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Wound Healing Acceleration by a Textile Dressing Containing Dibutyrylchitin and Chitin

Abstract
New textile dressings containing dibutyrylchitin (DBCH) or regenerated chitin (RC) were prepared in the process of coating a trade polypropylene non-woven material with films of DBCH or RC. The dry dressing material contained ca. 40% of DBCH and 30% of RC. The dressings obtained were cut into pieces of 5×5 cm, sterilised by ethylene oxide and then subjected to biological evaluation required for medical devices. The evaluation included cytotoxicity effects, levels of cytokines TNF-α and IFNs, synthesis of nitrogen oxides (NO_2/NO_3), intracutaneous irritation, and the influence of full thickness skin lesions on the healing process.DBCH and RC caused no cytotoxic effects or primary irritation either in vitro or in vivo, nor did the activity of TNF-α, IFNs or the nitrogen oxide levels increase, and both had a positive influence on the wound healing process. Both dibutyrylchitin and regenerated chitin used for coating trade polypropylene non-woven material can be regarded as valuable dressing materials that accelerate wound healing.

Key words: *dressing materials, wound healing, dibutyrylchitin, regenerated chitin, cytotoxicity, intracutaneous irritation, TNF-α, IFNs, NO.*

Introduction

Chitin, the second most widespread natural polymer after cellulose, can be extracted from seafood waste. It was found and confirmed in many experiments that chitin promotes the ordered healing of tissues, activation of macrophages, and works as a bacteriostatic, antistereoporotic and immunoadjuvant agent [1-16]. However, due to its high molecular weight and strong crystalline structure, chitin is insoluble in common organic solvents, which makes its direct application difficult and expensive. A thin chitin fleece-forming dressing material, which is manufactured and marketed in Japan, is produced from chitin fibre spun from 3-5% solutions of native chitin in the presence of huge amounts of coagulating and washing media [17-21]. Such a troublesome operation is one reason for the high cost of this chitin product: the price of one 120-cm^2 piece of Japanese chitin dressing material named Beschitin is ca. 30 USD. However, the recommended applications of chitin dressing materials are very wide, which may explain their high prices: Unitika Ltd. (Japan) offers such materials for successful and fast healing of burns, skin abrasions, postoperative wounds, bed sores, ulcers, and several other injuries. Chitin-based bioactive dressing materials have not yet been produced in Europe.

An original method for synthesising the ester derivative of chitin, dibutyrylchitin (DBCH), has been devised in Poland. DBCH is soluble in the common organic solvents and has both film and fibre forming properties [22-27]. The easy solubility of this chitin derivative makes it possible to manufacture a wide assortment of DBCH materials suitable for medical applications. It was also found that the alkaline treatment of the finished materials made from DBCH led to chitin regeneration without destroying their macrostructure. Moreover, the regeneration of DBCH to chitin resulted in the improved mechanical properties of newly obtained materials, despite the fact that regenerated chitin RC had a lower molecular weight than the native chitin [28].

The first investigations of biological properties of DBCH and regenerated chitin materials were made *in vitro* and *in vivo* using their fibrous forms. They included all tests fulfilling the EN ISO 10993 (Biological evaluation of medical devices) requirements [29-32]. It was found that DBCH and regenerated chitin fibres met all the basic EN requirements and showed good biocompatibility when they were used as implants into the gluteal muscles of rats of the Wistar breed.

The main objective of the present investigations was to determine the ability of DBCH and regenerated chitin to accelerate wound healing. DBCH and regenerated chitin RC were used in the form of films coating bilaterally commercial polypropylene non-woven materials. The biological properties of the dressing materials prepared were investigated according to EN ISO 10993, and their medical properties were studied *in vivo* using a representative group of albino rabbits of the New Zealand breed.

Experimental

Dressing material samples preparation

Krill chitin, a product of the Institute of Sea Fisheries (MIR) in Gdynia, with the intrinsic viscosity value of [η]=13.33 dl/g, determined at 25°C in solutions of dimethylacetamide (DMAc) containing 5% of LiCl, was used for the synthesis of DBCH. The chitin had been previously additionally purified from the remains of calcium carbonate. DBCH with the intrinsic viscosity value of [η]=2.41 dl/g, determined at 25°C in DMAc solutions, was received in accordance with the description given in Szosland et al. [32].

