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Topical Formulations of Water-Soluble Chitin as a Wound Healing Assistant –Evaluation on Open Wounds Using a Rabbit Ear Model–

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Abstract: Water-soluble chitin (WSC) was prepared by carefully deacetylating chitins to about 50% of N-acetyl content. Topical formulations based on WSC were prepared and their effects on wound healing were evaluated on a rabbit ear model. Full-thickness, open skin wounds were made on the ears of rabbits and WSC ointments were embedded in the open wounds. The application of WSC ointments significantly accelerated wound healing and wound contraction. The areas of epithelialization and granulation tissues in WSC ointment group are remarkably larger than those in control group (no treatment) and in placebo group (treated with ointment-base materials). A large number of grown granulation tissues including dense fibroblast deposition were observed under the thickened epithelium of the wound treated with WSC ointments. The number of inflammatory cells in WSC ointment group was significantly decreased compared with those in control and placebo groups, indicating that WSC would give low stimuli to wounds and prevent excessive scar formation. Neovascularization was the most prominent in WSC ointment group. Wound contraction in WSC ointment group was much larger than those in control and placebo groups. Overall results demonstrate that the topical formulation based on WSC is considered to become an excellent dressing as a wound healing assistant.

Keywords: Chitin, Chitosan, Water-soluble, Wound-healing, Ointment, Open wound, Epithelialization, Granulation, Neovascularization

Introduction

Chitin is a high molecular weight heteropolysaccharide composed mainly of $\beta(1\rightarrow4)$-2-deoxy-2-acetamido-D-glucopyranose and the second-most abundant natural polysaccharide next to cellulose. Chitin has excellent biocompatibility for most tissues including skin and bone. It has a similar structure to glycosaminoglycan (GAG), mostly components of the extracellular matrix. Human connective tissues do not actually contain chitin. However, its monomeric unit, N-acetylglucosamine, is found in the core of certain human glycoproteins such as heparin, heparan sulfate, keratosulfate, and hyaluronic acid, etc. [1].

The history of chitin as a wound healing assistant is of long standing. A pen of squid has been used for wound healing as a folk remedy in Asia. In 1957, Prudden *et al.* [2] reported that the shark cartilage accelerated wound healing. In 1970, the specific chemical agent responsible for striking biological effects on the acceleration of wound healing was isolated from the cartilage and identified as a polymeric N-acetylglucosamine, that is, chitin [3]. Different types of chitin/chitosan-based wound healing materials such as beads [4], fibers [5], films [6,7] and hydrogels [8,9] have been studied for a wide spectrum of applications from simple wound covering to sophisticated artificial skin matrices. A few wound medications were commercialized such as Beschitin® (Unitika, Japan), ChitipackS® (Eisai Co., Japan) and Tegasorb® (3M, USA).

Chitin obtained from the shell of crustacean is insoluble and even unswellable in water due to its highly ordered crystalline structure [1]. However, the chitin with about 50 % of degree of deacetylation (DD) is the most hydrophilic, even soluble in water [10-13]. In addition, the chitin with about 50 % DD has superior susceptibility to lysozyme [13]. The study on incisional wound healing in rats indicated that water-soluble chitin (WSC) with 50 % of DD is highly effective as a wound healing accelerator [13].

In the present study, a WSC ointment was prepared by mixing an aqueous WSC solution with white Vaseline, a stearyl alcohol, and a surfactant, and its accelerating effect on open wound healing in a rabbit ear was investigated. A few formulations based on chitin/chitosan have been used for the treatment of open wounds. Okamoto *et al.* [14] reported that granules of chitin and chitosan enhanced re-epithelialization and regenerated normal skins in open wounds. Ueno *et al.* [5] reported that cottenfiber-type chitosan had a function in the acceleration of infiltration of polymorphonulear cells at the early phase of wound healing, followed by the production of collagen by fibroblasts. However, in those studies, both the powder-type [14] and the cottenfiber-type [5] which are poorly suitable forms to be applied on open wounds were used. Furthermore, the open wounds were created on the backs of dogs, inevitably occurring a large physical contraction irrespective of wound healing.

