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Development of Artificial Skin from Chitosan Derivatives

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Abstract Chitosan derivitives, a sulfated N-acetylchitosan was synthesized, and artificial skin of sulfated N-acetyl chitosan and N-carboxyl butyl chitosan were investigated. Sulfated derivatives of chitosan were analyzed by ^{13}C-NMR and the structure of N-acetyl chitosan 3,6-O-disulfate were confirmed.

Rabbits underwent a midline laparotomy followed either by a bilateral peritoneal sidewall abraison(3.0×1.5cm). The injured surface was then covered with 0.2mm thick sulfated N-acetyl chitosan membrane.

Sulfated N-acetyl chitosan membrane was found to reduce postsurgical bleeding after abraison of peritoneal surface treated with sulfated N-acetyl chitosan membrane.

Sulfated N-acetyl chitosan implanted rabbit showed quick wound healing than N-carboxybutyl chitosan. With a sterilization procedure of chemical sterilization, sulfated N-acetyl chitosan seem to be better substitutes than N-carboxybutyl chitosan.

Key words: *Chitosan derivatives, Sulfated N-acetyl chitosan, N-carboxyl butyl chitosan, Artificial skin*

Introduction

Chitosan ((1→4)2-amino 2-deoxy β-D-glucan) is a unique polysaccharide derived from chitin. Chitosan, having structural characteristics similar to glucosaminoglycans, seems to mimic their functional behaviour into the extra cellular matrix and then cross-linked to guide tensile strength to the newly healing wound [2,15,16]. Much attention has been paid to chitosan due to its attractive characteristics including abundance in nature biogradability, cellulose like rigid structure, presence of amino group, and inherent bioactivity [14]. Several attempts have been made to use chitosan in biomedical field.

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Chitosan proposed a design for artificial skin, applicable to long-term chronic use, focuses on a nonantigenic membrane which performs as a biodegradable template for synthesis of neodermal tissue [1,13]. The chitosan, polysaccharide, having structural characteristics similar to glycosminoglycans, could be considered for developing such substratum for skin replacement [20]. Beschitin W, recently developed a new wound dressing, composed of chitin nonwoven fabric and has been proved to be beneficial in clinical practice [8]. It observed that this material accelerated wound healing property and attainment of good-looking healing skin surface [9].

Usually the efficacy of wound management product is determined either by clinical assessment, grided by empirical observations, or through the use of wound models [17,21]

The further development of chitosan derivatives requires a better understanding of their effects on the qualitative and qantitative aspects of the cellular response of the major phases of wound healing.

This work is to develop a new material in place of human skin which in all respects should suit for healing wound. This study also aimed at pointing out the probable mechanism, chitosan derivatives sulfated N-acetyl chitosan or N-acetyl butyl chitosan with sterilization procedures, and animal models used for a quicker wound healing process.

Materials and Methods

Chitosan (Flinac-N, commercial chitosan of crab shell, d.s. for HAC. [*a*], -5℃) was obtained from Kyowa yushi Co. Flonac -N was treated with aqueous 40% NaOH containing $NaNH_4$ (0.5g/500ml) at 110℃ for 5hrs to afford purified product (d.s. 0.05 for HAc) which had [*a*], at -10℃ 2% aqueous acetic acid.

N-carboxybutyl chitosan was obtained from Dr, Hirano (Tottori university, Japan)

Sulfation of N-acetylchitosan

Sulfation of chitosan was carried out according to the method of Hirano and Kinugawa [7]. N-acetyl chitosan (0.5g) was dissolved in methane sulfonic acid (5ml) in ice bath or swollen in N, N-dimethylformamide (5mL) at room temperature, and sulfated with SO_3-DMF complex (5-7 mole equivalent amounts to one hydroxyl group) by stirring at room temperature for overnight. The reaction mixture was poured into ice cooled water, and the solution was adjusted to pH 9 with 2.5 N-NaOH. Three volumes of 95% ethanol were then added, the precipitate produced was collected by centrifugation (2,000×g, at 20 min) and dissolved in a small volume of water. The soultion was dialyzed against distilled water, overnight, and lyophilized to afford the sulfated products of N-acetyl chitosan.