The commercial non-woven polypropylene material (PP) with a surface mass of

ca. 30 g/m^2 was washed in distilled water with flax shampoo, then rinsed several times with distilled water, and finally with ethanol. Finally, the non-wovens were dried and cut into rectangular 20×30-cm pieces. Each rectangular piece was weighed. Clean piece of PP non-woven material was covered bilaterally with 3% DBCH ethanol solution (3 g of DBCH in 100 ml of ethanol) using the polyurethane sponge roll, previously washed in ethanol and dried. The non-woven material was dried and coated for the second time with DBCH solution prepared as above, but this time containing dehydrated glycerin of analytical purity (1ml in 100 ml of solution). All non-woven pieces, double-covered with DBCH, were dried and weighed. The percentage of DBCH in the dry dressing material was ca. 40%.

One part of the DBCH-coated dressing materials was treated with 5% NaOH water solution to restore chitin in the coating layer. Alkaline treatment was carried out at 90°C over 15 minutes. The pieces of dressing materials treated with NaOH were washed with distilled water to remove any traces of alkali, then with ethanol, and dried. The percentage of regenerated chitin RC in the obtained dressing material was ca. 30%. Both dressing materials used for biomedical investigations were cut into 5×5 cm pieces and sterilised with ethylene oxide.

With the purpose of evaluating the change in RC molecular weight, 10 ml of 5% acetone solution of DBCH was dropped into 100 ml of 5% NaOH water solution at 90°C, sediment (regenerated chitin RC) was washed from any traces of alkali, then with ethanol, and dried. The intrinsic viscosity value of the obtained regenerated chitin RC determined in DMAc/5% LiCl solutions was 4.4 dl/g. The correlation between the intrinsic viscosity value [η] of chitin and its molecular weight $\bar{M}v$ is expressed in the Mark-Houwink equation: $[\eta]=K\cdot\bar{M}_v^a$, where $K=2.1\times10^{-4}$ and a=0.88 [33]. The decrease in the intrinsic viscosity value from 13.33 for initial chitin to 4.4 for regenerated chitin RC corresponded to a ca. 3.5 times decrease in RC molecular weight.

Biological tests and results

The polypropylene non-wovens covered bilaterally with the layer of DBCH and regenerated chitin RC were subjected to biological evaluation in accordance with the demands determined for the medical devices. We evaluated the cytotoxicity effects, the levels of the cytokines TNF-α and IFNs, the synthesis of nitrogen oxides (NO_2/NO_3), intracutaneous irritation and the influence on the healing process of the full thickness skin lesions.

Cytotoxicity effects

The evaluation of cytotoxicity effects was conducted on the reference cell line of mouse fibroblast 3T3/Balb, with the direct contact method. The cultures of mouse fibroblasts, with ca. 5×10^5 cells each, were established on Petri dishes. After 24 h the old culture medium was removed and the new one added. The culture cells were covered with the samples tested of 2 cm in diameter The changes in the cell cultures were recorded after 24, 48 and 72 h. Dyeing with the neutral red dye was employed for the count living cells, while the dead cells were dyed with the trypan blue dye. Both kinds of cells were counted in the Bürker chamber. The results of our cytotoxicity assessment are shown in Table 1. Direct contact of the mouse fibroblast cultures (3T3/Balb.) with non-woven dressing materials coated with DBCH and regenerated chitin RC did not show any cytotoxicity effect.

Immunological testing

In order to define the inflammatory and immunomodulation effects of DBCH and RC, an assessment of their influence on the induction of the cytokines TNF-α and IFNs, and on the synthesis of nitrogen oxides of human leukocytes was conducted. The leukocytes used for the assessment were extracted from the peripheral blood of healthy volunteers, which was collected for heparin after centrifugation at the gradient of Dextran-Uropolina (i.e. gradient G of the density 1.115 g/ml). For the purpose of this assessment, a leukocyte suspension in the RPMI medium with 2% foetal calf of serum, 100 u/ml of penicillin, 100 μg/ml of streptomycin and the required density of 2×10^6 cells/ml was prepared.

