Chitin Nanofibrils Linked to Chitosan Glycolate as Spray, Gel, and Gauze Preparations for Wound Repair

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ABSTRACT: Recent advances in process chemistry have made it possible to make chitosan and chitin nanofibril materials more flexible and useful for the development of new biorelated products. In this study, the effectiveness of three chitin nanofibril/chitosan glycolate-based preparations, a spray (Chit-A), a gel (Chit-B), and a gauze (Chit-C), in healing cutaneous lesions are assessed macroscopically and by light microscopy immunohistochemistry.
These evaluations are compared to the results obtained using a laser co-treatment. The wound repair provided by the three preparations is clearly evident even without the synergistic effect of the laser co-treatment. These results confirm the effectiveness of chitin nanofibril/chitosan glycolate-based products in restoring subcutaneous architecture. The spray seems to be most effective in healing superficial lesions, including extensive ones; the gel is more effective in repairing shallow lesions as well as an aesthetic factor while the gauze is effective in slow-healing dermo-epidermal wounds.

**KEY WORDS:** chitin nanofibrils, chitosan glycolate, wound healing, immunohistochemistry, cutaneous lesions, laser treatment.

**INTRODUCTION**

The selection of an ideal bio-artificial matrix for dermal regeneration requires an optimal combination of biological, chemical, physical, and mechanical properties. The matrix should be capable not only of inducing correct biological responses in terms of cell adhesion, proliferation, and extracellular matrix (ECM) production, but also of maintaining a suitable physical environment promoting formation of a viable, vascularized neo-dermis [1,2].

Since Prudden et al. [3] reported that chitosan enhanced wound healing in 1970, this polysaccharide has been studied extensively [4,8]. Its best-known quality is its ability to foster the formation of appropriate granulation tissue with angiogenesis stimulation and collagen fiber deposition properties that enhance dermo-epidermal lesion healing [2–7]. Recent advances in chemical processes have made the latest-generation chitosans and chitin nanofibrils more flexible and effective, enabling development of new wound healing products [9–11].

It is known that biological molecules employed in regenerative medicine can induce enhanced effects by co-administration with other therapeutic options [12]. In this study, the healing power of three different chitin nanofibril/chitosan glycolate-based preparations (a spray, a gel, and a gauze dressing) was tested alone and co-administered with a continuous-wave laser treatment. The laser treatment enhanced the biological and metabolic cell activity [13] while promoting both clinical and aesthetic healing of difficult-to-repair cutaneous wounds [14,15].
MATERIALS AND METHODS

Materials

The three products tested, Chit-A spray, Chit-B gel, and Chit-C gauze dressing, were prepared by Mavi Sud Srl, Italy. Chitosan glycolate was incorporated into chitin nanofibrils isolated from crustacean chitin (Katakura Chikkarin, Japan) [15], still in suspension after preparation, by adding the required amount of chitosan powder and glycolic acid crystals to the suspension. The chitin nanofibrils were characterized by X-ray diffraction and FTIR analysis [16].

Chit-A was an odorless, pearly liquid containing 70% glycolic acid (0.56%), chitin nanofibril suspension (97.5%), and chitosan (0.97%). The pH of the final product was 5.5.

Chit-B, was a transparent gel made of 70% glycolic acid (3.13%), chitin nanofibril suspension (91.1%), and chitosan (4.81%) and had a pH of 4.00.

Chit-C was a non-woven gauze made of dibutyryl chitin (0.8 g) and 1.0% chitosan glycolate solution (8 g) containing chitin nanofibrils (2 g/L). The dressing was obtained by freezing at -20°C and freeze-drying at -93°C.

EXPERIMENTAL PROCEDURE

Eight male Wistar rats aged 8–10 weeks and weighing 300 ± 20 g were subjected to comprehensive genetic and health quality controls (specific-pathogen free) by a veterinary surgeon before being housed in individual cages at the animal-care facilities of INRCA (Ancona, Italy), which is certified for all types of authorized animal experiments. Twenty-four hours before surgery and 36 h postoperatively, each rat received 800 mg amoxicillin powder (2.6 mg/g) dissolved in water. Throughout the study the animals were kept in a standard environment at a temperature of 20–22°C, 55% relative humidity, with a 12 h light/dark cycle and ad libitum access to water and pellet food.