Preparation of samples

Samples was dissolved in dimethyl formamide and spread over a clean dry glass and immediately dipped in ice cold distilled water. After 12hrs, the membrane was peeled off from the glass plate and washed with plenty of distilled water. It was then dried in a vaccum oven at 60℃ for 3hrs, The membrane was then cut out into suitable sizes, i.e., 3.0cm×1.5cm. The membrane was sterilized by mode of methods [17].

Modes of sterilization

Chemical sterilization

Each sample was exposed to 0.5% glutaraldehyde solution for 5 minutes and then rinsed many times with saline just before implantation.

Steam Sterilization

The samples were autoclaved under a pressure of 20 lbs at 120℃ for 10 minutes and then dried under vacuum.

Spectrum of IR and ^{13}C-NMR

Infra-red(KBr) and ^{13}C-NMR spectra were recorded Hitachi 125 grating spectrometer and a Jeol FX 200 FH-NMR, respectively.

Surgical proceduce

Normal adult albino rabbits (2Kg) were housed in a light : dark cycle (16:8) controlled vivarium and maintained with water and rabbit chow *albitum*. In the treatment groups, the area of the injured peritoneum was covered with N-carboxybutyl chitosan membrane or sulfated N-acetyl layers chitosan (membrane size was approximately same as the wound area). The abdominal wall was then closed in two layers with 3-0 nylon sutures (Ethicon, Raritan, NJ). In order to minimize any possible contamination by intraoperative bleeding, the peritoneal cavity was protected from the injury site by sterile gauze during the operative procedure.

The fur on the back of the animals was clipped and the area to be cut from the body was marked (3.0×1.5cm).

Surgical procedures were performed under the influence of Rompun (25mg/Kg) and Ketamine (130mg/Kg) anesthesia. Rabbit underwint a midline laparotomy followed by the indication of either a bilateral peritoneal sidewall abrasion with surgical blade (Fig. 1).

The postsurgical time course in number of red blood cells in abrasion area was initially determined, and then the treatment group which received the N carboxybutyl chitosan or sulfated N-carboxybutyl chitosan for few days was compared to the surgical control.

On the eighth day of grafting, the stitches were removed and on the tenth day, the materials were removed from the wound area with virtually zero peel strength. The wound area and animal weight were recorded every day. After healing the wound completely, the animals were observed for different periods of time.

Cell preparation

At a varying number of days after surgery, rabbits were sacrificed with an overdosages of pentobarbiturate (Western Medical Supply, Arcadia, CA). Fifty milliliters of phosphate buffered saline pH 7.4, (PBS) containing 20IU heparin/ml, was injected into the peritoneal cavity of the postsurgical and non-surgical control rabbits. Red blood cells (RBC) were recovered by PBS-heparin (50ml) lavage.

RBCs were counted using an improved Neubauer chamber. The lavage fluid was centrifuged at 200×g for 10min and WBCs were recovered after hypotonic red cell lysis with a PBS:H_2O (1:9) solution[4]. The cells were washed twice with cold PBS and total cell number was determined by a hemocytometer.

Statistics

Data were initially analyzed by a rankit analysis to determine the distribution. Difference within the groups was examined by the Mamm-Whitney U test. The results are

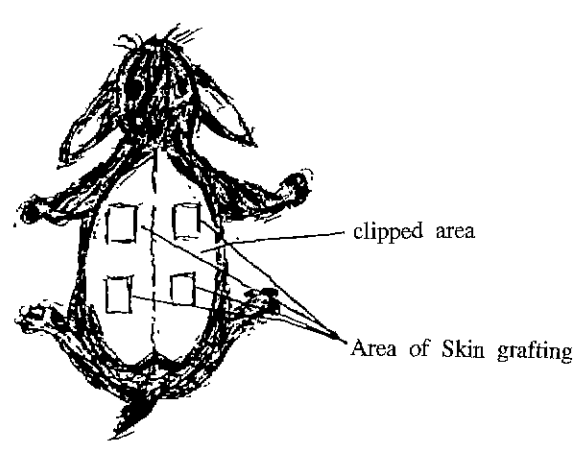

Fig. 1. Skin grafting in rabbits.

presented as the mean ±SEM.