In this work, the effect of WSC on wound healing was studied using a rabbit ear as an open wound model since it hardly shows a physical contraction during wound healing. Full-thickness skins in circles with a diameter of 2 cm were removed, and then a WSC ointment was applied in the open wounds. The accelerating effect of WSC on wound healing

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was evaluated by measuring re-epithelialization and granulation areas of the wounds in conjunction with macroscopic and microscopic observations.

Experimental

Materials

Chitin (DD of 8.60 %) was purchased from Tokyo Chemical Industry, Japan and purified with 1 N NaOH and 1 N HCl. WSC was prepared through alkaline treatment of chitin and depolymerization by ultrasonication [12,13]. Chitin was suspended in 40 % aqueous sodium hydroxide solution. Alkaline chitin was dissolved by stirring vigorously with crushed ice at 0 °C. The obtained solution was stirred at 25 °C for 60 h and then neutralized with aqueous hydrochloric acid solution. The solution was poured into acetone and the precipitate was washed with acetone/water (7/1) mixture and dried under vacuum. The regenerated chitin was dissolved in 2 % aqueous acetic acid solution. Depolymerization by ultrasonic treatment was conducted at 225 W for 1 h with a sonicator (Bransonic-221, Branson Co., USA).

Preparation of Water-Soluble Chitin Ointment

WSC (0.4 g) was dissolved at room temperature in water (20 m*l*) with a small amount of acetic acid (0.01 %). White Vaseline (6 g), stearyl alcohol (6 g) and a small amount of surfactant (HCO-60) were mixed and slowly heated up to 70 °C, and then stirred into the WSC solution at 70 °C to give an emulsion. The emulsion was slowly cooled down to room temperature to give a WSC ointment.

Creation of Wound

Figure 1 represents schematically the creation of open wounds on rabbit ears. Fifteen adult rabbits weighing 2.8-3.2 kg were used in this study. After they were anesthetized with 60 mg of ketamine hydrochloride and 5 mg of xylene, full-thickness skins in circles with a diameter of 2 cm were removed, and then a WSC ointment was applied on the wound. All wounds were covered with a polyurethane film (Tegaderm, 3M, USA). On the 7th day after initial wounding, each wound was taken for determining re-epithelialization and granulation areas.

The rabbits were divided into three groups by five rabbits. In the first group, the wound of one side ear was treated with a WSC ointment and that of the other side ear was not treated. In the second group, the wound of one side ear was treated with a placebo (composed of ointment-base materials without WSC) and that of the other side ear was not treated. In the third group, the wound of one side ear was treated with a WSC ointment and that of the other side ear was treated with a placebo.

Histological Observations

The tissues were taken form the wounds and fixed in 10 % phosphate-buffered formalin, and then embedded in paraffin following dehydration. The tissue was incised with the line including its center. The incised section of the tissue was stained by hematoxylin-eosin for histological examination.

