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Copazan Oral Gel and Wound Healing *In Vitro*: Assessment of the Functional Biomaterial

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Abstract

Objective: To evaluate performance of Copazan Oral Gel in wound healing process *in vitro* as well as to evaluate the free radical scavenging properties of the new materials, microbiological and bioadhesive capacity of the Copazan Oral Gel and the individual components of the gel.

Methods: Bio-adhesive studies, microbiology and free radical defense capacity were investigated in order to assess the suitability of these designer materials.

Results: The Copazan Oral Gel showed a high adhesive force and were only slightly swollen in the aqueous medium. All the test samples gave an average inhibition zone against *Staphylococcus aureu*s larger than the chlorhexidine gluconate control disc. The hydrogels also had significant free radical defense capability.

Conclusion: In this study we demonstrated that the Copazan Oral Gel are suitable bio-active material capable of efficiently counter-acting potential free radical damage generated during the wound healing treatment *in vitro*.

Keywords: *Copazan Oral Gel; Topical Application; Chitosan Hydrogels; Antimicrobial Systems; Wound Healing; Free Radical*

Introduction

Wound healing is a specific biological process related to the general phenomenon of growth and tissue regeneration [1]. Wound healing progresses through a series of interdependent and overlapping stages in which a variety of cellular and matrix components act together to reestablish the integrity of damaged tissue and replacement of lost tissue [2].

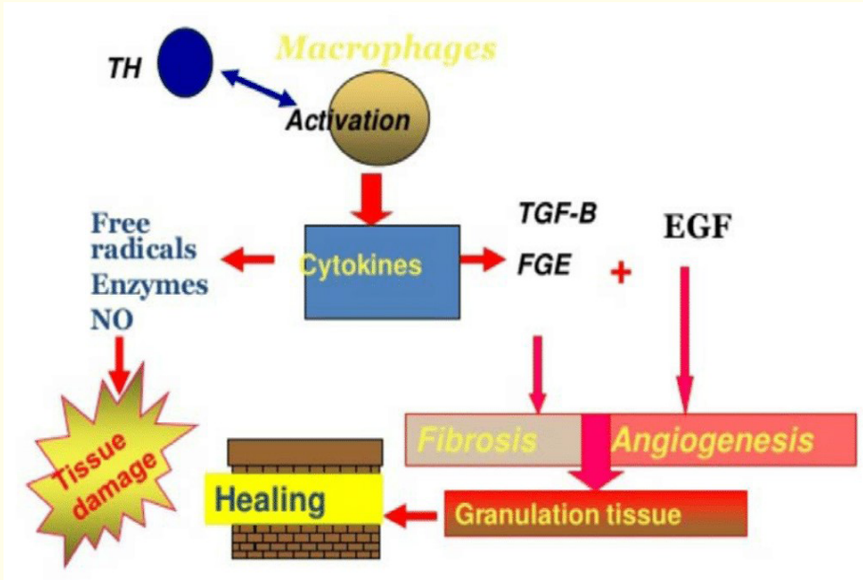

Figure 1: *Free radicals and tissue damage.*

Oral mucosa is covered with stratified squamous epithelium, and the connective underneath the epithelium consists of fibroblasts, collagens and capillaries [3]. The process of wound healing involves hemostasis, inflammation, cell proliferation and remodeling. The first phase of hemostasis begins immediately after wounding, with vascular construction and fibrin clot formation [4].

The inflammatory phase is characterized by the sequential infiltration of neutrophil, macrophages, and lymphocytes [5]. The proliferation phase overlaps with the inflammatory phase, and is characterized by epithelial proliferation and migration over the provisional matrix within the wound, vascularization, collagen synthesis and extracellular matrix formation. The collagen remodeling, and vascular maturation and regression occurs in the remodeling phase [6]. Multiple local and systemic factors impair their wound healing. Supply of oxygen, infection and foreign body are the local factors. Ageing, sex, stress, failure of circulation, obesity, medications such as steroid and anti-cancer drug, alcohol, smoking, immune suppressed state, nutrition and many types of systemic diseases can be a systemic factor for it [7].