Results and Discussion

Preparation of sulfted N-acetyl chitosan

Fig. 2. showed the IR spectrum of chitosan, and sulfated N-acetyl chitosan. Sulfated group was detected by infrared absorptions at 1240~1250 (S=O), and 800~1660cm (eq. C-O-S). N-acetyl group was also detected at 1640~1660, and 1540~1560cm (C=O, and NH of N- acetyl). Because the raw material, chitosan, generally contains several percent of unhydrolyzed acetyl group, the bonds for unhydrolyzed acetyl group were also observed in the upper spectrum. Fig. 3. shows the ^{13}C-NMR spect of sulfated N-acetyl chitosan.

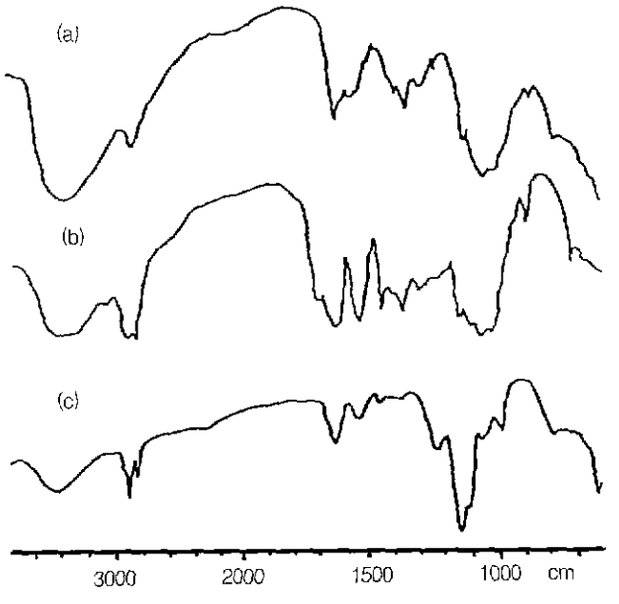

Fig. 2. Infrared spectra of chitosan(a), N-acetyl chitosan (b) and sulfated N-acetyl chitosan(c).

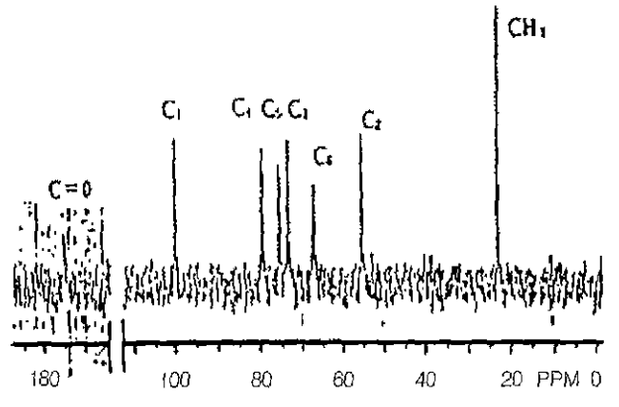

Fig. 3. ^{13}C-NMR of sulfated N-acetyl chitosan.

^{13}C signals of these compounds assigned as reported of chitosan. The C1 and C4 signals in N-acetyl β-D-glucosamine HCl appear at 90.0~91.1ppm and 70.5ppm, respectively. These signals displace at 97.3~101ppm and 75.9~76.4ppm due to β-D-glucosidation. The C6 signal appears at 67.5~67.3ppm, indicating the O-sulfated of the hexosamine moiety. This deplacement agrees with the C6 signals at 67~68ppm in heparine, and at 69.33 ppm in keratan sulfate which has O-sulfate at C6 in the N-acetyl D-glucosamine moiety. The unsulfated C3 signal in N-acetyl β-D-glucosamine HCl, appears at 70.9ppm and 70.5ppm, respectively. These data shows the formation of 3, 6-O-disulfated N-acetyl chitosan. The present method is applicable to analysis of the positions of substitution with O-sulfate at C6 and C3 in the hexosaminyl residue.