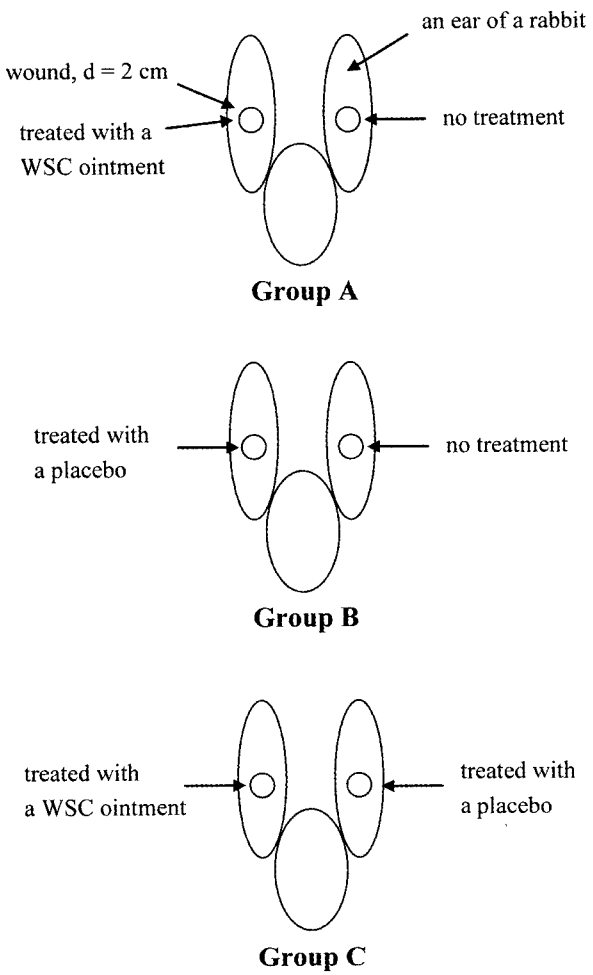

Figure 1. Schematic representation of the creation of open wounds on ears of rabbits.

Measurement of Epithelialization and Granulation Areas

The epithelialization and granulation areas of the incised sections of the tissues taken from wounds were determined by Image Analysis System (Kontron, Germany).

Statistical Analysis

All data were analyzed by Student's unpaired t-test. When the p value was less than 0.05, the difference was considered to be significant.

Results and Discussion

Macroscopic Observations

A small amount of exudate and granulation tissues were found in control and placebo groups. There were no significant

differences between the two groups in macroscopic observation. But, in the wound treated with a WSC ointment, a large amount of exudate and granulation tissues were found, and thickened wound margins were also observed. Greater wound contraction was shown in WSC ointment group than in control and placebo groups.

Measurement of Epithelialization and Granulation Areas

In examining the wound-healing process, an ideal model may be the external ear of the rabbit. When these appendages have been punched through, they produce a circular blastema around margins that grows centripetally to obliterate the aperture, and finally fill in full-thickness holes. It is prerequisite to exclude the possibility of physical wound contraction during wound-healing process. The rabbit ear may fulfill this condition because its skin is firmly adherent to an underlying skeletal substrate. Figure 2 schematically illustrates the open wound healing on a rabbit ear. In this study, the tissues taken from open wounds were incised with the line including its center, as shown in Figure 2, and were stained by hematoxylin-eosin. The epithelialization and granulation areas were determined by Image Analysis System. The effect of a WSC ointment on epithelialization in open wounds is shown in

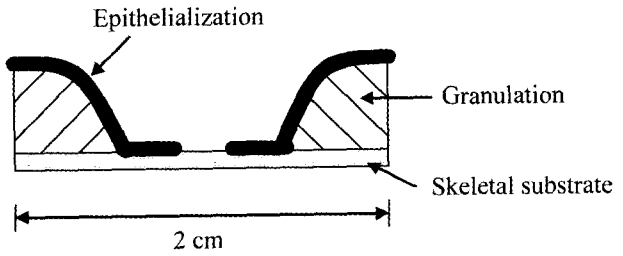

Figure 2. Schematic illustration of open wound healing on an ear of a rabbit.

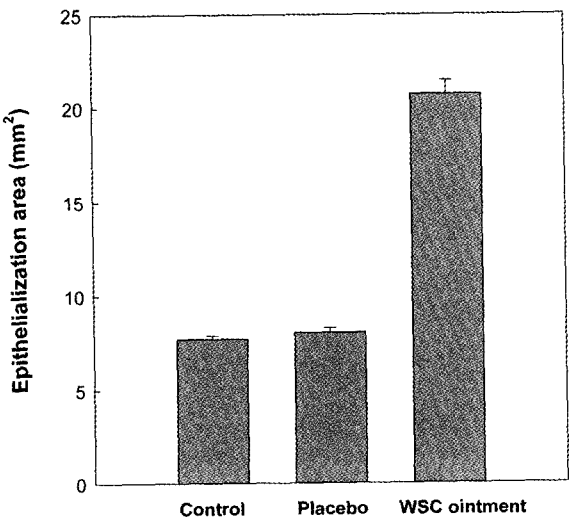

Figure 3. Effect of WSC ointments on epithelialization.

