

Copazan Herbal gel and Wound Healing *in vitro*: Assessment of the Functional Biomaterial for Veterinary Application



Victoria Tamara Perchyonok*

VTPCHEM PTY LTD, Australia

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***Corresponding author:** Victoria Tamara Perchyonok, VTPCHEM PTY LTD, Glenhuntly, 3163, Australia, Email: tamaraperchyonok@gmail.com

Abstract

Objective: To evaluate performance of Copazan Herbal 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 Herbal 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 Herbal 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 aureus* 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 Herbal Gel are suitable bio-active material capable of efficiently counteracting potential free radical damage generated during the wound healing treatment *in vitro*.

Keywords: Copazan herbal 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). Recently, much attention has been given to the use of chitosan in veterinary applications, as a wound healing agent, antimicrobial agent, bandage material, skin grafting template, hemostatic agent and drug delivery vehicle. In this section, various investigations carried out recently on veterinary applications of chitosan are discussed as well as their potential uses in veterinary medicine. The chemical structure of chitosan, which is similar to that of hyaluronic acid, suggested that it would act as a wound healing agent [3]. Mechanisms of acceleration of wound healing have been investigated *in vitro* and *in vivo* by several groups [4-6]. Since 1985, Minami and his group have been developing chitin and chitosan products for veterinary use and they have reported

their clinical effects on wound healing and their biological activities [7]. They found these polymers and products to accelerate wound healing, decrease treatment frequency, and give comfortable and painless wound surface protection. In addition to accelerating wound healing, chitosan is also capable of activating host defenses to prevent infection, thereby offering an alternative to the use of antibiotics. Chitosan enhances the functions of inflammatory cells such as polymorphonuclear leukocytes (PMN) (phagocytosis, production of osteopontin and leukotriene B4), macrophages (phagocytosis, production of interleukin (IL-1), transforming growth factor h1 and platelet-derived growth factor). As a result, chitosan promotes granulation and organization. Therefore, it is beneficial for healing of large open wounds in animals [8]. One of the most interesting effects of chitin and chitosan on wound healing is formation of granulation tissue with angiogenesis. Chitin and chitosan induce fibroblasts to release interleukin-8, which is involved in migration and proliferation of fibroblasts and vascular endothelial cells.

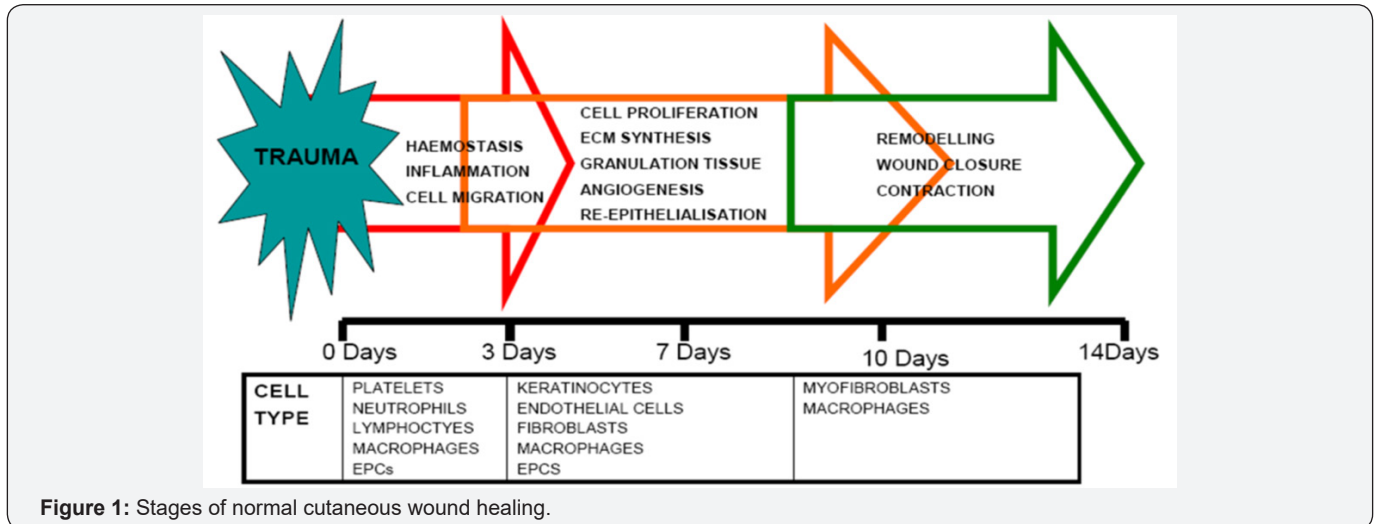


Figure 1: Stages of normal cutaneous wound healing.