Effect on postsurgical bleeding

Initially, Fig. 3 determined the course of red blood cells (RBC) accumulation in the peritoneal cavity after sidewall abrasion. As a control for RBC contamination at the time of cell harvest, RBC from non-surgical rabbits were lavaged and found to be less than 5% of the RBCs recovered on postsurgical day 1 ($3.8\pm1.7\times10^8$ cells/cavity), The number of RBC increased gradually reaching peak levels by day 3 ($6.0\pm1.5\times10^8$ cell/cavity) and thereafter gradually decreased.

These data indicated that postsurgical bleeding continued for at least 3 days in models. Therefore, the treatment group was given a sulfated N-acetyl chitosan membrane for 3 days. An abrasion only control group was used for comparison with N-carboxybutyl chitosan or sulfated N-acetyl chitosan. As shown in Fig. 4, the sulfated N-acetyl chitosan membrane significantly decreased postsurgical bleeding after abrasion of sidewall surface with sulfated N-acetyl chitosan membrane : 7.0 ± 0.8; without sulfated N-acetyl chitosan membrane : $24.9\pm5.0\times10^8$ cells/pertioneal cavity.

Although there was apparent reduction in postsurgical bleeding after peritoneal sidewall abrasion, the number of RBCs were signficantly less than the number of RBCs recovered from the peritoneal cavity as control.

Postsurgical bleeding is a common complication associated with abdominal surgery which may induce infection or impair healing through hematoma formation.

Bleeding may also facilitate peritoneal adhesion through the activation of coagulation and fibroblast proliferation [5]. Clinically, several compounds are normally used such as hemostatic agents, including fibrin glue and microfibril collagen [19]. Chitosan, which is a large molecular weight polysaccharide and a deacetylated form of chitin from crab shells, also posesses hemostatic activity and has been suggested for use as an adjuvant in tissue repair [2,3]. Solution of chitosan in contact with red cell membranes forms a hemostatic activity of chitosan on prosthetic vascular grafts [6,18]. In this study, we used sulfated N-acetyl chitosan membrane for hemostatic in a peritoneal injury model. The

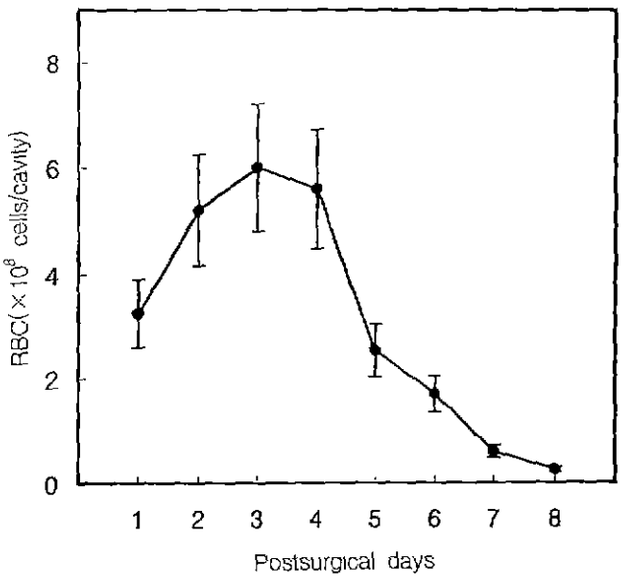

Fig. 4. Time courses of postsurgical bleeding after peridoneal sidewall abrasion. Rabbits underwent bilateral peritoneal sidewall abrasion (3.0×1.5cm). At various day after surgery, peritoneal lavage with 50ml PBS was performed and number of red blood cell (RBC) was counted (×10^8 cells/cavity).
Data are expressed as mean±SEM

sulfated N-acetyl chitosan membrane applied to the peritoneal cavity surface after abrasion signficantly reduced the amount of bleeding. This may result from the mechanical protection of injured surface. Sulfated N-acetyl chitosan may reacted also as a function of local hemostatics.

Thus, the sulfated N-acetyl chitosan membrane treatment reduced the bleeding of the abraded injured surface. In addition, the amounts of bleeding from the peritoneal cavity after sidewall abrasion might be too low to quantitate any hemostatic activity by the membrane.

Clearly, our data suggest that the sulfated N-acetyl chitosan membrane may be useful for hemostasis and may promote repair after abdominal injury. Further experiments to better elucidate the mechanism of action of sulfated N-acetyl chitosan are now in progress.

