulcus, with flat and elongated cells adhering to the enamel through hemidesmosomes, promoting the junction between the gingiva and the tooth [12]. The junctional epithelium ends in the cementum-enamel junction [5].

In processes such as inflammation, hyperplasia or in both, the junctional epithelium can recede apically or the gingiva can increase, making deeper the gingival sulcus [1]. In gingival hyperplasia the deepening of the sulcus occurs without loss of periodontal tissue, named false pocket, although when there is a loss of the support tissue and protection of the tooth, the sulcus is called periodontal pockets, which can be of two types: suprabone, when the bottom of the sulcus is coronal to the support alveolar bone, and intrabone, when the bottom is located apically in relation to the adjacent alveolar bone. The pocket depth can vary between regions of the mouth and even between neighbouring teeth [13]. According to the location, the plaque can be classified as supra or sub-gingival. The supragingival plaque corresponds mainly to microbial aggregates found on tooth surfaces (mostly in the gingival third of the crown), however may extend into the gingival sulcus, where they are in immediate contact with the marginal gingiva. The subgingival plaque corresponds to bacterial aggregates found entirely within the gingival sulcus or periodontal pockets [1]. The bacterial constituents present in dental plaque are modified according to the disease evolution. In healthy gingiva, the cocci represent nearly two-thirds of the bacteria, followed by non-motile small rods. The bacteria present are mostly gram-positive and there is no significant representation of more virulent bacterial types. At the stage of gingivitis, the gram- positive rods (non-motile) gradually increase, surpassing the cocci, and the number of gram- negative bacteria also grows. This change continues to go on until the periodontium involvement phase (periodontitis), when the more pathogenic gram-negative microorganisms become the majority, so that the spirochetes represent almost half of the bacteria, while gram-positives are underrepresented [14]. Without interference in the plaque formation an inflammatory process can occur, which marks the beginning of a periodontal disease and that provides a favourable environment for change in the microbial composition of the plaque, that become a biofilm with more pathogenic characteristics, continuing with later stages of the disease [15].

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Prevention of Periodontal Disease

Prevention is considered essential for the maintenance of the animals’ teeth throughout their lives, making it impossible for the formation of the periodontal disease process [15]. Brushing, chewable products, promoters of friction and the use of antimicrobial substances are considered preventive techniques that remove supra and sub-gingival plaque. The effectiveness of the preventive technique should be monitored by a veterinarian and in most cases will require their intervention, performing dental prophylaxis in order to eliminate residual plaque and calculus in places of difficult access in the teeth [16] (Figure 4).
The tooth brushing, which acts by removing the biofilm through friction, is considered a technique with greater effect to reduce the buildup of plaque on the tooth [17]. The frequency of tooth brushing in the animals should be daily, to constantly avoid the formation of dental plaque and to establish a routine between the owner and the animal [18]. However, because less than 10% of owners agree with these recommendations for the dental care of their dogs and because of the time required for the organisation of the plaque, dog tooth brushing procedure has been recommended three times a week with satisfactory results [19].

Various forms of introduction of these therapies in the animal management have been tested, including the use of cookies and chewing objects for oral hygiene, additives to drinking water with an inhibitory effect on the growth of bacteria and oral rinse solutions as well as leather and biscuits with the addition of antimicrobial agents [20,21].

Prevention of periodontal disease, taking into account the least harmful nature of bacteria in the onset of the disease and higher prevalence of supragingival plaque, it is recommended that the use of topical oral solutions such as mouthwashes is sufficient to combat bacteria in question with great advantage because of its easy application in most patients [22].

Among the chemicals, that can be used this way to reduce

![Figure 3: Periodontal disease progression: A) healthy periodontium; B) gingivitis; C) Initial periodontitis – begins with the loss of periodontal tissue; D) moderate and severe periodontitis.](image-url)
the accumulation of plaque on dental surfaces, the bisguanids, quaternary ammonia and phenols have been widely evaluated. Chlorhexidine appears as a substance that has the greatest efficacy in the inhibition of oral plaque (Hennet, 2002) and has good antiseptic activity against all oral pathogens, more directly on the bacterial plaque organisms.[1] Its main concentration is the commercial use of alcoholic solution at 0.12% and it is also found in alcohol-free solutions and in gel form [23].