Copaiba oleoresin is widely used as a popular medicine, through topical and oral administration. It has various ethnopharmacological 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 [9]. Copazan HerbalGel[®] 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 HerbalGel[®] as a non-adhesive wound dressing through evaluation of bioadhesion, inhibition zone and assessment of free radical capacity defense properties in vitro.

Methods

Microbiology investigation of the Copazan Herbal 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 [10]. Five to 6mm 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 10⁷-10⁸ colony forming units/ml with a throat cotton swab. For each test sample 500µg of hydrogel was applied to a 6mm diameter paper disc. The paper discs were placed on the inoculated Muller-Hinton agar medium and incubated at 37 °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 25µg of clohexadine gluconate per disc.

Bioadhesive investigation

Bioadhesion studies were done using Chatillon apparatus for force measurement [11]. 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 1cm² 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 (1min) under contact strength (0.5N) the 2 surfaces were separated at a constant rate of displacement (1mm/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 Kyselovaet 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 [12]. The incubation mixtures contained the following reagents: bovine serum albumin (0.8mg/ml), phosphate buffer, pH 7.4 (10mM), EDTA (4.8mM), Fe(NH₄)₂(SO₄)₂ (4mM), ascorbate (4mM) and H₂O₂ (0.2%) in water to reach a total volume of 2.5ml. The hydrogels were added and the reaction mixture was incubated for 20min at room temperature.

After completion of the reaction, the mixture was centrifuged at 3500rpm for 10min. The supernatant was precipitated with an equal volume of trichloroacetic acid (10%) at 0 °C. The precipitate thus obtained was re-dissolved in 1ml of Na₂CO₃ (10%) in NaOH (0.5M) and the final volume made up to 2.5ml by adding distilled water. An aliquot of the solution was used for protein determination using the method of Lowry et al. [13].

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

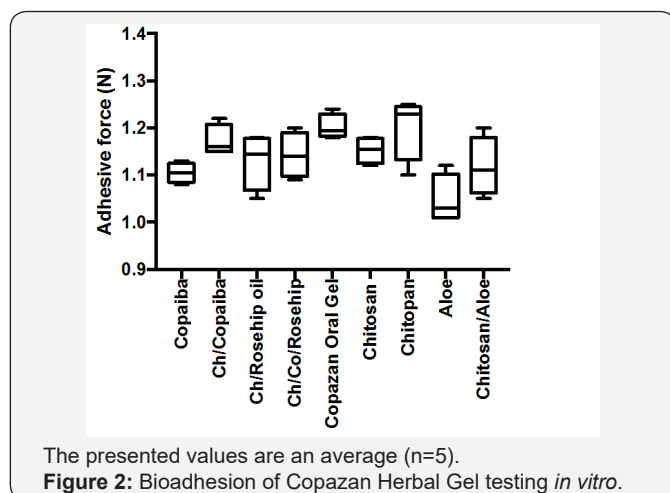
Free radical scavenging ability

We adopted the method of Kyselova et al. [14] 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 [14]. The incubation mixtures contained the following reagents: bovine serum albumin (0.8mg/ml), phosphate buffer, pH 7.4 (10mM), EDTA (4.8mM), Fe(NH₄)₂(SO₄)₂ (4mM), ascorbate (4mM) and H₂O₂ (0.2%) in water to reach a total volume of 2.5ml. The hydrogels were added and the reaction mixture was incubated for 20min at room temperature. After completion of the reaction, the mixture was centrifuged at 3500rpm for 10min. The supernatant was precipitated with an equal volume of trichloroacetic acid (10%) at 0 °C. The precipitate thus obtained was redissolved in 1ml of Na₂CO₃ (10%) in NaOH (0.5M) and the final volume made up to 2.5ml by adding distilled water. An aliquot of the solution was used for protein determination using the method of Lowry et al.