On a plate with 24 wells (Costar Ltd.) 1 ml of the leukocyte suspension of 2×10^6 cells/ml in the culture liquid RPMI with 2% calf serum was deposited into each well. Samples of the tested biomaterials in the amount of 10 mg of each were added to prepared cells, which were then incubated for 24 and 72 h at 37°C and 5% CO_2. The activities of TNF-α, IFNs, and nitrogen oxides were assessed in the supernatants.

TNF-α levels

The level of TNF-α was determined by the biological method on 96-well plates, on which the L_{929} cell cultures of mouse fibroblasts were established with the density of 2×10^5 /ml and incubated for 24h (37°C and 5% CO_2). The proper dilutions of the supernatant from above leukocyte cultures (from 1:2 to 1:256) in the Eagle medium containing 10% calf foetal of serum and actinomycine D (Sigma) (2,5 μg/ml) were prepared on a separate plate. Then the liquid from above the L_{929} cultures was removed, and the appropriate previously prepared dilutions were deposited on these cultures. The degeneration of the cells caused by TNF was observed in the inverted microscope after 24h and 72h incubation (at 37°C and 5% CO_2). In parallel, L_{929} cultures in the Eagle medium without actinomycine, with actinomycine and the standard control of rHu TNF-α were also established for comparison. The results of TNF-α level determination are collected in Table 2.

Table 1. *Changes in the mouse fibroblast cultures (3T3/Balb) after 24, 48 and 72 hours of contact with the samples of dressing materials manufactured from polypropylene non-woven (PP) double covered with dibutyrylchitin (DBCH) and regenerated chitin RC films (n.o. - not observed, s - single).*

Material	Morphological changes			Vacuolisation			Separation from medium, %			Agglutination, %			Dead cells, %			Number of living cells x 10^6			Level of toxicity		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
PP coated with DBCH	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	0	5	5	2	5	5	2	3	5	1.10	1.41	2.2	0	0	0
PP coated with RC	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	s	7	5	2	20	10	3	5	5	1.36	1.79	2.6	0	0	0
Control	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	0	s	s	0	0	0	2	2	3	1.20	1.75	2.8	0	0	0

Table 2. The levels of TNF-α, IFNs and NO_2/NO_3 produced by human leukocytes treated with the samples of the polypropylene non-wovens (PP) coated with DBCH and regenerated chitin RC.

Material	TNF-α level, unit/ml		IFNs, unit/ml		NO_2/NO_3, μM	
	24 h	72 h	24 h	72 h	24 h	72 h
PP coated with DBCH	16	8	4	4	2.08	1.85
PP coated with RC	8	2	2	<2	2.21	1.99
Control	16	2	2	<2	1.94	1.90

IFNs activity

The interferon level was assessed by the micro method of incubation of the cytopathological effect (CPE) of the EMC virus in the A_{549} cell culture of human lung adenocarcinoma. The interferon titration was conducted on plastic 96-well plates. An A_{549} cell culture of the 2×10^5/ml density was established on each plate and incubated for 24h at 37°C and 5% CO_2. The proper dilutions (i.e. from 1:2 to 1:256) of the tested materials in the Dulbecco medium with 10% calf foetal of serum were prepared on the additional plate. The supernatant from the above cell cultures was removed, and the prepared dilutions were deposited. After 24h of incubation (at 37°C and 5% CO_2) the culture was infected with the titre 10^2 $TCID_{50}$/ml of EMCV virus. The virus suspension was prepared in the liquid culture medium DMEM with 2% calf foetal serum. The control EMCV virus in the culture, the control-referenced interferon and non-infected A_{549} cell culture were left on the plate. The cytopathological effect was observed in the reverse microscope after 48 h incubation. The presence of interferon resulted in the protection of the cells against the cytopathological effects of the virus. The dilution of the interferon, which protected 50% of the cells, was adopted as one unit of IFNs. All results were corrected to the standard titre of IFN-s. The results obtained for the materials investigated are presented in Table 2.