Despite the above indications for the use of chlorhexidine in the fight against dental plaque, it presents a series of unpleasant effects when used for prolonged therapy, such as loss of taste by the patient, pigmentation of the enamel, burning and even ulceration of the buccal mucosa [24]. These effects justify the use of this material only for few days, which makes its application not recommended in the prevention of periodontal disease, which requires a prolonged use of the antimicrobial agent chosen for this purpose [25].

Recently, the ozonised sunflower oil was tested, with positive results on microbial reduction in human patients with periodontal disease and copaiba oil was applied topically on dogs and the results were equal to those obtained with chlorhexidine on the oral microbial population [26]. Additionally, some in vitro tests were performed to analyse the antimicrobial activity of Copaiba oil on plaque-forming bacteria (and the evaluation of the inhibition of Streptococcus sp. adherence in glass capillaries caused by the same phytotherapeutic [27], obtaining in both cases positive results.

Many researchers continue their analysis looking for natural drugs as propolis, Camellia sinensis, Mimoso tenuiflora, Vitisamurensis, Rhinacanthus nasutus, Murraya koenigii, Allium sativum and Melaleuca alternifolia to its use in the prevention of periodontal disease by inhibiting plaque formation [28]. It is important that this drug combine properties such as antimicrobial activity that does not induce bacterial resistance, and inhibition of microbial adherence on tooth surfaces that suggest a great potential for use in therapies in the oral cavity and as an aid in oral hygiene [29]. For the use in the treatment of domestic animals it is suggested the inclusion of this antimicrobial and non-adherent agent in formulations containing the base flavours of chicken, beef, fish, etc [30].

Periodontal Disease at the Molecular Level: From Understanding to Designer Biomaterial: Copazan Oral Gel in Vitro Investigations

Evidence for the presence and role of ROS in periodontal tissue damage

The idea that ROS are associated with the pathogenesis of a variety of inflammatory diseases and have a role (direct or indirect) in tissue damage has become a major area of research over the last decade as demonstrated by electronic searches of the literature [31]. However, supporting evidence for their role in tissue damage is often indirect and circumstantial. Indeed, few reports fulfill any, or all, of Halliwell’s Postulates, those being the criteria requires to be fulfilled before ROS can be concluded to be key mediators of tissue injury in a given disease [31] (Figure 5).

The four criteria proposed by Halliwell, similar to those proposed by Robert Koch in 1884 [90] to establish a causal relationship between an organism and a disease, are:

1. ROS or oxidative damage caused must be present at site of injury;
2. Time course of ROS formation or the oxidative damage caused should occur before or at the same time as tissue injury;
3. Direct application of ROS relevant time course to tissues at concentrations found in vivo should reproduce damage similar to that observed in diseased tissue;
4. Removing or inhibiting ROS formation should decrease tissue damage to an extent related to their antioxidant action in vivo.

Local presence of ROS in periodontal disease

There are no published studies investigating directly the presence and levels of ROS in periodontal tissues, gingival crevicular fluid, saliva or blood in periodontal health and disease [32]. Possibilities do exist for local detection of ROS using endogenous molecular spin traps such as urate. Allantoin is one of urate's oxidation products that have been shown to be elevated in conditions associated with oxidative stress and periodontal disease such as diabetes, lung disease in pre-term infants, rheumatoid arthritis, and chronic heart failure [32]. A second potential avenue of enquiry is the direct detection of hydrogen peroxide, a relatively stable ROS, by sampling the air within the oral cavity [32]. Studies have shown that hydrogen peroxide can be detected in exhaled air and breath condensate, and that levels appear to correlate with inflammation [33]. None of the biomarkers is absolutely specific for ROS damage (i.e. they can be generated by means other than reacting with ROS) or specific to the periodontal tissues or periodontitis. Measuring ROS and oxidative stress damage in biological samples Free radicals and other reactive species have extremely short half-lives in vivo (10^6 –10^8 s) and simply cannot be measured directly. In vitro systems called spin traps are used to measure radical species but there are currently no suitable spin traps/probes available for in vivo measurement of ROS production in the human, because of their unknown toxicity [33] (Figure 6).
Current treatments: Brief Introduction

The dental use of antibiotics is characterized by a number of particularities. In effect, antibiotic prescription is empirical, i.e., the clinician does not know what microorganism is responsible for the infection, since pus or exudate cultures are not commonly made. Based on clinical and bacterial epidemiological data, the germs responsible for the infectious process are suspected, and treatment is decided on a presumptive basis and probabilistic reasoning [33] (Figure 7).