Rats were anesthetized with an intraperitoneal injection of 242 ng of 2,2,2 tribromoethanol/kg body weight and four circular dermoepidermal dorsal incisions 0.5 cm in diameter were made. The four lesions (A–D) were medicated with (A) Chit-A; (B) Chit-B; (C) Chit-C, and (D) (control lesions) Phytostimuline (Damor S.P.A Italy). Phytostimuline is a gauze impregnated with aqueous Triticum vulgare extract. Protective dressings were applied to preserve the medications.
Eight days after surgery, four rats (50%) received one-pass (two animals) or two-passes (two animals) of laser treatment to all four wounds using a BIOLASER 810 (Creation S.r.l., Verona, Italy); laser beam parameters were as follows: Program: tissue regeneration; Length: 810 nm; Power: 1.5 W; Pulse: 20 s; Mode: continuous; Wand: at a non contact distance: ≈5 cm.

Wound healing was evaluated by:

1. Macroscopic examination on postoperative days 7, 9, and 15 (in laser-treated rats);
2. Histo-morphological and immunohistochemical examination of biopsies collected from all treated areas on postoperative day 15.

**MORPHOLOGICAL ANALYSIS**

Tissue fragments were fixed in 2% GTA in 0.1 M cacodylate buffer, dehydrated by raising ethanol concentrations and araldite-embedded. Semithin sections were obtained with an LKB ultramicrotome, stained with toluidine blue and examined under a Nikon Eclipse (Nikon-Italia, Italy) light microscope.

**IMMUNOHISTOCHEMISTRY**

Skin fragments were stored in liquid nitrogen at −70°C; 6-μm sections were obtained with a cryotome, dried overnight and then fixed in acetone for 10 min. Some sections were incubated overnight at 4°C with anti-VEGF (diluted 1:200, Santa Cruz, CA) and anti-CD34 (diluted 1:20, BD Biosciences, Belgium) monoclonal antibodies and processed by the streptavidin-biotin peroxidase technique; then incubated with 3,3 diaminobenzidine (Sigma-Aldrich, Italy), stained with Mayer’s hematoxylin and mounted in Paramount. The antibody activity was examined using a Nikon Eclipse light microscope.

For the fluorescence experiments, frozen sections were fixed in acetone at 4°C for 10 min and processed using FITC-conjugated phalloidin (to label polymerized cytoskeletal actin). After rinsing in PBS, sections were permeabilized with 0.1% Triton X-100 in PBS for 15 min and incubated in 0.1% BSA solution in PBS at 37°C for 5 min. Sections were then stained with 0.2 μM FITC-conjugated phalloidin at 37°C for 30 min, washed in PBS and finally mounted in glycerol-base mounting medium (DAKO, Denmark) for observation with a Nikon Eclipse fluorescent microscope.
At least five fields per sample were studied for each reaction (×250 magnification); the number of cells positive for the antibodies tested was expressed as the proportion of positive cells out of all counted cells.

RESULTS

Macroscopic Examination

Chitin nanofibril/Chitosan glycolate preparations
Postoperative microscopy was performed on days 7 and 15. After 7 days the four wounds exhibited different repair responses. The control lesions were blurred and had an irregular grayish surface. Wounds treated with Chit-A showed a large coagulum; the amount and persistence indicated that the spray medication induced significant coagulation. The lesions medicated with Chit-B exhibited superficial cutaneous reconstruction, as if the gel on the connective layer slowed the proliferative-migratory epidermal restoration. In the wounds dressed with Chit-C, the skin reconstruction was more advanced and the injured areas, which did not display re-epithelization, however, had shrunk and exhibited regular borders, indicating effective and correct repair and reconstruction.

Chitin nanofibril/chitosan glycolate preparations with laser treatment
Macroscopic evaluations were conducted on days 9 and 15 after surgery. On day 9, two days after laser treatment, tissue regeneration had advanced more in all the wounds compared with those treated only with the three preparations. The healing process was particularly evident in the wounds medicated with the gauze (Chit-C), which even without laser treatment, appeared macroscopically to be more effective than the spray or the gel. The effects of laser treatment were more evident in the wounds treated with a two-pass lasing than with one-pass. On day 15, seven days after laser treatment, tissue repair was good in all wounds and was also enhanced in the control lesions.

Histo-morphological Study

Chitin nanofibril/chitosan glycolate preparations
The biopsies collected 15 days after surgery confirmed fully regenerated cutaneous tissue with differentiated epithelium displaying different epidermal layers, including a thin horny layer in the lesions dressed with Chit-C gauze (Figure 1(a)). Some small vessels as well as a few inflammatory cells were also detected among collagen bundles.
Figure 1. Day 15 from surgery; wound medicated with Chit-C gauze without (a) and with laser treatment (b): a) Enhanced re-epithelization (\(\uparrow\)); b) Restoration of a correct dermo-epidermal interface after medication with Chit-C and two-pass lasing (basal lamina \(\uparrow\)).
These features were partially different from the wounds medicated with the other two preparations. In particular, Chit-A, which induced the deposition of healing tissue with more irregular histological features, including scattered chitosan globules and some inflammatory cells embedded in the regenerating epithelium. Neo-vascular dermal structures were also observed (Figure 2). Macroscopically, the Chit-B gel induced fairly good tissue repair that was comparable to the healing observed in the gauze-treated lesions.