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Results and Discussion

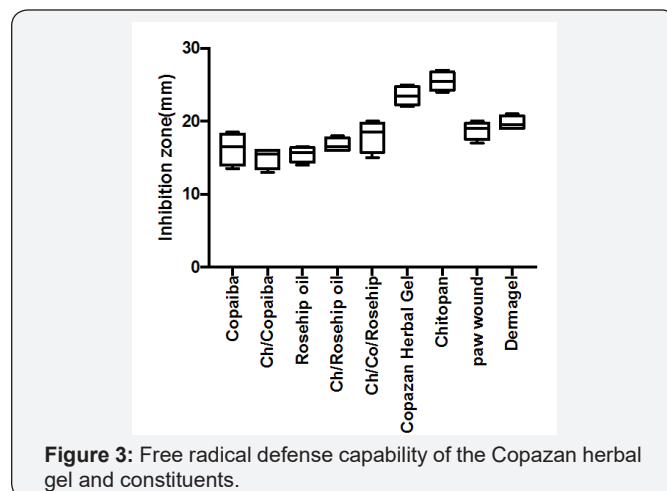
Bio-adhesion in vitro model and Copazan herbal gel



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 [15] 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 [16]. Appropriate swelling is important to guarantee adhesiveness; however, over hydration can form slippery non-adhesive hydrogels [3]. The correlation between the force and work of adhesion is noticeable in all the hydrogels (Figure 2).

Microbiology of Copazan herbal 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 Herbal 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 [4]. 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) [5]. 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 [6]. Upon application *in vitro*, chlorhexidine can kill nearly 100% of Gram-positive and Gram-negative bacteria within 30 seconds [7]. Since chlorhexidine formulations can destroy the majority of categories of microbes, there is limited risk for the development of an opportunistic infections [8].

A number of mechanisms explaining the antimicrobial activity of chitosan have been postulated [17]. 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 [18]. 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 [19]. 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 $\mu\text{g/ml}$. The oils showed bactericidal activity, decreasing the viability of these Gram-positive bacteria within 3h [20]. The antibacterial and antimicrobial properties of Calendula oil are well documented and support the traditional use of the plant in the therapy of bacterial infection [20].

Free radical defense capability of the Copazan herbal 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. The amount of uncontrolled ROS is the main cause of the inability of healing process to continue and therefore 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 for the irrigation of root canals as well as a medicament during root-canal treatment [1]. It has also been found that in combination with $\text{Ca}(\text{OH})_2$ and H_2O_2 , CHX it is more effective against *Streptococcus* species and in eliminating *Enterococcus faecalis* from root canal infections [6,7]. However, recent reports suggested CHX as a potential genotoxic agent toward leucocytes, oral mucosal cells and lymphocytes and the use of CHX on its own, or in combination with these products may be potentially genotoxic and cause tissue damage [8]. 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 $\text{HO}\cdot$ can be generated from a

reaction known as the Fenton reaction in the presence of H_2O_2 [17,18] and the generation of $\text{HO}\cdot$ has been shown to be a critical factor in various ROS-induced oxidative stresses [19,20]. Protein cross-linking can be used as a model for detection of free radical activity [8]. Water soluble Bovine Serum Albumin (BSA) can be polymerized by hydroxyl radicals generated by the Fenton reaction system of $\text{Fe}^{2+}/\text{EDTA}/\text{H}_2\text{O}_2/\text{ascorbate}$ [1]. 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 [9]. 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 $\text{Fe}^{2+}/\text{EDTA}/\text{H}_2\text{O}_2/\text{ascorbate}$ [6-8]. 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 “built 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 $\text{Fe}^{2+}/\text{EDTA}/\text{H}_2\text{O}_2/\text{ascorbate}$ 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 there for 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) [21-27].

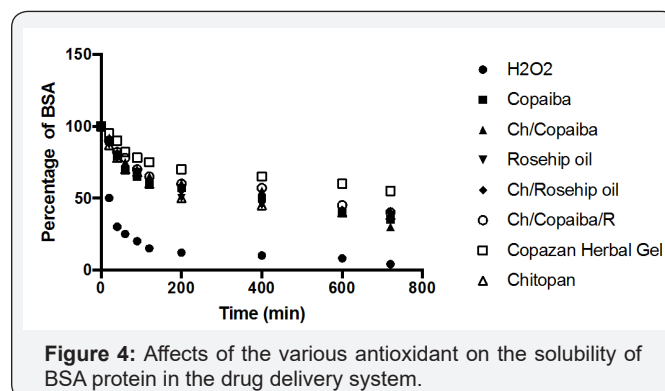


Figure 4: Affects of the various antioxidant on the solubility of BSA protein in the drug delivery system.

Conclusion

Copazan Herbal Gel[®] is a medical grade isotonic hydrogel made from high molecular-weight biopolymer that promotes wound healing *in vitro*. As part of in vitro evaluation of the

Copazan HerbalGel[®] the the parameters such as bio adhesion, 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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