Copazan Oral Gel® is a medical grade isotonic hydrogel made from high molecular-weight biopolymer that promotes wound healing in oral cavity, dry mouth management and to help reinforce your mouth’s own defense system. Additional benefits of the natural oils such as oleo di copaiba, calendula oil and aloe vera gel provide the additional anti-inflammatory, pain-management and wound healing properties to address all aspects of healing and wound management as well as providing additional benefits of natural oils.

Copazan Oral Gel® is scientifically formulated and extensively tested medical grade gel, which incorporates state of the art science and exclusive natural ingredients boosting the angiogenesis phase of tissue regeneration.

Performance of Copazan Oral Gel®

Bio-adhesion in vitro model and Copazan Herbal Gel

High adhesiveness of the gels is desired to maintain an intimate contact with the tooth structure. The chitosan hydrogels showed a high adhesive force and work of adhesion. This can be expected due to the well-known intrinsic bio-adhesive properties.
of chitosan. The adequate water absorption capacity together with the cationic nature which promotes binding to the negative surface of the dentin structure can also explain these results [34-40] (Figure 8 and Table 1). The correlation between the force and work of adhesion is noticeable in all the hydrogels.

Microbiology of Copazan Oral Gel and individual components

All the test samples gave an average inhibition zone larger than the chlorhexidine gluconate control disc, thereby confirming the antibacterial activity of the Copazan Oral Gel and individual combinations against *Staphylococcus aureus* (Figure 9). There was a significant difference between the rest of the samples when compared to each other and the positive control.

Chlorhexidine is a broad-spectrum biocide effective against Gram-positive bacteria, Gram-negative bacteria and fungi [34-40]. Chlorhexidine inactivates microorganisms with a broader spectrum than other antimicrobials (e.g. antibiotics) and has a quicker kill rate than other antimicrobials (e.g. povidone-iodine) [41].

A number of mechanisms explaining the antimicrobial activity of chitosan have been postulated [42]. One of the proposed mechanisms is that the cross-linker moieties incorporated into hydrophilic chitosan increase their solubility and ease of penetration of the hydrogels into the cells of microorganisms. The chitosan then binds to microbial DNA, inhibits the transformation of mRNA and protein synthesis, and thereby inhibits metabolism [42].

Another suggested antibacterial mechanism of chitosan is the interaction between positively charged protonated NH₃⁺ groups of the chitosan molecules and negatively charged microbial cell surfaces. The electrostatic interaction results in changes in the properties of the cell wall permeability with leakage of the intracellular electrolytes causing internal osmotic imbalance that inhibit the growth of the microorganism [43]. The antimicrobial activity of copaiba oils was tested against Gram-positive and Gram-negative bacteria, yeast, and dermatophytes. Oils obtained from Copaifera martii, Copaifera officinalis, and Copaifera reticulata (collected in the state of Acre) were active against Gram-positive

![Figure 7: Application and mode of action of chitosan based materials and wound healing.](image-url)
of the “designer hydrogels” to detect and fight the free radical excess. It is well established that HO• can be generated from a reaction known as the biologic Fenton reaction and this reaction requires the presence of H₂O₂. Chlorhexidine gluconate is an effective antimicrobial agent with potent antimicrobial and anti-inflammatory properties and has been widely used as an antiseptic agent [43].

The amount of uncontrolled ROS is the main cause of the inability of the healing process to continue and therefore it would be ideal to utilize the antioxidant capacity of the “designer hydrogels” to detect and fight the free radical excess. It is well established that HO• can be generated from a reaction known as the Fenton reaction in the presence of H₂O₂ and the generation of HO• has been shown to be a critical factor in various ROS-induced oxidative stresses [34-40].