Nitrogen oxides level

The concentration of nitrogen oxide ($N0_2$) was measured in the supernatant from the above-mentioned leukocyte cultures using the colorimeter method according to Ding et al. [34]. 100 μl of Griess reagent (i.e. 0.1% dihydrochloride naphthylenediamine in H_2O and 1% sulphanilamide in 5% H_3PO_4 at the ratio 1:1) was added to 100 μl of the tested material for one well. Then, the plate was incubated at room temperature for only 12 minutes, and optical density was measured in the Stat Fax 2100 counter (Awareness Technology Inc.) at the wavelength of 540 nm. The concentration of NO_2 was calculated in relation to the standard curve of $NaNO_2$ prepared within concentrations from 1 μM/ml to 100 μM/ml.

The results of the assessments of nitrogen oxide synthesis in the human leukocyte cultures are also shown in Table 2. It was found that neither the PP non-woven material coated with DBCH nor that coated with regenerated chitin RC stimulated human leukocytes to a greater activity of TNF-α and IFNs, nor to a greater synthesis of nitrogen oxides. The results obtained were comparable to those for the control samples. Neither inflammatory nor immunomodulation effects were observed.

Intracutaneous irritation test

This test was conducted using the extracts of the samples of PP non-wovens coated with DBCH and RC. The polar and non-polar extracts were prepared using the saline solution and sesame oil respectively, after sinking the tested materials (120 cm^2 each) in 20 ml of the corresponding liquid. Incubation was carried out at the temperature of 37°C for 72 h. The saline solution and pure sesame oil, which had no contact with the tested materials, were used as the control samples and were incubated under the same conditions as above.

The assessments of the extracts from each material were conducted on 3 albino rabbits of the New Zealand breed. On the back of each animal 5 intracutaneous injections of tested extract and 5 injections of control solution, each of 0.2 ml, were carried out.

Observations of skin were made 24, 48, and 72 h after the injections, and no skin changes were found. The Primary Irritation Index for the polar and non-polar extracts from the polypropylene non-woven materials coated with DBCH and regenerated chitin was equal to 0.00.

Influence on healing of skin defects

The evaluation of the influence of the polypropylene non-woven materials coated with DBCH and regenerated chitin on the healing process of skin wounds was conducted on 16 albino rabbits of the New Zealand breed of near-equal body mass of 3.2-3.5 kg.

The surgery was carried out under general anaesthesia and in fully aseptic conditions. Four oval wounds (ca. 12 mm in diameter) across the entire skin thickness were incised with the scalpel on the back of each rabbit. The wounds to the left of the backbone were covered with aseptic swaps as the controls. On the right side, the anterior wound was covered with the polypropylene non-woven material coated with regenerated chitin, while the posterior wound was covered with the polypropylene non-woven material coated with DBCH. In addition, all those dressings were protected by a gauze band. The wound healing was observed and the dressings were changed every 24 h until the wounds were covered with scabs. Later on, the wounds with scabs were protected only by a gauze band.

During the macroscopic observations, no significant differences were noted in the healing of full-thickness skin lesions covered with dressings containing either DBCH or regenerated chitin. The wound edges were flat, and the neighbouring skin showed no signs of inflammation. All the full-thickness skin lesions dressed with the tested materials were filled with white-yellowish, elastic tissue and all appeared to be more contracted, wetter and more elastic as compared to the control wounds covered only with gauze. The edges of control wounds were thickened and with significant areas of redness, while the skin was congested. By the sixth day after surgery, massive scabs covered the skin lesions. The photographs of the wounds taken on the 14^{th} day after surgery are shown in Figures 1-3.

The microscopic assessment showed that the wounds covered with the dressing containing DBCH healed fastest. By the tenth day a new, granulated tissue was observed in the site of the lesion, the epithelium almost totally covered by the squamous epithelium. On the fourteenth day, most of the wounds were filled with immature connective tissue with numerous blood vessels and collagenous fibres, and the connective scars were all completely covered by the epidermis. The healing of wounds covered with the dressing containing regenerated chitin was quite simi-

lar, except that the connective tissue that filled the wound remained in the granulated phase for longer. In the samples from the wounds dressed with gauze only (control), the exudative phase was significantly longer, the formation of granulated tissue lasted until the fourteenth day and this tissue was covered only patchwise by the epidermis which migrated from the wound edges. Microscopic views of the wounds at the 14th day after surgery are shown in Figures 4-6. Formation of the connective tissue scars with a fibrous texture and their complete covering by squamous epithelium in the case of the control wound occurred only after 21 days after surgery.