Chitosan, which is produced by deacetylation of chitin, is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) [8]. Chitosan is used in the treatment of wounds and burns due to its haemostatic effect [9]. It is thought that chitosan accelerates the formation of fibroblasts and increases early phase reactions related to healing [10]. Chitosan can be prepared in a variety of forms, namely films, hydrogels, fibres, powders and micro-/nanoparticles. Applications of this biopolymer in commercial and biomedical fields have increased due to the low toxicity of chitosan and its biodegradation products, and its biocompatibility with blood and tissues [11].

Copaiba oleoresin is widely used as a popular medicine, through topical and oral administration. It has various ethno-pharmacological indications, including: wounds, asthma, as an antiseptic for wounds, skin ulcers, aching joints, ovarian cysts, uterine myoma, weak uterus, vaginal discharge, ovarian problem, ulcers, sore throat, uterine infections, general inflammations, as a tonic and to treat ulcers and other digestive diseases, and cancer, and leishmanioses [12].

Copazan Oral Gel^{R} 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. The main objective is to evaluate the Copazan Oral GelR as a non-adhesive wound dressing through evaluation of evaluation of bioadhesion, inhibition zone and assessment of free radical capacity defense properties *in vitro*.

Methods

Microbiology investigation of the Copazan Oral gel and individual components of the gel

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 antibacterial activity of the prepared chitosan hydrogels were tested using the standard Kirby-Bauer agar disc diffusion method [13]. Five to 6 mm deep Muller-Hinton agar (Oxoid, Basingstoke, UK) plates were inoculated by streaking a standardized inoculum suspension that match a 0.5 McFarland standard and containing 10^{7} – 10^{8} colony forming units/ml with a throat cotton swab. For each test sample 500 µg of hydrogel was applied to a 6 mm diameter paper disc. The paper discs were placed on the inoculated Muller-Hinton agar medium and incubated at 37^{o}C for 24 hours. The diameter of the zones of growth inhibition was measured with a caliper. Each measurement was done in triplicate and the testing of each sample was repeated 3 times. The antibacterial efficacy of the prepared gels were compared to antibiotic sensitivity discs (Mast Laboratories, Merseyside UL) containing 25 µg of chlorhexidine gluconate per disc.

Bioadhesive investigation

Bioadhesion studies were done using Chatillon apparatus for force measurement [14]. This method determines the maximum force and work needed to separate two surfaces in intimate contact. The Copazan Herbal gel and combination of the starting materials (0.1g) were homogeneously spread on a 1 cm^2 glass disk and then the disks were fixed to the support of the tensile strength tester using double side adhesive. The gel was brought into contact with the commercially available band aid, in order to simulate the skin attachment or the contact with slice of pork skin was established in order to imitate adhesion of the gel to the oral mucosa structure, after a preset contact time (1 min) under contact strength (0.5N) the 2 surfaces were separated at a constant rate of displacement (1 mm/s). The strength was recorded as a function of the displacement, which allowed to determine the maximal detachment force, F_{max}, and the work of adhesion, W, which was calculated from the area under the strength-displacement curve.

Free radical scavenging ability: We adopted the method of Kyselova., *et al*. to test for radical scavenging ability of the new hydrogels. This method records changes in water solubility of the model protein bovine serum albumin (BSA) exposed to free radicals generated by the Fenton reaction system [15].

The incubation mixtures contained the following reagents: bovine serum albumin (0.8 mg/ml), phosphate buffer, pH 7.4 (10 mM), EDTA (4.8 mM), $Fe(NH_4)_2(SO_4)_2$ (4 mM), ascorbate (4 mM) and H_2O_2 (0.2%) in water to reach a total volume of 2.5 ml. The hydrogels were added and the reaction mixture was incubated for 20 min at room temperature. After completion of the reaction, the mixture was centrifuged at 3500 rpm for 10 min. 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 Na_2CO_3 (10%) in NaOH (0.5 M) and the final volume made up to 2.5 ml by adding distilled water. An aliquot of the solution was used for protein determination using the method of Lowry., *et al* [16].

Statistics: The Student's T-test was used to analyze the data.