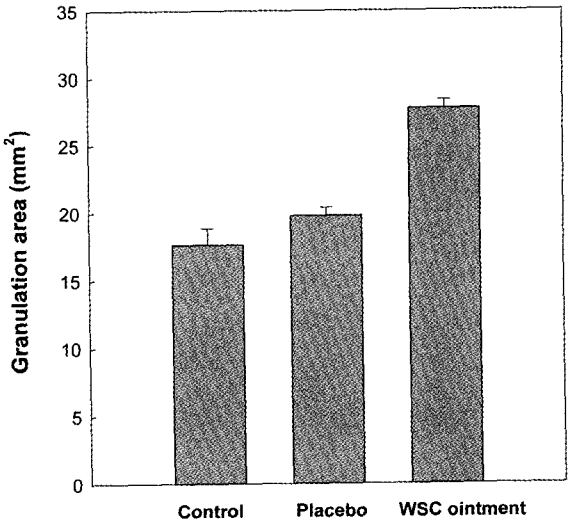

Figure 4. Effect of WSC ointments on granulation.

Figure 3. The epithelialization area in WSC ointment group was much larger than those in control and placebo groups. The p-value of WSC ointment group was extremely low (3.419×10^{-7} and 4.997×10^{-7} compared with control group and placebo group, respectively). There were no significant differences in epithelialization between control group and placebo group. The p-value of placebo group was 0.2859 compared with control group, indicating that ointment-base, containing no WSC, did not significantly accelerate re-epithelialization.

Figure 4 shows the effect of a WSC ointment on granulation in open wounds. The result gave the same tendency as shown in Figure 3. The granulation area in WSC ointment group was much larger than those in control and placebo groups. The p-values of WSC ointment group were 1.604×10^{-4} and 3.741×10^{-5} compared with control group and placebo group, respectively. The ointment-base materials alone did not affect granulation in open wounds. The areas of granulation tissues in control and placebo groups were not much different (p-value, 0.1916).

Histological Examination

In general, wound healing following trauma or burn occurs through several processes by inflammation, re-epitheliaization, granulation tissue formation, and matrix synthesis, leading to wound contraction [5,13-17]. Histological findings in the incised sections of the wounded skins on the 7th day after initial wounding are shown in Figure 5. The results were summarized in Table 1. The number of inflammatory cells in WSC ointment group was significantly decreased in comparison with those in control and placebo groups. In wound-healing process, there are two major stages at the early phase [5,13,16]. The first is the inflammatory stage and the second is the proliferative stage. The inflammatory stage is highly important and essential for repair and restoration of structural and