Discussion and Conclusions

An ideal dressing should induce no cytotoxicity effects or immunological reaction, nor cause any other morphological or cause enzymatic changes in the wound. It should adhere closely to the wound, have a good ability to stop bleeding, prevent the formation of secretion, and act as an antibacterial barrier. In addition an ideal dressing should keep the wound wet, be permeable to air, have good antibacterial and painkilling properties, and properly stimulate the damaged tissue to regeneration. An ideal dressing material should also be biodegradable during the time of wound healing to avoid any damage to the new, fresh tissues during the process of its removal.

Chitin, as well as several of its derivatives, has some of these properties, with a beneficial influence on the wound healing process. It was demonstrated in many experiments that chitin and its derivatives are biocompatible, have antibacterial and painkilling properties, induce faster wound healing, and stimulate the reconstruction of connective tissue [35-38].

Based on this data and on our own experience, we have conducted a biological evaluation of new dressing materials prepared from an ester chitin derivative, DBCH, and the regenerated chitin RC obtained from DBCH. We were seeking an answer to the following question: would DBCH and regenerated chitin accelerate the healing process, and could both these materials be regarded as new bioactive dressing materials? The results of the initial testing of dibutyrylchitin and regenerated chitin on mouse fibroblast cultures 3T3/Balb did not show any cytotoxicity effects. No fibroblast cultures after contact with both tested dressings showed any damage, and the cells had proper morphologies and showed good proliferation and good ability to form new colonies, in contrast to the control fibroblast. An increased agglutination of some fibroblasts after the contact with the regenerated chitin coated dressing was restricted only to the cells in the mitotic phase. Our testing of the dibutyrylchitin and regenerated chitin extracts did not demonstrate their primary irritation effect.

We assume that chitin and its derivatives used as dressing materials cause an increase in the activity of wound enzymes, mainly of the lysozyme, which is in turn responsible for the depolymerisation of chitin itself. It is probable that the products of chitin degradation are able to stimulate the granulation process, but this process is not yet sufficiently known. Many different factors, including cytokines and growth factors, influence the wound healing process. Many authors have demonstrated that some chitin derivatives (chitosan) could stimulate cells to the synthesis of mediators of immunological response, such as TNF-α, IL-1, IL-6, IL-8, growth factors, such as PDGF (Platelet Derived Growth Factor) and TGF-β

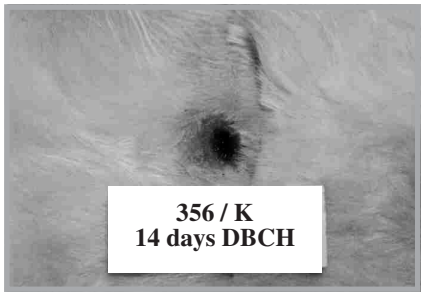

Figure1. *A wound treated with DBCH containing dressing material on the 14th day after surgery.*

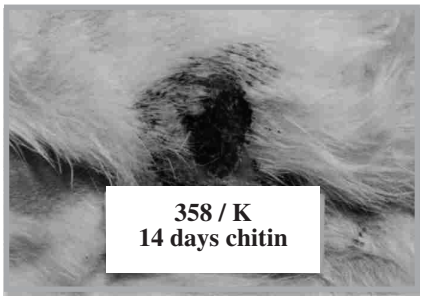

Figure 2. *A wound treated with regenerated chitin containing dressing material on the 14th day after surgery.*

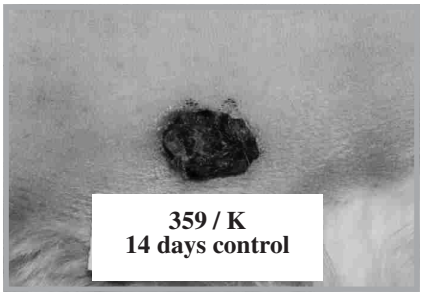

Figure 3. *A wound treated with gauze only (control) on the 14th day after surgery.*