Cutaneous repair in aminal

Table 1 are shown the wound healing periods of control and N-carboxybutyl chitosan or sulfated N-acetyl chitosan. The wound with sulfated N-acetyl chitosan implanted healed faster than the wound with chitosan implanted, irrespective of the mode of sterilization of the sample implanted.

Sulfated N-acetyl chitosan implanted animals showed quicker wound healing were sterilized by chemical methods rather than autoclaved.

Sulfated N-acetyl chitosan blend in a particular ratio and with a sterilization process of chemical sterilization gave satisfactory results when tried in rabbit. With a sterilization procedure of chemical sterilization, sulfated N-acetyl chitosan seem to be better substitutes than N-carboxybutyl chitosan absorbed chitosan samples, the latter giving only small variation with that of bare chitosan samples.

In order to assess the inductive effects of sulfated N-aceyl chitosan on skin repair processes in rabbits, the peritoneal cutis and subcutis were removed down to the surface bundles of furrier muscles (4×1.8cm). The animals were treated with sulfated N-acetyl chitosan (freeze-dried soft pads) and a control group was treated with bare chitosan (Fig. 5).

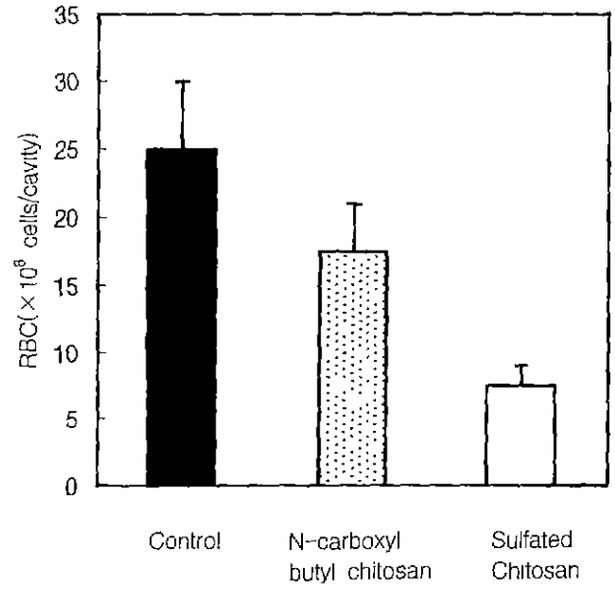

Fig. 5. The effect of chitosan derivatives membrane on surgical breeding after the abrasion of peritoneal sidewall, injured area were covered with N-carboxybutyl chitosan chitosan, and N-acetyl sulfated chitosan menbrane was compared to non-treated surgical controls.
Data are expressed as mean±SEM

Table 1. Wound healing patterns of chitosan and its derivatives and sterilization in rabbits

Samples implanted	Mode of sterilization	No. of days taken for complete wound healing
Chitosan	chemical sterilization	28.72±5.42
Deacetylated chitosan	chemical sterilization	24.64±1.63
Sulfated N-acetyl chitosan	chemical sterilization	30.33±4.15

Eeach value expressed as mian±SEM. of 3 animals

The morphological analysis on the sulfated N-acetyl chitosan animals showed that, after 15 days, keratinization was absent. The epithelium was still immersed and covered with sulfated N-acetyl chitosan residues. It ta pered progressively, showing a front of epithelialization composed of a few elongated cellular elements immersed in sulfated N-acetyl chitosan.

Sulfated N-acetyl chitosan is obtained sterile after the chemical manipulation and lyophilization, thus the freezed dried dressing can be package under sterile conditions with no further treatment. Thus, the resulting freeze dried wound dressing was found to be well suited for applications *in vivo* and it intreated in an active manner with cells migrating from adjacent tissues in the implant, while chemical structure of sulfated N-acetyl chitosan acted as a template for the extracellular matrix reconstruction.

It is quite a contrast from the observation made in our animal model, where a distinct change in the wound healing property of material was observed with differences in sterilization processes. Quick wound healing function in rabbit is found to be independent of the materials used and sterilization procedures adopted

In general, quickened wound healing is observed in the rabbit model, different types of samples and sterilization methods used.

Acknowlegement

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