Results and Discussion

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 [9] which promotes binding to the negative surface of the dentin structure can also explain these results.

According to Caffaggi, hydration of the polymer causes mobilization of the polymer chains and hence influences polymeric adhesion [17]. Appropriate swelling is important to guarantee adhesiveness; however, over hydration can form slippery non-adhesive hydrogels [18]. The correlation between the force and work of adhesion is noticeable in all the hydrogels.

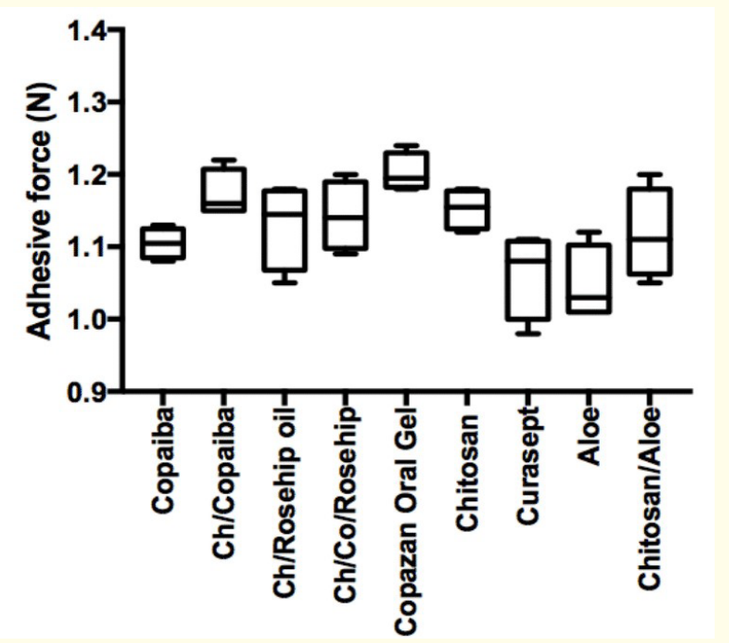

Figure 2: *Bioadhesion of Copazan Herbal Gel testing in vitro. The presented values are an average (n = 5).*

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 3). 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 [19]. 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) [20]. It has both bacteriostatic (inhibits bacterial growth) and bactericidal (kills bacteria) mechanisms of action, depending on its concentration. Chlorhexidine kills by disrupting the cell membrane [21]. Upon application *in vitro*, chlorhexidine can kill nearly 100% of Gram-positive and Gram-negative bacteria within 30 seconds [22]. Since chlorhexidine formulations can destroy the majority of categories of microbes, there is limited risk for the development of an opportunistic infections [23].

A number of mechanisms explaining the antimicrobial activity of chitosan have been postulated [24]. 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 [25].

Another suggested antibacterial mechanism of chitosan is the interaction between positively charged protonated NH_3^+ 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 microorganisms [26]. 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 species (*Staphylococcus aureus,* methicillin-resistant *S. aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, and *Enterococcus faecalis)* with minimum inhibitory concentrations ranging from 31.3 - 62.5 μg/ml. The oils showed bactericidal activity, decreasing the viability of these Gram-positive bacteria within 3h [27,28]. The antibacterial and antimicrobial properties of Calendula oil are well documented the and support the traditional use of the plant in the therapy of bacterial infection [29].