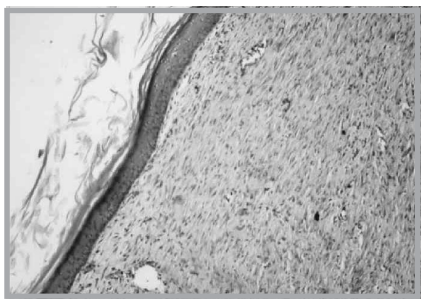

Figure 4. *A microscopic view of the skin lesion, dressed with the polypropylene nonwoven material coated with dibutyrylchitin on the 14th day after the surgery. On the left, the layer of epidermis covering the granulated tissue is seen. Dyed by HE. Magnification 120x.*

Figure 5. *A microscopic view of the skin lesion, dressed with the polypropylene nonwoven material coated with regenerated chitin on the 14th day after the surgery. Immature connective tissue covered by epidermis is visible. Dyed by HE. Magnification 120x.*

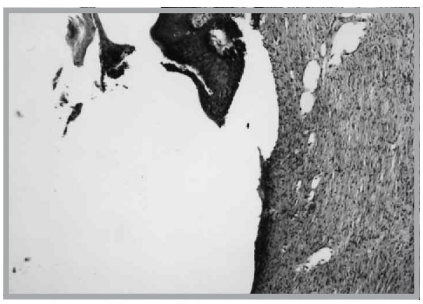

Figure 6. *A microscopic view of the skin lesion, dressed with the gauze only, (control) on the 14th day after the surgery. On the right, immature connective tissue partially covered by squamous epithelium is visible. Dyed by HE. Magnification 120x.*

(Transforming growth factor-beta), and NO (nitrogen oxide). A high level of pro-inflammatory factors such as TNF-α, IL-6, and NO is not desirable for the organism as a whole. That is why it is so important to discover such biomaterials which will not stimulate the cells to produce high amounts of inflammation factors [39-43].

In our *in vivo* evaluation, which was conducted with human leukocytes stimulated by polypropylene non-woven materials coated either with dibutyrylchitin or with chitin, we did not observe any significant differences in the levels of TNF-α, IFNs or NO. After 24 and 72 h contact with the dressings tested, the amounts of TNF-α, IFNs and NO in the supernatants from the above-mentioned leukocyte cultures were only slightly different from their levels received in the control test with the use of human leukocytes without any contact with the dressing materials.

There are other important factors that influence treatment wounds: wet environment, accessibility oxygen, antibacterial protection, and stimulation of the cells to proliferation, which in turn results in faster regeneration of the damaged tissues. It was demonstrated in experiments and observed in clinical observations that a liquid produced by the wound surface contains oxygen and nutritive elements which secure the proper growth of the tissues, provide a substrate for the proper growth of granulation tissue and correct the growth of the new epidermis [34,39]. The same experiments demonstrated that wounds in wet environments healed faster and in a more regular way than dry wounds. The formation of scabs makes the healing process more difficult.

In our evaluation of the influence of dibutyrylchitin and regenerated chitin on the healing process of the full skin-thickness wounds, we have found that the wounds dressed with those materials contracted more, were wetter and more elastic as compared to the control wounds, which were covered by massive scabs at a very early stage (after 5-6 days). In the microscopic assessment, it was shown that the skin lesions treated with the tested dressings after 10 days were filled with granulated tissue, which was almost completely covered with the new squamous epithelium. On the fourteenth day, most of the wounds were filled with immature connective tissue with numerous vessels, collagenous fibres, and small fascicles of elastic fibres. For similar wounds, which were covered only with gauze (control), we noted a long-lasting exudative phase (up to 10 days) and the presences in the centre of the wound, a homogenous, amorphous tissue, which later formed the scab. The formation of granulated tissue only partially covered by epidermis, and not so abundant in vessels, was also observed on the fourteenth day. The formation of connective tissue scars, and then covering with the squamous epithelium, ended no earlier than after 21 days.

In conclusion we can confirm that our investigation of the polypropylene non-woven materials coated with DBCH and regenerated chitin, conducted both *in vitro* and *in vivo*, showed that they do not demonstrate cytotoxicity or primary irritation effects, do not cause an increase of the activities of TNF-α, IFNs or the nitrogen oxide level, and both have an active positive influence on the wound healing process. Therefore, both the dibutyrylchitin and the regenerated chitin could be regarded as valuable dressing materials which accelerate wound healing.

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