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. As clearly demonstrated by the Scheme 3, upon exposure to standard H₂O₂ in the form of Fe²⁺/EDTA/ H₂O₂/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 (Figure 10).

### In Summary

Copazan Oral Gel® is a medical grade isotonic hydrogel made from high molecular-weight biopolymer that promotes wound healing in oral cavity, dry mouth management and to help reinforce your mouth’s own defense system. Additional benefits of the natural oils such as oleo di copaiba, calendula oil and aloe vera gel provide the additional anti-inflammatory, pain-management and wound healing properties to address all aspects of healing and wound management as well as providing additional benefits of natural oils. As part of in vitro evaluation of the Copazan Oral Gel® the the parameters such as bioadhesion, inhibition zone and assessment of free radical capacity defense properties of the materials were measure and potential clinical application s of this promising material are discussed.

### Table 1: Copazan Oral Gel®

<table>
<thead>
<tr>
<th>Properties</th>
<th>Copazan Herbal Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Viability percentage (1)</td>
<td>105%</td>
</tr>
<tr>
<td>Moist Environment (2)</td>
<td>Yes</td>
</tr>
<tr>
<td>Protective Film Effect</td>
<td>Yes</td>
</tr>
<tr>
<td>Anti-inflammatory and analgesics</td>
<td>Yes</td>
</tr>
<tr>
<td>Cell multiplication enhancement</td>
<td>Yes</td>
</tr>
<tr>
<td>Wound Contraction (3)</td>
<td>Yes</td>
</tr>
<tr>
<td>Bacteriocidal</td>
<td>Yes</td>
</tr>
<tr>
<td>Bacteriostatic</td>
<td>Yes</td>
</tr>
<tr>
<td>Stays where applied (4)</td>
<td>Yes</td>
</tr>
<tr>
<td>Non toxic</td>
<td>Yes</td>
</tr>
</tbody>
</table>

(1) Standardized MTT assay determining cell viability percentage and viable cell counting
(2) Hydrophilic: Copazan Oral Gel® absorbs 6 times its own weight in wound exudate
(3) With its involvement in 4 stages of wound healing and tissue regeneration. Copian OralGel® accelerates the wound healing contraction phase
(4) Stays where applied product application on vertical wound exposed to wound exudate without the use of bandage. Copazan Oral Gel® poses excellent bio adhesive properties.

### Figure 9: Microbiology and Copazan Oral Gel and substituents.

![Figure 9: Microbiology and Copazan Oral Gel and substituents.](image)

Free radical defense capability of the Copazan Oral Gel and constituents

When wound occurs, it is generally accompanied by classical symptoms of inflammation, such as pain, redness and edema. In the inflammation stage, the main aim is the removal of debris, damage tissue, and bacteria by neutrophils and macrophages, which have a role in antimicrobial defense and debridement of devitalized tissue by production of proteolytic enzyme and reactive oxygen species [34-40]. The amount of uncontrolled ROS is the main cause of the inability of healing process to continue and therefor it would be ideal to utilize the antioxidant capacity of the “designer hydrogels” to detect and able to “fight the free radical excess” have been assessed using previously described model using well-established that HO radical can be generated from a reaction known as the biologic Fenton reaction and this reaction requires the presence of H₂O₂. Chlorhexidine gluconate is an effective antimicrobial agent with potent antimicrobial and anti-inflammatory properties and has been widely used as an antiseptic agent [43].

Further investigations and fine-tuning of the system are currently on the way in our laboratory.

### Figure 10: Influence of the various antioxidant on the solubility of BSA protein in the drug delivery system: in vitro approach

![Figure 10: Influence of the various antioxidant on the solubility of BSA protein in the drug delivery system: in vitro approach](image)
General Conclusion

Periodontal disease is considered an inflammatory disorder that damages tissue through the complex interactions between periodontopathic bacteria and host defense systems. It is likely that the role of Reactive Oxygen Species (ROS) is common to both bacterial- and host-mediated pathways of tissue damage. In recent years, there has been a tremendous expansion in the medical and dental research concerned with free radicals, ROS and antioxidant defense mechanisms. We demonstrated that the newly prepared Copazan Oral gel is a designer bio-materials capable to act as functional drug delivery systems which can be successfully applied for various applications in periodontal treatment of the future.

References