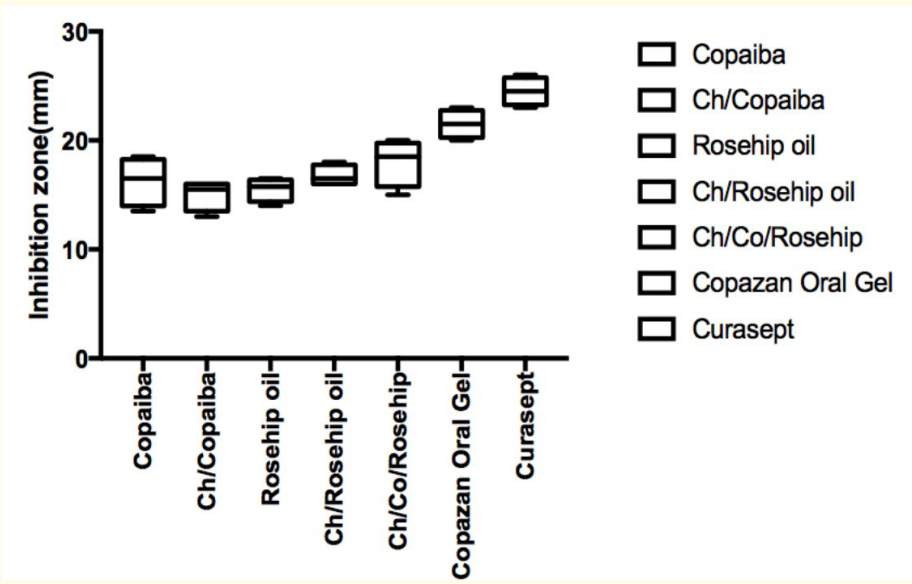

Figure 3

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 [30]. 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 H_2O_2.

Chlorhexidine gluconate is an effective antimicrobial agent with potent antimicrobial and anti-inflammatory properties and has been widely used as an antiseptic agent [31].

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 $HO^{\bullet}$ can be generated from a reaction known as the Fenton reaction in the presence of H_2O_2 [32,33] and the generation of $HO^{\bullet}$ has been shown to be a critical factor in various ROS-induced oxidative stresses [34,35].

Protein cross-linking can be used as a model for detection of free radical activity [36]. Water soluble Bovine Serum Albumin (BSA) can be polymerized by hydroxyl radicals generated by the Fenton reaction system of Fe^{2+}/EDTA/ H_2O_2/ascorbate [36]. 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 then be detected [37].

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 Fe^{2+}/EDTA/ H_2O_2/ascorbate [36]. 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. We considered worthwhile to study the chitosan as a "build in defense mechanism" for the *in-vitro* generated free radical production and "site specific" *in vitro* model counter reaction of the hydrogel.

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 figure 4, upon exposure to standard H_2O_2 in the form of Fe^{2+}/EDTA/ H_2O_2/ascorbate solution as a base line determinate free radical generation under "prototype *in-vitro* free radical damage", upon incorporation of the chitosan substituted hydrogels, the build in antioxidant capacity and therefor 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.

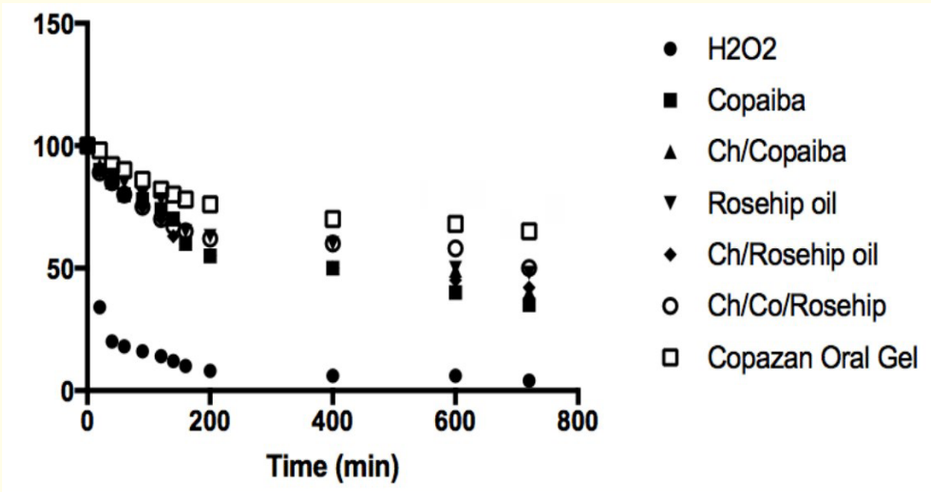

Figure 4: *Influence of the various antioxidant on the solubility of BSA protein in the drug delivery system: in vitro approach.*

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

Conclusion

Copazan Oral Gel[R] 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[R] the parameters such as bioadhesion, inhibition zone and assessment of free radical capacity defense properties of the materials were measure and potential clinical applications of this promising material are discussed.

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