This diverse efficacy by the chitin nanofibril/chitosan glycolate-based preparations should be considered when treating different types of wounds. Control wounds exhibited more marked scarring, hence failed restoration of the skin structure (Figure 3(a), (b)).

Chitin nanofibril/chitosan glycolate medications with laser treatment

Histological observations were performed on the 15th day after surgery. The two-pass laser treatment proved to be more effective than the one-pass lasing (Figure 3(c), (d)). The wounds medicated with the three preparations alone exhibited a significantly less
ordered epithelial histo-architecture, and fewer polarized cells. However, the gauze bandage consistently induced better epithelial differentiation-keratinization, both with and without laser application (Figure 3(b), (c)).

Therefore, laser treatment should be viewed favorably, especially when lesions involve both dermis and epidermis, particularly in 'fragile' patients, who may also be suffering from a systemic vascular disease. This application was particularly effective in conjunction with the gauze (Chit-C) medication, while the slowly resorbing gel (Chit-B) seemed to retard the laser treatment, thus offsetting its positive action.

**IMMUNOHISTOCHEMICAL STUDY**

Fifteen days after surgery, the presence of F-actin in the cytoskeleton of dermal and epidermal cells was clearly evident, which was more...
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marked in the wounds treated with the gel and the gauze, independently of laser treatment (Figure 4).

The bio-trophism of the medicated wounds was also assessed using two angiogenic markers; CD34, found in newly formed vessels, and VEGF. In particular, the angiogenic response induced by the gauze, compared to the phytostimuline, although not very great, did reflect satisfactory vascular dermal trophism independent of the laser treatment (Figure 5).

DISCUSSION

An increasingly wide array of bio-design technologies are being harnessed to promote skin repair [17]. In the present study, chitin nanofibril/chitosan glycolate-based products were formulated using the latest molecular biology research techniques [16]. An analysis of the healing power of the three chitin nanofibril/chitosan glycolate based preparations indicated that liquid Chit-A could be a useful first-aid tool for superficial lesions, such as abrasions that bleed little but involve a fairly broad skin area (i.e., skin abrasion from falls on concrete or gravel). The Chit-B gel was the longest to induce tissue repair, however, it proved capable of stimulating physiological lesion repair and thus could be ideal for treating aesthetically vulnerable areas with thin epidermal layers (i.e., ears, eyes, etc.). The Chit-C gauze bandage was more effective in repairing dermo-epidermal lesions by promoting vascular bed formation and preventing scarring. The gauze also effectively repaired slow-healing lesions or ulcers in a para-physiological manner, which prevented irregular scarring. All three chitosan treatments, alone, enhanced tissue bio-trophism by virtue of their angiogenic properties [18-20] and have been used clinically for diabetic wound healing [20,21].

Based on the nature of the reaction exerted, skin-repair enhancers are generally classed as: (a) passive, (b) interactive, and (c) bioactive. Traditional dressings, such as large-mesh gauze (which accounts for a large proportion of applications), are passive products. Interactive products include polymeric films that are usually transparent, permeable to water vapor and oxygen, but impermeable to bacteria; these are recommended for wounds with sparse exudation [22]. In contrast, bioactive tools are capable of delivering bio-active substances (proteoglycans, collagen, non-collagen proteins, alginites, and chitosans) into the wound. In the recent literature, the application of chitosan as a hydrogel on dorsal incisions enhanced the rate of tissue repair in rats [22-24].

In several studies, laser treatment has been shown to enhance skin repair [25-29]. This may be due to the fact that laser biostimulation
increases cell metabolic demand, inducing endocellular activation and thus promotes skin regeneration [29,30]. The present study supports the effectiveness of chitin nanofibril/chitosan glycolate products and the benefits of co-treatment with laser therapy in repairing cutaneous wounds.
Figure 5. Postoperative day 15; immunohistochemical detection of CD34 and VEGF immunoreactivity in wounds: a) Small CD34+ vessel in endothelium in a wound medicated with Chit-C gauze. Inset: focal dermal staining for VEGF (→); b) Weak CD34 staining in wound medicated with Phytostimuline without laser treatment (E = epithelium).

The results indicate that the three formulations in this study appear to provide the possibility for differential applications. The chitin nanofibrils/chitosan glycolate spray seems to be more suitable to treat superficial abrasions, as well as extensive; the gel could be used for the
more shallow lesions in aesthetically vulnerable areas, while the gauze dressing would be applicable for slower-healing dermo-epidermal wounds.

REFERENCES


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