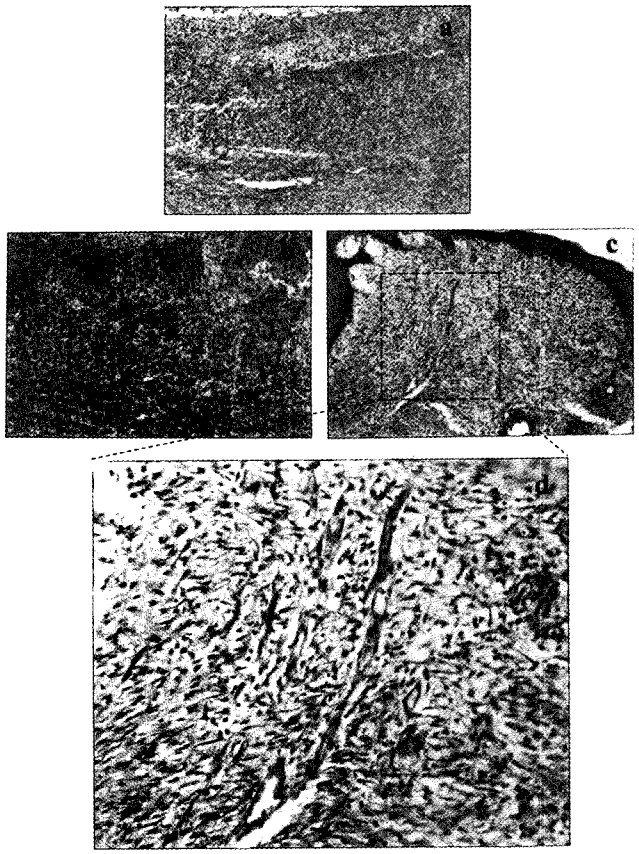

Figure 5. Histological findings in the incised sections of the wounds on the 7th day after initial wounding by hematoxyline and eosin; (a) control (×50), (b) placebo (×50), (c) WSC ointment (×50), (d) WSC ointment (×200).

Table 1. Histological findings in open wounds

	Control	Placebo	WSC ointment
Re-epithelialization	10–30 %	10–30 %	70–90 %
Granulation tissue	thin	thin	full thickness
Number of inflammatory cells	severe	severe	slight
Number of fibroblasts	slight	slight	severe
Neovascularization	slight	slight	severe

functional integrity of damaged tissues [5,13,16]. Inflammation occurs in response to tissue injury induced by trauma. This stage involves exudation of plasma components, hemostasis, inward migration of inflammatory cells, a clearance of necrotic tissues, etc. If this stage lasts too long, the next proliferative stage is retarded. While most of the wounds in control and placebo groups still remained in the inflammatory stage, the WSC ointment-treated wound mostly proceeded to the next proliferative stage.

The wounds of control and placebo groups were partially re-epithelialized at the wound margin, but those in WSC ointment group were completely re-epithelialized except for their centers. In the wounds of control and placebo groups, a small amount of granulation tissue was found under the regenerated epithelium at wound margin. However, in the wounds of WSC ointment group, a large amount of granulation tissue was observed under the thickened epithelium at wound margin. In general, fibroblasts are one of the key factors involved in remodeling tissue defects [13]. After injury, fibroblasts migrate following inflammatory cells from adjacent tissues into the wound sites, where they proliferate and produce collagen, elastin, and proteoglycan, which will reconstruct and reorganize the extracellular matrix [5,16,17]. In the wound treated with a WSC ointment, it was found that many fibroblasts were deposited densely in granulation tissues. Neovascularization occurred most prominently in WSC ointment group, as shown in Figure 5. In the proliferative phase, capillary sprouts into the wound behind macrophages. Shortly thereafter, new capillary loops and fibroblasts divide and deposit quantities of fibrillar collagen. When the wound is filled with new tissues, some capillaries with blood flow differentiate into arterioles and venules while others without blood flow involute. Figure 5(d) clearly shows that newly formed capillaries proliferated in WSC ointment group, indicating that wound healing in WSC ointment group much more developed than those in control and placebo groups.

Wound contraction is a basic biological process necessary for survival. The extent of wound contraction is an overall indicator representing the degree of wound healing. Wound contraction in WSC ointment group was larger than those in control and placebo groups. All results clearly demonstrate that the WSC ointment accelerated wound healing in a rabbit ear model.

Conclusions

WSC ointments were highly effective as a wound-healing assistant in open wounds. The rabbit ear model was suitable for observing the effects of medications on open wound healing. The topical formulations based on WSC accelerated epithelialization and granulation, and greatly induced wound contraction and wound closure. Notably, some areas of the wounds treated with WSC ointments were filled with new tissues, therein newly formed capillaries proliferated. Overall results indicate that the WSC ointment is a promising candidate as a wound dressing due to its easy application and high effectiveness.

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